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Characterization of Prolactin Receptor

in *Meleagris gallopavo*

by

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ABSTRACT

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Prolactin receptor (PRLR) is a membrane anchored protein mediating the biological actions of prolactin. The turkey PRLR (tPRLR) cDNA was isolated and characterized. The open reading frame (ORF) of tPRLR predicted 831 amino acid residues composed of a signal peptide, an extracellular domain (ECD), a single transmembrane domain and an intracellular domain. The deduced amino acid sequence of the turkey prolactin receptor is 53.8%, 51.7%, 49.8%, 49.8%, 80.3% and 89.9% identical to that of the rabbit, bovine, human, long form of the rat, pigeon and chicken PRLRs, respectively. The extracellular domain contains two homologous repeat units with 63% amino acid sequence identity to each other. The membrane-distal and membrane-proximal repeats were 53-60% and 62-70% identical to the ECDs of the mammalian PRLRs, respectively. A tPRLR transcript with a molecular size of 3 kilo nucleotides was identified and was detectable in 26 tissues examined. The pituitary gland, crop sac, duodenum and gizzard were found to express the highest levels of tPRLR mRNA among the 26 tissues. In most tissues examined there was no obvious relationship between blood levels of PRL, reproductive states and estimated concentrations of the receptor mRNA. However, in the hypothalamus, increasing blood levels of PRL were associated with decreasing levels of receptor transcripts ($p<0.05$), whereas, the opposite relationship was observed in the pituitary gland ($p<0.05$). The extracellular domain of tPRLR (tPRLR-ECD) was expressed as a GST fusion protein (tPRLR-ECD-GST)

in *E.coli*. The expression of tPRLR-ECD-GST in BL21 strain yielded a protein with a molecular mass of 76 kDa. About 99% of the fusion protein was present in inclusion bodies and about 50% of the total protein in inclusion bodies was the fusion protein. The insoluble fusion protein was denatured, refolded and purified using GST affinity chromatography. The yield of the purified fusion protein was 20 mg per liter with an estimated purity of 90%. The tPRLR-ECD was released from the fusion protein by thrombin cleavage and the yield was 0.2 mg per liter with about 95% purity. The estimated molecular mass of the purified ECD was 48 kDa. An antisera against the purified ECD was raised in rabbits and was able to recognize both the fusion protein, the tPRLR-ECD protein and the tPRLR protein derived from turkey kidney membranes. The effects of the active immunization against the recombinant derived tPRLR-ECD-GST fusion protein on body weight, egg laying, levels of plasma PRL and the incidence of incubation behaviour in turkey hens were investigated. When hens were confined in individual cages, the egg laying intensity and the level of serum PRL were significantly higher in the group immunized with the fusion protein using mineral oil as adjuvant than the control group. When the nesting environment was provided, these hens had a higher incidence of incubation behaviour than the control group.

RÉSUMÉ

Le récepteur de la prolactine (PRLR) est une protéine membranaire ancrée qui transmet les actions de la prolactine. L'ADN complémentaire du PRLR de la dinde (tPRLR) a été isolé et caractérisé. La séquence codante (ORF) du tPRLR correspond à une séquence de 831 acides aminés et est composée d'un peptide signal, d'un domaine extracellulaire (ECD), d'un unique domaine transmembrannaire et d'un domaine intracellulaire. La séquence d'acides aminés déduite possède 53,8% d'identity avec celle du lapin, 51,7% avec celle du bovin, 49,8% avec celle de l'humain, 49,8% avec la longue forme du rat, 80,3% avec celle du pigeon et 89,9% avec celle de la poule. Le domaine extracellulaire contient deux séquences répétées possèdant 63% d'homologie entre elles. Les répétitions au niveau de la membrane distale et de la membrane proximale possèdent 53-60% et 62-70% d'homologie par rapport aux ECDs des PRLRs des mammifères. Un produit de la transcription du tPRLR de 3 kilo nucléotides a été identifié et était détectable dans 26 tissus examinés. Parmis ces tissus, l'hypophyse, le jabot, le duodénum et le gésier exprimaient les taux les plus élevés d'ARN messager (ARNm) du tPRLR. Dans la plupart des tissus examinés, aucune relation apparente entre les taux de PRL sanguins, l'état physiologique et les concentrations estimées d'ARNm du recepteur n'ont été détectées. En revanche, au niveau de l'hypothalamus, l'augmentation des taux sanguins en PRL était associée à une diminution des taux de transcription du récepteur ($p<0,05$), alors qu'une relation inverse a été observée au niveau de l'hypophyse ($p<0,05$). Le domaine extracellulaire du tPRLR (tPRLR-

ECD) a été exprimé chez *E.Coli* sous forme d'une protéine de fusion avec la GST (tPRLR-ECD-GST). Le résultat de l'expression de la tPRLR-ECD-GST chez BL 21 correspond à une protéine d'un poids moléculaire de 76 kDa. A peu près 99% de la protéine de fusion était présent dans les corps d'inclusion et environ 50% des protéines totales de ces corps étaient représentées par de la protéine de fusion. La protéine de fusion insoluble a été solubilisée, récupérée et purifiée grace à l'utilisation d'une chromatographie d'affinité à la GST. Le rendement d'obtention de la protéine de fusion était de 20 mg par litre de culture avec un tau de pureté de 90%. Le tPRLR-ECD a été séparé de la protéine de fusion par digestion avec de la thrombine, le rendement était alors de 0,2 mg par litre de culture avec une pureté d'environ 95%. Le poids moléculaire de l'ECD purifié a été estimé à 48 kDa. Un anticorps de lapin anti-ECD a été développé. Le sérum ainsi produit était capable de reconnaitre la protéine de fusion, le tPRLR-ECD ainsi que du tPRLR provenant de membranes de reins de dinde. Les effets chez la dinde d'une immunisation active contre la tPRLR-ECD-GST sur le poids vif, le taux de ponte, les concentrations plasmatiques en PRL et sur l'expression du comportement de couvaison ont été analysés. Quand les dindes étaient confinées en batteries, le taux de ponte et les concentrations plasmatiques en PRL étaient significativement supérieurs chez les animaux immunisés en comparaison des animaux témoins. Quand les dindes étaient placées dans un environnement favorable a l'expression de la couvaison, les animaux immunisés ont davantage exprimé ce comportement que les témoins.

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CONTRIBUTIONS TO KNOWLEDGE

1) The cDNA coding for tPRLR was cloned and characterized for the first time. It is also the first report showing that multiple transcripts of PRLR may exist in avian species and that at least one tPRLR transcript has a molecular size of about 3000 nucleotides. The nucleotide sequence of the cDNA has been submitted to Genbank (Accession number L76587). The amino acid sequence of the receptor has been deduced from the cDNA sequence.

2) The mRNA coding for tPRLR was detected in all 26 tissues examined in the study. The ubiquitous expression pattern of tPRLR was reported for the first time in avian species.

3) In most tissues tested, the levels of tPRLR mRNA were independent of the levels of plasma PRL and the reproductive status of the hen. In the hypothalamus, increasing levels of serum PRL was associated with decreasing levels of the receptor, whereas, the opposite was observed in the pituitary gland. These data, reported for the first time, suggest that PRL itself may participate in the neuro-endocrine control of incubation behaviour through actions at both the hypothalamus via a short loop feed back mechanism and the pituitary gland via autocrine and/or paracrine effects.

4) The extracellular domain of tPRLR was expressed in *E.coli* as a GST fusion protein, purified and characterized. It is the first reported recombinant PRLR produced in avian species.

5) A polyclonal antibody against the extracellular domain of tPRLR was raised in rabbits and characterized. The antibody could recognize the endogenous tPRLR from kidney membrane. This is the first anti avian PRLR ever produced.

6) The effects of active immunization against tPRLR on body weight, levels of plasma PRL, egg laying performance and incidence of incubation behaviour were investigated for the first time. The incidence of incubation behaviour could not be reduced, but egg laying performance might be increased by active immunization.

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MANUSCRIPTS AND AUTHORSHIP

The following text, from the "Guidelines Concerning Thesis Preparation" of the Faculty of Graduate Studies and Research, must be "cited in full in the introductory sections of any thesis to which it applies":

*"**2/Manuscripts and Authorship:** Candidates have the option, subject to the approval of their department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that thesis copies are bound as an integral part of the thesis. If this option is chosen, connecting texts, providing logical bridges between the different papers, are mandatory. The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary. Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. In appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis. In the case of manuscripts co-authored by the*

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CONTRIBUTIONS OF CO-AUTHORS TO MANUSCRIPTS FOR PUBLICATION

The main body of this thesis consists of 3 manuscripts. Manuscript I (chapter 3 and 4) has been published in the *Biology of Reproduction* (55:1081-1090; 1996). Manuscript II (chapter 5) and manuscript III (chapter 6) will be submitted to *Biology of Reproduction* and *Poultry Science* for publication, respectively. Dr. D. Zadworny, Dr. D. Guémené and Dr. U. Kuhnlein are co-authors on all manuscripts. Dr. D. Zadworny, my supervisor, was responsible for the overall design and supervision of the research project. Dr. U. Kühnlein and Dr. D. Guémené, co-investigators of the project, helped supervising and correcting the manuscripts. Dr. D. Guémené helped in obtaining the bird samples and supervised the active immunization experiment. Miss C. Morvan is a co-author on manuscript III. She helped taking care for the birds and collecting data during the study.

CHAPTER 1. INTRODUCTION

A problem of considerable economic importance to the poultry producer is the occurrence of incubation behaviour in the breeder hen. Incubation behaviour is characterized by increased nesting frequency, incubation of eggs, gonadal involution, anorexia and dramatic changes in intermediary metabolism. Despite intensive selection against the trait, its incidence in broiler chickens and particularly in domestic turkeys may result in up to a 30 percent decrease in egg production by the flock (Zadworny *et al.*, 1986). Many empirical methods of disrupting incubation behaviour have been tested (Guémené 1992), but none has provided a consistent means of altering this aspect of maternal behaviour in commercial breeding flocks.

Levels of prolactin (PRL) increase at the onset of incubation behaviour and are maintained at high levels through the incubation phase in both the pituitary gland (Burrow and Byerly, 1936; Saeki and Tanabe, 1955; Nakajo and Tanaka, 1956) and the peripheral blood circulation (Sharp *et al.*, 1979; Burke and Dennison, 1980; Proudman and Opel, 1980) in galliform birds. The levels of PRL mRNA in the pituitary glands of turkeys (Wong *et al.*, 1991; Karatzas *et al.*, 1997) and bantam hens (Shimada *et al.*, 1991; Talbot *et al.*, 1991) have similar trends. Recently, it has been shown that active immunization against recombinant-derived PRL has reduced the incidence, delayed the development (March *et al.*, 1994) or prevented the occurrence (Guémené *et al.*, 1995) of incubation behaviour in birds. Thus, PRL has been considered as a possible regulator of the onset and/or maintenance of incubation behaviour in birds. However, how levels of PRL may influence and/or

be influenced by this behaviour is not known. In addition, the functional role(s) of PRL in the physio-behavioural process associated with incubation activity has not been clearly defined.

Prolactin, in addition to the possible role in incubation behaviour, elicits a variety of biological actions on different target tissues. These actions are mediated by an interaction of PRL with specific high affinity receptors located on the cell membranes. Although receptors for PRL (PRLR) have been cloned and characterized in several mammalian and avian species (Boutin *et al.*, 1988; 1989; Edery *et al.*, 1989; Scott *et al.* 1992; Tanaka *et al.*, 1992; Chen and Horesman, 1994), the mechanism by which the hormonal signal is transferred inside the cell remains poorly characterized. The mammalian PRLR consists of a ligand binding domain, a transmembrane domain and an intracellular domain. The structure of the avian PRLRs is similar to that of mammals with the notable exception that the extracellular domain consists of two tandem repeats (Tanaka *et al.*, 1992; Chen and Horseman, 1994). Each repeat corresponds to a singular mammalian extracellular ligand binding domain and has all the conserved ligand binding characteristics of the mammalian PRLRs. How this unique structure influences PRL signal transduction in birds has not been comprehensively investigated. In addition, neither the receptor tissue distribution nor the changes of the receptor number in specific tissues at various physiological stages during the reproductive cycle of birds have been determined.

In this study, we cloned and characterized the turkey PRLR (tPRLR) at the

DNA level. Cloning and characterization of tPRLR enabled us to study the ontogeny and the phylogeny of the receptor and to contribute to an understanding of the PRL signalling mechanism. The availability of this clone also allowed us to examine the target tissues of PRL and to study the expression of the receptor at different physiological stages during the turkey reproductive cycle. Analysis of correlation among plasma PRL, receptor levels and physiological stages in the turkey is important for understanding the relationship between PRL and incubation behaviour in birds. In the study, we also expressed and purified the extracellular domain (ECD) of the tPRLR in *E.coli*. An attempt to develop an effective means of preventing the incubation behaviour was also carried out using active immunization against the ECD of tPRLR in turkeys.

CHAPTER 2. LITERATURE REVIEW

2.1. Prolactin

Prolactin is an adenohypophyseal hormone and is also referred to as lactin due to its apparent stimulating effect on lactation in mammals. Prolactin was first identified as a substance in the extract from the anterior pituitary gland that was able to induce lactation in pseudopregnant rabbits. In 1933, this substance was also found to be responsible for the production of crop "milk" in Columbiformes (Riddle *et al.*, 1933). Since then, more than 100 biological actions have been attributed to PRL (Nicoll, 1974). In general, PRL is involved in the differentiation and proliferation of cells.

2.1.1. Sources of Prolactin

The anterior pituitary gland is the predominant source of PRL (Simmons *et al.*, 1990). In the pituitary gland, PRL is synthesized in the membrane bound polyribosome in lactotroph cells (Tashjian, 1977) and secreted into the blood circulation by a process of exocytosis (Pelletier, *et al.*, 1972). The turn over rate of PRL within the pituitary is very rapid; PRL released within one hour after an injection of thyrotropin releasing hormones (TRH), a PRL stimulating factor, exceeds the total PRL content of the pituitary gland (Noel *et al.*, 1974).

Recent studies have shown that other cell types can also produce PRL or PRL-like proteins. It has been reported that lymphocytes synthesize PRL or PRL-like proteins, which can then act to regulate immune cell proliferation in an autocrine or paracrine fashion (Montgomery *et al.*, 1987; Hartmann *et al.*, 1989).

The rat brain (Emanuele *et al.*, 1992), hypothalamus (DeVito, 1989) and human myometrium (Gellersen *et al.*, 1991) have also been reported to produce PRL at low levels. Using the polymerase chain reaction (PCR), mRNA encoding PRL was also detected in normal breast tissues and breast carcinomas, indicating that gene expression of PRL occurs in both the normal and neoplastic mammary gland (Fields, *et al.*, 1993). Several PRL related proteins have also been identified in the placenta of rodents and ruminants (Soares *et al.*, 1991; Schuler and Kessler, 1992; Wallis 1992; Byatt *et al.*, 1992). These proteins have been purified, characterized and termed placental lactogens (PLs). In addition, another group of proteins, proliferins (PLF), which share high structural homology with pituitary PRL have been identified in the mouse placenta (Soares *et al.*, 1991). The high degree of amino acid homology among PRL, PL, PLF and growth hormone (GH) of various species has suggested that these hormones may have evolved from a common ancestral gene (Miller and Eberhardt, 1983; Soares *et al.*, 1991; Walker *et al.*, 1991; Wallis, 1992).

2.1.2. Chemical and Biological Properties of Prolactin

The chemical and biological properties of PRLs have been well studied since its discovery about 60 years ago. The first PRL to be highly purified and the primary amino acid sequence determined was ovine PRL (Li *et al.*, 1969). Subsequently, PRLs in many vertebrate species have been purified and the structures have been determined (eg. human PRL, Hwang *et al.*, 1974). Prolactin in mammalian species was found to be a single peptide protein of about 22 - 24

kDa with 190 - 200 amino acid residues and approximately a 50% α-helix content. It contains six cysteine residues within the sequence forming three disulfide bridges. The structure of PRL closely resembles that of GH, suggesting that they may have evolved originally from a common gene.

Avian PRLs were also purified and characterized from the turkey (Burke and Papkoff, 1980; Proudman and Corcoran, 1981, Etches and Cheng, 1982) and the chicken (Scanes *et al.*, 1975). The chemical and biological properties of the avian PRLs are similar to those of the mammalian PRLs (Scanes *et al.*, 1975). Although PRL is encoded by a single copy gene (Miller and Eberhardt, 1983), several structural variants of PRL, with different bioactivities and immunoreactivities, have been recently identified (Sinha, 1992). The variants of PRL appear to be the result of post-translational modifications and post-release processes. Some of these variants arise as the result of regulated proteolytic cleavage and are thought to have biological activities distinct from native PRL (Anthony *et al.*, 1993; Clapp *et al.*, 1993). So far, cleaved isoforms of PRL of 8 kDa (Sinha, 1992), 16 kDa (Sinha, 1992), 21 kDa (Oetting *et al.*, 1989; Sinha 1992;), 23.5 kDa (Oetting *et al.*, 1989) and 24 kDa (Sinha and Gilligan, 1985) have been reported. Other variants arise as the result of aggregation and/or formation of disulfide-linked polymers, or due to post-translational modification of PRL by glycosylation, or phosphorylation (Larrea *et al.*, 1993). The physiological significance of these variants is not clear although they likely contribute to the pleiotropic effects of PRL.

2.1.3. Molecular Cloning of Prolactin cDNAs

Since the early 1980's, PRL has also been studied at the DNA level. The analysis of deduced amino acid sequences of vertebrate PRLs revealed 5 highly conserved domains among all vertebrate PRLs (domain A: residues 11-51; B: residues 58-71; C: residues 80-98; D: residues 108-133 and E: residues 160-192) (Kawauchi and Yasuda, 1988). The conservation of these domains in PRLs of various species suggests that they are essential for their biological activity. Comparison of PRL sequences with GH and PL suggested that these three hormones may have commonality in their evolution, since they share high DNA sequence similarity (Soares *et al.*, 1991).

Several avian PRLs including chicken (cPRL) (Hanks *et al.*, 1989a; Watahiki *et al.*, 1989) and turkey PRL (tPRL) (Karatzas, *et al.*, 1990; Wong *et al.*, 1991) cDNAs were also cloned and characterized. The open reading frames (ORF) of chicken and turkey PRL cDNAs encode for a pre-prolactin composed of 229 amino acids residues. The putative mature PRL consists of 199 amino acids with a calculated molecular weight of about 23 kDa. Analysis of sequence homology between avian PRL and other species demonstrates the phylogenetic relationship between birds and other vertebrate classes. The avian PRLs share about 90%, 70%, and 30% cDNA sequence identity to reptiles, amphibians, and fish PRLs, respectively (Cook and Baxter, 1982; Truong *et al.*, 1984; Karatzas *et al.*, 1990; Noso *et al.*, 1993). Both chicken and turkey PRL cDNAs have been used to produce recombinant proteins, which have been shown to be biologically active (Hanks *et al.*, 1989b; Karatzas *et al.*, 1993).

2.1.4. Regulation of Prolactin Secretion in Birds

The secretion of PRL in birds is regulated by a variety of stimulatory and inhibitory influences of hypothalamic and/or higher origin and by the feedback effects of gonadal steroids (Sharp *et al.*, 1984). The hypothalamus secretes one or more PRL releasing factor(s) (PRFs) and PRL inhibiting factors (PIFs) (Hall and Chadwick, 1983) to influence the release of PRL in the pituitary gland.

2.1.4.1. Hypothalamic Neuropeptide-Mediated Prolactin Release

A number of peptides isolated from the hypothalamus have shown PRF-like activity, including TRH, vasoactive intestinal polypeptide (VIP) and substance P (Hall, 1984).

Thyrotropin releasing hormone in mammals is a potent stimulator of PRL secretion by a direct action on the pituitary, but its role on avian PRL release is controversial. It has been shown that TRH enhances PRL release *in vitro* in chickens, but not in turkeys (Hall *et al.*, 1986). *In vivo*, the administration of endogenous TRH does not stimulate the secretion of PRL (Harvey *et al.*, 1978), but does increase the amount of PRL mRNA in the pituitary gland, indicating TRH enhances transcription, but not translation (Frawely and Miller, 1989).

Vasoactive intestinal polypeptide has been clearly established to be the major avian PRL releasing factor. Administration of VIP *in vivo* induces dramatic increases in the serum levels of PRL in the ring dove, chicken and turkey (Lea and Vowles, 1986; Macmamee *et al.*, 1986; El Halawani *et al.*, 1990b). Passive immunization with anti-chicken VIP serum results in an immediate decrease of PRL

in the plasma of incubating bantam hens (Sharp *et al.*, 1989) and impaired development of the crop sac in incubating ring doves (Lea *et al.*, 1991). In turkeys, the number of VIP containing cell bodies in the hypothalamus and levels of immunoreactive VIP in the median eminence are increased and are correlated with circulating levels of PRL during egg laying and incubating (Mauro *et al.*, 1989).

2.1.4.2. Neurotransmitters and Prolactin Release

Neurotransmitters, particularly dopamine (DA) and serotonin may regulate the secretion of PRL in birds by stimulating / inhibiting the release of PRF and PIF into the hypophyseal portal circulation or by acting directly on the pituitary gland itself.

Dopamine in birds exerts an inhibitory effect on the secretion of PRL. In part, DA acts at the hypothalamic level by reducing the secretion of PRF in laying hens (El Halawani *et al.*, 1991a). During incubation, this inhibition is repressed allowing for increased secretion of PRL. In addition, DA acts directly at the pituitary and inhibits the release of PRL from chicken and turkey pituitary cells in culture (El Halawani *et al.*, 1984; 1988b; 1990a). Furthermore, dopaminergic agonists administered centrally appear to stimulate release of prolactin in the turkey (El Halawani *et al.*, 1984), providing further evidence for the inhibitory effect of DA on PRL secretion.

Serotonin, on the other hand, is a potent stimulator of PRL release in the chicken (Rabii *et al.*, 1981; Sharp *et al.*, 1984), in the pigeon (Hall 1982), and the turkey (El Halawani *et al.*, 1984). Since serotonin did not stimulate PRL release in

pituitary cells in culture (Proudman and Opel, 1988; El Halawani *et al.*, 1988b) and its stimulating effect could occur without changes in DA turnover in the hypothalamus (Hall *et al.*, 1984a), it may stimulate the secretion of PRL by enhancing the release of a PRF.

2.1.4.3. Feedback Effects of Steroids on Prolactin Secretion

Gonadal steroids may exert a positive feedback effect on PRL release from the anterior pituitary gland (Sharp *et al.*, 1984; El Halawani *et al.*, 1988a). The circulating levels of PRL were decreased in ovariectomized turkeys and increased following an estrogen replacement treatment (El Halawani *et al.*, 1988a; Hall *et al.*, 1986). Estrogen pretreatment increases PRL release in the chicken and increases the responsiveness of the pituitary to other release factor of PRL (Hall *et al.*, 1984b). Conversely, testosterone and progesterone could decrease the sensitivity of the pituitary prolactin cells to the stimulatory action of the hypothalamus or TRH *in vitro* (Hall *et al.*, 1984c; Hall *et al.* 1984d).

2.2. Broodiness

Broodiness, a condition during which the female bird stops laying eggs and exhibits "maternal behaviour" involves both incubation of the eggs and involution of the reproductive tract.

2.2.1. Association of Prolactin with Broodiness

Prolactin, since its discovery in 1933, has been ascribed as a possible cause of broodiness in birds (Riddle *et al.*, 1933). This hypothesis is mainly based on the antigonadal effects of PRL, the demonstration of successful induction of

broodiness in some avian species and the correlation of broodiness with elevated levels of blood PRL as well as enhanced synthesis of PRL in the pituitary gland.

The most obvious effect of PRL in birds is its antigonadal effect. Administration of PRL reduced the ovarian weight of laying domestic fowl. In out-of-lay hens, PRL reduced comb size, oviduct weight and the gap between the pelvic bones. Rapid involution of the mature testes of the ring dove and pigeon in response to the injection of PRL was also observed (Bates *et al.*, 1937). Testicular regression induced by injections of PRL in cockerels was associated with a regression of basophilic cells in the pituitary gland thought to synthesize gonadotrophins. Prolactin was also observed to exert an anti-gonadal effect in the male pigeon (Bates *et al.*, 1937).

Prolactin may suppress the gonads by inhibiting steroid production (El Halawani *et al.*, 1988a; Porter *et al.*, 1991a). Studies have shown that PRL inhibits luteinizing hormone (LH) induced premature ovulation, suggesting that PRL may act directly on the ovary to suppress the ovulatory surge of steroid in the hen (Tanaka, *et al.*, 1971). In addition, high levels of endogenous PRL suppress gonadotropin-stimulated steroidogenesis *in vivo* (Camper and Burke, 1977). However, PRL at levels close to physiological concentrations, did not affect progesterone synthesis *in vitro* (Hammond, *et al.*, 1982). Ovine PRL suppresses LH-stimulated production of estrogen from the very small white follicles of the chicken *in vitro* (Zadworny *et al.*, 1989) and reduced production of estrogen by these follicles may mediate ovarian regression (Etches 1990).

The results of experiments attempting to induce incubation behaviour by injection of exogenous PRL remains controversial. Riddle *et al.* (1935) claimed that the administration of a purified PRL induced expression of broodiness in "broody strains" of actively laying chickens, but in non-laying fowl, cockerels and laying fowl of a non-broody strain, PRL only caused partial broodiness (i.e., clucking and caring for young). Saeki and Tanabe (1955) have shown that exogenous PRL could induce incubation behaviour in molting, non laying hens, which had previously shown incubation activity, but not in laying hens. The partial expression of broodiness observed in cockerels, which brooded chicks might be due to the absence of circulating androgens and the presence of a conducive environment. Indeed, the onset of the broody behaviour in all of the above studies necessitated the presence of environmental stimuli such as dim light and high temperature.

However, the ability of exogenous PRL to induce broodiness in early studies has come under suspicion in recent years. A few studies have been reported that the injection of a highly purified mammalian PRL failed to induce incubation behaviour in domestic hens (Opel and Proudman, 1980; Sharp, 1980). Although all the environmental cues known to promote nesting activity were provided and PRL was injected several times throughout the photophase, the only effect observed was ovarian regression. The reasons for the failure of PRL to induce incubation activity are not clear. Many factors could be involved including the possibility that the preparations of mammalian PRL used in early studies might have been contaminated with different amounts of other pituitary hormones, which may

have modulated any putative actions of PRL. However, administration of ovine PRL in a slow fashion induced incubation behaviour in laying and in ovariectomized turkey hens pretreated with estradiol and progesterone (El Halawani *et al.*, 1986).

A role for PRL in the regulation of broodiness was also supported by the demonstration of increased PRL synthesis in the anterior pituitary gland (Saeki and Tanabe, 1955; Nakajo and Tanaka, 1956) and by elevated levels of blood PRL (Sharp *et al.*, 1979; Burke and Dennison, 1980; Proudman and Opel, 1980; Lea *et al.*, 1981; Etches and Cheng, 1982) at the onset and during incubation behaviour. While the caudal lobe of the non-broody hen contained a smaller PRL content than the cephalic lobe, the caudal lobe of a pituitary from a broody hen contained as much PRL, or more, than that of the cephalic lobe (Nakajo and Tanaka, 1956). In the turkey pituitary it was suggested that PRL synthesis occur during broodiness in the acidophilic cells (Cherms *et al.*, 1962).

Serum PRL levels are influenced by the photoperiod; increasing when hens are exposed to a long photoperiod and decreasing when the photoperiod is decreased (Burke and Dennison, 1980). Prolactin levels significantly increase about 7 to 10 days after photostimulation. Levels of PRL tend to rise during the egg laying period and are higher than those of sexually quiescent hens (Scanes *et al.*, 1979), whereas, ovariectomized hens fail to show this associated rise in PRL levels. As broodiness approaches there is a significant rise in plasma PRL, which preceded the increase in nesting time and frequency (Proudman and Opel, 1980; Burke *et al.*, 1981). Once the hen has become broody, levels of PRL continue to

rise and remain at high levels throughout the incubation period (Cogger *et al.*, 1979; Burke and Dennison, 1980; El Halawani *et al.*, 1980; Zadworny *et al.*, 1985a). The disruption of broodiness by nest deprivation (El Halawani *et al.*, 1980; Burke *et al.*, 1981; Zadworny *et al.*, 1985b) or by pharmacoiogical means (El Halawani *et al.*, 1983; Guémené and Etches, 1989) induced a fall in levels of blood PRL and the resumption of incubation activity increased PRL to the original levels (El Halawani *et al.*, 1991b). Upon the hatching of poults, levels of PRL in the blood fall rapidly (Burke and Dennison, 1980; Burke and Papkoff, 1980).

2.2.2. Control of broodiness

The expression of broodiness remains a source of economic loss for the modern poultry industry due to its negative correlation with egg production (Guèmenè 1992). Effective methods are desirable to prevent or treat the maternal behaviour. Although the expression of the behaviour can be controlled to a certain extent by genetic selection, changing the environmental factors to discourage the behaviour, physical means and using pharmacological treatments, none of these methods are practically satisfactory.

Broodiness is an inherited trait and the heritability of broodiness has been estimated by Nestor (1972). Both sex-linked and autosomal factors are involved in the expression of broodiness (Nueller, 1952; Saeki, 1957). The incidence of broodiness has been reduced partly by the application of selective breeding. This is especially true in light weight laying strains of hens. However, genetic selection has been less successful in turkeys since reduction in the expression of the trait are

associated with decreased body weight. Depending on the breed, 10 to 70% of the turkey hens from a typical laying flock become broody even today (Cogger *et al.*, 1979; Harvey and Bedrak, 1984; Guèmenè 1990).

The inhibiting properties of changes in the environmental conditions on broodiness are well studied (Nixey, 1973; Nestor *et al.*, 1986). The incidence of broodiness can be minimized by changing the environmental factors to discourage birds from expression the behavior, such as uniform lighting, adequate ventilation and frequent collection of eggs. Early identification of the behavior and subsequent treatment using physical means are effective to disrupt broodiness. The interruption of broody episodes in laying hens is frequently achieved by confining the broody birds in broody pens. However, early identification of the behaviour requires experience and is time-consuming. Farm managers could spend more than 50% of their labour costs in attempts to prevent and disrupt broodiness expression. Moreover, physical disruption of broodiness may result in decreased egg production since the incubation period always overlaps with the egg laying phase.

Early successful pharmacological treatments of broodiness were based on the administration of gonadal steroids or gonadotrophins (Haller and Cherms, 1961; Sharp and Sterling, 1986). Though this method is effective to disrupt broodiness and resume egg-laying, it is not practically useful due to its high cost. Studies using synthetic gonadotrophin releasing hormone (GnRH) failed to stimulate ovarian growth in broody bantams (Sharp and Sterling, 1986). Estrogen may have

an inhibitory feedback action on gonadotrophin release, therefore, anti-estrogen agents may be potentially useful for the treatment of broodiness. However, results from different studies were controversial (Robinzon *et al.*, 1984; Renner *et al.*, 1987).

Immunological approaches to control broodiness by reducing the plasma levels of prolactin have been successful (Sharp 1997). Studies using anti-prolactin serum could rapidly reduce the plasma PRL levels and increase the levels of LH, but it is not effective to disrupt broodiness in chickens (Lea *et al.*, 1981). However, in turkeys passive immunization against PRL has proven to be effective (Crisóstomo *et al.*, 1997). Active immunization against tPRL has been reduced the incidence, delayed the development (March *et al.*, 1994, or prevented the occurrence (Guèmenè *et al.*, 1995) of incubation behaviour in galliforms.

Studies on the role of dopamine, serotonin and VIP in the regulation of PRL secretion suggest other pharmacological treatments of broodiness. Treatment of turkeys using a dopamine receptor blocking agent has decreased the incidence of broodiness and increased egg production (Millam *et al.*, 1980). Daily treatment of incubating turkeys with the serotonin synthesis inhibitor, p-chlorophenylalanine, could abolish nesting behaviour and stimulate the resumption of egg production(El Halawani *et al.*, 1983). Active immunization against VIP increased egg production in turkeys (El Halawani *et al.*, 1995).

2.3. Prolactin Receptors

Numerous biological actions have been described for PRL in the various

vertebrates in which PRL is produced (Nicoll and Bern 1972). These actions are initiated by an interaction of PRL with specific high affinity receptors located on the cell membrane.

2.3.1. Molecular Cloning and Structure of Prolactin Receptors

The primary structure of PRLRs of several species in mammals and birds has been identified, and characterized.

Prolactin receptor was initially purified from rabbit mammary gland (Katoh *et al.*, 1984) and monoclonal antibodies against it have been developed (Katoh *et al.*, 1985). It has been shown that the PRLR has a relative molecular mass of about 40 kDa and is not linked by disulfide bonds to itself or to other subunits. Using immunoaffinity chromatography and preparative electrophoresis, PRLR was purified to homogeneity in rat mammary gland (Boutin *et al.*, 1988) and the amino acid sequences of various tryptic digests were determined. Based on the amino acid sequence, an oligonucleotide probe was synthesized and used for screening a cDNA library. The full length cDNA for the rat PRLR was identified (Boutin *et al.*, 1988). Soon after, PRLR cDNAs were cloned and sequenced in several mammalian species including human (Boutin *et al.*, 1989), rabbit (Edery *et al.*, 1989), mouse (Davis and Linzer, 1989a) and bovine (Scott *et al.*, 1992).

The characterization of the PRLRs in these species has revealed that the mature PRLR has a large extracellular domain consisting of about 210 amino acid residues, a single transmembrane region of 24 amino acids and intriguingly, a variable length cytoplasmic domain dependant on the tissue from which the

receptor was cloned from. There are five extracellular cysteine residues, the first four of which are located near the N-terminal region, with a fifth near the transmembrane domain. Three potential N-linked glycosylation sites are present, the first two of which have been confirmed by amino acid sequence analysis to be glycosylated (Kelly *et al.*, 1992) in rats. Comparison of the PRLR with the GH receptor (GHR) identified several highly conserved domains in the extracellular and intracellular regions (Boutin *et al.*, 1988). This may suggest that the PRLR and GHR are evolutionary related and have a commonality in signal transduction mechanism.

Southern-blot analysis of restriction fragments of genomic DNA suggests that the PRLR is encoded by a single gene. The PRLR and GHR genes were co-localized to chromosome 5p13-p14 (Arden *et al.*, 1990). In the rat, the PRLR gene consists of 11 exons spanning more than 70 kilo base pairs (Kelly *et al.*, 1992). The first nine exons of the PRLR gene are homologous with the first nine exons of the GHR gene. The 10th and 11th exons encode the majority of the cytoplasmic domain of the PRLR and various forms of receptor protein arise by a mechanism of alternative splicing within this region of the pre-mRNA molecule (Kelly *et al.*, 1992).

2.3.2. Multiple Forms of the Prolactin Receptor in Rats

Three forms of PRL receptor with various length of cytoplasmic domain have been found (Boutin *et al.*, 1988; Davis and Linzer, 1989; Shirota *et al.*, 1990; Ali *et al.*, 1991). The short form was the first PRLR to be identified (Boutin *et al.*, 1988).

It contains 291 amino acid residues with a short cytoplasmic domain (96 amino acids). The mature long form of the rat PRL receptor is a peptide with 591 amino acid residues with a cytoplasmic domain of 396 amino acids. The intermediate form with 398 amino acids was only found in Nb2 lymphoma cells. This latter form appears to be due to a deletion in the PRL receptor gene within the cytoplasmic region (Ali *et al.*, 1991).

The short and long forms of PRLR have been shown to be differentially expressed in the rat mammary gland and liver during pregnancy and lactation. The mammary gland contained about 70% short form and 30% long form, while in the liver the short form was more dominant (90%) (Kelly *et al.*, 1991, 1992). By transfection of the long and short forms of PRLR into eukaryotic cells, it was found that only the long form was able to transduce a hormonal signal to milk protein genes (ß-lactoglobulin and ß-casein) (Kelly *et al.*, 1991; 1992). No study has been reported on the genes, which may be regulated through the short form of the rat PRLR.

The biological significance of multiple forms of the PRL receptor is not known, though it is possible that the various forms of the PRLR may contribute to the diverse biological functions of PRL.

2.3.3. Avian Prolactin Receptors

Prolactin receptors in birds including chicken (Tanaka *et al.*, 1992), pigeon (Chen and Horseman, 1994) and turkey (Zhou *et al.*, 1996) have been recently cloned and characterized. The putative mature avian PRLR consists of 830 - 831

amino acid residues. The structure of the receptor is similar to that of the mammalian receptors except that the extracellular domain is larger.

The extracellular domain of the avian PRLR has a unique double antenna structure (Tanaka *et al.*, 1992). This double antenna structure consists of two tandem repeated units with about 67% amino acid sequence identity, which may have arisen from a duplication event during evolution. Each of these units corresponds to the extracellular domain of mammalian PRLRs (Boutin *et al.*, 1988; Ali *et al.*, 1991; Scott *et al.*, 1992). All of the characteristic cysteine pairs and a WSXWS motif, which are conserved in the cytokine receptors, are present in each unit. When compared with the extracellular domains of mammalian PRLRs, the membrane distal unit of the avian extracellular domain is less similar than the membrane proximal unit, indicating that duplication of this unit may have occurred before the evolutionary divergence of birds and mammals. The biological significance of this unique antenna structure is not yet known, though it has been proposed that each of the repeat units could form an independent ligand binding site (Chen and Horseman, 1994). Mutational analysis of the repeat structure has shown that hormone molecules do not bind to one receptor cooperatively, since the binding affinities of the rat PRL to the wild-type pigeon PRLR is equivalent to a mutated receptor, which only contained the membrane proximal repeat unit (Chen and Horseman, 1994).

2.3.4. Tissue Distribution of Prolactin Receptors

The specific receptor for PRL has been found in numerous organs in

mammals (Posner *et al.*, 1974; Djiane *et al.*, 1977). In mammals, the mammary gland is the classic target of PRL action and contains PRLR. Prolactin binding sites are also found in the ovary, uterus, placenta, testis, prostate, pancreas, intestine, liver, adrenal, kidney, and specific brain centres, eye and cells of the immune system (Kelly *et al.*, 1991; 1992; Soares *et al.*, 1991).

The expression of the PRLR gene in different tissues has not been systematically surveyed, though fragmented information has shown that in mammalian species the PRLR gene is expressed in cerebral cortex, choroid plexus, hypothalamus, pituitary, heart, lung, thymus, spleen, liver, pancreas, kidney, adrenal, ovary, uterus, skeletal muscle, skin and mammary gland (Nagano and Kelly, 1994; Brown-Borg *et al.*, 1996). Other tissues including human bone marrow stroma cells (McAveney *et al.*, 1996), osteosarcoma cells (Bataille-Simoneau *et al.*, 1996), decidua (Tanaka *et al.*, 1996) and prostate (Navalainen *et al.*, 1997) express the receptor.

In avian species, binding sites for PRL have been identified in the pigeon crop sac, forebrain, hypothalamus and choroid plexus of the ring-dove (Buntin and Walsh, 1988; Buntin *et al.*, 1993) and the kidney of the chicken and turkey (Krishnan *et al.*, 1991). The distribution of the PRLR mRNA has been detected in the kidney, liver, oviduct, intestine, crop sac and heart (Tanaka *et al.*, 1992; Chen and Horseman, 1994).

2.3.5. Regulation of the Expression of Prolactin Receptor

Prolactin receptor levels are differentially regulated depending on the tissue

studied. In rat liver, one of the tissues with the highest PRL binding, levels of PRLR vary during the oestrous cycle, increase during pregnancy (Kelly *et al.*, 1975) and are markedly stimulated by estrogens (Posner *et al.*, 1974). Prolactin plays a major role in the regulation of its own receptor, inducing both up and down regulation depending on the concentration and duration of exposure to PRL (Posner *et al.*, 1975; Manni *et al.*, 1978; Djiane *et al.*, 1979).

Not only the number of receptor but also the form of receptor is regulated. Using the polymerase chain reaction, the long and short forms of mRNA coding for the PRLR in various rat tissues were measured. The thymus and kidney expressed both forms equally and the liver expressed the short form predominantly. In the liver, the level of mRNA of the short form was about 2 fold higher in proestrus than in diestrus compared to the long form. Conversely, in the ovary, uterus, and cerebral cortex, the expression of the long form transcript was higher in proestrus than in diestrus. In contrast, the hypothalamus and the pituitary expressed more long form transcript in diestrus than in proestrus (Nagano and Kelly, 1994). These results indicate that the form of the PRLR is expressed in a tissue-specific and regulated manner.

In avian species, no study has been done to examine the expression of PRLR in various tissues at different physiological stages during reproductive cycles.

2.3.6. The Hematopoietin Receptor Superfamily

Based on sequence homologies, the PRLR belongs to the recently recognized hematopoietin receptor superfamily (Cosman *et al.*, 1990; Kelly *et al.*,

1991). The hematopoietin receptor superfamily also includes interleukin (IL)-2, IL-3, IL-4, IL-6, IL-7, granulocyte-macrophage colony stimulating factor (GM-CSF), GH and erythropoietin receptors. The receptors for GH (Leung *et al.*, 1987) and PRL (Boutin *et al.*, 1988) were the first members of this family to be identified. The cloning of these receptors established a prototype for the subsequent identification of new members of transmembrane hormone receptors. Shortly thereafter, the IL-6 receptor (Yamasaki *et al.*, 1988), murine erythropoietin receptor (D'Andrea *et al.*, 1988), IL-2 receptor ß-subunit (Hatakeyama *et al.*, 1989), murine (Mosley *et al.*, 1989) and human (Idzerda *et al.*, 1990) IL-4 receptors, human GM-CSF receptor (Gearing *et al.*, 1989) and murine IL-3 (Bazan, 1989), human (Itoh *et al.*, 1990) and murine IL-7 (Goodwin *et al.*, 1990) receptors were cloned. With identification of new cytokine receptors, the hematopoietin receptor superfamily are expanding further.

All the receptors in the superfamily are membrane glycoproteins, oriented with their N-termini outside the plasma membrane. The N-terminal signal sequence directs the translocation of the nascent polypeptide across the membrane of the endoplasmic reticulum, and this signal peptide is subsequently removed. The receptor molecules then anchor in the cell membrane at a single hydrophobic transmembrane domain.

The deduced amino acid sequences of these cloned receptors show significant homology, primarily in their extracellular, ligand-binding domains. The characteristic, highly conserved features of this region are four cysteine residues

located in the N-terminal half of the region, which for the GHR have been shown to be linked sequentially (C1-C2 and C3-C4) (Fuh *et al.*, 1990) and a WSXWS motif (tryptophan, serine, any amino acid, tryptophan and serine) located just outside the membrane-spanning domain. The codon usage of serine in the WSXWS motif was found to be limited to AGC and AGT out of the six possible serine codons, implying a common evolutionary origin for these receptors. These features are found in all of the receptors with exception of the IL-7 receptor which has only two of the conserved cysteines and the GHR which lacks the WSXWS motif. This conserved extracellular region appears to have been duplicated in the IL-3 (Bazan, 1989) and avian PRLR (Tanaka *et al*, 1992; Chen and Horseman, 1994; this study). However, the N-terminal half of the IL-3 receptor lacks the WSXWS motif. Whether the structure defined by the WSXWS motif has any functional role in hematopoietin receptors has not clearly been determined, although it has been suggested that WSXWS motif may interact with other factors to assist ligand binding. It has also been suggested that the extracellular domains of these receptors form seven antiparallel ß-strands in each of the two 100 amino acid subdomains (Bazan, 1990).

The length of the cytoplasmic domains ranges from 54 amino acids (GM-CSFR) to 568 amino acids (human IL-4R). There is not much sequence similarity between the cytoplasmic domains of these receptors, with the exeption of the IL-2 and erythropoietin receptors. There are, however, some overall similarities in the amino acid composition of this domain. The IL-2 receptor and IL-4 receptor have a relatively large proportion of acidic residues, and the cytoplasmic domains of the

IL-2, IL-3, IL-4, IL-7 and erythropoietin receptors are rich in proline and serine residues. Whether these similarities in amino acid composition reflect common signal transduction mechanisms remains to be seen.

The transmembrane domain in these receptors is a single hydrophobic peptide consisting of about 24 amino acids. It divides the receptor into the intracellular and extracellular domains. No sequence similarity was found within the transmembrane domain among the receptor members.

2.3.7. Mechanism of Prolactin Actions

The numerous biological actions of PRL are initiated by an interaction of PRL with its cell membrane-anchored receptor. However, the mechanism by which the hormonal signal is transferred inside the cell remains poorly characterized.

Although the signal transduction mechanism of PRL is not completely elucidated yet, emerging evidence indicates that sequential dimerization of signalling subunits after ligand binding is the first step in signal activation for both GH and PRL. High resolution functional (Cunningham and Wells, 1989, Cunningham *et al.*, 1991) and structural (DeVos *et al.*, 1992) analyses have shown that there are two receptor-binding sites on human GH, called sites 1 and 2. These two sites bind to the same site on the human GHR to give a human GH-$(receptor)_2$ complex. This complex forms sequentially, a first receptor binds to site 1 on human GH followed by binding of a second receptor to site 2. A 1:2 stoichiometry between PRL and soluble PRL receptors has recently been reported (Hooper *et al.*, 1993; Gertler *et al.*, 1996; Sakae *et al.*, 1996). Analogs of human GH, which only possess

one receptor binding site have been shown to be potent antagonists to human GH- or human PRL-induced cell proliferation (Fuh *et al.*, 1992; 1993) indicating that receptor dimerization is a necessary prerequisite to signal transduction. Furthermore, monoclonal antibodies to the rat PRLR which can dimerize the receptor are known to be weak agonists of PRL (Elberg *et al.*, 1990) and pairwise cross-linking of PRLRs by bivalent, but not monovalent, anti-PRLR antibody mimicked the mitogenic end effect of PRL (Rui *et al.*, 1994a). Similar results have also been reported with monoclonal antibodies to GHR (Fuh *et al.*, 1993). These data provide further support for ligand-induced receptor homodimerization as a common activation mechanism of PRL and GH receptors.

After binding of the ligand to its receptor, little is known about the down stream events of the signal transduction pathway. Sequence analysis of all characterized PRLRs has shown the cytoplasmic domains of these receptors to have substantial diversity in size and structure, implying unique interaction capacities with primary and secondary effector proteins. However, two conserved regions have been identified, denoted homology box 1 and 2 (Murakami *et al.*, 1991; Colosi *et al.*, 1993). The membrane proximal box (box 1), also referred to as the proline-rich motif, has been proposed to participate in signal transduction (O'Neal and Yu-Lee, 1993). Indeed, mutational analysis of these two boxes has shown that box 1 is essential for PRL signal transduction, but not box 2 (Edery *et al.*, 1994). No intrinsic enzymatic activity of the receptor was found at the cytoplasmic domain (Boutin *et al.*, 1988; 1989), but there is an association with

cellular tyrosine kinases. Recently, PRLR was demonstrated to induce tyrosine phosphorylation of itself (Lebrun *et al.*, 1994) and activation of the receptor-associated tyrosine kinase, JAK2 (Rui *et al.*, 1994b) as well as JAK1 (Dusanter *et al.*, 1994). The protein tyrosine kinase $p59^{fyn}$ has also been reported to be associated with PRLR and activated by PRL stimulation in T-lymphocytes (Clevenger and Medaglia, 1994). These data suggest that tyrosine kinase JAK family, *fyn* and other tyrosine kinases, to be identified, may serve during PRL stimulation as a signalling intermediary necessary for PRL-induced tyrosine phosphorylation.

In addition, several reports have alternatively suggested that GTP binding proteins (Too *et al.*, 1989), adenylate cyclase (Rayhel *et al.*, 1990), protein kinase-C (Banarjee and Vonderhaar, 1992; Buckley *et al.*, 1986), or the sodium/hydrogen antiport (Rillema *et al.*, 1989; Too *et al.*, 1987), may participate in the signal transduction of PRL. It has been reported that PRL-stimulated mitogenesis in Nb2 cells could be modulated by cAMP (Larsen and Dufau, 1988), cholera and pertussis toxins (Too *et al.*, 1989; 1990), suggesting that G-protein may be implicated in the PRL response. In the rat liver, the cell response of PRL has been shown to be linked to diacylglycerol generation and protein kinase C activation (Buckley and Buckley, 1991) as well as a Ca^{2+} mobilization from intracellular or extracellular storage sites (Villalba *et al.*, 1991; Vacher *et al.*, 1994).

Structural polymorphism of the PRLR combined with the variations in the ligands suggests that different signal transduction mechanisms may be involved in

mediating the action of PRL in its various target tissues. However, further research is required to clarify the relationship of receptor and PRL variants to the signal transduction pathway.

Connecting Statement I

In the turkey hen, reproduction is associated with large changes in circulating levels of prolactin. However, neither the target tissues nor the receptor for this hormone have been well characterized. Accordingly, in this chapter, we describe the molecular cloning and characterization of turkey prolactin receptor.

CHAPTER 3

Molecular Cloning and Characterization of the Turkey Prolactin Receptor cDNA

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3.1. ABSTRACT

The turkey prolactin receptor (tPRLR) cDNA was isolated by screening a turkey kidney cDNA library and using the polymerase chain reaction (PCR). The open reading frame (ORF) of tPRLR predicted 831 amino acid residues composed of a signal peptide, an extracellular domain, a single transmembrane domain and an intracellular domain. The deduced amino acid sequence of tPRLR is about 53.8%, 51.7%, 49.8%, 49.8% 80.3% and 89.9% identical to that of the rabbit, bovine, human, long form of the rat, pigeon and chicken PRLRs, respectively. The extracellular domain of the turkey prolactin receptor contains two homologous repeat units with 63% amino acid sequence identity to each other. The membrane-distal and membrane-proximal repeats were 53-60% and 62-70% identical to the corresponding regions of the mammalian prolactin receptors, respectively. Each repeat unit contains two conserved cysteine pairs and a WSXWS motif found in mammalian PRLRs. The cytoplasmic domain of tPRLR was similar to the long form of mammalian PRLRs in both length and sequence characteristics. A tPRLR transcript with a molecular size of about 3000 nucleotides was identified by Northern blot analysis.

3.2. INTRODUCTION

Prolactin is an adenohypophyseal hormone with more than 100 physiological actions attributed to it (Nicoll, 1974). In mammals, the best known action of PRL is lactogenesis. It stimulates the expression of milk protein genes by increasing both gene transcription and mRNA half-life (Guyette *et al.*, 1979). In avian species, PRL has long been associated with the onset and/or maintenance of incubation behaviour (Sharp *et al.*, 1979; Burke and Dennison, 1980; Etches and Cheng, 1982; El Halawani *et al.*, 1980). The actions of PRL on different target tissues are initiated by an interaction of PRL with its cell membrane anchored receptor. These receptors have been cloned and characterized in a number of mammalian (Boutin *et al.*, 1988, 1989; Sharito *et al.*, 1991; Scot *et al.*, 1992; Edery *et al.*, 1993) and avian species (Tanaka *et al.*, 1992; Chen and Horseman, 1994).

The characterization of the PRLR in mammals has revealed that the PRLR has a large extracellular domain, a single transmembrane domain and intriguingly, a variable length cytoplasmic domain depending on the tissue from which the receptor was cloned from. The extracellular domain of the PRLR was highly conserved among mammalian species. Two pairs of cysteines near the amino terminus region and a WSXWS motif located near the transmembrane region are conserved and thought to participate in ligand binding. Three forms of PRLRs differing in the length of the intracellular domain have been detected (Boutin *et al.*, 1988; 1989; Sharito *et al.*, 1991; Ali *et al.*, 1991). The long and short forms appear to be the result of the alternative splicing of a single gene transcript, whereas, the

intermediate form found only in Nb2 cells was due to a deletion within the PRL receptor gene (Ali *et al.*, 1991). The biological significance of these different forms of tPRLRs is not clear, although studies have found that the expression of short and long forms were regulated in a tissue-specific fashion in rat and mouse in concert with the hormonal milieu associated with the reproductive status of the animal (Jahn *et al.*, 1991, Clarke and Linzer, 1993, Nagano and Kelly, 1994).

Specific receptors for PRL in birds have not been extensively studied at either the protein or molecular level although recently cDNAs coding for the chicken (Tanaka *et al.*, 1992) and pigeon (Chen and Horseman, 1994) PRLRs have been cloned. In these species, only a single form of receptor has been discovered and the overall structure of the avian PRLRs is similar to that of mammals. The avian PRLRs contain a single transmembrane domain, a cytoplasmic domain which has a similar size with the long form of mammalian PRLR, and an extracellular domain. Uniquely, the extracellular domain of the avian PRLR contains two homologous repeat units. Each repeat has all the conserved cysteine pairs and a WSXWS motif characteristic of the conserved ligand binding domains of mammalian PRLRs. The biological significance of this unique feature was not known, although it has been proposed that two ligand binding sites may exist in the extracellular domain (Tanaka *et al.*, 1992; Chen and Horseman, 1994).

In this study, we cloned and characterized the tPRLR cDNA. Cloning of tPRLR enabled us to study the ontogeny and phylogeny of the receptor. It also provided the starting material for production of the recombinant tPRLR protein and

structural/functional analysis of the receptor. In addition, cloning of the tPRLR would be importance of studying the mechanism of tPRL actions.

3.3. MATERIALS AND METHODS

3.3.1. Purification of Poly(A)$^+$RNA

To construct the cDNA libraries, tissue samples of liver and kidney were collected at the abattoir from an 18 week old large white female turkey and frozen in liquid nitrogen. Poly(A)$^+$RNA was extracted from the kidney or liver using a QuickPrep^{TM}mRNA purification kit (Pharmacia, Baie d'Urfé, Québec, Canada) according to the manufacturer's protocol.

Briefly, 0.5 gram tissue was removed from liquid nitrogen storage and immediately homogenized in an extraction buffer (containing guanidine thiocyanate, sodium citrate, and sodium lauryl sarcosine, pH 7.4) using a homogenizer (Tissue-Tearor^{TM}, Biospec, Bartlesville, Oklahoma, USA). After brief centrifugation, the supernatant containing poly(A)$^+$RNA was diluted with two extraction buffer volumes of TE buffer (containing 10 mM Tris.HCl and 1 mM EDTA, pH 7.4) and incubated with oligo(dT)-cellulose beads, equilibrated with TE buffer, for 10 minutes with gentle inverting. The oligo(dT)-cellulose beads were then washed with 5 bead volumes of a buffer containing 0.5 M NaCl, 10 mM Tris.HCl and 1 mM EDTA, pH 7.4 for 5 times, a buffer containing 100 mM NaCl, 10 mM Tris.HCl and 1 mM EDTA, pH 7.4 for 4 times and TE buffer once. Poly(A)$^+$RNA was eluted in TE buffer at 65 °C, and cooled on ice. Sodium chloride was added to the elutent to a final concentration of 0.5 M.

Poly(A)$^+$RNA in the elutent was purified again by oligo-(dT) cellulose beads using the same procedure as described above. Poly(A)$^+$RNA eluted in TE buffer was precipitated with 2 volumes of 100% ethanol for 30 minutes at -20 °C, washed with 70% ethanol, air-dried and redissolved in RNAse free H_2O. The recovery of poly(A)$^+$RNA was measured by a spectrophotometer (Milton Roy, Rochester, New York, USA) at a wave length of 260 nm. The concentration of Poly(A)$^+$RNA was adjusted to 0.5 µg/µl with RNAse free H_2O and stored in 20 µl aliquots at -70 °C until use.

3.3.2. Extraction of total RNA

Total RNA for Northern blot analysis was extracted from the turkey liver. About 100 mg slices were extracted using RNAzol^{TM}-B (TEL-TEST, INC. Friendswood, Texas, USA) according to the manufacturer's protocol. Tissue slices were first homogenized with 1 ml RNAzol^{TM}-B using a homogenizer (Tissue-Tearor^{TM}, Biospec, Bartlesville, Okalahoma, USA). Chloroform (0.2ml) was then added and the suspension was shaken vigorously for 30 seconds followed by incubation for 5 minutes at room temperature. After centrifugation at 12,000 x g for 10 minutes, the aqueous phase was recovered and the RNA was precipitated with an equal volume of 100% isopropanol. After 15 minutes incubation at -20 °C, total RNA was recovered by centrifugation at 10,000 x g for 10 minutes, washed with 70% ethanol, air-dried and dissolved in 200 µl of RNAse free H_2O. The recovery of total RNA was measured by a spectrophotometer (Milton Roy, Rochester, New York, USA) at a wave length of 260 nm. The RNA was stored in 20 µl aliquots at

-70 °C until use.

3.3.3. Design of primers

A sense primer (P1, 5'-AGGAAACATTCACCTG(C/T)TGGT-3', bp 767-787, Figure 3.1and 3.2) and an antisense primer (P2, 5'-AAGCCATCCAG(A/T)T(T/C) T(G/C)ACATC-3', bp 1117-1137, Figure 3.1 and 3.2) were designed on the basis of the conserved sequences of the human (bp 406-426 and 991-1011, respectively, Boutin *et al.*, 1990), rabbit (bp 598-618 and 948-968, respectively, Edery *et al.*, 1989), and chicken (bp 953-973 and 1303-1323, respectively,Tanaka *et al.*, 1992) PRLRs for the amplification of a cDNA fragment from tPRLR mRNA. Two antisense tPRLR-specific primers (P3, 5'-AATTGGGGCCTGCAGTT CTGT-3'; bases 878-898, Figure 3.1 and 3.2 and P4, 5'-TCCATCTGAACCAGGCTTCC-3'; bases 179-199, Figure 3.1 and 3.2), an adaptor primer (5'-CCAAGCTTGGATCCGAATTC-3') and adaptor-(dT)18 were synthesized to amplify the 5' region of tPRLR cDNA. All oligonucleotides were synthesized by the Sheldon Biotechnology Centre (McGill University, Montréal, Québec, Canada).

3.3.4. Probe preparation for library screening and Northern blot analysis

In order to screen the cDNA libraries and perform the Northern analysis, a 371-bp fragment of tPRLR cDNA was initially amplified by reverse transcriptase polymerase chain reaction (RT PCR) using P1 and P2 primers. Briefly, about 1 µg poly(A)$^+$RNA was reverse-transcribed into first strand cDNA in a reaction containing 50 mM Tris-HCl (pH8.3), 60 mM KCl, 3 mM $MgCl_2$, 2 µg of oligo-$dT_{(18)}$, 1 mM each of dNTPs, 10 mM dithiothreitol (DTT), 1 unit RNAguard (Pharmacia, Baie d'Urfé,

Québec, Canada), 0.1 µg/µl bovine serum albumin (BSA), and 20 units Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (Pharmacia, Baie d'Urfé, Québec, Canada). The reaction was incubated at 37 °C for 2 hours. One tenth of the first strand cDNA was used as template in the PCR. The PCR was performed in a volume of 100 µl containing 10 mM Tris-HCl (pH9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 50 pmol of each primer, and 2.5 units of Tth DNA polymerase for 35 cycles. Each cycle consisted of 45 seconds at 93 °C, 2 minutes at 55 °C and 90 seconds at 72 °C. The PCR product was separated on 1 % agarose gel. A band corresponding to about 371 bp was observed. About 1 µg of double strand DNA was purified from the band using DEAE-cellulose membrane (Schleicher & Schuell, Keene, New Haven, USA) procedure (Dretzen *et al.*, 1981).

The 371-bp RT PCR product was first subcloned into a pUC18 plasmid vector and then confirmed to be a partial tPRLR cDNA by sequence analysis. Briefly, about 0.2 µg purified RT PCR product was blunt-ended and phorsphorylated in a 10 µl reaction containing 10 mM Tris.HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 1 mM each of dNTPs, 1 mM ATP, 5 units of Klenow fragment of DNA polymerase I (Pharmacia, Baie d'Urfé, Québec, Canada) and 10 units of polynucleotide kinase (Pharmacia, Baie d'Urfé, Québec, Canada) for 30 minutes at 37 °C. In parallel, about 100 ng of pUC18 plasmid was digested and dephorsphorylated in a 10 µl reaction containing 10 mM Tris.HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 1 unit of SmaI restriction enzyme and 0.1 units of alkaline phosphatase at 37 °C for 1 hour. Both reactions were further incubated for 10 minutes at 75 °C and then cooled to

room temperature. Two reactions were pooled and about 8 units of T4 DNA ligase (Pharmacia, Baie d'Urfé, Québec, Canada) were added to the mixture. The reaction mixture was incubated overnight at 16 °C. One µl of the mixture was used to transform 100 µl competent DH5α bacteria. Plasmid was prepared according to Sambrook *et al.* (1989). Briefly, a single colony was inoculated into 2 ml of Luria-Bertani (LB) broth (containing 1% bacto-tryptone, 0.5% bacto-yeast extract and 1% NaCl) and grown overnight at 37 °C with shaking. Cells were pelleted at 12,000 x g for 20 seconds at room temperature and resuspended in 100 µl buffer containing 50 mM glucose, 25 mM Tris (pH 8) and 10 mM EDTA (pH 8). Bacteria were lysed by adding 200 µl buffer (containing 0.2 M NaOH and 1% SDS). Proteins and large molecular weight DNA were precipitated by adding 150 µl buffer (containing 3 M potassium acetate and 2 M acetic acid) and pelleted by centrifugation at 12,000 x g for 5 minutes. The supernatant was extracted with an equal volume of phenol:chroloform:isoamyl alcohol (25:24:1) once. Plasmid was precipitated with 2 volumes of 100% ethanol, washed with 70% ethanol, air-dried and dissolved in 50 µl of TE buffer. One µg of RNAse I was then added to the plasmid solution and incubated at room temperature for 30 minutes. The purified plasmid was sequenced and used as a probe in the library screening and Northern blot analysis.

The amplified 371-bp DNA fragment was radio-labelled with [α^{32}P]dCTP using a T7 Quickprime™ kit (Pharmacia, Baie d'Urfé, Québec, Canada) according to the manufacturer's protocol. Briefly, about 50 ng purified 371-bp DNA fragment

was dissolved in 29 µl H_2O, boiled for 10 minutes and chilled on ice for 2 minutes. Five µl of [^{32}P]dCTP (3000 ci/mmole, ICN, Irvine, California, USA) and 10 µl of reaction buffer (containing 200 mM Tris.HCl, pH 7.5, 50 mM $MgCl_2$, 5 mM DTT, 0.1 mg/ml BSA and 1.5 mM each of dNTPs) were then added to the DNA and mixed thoroughly. The reaction was initiated by adding 1 µl of T7 DNA polymerase (1 unit/µl) and incubated at 37 °C for 1 hour. Unincorporated [^{32}P]dCTP was removed by Sephadex G-25 chromatography (Pharmacia, Baie d'Urfé, Québec, Canada). The labelling specificity was estimated using a liquid scintillation counter (Pharmacia LKB Biotechnology AB, Uppsala, Sweden).

3.3.5. Northern blot analysis

Northern blot analysis was performed according to Sambrook *et al.* (1989). Briefly, 100 µg of total RNA was denatured at 65 °C for 10 minutes, chilled on ice for 2 minutes, fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde, 20 mM morpholinopropanesulfonic acid (MOP), 5 mM sodium acetate and 1 mM EDTA and transferred to a Zeta-probe membrane (BIO-RAD, Richmond, California, USA) in 50 mM NaOH. The membrane was briefly rinsed in RNAse free H_2O, baked at 80 °C for 2 hours and prehybridized in 10% dextran sulphate, 40% deionized formamide, 5x Denhardt's solution (containing 0.1% ficoll, 0.1% polyvinylpyrrolidone and 0.1% BSA), 4x SSPE (1x SSPE contains 0.18 M sodium chloride, 5 mM sodium phosphate, pH 7.4, and 0.5 mM EDTA), 1% SDS and 100 µg/ml of Herring sperm DNA at 42 °C for 2 hours. The 371-bp PCR fragment radio-labelled with [α^{32}P]dCTP at previous step ($1x10^6$ cpm) was then added to the

prehybridization solution and the membrane was further incubated at 42 °C for 24 hours. After hybridization, the membrane was sequentially washed with 1x, 0.5x and 0.1x SSC (1x SSC contains 0.15 M sodium chloride and 0.15 M sodium citrate, pH 7.0) and 0.1% SDS at 50 °C for 15 minutes. The membrane was air-dried, wrapped with saran wrap and exposed to X-ray film (Eastman Kodak, Rochester, New York, USA) for 24 hours at -70°C with 2 intensify screens (Du Pont-New England Nuclear, USA).

3.3.6. Construction and screening of cDNA libraries

Turkey liver and kidney cDNA libraries were constructed using the lambda Uni-Zap cloning system (Stratgene, La Jolla, California, USA) according to the manufacturer's protocol. Briefly, 5 µg of poly(A)$^+$ RNA was primed with a primer (5'-GAGAGAGAGAGAG AGAGAGAGAACTAGTCTCGA G$(T)_{18}$-3') containing a Xho restriction site (5'-C↓TCGAG-3') and reverse-transcribed into first strand cDNA with M-MuLV reverse transcriptase in the presence of methylated dCTP and absence of dCTP. One-tenth of the first strand cDNA mixture was reserved for quality control and later was used as template to amplify the 5' region of tPRLR. The RNA:DNA hybrid cDNA was then converted into double strand cDNA using RNAse H and DNA polymerase I. The ends of the double strand cDNA were blunt-ended with T4 DNA polymerase in the presence of dNTPs and ligated with linkers containing EcoRI restriction overhangs. After phosphorylation with polynucleotide kinase, double strand cDNAs were digested with XhoI restriction enzyme and fractionated with a CL-4B sepharose column (1 mm x 10 cm) equilibrated with STE

buffer (containing 150 mM NaCl, 10 mM Tris.HCl and 1 mM EDTA, pH 7.5). cDNAs with size greater than 600 bp were pooled and co-precipitated with Uni-Zap™ XR vector digested with EcoRI and XhoI restriction enzymes, ligated using T4 DNA ligase and packaged *in vitro* using the GigapackII Gold Packing extract (Stratagene, La Jolla, California, USA). The libraries were subsequently titrated and amplified to make large, stable stocks.

Approximately, $1X10^6$ plaques from the kidney cDNA library and $2X10^6$ plaques from the liver cDNA library were screened with the radio-labelled 371-bp PCR fragment, respectively. About $2.5X10^4$ phage particles were plated with 0.6 ml XL1-Blue bacteria (OD_{600} = 0.5) in a 137 mm petri dish and grown for 12 hours at 37 °C. Plaques were lifted onto a 137 mm Hybond-N nylon filters in duplicate (Amersham, Arlington Heights, IL, USA). The membranes were denatured with a buffer (containing 0.5 M NaOH and 1.5 M NaCl), neutralized in a buffer (containing 0.5 M Tris.HCl and 1.5 M NaCl, pH 8.0), rinsed in a buffer (containing 0.2 M Tris.HCl, 2x SSC, pH 7.5) and baked at 80 °C for 2 hours. Before hybridization, the membranes were soaked in 2x SSC at 50 °C for 10 minutes. Prehybridization was performed in 50% formamide, 4x SSPE, 1% SDS, 0.5% skimmed milk powder and 100 µg/ml denatured herring sperm DNA at 42 °C for 2 hours. Hybridization was carried out in 50% formamide, 4x SSPE, 1% SDS, 0.5% skimmed milk powder, 10% Dextran and $1x10^6$ cpm probe per filter at 42 °C for 24 hours. The membranes were then washed with 2x SSC, 0.1% SDS at 42 °C for 15 minutes, followed by 0.5x SSC, 0.1% SDS, and finally with 0.1x SSC, 0.1% SDS at 52 °C for 30 minutes. The

membranes were then air-dried, wrapped with saran wrap and exposed to X-ray film (Eastman Kodak, Rochester, New Haven, USA) with 2 intensifying screens (Du Pont-New England Nuclear, USA) at -70 °C for 24 hours. Clones with positive signal at the same location on both filters were selected, converted into phagemid and sequenced.

3.3.7. Amplification of the 5' region of the tPRLR cDNA

Since we were not able to obtain a full length tPRLR cDNA from the libraries, we used the rapid amplification of cDNA ends (RACE) method to clone the 5' region of tPRLR cDNA. First strand cDNAs synthesized from about 0.5 µg mRNA were tailed with poly(dA) in a reaction containing 10 mM Tris (pH 7.5), 50 mM potassium acetate, 10 mM $MgCl_2$, 0.3 mM $ZnSO_4$, 0.5 mM dATP and 30 units of terminal deoxynucleotidyl transferase (Pharmacia, Baie d'Urfe, Québec, Canada) at 37 °C for 30 minutes.

The poly(dA)-tailed cDNAs were then amplified by 30 cycles of PCR using adaptor-$(dT)_{18}$ and P2 primers. The PCR product was diluted 10^4 times with distilled H_2O. Next, the adaptor primer was paired with tPRLR specific primers P3 or P4 to amplify the 5' region of tPRLR using the diluted PCR product as templates. Both PCRs were performed in a volume of 100 µl containing 10 mM Tris-HCl (pH 9.0), 50 mM potassium chloride, 1.5 mM magnesium chloride, 200 µM dNTPs, 50 pmol of each primer, 5% formamide and 2.5 units of Tth DNA polymerase. Each cycle consisted of 45 seconds at 93 °C, 2 minutes at 58 °C and 90 seconds at 72 °C. The latter PCR product was separated on 1% agarose gel, gel-purified, cloned

into pUC18 plasmid at the SmaI site and sequenced.

3.3.8. DNA sequence analysis

Positive recombinant lambda phage (Uni-Zap™ XR) identified from the kidney cDNA library was converted into pBluescript phagemid (Stratagene, La Jolla, California, USA) according to the manufacturer's protocol. Briefly, 200 µl of positive recombinant lambda phage stock (containing $1X10^5$ phage particles) and 1 µl of R408 helper phage (containing $1X10^5$ pfu/ml) were co-infected with 200 µl of XL1-Blue *E.coli* cells (OD_{600} = 1.0) for 15 minutes at 37 °C. Five ml of 2x YT media (containing 1% NaCl, 1% yeast extract and 1.6% bact-tryptone, pH 7.0) was then added and incubated for 3 hours at 37 °C with shaking. Any alive bacteria were killed by heating at 70 °C for 20 minutes and the solution was cleared by centrifugation at 4000 g for 5 minutes. One hundred µl of supernatant was mixed with 200 µl of XL1-Blue cells (OD_{600} = 1), incubated for 15 minutes at 37 °C and plated on a LB plate containing 100 µg/ml ampicillin. The plate was incubated overnight at 37 °C and colonies appearing on the plate were inoculated for phagmid preparation.

For DNA sequencing, serial deletions of phagemid containing tPRLR insert or pUC18 plasmid containing PCR products (5' end of tPRLR) were made using unique restriction enzymes (Figure 3.1) to generate overlapping fragments. About 500 ng of phagemid or pUC18 plasmid which contained insert was digested to completion with one of the restriction enzymes using appropriate conditions according to the manufacturer's protocol. Then the restriction enzyme was denatured by heating to 85 °C for 30 minutes. If the restriction enzymes used to digest the clone were compatible with the multiple cloning sites (MCS) on the

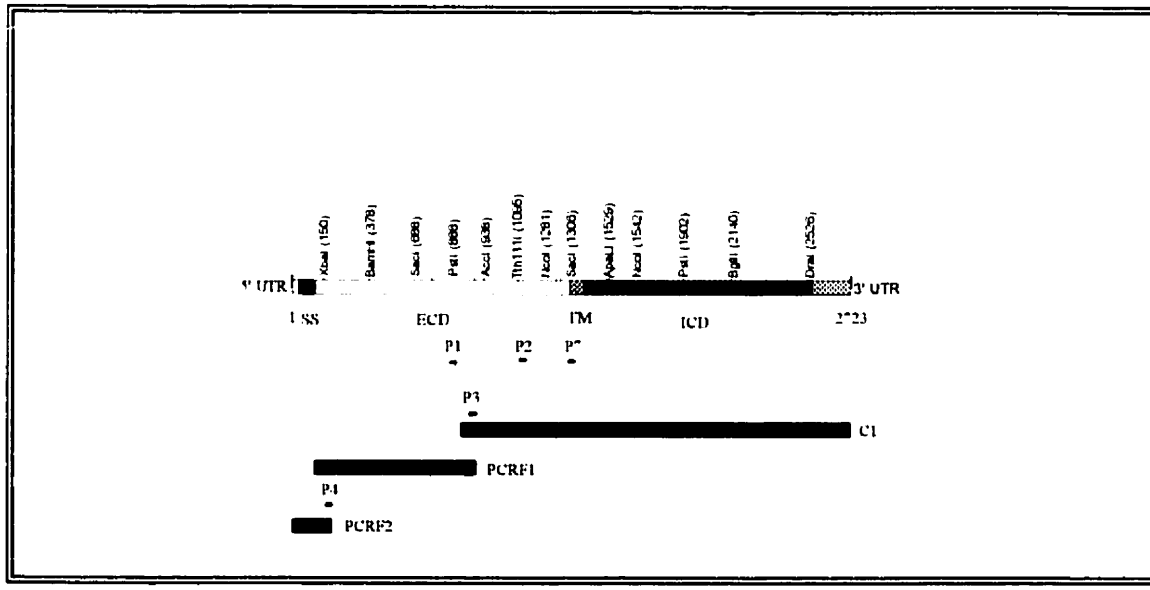

Figure 3.1. Schematic representation of the tPRLR cDNAs. The yellow and sky-blue areas represent the 5' and 3' UTR regions. The blue, white, green and red areas indicate the signal peptide (SS), extracellular domain (ECD), transmembrane domain (TM), and intracellular domain (ICD). The black solid bars are cDNA clone 1 (C1) and PCR products PCRF1 and PCRF2. Short arrows are the location of primers used in RT PCR.

vector, the deleted clone was re-circularized using T4 DNA ligase (Pharmacia, Baie d'Urfé, Québec, Canada) and transformed into $DH5_{\alpha}$ competent cells. Where the restriction enzyme was not compatible with any site at the MCS, the digested clone was subjected to a second restriction enzyme (site was located in MCS) digestion. Ends of the linerized vector were polished with S1 nuclease (Pharmacia, Baie d'Urfé, Québec, Canada), self-ligated and transformed into $DH5_{\alpha}$ competent cells.

The full length tPRLR was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using alkali denatured double stranded DNA as template. All sequence reactions were performed using a T7 sequencing kit (Pharmacia, Baie d'Urfé, Québec, Canada) according to the manufacturer's instruction. Briefly, about 2 µg of double strand plasmid or phagmid DNA was denatured with 0.5 M NaOH, precipitated with 3 volumes of 100% ethanol, washed with 70% ethanol, air-dried and dissolved in 10 µl of H_2O. About 1.6 pmole sequencing primer was annealed to the denatured DNA at 37 °C for 20 minutes. The primer was extended using T7 DNA polymerase in the presence of dCTP, dGTP, dTTP and [^{35}S]dATP at room temperature. The reaction was then separated into 4 reactions, each of which was terminated with one of the 4 dideoxynucleotides. Chain terminated products of 4 separate reactions were electrophoresized through 8% polyacrylamide gels (PAG) containing 7 M urea in 4 adjacent lanes. Gels were fixed in 10% methanol and 10% acetic acid, dried and exposed to X-ray film (Eastman Kodak, Rochester, New York, USA). Both strands of DNA were sequenced using either vector- or tPRLR-specific primers. DNA

sequencing data were processed and analyzed using the DNASIS and PROSIS programs (HIBIO, Brisbane, California, USA).

3.4. RESULTS AND DISCUSSION

3.4.1. Construction and screening of turkey kidney and liver cDNA libraries

In mammalian species, the liver and kidney were among the tissues with highest PRLR levels (Jahn *et al.*, 1991; Boutin *et al.*, 1988), but in avian species, no studies have been reported on the abundance of the PRLR in different tissues. In this study, we chose the turkey liver and kidney to extract the poly(A)$^+$RNA for the construction of cDNA libraries. The kidney cDNA library contained $5X10^5$ independent recombinants with an average insert size of 1.2 kb, whereas, the liver cDNA library had $1X10^6$ independent recombinants with smaller inserts (0.6 kb).

In comparison to rabbit (Edery *et al.*, 1989), rat (Boutin *et al.*, 1988), human (Boutin *et al.*, 1989), bovine (Scott *et al.*, 1992) and chicken (Tanaka *et a.*, 1992) PRLRs, 2 highly conserved regions at the extracellular domains of PRLRs were identified. Two degenerate oligonucleotide primers (P1 and P2) were designed based on the sequence information at the corresponding regions and used to amplify a fragment from tPRLR mRNA by RT PCR. Later, sequencing analysis showed that one of the 8 possible combinations of degenerate P1 primer matched exactly the tPRLR sequence at the corresponding region, whereas, a nucleotide at position 11 in P2 primer (dCTP) mismatched with a dTTP in tPRLR sequence (base 827, Figure 3.2). Nevertheless, using a low stringency annealing condition (55 °C annealing temperature versus 62 °C theoretical primer melting temperature), a 371-bp fragment was amplified from the turkey liver and kidney mRNA using RT PCR.

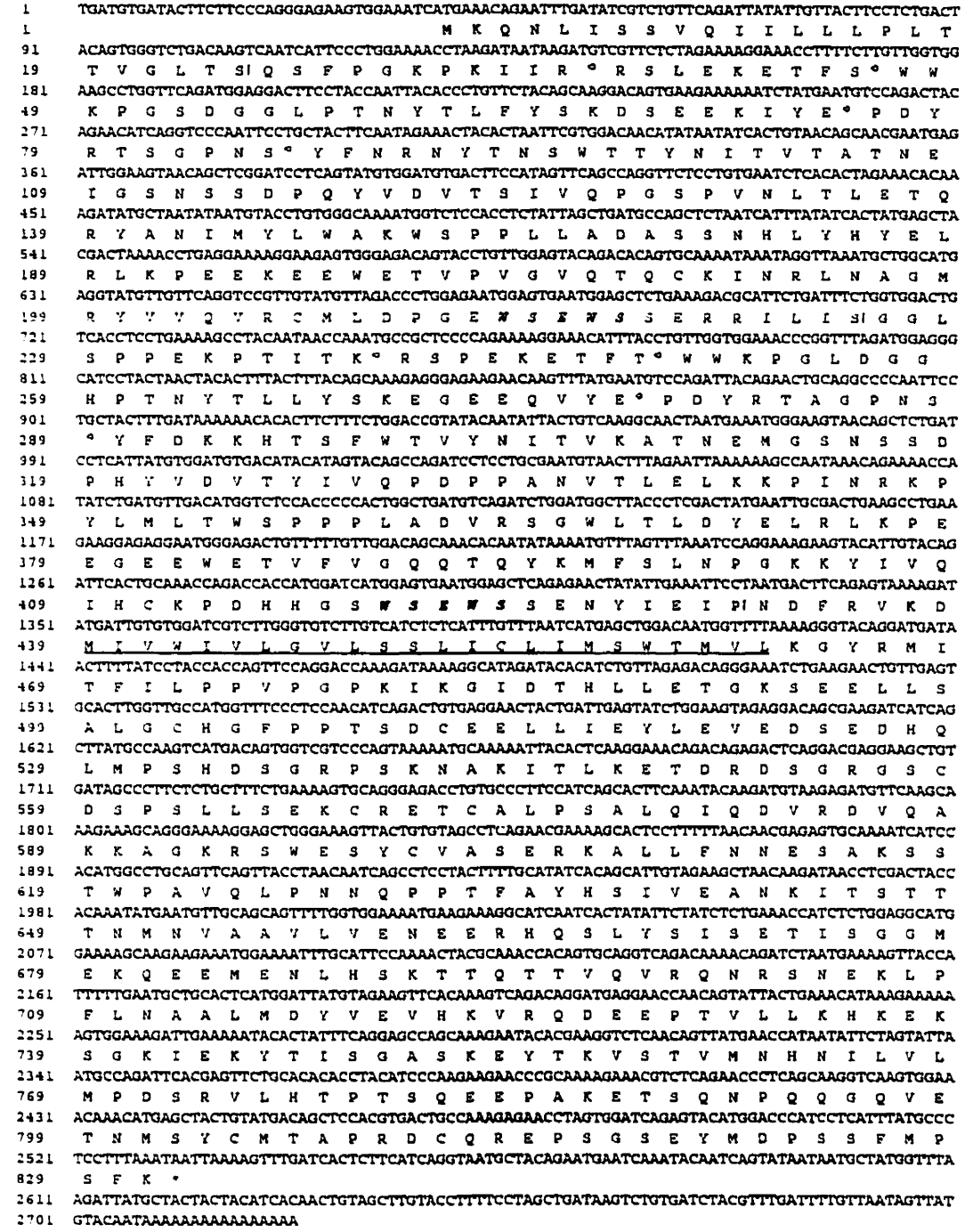

Figure 3.2. Nucleotide and deduced amino acid sequences of tPRLR(GenBank accession L76587). The transmembrane region is underlined. The two homologous repeat units in the extracellular domain are indicated by arrows. Two pairs of cysteine residues in the N-terminal region of each repeat unit are circled, and the WSXWS sequences are in italics. The asterisk indicates the stop codon.

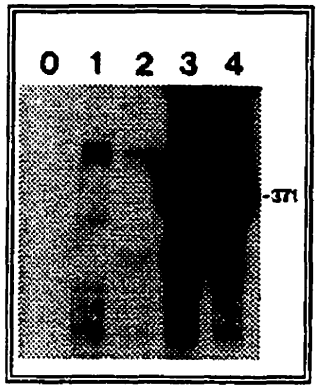

Figure 3.3. Southern blot analysis of the PCR product amplified using P1 and P2 primers. The full length of the rat PRLR cDNA was used as a probe. Lane 0 is the zero DNA negative control; lane 1 is the un-transcribed mRNA control, lane 2 is the PCR product using turkey genomic DNA as template, lane 3 and 4 are the RT PCR products amplified from the liver and kidney mRNA. The size of the molecular weight marker is indicated (bp).

Southern blot analysis of this 371-bp fragment using full length rat PRLR (kindly provided by Dr. Kelly, Royal Victoria Hospital, McGill) showed strong signals (Figure 3.3), indicating that this DNA fragment might be directly amplified from the tPRLR mRNA. For further confirmation of the DNA identity, this DNA fragment was subcloned into pUC18 at the SmaI site. Sequence analysis of this fragment showed that it had 71.4%, 72.8%, 70.3%, 72.7%, 89.9% and 96.5% DNA sequence homology with rabbit (Edery *et al.*, 1989), rat (Boutin *et al.*, 1988), human (Boutin *et al.*, 1989), bovine (Scott *et al.*, 1992) and chicken (Tanaka *et al.*, 1992) PRLRs, respectively, at the corresponding region. The high degree of sequence homology suggested that this fragment represented part of the tPRLR cDNA.

Using this DNA fragment as probe to screen the turkey kidney and liver cDNA libraries, only one positive clone (Clone TPRLR01, base 821 to 2723, Figure 3.1 and 3.2) with a 1903 bp insert was identified. However, Northern blot analysis indicated that the full length of tPRLR contained about 3 kilo nucleotides which suggested that clone TPRLR01 did not contain the entire coding sequence for tPRLR. Indeed, preliminary sequence analysis of this clone showed it contained

the 3' end of the tPRLR but lacked the 5' untranslated region (UTR), signal sequence and most of the extracellular domain sequence. Further screening of about $3x10^6$ plaques using this clone as probe did not reveal any additional positive clones. The estimated abundance of the PRLR in mammalian liver was about 0.0002% (Boutin *et al.*, 1988). If the abundance of tPRLR was similar to the mammalian PRLR in the liver, the libraries might not contain any additional clones with tPRLR insert.

3.4.2. Cloning the 5' end of the tPRLR cDNA

Since we did not obtain full length tPRLR cDNA from the libraries, we employed the 5' rapid amplification of cDNA end (RACE) method to clone the 5' region of the receptor. Initially, the P2 primer was coupled with the adaptor-$(dT)_{18}$ primer to amplify the 5'-region of tPRLR. No specific band was observed in 7.5% polyacrylamide gel electrophoresis. A tPRLR specific antisense primer P3 located at the 5' end of clone TPRLR01 (from 878 to 898, Figure 3.2) was designed and used to amplify the 5'-region of tPRLR coupled with the adaptor primer. The PCR product amplified using P2 and adaptor-$(dT)_{18}$ primers was used as template in the reaction, thus P3 primer served as a nested primer. A 796 bp fragment (PCRF1, base 103 to 898, Figure 3.1 and 3.2) was generated, subcloned and sequenced. Sequencing analysis showed this PCR product had a 77 bp overlaping region with the 5' end of clone TPRLR01. Combined sequence information from this PCR fragment together with clone TPRLR01 showed that these two pieces of DNA contained the cDNA sequence coding for the mature peptide but the leader peptide

and 5'-UTR region were not present. Therefore, another tPRLR specific primer (P4) located near the 5' end of PCRF1 was synthesized and paired with adaptor primer in order to generate DNA sequences of tPRLR located further up stream of the PCRF1. An additional PCR fragment (PCRF2, base 1 to 199, Figure 3.1 and 3.2) was amplified, cloned and sequenced. This fragment contained the 5'-UTR as well as leader peptide sequence and had 96 bp overlapping with the 5' end of PCRF1 (Figure 3.1 and 3.2). Thus, total sequence information of PCRF1, PCRF2 and clone TPRLR01 covered the whole ORF of the tPRLR cDNA and part of the 5'- and 3'-UTR regions.

3.4.3. Nucleotide sequence of the tPRLR cDNA

Figure 3.2 shows the nucleotide and deduced amino acid sequence of tPRLR. The cloned tPRLR cDNA was 2723 base pair long with 40.32% GC content. Overall, the tPRLR shares a total of 55%, 57%, 59%, 57.3%, 85.8%, 93.1% cDNA identity with rabbit (Edery *et al.*, 1989), human (Boutin *et al.*, 1989), long form of rat (Shirota *et al.*, 1991), bovine (Scott *et al.*, 1992), pigeon (Chen and Horseman 1994) and chicken (Tanaka *et al.*, 1992) PRLRs, respectively. A putative initiation codon for translation was identified as ATG from position 37 to 39, and this codon was preceded by nucleotides ATC. Thus the putative initiation recognition sequence was ATCATG, which was in agreement with the proposed initiation consensus sequence (A/GNCATG) (Cavener and Ray, 1991). The translation of tPRLR cDNA is terminated with the TAA codon common to other avian PRLRs (Tanaka *et al.*, 1992; Chen and Horseman, 1994), but which differs from mammalian

PRLRs (TAG; Boutin *et al.*, 1988, 1989; Edery *et al.*, 1992).

The 5'-UTR region of the cloned tPRLR was 36 bp, which was shorter than its characterized counterparts in the chicken (222 bp, Tanaka *et al.*, 1992) and pigeon (178 bp, Chen and Horseman 1994). This 36 bp sequence may not be the full length 5'-UTR of tPRLR and further analysis such as primer extension should be carried out to estimate the size of the full length. In the pigeon, the 5' UTR has been reported to be at least 300 nucleotides (Chen and Horseman, 1994). The cloned 5'-UTR region of tPRLR shared 47%, 51%, 55%, 46%, 78% and 89% DNA homology with that of the rabbit (Edery *et al.*, 1989), bovine (Scott *et al.*, 1992), human (Boutin *et al.*, 1989), rat (Boutin *et al.*, 1988), pigeon (Chen and Horseman 1994) and chicken (Tanaka *et al.*, 1992) PRLRs, respectively.

The ORF started with the ATG codon was 2493 bp long. Two homologous repeat units (from base 109 to 711; base 712 to 1329, Figure 3.2) were found near the 5'-end of the ORF with 72% cDNA identity to each other. This feature was also found in other avian PRLRs (Tanaka *et al.*, 1992; Chen and Horseman, 1994) and human interleukin 3Rß (IL-3Rß) (Itoh *et al.*, 1990). Table 3.1 lists the cDNA identity of each repeated unit to other PRLRs at corresponding regions. The membrane distal repeat unit is less homologous (64-67%) to the extracellular domains of mammalian PRLRs than the membrane proximal repeat unit (71-72%). The repeat units might be a result of a duplication event during evolution. The fact that the higher cDNA identity of the membrane proximal repeat unit than the membrane distal unit with mammalian PRLRs suggested that the duplication of these units may

have occurred before the evolutionary divergence of birds and mammals. The membrane distal unit was lost in mammals, but kept in birds.

The 3'-UTR region of tPRLR consisted of 191 bp. One polyadenylation signal sequence (AATAAA) was found from base pair 2705 to 2710. However, we were not sure whether this AATAAA was involved in the polyadenylation or not, since this sequence was followed by 13 $dATP_s$ and it was possible these 13 $dATP_s$ plus 3 $dATP_s$ in the AATAAA sequence were added by polyadenylation event. No putative polyadenylation signal sequences were found in other avian PRLRs. The overall DNA homology of tPRLR at 3'-UTR region with rabbit (Edery *et al.*, 1989), bovine (Scott *et al.*, 1992), human (Boutin *et al.*, 1989), rat (Boutin *et al.*, 1988), pigeon (Chen and Horseman 1994) and chicken (Tanaka *et al.*, 1992) PRLRs were 58%, 55%, 55%, 52%, 61% and 87%, respectively.

3.4.4. Deduced amino acid sequence of tPRLR cDNA

The 2493 bp ORF encoded a propeptide of 831 amino acid residues with a theoretical molecular weight of 94,389 kDn. According to the size of its cytoplasmic domain (369 amino acids), the turkey PRLR was equivalent to the long form of mammalian PRLRs. The overall amino acid identity of deduced tPRLR with rabbit (Edery *et al.*, 1989), bovine (Scott *et al.*, 1992), human (Boutin *et al.*, 1989), long form of rat (Shirota *et al.*, 1991), pigeon (Chen and Horseman 1994) and chicken (Tanaka *et al.*, 1992) PRLRs were 53.8%, 51.7%, 49.8%, 49.8% 80.3% and 89.9%, respectively.

The hydropathy profile (Figure 3.4) of the deduced tPRLR amino acid sequence revealed 2 strongly hydrophobic regions. The first hydrophobic region, following the initial methionine, is a 24 amino acid stretch of hydrophobic residues

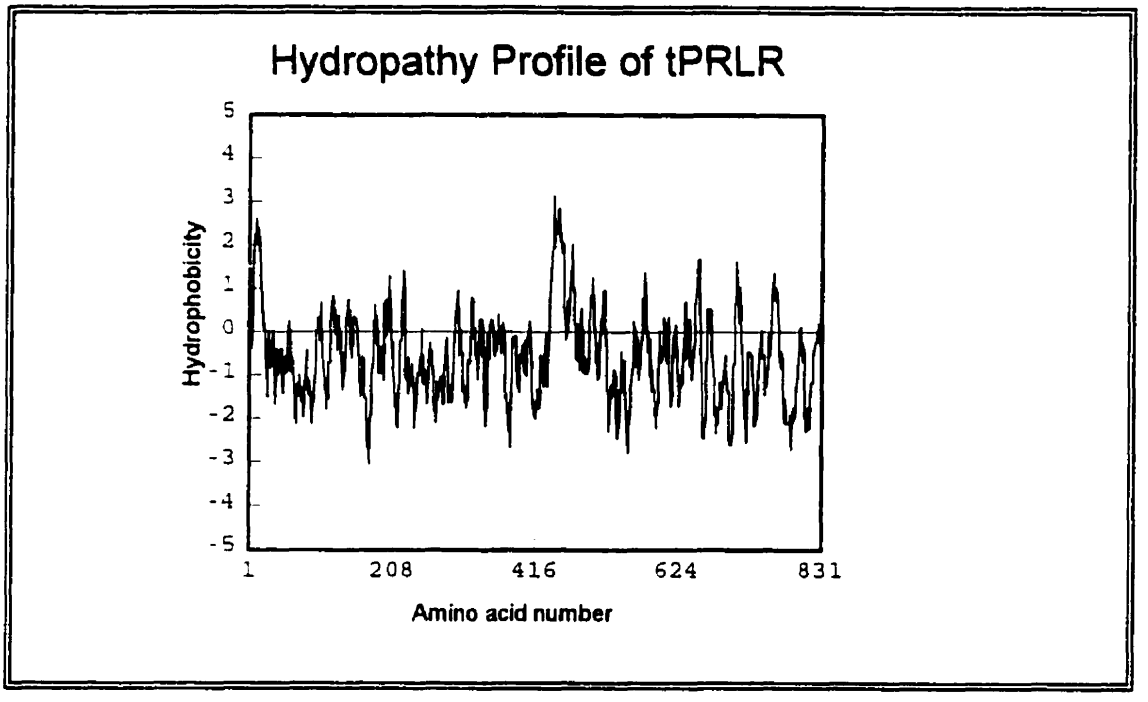

Figure 3.4. Schematic representation of the tPRLR hydropathy profile calculated according to Kyte and Doolittle (1982). The hydropathy profile was calculated with a window of 9 residues; positive values indicate increasing hydrophobicity.

(from amino acid 1 to 24) (Figure 3.4), indicative of a signal peptide sequence (Gascuel and Danchin, 1986). The other hydrophobic region also contained 24 amino acids and was located at the centre of the peptide (from amino acid 439 to

462, Figure 3.4). This region presumably corresponded to the transmembrane region of the tPRLR, separating the N-terminal, extracellular tPRL binding region from the C-terminal cytoplasmic region (Boutin *et al.*, 1988).

The putative mature extracellular domain consisted of 415 amino acid residues which could be divided into two homologous units. The membrane-distal domain (from amino acid 25 to 225, Figure 3.2) and -proximal domain (from amino acid 228 to 431, Figure 3.2) consisted of 201 and 204 amino acids, respectively. Each unit was similar to the singular extracellular domain of the mammalian PRLRs.

Table 3.1. Amino Acid Sequence and cDNA Sequence Homology of tPRLR Repeat Units with Others at Extracellular Regions

tPRLRs		tPRLR (M.P. Repeat)		tPRLR (M.D.Repeat)	
		A.A.	cDNA	A.A.	cDNA
Turkey	M.P. Repeat	-	-	63 %	71%
	M.D. Repeat	63 %	72%	-	-
Chicken	M.P. Repeat	95 %	96%	63%	72%
	M.D. Repeat	63 %	71%	93%	96%
Pigeon	M.P. Repeat	89 %	90%	66%	72%
	M.D. Repeat	63 %	71%	82%	90%
Bovine (Scott *et al.*, 1992)		67 %	72%	56%	63%
Human(Boutin *et al.*, 1989)		62 %	71%	53%	65%
Mouse (Davis & Linzer, 1989)		64 %	72%	57%	66%
Rabbit (Edery *et al.*, 1989)		70 %	71%	60%	66%
Rat (Boutin *et al.*, 1988)		64 %	72%	57%	66%

Figure 3.6 shows a comparison of the amino acid sequences of the tPRLR repeat units with other PRLRs at the extracellular domains. The characteristic extracellular cysteines (amino acids 36, 46, 75, 86, 239, 249, 278 and 289, Figure 3.2) and the WSXWS motif (amino acids 213-217 and 419-423, Figure 3.2) of the hemapoietin family of receptors are conserved in both repeat units. The feature of two repeat units was found in all characterized avian PRLRs (Tanaka *et al.*, 1992; Chen and Horseman, 1994). The biological significance of these repeat units in avian species remains to be identified although it has been proposed that avian PRL receptors may bind 2 ligands (Tanaka *et al.*, 1992). Data from a mutation experiment where the membrane-distal repeat was deleted indicated that binding of a heterologous ligand to the mutated and wild type receptor is equivalent (Chen and Horseman, 1994). More detailed stoichometric analysis of receptor binding kinetics to date have not been carried out. Thus the possibility of 2 ligand binding sites has not been excluded. In addition, PRL in turkeys is known to be present in 3 isoforms (Corcoran and Proudman, 1991). The ratio of these isoforms varies with physiological states (Bedecarrats *et al.*, 1995) with the glycosylated forms dominant during hyperprolactinemia states (i.e. incubation behaviour). Whether or not the variant isoforms bind with equal affinity to the receptor is currently not known. However, in view of the receptor dimerization mechanism associated with signal transduction for PRL (Fuh *et al.*, 1992) and the observation that the form of PRL in turkeys changes with reproductive state, it is open to speculation that the multiplicity of actions of PRL could be modulated by various interactions between

the receptor and the ratio of isoforms.

The intracellular domain which spanned from amino acid 462 to 831 (Figure 3.2), shared less overall structure identity with mammalian PRLRs than the extracellular domain. However, several regional segments that had higher levels of homology to other PRLRs were identified. A membrane proximal region between amino acids 462 and 533 (Figure 3.2) shared 77.8%, 72.2%, 76.4%, 79.2%, 97.2% and 95.8% amino acid sequence identity with rabbit (Edery *et al.*, 1989), bovine (Scott *et al.*, 1992), human (Boutin *et al.*, 1989), long form of the rat (Shirota *et al.*, 1990), pigeon (Chen and Horseman 1994) and chicken (Tanaka *et al.*, 1992), respectively. A proline-rich 8-amino acid motif (amino acids 473 - 480; PPVPGPKI, Figure 3.2) (O'Neal and Yu-Lee, 1993) was also identified within this region. This motif has been suggested to be important for the association with JAK2 in the signal transduction of PRL (Argetsinger *et al.*, 1993). An additional region spanning amino acids 553 and 571 (Figure 3.2) shared 94.7%, 94.7%, 94.7%, 78.9%, 100% and 100% amino acid sequence identity with that of rabbit (Edery *et al.*, 1989), bovine (Scott *et al.*, 1992), human (Boutin *et al.*, 1989), long form of the rat (Shirota *et al.*, 1990), pigeon (Chen and Horseman 1994) and chicken (Tanaka *et al.*, 1992). The conservation of amino acid sequences in these regions may indicate a conservation of functions, such as association with intracellular factors or membrane proteins necessary for signal transduction, among PRLRs of different species. Indeed, mutational analysis of these regions indicated that fragment between amino acids 462 and 533 of the rat PRLR was essential for the cell response upon stimulation

by PRL (Edery *et al.*, 1994).

3.4.5. Northern blot analysis of tPRLR mRNA

In order to characterize the transcripts of tPRLR, total RNA isolated from the liver of an 18 week old turkey hen was analyzed by Northern blotting using PCR product (amplified using P1 and P2 primers) as a probe. A band corresponding to about 3 kilo nucleotides(kn) and a broad smear extended from about 0.8 to 2.6 kn were detected (Figure 3.5).

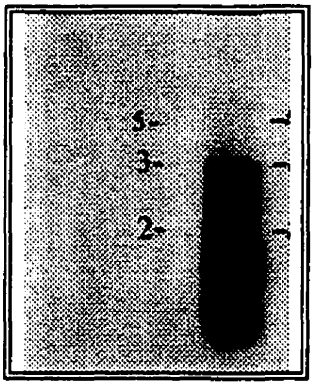

Figure 3.5. Northern blot analysis of tPRLR. About 100 µg of total RNA isolated from the turkey liver was separated on a denaturing agarose gel, transferred to nylon membrane, and hybridized with the radiolabeled 371-bp PCR product. the size of the molecular weight marker is indicated (kn).

In mammals, multiple forms of PRLR have been found, which represent alternative splicing products or gene deletions and which are regulated in tissue specific fashion (Nagano and Kelly *et al.*, 1994). In the present study, a PRLR mRNA with a size of 3 kn was detected by Northern blotting in turkeys; this is in accord with data reported in the chicken (Tanaka *et al.*,1992), and pigeon (Chen and Horseman 1994). In addition, the possibility of multiple forms of tPRLR mRNA could not be excluded, since a smear extending from about 0.8 to 2.6 kb was consistently detected. In pigeons (Chen and Horseman 1994), multiple bands were detected when the pigeon PRLR mRNA at the region coding for the extracellular

domain was amplifed. One of the three tPRLR fragments cloned recently (Pitts *et al.*, 1995) has only 84% sequence identity with the corresponding sequence in this study (Figure 3.2, bp1935 to 2075). However, further studies are required to determine whether more than one form of receptor is present in turkeys.

```
TPRLRMP   G.LSPPEKPTITKCRSPEKETFTCWWKPGLDGGHPTNYTLLYSKEGEEQVYECPDYRTAG   59
CPRLRMP   -.q--------i-------------------------------------------------   59
PGNMP     -.e--------i----------------s----------------r-------k---      59
TPRLRMD   .sq-f-g--k-ir---l-----s------s---l------f---ds--ki--------s-   59
CPRLRMD   ..q-f-g--k-ir---l-----s------s---l------f---ds--ei--------s-   58
PGNMD     -.q-y-g--k-ir---l-----s------s---l------f---ds--ki------gms-   59
HUMED     -.ql--g--e-f-----n--------r--t---l----s-t-hr---tlmh-----i-g-   59
BOVED     -.q------klv-----g--------e--a---l------t-h----tlih-----k-g-   59
RABED     -.q---g--f-f-------------r--a---l------t-h----tith-----k-g-   59
MUSED     -.q---g--e-h-----d--------n--s---l----s-t------knt------k-s-   59
RATED     -.q---g--e-h-----d--------n--t---l----s-t------ktt------k-s-   59

TPRLRMP   PNSCYFDKKHTSFWTVYNITVKATNEMGSNSSDPHYVDVTYIVQPDPPANVTLELKKPIN  119
CPRLRMP   ---------------i-----r-------------------------v----------   119
PGNMP     ---------------i---------i---v---l---------t---v--------tv-   119
TPRLRMD   ------nrny-ns--t-----t----i-------q-----s----gs-v-l---tqrya-   119
CPRLRMD   ------n-n---p--tf----t----i-------q-----s----gs-v-l---t-rsa-   118
PGNMD     --------n--np--t-----m-m--i-------q-----s-----a-v-ls--t-tsas   119
HUMED     ----h-g-qy--m-rt-imm-n---q---sf--el-------------lelav-v-q-ed   119
BOVED     ------s-----i-km-v---n-i-q--is----l--h-----e-e----l-----h-ed   119
RABED     ------s-----i--i-i---n---q---sv---r--------e----v-l---v-h-ed   119
MUSED     ----f-s-qy--i-ki-i---n-------st---l--------e-e--r-l---v-qlkd   119
RATED     ----f-s-qy--i-ki-i---n---q---s----l--------e-e--r-l---v-qlkd   119

TPRLRMP   RKPYLMLTWSPPPLADVRSGWLTLDYELRLKPEEGEEWETVFVGQQTQYKMFSLNPGKKY  179
CPRLRMP   -----v-----------------e--------------i------------------   179
PGNMP     -----v----------------------------a-----i------h-----------   179
TPRLRMD   im.--wak----l---as-nh-yh.---------k------p--v---c-inr--a-mr-   177
CPRLRMD   im.--wak----l---as-nh-yh.----i----k-----is--v---c-inr--a-mr-   176
PGNMD     tt.--lak---------t-nshvyr---------k------s--v-----vnr-qa-v--   178
HUMED     -----wik----t-i-lkt--f--l--i-----kaa---ih-a----ef-il--h--q--   179
BOVED     -----wik----tmt--k---fiiq--i-----katd---h-tlk---l-i-n-y--q--   179
RABED     -----wvk-l--t-v---------q--i-----kaa----h-a-----f-il--y--q--   179
MUSED     k-t--wvk-l--tit--kt--f-me--i---s--ad---ih-t-h---f-v-d-y--q--   179
RATED     k-t--wvk----tit--kt--f-me--i------a----ih-t-h---f-v-d-y--q--   179

TPRLRMP   IVQIHCKPDHHGSWSEWSSENYIEIP                                    205
CPRLRMP   -i--------------------q--                                     205
PGNMP     ------------------l-k-lq--                                    205
TPRLRMD   v--vr-ml-pge.-------rr-l-s                                    202
CPRLRMD   v--vr-tl-pge.-------rh-l--                                    201
PGNMD     v--vr-vl-ige.-------rh-h--                                    203
HUMED     l--vr----.--y--a--patf-q--                                    204
BOVED     l---r----.--y-----p-ss-q--                                    204
RABED     l--vr----.--f--v--p-ss-q--                                    204
MUSED     l--tr----.--y--r-gq-ks----                                    204
RATED     l--tr----.--y--r--q-ssv-m                                     203
```

Figure 3.6. Comparison of amino acid sequences of the repeat units of the extracellular domain of tPRLR with other PRLRs. Matching residues are shown by dashes. Gaps are introduced to maximize the matching and indicated by periods. Mismatching residues are shown in lower case. Abbreviations are: TPRLRMP (turkey PRLR membrane-proximal repeat unit), CPRLRMP (chicken PRLR membrane-proximal repeat unit), PGNMP (pigeon PRLR membrane-proximal repeat unit), TPRLRDP (turkey PRLR membrane-distal repeat unit), CPRLRDP (chicken PRLR membrane-distal repeat unit), PGNDP (pigen PRLR membrane-distal repeat unit), HUMED (human PRLR extracellular domain, BOBED (bovine PRLR extracellular domain), RABED (rabbit PRLR extracellular domain), RATED (rat PRLR extracellular domain) and MUSED (mouse PRLR extracellular domain).

Connecting Statement II

In the previous chapter, we described the cloning and characterization of a cDNA coding for turkey prolactin receptor. However, how the expression of the turkey prolactin receptor gene is regulated is not known. The following research was undertaken to study the tissue distribution of turkey prolactin receptor mRNA and the expression of the turkey prolactin receptor gene in various tissues at different stages of the reproductive cycle. Since PRL may play a key role on the manifestation of the incubation behaviour in galliforms, the study of the expression of turkey prolactin receptor associated with the physiological status of the hen and the levels of plasma prolactin may provide further insight into the mechanism of incubation behaviour.

Chapter 4

Tissue Distribution and Expression of the Prolactin Receptor During Various Reproductive States in *Meleagris gallopavo*

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4.1. ABSTRACT

Using RT PCR, the tissue distribution of the tPRLR mRNA and its expression at various reproductive states in *Meleagris gallopavo* were investigated. The tPRLR transcripts were detected in all 26 tissues examined. The pituitary gland, hypothalamus, crop sac, duodenum and gizzard were found to express the highest levels of tPRLR mRNA among the 26 tissues. The levels of tPRLR mRNA in 17 tissues were compared using semi-quantitative RT PCR in non-photostimulated, laying, out-of-lay, incubating, maternal hens and male birds. In most tissues there was no obvious relationship between levels of plasma PRL, reproductive states and estimated concentrations of the receptor mRNA. In the pituitary gland and hypothalamus, levels of plasma PRL and levels of tPRLR transcript were inversely correlated. In the hypothalamus, increasing levels of plasma PRL were associated with decreasing levels of the receptor transcript ($p < 0.05$), whereas, the opposite relationship was observed in the pituitary gland ($p < 0.05$). These findings support the hypothesis that PRL itself may participate in the neuroendocrine control of incubation behaviour through actions on both the hypothalamus via a short-loop feedback mechanism and the pituitary gland via autocrine and/or paracrine effects.

4.2. INTRODUCTION

In galliform species, the levels of PRL increase at the onset of incubation behavior and are maintained at high levels throughout the incubation phase in both the pituitary gland (Burrows and Byerly, 1936; Saeki and Tanabe, 1955; Nakajo and Tanaka, 1956) and circulating blood (Sharp *et al.*,1979; Burke and Dennison, 1980; Proudman and Opel, 1980). The levels of PRL mRNA in the pituitary glands of turkeys (Wong *et al.*, 1991; Karatzas, 1997) and bantam hens (Shimada *et al.*, 1991; Talbot and Hanks, 1991) show a similar trend. Recently, it has been shown that active immunization against recombinant-derived PRL reduced the incidence, delayed the development (March *et al.*, 1994), or prevented the occurrence (Guémené *et al.*, 1995) of incubation behaviour in birds. These observations suggest that PRL regulates the onset and/or maintenance of incubation behaviour in galliforms. However, how levels of PRL may influence and/or be influenced by this behaviour are not clearly understood. Furthermore, neither the target tissues nor the way in which the PRL receptor interacts with its ligand have been well characterized in these species.

The actions of PRL in its target tissues are initiated through interaction with specific, high affinity receptors at the cell membrane. Receptors for PRL have been cloned and characterized in a number of mammals and avian species (Boutin *et al.*,1988, 1989; Edery *et al.*, 1991, Scott *et al.*, 1992; Tanaka *et al.*, 1992; Chen and Horseman, 1994, Zhou *et al.*, 1996). Prolactin receptor belongs to the cytokine receptor superfamily and its structure is highly conserved among different species.

It consists of a cytoplasmic domain, a single transmembrane domain and an extracellular domain. In mammals, two major forms of PRLR differing in the length of the intracellular domain have been detected (Boutin *et al.*, 1988, 1989; Edery *et al.*, 1989; Davis and Linzer, 1989b) and refered as long and short forms. The ratios of "short" and "long" forms in various tissues associated with various stages in the estrous cycle, pregnancy and lactation have been described in the rat (Jahn *et al.*, 1991; Nagano and Kelly, 1994) and mouse (Clarke and Linzer, 1993). Levels of the two PRLR mRNAs were shown to vary in a tissue-specific fashion and in concert with the hormonal milieu associated with the reproductive status of the animal. In avian species, only a single form of PRLR has been identified and receptors have been detected in the liver, kidney, intestine, oviduct, crop sac, heart and various areas of the brain (Krishnan *et al.*, 1991; Tanaka *et al.*, 1992; Chen and Horseman 1994) and in some cells of the immune system (Skwarlo-Sonta, 1992). Although incomplete, this distribution is consistent with the broad categories of actions of PRL and the metabolic and reproductive adjustments that the hen makes during the hyperprolactinemia associated with incubation behaviour. However, how the expression of the PRLR gene is regulated is not known.

In this study, we examined the tissue distribution of tPRLR mRNA and the expression of tPRLR gene in various tissues at different physiological stages during the reproductive cycle. Since PRL may play a key role in the manifestation of the incubation behaviour in galliforms (Sharp *et al.*, 1979; Burke and Dennison, 1980; Etches and Cheng, 1982; El Halawani *et al.*, 1986), the study of the expression of

PRLR associated with the physiological status of the hen and the levels of blood PRL may provide further insight into the relationship between PRL and incubation behaviour.

3.3. MATERIALS AND METHODS

4.3.1. Animals and sample collection

All turkeys used for the gene expression experiment were of the Betina strain (BETINA, Saint Nolff, Elven, France) and were 56 weeks old at the time of necropsy. These birds were kept in collective floor pens (6L:18D) and photo-stimulated (14L:10D) at various ages from 30 to 53 weeks in order to have different physiological status at the same physical age. One group of hens was maintained in individual battery cages under a short photoperiod (6L:18D) throughout the experiment. Table 4.1 lists the physiological stages and characteristics of the birds

Table 4.1. Physiological Stages and Characteristics of the Turkeys Used in the Gene Regulation Experiment[a]

Physiological Stages	No.	Characteristics
Non-photo-stimulated	2	Maintained 6L:18D.
Laying	6	Laid eggs for 1 to 24 weeks before necropsy and had normal follicular hierarchy.
Out-of-lay	3	Ceased laying eggs 1 to 5 weeks prior to necropsy and lacked follicular hierarchy.
Incubating	3	Laid eggs for 9 to 14 weeks prior to incubating eggs for 1 to 6 weeks.
Maternal	2	Laid eggs for 14 weeks and had incubated mock eggs for 8 weeks. They had provided maternal care for poults for 3 days prior to necropsy.
Male	2	Sexually active.

[a]All birds were 56 weeks of age at necropsy.

at the time of necropsy. Hens which had laid eggs for 1, 12 or 24 weeks were grouped together for analysis since no differences were noted between the weeks of egg production. The birds were killed by cervical dislocation. Tissue samples were immediately dissected, weighed and frozen in liquid nitrogen until further processed. Most of the samples were collected within 10 minutes following death. For larger tissues such as the brood patch, cerebral cortex, duodenum, liver, shell gland etc. only a portion of the tissues was retained for analysis. For example, an area of approximately 2 cm^2 of epidermis and dermis from the central part of the brood patch was excised for analysis. Brain tissue was divided into the cerebellum, hypothalamus and the cerebral cortex. A section of muscle tissue from the middle of the pectoralis major was analysed. The ovary was excised and separate measurements were made on the small follicles (<3 mm diameter), the ovarian stroma and the large follicles (theca plus granulosa cells). For the survey of tissues which express the PRLR, equal amounts of reverse transcribed RNA was pooled from 18 animals per tissue with the exception of the testes which was a pool of 2 males and the large follicles of the ovary which was a pool of 6 hens. Blood samples (5 ml) were obtained by brachial venipuncture into heparinized tubes before cervical dislocation. Following centrifugation, plasma was stored at -20 °C until required for radioimmunoassay (RIA).

4.3.2. Radioimmunoassy for measuring of plasma prolactin level

The radioimmunoassy used in the present study was previously described (Guémené *et al.*, 1994). Briefly, 5 µg of recombinant turkey PRL (rctPRL) was

radiolabelled using 16.5 MBq of ^{125}I and 20 to 40 µl of chloramine T (60 µg/ml in 0.25 M PBS). Incorporation rate ranged from 45 to 68%. Free iodine and labelled rctPRL were separated by chromotography on a poly-acrylamide biogel column (Bio-Rad Laboratories, Richmond, California, USA).

Assay tubes (12x75 mm disposable polypropylene tubes) contained rctPRL standards or samples diluted in PBS assay buffer (300 µl total volume, containing 20 mg/ml equine serum albumin and 30 mg/ml EDTA). The standards were serial exponential dilutions of rctPRL and ranged from 50 to 0.2 ng. One hundred µl of antibody (1:16000 dilution) was added on the first day to all tubes except in tubes used to assess non-specific binding. After an overnight incubation at 4 °C, 200 µl of the labelled hormone (15000 cpm) was added and the mixture was incubated for an additional 24 hours at 4 °C. One hundred µl of sheep anti-rabbit serum (1:10 in PBS) and 100 µl of normal rabbit serum (1:200 in PBS) were added to each tube and the mixture was further incubated for 72 hours at 4 °C prior to the addition of 750 µl of assay buffer and centrifugation at 3000 g for 25 minutes at 4 °C. The pellets were resuspended with 750 µl of PBS and centrifugated at 3000 g for 15 minutes at 4 °C. The radioactivity present in the pellets was counted for 1 minute using a gamma counter (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). All samples were assessed in triplicate in a single RIA and the intra assay coefficient of variation was 4.5%. Average levels of plasma PRL are listed in Table 4.2 and 4.3.

4.3.3. Extraction of total RNA

Prior to RNA extraction, tissues were weighed to the nearest mg. For larger tissues about 100 mg slices were extracted using RNAzol™-B (TEL-TEST, INC. Friendswood, Texas, USA) according to the manufacturer's protocol. Tissue slices were first homogenized with 1 ml RNAzol™-B using a homogenizer (Tissue-Tearor™, Biospec, Bartlesville, Oklahoma, USA). Two tenth of a ml of chloroform was then added and the suspension was shaken vigorously for 30 seconds followed by incubation for 5 minutes at room temperature. After centrifugation at 12,000 x g for 10 minutes, the aqueous phase was recovered and the RNA was precipitated with an equal volume of 100% isopropanol. After 15 minutes incubation at -20 °C, total RNA was recovered by centrifugation at 10,000 x g for 10 minutes, washed with 70% ethanol, air-dried and disolved in 200 µl of RNAse free H_2O. The recovery of total RNA was measured by a spectrophotometer (Milton Roy, Rochester, New York, USA) at a wave length of 260 nm. Total RNAs were diluted to 0.5 µg/ml in H_2O and stored in 20 µl aliquots at -70 °C until use.

4.3.4. Reverse transcriptase PCR

One µg of total RNA from each tissue was primed with an antisense tPRLR-specific primer (P7, 5'-CCCAAGACGATCCACACAATC-3'; bases 1353-1373, Figure 3.2) and reverse-transcribed into first strand cDNA. The reaction was performed in a final volume of 6.6 µl containing 50 mM Tris-HCl (pH8.3), 60 mM KCl, 3 mM $MgCl_2$, 25 pmol P7 primer, 1 mM each of dNTPs, 10 mM dithiothreitol (DTT), 1 unit RNAguard (Pharmacia, Baie d'Urfé, Québec, Canada), 0.1 µg/µl BSA,

and 20 units MuMLV-reverse transcriptase (Pharmacia, Baie d'Urfé, Québec, Canada). The reaction mixture was incubated for 1 hour at 37 °C and 10 minutes at 85 °C. The volume of the reaction was then brought up to 20 µl with sterile distilled water.

Reverse transcriptase PCR was used to detect the expression of the tPRLR in various tissues using TPRLR-specific primers (P5, sense, 5'-AGGAAACATTTACCTGTTGGT-3' ,bases 767 -787, Figure 3.2) and (P6, antisense, 5'-AAGCCATCCAGATCTGACATC-3', bases 1117 - 1137, Figure 3.2). An aliquot of 2 µl of the diluted first strand cDNA mixture synthesized as above was amplified by PCR in a volume of 20 µl containing 10 mM Tris-HCl (pH9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 10 pmol each of P5 and P6 primers, 5% formamide and 0.5 units of Tth DNA polymerase. The optimal number of PCR cycles was determined based on the abundance of the tPRLR in specific tissues and ranged from 25 to 35 cycles. Each cycle consisted of 45 seconds at 93 °C, 2 minutes at 56 °C and 90 seconds at 72 °C.

Two control reactions were also included in the assay. First, total RNA pooled from each tissue examined without reverse transcription was used as template in the PCR. Second, a PCR mixture was prepared without first strand cDNA and total RNA. The first control confirmed that there was no DNA contamination, which could result in false positives, and the second control ensured no contamination occurred during the course of the manipulation.

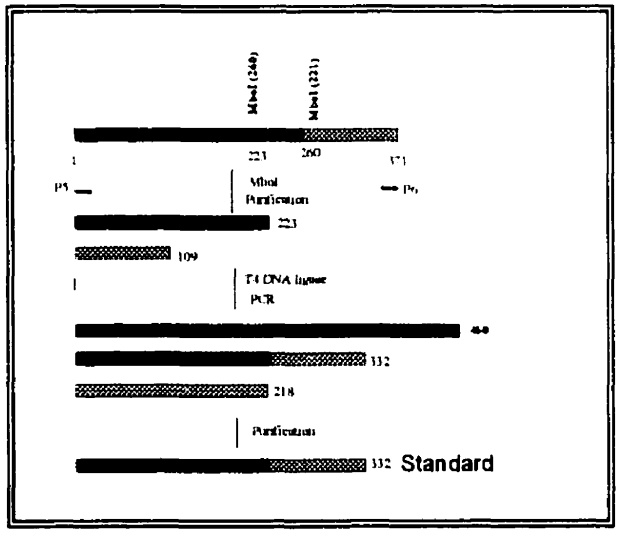
The identity of the RT PCR product was confirmed by Southern blot analysis.

Ten µl of above RT PCR product was separated on 1% agarose gel, stained with 0.5 µg/ml ethidium bromide, visualized under U.V. light and transferred to Zeta-probe membrane (Bio-Rad, Richmond, California, USA) using 0.4 M NaOH. The full length tPRLR was radio-labelled with [^{32}P]dCTP and hybridized with the membrane. The radio-labelling, prehybridization, hybridization, washing and autoradiography conditions were the same as the conditions used for the probe preparation and the screening of cDNA libraries

4.3.5. Construction of an internal standard for semi-quantitative RT PCR

An internal standard was constructed to calibrate the efficiency of PCR amplification (Figure 4.1). A 371-bp DNA fragment from the turkey PRLR cDNA

Figure 4.1. Schematic presentation of the construction of the internal standard. The bar indicates the PCR product, and the red area represents the deleted 39-bp sequence. Short arrows indicate the PCR primers. The size (bp) of each fragment is shown.



was first amplified using primer P5(5'-AGGAAACATTTACCTGTTGGT-3', bp 767 -787, Figure 3.2) and P6 (5'-AAGCCATCCAGATCTGACATC-3', bp 1117 - 1137, Figure 3.2). The PCR was performed in a volume of 100 µl containing 10 mM Tris-

HCl (pH9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 50 pmol of each P5 and P6 primers, 5% formamide and 2.5 units of Tth DNA polymerase for 35 cycles. Each cycle consisted of 45 seconds at 93 °C, 2 minutes at 55 °C and 90 seconds at 72 °C. Ten µl of the PCR product was then digested with 5 units of MboI restriction enzyme and separated on 12% PAG. Three fragments corresponding to 223, 109 and 39 bp were identified on the gel. Gel slices containing the 223-bp and 109-bp fragments were excised. The double-stranded 223-bp and 109-bp fragments were eluted from the gel slices in TE buffer at 65 °C overnight, recovered by ethanol precipitation and ligated using T4 DNA ligase (Pharmacia, Baie d'Urfé, Québec, Canada). One-thousandth of the ligation mixture was then amplified using P5 and P6 primers to increase the concentration of the ligation product. The PCR conditions were the same except the number of cycles was 25. The PCR product was then separated on 12% PAG. A 332-bp fragment was excised from the gel, reamplified for 25 cycles using P5 and P6 primers and used as an internal standard in the semi-quantitative PCR. The copy number of molecules in the internal standard solution was determined by U.V. absorbance at a wave length of 260 nm.

4.3.6. Semi-quantification of tPRLR mRNA in tissues

One µg of total RNA from each tissue was primed with an antisense tPRLR-specific primer (P7, 5'-CCCAAGACGATCCACACAATC-3'; bases 1353-1373) and reverse-transcribed into first strand cDNA. The reaction was performed in a final volume of 6.6 µl containing 50 mM Tris-HCl (pH8.3), 60 mM KCl, 3 mM $MgCl_2$, 25 pmol P7 primer, 1 mM each of dNTPs, 10 mM dithiothreitol (DTT), 1 unit RNAguard

(Pharmacia, Baie d'Urfé, Québec, Canada), 0.1 µg/µl BSA, and 20 units MuMLV-reverse transcriptase (Pharmacia, Baie d'Urfé, Québec, Canada). The reaction mixture was incubated for 1 hour at 37 °C and 10 minutes at 85 °C. The volume of the reaction was then brought up to 20 µl with sterile distilled water.

Before the semi-quantitative PCR, a rough estimation of the tPRLR mRNA level in a particular type of tissue and the number of PCR cycles to maintain exponential amplification was necessary. Therefore a titration assay was performed prior to the semi-quantitative PCR. Ten-fold serial dilutions of the internal standard were prepared to cover the possible range of tPRLR mRNA levels in various tissues. Two µl of reverse-transcribed first strand cDNA mixture from each tissue of the 15 individual samples were pooled, separately. An aliquot of 2 µl of the diluted first strand cDNA mixture synthesized at previous step was co-amplified with 2 µl of diluted internal standard by PCR in a volume of 20 µl containing 10 mM Tris-HCl (pH9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 10 pmol of each P5 and P6 primers, 5% formamide and 0.5 units of Tth DNA polymerase. PCR product was then separated on 12% PAG, stained with ethidium bromide and visualized under U.V. light. Based on the ratio of intensity of wild-type PCR product to that of the internal standard PCR product, the optimal amount of the internal standard was determined for a given tissue. Therefore, the above assay was repeated using differing number of cycles to determine the optimal number of cycles for a given tissue in order to maintain exponential amplification. Based on the intensity of the PCR product, the minimum number of

cycles was chosen to generate adequate amounts of PCR product for visualization and quantification.

For the semi-quantification of the tPRLR in tissues, assays were grouped according to the types of tissues. For each type of tissue, 15 individuals were tested under the same conditions (constant amount of internal standard and number of PCR cycles, which were determined in the titration assay). The PCR products were separated on 12% PAG, stained with ethidium bromide and visualized under U.V. light. The image of the gel was digitalized using the Gel Print 2000i system (Bio/Can Scientific, Mississauga, Ontario, Canada) and analyzed using the Gel Printing Tool Box software (BioPhotonic Corporation, Michigan, USA).

4.3.7. Statistical analysis

Data on concentrations of tPRLR mRNA were first grouped according to physiological stages (Table 4.2) and variances of percent rank were analysed using non-parametric tests. When appropriate ($p \leq 0.05$ Kruskal-Wallis), these data were further compared using the Mann-Whitney U test.

Data from the hens were also grouped according to levels of the plasma RLR (Table 4.3). These data were compared using a factorial ANOVA and a Fisher Protected Least Significant Difference test where appropriate ($p \leq 0.05$; ANOVA). All data is reported as the group mean ± the standard error of the mean.

4.4. RESULTS

4.4.1. Tissue distribution of the tPRLR mRNA

Tissue specific expression of tPRLR mRNA was analyzed using RT PCR. A 371 bp cDNA fragment from extracellular domain of tPRLR was amplified from mRNA prepared from 26 tissues using gene specific primer P5 and P6. The location of the P5 and P6 primer was the same as P1 (Figure 3.1 and 3.2) and P2 (Figure 3.1 and 3.2) primers, except that the P5 and P6 were tPRLR gene specific

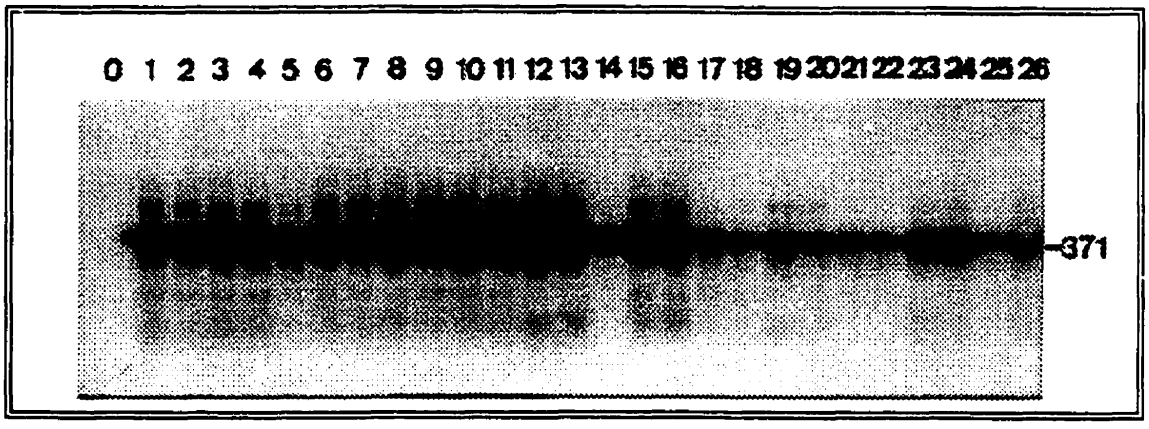


Figure 4.2. Southern blot analysis of PCR product amplified from the tPRLR mRNA using RT PCR. Lanes 0-26 are the Southern blot signal of the PCR product amplified from the negative control (lane 0), anterior pituitary (lane 1), posterior pituitary (lane 2), hypothalamus (lane 3), cerebral cortex (lane 4), cerebellum (lane 5), brood patch (lane 6), kidney (lane 7), crop sac (lane 8), gizzard (lane 9), duodenum (lane 10), caeca (lane 11), shell gland (lane 12), small follicle (<3 mm; lane 13), stroma (lane 14), isthmus (lane 15), infundibulum (lane 16), vagina (lane 17), large follicle (lane 18), liver (lane 19), leukocyte (lane 20), spleen (lane 21), pancreas (lane 22), heart (lane 23), lung (lane 24), testes (lane 25), and pectoralis (lane 26). The size (bp) of the RT PCR product is indicated. Lanes 1-11, 12-18, and 19-26 were amplified for 25, 30, and 35 cycles, respectively.

primers. The PCR condition was the same as the reaction using P1 and P2, except that the annealing condition was more stringent (56 °C, 5% formamide). In order to control for possible DNA contamination, a control reaction using total RNA, which was not reverse transcribed, as template was included in the test. No visible bands were amplified. The number of PCR cycles ranged from 25 to 35 and was determined according to the abundancy of the tPRLR mRNA in the different tissues. The identity of the amplified product was confirmed by Southern blot analysis using the full length tPRLR as probe. The PRLR gene is expressed in a broad range of tissues in turkeys and mRNA was detected in all 26 tissues examined.

4.4.2. Semi-quantitative PCR

The use of RT PCR to compare the level of expression of tPRLR in different physiological stages during the turkey reproductive cycle required the construction of an internal standard with similar base composition and distinguishable length. The internal standard constructed here was the 371 bp PCR product amplified by P5 and P6 with a 39 bp internal deletion. The internal standard could be distinguished

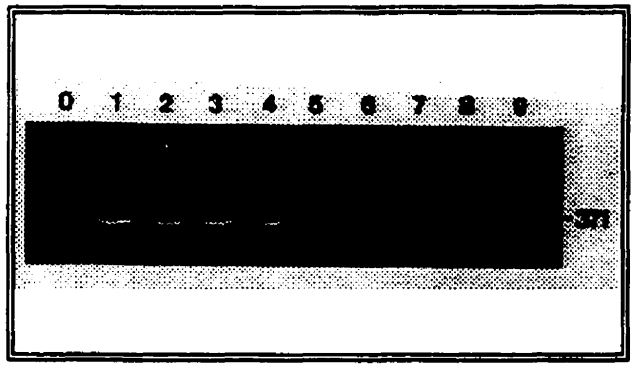


Figure 4.3. Coamplification of internal standard and sample first-strand cDNA. The PCR product was separated on 12% polyacrylamide gel and stained with ethidium bromide. Lane 0 indicates the negative control. Lanes 1-9 show the coamplification of the exponentially diluted internal standard and a constant amount of sample cDNA.

from its wild-type PCR product on 12% polyacrylamide gel (Figure4.3).

The estimation of the abundance of tPRLR in specific tissues prior to quantitative PCR is important since the ratio of the standard to the target affects the reliability of the quantification reaction. Hence, mRNA from individual tissues was titrated to determine the optimal amount of internal standard required for particular tissues and the number of PCR cycles to produce exponential amplification. Figure 4.3 shows an example of the titration assay where constant amounts of first strand cDNA was mixed with different amounts of internal standard and co-amplified. When the log of internal standard copy number was plotted against the log of the ratio of the intensity of sample PCR product to internal standard PCR product (measured in densitometric units), a linear relationship (Y = 1.27-0.33X; r=0.99) was observed (Figure 4.4). Such curves were generated for each tissue examined.

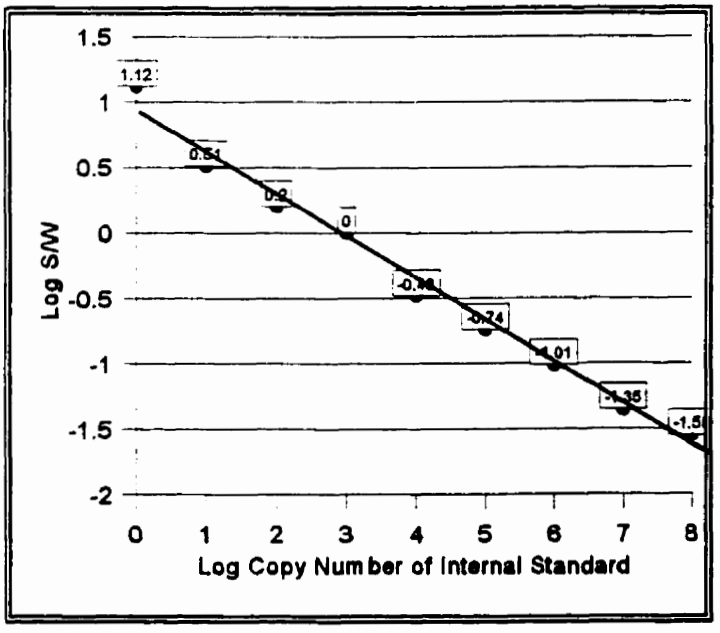


Figure 4.4. Relationship between the number of the internal standard molecules and the ratio of the sample PCR product intensity to internal standard PCR product intensity (measured in densitometric units). Constant amounts of first strand cDNA were mixed with different amounts of internal standard and coamplified. Y=1.27-0.33X r=0.99.

Thus, the expression level of tPRLR in various tissues could be compared by estimating the ratio of the intensity of sample to the internal control when a constant amount of internal standard was used in the analysis of samples from the same tissue at different physiological stages. A total of 17 tissues were assessed. As estimated from the titration data, the levels of transcript were: pituitary gland > crop sac > hypothalamus > gizzard, brain, duodenum > ovary, abdominal skin, kidney > uterus (shell gland) > liver > pancreas > spleen > heart > lung, leucocytes > pectoralis muscle.

4.4.3. Expression of tPRLR in specific tissues during various reproductive states

Table 4.2 lists the relative concentrations of tPRLR mRNA in various tissues at 6 different physiological stages. In the majority of tissues (kidney, lung, ovary, pancreas, spleen, liver, duodenum, gizzard, muscle, heart and leucocytes), there was no significant variation in the level of tPRLR transcript in association with the different physiological stages assessed. In general, the levels of receptor in male birds were comparable to those observed in reproductively active females. In certain tissues (the hypothalamus, pituitary, crop sac and shell gland), significant differences in levels of receptor were observed. In the pituitary gland and shell gland, the highest levels of transcript were observed in incubating hens ($p < 0.05$), whereas, hens which were not photostimulated had the lowest levels. In the hypothalamus, the opposite relationship was observed. In the crop sac, highest levels of transcript were detected in incubating hens and lowest levels were

observed in hens showing maternal behaviour.

Data from the hens were also grouped according to the levels of plasma PRL (Table 4.3). Three levels of plasma PRL were compared (< 50, 50 - 100 and > 100 ng/ml). In the majority of the tissues, levels of tPRLR transcript did not vary significantly in association with the levels of plasma PRL. However, in the hypothalamus and pituitary gland, levels of transcript were inversely correlated with levels of plasma PRL ($p<0.05$). As the concentration of PRL increased in the plasma, levels of receptor increased in the pituitary gland, whereas, they decreased in the hypothalamus. The levels of tPRLR mRNA in the shell gland increased at only the highest level of plasma PRL.

4.5. DISCUSSION

The adenohypophyseal hormone, prolactin has long been associated with the onset and maintenance of incubation behaviour in galliformes. However, whether, the associated hyperprolactinemia is a cause or a consequence of the physio-behavioural adjustments which the hen makes to support the behaviour is not clear. The cloning of the PRLR and subsequent development of an assay for tissues which transcribe the receptor allowed us to characterize tissues which may be important target tissues for prolactin.

The tPRLR was expressed in a broad range of tissues and transcripts were detected in all 26 tissues examined (Figure 4.2). Within tissues, there were apparent variations in content of tPRLR mRNA. For example, the small white follicles of the ovary had higher levels of receptor than larger follicles or the ovarian stroma; an observation consistent with the biological effects of PRL on suppressing estrogen production in turkeys (Zadworny *et al.*, 1989). In addition, the crop sac, gizzard and duodenum had higher levels of receptor than more distal parts of the digestive system. This is consistent with the distribution of receptor observed in the mammalian gastrointestinal system (Nagano *et al.*, 1995). The apparent ubiquity of the receptor is consistent with the multiplicity of physiological processes that PRL is known to be involved in. These actions have been grouped into those which affect reproduction, growth and development, osmoregulation and metabolism, behaviour and immunoregulation (Meites 1988).

In the majority of tissues which were assessed by semi-quantitative RT PCR,

the levels of receptor transcript did not vary with the reproductive states or the concentration of PRL in the hens. Thus, in these tissues there were no apparent relationships which suggest a major physiological function associated with hyperprolactinemia and the expression of incubation behaviour. Since hens alter their intermediary metabolism to adjust to the adipsic and aphagic states associated with incubation behaviour (Zadworny *et al.*, 1985a) , it is notable that levels of receptor in tissues associated with metabolism, digestion and osmoregulation did not markedly change (Tables 4.2 and 4.3). A more detailed analysis of these tissues, however, is required since PRL is likely to have regulatory roles in digestive functions as has been observed in mammals (Nagano *et al.*, 1995) and in the ring dove (Li *et al.*, 1995). In addition, in mammals, multiple forms of the receptor have been found (Boutin *et al.*, 1988, 1989; Edery *et al.*, 1989), which represent alternative splicing products and which are regulated in tissue specific fashion (Jahn *et al.*, 1991; Clarke and Linzer 1993; Nagano and Kelly 1994). In the present study, only a single PCR product was amplified when 2 primers located at the extracellular domain of the receptor (P6 and P7, Figure 4.2) were used. Since these primers only span 371 bp, the possibility of multiple forms of tPRLR mRNA could not be excluded. In pigeon, multiple bands were detected when the entire extracellular domain of the pigeon PRLR mRNA was amplified (Chen and Horseman). In additon, Pitts *et al* (1995) have recently reported a DNA fragment, which shares 84% DNA sequence identity with the tPRLR in the extracellular domain (Figure 3.2). In our previous report, a smear extending from about 0.8 kb

to 2.6 kb was detected on the Northern blot analysis. Further studies are required to determine if more than one form of receptor is present in turkeys.

In the hypothalamus and pituitary gland, plasma levels of PRL appeared to down-and up-regulate, respectively, the concentration of its own receptor (Table 4.3). The presence of receptors in the hypothalamus suggests that a short-loop feedback mechanism may operate in the brain, whereas, receptors in the pituitary gland suggests a possible autocrine and/or paracrine role of PRL in the regulation of pituitary hormone secretion. Whether PRL circumvents the blood-brain barrier to reach the brain via retrograde flow, transport via the choroid plexus, or is locally produced is not currently known, however, a hyperprolactinemic state is invariably associated with incubation behaviour in galliforms which would likely affect transport kinetics across the barrier. In the current study levels of plasma PRL in incubating hens were 8 to 30 fold higher than in out of lay or laying hens. The observation that the level of expression of PRLR mRNA decreased in the hypothalamus (>4 fold) when the levels of plasma PRL were high strongly supports the hypothesis that PRL may feedback on hypothalamic neurons to influence its own secretion as has been observed in mammals (Nagano and Kelly 1994; Ouhtit *et al.*, 1993). The type or precise location of the binding sites in the hypothalamus was not addressed in the current study. However, since VIP appears to be a major hypothalamic releasing factor for PRL in galliforms (Sharp *et al.*, 1989; Mauro *et al.*, 1989; Youngren *et al.*, 1994), it is possible that VIP containing neurons may be a target tissue for PRL. Further investigations to clarify the localization of these

receptors may provide further insight into their functional role. Similarly, in the hypophysis, there is a relationship between VIP and PRL since both VIP receptors (Rozenboim and El Halawani 1993) and expression of PRLR (>2 fold increase: Table 4.3) appear to increase in association with hyperprolactinemia. Taken together, these data support the hypothesis that PRL, itself may participate in the neuroendocrine control of incubation behaviour.

Table 4.2. Levels of tPRLR mRNA (arbitrary units) in Various Tissues in Non-photostimulated (IM), Laying (LA), Out-of-lay (OT), Incubating (IN), Maternal (MA) and Male (ML) Turkeys. The plasma levels of PRL (ng/ml) are shown for each group.

Tissues	PRLR mRNA concentration (arbitrary units)					
	IM	LA	OT	IN	MA	ML
PRL	9.1±0.4[a]	65.0±8.4[b]	125.0±82.3[c]	665.0±251.0[d]	127.0±39.0[c]	20.6±3.0[e]
Leucocyte	2.02±0.36	2.27±0.40	3.04±0.23	3.11±0.22	2.83±0.45	2.41±0.31
Brood patch	0.72±0.07	1.32±0.14	1.25±0.21	0.90±0.18	0.64±0.07	1.13±0.36
Brain	0.36±0.01	0.47±0.07	0.41±0.03	0.44±0.14	0.73±0.01	0.62±0.13
Crop sac	1.36±0.26[a]	1.28±0.14[a]	0.96±0.10[a]	1.51±0.05[a]	0.66±0.01[b]	1.07±0.06[a]
Gizzard	0.92±0.06	1.14±0.03	1.03±0.01	0.95±0.06	0.73±0.13	0.80±0.03
Heart	2.0±0.25	2.41±0.34	2.79±0.55	2.30±0.42	1.38±0.04	1.88±0.28
Hypothalamus	14.88±3.09[a]	5.61±0.68[b]	4.31±0.81[b]	2.15±0.33[c]	3.21±0.32[d]	3.14±0.93[d]
Duodenum	2.09±0.57	2.86±0.28	2.56±0.10	2.66±0.08	2.45±0.65	1.66±0.34
Kidney	4.89±0.21	4.09±0.27	3.80±0.24	4.57±0.38	4.05±0.05	3.50±0.45
Lung	1.20±0.19	0.93±0.08	0.89±0.07	1.00±0.15	0.85±0.01	1.00±0.29
Pectoralis	1.15±0.23	1.14±0.11	1.06±0.06	1.24±0.08	1.17±0.02	0.93±0.23
Ovary	2.29±0.58	2.30±0.30	2.74±0.33	1.90±0.06	2.28±0.37	n/a
Pancreas	2.47±0.24	2.50±0.21	2.53±0.23	2.12±0.03	1.97±0.07	2.46±0.31
Pituitary	0.24±0.05[a]	0.71±0.15[b]	1.12±0.07[c]	2.09±0.10[d]	1.51±0.08[cd]	1.10±0.21[c]
Spleen	2.86±0.05	2.40±0.32	2.66±0.05	2.15±0.23	1.99±0.18	2.45±0.18
Shell gland	1.63±0.38[a]	2.84±0.51[b]	2.32±0.11[b]	9.30±1.4[c]	3.49±0.95[b]	n/a
Liver	1.53±0.12	2.33±0.23	1.78±0.13	2.47±0.22	2.21±0.13	1.77±0.41

[a,b,c] Mean levels with the same superscript are not significantly different ($p>0.05$).

Table 4.3. Levels of tPRLR mRNA (arbitrary units) in Different Tissues Corresponding to the Concentrations of Blood PRL. Data are expressed as means±S.E.M. for each group of hens (n=5).

Tissues	PRLR mRNA concentration (arbitrary units)		
	I (< 50)	II (50 - 100)	III (> 100)
PRL(ng/ml)	19.4±3.9[a]	73.4±2.7[b]	615.6±201.3[c]
Leucocyte	2.23±0.31	2.68±0.45	3.02±0.20
Broody patch	0.94±0.18	1.29±0.19	0.93±0.13
Brain	0.37±0.01	0.5±0.08	0.51±0.10
Crop sac	1.08±0.15	1.12±0.14	1.25±0.16
Gizzard	0.98±0.03	1.12±0.05	0.89±0.09
Heart	2.56±0.35	1.96±0.26	1.98±0.31
Hypothalamus	10.10±2.86[a]	4.76±0.51[b]	2.31±0.22[c]
Intestine	2.45±0.27	3.00±0.35	2.39±0.20
Kidney	4.61±0.33	3.78±0.10	4.3±0.28
Lung	1.04±0.10	0.88±0.08	0.91±0.01
Muscle	1.05±0.12	1.29±0.06	1.18±0.01
Ovary	2.15±0.32	2.45±0.27	2.16±0.27
Pancreas	2.53±0.19	2.36±0.20	2.26±0.19
Pituitary	0.71±0.24[a]	1.04±0.19[b]	1.74±0.21[c]
Spleen	2.63±0.12	2.41±0.39	2.29±0.17
Shell gland	2.55±0.60[a]	2.53±0.31[a]	7.06±1.52[b]
Liver	1.68±0.11	2.54±0.22	2.25±0.18

[a,b,c] Mean levels with the same superscript are not significantly different ($p>0.05$).

Connecting Statement III

In the previous two chapters, we described the cloning and characterization of the turkey prolactin receptor cDNA. In addition, we investigated the tissue distribution of the turkey prolactin receptor mRNA and the relative abundance of the mRNA in these tissues. However, the mechanism by which the turkey prolactin receptor mediates the hormonal signal is not clear. No effective method has been available yet to alter incubation behaviour in turkeys, which is possibly regulated by prolactin. The availability of a highly purified and biologically active prolactin receptor would facilitate the process to solve these problems. The following study is undertaken to express, purify and characterize recombinant turkey prolactin receptor extracellular domain - GST fusion protein and turkey prolactin receptor extracellular domain protein.

Chapter 5

Expression and Purification of the Extracellular Domain of the Turkey Prolactin Receptor in *Escherichia coli*

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The manuscript of this paper is in preparation and will be submitted for publication.

5.1. ABSTRACT

The extracellular domain of the turkey prolactin receptor was amplified using polymerase chain reaction, cloned into the pGEX-2TK fusion expression vector and expressed in *E.coli*. The expression of fusion protein in $DH5_{\alpha}$ strain resulted in 26, 28, 40, 45 and 60 kDa proteins, while the expression of the fusion construct in a protease-deficient (*ompT*) strain BL21 yielded a protein with a molecular mass of 76 kDa. About 99 % of the fusion protein expressed in BL21 was present as an insoluble form in inclusion bodies and about 50 % of the total protein in inclusion bodies was the fusion protein. The fusion protein contained in inclusion bodies was solubilized in the presence of 4 M urea, refolded by the removal of the urea and purified using GST affinity chromatography. The yield of the purified fusion protein was 20 mg per litre bacterial culture with an estimated purity of 90 %. The extracellular domain of tPRLR was released from the fusion protein by thrombin cleavage and the yield was 0.2 mg per litre with about 95 % purity. The estimated molecular mass of the purified extracellular domain was 48 kDa. An antisera against the purified extracellular domain was raised in rabbit and was able to recognize both the fusion protein and the released extracellular domain of tPRLR.

5.2. INTRODUCTION

Prolactin is mainly synthesized in the lactotroph cells of the pituitary gland, released into plasma and elicits a variety of physiological actions. In birds, PRL is considered to participate in osmoregulation (Bern and Nicoll, 1968; Arad *et al.*, 1986), crop-milk production (Scanes *et al.*, 1975) and the manifestation of incubation behaviour (Riddle *et al.*, 1935; Saeki and Tanabe 1955). The physiological effects of PRL are mediated by its interaction with specific cell-surface receptors. PRL receptor cDNAs in avian species have been cloned and characterized in the chicken (Tanaka *et al.*, 1992), pigeon (Chen and Horseman 1994) and turkey (Zhou *et al.*, 1996).

The general structure of the avian PRLRs is similar to its counterparts in mammals and is composed of an extracellular domain (ECD), a short transmembrane segment and an intracellular domain. In avian species, however, the ECD is about 2 times larger than that observed in mammals and consists of two homologous units (201 and 204 amino acids for the membrane distal and proximal units, respectively), each of which corresponds to the extracellular domain of mammalian PRLRs (about 200-250 amino acids). Both extracellular units of avian PRLRs possess two structural features characteristic of the ligand binding domain of cytokine receptor superfamily, namely two pairs of cysteine residues at the "N-termini" and a WSXWS motif. Thus, it has been proposed that the avian PRLR may contain two ligand binding domains (Tanaka *et al.* 1992; Chen and Horseman, 1994) per receptor molecule. Moreover, in mammals, PRL has been found to

initiate its biological actions through interaction with 2 receptor molecules (Fuh *et al.*, 1993). Therefore, in avian species there is the possibility of multiple interactions both with respect to the binding of PRL and the receptor dimerization model. To date, however, very little is known about the mechanism of action in avians.

The ECD of the PRLR from the rabbit (Gignon *et al.*, 1994), the bovine (Tchelet *et al.*, 1995) and the rat (Sandowski *et al.*, 1995) have been prepared using recombinant protein expression systems. These proteins have proved to be useful for studying the stoichiometry of interaction of a variety of lactogenic hormones using gel filtration (Bignon *et al.*, 1994; Sandowski *et al.*, 1995) or the BIAcore apparatus (Gertler *et al.* 1996). Availability of a purified PRLR protein from turkeys would facilitate such studies in an avian species. The latter may be particularly interesting since 1) the turkey PRLR ECD may interact with more than 1 PRL molecule 2) the degree of glycosylation of PRL which varies with the physiological state of the hen (Bedecarrats *et al.*, 1995) may affect binding kinetics to the ECD and 3) an antibody against the ECD could be developed which might be useful for blocking the physiological effects of PRL *in vivo*.

Accordingly, in this study, we describe the construction of a Glutathione-S-Transferase fusion expression system for the production of recombinant ECD of the PRLR in *E. coli*. The majority of the fusion protein was expressed in an insoluble form hence the protein was purified and refolded from inclusion bodies followed by purification to homogeneity. Cleavage of the fusion protein with thrombin yielded

the ECD which was used to produce an antisera in rabbits.

5.3. MATERIALS and METHODS

5.3.1. Construction of the Expression Vector

The ECD of the tPRLR cDNA was expressed in a Glutathione-S-Transferase fusion system (Pharmacia, Baie d'Urfé, Québec, Canada) (Figure 5.1). Briefly, a sense primer 5'-TCAATCATTCCCTGGAAAACC-3' (P8, bp110 - 131, Figure 3.2) and an antisense primer 5'-CCCAAGACGATCCA CACAATC-3' (P9, bp 1353 - 1373, Figure 3.2) were designed to amplify a tPRLR cDNA fragment coding for the

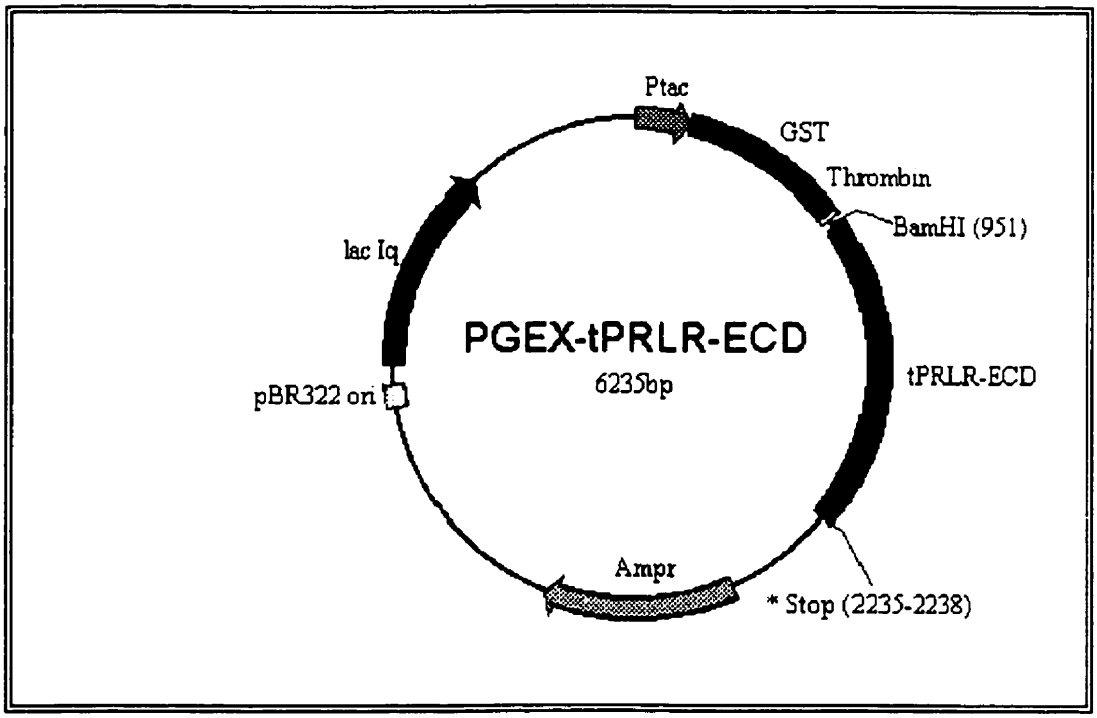

Figure 5.1. Schematic representation of pGEX-tPRLR-ECD construct. The thrombin cleavage site is located between GST and tPRLR-ECD domains. The stop codon is located after the tPRLR-ECD and is derived from the pGEX--2TK vector.

ECD of the putative mature tPRLR. About 100 pg of pBluescript plasmid containing tPRLR cDNA was amplified for 25 cycles using P8 and P9 primers. The reaction was performed in a final volume of 100 µl containing 10 mM Tris-HCl (pH9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol of each primers, 5% formamide and 2.5 units of Tth DNA polymerase (Pharmacia, Baie d'Urfé, Quebec, Canada). Each cycle consisted of 45 seconds at 93 °C, 2 minutes at 56 °C and 90 seconds at 72 °C. The PCR product was separated on 1 % agarose gel and purified using DEAE-cellulose paper. Concurrently, about 100 ng of pGEX2TK plasmid (Pharmacia, Baie d'Urfé, Quebec, Canada) was digested with 1 unit of SmaI restriction enzyme in 10 mM Tris.HCl (pH 7.5), 50 mM KCl and 10 mM $MgCl_2$ at 30 °C. After 2 hours of digestion, 0.1 unit of alkaline phosphatase (CIP) was added in the reaction and incubated for 30 minutes at 37 °C. The reaction was heated to 85 °C for 10 minutes and cooled to room temperature. About 200 ng of purified PCR product was then mixed with the digested and dephosphorylated pGEX2TK plasmid. The final reaction was adjusted to contain 10 mM Tris.HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP and 10 units of T4 DNA ligase. The ligation reaction was incubated at 16 °C overnight and transformed with competent *E.coli* $DH5_\alpha$. Clones containing insert were inoculated in 2 ml of LB containing 100 µg/ml of ampicillin and grown at 37 °C overnight. Plasmid was prepared and analyzed by restriction digestion. Only those, whose corresponding N-terminals were connected to the C-terminal of GST, were selected for sequencing analysis. Clones with correct ORF confirmed by sequencing analysis were chosen for the expression

analysis.

5.3.2. Optimization of the Extraction of tPRLR-GST Fusion Protein

5.3.2.1. Expression and purification of the fusion protein in DH5α strain

The expression construct was first transformed into an *E.coli* bacterial strain DH5α (GIBCO/BRL, Grand Island, New York, USA) and expressed. The pGEX-2TK vector was also transformed into DH5α strain as a control. The bacterial strain DH5α containing the expression construct or pGEX-2TK vector was grown in 2 ml 2xYT medium containing 100 µg/ml ampicillin at 37 °C to OD_{600} 1. The culture was then induced by 1 mM isopropyl-ß-thiogalactoside (IPTG) for 4 hours at 37 °C. Cells were harvested by centrifugation at 5000 x g for 2 minutes at 4 °C and resuspended in 0.4 ml ice cold extraction buffer containing PBS, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.7 µg/ml pepstatin, 0.5 µl/ml leupeptin. Bacterial cells were lysed by sonication on ice for 20 seconds using a half-inch probe at a power output of 5 using a sonicator (Vibra Cell™, Sonics and Materials Inc, Danbury, CT, USA). Samples were centrifuged at 12,000 x g force for 2 minutes. The supernatant was mixed with 25 µl of 50 % GST sepharose bead slurry equilibrated with PBS and incubated for 10 minutes. The GST sepharose beads were then washed with PBS 5 times and resuspended in 0.2 ml 1x gel loading buffer containing 50 mM Tris.HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol. The pellet was also resuspended in 0.4 ml 1x gel loading buffer. The effects of growth and induction conditions on the yield and quality of the soluble fusion protein were assessed by 8% SDS-PAGE

(Laemmli, 1970). Briefly, the 8% separation gel was prepared by mixing 2.7ml 30% acrylamide:bis mix, 2.5ml of 1.5 M Tris (pH 8.8), 0.1 ml 10% SDS, 0.1 ml 10% ammonium persulfate, 6 µl of TEMED and 4.6 ml of distilled water. The 5% stacking gel was prepared by mixing 0.67 ml 30% acrylamide:bis mix, 0.5 ml of 1.0 M Tris (pH 6.8), 0.04 ml 10% SDS, 0.04 ml 10% ammonium persulfate, 4 µl of TEMED and 2.7 ml of distilled water. Samples including the whole bacterial lysate before induction, the lysate after induction by IPTG, the lysate after GST beads absorbtion and the GST beads were loaded onto the gel. Samples were first boiled for 10 minutes in a water bath, cooled at room temperature for 10 minutes, centrifuged at 15,000 x g for 5 minutes and loaded on the 8% SDS-PAGE gel. The samples were then separated using the Miniprotean II System (Bio-Rad, Mississauga, Ontario, Canada) at 100 volts for 2 hours in Tris-glycine buffer containing 25 mM Tris, 250 mM glycine (pH8.3) and 0.1% SDS. The gel was stained in the Coomassie brilliant blue staining buffer containing 45% methanol, 10% acetic acid and 0.25% Coomassie brilliant blue for 30 minutes and destained in the buffer containing 45% methanol and 10% acetic acid for about 8 hours with gentle agitation. The band pattern and intensity were assessed using a densitometer.

Since the majority of the fusion protein was degraded into smaller proteins (see results and Figure 5.3) using the above culture and purification conditions, three other procedures were tested to purify the intact fusion protein. The conditions in these procedures are the same as above with modifications. First, the

culture media was supplemented with 2% glucose to suppress the basal expression of the vector and the induction time was reduced to 1 hour at a cell density of OD600 2. Second, the lysis procedure was modified by including 1 mg/ml lysozyme, 1 ug/ml DNAse and omitting the sonication. The third procedure was the combination of the first and second.

5.3.2.2. Expression of the construct in BL21 bacterial strain

Although the modification of the culture and purification procedure by suppressing the basal expression, reducing the induction time and increasing the cell density at induction and mild lysis process improve the profile of the intact fusion protein (see results and Figure 5.4), its yield was still far below a desirable level. Therefore, the fusion construct and pGEX-2TK were also transformed into a protease deficiency *E.coli* bacterial strain BL21 (*ompT*, Pharmacia, Baie d'Urfé, Québec, Canada), and used throughout the rest of the purification experiment.

The bacterial strain BL21 containing the expression construct was grown in 2 ml 2xYT medium containing 100 µg/ml ampicillin at 37 °C to OD_{600} 1. The culture was then induced by 1 mM isopropyl-ß-thiogalactoside (IPTG) for 4 hours at 37 °C. Cells were harvested by centrifugation at 5000 x g for 2 minutes at 4 °C and resuspended in 0.4 ml ice cold extraction buffer containing PBS, 1 mM phenylmethylsulfomylfluoride (PMSF), 0.7 µg/ml pepstatin, 0.5 µl/ml leupeptin. Bacterial cells were lysed by sonication on ice for 20 seconds using a half-inch probe at a power output of 5 using a sonicator (Vibra Cell™, Sonics and Materials Inc, Danbury, CT, USA). Samples were centrifuged at 12,000 x g for 2 minutes.

The supernatant was mixed with 25 µl of 50 % GST sepharose bead slurry equilibrated with PBS and incubated for 10 minutes. The GST sepharose beads were then washed with PBS 5 times and resuspended in 0.2 ml 1x SDS-PAGE gel loading buffer. The pellet was also resuspended in 0.4 ml 1x gel loading buffer. The effects of growth and induction conditions on the yield and quality of the soluble fusion protein were assessed by 8 % SDS-PAGE using the same condition as before.

The majority of the fusion protein was present in the inclusion body (see results). It has been reported that the solubility of the fusion protein may be increased by growing the bacteria at 30 °C (Schein, 1989; Schein and Noteborn, 1988), or by altering the time of induction and concentration of IPTG (Pharmacia Biotech, 1993). Therefore, we tested the effect of induction duration and the concentration of IPTG on the yield of the soluble fusion protein.

Bacteria BL21 containing the expression construct was grown in 20 ml 2x YT medium supplemented with 100 µg/ml ampicillin and 2% glucose to OD600 1 at 30 °C. Aliquots of 2 ml of the above culture was then induced by 0.25, 0.5, 1 and 2 mM IPTG for 1, 2, 4 hours, respectively. Cells were harvested by centrifugation at 5000 x g for 2 minutes at 4 °C and resuspended in 0.4 ml ice cold extraction buffer containing PBS, 1 mM PMSF, 0.7 µg/ml pepstatin, 0.5 µl/ml leupeptin. Bacteria were disrupted by sonication on ice for 20 seconds using a half-inch probe at a power output of 5 using a sonicator (Vibra Cell™, Sonics and Materials Inc, Danbury, CT, USA). Samples were centrifuged at 12,000 x g for 2 minutes. The

supernatant was mixed with 25 µl of 50 % GST sepharose bead slurry equilibrated with PBS and incubated for 10 minutes. The GST sepharose beads were then washed with PBS for 5 times. The effects of growth and induction conditions on the yield and quality of the soluble fusion protein were assessed by 8 % SDS-PAGE using the same condition as before.

5.3.2.3. Purification of fusion protein from inclusion body

The SDS-PAGE analysis of the purified fusion protein using the GST bead, bacterial crude lysate and the inclusion body showed that the culture temperature, induction time and concentration of IPTG have no noticeable effect on the yield of the soluble fusion protein (see results, Figure 5.6). We, therefore, decided to purify the fusion protein from inclusion bodies.

Two ml bacteria containing the expression structure were grown at 30 °C to OD_{260} 1 with 2% glucose and induced by 0.5 mM IPTG for 2 hours. Cells were then harvested by centrifugation, resuspended in the extraction buffer B (containing 25 mM Tris.HCl, pH 9.0, 1 mM PMSF, 0.7 µg/ml pepstatin and 0.5 µl/ml leupeptin), lysed by sonication and centrifuged at 12,000 g for 15 minutes at 4 °C. The pellet was resuspended in 400 µl extraction buffer B by 20 seconds sonication on ice. Six aliquots of 50 µl were distributed into Eppendorf tubes and urea was added to a final concentration of 0, 3 M, 3.5 M, 4 M, 4.5 M and 5 M by mixing equal volume of extraction buffer B supplemented with 0 M, 6 M, 7 M, 8 M, 9 M and 10 M urea, respectively. After 5 minutes incubation on ice, the tubes were centrifuged at 12,000 x g for 5 minutes. The supernatant was saved and the pellets were washed

and resuspended in 100 µl of extraction buffer B. Ten µl aliquots of supernatant and pellet resuspension were analysed on 8% SDS-PAG, respectively.

When the inclusion body was incubated with the solution containing 4 M urea, the majority of the fusion protein was solubilized, whereas the other cellular proteins in the inclusion body remained insoluble (Figure 5.7a and 5.7b). Therefore, the supernatant of the sample with 4 M urea was chosen for the further purification. About 90 µl of this supernatant was buffer exchanged with extraction buffer B supplemented with 0.1 mM cysteine, incubated at 4 °C for 2 hours with occasional stirring and centrifuged at 12,000 x g for 5 minutes. The supernatant was mixed with 20 µl of 50 % GST-Sepharose bead slurry equilibrated with 25 mM Tris.HCl (pH 9.0) and incubated at 4 °C for 10 minutes with gentle mixing. The GST-Sepharose beads were washed with 1 ml 25 mM Tris.HCl (pH 9.0) for 5 times. Samples were then analysed on 8% SDS-PAG in the presence of ß-mercaptoethanol using the same condition as before.

5.3.2.4. Large-scale preparation and purification of tPRLR-ECD-GST fusion protein from inclusion bodies

Based on the results of the above assay, the extraction of the fusion protein was scaled up. A single BL21 colony containing the expression construct was inoculated into 100 ml 2x YT medium supplemented with 100 µg/ml ampicillin and 2% glucose and incubated at 30 °C overnight. Then the overnight culture was diluted in one litre of 2x YT medium supplemented with 100 µg/ml ampicillin and 2% glucose and incubated at 30°C until the OD600 reached 1. Isopropyl ß-

thiogalactoside was then added to a final concentration of 0.5mM. After an additional 2 hours of incubation, cells were collected by centrifugation at 5,000 x g for 10 minutes at 4 °C. The cells were resuspended in 500 ml extraction buffer B supplemented with 1 mg/ml lysozyme and incubated on ice for 30 minutes. The suspension was sonicated for 30 seconds twice on ice and centrifuged at 12,000 x g for 30 minutes at 4°C. The supernatant was discarded. The pellet was resuspended in 500 ml H_2O by sonication and centrifuged at 20,000 x g for 15 minutes at 4°C. The suspension and centrifugation procedures were repeated 3 times. After the final centrifugation, the pellet was resuspended in 500 ml buffer containing 25 mM TrisHCl (pH 9.0) and 4.0 M urea. The solution was incubated at 4 °C for 10 minutes with gentle shaking and centrifuged at 12,000 x g for 15 minutes. The supernatant was then dialysed to remove the urea. Briefly, a dialysis tube (Sigma, St. Louis, MO, USA) with a molecular weight cut off of 12 kDa was treated with a 0.3% (w/v) solution of sodium sulfide at 80°C for 1 minute, washed with distilled water at 60 °C for 2 minutes, followed by acidification with a 0.2% (v/v) solution of sulfuric acid, and rinsed with distilled water at 60 °C. The solubilized inclusion body was loaded into the treated dialysis tube and dialysed against 5 x 5 litres of 25 mM Tris-HCl (pH 9.0) for 24 hours. The dialysed solution was transferred to a beaker and cysteine was added to a final concentration of 0.1 mM and then incubated at 4 °C for 2 hours. Fifty ml solution was loaded at 25 ml per hour onto a 2 ml GST-sepharose column pre-equilibrated with 25 mM Tris-HCl (pH 9.0), at 4 °C. The column was washed with 50 ml 25 mM Tris-HCl (pH 9.0). For

preparation of the fusion protein, the bound protein was eluted in 6 ml elution buffer containing 10 mM reduced glutathione and 25 mM Tris-HCl (pH 9.0). The eluted protein was dialysed with a 15 ml Amicon concentrator with molecular weight cut off 30 kDa against 25 mM Tris-HCl (pH 9.0), measured by the chromogenic method (Bradford, 1976) and lyophilized.

5.3.3. Release and purification of the ECD protein from the fusion protein

For preparation of soluble recombinant ECD of the tPRLR, the bound protein from the previous step was subjected to thrombin cleavage. After the final wash with 25 mM Tris-HCl (pH 9.0), the GST-sepharose beads were washed twice with thrombin cleavage buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8), 2.5 mM $CaCl_2$. The beads were then suspended in an equal volume of thrombin cleavage buffer containing 100 cleavage units of thrombin and incubated at 4 °C overnight. The released protein was recovered by washing the beads with 25 mM Tris.HCl (pH 9.0) and the thrombin was removed by passing through a heparin column (Pharmacia, Baie d'Urfé, Québec, Canada) equilibrated with 25 mM Tris.HCl (pH 9.0). The concentration of purified extracellular domain was determined by the method of Bradford (1976). The protein was aliquoted and immediately lyophilized.

5.3.4. N-terminal amino acid sequencing

The N-terminal sequence of the released ECD domain of the receptor was determined by the Edman degradation method. Approximately 20 pmol of purified ECD protein was separated on 8% SDS-PAGE using the same conditions as

previously described. The protein was then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Mississauga, Ontario, Canada). The membrane was stained with a solution containing 40% methanol and 0.25% Coomassie brilliant blue for 5 minutes, destained with 40% methanol for 4 hours and washed 3 times with 1 litre of distilled water for 1 hour. The sequencing analysis was provided by the Sheldon Biotechnology Centre (McGill University, Montreal, Canada) using a Model 470A Gas--Phase Sequencer equipped with an on-line Model 120A high-pressure liquid chromatography analyser (Applied Biosystems, Boston, MA, USA) for quantitation of phenylthiohydantoin amino acid residues.

5.3.5. Western analysis

5.3.5.1. Immunization against the ECD of tPRLR in rabbits

Antisera against the ECD of tPRLR was prepared by immunizing rabbits intradermally by the multiple site method (Vaitukaitis *et al.*,1971). About 200 µg of the purified fusion protein was resuspended in 0.9 ml saline and emulsified with an equal volume of Freund's incomplete adjuvant (CFA). The emulsion was injected intradermally over 10 to 20 sites. One week after the first injection, another 200 µg of fusion protein emulsified in 0.9 ml of Freund's incomplete adjuvant (IFA) were injected. A booster injection into the ear vein with 50 µg of the ECD of tPRLR emulsified on 0.9 ml IFA was given on the fifth week. Two weeks after the booster injection, 5 ml blood was collected from an ear vein once a week for 8 consecutive weeks. Blood was allowed to coagulate overnight at room temperature and the

serum was aliquoted and lyophilized.

5.3.5.2. Western blotting analysis of the fusion and the cleaved tPRLR-ECD proteins

The antisera of the last collection against the recombinant tPRLR-ECD raised in rabbits were characterized for its specificity and optimal dilution factors for the detection of the tPRLR. First, serial dilutions containing 0, 100 pg, 1 ng, 10 ng and 100 ng of fusion protein were spotted on 6 pieces of nitrocellulose membranes (0.45 µm pore size, Bio-rad). Each membrane was hybridized with antisera with 1:125, 1:250, 1:500, 1:1000, 1:2000 and 1:4000 dilutions, respectively. To check the specificity of the antisera, about 1 µg of the unpurified total protein from inclusion body, 0.5 µg and 1 µg of purified fusion protein cleaved with thrombin were separated on 8% SDS-PAGE using the same conditions as before, transferred to nitrocellulose membrane (0.45 µm pore size, Bio-Rad, Mississaauga, Ontario, Canada) using the Mini Trans-Blot apparatus (Bio-Rad) at 100 volts for 1 hour at 4 °C and hybridized with 1:500 diluted anti-ECD serum raised in rabbits. A turkey kidney membrane preparation was also used in the Western analysis using the antisera. Briefly, about 2 grams of turkey kidney tissue was homogenized in 2 ml buffer containing 50 mM Tris (pH 7.5) and 10 mM $MgCl_2$. The mixture was then centrifuged at 200 x g for 1 minute. Fifty µl of supernantant was mixed with equal volume of RIPA buffer containing 1% sodium deoxycholate and 0.1% SDS, incubated on ice for 15 minutes, vortexed vigorously and centrifuged at 13,000x g for 10 minutes at 4°C. Fifteen µl of supernantant was subjected to Western

analysis.

For the detection, the membranes were wetted with TBST solution containing 20 mM Tris.HCl (pH 7.6), 137 mM sodium chloride, and 0.1% Tween-20 and incubated in blocking buffer containing TBST and 5% skimmed milk powder at room temperature for 1 hour. The membrane was then washed three times with 20 ml TBST at room temperature with gentle agitation and incubated with the antiserum at appropriate dilution in TBST buffer at room temperature for 1 hour. The membrane was washed three times as before and incubated with an anti-rabbit secondary antibody (Amershan Life Science, Little Chalfont, Buckinghamshire, England) at room temperature for 1 hour. The membrane was then washed three times with TBST and subjected to chemiluminescence detection using a ECL kit according to the manufacturer's protocol (Amersham Life Science, Little Chalfont, Buckinghamshire, England). The signal was recorded on an X-ray film (Kodak).

5.4. RESULTS

5.4.1. Construction of the fusion vector

The construct used to produce the recombinant extracellular domain of tPRLR is shown in Figure 5.1. DNA coding for the extracellular domain of the putative mature tPRLR plus the first 8 amino acids of the transmembrane domain (amino acid 25 to 446, Zhou *et al.*, 1996) was amplified using PCR and subcloned into the SmaI site of pGEX-2TK vector (Pharmacia, Baie d'Urfé, Québec, Canada). The sense primer used in PCR was designed so that the PCR product remained in frame after cloning into the SmaI restriction site. The orientation of the construct in positive transformants was first confirmed with restriction mapping analysis and subsequently, a single clone was chosen for sequence analysis. The entire tPRLR-ECD including the junction between the GST coding region and the tPRLR-ECD were sequenced and no changes in sequence occurred as a result of the cloning strategy. This clone was used for the expression studies. The predicted molecular mass of the fusion protein was 76.36 kDa, whereas, the cleaved ECD was 49.97 kDa after digestion with thrombin. The amino terminus of the ECD is modified as

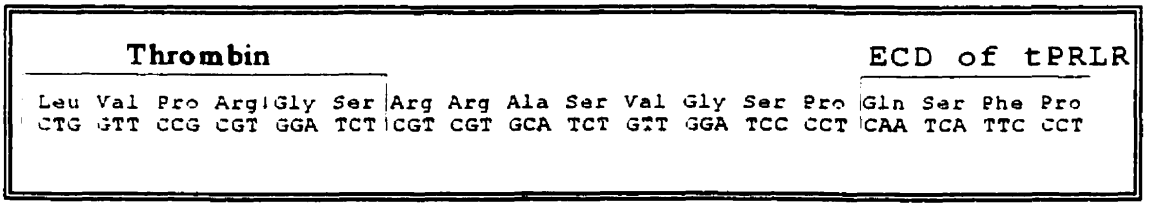

Figure 5.2. The sequence of tPRLR-ECD-GST construct at the junction between the 3' end of GST and the 5' end of tPRLR-ECD.

a result of the cloning strategy and contains 10 additional amino acids (Figure 5.2). These 10 amino acids are part of the multiple cloning site of the expression vector and contain a protein kinase site (Arg Arg Ala Ser Val) to facilitate radiometric labelling of the protein.

5.4.2. Expression of recombinant ECD of tPRLR in DH5α Strain.

The construct was first used to transform *E. coli* strain $DH5_{\alpha}$ and tested for the ability to produce an IPTG induced fusion protein (Fig. 5.3). Induction with IPTG resulted in the expression of a 76 kDa protein by the cells. When the cell lysate was subjected to affinity chromatography using a GST-Sepharose matrix, three proteins with molecular masses of 26, 60, and 76 kDa were detected (Figure 5.3). The 76 kDa protein had the expected size of the fusion protein but represented only about 5% of the total purified protein estimated by densitometry. Growth of the bacteria in media supplemented with glucose to suppress the

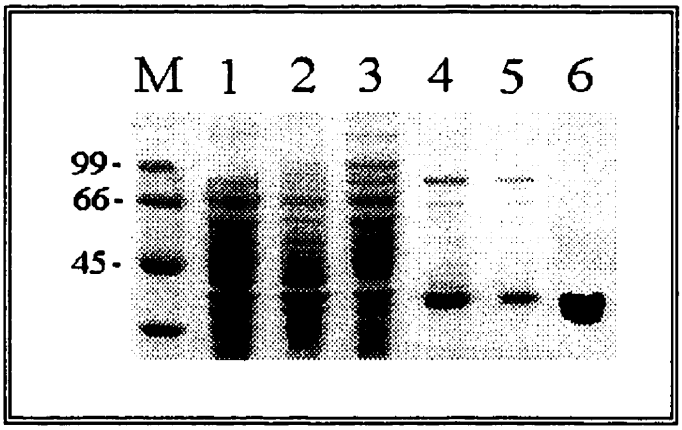

Figure 5.3. Expression of the tPRLR-ECD-GST fusion protein in DH5α strain. Lane 1 is the lysate of bacterial cells induced by IPTG. Lane 2 is the lysate of the uninduced bacteria. Lane 3 is the lysate after incubation with GST-sepharose beads. Lane 4 and 5 are proteins bound on the GST sepharose beads, which have been incubated in the lysate of the IPTG induced bacteria. Lane 6 is a GST control sample.

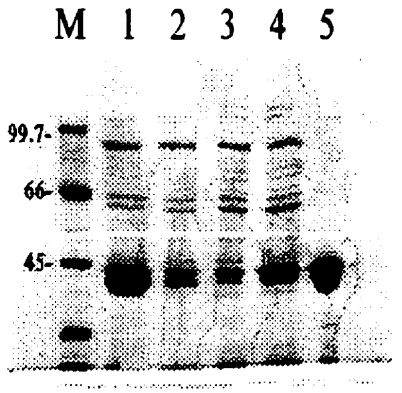

Figure 5.4. The expression and purification of recombinant tPRLR-ECD-GST in DH5α strain under various conditions. Lane M is the molecular weight marker. Lane 1 is the GST-sepharose bound proteins from normal growth condition with mild lysis procedure (1mg/ml lysozyme and 1 µg/ml DNAse). Lane 2 is the proteins from media supplemented with 2% glucose, grown at 30 °C, induced for 1 hour at OD600 2. Lane 3 is the combinatorial conditions in lane 1 and 2. Lane 4 is the protein from normal growth and lysis condition. Lane 5 is the protein expressed from GST vector.

basal expression of the construct prior to the induction, a short induction time (1 h) with IPTG and a mild lysis procedure improved the yield of the 76 kDa protein to about 10% of total purified protein. However, these modifications also resulted in three additional proteins of 28, 40, and 45 kDa being produced (Figure 5.4).

5.4.3. Expression of recombinant ECD of tPRLR in BL21

The construct was also expressed in BL21 strain which is a protease-deficient (*ompT*) host. The bacteria were grown in the presence of 2% glucose at 30 °C. The fusion protein was inducible by IPTG and showed the expected size of 76 kDa (Figure 5.5). It constituted about 25% of the total bacterial protein after IPTG induction as determined by densitometry (Figure 5.5, Lane 2). However, over

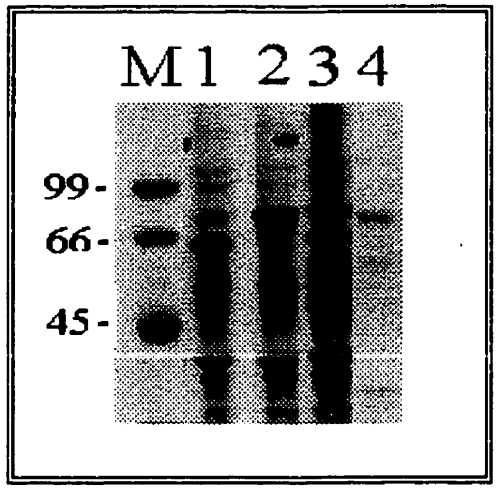

Figure 5.5. Expression of the fusion construct in BL21 strain. Lane M is the molecular marker. Lane 1 to 4 are the cell lysate before induction, lysate after induction, lysate after absorbtion and the GST beads incubated with lysate. Molecular weight are indicated (kDa).

99% of the fusion protein was present in the inclusion body in an insoluble form. The fusion protein constituted about 50% of the total insoluble protein within the inclusion body (Figure 5.7a, lane 1 and 2). With the increase of induction duration from 1 to 4 hours by IPTG, the total amount of fusion protein increased from 25% to about 35%, however, a concurrent increase in lower molecular weight proteins was also observed with the increase of incubation time (Figure 5.6). Modification of the concentration of IPTG from 0.25 to 2 mM had

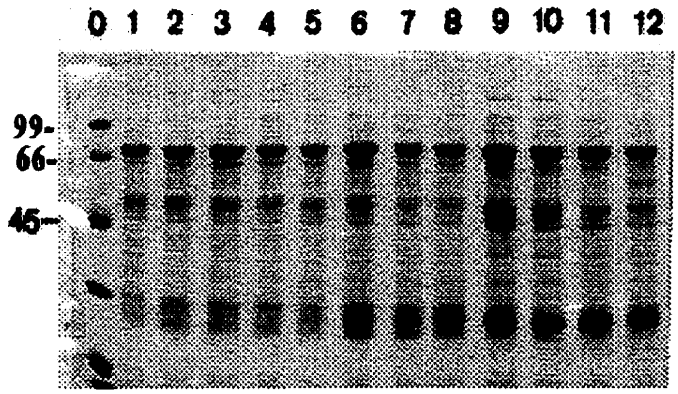

Figure 5.6. The effects of induction duration and concentration of IPTG on the yield of the soluble fusion protein. Lane 1to 4, 5 to 8 and 9 to 12 are induced for 1, 2 and 4 hours, respectively. Lane 1, 5 and 9 are induced by 0.5 mM IPTG. Lane 2, 6 and 10 are induced by 1 mM IPTG.

no apparent effect on either the yield or solubility of the fusion protein. The estimated yield of the fusion protein purified using affinity chromatography was less than 1% of the total fusion protein in the lysate.

5.4.4. Purification of recombinant tPRLR-ECD-GST from inclusion bodies

The inclusion bodies were first solubilized in buffer containing urea. The urea concentration ranged from 2.5 to 5 M to determine the condition in which the fusion protein and bacterial proteins could be differentially separated. At 4 M urea and 25 mM Tris.HCl (pH 9.0) (Figure 5.7a,b), most of the fusion protein was solubilized, while the bacterial protein remained insoluble (Figure 5.7a) and was removed by centrifugation at 12,000 x g for 5 minutes at 4 °C. The solubilized fusion protein was dialysed against 25 mM Tris.HCl (pH 9.0), refolded at pH 9.0 in the presence of catalytic amounts of cysteine (0.1 mM) and purified using GST-Sepharose affinity chromatography. About 40% of the solubilized fusion protein could bind to the GST-sepharose beads

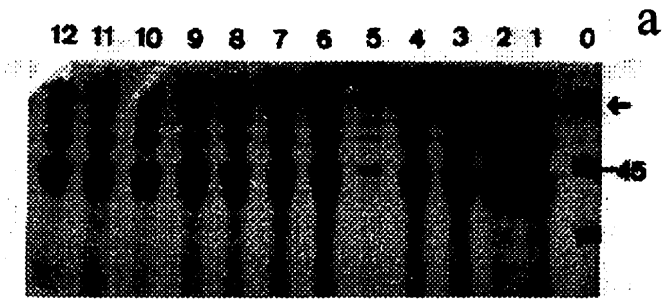

Figure 5.7a. Solubilization of inclusion bodies by urea. Lane 1, 3, 5, 7,9 and 11 are unsolublized material of inclusion bodies after incubation with 0, 3, 3.5, 4, 4.5 and 5 M urea. Lane 2, 4, 6, 8, 10 and 12 are duplicates of lane 1, 3, 5, 7, 9 and 11. The band indicated by the arrow is the fusion protein. Lane 0 is the molecular weight marker. The size is indicated (kDa).

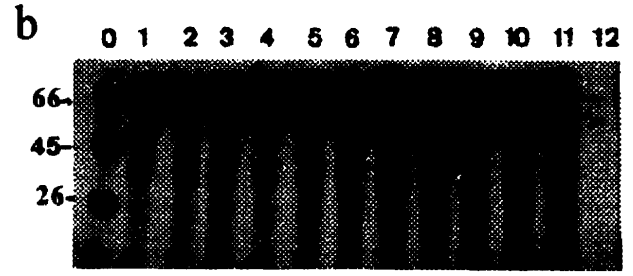

Figure 5.7b. Solubilization of fusion bodies by urea. Lane 1, 3, 5, 7, 9 and 11 are the supernatant of inclusion bodies after incubation with 0, 3, 3.5, 4, 4.5 and 5 M urea. Lane 2, 4, 6, 8, 10 and 12 are duplicates of lane 1, 3, 5, 7, 9 and 11. Band indicated by the arrow is the fusion protein. Lane 0 is the molecular weight marker. The size is indicated (kDa).

using batch absorbtion. The purity of the fusion protein is about 90% on SDS-PAGE (Figure 5.8). The major contaminating protein was about 26 kDa.

Based on the conditions above, a large scale purification from 10 X 1 litre culture was carried out. The concentration of the fusion protein in the bacteria was about 50 mg per litre as estimated by SDS-PAGE and the yield of purified fusion protein was estimated to be about 20 mg per litre culture by the Bradford method (1976).

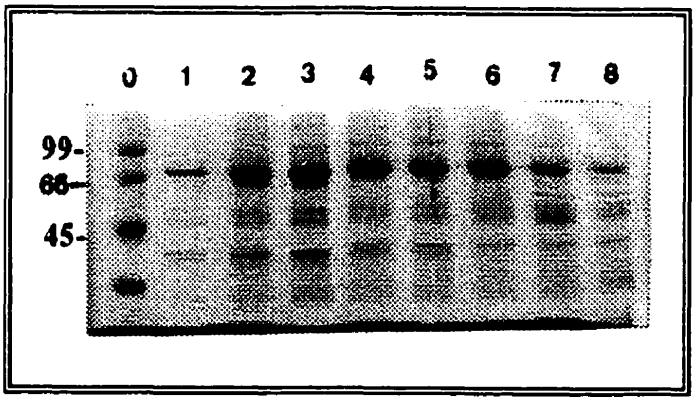

Figure 5.8. SDS-PAGE analysis of tPRLR-ECD-GST fusion protein purified using the GST-sepharose matrix. Lane 1 to 8 are 8 different preparations. Lane 0 is the molecular weight marker. The size is indicated (kDa).

5.4.5. Release of the soluble ECD protein from the purified fusion protein

The ECD of tPRLR was released from the GST moiety by incubating the fusion protein bound on the GST-Sepharose beads by thrombin cleavage overnight at 4 °C. Under this condition, the cleavage of fusion protein was complete as shown by SDS-PAGE analysis (Figure 5.9). The released extracellular domain of tPRLR was concentrated to 1 mg/ml. The total yield of the ECD was about 0.2 mg per litre of bacterial culture.

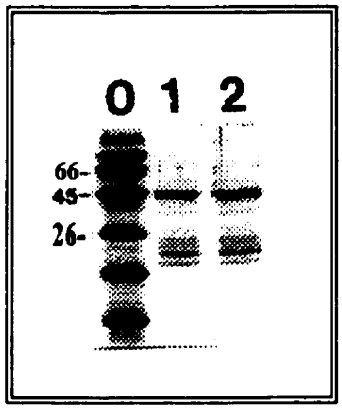

Figure 5.9. SDS-PAGE analysis of the released tPRLR-ECD protein. Lane 1 and 2 are two different preparations. Lane 0 is the molecular weight marker. The size is indicated (kDa).

5.4.6. Characterization of the purified recombinant tPRLR-ECD-GST and tPRLR-ECD by Western analysis

The antiserum raised against the ECD of the tPRLR was first characterized for its optimal titer for use in the Western blotting analysis (Figure 5.10). The pre-immunization fraction did not react with the purified fusion protein. At the highest dilution, only 100 ng of the fusion could be detected, whereas, at the lowest dilution (1:125), as little as 0.1 ng of the purified fusion protein could be detected. The 1:500 dilution was used in the subsequent Western blot analysis since at this concentration 0.1 ng could be detected but very litle background staining was

observed.

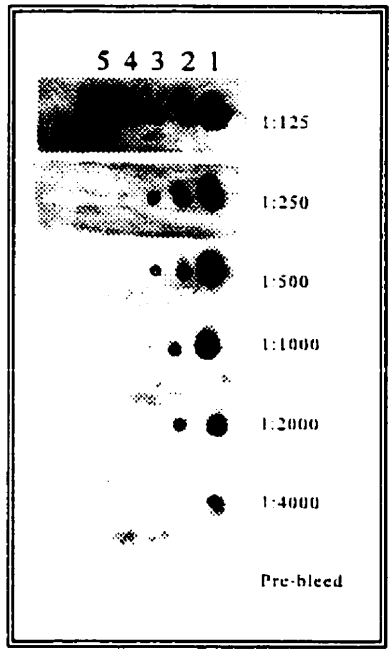

Figure 5.10. Dot blot analysis of the anti tPRLR-ECD raised in rabbits. Dot 1, 2, 3, 4 and 5 represent 100ng, 10 ng, 1 ng, 0.1ng and 0 ng of the fusion protein. The numbers on the right side indicate the dilution of the antiserum used.

Western blot analysis of the purified fusion protein and the cleaved ECD are shown in Figure 5.11. In addition to the 76-kDa fusion protein, 4 proteins with estimated molecular sizes of 26, 28, 45, and 60 kDa were observed in the fusion preparation (Figure 5.11, lane 1). These proteins have identical molecular weight to the proteins observed when the construct was expressed in the DH5α (Figure 5.3). They may represent partially degraded fusion proteins produced in the BL21 strain. Cleavage of

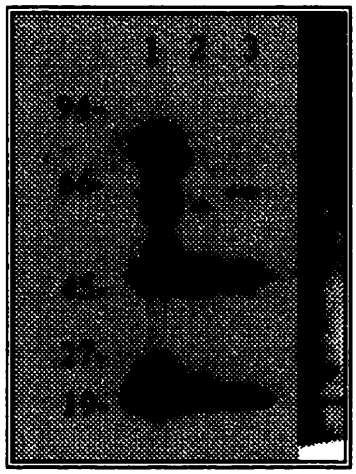

Figure 5.11. Western blot of GST/tPRLR-ECD fusion protein and tPRLR-ECD hybridized with anti-tPRLR-ECD serum. Lane 1 is the fusion protein. Lane 2 and 3 corresponding to 1 μg and 0.5 μg of fusion proteins cleaved with thrombin, respectively. Lane 4 is a membrane preparation from turkey kidneys. Molecular size is indicated (kDa).

the fusion protein with thrombin produced a 48 and a 19 kDa protein which strongly cross-reacted with the anti-sera (Figure 5.11, lane 2 and 3). The former is the ECD, whereas, the latter may be a derivative of the ECD. Separate blotting using anti-GST antisera showed only the 76 kDa fusion protein and the 26 kDa GST moiety post-thrombin cleavage (data not shown). No other bands were observed with this anti-sera. Two bands corresponding to approximately 95 kDa and 65 kDa were also detected by the antisera in the turkey kidney membrane preparation. The 95 kDa protein has a similar molecular mass with the calculated intact tPRLR and may represent the native tPRLR, whereas, the identity of the 65 kDa protein remains to be identified.

5.4.7. Characterization of the purified recombinant tPRLR-ECD

The purified ECD of the tPRLR migrated with an apparent molecular weight of 48 kDa on SDS-PAGE under reducing conditions (Figure 5.9), close to the size of 49.97 kDa predicted from the cDNA sequence. The purity of the recombinant tPRLR based on densitometric analysis of SDS-PAGE stained with Coomassie brilliant blue, was about 95% (Figure 5.9). The two major contaminants (2-4%) were proteins of about 24 and 28 kDa. They may represent the product of nonspecific thrombin cleavage.

Proper cleavage of the purified recombinant ECD of the tPRLR by thrombin was verified by amino terminal sequencing. The first 14 amino acids (Gly Ser Arg Arg Ala Ser Val Gly Ser Pro Gln Ser Phe Pro) were identical to the sequence expected from the cloning strategy (Figure 5.2), confirming the fidelity of the

thrombin cleavage reaction. In addition, minor peaks corresponding to amino acid Ser, Ala, Phe, Gln, Lys, Pro, Glu, Pro, Leu, Trp, Ile and Arg were also observed in cycle 1, 2, 3, 4, 5, 6, 8, 9, 10, 12, 13 and 14, respectively. Cycle 7 and 11 did not yield additional peaks. It was assumed that at cycle 7 and 11, the minor amino acids were the same as the major amino acids. Comparison of the minor amino acid sequences to the GST and tPRLR-ECD proteins did not reveal any sequence homology. Futhermore, homology to other proteins available in the Genbank was not found. The minor amino acid sequence may represent products of multiple contaminants from bacterial proteins or multiple nonspecific cleavage of the fusion protein by thrombin.

4.5. DISCUSSION

This is the first reported study on the production of PRL receptor using recombinant DNA technology in an avian species. The production of large quantities of recombinant tPRLR is desirable for the study of the PRL ligand binding and signalling mechanisms as well as facilitating the development of inhibitors against incubation behaviour in gallinaceous birds.

In this study we expressed and purified a recombinant tPRLR ECD using the GST-fusion system in *E.coli*. The PCR primers were designed to amplify a 1264 bp fragment from the ECD of tPRLR cDNA (nucleotide positions from 110 to 1373 bp: Zhou *et al.*, 1996). This product excluded the cDNA encoding the hydrophobic leader peptide, the transmembrane domain and the cytoplasmic domain and the protein was produced as a GST fusion protein (Smith and Johnson, 1988) with a molecular mass of 76.36 kDa. Expression as a GST fusion protein not only had the advantage of simplifying the purification by binding to an affinity matrix but fusion proteins are often more stable and soluble than the protein of interest itself (Marston, 1986). At first, we expressed the ECD using *E.coli* DH5α, however, the majority of the protein was present in insoluble form and the soluble fraction was degraded into smaller peptides. Although the addition of 1 mM PMSF to the lysis solution (Mintz 1993), suppression of the basal expression of the construct prior to the induction, short induction time with IPTG and the use of a mild lysis procedure improved the yield of the intact 76 kDa protein, over 90% of the soluble fusion protein was partially degraded. This was probably due to the protease

activity of the DH5α strain and thus, the IPTG-induced fusion protein was degraded. The use of a protease-deficient strain (BL21) did overcome the problem, however, the majority of the fusion protein was present in inclusion bodies in insoluble form. Similarly, in a study by Bignon *et al.* (1994), recombinant rabbit PRLR-ECD represented 70% of total cell protein but the soluble form was merely detectable by Western blotting analysis. It is not known why the majority of the recombinant protein was present in the inclusion body, however, the hydrophobicity of the receptor ECD may play a role in the insolubility of the recombinant protein. Indeed, since studies on the direct expression of recombinant ECD in mammals (Bignon *et al.*, 1994; Sandowski *et al., 1995)* indicated that the majority of the protein was localized in inclusion bodies, we chose a fusion protein system for expression. Although this strategy was very effective for the expression and purification of soluble recombinant tPRL (Karatzas *et al.*, 1993), the fusion of GST to the ECD of tPRLR did not improve solubility since less than 1% of the expressed fusion protein was in soluble form. Various studies have been reported that the formation of inclusion bodies can be reduced or eliminated by lowering the growing temperature of bacteria (Schein 1989; Schein and Noteborn, 1988), decreasing the concentration of IPTG and duration of induction (Pharmacia GST gene fusion system protocol) and increasing aeration (Schein 1989); presumably by affecting the kinetics and pathways of protein-folding (Schein and Noteborn, 1988; Mitraki and King, 1989). However, these alterations did not significantly improve the yield of the soluble fusion protein in the present study.

Soluble and active proteins have been successfully purified from inclusion bodies (Marston *et al.*, 1984; Schoner *et al.*, 1985). Therefore, we decided to purify the fusion protein from inclusion bodies by denaturing and refolding the fusion protein, followed by GST affinity chromatography. The fusion protein in inclusion bodies was solubilized using 4.0 M urea at pH 9. At this condition, the majority of the bacterial insoluble protein remained insoluble, whereas, the yield of the fusion protein in the supernatant fraction increased about 7.5 fold. The tPRLR contains two tandem repeats in the ECD and each repeat has two pairs of cysteine residues that are conserved among the cytokine receptor superfamily (Kelly *et al.*, 1992). In addition, repeat 1 and 2 have an additional 1 and 2 cysteine residues, respectively, leading to at least one cysteine residue being unpaired. It is well-known that unpaired cysteine residues can have an adverse effect on proper refolding (Martson 1988). To overcome this difficulty, we exposed the fusion protein to urea for only a short time and at high pH in the presence of free cysteine to facilitate the refolding process. The refolded fusion protein was then purified by GST affinity chromatography and the tPRLR-ECD protein was released by proteolytic cleavage. The yield of the fusion protein was about 20 mg per litre culture with an estimated purity of about 90%. A comparison of this material under both non- and denaturing conditions indicated that about 20 % was present in monomeric form. Thus, it is is likely that oligomeric forms are present which may arise from the formation of incorrect disulfide bonds. The latter can be easily separated using gel filtration chromatography (Sandowski *et al.*, 1995).

Proper cleavage of the expressed tPLR-ECD was verified by amino terminal sequencing and the first 11 amino acids were identical to the sequence expected from the cloning strategy. However, a minor sequencing signal indicating the presence of a contaminating protein was evident from the analysis. The identity of this peptide is unknown though it is unlikely to be related to the GST moiety since no hybridizable signal was detectable using anti-GST antisera in Western blots.

Although the overall yield of the fusion protein was 20 mg/l, the yield of the ECD was only 0.2 mg per litre with 95% purity. The majority of the tPRLR-ECD protein precipitated in the column following digestion of the fusion protein with thrombin. Since about 80 % of the fusion protein did not refold properly, the latter may not be surprising. In addition, additional tPRLR-ECD protein was lost during the concentration process. Similarly, Sandowski *et al.* (1995) reported that about 90 % of the recombinant PRLR-ECD produced from the rat did not refold properly and that additional protein losses occurred when the monomeric protein was concentrated. In the case of the turkey, the ECD is about 2 fold larger than in mammals and has a structure which may acerbate solubility problems. Nevertheless, preliminary measurements of binding activity to PRL using both the BIA COR apparatus (personal communication; Morvin *et al.*) and radiolabelled tPRL indicate that the recombinant ECD has specific binding.

The purified recombinant tPRLR-ECD was used to produce polyclonal antibodies in a rabbit. The antisera was able to react with both the tPRLR-ECD-GST fusion protein and the released tPRLR-ECD protein. No cross reactivity with

BL21 bacterial proteins was observed. In addition, Western blotting of kidney membrane preparations from turkeys indicated that a band corresponding to the predicted molecular weight of the intact receptor was detectable. An additional band with an estimated molecular mass of 65 kDa was detected by the antisera. In mammals, multiple forms of PRLRs have been found (Boutin *et al.*, 1988; 1989; Edery *et al.*, 1989). They differ in the amino acid composition and size of their cytoplasmic domain. A recent study (Berlanga *et al.*,1997) has shown that these forms of PRLRs have distinct biological functions and are regulated in a tissue-specific manner. Our previous study (Zhou *et al.*, 1996) and data from other reports (Chen and Horseman, 1994; Pitts *et al.*, 1995) have suggested that multiple forms of tPRLR transcripts may exist in turkeys. However, further studies are required to determine whether the additional protein on the Western blot of turkey kidney membrane preparation is a second form of tPRLR. The antibody developed in this study maybe proven useful for clarifying the possibility of multiple forms of avian PRLRs and for *in situ* staining proceedures, especially in the brain. PRLRs are known to exist and to be regulated by blood levels of PRL in the brain (Zhou *et al.*, 1996). The discovery of which neurons have the receptor would provide considerable knowledge as to how PRL is regulated and how behavioural pathways interact with PRL.

The expression and purification method reported here will allow the production of large amount of recombinant tPRLR-ECD-GST fusion protein and tPRLR-ECD protein. The availability of the fusion protein and the ECD protein will

provide necessary material for the study of the receptor binding characteristics and for searching for effective inhibitors to prevent the expression of incubation behaviour in galliforms.

Connecting Statement IV

In the previous chapter, we have described the expression, purification and characterization of the turkey prolactin receptor extracellular domain and its fusion protein with GST in *E.coli*. The availability of large amounts of tPRLR extracellular domain GST fusion protein provided the starting material for searching potential inhibitors of broodiness in turkeys. The following chapter describes the effects of active immunization against the recombinant derived tPRLR extracellular domain in turkey hens on egg laying, body weight, levels of plasma prolactin and incidence of broodiness.

Chapter 6

Active Immunization Against the Recombinant-derived Extracellular Domain of the Prolactin Receptor in the Turkey

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6.1. Abstract

Prolactin may play a key role in the manifestation of incubation behaviour in galliforms. In this experiment, we tested the effects of active immunization against a recombinant derived tPRLR-ECD fusion protein in turkeys on body weight, egg laying, levels of serum PRL and incidence of incubation behaviour. Two adjuvants (mineral oil vs metabolizable oil) were tested for their effectiveness in hens raised in cages. No significant difference was found among all parameters when the metabolizable oil was used as the adjuvant. The active immunization did not affect body weight and onset of egg laying in hens raised in cages or floor pens and immunized using mineral oil as adjuvant. However, the egg laying intensity and the level of plasma PRL were higher in the experimental group than the control group when hens were confined in individual cages. Hens also had a higher incidence of incubation behaviour when the nesting environment was provided. These results suggest that active immunization against the tPRLR-ECD may not prevent the occurrence of incubation behaviour in turkeys, but could increase egg production when the expression of the behaviour was prevented.

6.2. Introduction

Broodiness is one of the major phases of the reproductive cycle in gallinaceous birds. Although the broody trait was traditionally selected for in domestic birds, it is no longer of practical interest since artificial incubation is exclusively used in the poultry industry. In fact, the occurrence of broodiness represents a major source of economic loss in the modern poultry industry due to its negative correlation with egg production (Zadworny *et al.*, 1986). Therefore, an effective method to prevent or treat this behaviour is highly desirable.

The incidence of broodiness might be reduced partly by genetic selection against the trait. However, 10 to 70% of the turkey hens from a typical laying flock still become broody in today's poultry industry (Guémené, 1992). Traditional management programs are effective to prevent or disrupt broodiness, but are time consuming, labour intensive and expensive. In addition, the disruption of broodiness by physical methods may decrease egg production since the broody phase of many hens overlaps with the egg laying phase (Guémené and Etches, 1990). Pharmacological approaches including enhancing steroidogenic activities (Haller and Cherms, 1961; Sharp and Sterling, 1986), increasing LH secretion (Robinzon *et al.*,1984; Renner *et al.*, 1987; Guémené and Etches, 1989) or decreasing PRL secretion (Millam *et al.*, 1980; El Halawani *et al.*, 1983) have been tested to prevent or treat this maternal behaviour, but none has been proven satisactory.

Since PRL may play a key role in the manifestation of the incubation

behaviour in galliforms (Riddle *et al.*, 1933; Saeki and Tanabe 1955; El Halawani *et al.*, 1986), novel trials have been conducted to block the PRL signalling pathway in order to prevent the occurrence of the behaviour. An early study using anti-prolactin serum successfully lowered the plasma PRL and increased the level of serum LH, but did not disrupt broodiness in bantams (Lea *et al.*, 1981). More recently, it has been shown that active immunization against recombinant-derived PRL reduced the incidence, delayed the development (March *et al.*, 1994), or prevented the occurrence (Guémené *et al.*, 1995) of incubation behaviour in galliforms. However, the underlining mechanism by which the neutralization of serum PRL prevents the hens from expressing broodiness is not known and the effectiveness of the method has not been compared with traditional methods. To date, no study has been reported to prevent or treat broodiness by blockage of the PRL signal transduction pathway at its receptor level.

In this study, we investigated the effects of the active immunization against the recombinant tPRLR-ECD-GST fusion protein derived from *E.coli* on egg laying performance, body weight, levels of plasma PRL and incidence of broodiness in turkey hens.

6. 3. MATERIALS AND METHODS

6.3.1. Animals and antigen

Experimental animals in this study were British United Turkeys. They were raised under standard conditions in floor pens. At 22 weeks of age the hens were randomly assigned to treatment groups. Food and water were provided *ad libitum.*

The preparation of the tPRLR-ECD-GST fusion protein was described in the previous chapter. The purified fusion protein was lyophilized in aliquotes of 5 mg from 1 ml solution containing 25 mM Tris.HCl (pH 9.0). The protein was kept in the lyophilized powder form at 4 °C till use. A mineral oil based (ISA70) and metabolizable oil based (ISA763) adjuvants were used in the immunization trials.

6.3.2. Experiment 1

The purpose of this experiment was to study the effects of active immunization against the recombinant derived tPRLR-ECD fusion protein on the egg laying, body weight and secretion of prolactin in an environment where incubation behaviour was prevented (i.e. cages). Twenty two-week-old hens were caged individually and maintained on a short day photoperiodic regime (6 hours light:18 hours darkness). Two groups of hens (n=6) were intradermally injected with 100 µg of fusion protein emulsified with 500 µl of mineral oil based and metabolizable oil based adjuvants, respectively. Two control groups (n=6) were injected with 500 µl of the respective adjuvants. A total of four injections were carried out during 4 consecutive weeks (22-25 weeks of age). The hens were photostimulated (14 hours light: 10 hours darkness) to induce egg laying at 28

weeks of age. Three booster injections with a dosage of 100 µg of the fusion protein were performed at 30-, 34- and 38-weeks of age intramuscularly. The body weight of the hens was measured at 22-, 28-, 32-, 36-, 40-, 45-, 52- and 56-weeks of age (±0.01kg), whereas, egg production was recorded daily. Blood samples (5ml) were collected by brachial venipuncture into heparinized tubes at 22-, 25, 28-, 30-, 32-, 34-, 36-, 38-, 40-, 43-, 46-, 49-, 52- and 55-weeks of age. Following centrifugation, the plasma was collected and stored at -20 °C until radioimmunoassay for PRL.

The radioimmunoassy used in the present study was previously described (Guémené *et al.*, 1992). Briefly, 5 µg of recombinant turkey PRL (rctPRL) was radiolabelled using 16.5 MBq of ^{125}I and 20 to 40 µl of chloramine T (60 µg/ml in 0.25 M PBS). Incorporation rate ranged from 45 to 68%. Free iodine and labelled rctPRL were separated by chromatography on a poly-acrylamide biogel column (Bio-Rad Laboratories, Richmond, California, USA). Assay tubes (12x75 mm disposable polypropylene tubes) contained rctPRL standards or samples diluted in PBS assay buffer (300 µl total volume, containing 20 mg/ml equine serum albumin and 30 mg/ml EDTA). The standards were serial exponential dilutions of rctPRL and ranged from 50 to 0.2 ng. One hundred µl of antibody (1:16000 dilution) was added on the first day to all tubes except in tubes used to assess non-specific binding. After an overnight incubation at 4 °C, 200 µl of the labelled hormone (15000 cpm) was added and the mixture was incubated for an additional 24 hours at 4 °C. One hundred µl of sheep anti-rabbit serum (1:10 in PBS) and 100 µl of

normal rabbit serum (1:200 in PBS) were added to each tube and the mixture was further incubated for 72 hours at 4 °C prior to the addition of 750 µl of assay buffer and centrifugation at 3000 x g for 25 minutes at 4 °C. The pellets were resuspended with 750 µl of PBS and centrifuged at 3000 x g for 15 minutes at 4 °C. The radioactivity present in the pellets were counted for 1 minute using a gamma counter (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). All samples were assessed in triplicate in a single RIA and the intra assay coefficient of variation was 4.5%.

6.3.3. Experiment 2

The objective of this experiment was to study the effect of the active immunization against the recombinant derived tPRLR-ECD fusion protein on body weight, egg laying and incubation behaviour of hens in an environment where incubation behaviour was encouraged. Twenty two-week-old hens (n=16) were kept in floor pens (18 m^2) covered with wood shavings. Five nests were placed in the pen and 4 mock eggs were provided in each nest. Hens were subjected to a short day photoperiodic regime with 6 hours light and 18 hours darkness per day. One group of hens (n=16) was intradermally injected with 100 µg of fusion protein emulsified with 500 µl of mineral oil based adjuvant and one control group (n=16) were injected with 500 µl of the adjuvant only. A total of four injections were carried out during 4 consecutive weeks (22-25 weeks of age). The hens were photostimulated (14 hours light:10 hours darkness) to induce egg laying at 28 weeks of age. Three booster injections with the dosage of 100 µg of the fusion

protein were performed at 30-, 34- and 38-weeks of age intramuscularly. The body weight of the hens was measured at 22-, 28-, 32-, 36-, 40-, 45-, 52- and 56-weeks of age (±0.01kg), whereas, egg production was recorded daily. The nesting behaviour was recorded 4 times a day at 09:00 h, 11 :00 h, 14:00 h and 16:00 h. Hens were determined to be in incubating if they nested at least 3 times out of the 4 daily inspections during 3 consecutive days.

6.3.4. Statistical analyses

Statistical analyses were carried out by standard analyses of variance or Student's *t* test as appropriate. The Fisher exact probability test was used for a comparison of the number of birds displaying incubation behaviour in each treatment group.

6.4. RESULTS

6.4.1. Experiment 1

6.4.1.1. Effect of active immunization with the recombinant tPRLR-ECD-GST protein on the growth and physical conditions of the hens in individual cages.

Hens kept in individual cages were weighed at the age of 22, 28, 32, 36, 40, 45, 52 and 56 weeks. The body weight of the experimental birds was not significantly different from that of the control birds. No difference in body weight was observed between the two adjuvant injected groups. All hens remained healthy and no detrimental effects of the immunization on the birds was observed throughout the experiment. Table 6.1 lists the body weight of hens raised in individual cages.

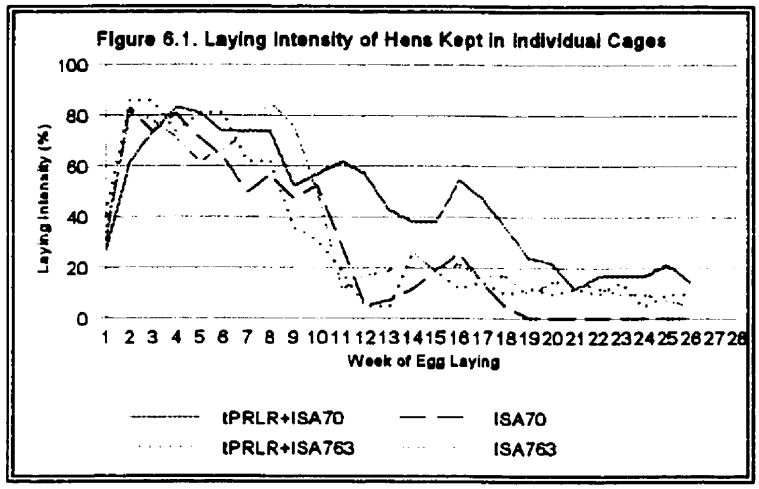
Table 6.1. Body Weight (kg) of Hens Raised in Individual Cages

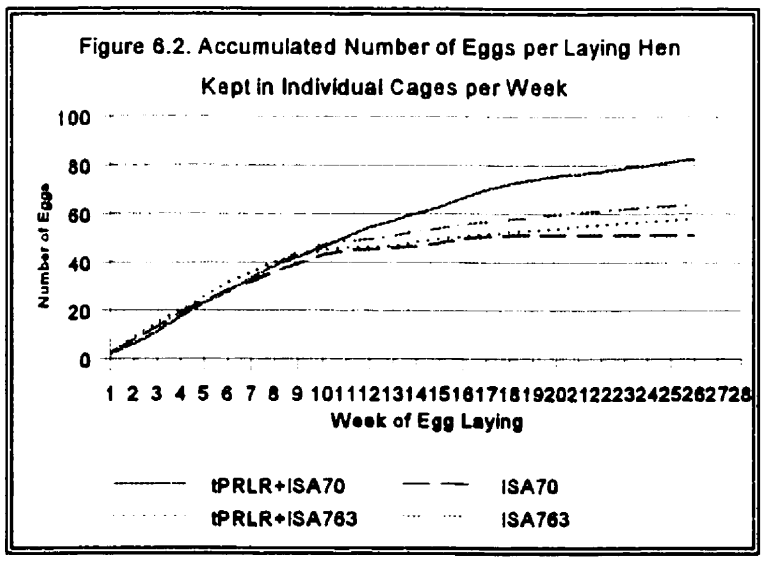
		Age of Hens (week)							
		22	28	32	36	40	45	52	56
Treatment (n=6)	ISA70	7.63±0.62	9.4±0.2	9.9±0.2	9.3±0.1	9.2±0.1	9.7±0.2	10.5±0.2	10.6±0.3
	Fusion+ ISA 70	7.88±0.50	9.3±0.2	10.0±0.2	9.8±0.2	9.7±0.3	9.6±0.2	9.8±0.2	9.5±0.5
	ISA763	8.12±0.41	9.4±0.2	9.9±0.2	9.5±0.1	9.1±0.2	9.2±0.4	9.5±0.4	10.2±0.2
	Fusion+ ISA 763	8.0+0.3	9.4±0.2	9.7±0.2	9.3±0.3	9.0±0.3	9.1±0.2	10.0±0.3	10.6±0.3

6.4.1.2. Effect of active immunization with the recombinant tPRLR-ECD-GST fusion protein on egg laying performance of hens kept in individual cages.

All hens laid eggs after photostimulation, with the first eggs being laid around day 17 after photostimulation. When hens were raised in individual cages, hens

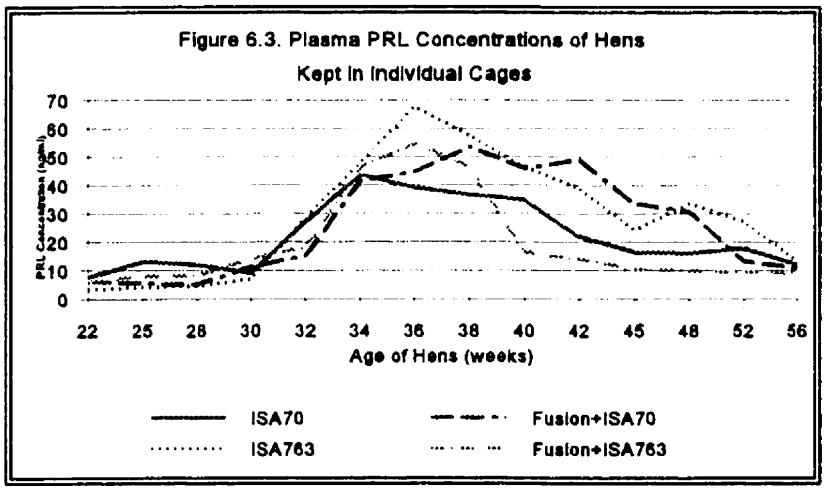
immunized with the fusion protein using the mineral oil based adjuvant had higher ($p < 0.05$) laying intensity (Figure 6.1.) than the hens injected with the mineral oil alone from 41 weeks of age (11th week of egg laying) to the end of the experiment, whereas no difference was observed between the two groups from 31 to 40 weeks of age. As a result of the higher laying intensity, total egg production in the group immunized with the fusion protein was higher than that in the control group during the same period ($P < 0.05$)(Figure 6.2.). The laying intensity and accumulated egg numbers in the hens immunized with the fusion protein using the metabolizable oil based adjuvant were not significantly different from the control group which was only injected with the adjuvant (Figure 6.1. and 6.2.).





6.4.1.3. Effects of active immunization with the recombinant tPRLR-ECD-GST fusion protein on the levels of plasma PRL in hens kept in individual cages.

The mean levels of plasma PRL were 5.75±0.37, 10.71±2.87, 6.87±1.38 and 3.56±0.65 ng/ml in hens injected with the fusion protein emulsified with mineral oil, mineral oil alone, the fusion protein emulsified with metabolizable oil and metabolizable oil alone, respectively. The levels of plasma PRL started to increase when the hens were at the onset of egg laying. Around 36 weeks of age, hens reached the highest levels of plasma PRL. The levels of plasma PRL are not significantly different between experimental and control groups (Figure 6.3).



6.4.2. Experiment 2

6.4.2.1. Effect of active immunization with the recombinant tPRLR-ECD-GST protein on the growth and physical conditions of the hens kept in floor pens.

Hens kept in the floor pens were weighed at the age of 22, 28, 32, 36, 40, 45, 52 and 56 weeks. The body weight of the experimental birds was not significantly different from that of the control birds. All hens remained healthy and

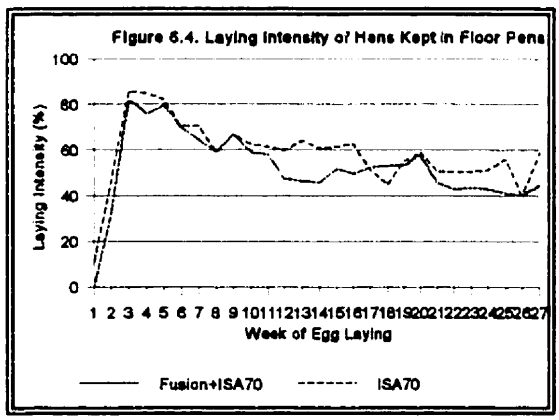
no detrimental effects of the immunization on the birds was observed throughout the experiment. Table 6.2 lists the body weight of hens kept in floor pens.

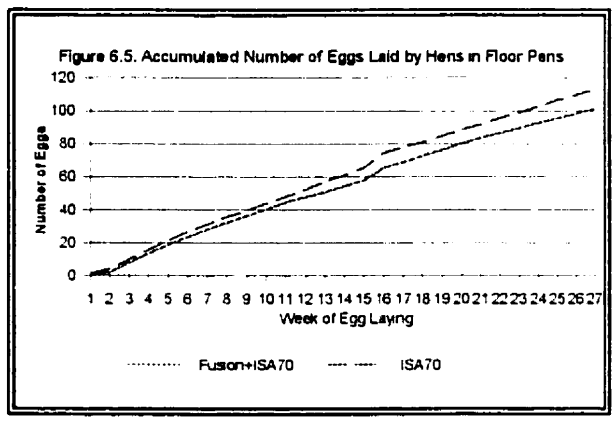
Table 6.2. Body Weight (kg) of Hens Kept in Floor Pens

		Age of hens (week)							
		22	28	32	36	40	45	52	56
Treatment (n=16)	Fusion +ISA70	7.73±0.14	9.18±0.15	9.58±0.17	9.26±0.19	8.83±0.24	8.62±0.26	9.12±0.24	9.54±0.18
	ISA70	7.84±0.14	9.31±0.14	9.69±0.15	9.21±0.17	8.75±0.23	8.73±0.26	9.36±0.2	9.77±0.18

6.4.2.2. Effect of active immunization with the recombinant tPRLR-ECD-GST fusion protein on egg laying performance of hens kept in floor pens.

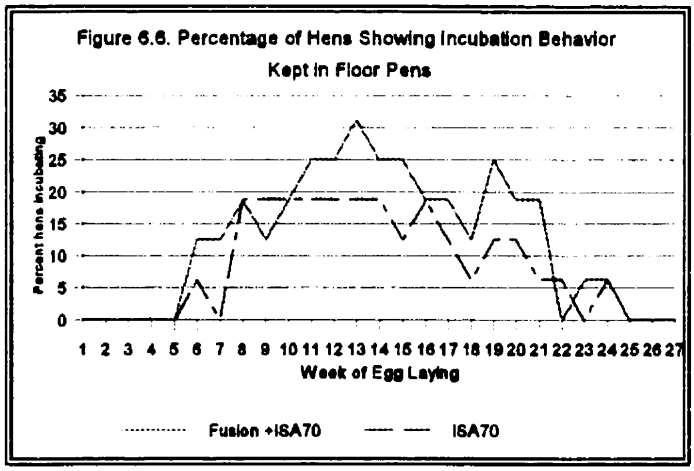
At week three, both experimental and control groups reached the maximum egg laying intensity (80% of hens). There were no significant differences between the two groups in terms of either egg laying intensity (Figure 6.4) or the accumulated numbers of eggs (Figure 6.5).





6.4.2.3. Effect of active immunization on the expression of incubation behaviour

To assess the effect of the immunization with the recombinant derived fusion protein on the incidence of incubation behaviour, birds were kept in floor pens. Broodiness started at 35 weeks of age (after the 4th week of egg laying) in both the experimental and control groups. In the experimental group, broodiness reached the highest level at 42 weeks of age (week 13 of egg laying) with 31% of the hens showing incubation behaviour. From 40 to 50 weeks of age (week 11 to 21 of egg laying), hens in the experimental group had higher incidence of broodiness than the control group ($p < 0.05$).



6.5. DISCUSSION

Generating antibodies against specific endogenous protein has been an often applied strategy to study protein function in animals. The major requirement for the success of this type of approach is that the target protein has to be immunogenic for the animal. Many factors could affect the immunogenicity of the antigen, such as the conformation of the protein and the adjuvant used for the immunization. Since turkey PRLR is in essence a 'self-protein', it would not be an effective immunogen for turkeys. For this reason, the tPRLR ECD was produced as a fusion protein with GST to enhance the immunogenicity of the protein. Such an approach to enhance the immunogenicity by linking the antigen to a foreign protein has successfully generated antibody against many proteins of interest (Nash *et al.*, 1980; Marsh *et al.*, 1994; Guémené *et al.*, 1994). Previous studies (Guémené *et al.*, personal communication) on the active immunization against tPRL fused with GST also indicated that different adjuvants may elicit different immune responses. In their study, the mineral oil based adjuvant (ISA70) had higher and longer immuno-stimulating effects on the response to the fusion protein than the metabolizable oil based adjuvant (ISA 763). Similarly, we noted a difference in response to the 2 adjuvants used in the current experiment. Only the injection of fusion protein emulsified in the mineral based adjuvant produced a significant effect on the rate of egg production (Figure 6.1), accumulated number of eggs (Figure 6.2) and levels of plasma PRL (Figure 6.3) in hens which were maintained in cages. Whether or not these effects are due to differences in the titre of antibodies to the

tPRLR-ECD is currently unknown. In addition, 80% of the purified fusion protein was present in ologomeric for, which may have different antigenic properties from the monomeric form. Antibodies raised against these forms of the fusion protein may not recognize the natural tPRLR *in vivo.* However, antibodies against the tPRLR-ECD raised in the rabbit has been shown to cross-react with the endogenous tPRLR from kidney membranes (Chapter 5), thus it is likely that the fusion protein would result in an antigenic response in the turkey hens. Currently, an assay is under development to assess the structural and functional properties of the antibody raised in turkey hens.

It was anticipated that the neutralization of the tPRLR might result in detrimental effects on the turkey, since PRL has been found to have more than 100 biological functions and some of the functions are essential for the turkey. However, no adverse effects were observed in all experimental animals. Although one hen died in the group which was injected with the fusion protein, the death of this animal may not be the result of the fusion protein, since one hen also died in the control group where no fusion protein was injected. The onset of the egg laying and the body weights of the experimental animals were not different from control birds. However, the intensity of egg laying in the experimental animals was significantly increased when they were confined in individual cages and incubation behaviour was enhanced when birds were raised in the environment that encourages the expression of incubation behaviour. These findings indicate that the active immunization against the PRLR may enhance the PRL signalling

capacity rather than block the pathway. Although the result is unexpected, the findings were in agreement with the recent finding regarding the PRL and GH signalling mechanism in mammalian species (Fuh *et al.*, 1992, 1993). In the PRL and GH signalling process, it is essential that one molecule of ligand first binds with 2 receptor molecules and forms a dimer complex. Therefore, the ratio of the ligand to the receptor is an important factor in the cell response to the hormone (Fuh *et al.*, 1992, 1993). The partial neutralization of the receptor could change the ratio of hormone to the available receptors on the cell surface. Consequently, the cell activities in some tissues may be enhanced if the ratio of the hormone to the receptor favours signal transduction, whereas, cell activities in other tissues may be decreased. As a result, separate pathways associated with either incubation behaviour or ovarian folliculogenesis could be stimulated dependent on the environment cues presented to the hen. In addition, it is possible that the neutralization of the receptor itself may act directly as a stimulus to the cell response. It has been reported that some monoclonal antibodies to the rat PRL receptor which can dimerize the receptor are known to be weak agonists of PRL (Elberg *et al.*, 1990).

Since the increased intensity of egg laying is correlated with the elevation of the serum PRL in the same period, it is also possible that the enhanced physiological activity of hens in terms of egg laying intensity is the result of elevated serum PRL. Whether the higher incidence of incubation behaviour in the hens kept in floor pens also correlated with the elevation of the serum PRL in the cage group

has to be confirmed.

Although positive correlation between egg laying intensity, serum PRL or incubation behaviour and the active immunization against the tPRLR-ECD was observed in this experiment, the results are preliminary. Further studies are required to confirm the presence of the anti-tPRLR-ECD antibody, measure the level of such antibody and determine the functionality of the antibody.

CHAPTER 7. GENERAL CONCLUSIONS

The occurrence of incubation behaviour in galliforms represents a major source of economic loss in the modern poultry industry due to its negative correlation with egg production. The search for efficient methods to prevent or treat this behaviour is hampered by the lack of understanding of the cause. Although elevated levels of PRL have long been associated with the manifestation of the behaviour, it is not known whether high levels of PRL are the cause or the consequence of incubation. In addition, the mechanism by which PRL elicits numerous biological actions through its cell membrane anchored receptors is not well understood. Therefore, we characterized the turkey prolactin receptor cDNA in this study.

Cloning of the turkey prolactin receptor cDNA revealed detailed information of tPRLR at the molecular level. The general structure of tPRLR is similar to its counterparts in mammalian species. It consists of an intracellular domain, a transmembrane domain and an extracellular domain. Like other avian PRLRs, the extracellular domain of the receptor contains two tandem repeats. Each repeat unit has all structural characteristics similar to the singular mammalian PRLR-ECD. The importance of this unique structure is not known.

The characterization of the tPRLR cDNA also provided a valuable tool for studying the tissue distribution and expression of tPRLR in turkeys. It was found that tPRLR is ubiquitously expressed in turkey tissues, however, Northern blotting

was not sensitive enough to assess variations in the level of tPRLR transcripts. We therefore developed a semi-quantitative PCR based assay to compare the levels of tPRLR expression in various tissues at different physiological stages during the reproductive cycle in turkeys. The data obtained in this study supported the hypothesis that PRL itself may participate in the neuroendocrine control of incubation behaviour through actions on both the hypothalamus via a short-loop feedback mechanism and the pituitary gland via autocrine and/or paracrine effects.

The isolation of the tPRLR cDNA allowed us to produce a recombinant tPRLR-ECD protein. Methodologies have been developed to express the tPRLR-ECD as a cleavable GST fusion protein in *E.coli*. We have also successfully purified the fusion protein and tPRLR-ECD protein to high purity. The availability of large amounts of fusion protein and tPRLR-ECD could provide necessary material for the study of receptor binding characteristics and searching for effective inhibitors to prevent incubation behaviour in birds. An antisera against the recombinant tPRLR-ECD was produced in rabbits and was able to recognize the endogenous tPRLR from the turkey kidney. This antibody may be proved useful for the further study of the PRL signal transduction pathway.

In this study, we also tested the effects of active immunization with the recombinant tPRLR-ECD-GST protein on the body weight, physiological conditions, egg laying performance and incidence of the incubation behaviour in turkeys. Preliminary data suggested that active immunization against the tPRLR-ECD may not prevent the occurrence of incubation behaviour in turkeys, but could increase

egg laying production if incubation behaviour was discouraged.

The results of this study have contributed to the understanding of the mechanism by which PRL elicits its biological actions in avian species. However, further studies are necessary for complete delineation of the signal transduction pathway. Future studies may focus on the receptor binding characteristics, the importance of the unique structure in the extracellular domain and the search for effective inhibitors of incubation behaviour in galliforms.

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