Transcriptional Regulation of the Prolactin Gene in Turkeys

by

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Abstract

TRANSCRIPTIONAL REGULATION OF THE PROLACTIN GENE IN TURKEYS

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Poor reproductive performance by turkey hens compared with chickens is partially due to the early cessation of egg production associated with the onset of incubation behavior. Prolactin (Prl) is involved in the induction and maintenance of incubation behavior in birds, and understanding the regulatory mechanism(s) of Prl gene expression will provide fundamental information to manipulate Prl production for better reproductive performance in turkey hens.

To better understand the regulatory mechanism of Prl gene expression, the turkey Prl gene was isolated from a λ phage genomic library using a turkey Prl cDNA probe. The turkey Prl gene consists of five exons and spans approximately 6.7 kilobases (kb). The arrangement of the exons was found to be nearly identical to the rat and human Prl genes, showing the conserved feature of the gene among these species.

Two regions similar to the binding sites for the transcription factor Pit-1/GHF-1 were found within 2 kb of the 5'-flanking region of the Prl gene, while no estrogen response element (ERE) was found. This suggests that transcription of the turkey Prl gene may be directly regulated by Pit-1/GHF-1, and not by the estrogen receptor.

In order to identify regulatory elements for turkey Prl gene expression, a rat pituitary-derived tumor cell line, GH_3 and primary turkey pituitary cells were transiently transfected with luciferase reporter gene constructs containing the 5'-flanking region of the

turkey Prl gene. In the GH_3 cell system, the results indicated that negative-acting elements may be present between the -2.0 and -1.3 kb region and a positive-acting element between the -1.3 and -1.0 kb region of the turkey Prl gene. The decreased promoter activity resulting from the elimination of one or two Pit-1/GHF-1 binding sites strongly suggests that the Pit-1/GHF-1 transcription factor plays an essential role for turkey Prl gene expression. To test the regulatory elements in a native system, a primary turkey pituitary cell culture was prepared for transient transfection. Despite repeated trials, no consistent reporter gene expression was obtained from cells transfected with the reporter gene constructs containing the turkey Prl promoter.

The results of the GH_3 cell transfection experiment indicated that the Pit-1/GHF-1 transcription factor plays an essential role for turkey Prl gene expression. Three cDNAs encoding turkey Pit-1/GHF-1 isoforms, referred to as tPit-1*, tPit-1β* and tPit-1W*, were isolated from anterior pituitaries of turkey hens, and the ability of each tPit-1 isoform to activate the turkey Prl promoter was examined in mouse Ltk- cells. Both tPit-1* and tPit-1β* isoforms use the transcription start site (tss) of exon 1, while tPit-1W* uses the tss of exon 2, thus, lacking exon 1. Relative to tPit-1*, tPit-1β* has an additional 28 amino acids at the N-terminal domain due to the usage of an alternative splicing site 84 nt upstream from the splicing site of exon 2 used for tPit-1*. All three isoforms appeared to promote transcription of the turkey Prl gene, but tPit-1β* and tPit-1W* activated the Prl promoter to a lesser extent than tPit-1*. Clearly defined differential trans-activation effects of these tPit-1 isoforms were not obtained in the mouse Ltk- cell system.

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5HT	serotonin
5HTP	5-hydroxytryptophan
ACTH	adrenocorticotropic hormone
AMV	avian myeloblastosis virus
APO	apomorphin
ASV-LTR	avian sarcoma virus-long terminal repeat
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CMF-HBSS	Ca^{2+} and Mg^{2+} free-Hank's basic saline solution
CMV	cytomegalovirus
DA	dopamine
DAG	1,2-diacylglycerol
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle medium,
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol tetraacetic acid
ER	estrogen receptor
ERE	estrogen response element
FSH	follicle stimulating hormone
GH	growth hormone
GRF	growth hormone releasing factor
IP3	inositol triphosphate
LH	luteinizing hormone
Luc	luciferase
MMLV	moloney murine leukemia virus
PCPA	p-chlorophenylalanine
PKA	protein kinase A
PKC	protein kinase C
PL	placental lactogen
Prl	prolactin
RACE	rapid amplification of cDNA ends
RLU	relative light unit
RT-PCR	reverse transcriptase-polymerase chain reaction
SV40	simian virus 40
TPA	tetradecanoylphorbol acetate
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
VIP	vasoactive intestinal peptide
VIP-IR	vasoactive intestinal peptide-immunoreactivity

CHAPTER I

Literature Review

A. Introduction

Biological actions of prolactin (Prl) among the vertebrates are diverse, ranging from lactogenesis in mammals to osmoregulation in fish to induction of incubation behavior in avian species. Yet, phylogenetic conservation of Prl can be found in its tissue-specific expression, molecular structure and regulatory mechanism of gene expression. This review will discuss molecular aspects of Prl, the physiological function of Prl in avian species, and the regulatory mechanism of Prl gene expression, as well as the characteristics of Pit-1/GHF-1, a key transcription factor for Prl gene regulation.

B. Prolactin and Prolactin Genes.

The anterior pituitary gland develops from an ectodermal bud that protrudes from the roof of the oral cavity and becomes juxtaposed to a neuroectodermal extension from the base of the brain, the posterior pituitary. Endocrine cells develop within the anterior pituitary that are specialized in the synthesis and secretion of polypeptide hormones; growth hormone (GH), prolactin (Prl), luteinizing hormone (LH), follicle stimulating hormone

(FSH), adrenocorticotropic hormone (ACTH), and thyroid stimulating hormone (TSH). GH-producing somatotrophs and Prl-producing lactotrophs are the last two phenotypically distinct cell types to appear during development, and transient co-expression of GH and Prl occurs in a subset of somatotrophs prior to the appearance of distinct lactotrophs (Frawley and Boockfor, 1991; He *et al.*, 1989; Simmon *et al.*, 1990; Watanabe and Daikoku, 1979). In addition to lactotrophs in the anterior pituitary, Prl or Prl-like molecules are found in a variety of tissues, including the placenta, uterus, brain and spinal cord (Sinha, 1995). Prl is also produced in various cell types of the immune system and serves as an autocrine growth factor to modulate the function of the immune system (Gala, 1991). Nevertheless Prl found in the circulation is mostly of pituitary origin (Sinha, 1995).

Prolactin is structurally similar to growth hormone (GH) and placental lactogen (PL), and these hormones are thought to have arisen from a common ancestral gene by gene duplication at least 400 million years ago (Miller and Eberhardt, 1983). These hormones are similar in size (190-199 amino acids) and have similar protein structures with four homologous internal regions and two homologous disulfide bonds (Niall *et al.*, 1972). An identical number of exons, introns and similar splice sites among the known mammalian Prl, GH and PL genes provide additional evidence that these three genes evolved from a common precursor (Miller and Eberhardt, 1983). Human PL (hPL) appears to have been derived from one of the hGH genes, while rat and bovine PLs appear to have been derived from Prl genes rather than GH genes (Nicoll *et al.*, 1986).

The complete amino acid sequences of Prls from more than 25 vertebrate species have been determined either by protein sequencing or by complementary DNA sequencing (reviewed by Sinha, 1995). In mammalian species, Prls consist of 197-199 amino acids. A cDNA encoding turkey Prl has been isolated and the deduced amino acid sequence has been compared with chicken and other mammals (Wong *et al.*, 1991). Turkey Prl consists

of a 30 amino acid signal peptide followed by a 199 amino acid mature Prl and shows 90% homology to chicken Prl and 54-78% homology to other mammalian Prls.

C. Physiological Role of Prolactin in Birds

C-1. Prolactin and Incubation Behavior.

Sexually mature domestic turkey hens begin laying eggs after approximately 3 weeks of photostimulation (e.g., increase in day light length from 6 h to 14 h per day) Studies on physiological changes during early photostimulation revealed increased plasma levels of gonadotropins (i.e., luteinizing hormone (LH) and follicle stimulating hormone (FSH)) within 2 days of photostimulation (reviewed by Etches, 1990; Sharp, 1989a). The increased gonadotropins stimulate the growth of the ovarian follicles, which in turn secrete estrogens and progesterone. Establishment of the positive feedback between the ovarian steroids and gonadotropins further augments the growth of the ovarian follicles, leading to the onset of egg production. Egg production increases rapidly during the first 5 to 8 weeks after the first egg is laid, reaches a maximum and thereafter declines. The decline in egg production is often associated with lower levels of plasma gonadotropins and steroids. The lower gonadotropin levels in laying hens maintained under stimulatory daylight is likely due to the development of photorefractoriness. The ovarian steroids/gonadotropins positive feedback mechanism, which supports continuous egg production progressively disappears and the gradual decline in circulating gonadotropins leads to the end of egg production.

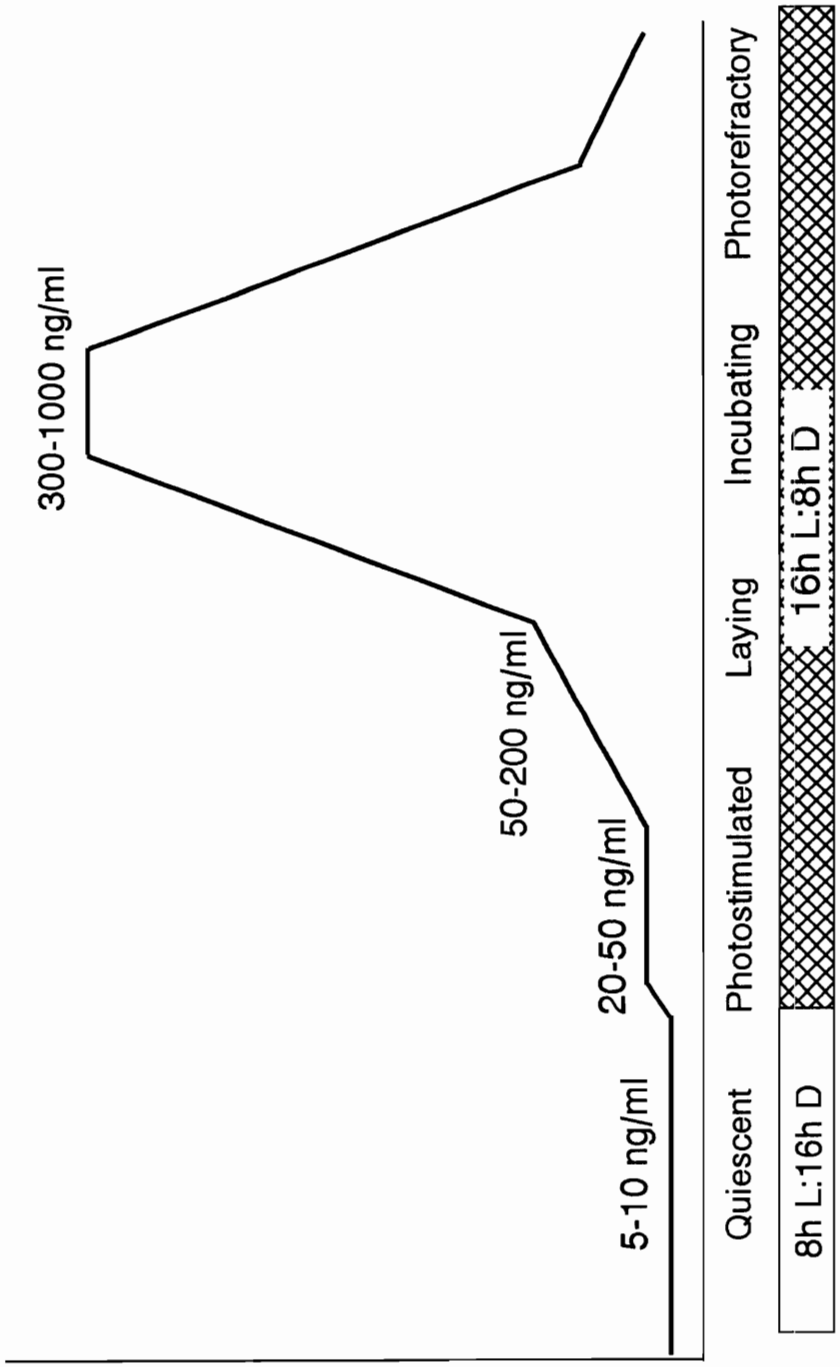
The decline in egg production in turkey hens is often associated with an increase in nest visits. When frequent nest visits are allowed, the hens cease egg production and devote their time to the nest, entering the incubation phase. Studies on physiological changes during the transition period from the laying to the incubation phase have suggested

that Prl is involved in the induction and maintenance of incubation behavior (El Halawani *et al.*, 1988a).

Changes in circulating plasma Prl levels are associated with progression through stages in the reproductive cycle in chickens and turkeys (Fig. 1-1) (reviewed by El Halawani *et al.*, 1988a; El Halawani and Rozenboim, 1993; Hall *et al.*, 1986; Sharp, 1989a). Burke and Dennison (1980) have reported that plasma Prl levels were low (22 ng/ml) in turkey hens before and during the first week of photostimulation. During the early laying period, Prl levels were increased to 100 ng/ml or more, and then were markedly increased (>1000 ng/ml) around the onset of intense incubation behavior. Similar increases in plasma Prl concentrations, associated with incubation behavior, were found by Proudman and Opel (1981), with slightly lower concentrations. In their study, plasma Prl levels of poults were 9.3 ng/ml, and increased to 41.8 ng/ml in laying hens. Highest plasma Prl levels (377.2 ng/ml) were found in hens showing incubation behavior.

The hypothesis that Prl plays a key role in regulating incubation behavior is supported by a number of studies. A dramatic rise in circulating Prl levels was associated with the onset of incubation in chicken and turkey hens (Lea *et al.*, 1981; Proudman and Opel, 1980). Exogenous Prl administration induced incubation behavior in laying turkey hens (El Halawani *et al.*, 1986; Youngren *et al.*, 1991), whereas a decline in plasma Prl levels by pharmacological manipulation caused the cessation of incubation behavior in turkeys (El Halawani *et al.*, 1980b; 1983). Disruption of incubation behavior by removing the nest resulted in a decline in plasma Prl levels (El Halawani *et al.*, 1980a). The manifestation of incubation behavior is likely triggered by the Prl surge observed at the end of the laying phase, however, ovarian steroid hormones appear to be essential for both the rise of plasma Prl and the Prl-induced incubation behavior. When ovariectomized turkey

Figure 1-1. Plasma Prl profiles during the reproductive cycle in turkey hens (adapted from Burke and Dennison, 1980; Proudman and Opel, 1981).


Plasma Prolactin (ng/ml)
300-1000 ng/ml
50-200 ng/ml
20-50 ng/ml
5-10 ng/ml
Quiescent
Photostimulated
Laying
Incubating
Photorefractory
8h L:16h D
16h L:8h D

hens were given estradiol benzoate followed by progesterone treatment, the plasma Prl level increased and the hens displayed incubation behavior (El Halawani *et al.*, 1986). Since single treatment with estradiol benzoate, progesterone, or Prl, or a combination of estradiol benzoate and progesterone did not induce incubation behavior, the onset of incubation behavior seemed dependent on estradiol benzoate priming. In the normal ovulatory cycle, ovulation is preceded by a preovulatory surge of progesterone (Lague *et al.*, 1975; Johnson and Van Tienhoven, 1980). Therefore, it appears that estrogens secreted from rapidly growing follicles primes a neural mechanism controlling incubation behavior, thus making it responsive to a pre-ovulatory surge of progesterone.

Prolactin is also required for maintenance of incubation behavior. Short term nest deprivation (48 hours) in incubating turkey hens caused a decrease in serum Prl levels, whereas reinitiation of nesting activity increased serum Prl levels (El Halawani *et al.*, 1980a). Therefore, a positive feedback relationship appears to exist in which Prl stimulates nesting, and nesting activity, in turn, stimulates Prl release at the transition from the laying phase to the incubation phase.

C-2. Neuroendocrine Regulation of Prolactin Secretion

The regulatory mechanism of Prl secretion in avian species is distinctively unique from the mammalian system. Prl secretion in mammals is mainly under inhibitory hypothalamic control, while it is stimulatory in avian species. The inhibitory regulation of Prl secretion in mammals is characterized by its uncontrollable Prl release when the anterior pituitary was removed from hypothalamic control (Chen *et al.*, 1970). Prl release from a removed pituitary can be inhibited by dopamine (DA), demonstrating the dopaminergic inhibition of Prl secretion (Macleod and Lehmeyer, 1974). The avian pituitary does not secrete Prl in the absence of hypothalamic control, and hypothalamic stimulation by Prl releasing factor(s) is required in avian species.

The most potent hypothalamic Prl releasing factor in avian species is vasoactive intestinal peptide (VIP). Exogenous porcine VIP administration elevated plasma Prl levels in the chicken (Macnamee *et al.*, 1986) and in ovariectomized turkey hens (Opel and Proudman, 1988). Passive or active immunization against VIP resulted in the absence of VIP-induced elevation of plasma Prl and reduced the incidence of incubation behavior in both chickens and turkeys (Sharp *et al.*, 1989b; El Halawani *et al.*, 1995). Changes in VIP immunoreactivity (VIP-IR) numbers during different stages of the reproductive cycle revealed that plasma Prl levels were correlated with the number of VIP-IR cells (Mauro *et al.*, 1989). VIP stimulates Prl release from dispersed anterior pituitary cells in both chickens and turkeys (Macnamee *et al.*, 1986: Proudman and Opel, 1988; El Halawani *et al.*, 1990). Hypothalamic extract-induced Prl release was suppressed by preincubation of pituitary cells with VIP antiserum and a VIP receptor antagonist, demonstrating that the hypothalamic stimulation of Prl secretion is mediated by receptors specific to VIP (El Halawani *et al.*, 1990). Administration of VIP to laying bantam hens increased pituitary Prl mRNA, while passive immunoneutralization of VIP resulted in a decrease in pituitary Prl mRNA (Talbot *et al.*, 1991), implying that VIP is involved in both Prl secretion and Prl synthesis.

A neurotransmitter, serotonin (5-HT), appears to have a stimulatory effect on Prl secretion by stimulating VIP secretion in the hypothalamus. Treatment of incubating turkey hens with p-chlorophenylalanine (PCPA), an inhibitor of 5-HT synthesis, resulted in a decrease in plasma Prl levels (El Halawani *et al.*, 1980b), while the systemic administration of a 5-HT precursor, 5-hydroxytryptophan (5-HTP) restored plasma Prl levels in PCPA-treated turkey hens. When chicken or turkey anterior pituitary cells were treated with 5-HT *in vitro*, 5-HT failed to directly stimulate Prl secretion from anterior pituitary cells (Fehrer *et al.*, 1985). Incubation of anterior pituitary cells with 5-HT in the

presence of hypothalamic extracts resulted in the release of Prl (Proudman and Opel, 1988), demonstrating that the 5-HT stimulatory effect on Prl secretion is mediated by releasing factor(s), presumably VIP, in the hypothalamus.

Even though stimulatory control determines the rate of Prl secretion, inhibitory action of DA under stimulatory conditions have been reported in avian species. Treatment of both chicken and turkey anterior pituitaries *in vitro* with DA, its precursor 3,4-dihydroxyphenyalanine, DA agonists apomorphin (APO) or bromocriptine suppressed hypothalamic extract-induced Prl release (Harvey *et al.*, 1982; Fehrer *et al.*, 1985), demonstrating that DA acts directly on the pituitary and inhibits Prl release. Recently, Youngren *et al.* (1995) reported a possible biphasic action of DA on Prl secretion in turkey. DA, infused intraventricularly at high doses (100 and 500 nM/min) inhibited the secretion of Prl induced by electrical stimulation, while DA at lower doses (1 and 10 nM/min) enhanced the electrically stimulated Prl secretion. The physiological significance of the inhibitory or biphasic actions of DA on pituitary Prl secretion remains unclear in avian species.

C-3. Regulatory Mechanisms of Prl Gene Expression

Limited information is available on the regulatory mechanism of Prl gene expression in avian species. Talbot *et al.* (1991) monitored the steady state Prl mRNA levels in the anterior pituitary of laying and incubating Bantam hens, and in incubating hens deprived of their nests for 1, 3 or 6 days using dot blot hybridization. Prl mRNA levels were three fold higher in incubating hens than laying hens, and nest deprivation resulted in a decrease in Prl mRNA levels to the levels observed in laying hens. When laying hens were injected with VIP, plasma Prl levels were increased by 6-7 fold within 10 min and pituitary Prl mRNA levels were also significantly increased at 90 min post-injection (Talbot *et al.*, 1991). The injection of anti-VIP antiserum to incubating hens resulted in a decline

in plasma Prl levels and pituitary Prl mRNA levels by 3-fold, 24 h post-injection. Similarly, in turkeys, Prl mRNA levels increased approximately 10-, 20- and 100- fold in photostimulated, laying and incubating hens, respectively, relative to that found in non-photostimulated hens (Wong *et al.*, 1991). Both studies demonstrated that the steady state Prl mRNA levels are correlated with plasma Prl levels, suggesting that transcriptional regulation of the Prl gene and/or the stability of the transcripts may play a major role in regulating plasma Prl levels.

D. Regulatory Mechanisms of Prolactin Gene Expression in Mammals.

Regulatory mechanisms governing Prl gene expression have been extensively studied in mammals, while little information on Prl gene regulation in avian species is available. This section reviews, therefore, studies on the regulatory mechanisms of Prl gene expression in mammals

D-1. Hormonal Regulation of Prolactin Gene Expression.

Prolactin synthesis and release from lactotrophs are influenced by a number of hormones, either stimulatory or inhibitory, acting through diverse intracellular signaling pathways.

The tonic inhibition of Prl release from the anterior pituitary in mammals can clearly be demonstrated by the uncontrollable release of Prl when the anterior pituitary gland is isolated from the original anatomical location (Chen *et al.*, 1970). Investigation for the inhibitory factor(s) identified dopamine (DA) in the hypothalamus as a physiologically potent inhibitory factor for Prl release (Ben-Jonathan, 1985; Lamberts and MacLeod, 1990). Dopaminergic inhibition of Prl secretion from rat primary pituitary cells and human pituitary adenomas is associated with decreases in Prl synthesis and steady state Prl mRNA levels (DeCamilli *et al.*, 1979; Maurer, 1980; 1981). DA receptor in the anterior pituitary

is characterized as a D2 receptor, which interacts with a guanine nucleotide-binding (Gi) proteinto reduce adenylate cyclase activity and cytoplasmic cAMP levels (Ben-Jonathan, 1985).

Vasoactive intestinal peptide (VIP) is a neuropeptide stimulatory for Prl secretion. A VIP-stimulated increase in Prl secretion was associated with an increase in steady state Prl mRNA levels in a rat pituitary cell line, GH_3 (Gourdji *et al.*, 1979: Carrillo *et al.*, 1985). Direct observation of Prl gene expression upon changes in cytoplasmic cAMP levels in GH_4 cells clearly demonstrated the direct correlation between Prl gene expression and cytoplasmic cAMP levels (Murdoch *et al.*, 1982). Therefore, it is likely that VIP activates adenylate cyclase and increases cytoplasmic cAMP levels, while DA counteracts the increased cAMP levels elicited by VIP (Onali *et al.*, 1981). The cAMP-dependent protein kinase A (PKA) has been shown to mediate the stimulatory effect of cAMP on Prl gene activation (Maurer, 1981).

Thyrotropin releasing hormone (TRH) also stimulates Prl secretion from the anterior pituitary in mammals, and a stimulatory effect of TRH on steady state Prl mRNA levels has been shown (Evans *et al.*, 1978). TRH treatment of GH_4 cells resulted in a rapid increase in larger Prl mRNA species, followed by slower accumulation of the 1.0 kb mature cytoplasmic Prl mRNA (Potter *et al.,* 1981; Murdoch *et al.,* 1983), indicating that TRH increases the steady state Prl mRNA levels by activating Prl gene expression. TRH receptor in the pituitary cells is coupled with a family of G proteins, Gα11 and Gαq (Lucas *et al.*, 1985; Hsieh and Martin, 1992; Aragay *et al.*, 1992), which activate phospholipase C (Martin *et al.*, 1986; Straub and Gershengorn, 1986). Activation of phospholipase C causes phosphotidylinositol 4'5-bisphosphate hydrolysis, leading to the production of inositol trisphosphate (IP3) and 1,2-diacylglycerol (DAG) (Straub and Gershengorn, 1986). Elevated IP3 levels signal the release of stored Ca^{2+} into the cytosol, and the

binding of Ca^{2+} to calmodulin results in the activation of a Ca^{2+}/calmodulin-dependent kinase, while DAG activates protein kinase C (PKC) (White, 1985). The stimulatory effects of TRH on the steady state Prl mRNA levels of GH_3 cells were attenuated in Ca^{2+} free media with EGTA, demonstrating the Ca^{2+}-dependent actions of TRH stimulation on Prl gene expression (White *et al.*, 1981; White and Bancroft, 1983).

Epidermal growth factor has been shown to increase steady state Prl mRNA levels through both the Ca^{2+}-dependent and independent pathways (White, 1985). The EGF receptor possesses protein tyrosine kinase activity, however, the specific substrate for the EGF receptor-tyrosine kinase in lactotrophs remains unclear.

Estrogens are important physiological regulators of Prl gene expression in mammals. Estradiol-17β treatment of male and ovariectomized female rats resulted in an increase in Prl mRNA levels in the anterior pituitary (Stone *et al.*, 1977: Ryan *et al.*, 1979). The increase in larger size Prl mRNA species by estradiol-17β indicated that estradiol activated Prl gene expression (Maurer, 1982).

In summary, studies of the cytoplasmic signal transduction mechanisms have suggested several cytoplasmic signaling pathways for Prl gene expression. DA and VIP alter Prl gene expression through a cAMP-dependent protein kinase, PKA, by activating G protein-coupled adenylate cyclase, while TRH is involved in phospholipase C-mediated hydrolysis of phosphatidylinositol, resulting in calcium release from intracellular stores and activation of calcium-lipid dependent protein kinase, PKC. The effects of EGF are probably mediated through the activation of a tyrosine kinase associated with EGF receptor. Unlike peptide hormone signal transductions, estrogens and its receptor directly bind to the estrogen response element to exert the estrogen effects on Prl gene expression.

D-2. Regulatory Elements in the Prolactin Gene for Gene Activation

While hormonal activation of various intracellular pathways leads to specific gene expression, intricate interactions between specific regulatory elements of a given gene and transcription factors mediate hormonal stimulation and determine the rate of transcription.

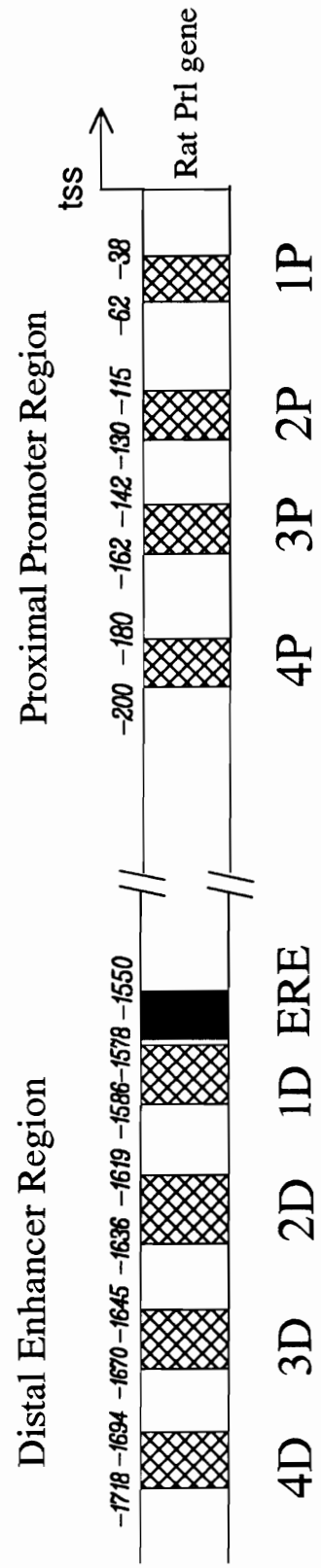
Regulatory elements in the 5'-flanking region of the rat Prl gene have been identified using various rat pituitary tumor cell lines transfected with a series of deletion sets of the 5'-flanking region linked with reporter genes. Using a GH_4 cell line, Nelson *et al.* (1986) first demonstrated tissue-specific activation with at least -1831 bp of the rat Prl 5'-flanking region. A deletion of the region between -1831 to -1530 virtually eliminated reporter gene expression, although a small residual activity was observed with a 5'-flanking region as short as -172 bp, but not -70 bp. Position- and orientation-independence of the region between -1831 to -1530 bp for gene activation was characteristic of an enhancer element in this region. Based on GH_4 cells, this distal enhancer region accounted for 98 - 99% of the tissue-specific transcription activity while the proximal region, between -442 to -36 bp, for the remaining 1 to 2%, (Nelson *et al.*, 1988). Transgenic mice with the rat Prl 5'-flanking region linked with reporter genes showed that the distal enhancer or the proximal promoter regions alone seemed capable of directing gene activation only in the anterior pituitary, while synergistic interactions of both regions were necessary for high level tissue-specific expression (Crenshaw *et al.*, 1989). Moreover, in transgenic mice containing the distal enhancer and the proximal promoter regions clustered together, the overall expression was less than the intact 3 kb construct.

Expression of the transgene in thyrotrophs was also detected, suggesting that either some other elements or relative position of the two regions are necessary for full expression and cell type-specific expression of the Prl gene.

Precise locations of regulatory elements within the proximal promoter as well as the distal enhancer regions have been shown using DNase I footprinting assays detecting direct interactions of pituitary specific transcription factor(s) with the DNA elements. Gutierrez-Hartmann *et al.* (1987) identified at least four DNase I-protected sites using GH_3 whole cell extracts in the proximal promoter region of the rPrl gene: FP1 (-44 to -65); FP2 (-120 to -130); FP3 (-145 to -173), and FP4 (-190 to -210). Whole cell extracts from GC cells also protect nearly identical sites: 1P (-38 to -62) ; 2P (-115 to -130); 3P (-142 to -162) and 4P (-180 to -200), as well as four sites in the distal enhancer region (1D, -1579 to -1596; 2D, -1619 to -1636; 3D, -1645 to -1670; 4D, -1694 to -1718) (Nelson *et al.*, 1988) (Fig. 1-2). Highly conserved sequence homology among the protected sites in both the proximal and the distal enhancer regions of the Prl gene and the two protected sites in the GH gene promoter region suggested that a common or related factor binds to the consensus sequence, (5'-A ($^{A}/_{T}$) ($^{A}/_{T}$)TATNCAT-3') (Nelson *et al.*, 1988). A transcription factor, named Pit-1 (Ingraham *et al.*, 1988) or GHF-1 (Bodner *et al.*, 1988), was later identified as a pituitary-specific transcription factor that interacts with the DNase I-protected sequences in the Prl and GH promoter regions.

An estrogen response element (ERE) mediating estrogenic effects on Prl gene activation was found in the regulatory region of the rat Prl gene, located between 1.7 and 1.5 kb upstream from the tss (Maurer, 1985). A high affinity binding sequence for an estrogen receptor, (5'-TGTCACTATGTCC-3'), was subsequently mapped at -1580 to -1568, adjacent to the 1D binding site of the distal enhancer region (Maurer and Notides, 1987) (Fig. 1-2). This sequence is an imperfect palindrome, which resembles the consensus estrogen receptor (ER)-binding sequence, (5'-GGTCANNNTGACC-3'), and mutations of this ERE resulted in a lack of an

Figure 1-2. Locations of DNase I-protected regions and an estrogen responsive element in the 5'-flanking region of the rat prolactin gene (Nelson *et al.*, 1988; Maurer and Notides, 1987).


Distal Enhancer Region
Proximal Promoter Region
tss
Rat Prl gene
-1718 -1694 -1670 -1645 -1636 -1619 -1586 -1578 -1550
-200 -180 -162 -142 -130 -115 -62 -38
4D 3D 2D 1D ERE 4P 3P 2P 1P

estrogenic responsiveness of Prl gene activation (Maurer and Notides, 1987).

Subsequent studies implicated that interaction between an estrogen receptor and Pit-1/GHF-1 are necessary for the estrogenic effects on Prl gene expression. Mutations of each Pit-1/GHF-1 binding site at the distal enhancer region or the deletion of 3D and 4D resulted in decreases in estrogen-mediated Prl gene activation (Day *et al.*, 1990; Nowakowski and Maurer, 1994). When the perfect palindrome sequence for ERE was introduced, mutations of the 1D site did not influence the estrogenic responsiveness of Prl gene activation (Nowakowski and Maurer, 1994), suggesting that the imperfect ERE palindrome of the Prl gene allows the interactions between ER and Pit-1/GHF-1 for estrogenic effects on Prl gene activation.

D-3. Transcription Factors for Prolactin Gene Activation.

Purification of the pituitary-specific transcription factor using DNA affinity chromatography with a DNase I-protected element allowed two laboratories to isolate a common transcription factor, referred to as Pit-1 by Ingraham *et al.* (1988) and GHF-1 by Bodner *et al.* (1988). Pit-1/GHF-1 protein has been shown to interact with several DNase I-protected elements in both the proximal promoter and the distal enhancer regions of the Prl gene, as well as the GH promoter region (Bodner *et al.*, 1988: Ingraham *et al.*, 1988). Expression of Pit-1/GHF-1 in heterologous cells resulted in the specific stimulation of both the GH and Prl promoters containing the DNase I-protected sequences (Ingraham *et al.*, 1988).

Structural characteristics of Pit-1/GHF-1, and its roles in Prl gene activation have since been extensively studied. Thus, in the following section, characteristics of Pit-1/GHF-1 and its role in Prl gene activation are discussed.

E. Role of Pit-1/GHF-1 for Prolactin Gene Activation.

E-1. Pit-1/GHF-1 is a POU-domain Containing Transcription Factor.

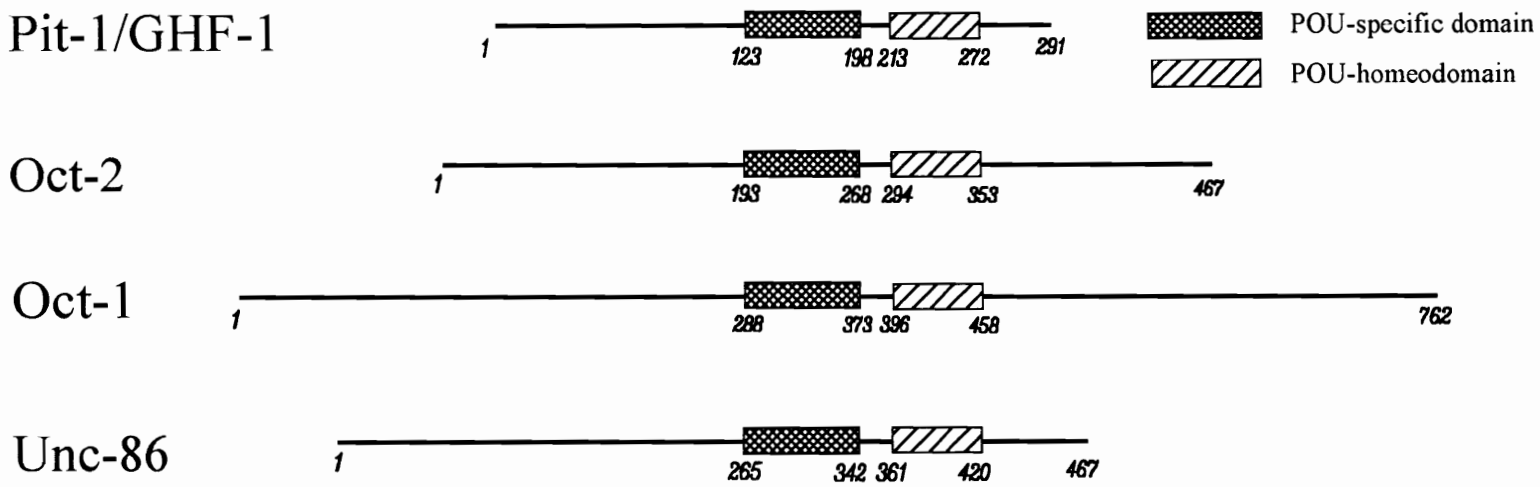
The cDNA sequence encoding Pit-1/GHF-1 predicted a 291 amino acid protein with a highly conserved domain at the carboxyl terminus, referred to as the POU domain (Herr *et al.,* 1990). The POU domain consists of the POU-specific domain and the POU homeodomain connected by a hypervariable linker. The POU-specific domain has initially been found in three mammalian proteins, Pit-1/GHF-1, Oct-1, Oct-2 and in *unc*-86 of the nematode *Caenorhabditis elegans.* (Ingraham *et al.*, 1990b) (Fig. 1-3). Oct-1 and Oct-2 recognize the same octamer sequence, 5'-ATGCAAAT-3', and Oct-1 is expressed ubiquitously, while Oct-2 is a B lymphocyte-specific transcription factor involved in immunoglobulin gene transcription. *Unc*-86 in *C. elegans* is involved in cell lineage determination and cell differentiation. A 60 amino acid POU homeodomain shares high homology with several *Drosophila* and vertebrate regulatory transcription factors involved in controlling body segmentation during development. The POU-specific domain and the POU homeodomain are structurally independent, and yet both contain the helix-turn-helix (HTH) motif, a motif common to a broad class of DNA binding domains. Since the proteins with the POU-specific domain always contain the POU-homeodomain, the cooperation of both domains seems essential for DNA-binding (Fig. 1-3).

X-ray crystallographic studies of the POU domain of Oct-1 have revealed the three dimensional structure of the POU domain/DNA interaction (Klemm *et al.*, 1994). The POU-homeodomain and the POU-specific domain interact on opposite sides of DNA, with the POU-specific domain contacting the 5' ATGC segment and the POU homeodomain the 3'AAAT segment of the octamer sequence.

The amino-terminal domain (residues 8-80) is critical for trans-activation function of Pit-1/GHF-1 (Ingraham *et al.*, 1990a), and differential Prl promoter stimulation by

Figure 1-3. Approximate locations of the POU-specific domain and POU homeodomain among the original POU domain protein family (adapted from Ingraham *et al.*, 1990b).

POU-domain protein family



several Pit-1/GHF-1 isoforms variable at the amino-terminus have been reported. Furthermore, a chimeric DNA binding protein with the amino-terminal domain of Pit-1/GHF-1 was able to mediate dopaminergic inhibition of Prl gene expression, showing that the amino-terminal domain of Pit-1/GHF-1 can mediate hormonal regulation of gene expression, independent of the POU domain (Lew and Elsholtz, 1995). However, Pit-1/GHF-1 mutants at the POU domain with moderately reduced DNA binding affinity showed significantly reduced trans-activation efficiency, emphasizing the intricate interaction between the POU domain and DNA also influences the trans-activation efficiency of Pit-1/GHF-1 (Pfaffle *et al*, 1992). Little is known about the structure of the amino-terminal domain, and the answer as to how it interacts with the transcriptional initiation complex remains to be discovered.

E-2. Pit-1/GHF-1 Regulates the Transcription of the Prolactin and Other Pituitary Genes.

The role of Pit-1/GHF-1 in Prl gene activation was first demonstrated by co-transfection of HeLa cells with a rat Pit-1/GHF-1 expression vector and a reporter fusion gene containing the rat Prl promoter (Ingraham *et al.*, 1988). Pit-1/GHF-1 has since been shown to activate several pituitary specific genes, including the GH gene (Ingraham *et al.*, 1988), the thyroid stimulating hormone (TSH) β-subunit gene (Mason *et al.*, 1992), the growth hormone releasing factor (GRF) receptor gene (Lin *et al.*, 1993), and its own gene (Chen *et al.*, 1990; McCormick *et al.*, 1990).

Pit-1/GHF-1 interacts with the binding sites located in the 5'-flanking regions of the Pit-1/GHF-1-dependent genes. The rat Prl gene has four binding sites at the proximal promoter region and four binding sites at the distal enhancer region (Nelson *et al.*, 1988), while the rat GH gene contains two Pit-1 binding sites. The TSH β-subunit promoter contains three Pit-1/GHF-1 binding sites within 400 nt upstream from the tss,

and at least two Pit-1/GHF-1 binding elements have been shown to confer thyrotropin releasing hormone (TRH) responsiveness (Mason *et al.*, 1992). In the rat Pit-1/GHF-1 promoter region, two Pit-1 binding sites were identified. One downstream from the major tss has been shown to act as a negative element for gene activation, while a second binding site at -38 to -70 nt of the tss as a positive element (Chen *et al.*, 1990). The GRF receptor gene has been shown to be under Pit-1/GHF-1 control, however, the mapping of Pit-1/GHF-1 binding site(s) has not been reported (Lin *et al.*, 1993).

Mutants in Pit-1/GHF-1 have provided direct evidence that Pit-1 has a critical function in the proliferation of specific endocrine cell types and gene activation in the anterior pituitary. In Snell dwarf mice, the anterior pituitary lacks somatotrophs, lactotrophs and thyrotrophs, and plasma GH and Prl are undetectable (Slabaugh *et al.*, 1981). Genetic analysis found a single mutation in the POU homeodomain of the Pit-1/GHF-1 gene (Li *et al.*, 1990). The mutated Pit-1/GHF-1 was able to bind a Pit-1/GHF-1 binding site in both the GH and Prl genes, yet, failed to stimulate the promoters (Li *et al.*, 1990). The lack of GRF receptor expression in Snell mice suggested that the mutated Pit-1/GHF-1 also failed to activate the GRF receptor gene, affecting somatotroph cell proliferation (Lin *et al.*, 1992). In humans, a point mutation at either the POU-specific domain (Pfaffle *et al.*, 1992) or the POU homeodomain (Radovick *et al.*, 1992) have been found responsible for patients with GH, Prl and TSH deficiency.

E-3. Mechanism(s) of Hormonal Regulation of Prolactin Gene Activation by Pit-1/GHF-1.

Both the proximal promoter and the distal enhancer regions contain multiple Pit-1/GHF-1 binding sequences and have been shown to mediate several intracellular signaling pathways, demonstrating that Pit-1/GHF-1 is a critical nuclear target of the hormonal regulation for Prl gene expression. Kapiloff *et al.* (1991) demonstrated the *in vivo*

phosphorylation at Ser 115 and Thr 220 of Pit-1/GHF-1 in response to cAMP or phorbol ester (TPA) treatment of a rat pituitary cell line GC. Pit-1/GHF-1 phosphorylation resulted in changes in DNA binding affinity to the Prl promoter target sequences, implicating a possible mechanism mediating intracellular signaling pathways by Pit-1/GHF-1.

However, recent studies have demonstrated that Pit-1/GHF-1 mutants lacking the phosphorylation sites can equally mediate the cAMP-dependent PKA and PKC pathways for Prl gene activation (Okimura *et al.*, 1994; Fischberg *et al.*, 1994), suggesting that phosphorylation of Pit-1/GHF-1 may not be involved in Prl gene activation induced by cAMP, forskolin, or TPA. It has been hypothesized that Pit-1/GHF-1 may recruit another factor, in a PKA- or PKC-dependent manner, to the Pit-1/GHF-1 binding sites for Prl gene activation (Gutierrez-Hartmann, 1994).

A member of the transcription factor Ets family mediates an intracellular signal pathway for Prl gene activation. Using chicken Ets-1 (c-Ets-1), Bradford *et al.* (1995) demonstrated that c-Ets-1 and Pit-1/GHF-1 synergistically stimulate the Prl promoter to mediate Ras stimulation, a pathway that has been shown to activate the Prl gene. The site for c-Ets-1-mediated Ras stimulation was mapped to -212 to -209 nt of the rat Prl promoter, adjacent to a Pit-1 binding site, previously identified as 4P (Nelson *et al.*, 1988) (Fig. 1-2). The proximity of the Pit-1/GHF-1 binding site and the Ets response element support the notion that Pit-1/GHF-1 may recruit another factor to the Pit-1/GHF-1 binding sites for gene activation (Gutierrez-Hartmann, 1994).

Other possible nuclear proteins that cooperatively function with Pit-1/GHF-1 are Oct-1 and P-Lim. Both transcription factors have been shown to interact with Pit-1/GHF-1 and/or its binding sites to synergistically activate Prl gene expression (Voss *et al.*, 1991a; Bach *et al.*, 1995).

As more transcription factors are defined for Prl gene regulation, it becomes apparent that a greater diversity of gene regulation can be achieved by combinatorial interactions among transcription factors, and it may be these combinatorial interactions among the transcription factors that dictate the spatial and temporal expression of the Prl gene.

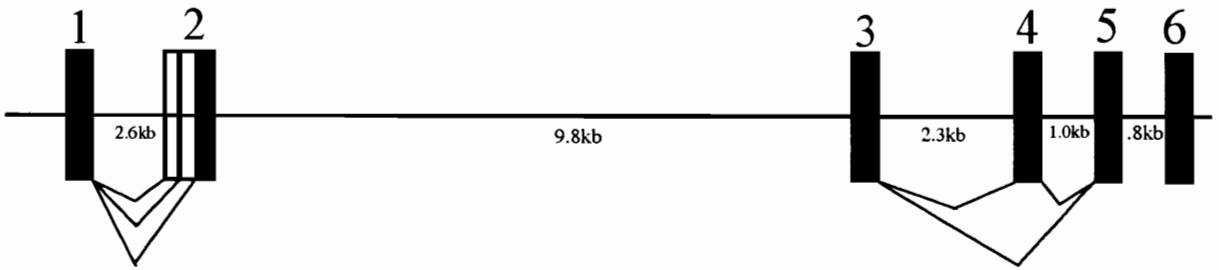
E-4. Pit-1/GHF-1 Isoforms

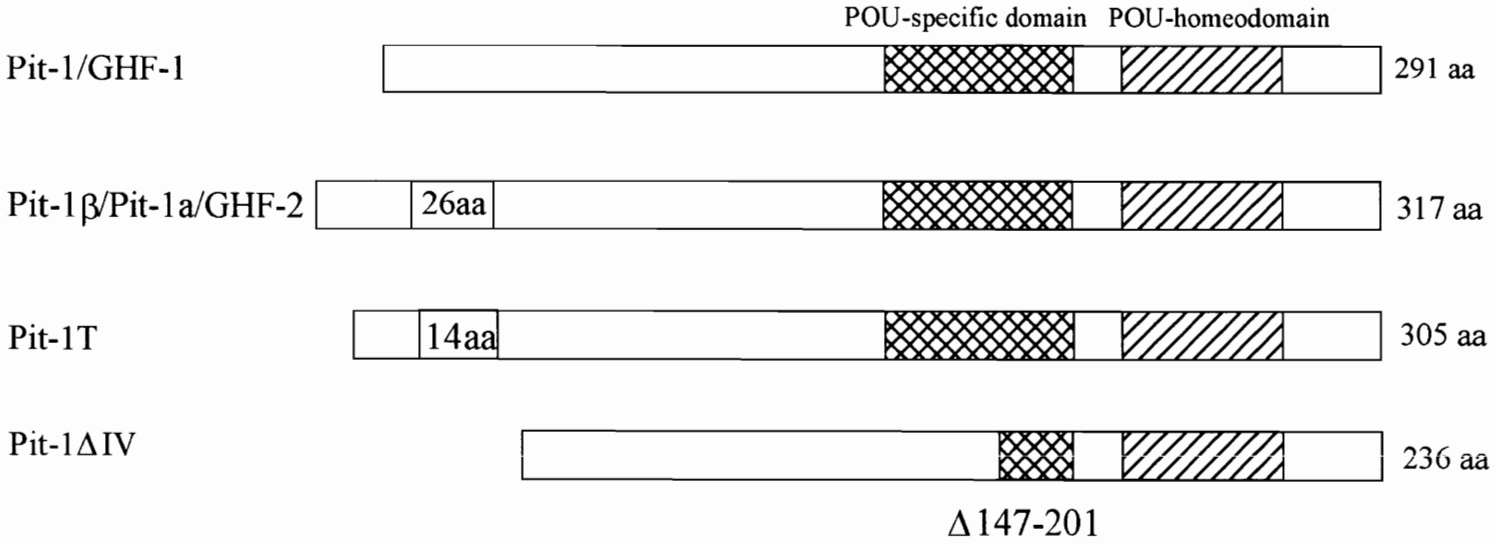
Several Pit-1/GHF-1 isoforms and their abilities to trans-activate Pit-1-dependent genes have been reported (Fig. 1-4). The original Pit-1/GHF-1 cDNA isolated from a rat pituitary cell line predicted a 291 amino acid protein that is 33-kDa in size (Ingraham *et al.*, 1988; Bodner *et al.*, 1988). However, Western blot analysis using Pit-1 antiserum identified 33- and 31-kDa forms in rat pituitary or in rat pituitary-derived cell lines. Detailed analysis of the two isoforms indicated that both isoforms arise from a single RNA transcript but use two translation initiation sites within exon 1 of the Pit-1/GHF-1 transcript, and yet, no differential trans-activation efficiency on Prl promoter stimulation was observed for each isoform (Voss *et al.*, 1991b).

Another Pit-1/GHF-1 isoform was isolated from a rat pituitary cell line cDNA library referred to as Pit-1β (Konzak and Moore, 1992), Pit-1a (Morris *et al.*, 1992) or GHF-2 (Theill *et al.*, 1992) (referred to as Pit-1β, hereafter). The cDNA sequence for the Pit-1β isoform has an additional 26 amino acids inserted into the trans-activation domain due to alternative splicing to a splice acceptor site located 78 bp upstream from the major exon 2 splice site. Even though there was no difference in binding affinity to a Prl promoter Pit-1/GHF-1 binding sequence between Pit-1β and Pit-1/GHF-1, this isoform failed to activate a co-transfected Prl promoter reporter gene construct either in Rat16 cells

Figure 1-4. Rat Pit-1/GHF-1 gene and the isoforms.

Rat Pit-1/GHF-1 gene and the isoforms





(Theill *et al.*, 1992), CHO cells (Morris *et al.*, 1992) or GH_4 cells (Bradford *et al.*, 1995). Since Western blot analysis failed to detect predicted the 36-kDa Pit-1β isoform in rat pituitary and rat pituitary-derived cell lines, physiological significance of this isoform is less clear. Pit-1/GHF-1 isoform with a 55 amino acid truncation in the POU-specificdomain has been identified from the rat anterior pituitary (Voss *et al.*, 1993) and from transplanted GH_3 cells (Day and Day, 1994), referred to as Pit-1ΔIV (Fig. 1-4). When GH_3 cells were transplanted in rats, the Prl gene was selectively silenced. Western blot analysis with a Pit-1 antiserum revealed that, in addition to the expected Pit-1 proteins of 33- and 31-kDa due to alternative translation start site usage, Pit-1/GHF-1 proteins with 27- and 24- kDa were detected from the transplanted cells, and these smaller forms quickly disappeared as the cells were returned to culture. RT-PCR analysis of Pit-1/GHF-1 transcripts from the transplanted cells identified a Pit-1/GHF-1 transcript shorter than the expected Pit-1/GHF-1 cDNA due to the lack of 165 bp sequence corresponding to exon IV of the Pit-1/GHF-1 gene, demonstrating alternative splicing of the Pit-1/GHF-1 transcript in the transplanted GH_3 cells. Pit-1ΔIV isoform stimulated transcription from the GH promoter, but not the Prl promoter, reflecting the fact that the transplanted GH_3 cells do not produce Prl but continue to produce GH. Expression of the Pit-1ΔIV form could represent a possible mechanism to negatively regulate Prl gene expression.

A thyrotroph-specific Pit-1 isoform (Pit-1T) has been isolated in mice and appears to have significant trans-activation activity for the thyrotropin β-subunit (TSHβ) gene (Haugen *et al.*, 1993). A cDNA encoding for Pit-1T, isolated from the mouse thyrotroph-derived tumor cell line TtT-97, contains an additional 42 nucleotides, generating a Pit-1 with a 14 amino acid insertion in the trans-activation domain. Pit-1T showed a 5- to 6-fold stimulation of the TSHβ promoter-reporter gene compared with Pit-1 and Pit-1β. In another thyrotroph-derived mouse cell line, αTSH, which has lost the ability to express the

endogenous TSHβ gene, only the combination of Pit-1T and Pit-1 could stimulate the TSHβ promoter, suggesting that both Pit-1 and Pit-1T are essential for TSHβ promoter stimulation (Haugen *et al.*, 1994).

Even though physiological significance of each isoform has not been clearly defined, it is likely that each isoform is co-expressed and the ratio of each isoform can modulate gene activation. Differential expression of each Pit-1/GHF-1 isoform may play an important role for spatial and temporal activation of the Pit-1/GHF-1-dependent genes. Variability of Pit-1/GHF-1 isoforms at the amino-terminal trans-activation domain suggests that Pit-1/GHF-1 interacts with other nuclear proteins for gene activation through the trans-activation domain. Alternatively, the modification at the amino-terminal trans-activation domain may affect gene activation through changes in the DNA-binding property of the POU-domain. The mechanism which controls the generation of each isoform is largely unknown.

F. Conclusion

Prolactin is involved in the induction and maintenance of incubation behavior in avian species. While hormonal regulation on Prl secretion has been studied, little is known about regulatory mechanism(s) governing Prl gene expression in avian species. In mammals, intricate interactions between specific DNA sequence motifs in the 5'-flanking region of the Prl gene and transcription factors for the tissue-specific and hormone-responsive expression of the Prl gene have been demonstrated. Molecular analysis of the Prl gene and its regulatory mechanism is necessary in avian species.

G. References Cited

Aragay, A., A. Katz, and M. I. Simon. 1992. The Gαq and Gα11 proteins couple the thyrotropin-releasing hormone receptor to phospholipase C in GH_3 rat pituitary cells. J. Biochem. 267:24983-24988.

Back , I., S. J. Rhodes, R. V. Pearse, II, T. Heinzel, B. Gloss, K. M. Scully, P. E. Sawchenko, and M. G. Rosenfeld. 1995. P-Lim, a LIM homeodomain factor, is expressed during pituitary organ and cell commitment and synergizes with Pit-1. Proc. Natl. Acad. Sci. USA. 92:2720-2724.

Ben-Jonathan, N. 1985. Dopamine: a prolactin-inhibiting hormone. Endocrine Rev. 6:564-589.

Bodner, M., J. -L. Castrillo, L. E. Theill, T. Deerinck, M. Ellisman, and M. Karin. 1988. The pituitary specific transcription factor GHF-1 is a homeobox-containing protein. Cell 55:515-518.

Bradford, A. P., K. E. Conrad, C. Wasylyk, B. Wasylyk, and A. Gutierrez-Hartmann. 1995. Functional interaction of c-Ets-1 and GHF-1/Pit-1 mediates Ras activation of pituitary-specific gene expression: Mapping of the essential c-Ets domain. Mol. Cell. Biol. 15:2849-2857.

Burke, W. H., and P. A. Dennison. 1980. Prolactin and luteinizing hormone levels in female turkeys (Meleagris gallapavo) during a photoinduced reproductive cycle and broodiness. Gen. Comp. Endocrinol. 41:92-100.

Carrillo, A. J., T. B. Pool, and Z. D. Sharp. 1985. Vasoactive intestinal peptide increase prolactin messenger ribonucleic acid content in GH_3 cells. Endocrinol. 116:202-206.

Chen, C. L., Y. Amenomori, K. H. Lu, J. L. Voogt, and J. Meites. 1970. Serum prolactin levels in rats with pituitary transplants or hypothalamic lesions. Neuroendo. 6:220-227.

Chen, R., H. A. Ingraham, M. N. Treacy, V. R. Albert, L. Wilson, and M. G. Rosenfeld. 1991. Autoregulation of Pit-1 gene expression mediated by two cis-active promoter elements. Nature 364:583-586.

Crenshaw, E. B., III., K. Kalla, D. M. Simmons, L. W. Swanson, and M. G. Rosenfeld. 1989. Cell-specific expression of the prolactin gene in transgenic mice is controlled by synergistic interactions between promoter and enhancer elements. Genes Dev. 3:959-972.

Day, R. N., and K. H. Day. 1994. An alternatively spliced form of Pit-1 represses prolactin gene expression. Mol. Endocrinol. 8:374-381

Day, R. N., S. Koike, M. Sakai, M. Muramatsu, and R. A. Maurer. 1990. Both Pit-1 and the estrogen receptor are required for estrogen responsiveness of the rat prolactin gene. Mol. Endocrinol. 4:1964-1971.

De Camilli, P., and D. Macconi. 1979. Dopamine inhibits adenylate cyclase in human prolactin-secreting pituitary adenomas. Nature 278:252-254.

El Halawani, M. E, W. H. Burke and P. E. Dennison. 1980a. Effect of nest deprivation on serum prolactin level in nesting female turkeys. Biol. Reprod. 23:118-123.

El Halawani, M. E., W. H. Burke and P. T. Dennison. 1980b. Effects of p-chlorophenylalanine on the rise in serum prolactin associated with nesting in broody turkeys. Biol. Reprod. 23:815-819.

El Halawani, M. E., S. Fehrer, B. M. Hargis and T. E. Porter. 1988a. Incubation behavior in the domestic turkey: physiological correlates. CRC, Critical Review in Poultry Biology. 1:285-314.

El Halawani, M. E. and I. Rozenboim. 1993. The ontogeny and control of incubation behavior in turkeys. Poult. Sci. 72:906-911.

El Halawani, M. E., J. L. Silsby and S. Pitts. 1995. Increased egg production by active immunization against vasoactive intestinal peptide in the turkey (Meleagris gallapavo). Biol. Reprod. 52:179-183.

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

El Halawani, M. E., J. L. Silsby, S. C. Fehrer and E. J. Behnke. 1983. Reinitiation of ovulatory cycles in incubating female turkeys by an inhibitor of serotonin synthesis, p-chlorophenylalanine. Biol. Reprod. 28:221-228.

El Halawani, M. E., J. L. Silsby, and L. J. Mauro. 1990. Enhanced vasoactive intestinal peptide-induced prolactin secretion from anterior pituitary cells of incubating turkey (*Meleagris gallopavo*). Gen. Comp. Endocrinol. 80:138-145.

El Halawani, M. E., O. M. Youngren, J. L. Silsby, and R. E. Phillips. 1988b. Involvement of serotonin in prolactin release induced by electrical stimulation of the hypothalamus of the turkey (*Meleagris gallopavo*). Gen. Comp. Endocrinol. 72:323-328.

Etches, R. J. 1990. The ovulatory cycle of the hen. Poultry Biology. 2:293-318.

Evans, G. A., D. N. David, and M. G. Rosenfeld. 1978. Regulation of prolactin and somatotropin mRNAs by thyroliberin. Proc. Natl. Acad. Sci. USA. 75:1294-1298.

Fehrer, S. C., J. L. Silsby, E. J. Behnke and M. E. El Halawani. 1985. Hypothalamic and serum factors influence on prolactin and luteinizing hormone release by the pituitary gland of the young turkey (Meleagris gallopavo). Gen. Comp. Endocrinol. 59:73-81.

Fischberg, D. J., X. -H. Chen, and C. Bancroft. 1994. A Pit-1 phosphorylation mutant can mediate either basal or induced prolactin or growth hormone promoter activity. Mol. Endocrinol. 8:1566-1573.

Frawley, L. S., and F. R. Boockfor. 1991. Mammosomatotrophs: presence and functions in normal and neoplastic pituitary tissue. Endocrine Rev. 12:337-355.

Gala, R. R. 1991. Prolactin and growth hormone in the regulation of the immune system. Proc. Soc. Exp. Biol. Med. 198:513-527.

Gourdji, D., D. Bataille, N. Vauclin, D. Grouselle, G. Rosselin, and A. Tixier-Vidal. 1979. Vasoactive intestinal peptide (VI) stimulates prolactin (PRL) release and cAMP production in a rat pituitary cell line (GH3/B6). Additive effects of VIP and TRH on PRL release. FEBS Letters. 104:164-168.

Gutierrez-Hartmann, A., S. Siddiqui, and S. Loukin. 1987. Selective transcription and DNase I protection of the rat prolactin gene by GH_3 pituitary cell-free extracts. Proc. Natl. Acad. Sci. USA. 84:5211-5215.

Gutierrez-Hartmann, A. 1994. Pit-1/GHF-1: a pituitary-specific transcription factor linking general signaling pathway to cell-specific gene expression. Mol. Endocrinol. 8:1447-1449.

Hall, T. R., S. Harvey and A. Chadwick. 1986. Control of prolactin secretion in birds: A review. Gen. Comp. Endocrinol. 62:171-184.

Harvey, S., A. Chadwick, G. Border, C. G. Scanes and T. G. Phillips. 1982. Neuroendocrine control of prolactin secretion. *In* "Aspects of avian endocrinology: Practical and theoretical implications" (C. G. Scanes, M. A. Ottinger, A. D. Kenny, J. Balthazart, J. Cronshaw, and I. C. Jones., Eds., Texas Tech Press, Lubbock, TX).

Haugen, B. R, W. M. Wood, D. F. Gordon, and E. C. Ridgway. 1993. A thyrotrope-specific variant of Pit-1 transactivates the thyrotropin β promoter. J. Biol. Chem. 268:20818-20824.

Haugen, B. R., D. F. Gordon, A. R. Nelson, W. M. Wood and E. C. Ridgway. 1994. The combination of Pit-1 and Pit-1T have a synergistic stimulatory effect on the thyrotropin β-subunit promoter but not the growth hormone or prolactin promoters. Mol. Endocrinol. 8:1574-1582.

He, X., M. N. Treacy, D. M. Simmons, H. A. Ingraham, L. W. Swanson, and M. G. Rosenfeld. 1989. Expression of a large family of POU-domain regulatory genes in mammalian brain development. Nature 340:35-42.

Herr, W., R. A. Sturm, R. G. Clerc, L. M. Corcoran, D. Baltimore, P. A. Sharp, H. A. Ingraham,, M. G. Rosenfeld, M. Finney, G. Ruvkun, and H. R. Horvitz. 1990. The POU domain: a large conserved region in the mammalian pit-1, oct-1, oct-2, and Caenorhabditis elegans unc-86 gene products. Genes Dev. 2:1513-1516.

Hsieh, K. -P., and T. F. J. Martin. 1992. Thyrotropin-releasing hormone and gonadotropin-releasing hormone receptors activate phospholipase C by coupling to the guanosine triphosphate-binding proteins Gq and G11. Mol. Endocrinol. 6:1673-1681.

Ingraham, H. A., R. Chen, H. J. Mangalam, H. P. Elsholtz, S. F. Flynn, C. R. Lin, D. M. Simmons, L. Swanson, and M. G. Rosenfeld. 1988. A tissue-specific factor containing a homeodomain specifies a pituitary phenotype. Cell 55:519-529.

Ingraham, H. A., S. E. Flynn, J. W. Voss, V. R. Albert, M. S. Kapiloff, L. Wilson, and M. G. Rosenfeld. 1990a. The POU-specific domain of Pit-1 is essential for sequence-specific, high affinity DNA binding and DNA-dependent Pit-1-Pit-1 interactions. Cell 61:1021-1033.

Ingraham, H. A., V. R. Albert, R. Chen, E. B. Crenshaw., III, H. P. Elsholtz, X. He, M. S. Kapiloff, H. J. Mangalam, L. W. Swanson, M. N. Treacy, and M. G. Rosenfeld. 1990b. A family of POU-domain and Pit-1 tissue-specific transcription factors in pituitary and neuroendocrine development. Annu. Rev. Physiol. 52:773-791.

Johnson, A. L., and A. Van Tienhoven. 1980. Plasma concentrations of six steroids and LH during the ovulatory cycle of the hen, *Gallus domesticus*. Biol. Reprod. 23:386-393.

Kapiloff, M. S., Y, Farkash, M. Wegner, and M. G. Rosenfeld. 1991. Variable effects of phosphorylation of Pit-1 dictated by the DNA response elements. Science. 253:786-789.

Klemm, J. D., M. A. Rould, R. Aurora, W. Herr, and C. O. Pabo. 1994. Crystal structure of the Oct-1 POU domain bound to an octamer site: DNA recognition with tethered DNA-binding modules. Cell 77:21-32.

Konzak, K. E., and D. D. Moore. 1992. Functional isoforms of Pit-1 generated by alternative messenger RNA splicing. Mol. Endocrinol. 6:241-247.

Lague, P. C., A. Tienhoven, and F. J. Cunningham. 1975. Concentrations of estrogens, progesterone and LH during the ovulatory cycle of the laying chickens (*Gallus domesticus*). Biol. Reprod. 12:590-598.

Lamberts, S. W. J., and R. M. MacLeod. 1990. Regulation of prolactin secretion at the level of the lactotroph. Physiol. Rev. 70:279-318.

Lea, R. W., A. S. M. Dods, P. J. Sharp, and A. Chadwick. 1981. The possible role of prolactin in the regulation of nesting behavior and the secretion of luteinizing hormone in broody bantams. J. Endocrinol. 91:89-97.

Lew, A. M., and H. P. Elsholtz. 1995. A dopamine-responsive domain in the N-terminal sequence of Pit-1. J. Biol. Chem. 270:7154-7160.

Li, S., E. B. Crenshaw, III., E. J. Rawson, D. M . Simmons, L. W. Swanson, M. G. Rosenfeld. 1990. Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene Pit-1. Nature 347:528-533.

Lin, C., S-C. Lin, C-P. Chang, and M. G. Rosenfeld. 1992. Pit-1-dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth. Nature 360:765-768.

Lin, S-C., C. R. Lin, I Gukovsky, A. J. Lusis, P. E. Sawchenko, and M. G. Rosenfeld. 1993. Molecular basis of the little mouse phenotype and implications for cell type specific growth. Nature 364:208-213.

Lucas, D. O., S. M. Bajjalieh, J. A. Kowalchyk, T. F. J. Martin. 1985. Direct stimulation by thyrotropin-releasing hormone (TRH) of polyphosphoinositide hydrolysis in GH3 cell membranes by a guanine nucleotide-modulated mechanism. Biochem. Biophys. Res. Comm. 132:721-728.

Macleod, R. M., and J. E. Lehmeyer. 1974. Studies on the mechanism of the dopamine-mediated inhibition of prolactin secretion. Endocrinol. 94:1077-1085.

Macnamee, M. C., P. J. Sharp, R. W. Lea, R. J. Sterling and S. Harvey. 1986. Evidence that vasoactive intestinal peptide is a physiological prolactin-releasing factor in the bantam hen. Gen. Comp. Endocrinol. 62:470-478.

Martin, T. F. J., S. M. Bajjalieh, D. O. Lucus, and J. A. Kowalchyk. 1986. Thyrotropin-releasing hormone stimulation of polyphosphoiositide hydrolysis in GH_3 cell membranes is GTP-dependent but insensitive to cholera or pertusis toxin. J. Biol. Chem. 261:10141-10149.

Mason, M. E., K. E. Friend, J. Copper, and M. A. Shupnik. 1993. Pit-1/GHF-1 binds to TRH-sensitive regions of the rat thyrotropin β gene. Biochem. 32:8932-8938.

Maurer, R. A. 1980. Dopaminergic inhibition of prolactin synthesis and prolactin messenger RNA accumulation in cultured pituitary cells. J. Biol. Chem. 255:8092-8097.

Maurer, R. A. 1981. Transcriptional regulation of the prolactin gene by ergocryptine and cyclic AMP. Nature 294:94-97.

Maurer, R. A. 1982. Estradiol regulates the transcription of the prolactin gene. J. Biol. Chem. 257:2133-2136.

Maurer, R. A. 1985. Selective binding of the estradiol receptor to a region at least one kilobase upstream from the rat prolactin gene. DNA 4:1-9.

Maurer, R. A., and A. C. Notides. 1987. Identification of an estrogen-responsive element from the 5'-flanking region of the rat prolactin gene. Mol. Cell. Biol. 7:4247-4254.

Mauro, L. J., R. P. Elde, O. M. Youngren, R. E. Phillips and M. E. El Halawani. 1989. Alterations in hypothalamic vasoactive intestinal peptide-like immunoreactivity are associated with reproduction and prolactin release in the female turkey. Endocrinol. 125:1795-1804.

McCormick, A., H. Brady, L. E. Theill, and M. Karin. 1990. Regulation of the pituitary-specific homeobox gene GHF1 by cell-autonomous and environmental cues. Nature 345: 829-832.

Miller, W. L., and N. L. Eberhardt. 1983. Structure and evolution of the growth hormone gene family. Endocrine Rev 4:97-130.

Morris, A. E., B. Kloss, R. E. McChesney, C. Bancroft, and L. A. Chasin. 1992. An alternatively spliced Pit-1 isoform altered in its ability to trans-activate. Nucleic Acids Res. 20:1355-1361.

Murdoch, G. H., E. Potter, A. K. Nicolaisen, R. M. Evans, and M. G. Rosenfeld. 1982. Epidermal growth factor rapidly stimulates prolactin gene transcription. Nature 300:192-194.

Murdoch, G. H., R. Franco, R. M. Evans, and M. G. Rosenfeld. 1983. Polypeptide hormone regulation of gene expression. J. Biol. Chem. 258:15329-15335.

Nelson, C., E. B. Crenshaw, III, R. Franco, S. A. Lira, V. R. Albert, R. M. Evans, and M. G. Rosenfeld. 1986. Discrete cis-active genomic sequences dictate the pituitary cell type-specific expression of rat prolactin and growth hormone gene. Nature 322:557-562.

Nelson, C., V. R. Albert, H. P. Elsholtz, L. I.-W. Lu and M. G. Rosenfeld. 1988. Activation of cell specific expression of rat growth hormone and prolactin gene by a common transcription factor. Science 239:1400-1405.

Niall, H. D., M. L. Hogan, R. Sayer, I. Y. Rosenblum, and F. C. Greenwood. 1972. Sequences of pituitary and placental lactogenic and growth hormones: evolution from a primordial peptide by gene duplication. Proc. Natl. Acad. Soc. USA. 68:866.

Nicoll, C. S., G. L. Mayer, and S. M. Russell. 1986. Structural features of prolactins and growth hormones that can be related to their biological properties. Endocrine Rev. 2:169-203.

Nowakowski, B. E., and R. A. Maurer. 1994. Multiple pit-1-binding sites facilitate estrogen responsiveness of the prolactin gene. Mol. Endocrinol. 8:1742-1749.

Okimura, Y., P. W. Howard, and R. A. Maurer. 1994. Pit-1 binding sites mediate transcriptional responses to cAMP through a mechanism which does not require inducible phosphorylation of Pit-1. Mol. Endocrinol. 8:1559-1565..

Onali, P., J. P. Schwartz and E. Costa. 1981. Dopaminergic modulation of adenylate cyclase stimulation by vasoactive intestinal peptide in anterior pituitary. Proc. Natl. Acad. Sci. USA. 78:6531-6534.

Opel, H. and J. A. Proudman. 1988. Stimulation of prolactin release in turkeys by vasoactive intestinal peptide. Proc. Soc. Exp. Biol. Med. 187:455-460.

Pfaffle, R. W., G. E. DiMattia, J. S. Parks, M. R. Brown, J. M. Wit, M. Jansen, H. Van der Nat, J. L. Van den Brande, M. G. Rosenfeld, and H. A. Ingraham. 1992. Mutation of the POU-specific domain of Pit-1 and hypopituitarism without pituitary hypoplasia. Science 257:1118-1121.

Potter, E., A. K. Nicolaisen, E. S. Ong, R. M. Evans, and M. G. Rosenfeld. 1981. Thyrotropin-releasing hormone exerts rapid nuclear effects to increase production of the primary prolactin mRNA transcript. Proc. Natl. Acad. Sci. USA. 78:6662-6666.

Proudman, J. A. and H. Opel. 1980. Plasma prolactin levels in broody turkeys. Poult. Sci. 59:1653.

Proudman, J. A. and H. Opel. 1981. Turkey prolactin: validation of a radioimmunoassay and measurement of changes associated with broodiness. Biolo. Reprod. 25:573-580.

Proudman, J. A. and H. Opel. 1988. Stimulation of prolactin secretion from turkey anterior pituitary cells in culture. Proc. Soc. Exp. Biol. Med. 187:448-454.

Radovick, S., M. Nations, Y. Du, L. A. Berg, B. D. Weintrsub, and F. E. Wondisford. 1992. A mutation in the POU-homeodomain of Pit-1 responsible for combined pituitary hormone deficiency. Science 257:1115-1118.

Ryan, R., M. A. Shupnik and J Gorski. 1979. Effect of estrogen on preprolactin messenger ribonucleic acid sequences. Biochem. 18:2044-2048.

Sharp, P. J. 1989a. Physiology of egg production. *In* "Recent advances in turkey science". (C. Nixey and T. C. Gray, Eds). Battetworths, England.

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

Simmons, D. M., J. W. Voss, H. A. Ingraham, J. M. Holloway, R. S. Broide, M. G. Rosenfeld, and L. W. Swanson. 1990. Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. Genes Dev. 4:695-711.

Sinha, Y. N. 1995. Structural variants of prolactin: occurrence and physiological significance. Endocrine. Rev. 16:354-369.

Slabough, M. B., M. E. Lieberman, J. J. Rutledge, and J. Gorski. 1981. Growth hormone and prolactin synthesis in normal and homozygous Snell and Ames dwarf mice. Endocrinol. 109:1040-1046.

Stone, R. T., R. A. Maurer and J. Gorski. 1977. Effect of estradiol-17β on preprolactin messenger ribonucleic acid activity in the rat pituitary gland. Biochem. 16:4915-4921.

Straub, R. E., and M. C. Gershengorn. 1986. Thyrotropin-releasing hormone and GTP activate inositol triphosphate formation in membranes isolated from rat pituitary cells. J. Biol. Chem. 261:2712-2717.

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

Theill, L. E., K. Hattori, D. Lazzaro, J-L, Castrillo, and M. Karin. 1992. Differential splicing of the GHF1 primary transcript gives rise to two functionally distinct homeodomain proteins. EMBO J. 11:2261-2269.

Voss, J. W., L. Wilson, and M. G. Rosenfeld. 1991a. POU-domain proteins Pit-1 and Oct-1 interact to form a heterodimeric complex and can cooperate to induce expression of the prolactin promoter. Genes Dev. 5:1309-1320.

Voss, J. W., T. -P. Yao, and M. G. Rosenfeld. 1991b. Alternative translation initiation site usage results in two structurally distinct forms of Pit-1. J. Biol. Chem. 266: 12832-12835.

Voss, J. W., L. Wilson, S. J. Rhodes, and M. G. Rosenfeld. 1993. An alternative Pit-1 RNA splicing product reveals modular binding and nonmodular transcriptional activities of the POU-specific domain. Mol. Endocrinol. 7:1551-1560.

Watanabe, Y. G., and S. Daikoku. 1979. An immunocytochemical study on the cytogenesis of adenohypophysial cells in fetal rats. Develop. Biol. 68:557-567.

White, B. A. 1985. Evidence for a role of calmodulin in the regulation of prolactin gene expression. J. Biol. Chem. 260:1213-1217.

White, B. A., and F. C. Bancroft. 1983. Epidermal growth factor and thyrotropin-releasing hormone interact synergistically with calcium to regulate prolactin mRNA levels. J. Biol. Chem. 258:4618-4622.

White, B. A., L. R. Bauerle, and F. C. Bancroft. 1981. Calcium specifically stimulates prolactin synthesis and messenger RNA sequences in GH_3 cells. J. Biol. Chem. 256:5942-5945.

Wong, E. A., N. H. Ferrin, J. L. Silsby, and M. E. El Halawani. 1991. Cloning of a turkey prolactin cDNA: Expression of prolactin mRNA throughout the reproductive cycle of the domestic turkey (Meleagris gallapavo). Gen. Comp. Endocrinol. 83:18-26.

Youngren, O. M., G. R. Pitts, R. E. Phillips and M. E. Halawani. 1995. The stimulatory and inhibitory effects of dopamine on prolactin secretion in turkeys. Gen. Comp. Endocrinol. 98:111-117.

Youngren, O. M., M. E. Halawani, J. L. Silsby and R. E. Phillips. 1991. Intracranial prolactin perfusion induces incubation behavior in turkey hens. Biol. Reprod. 44:425-431.

CHAPTER II

Cloning of the Turkey Prolactin Gene and its 5'-Flanking Region

Abstract

To better understand the regulatory mechanism of prolactin (Prl) gene expression, the turkey Prl gene was isolated from a λ phage genomic library using a turkey Prl cDNA probe. The turkey Prl gene consists of five exons and spans approximately 6.7 kilobases (kb). The distribution of the exons was found to be nearly identical to the rat and human Prl genes, showing conservation of gene organization among these species.

The major transcription start site (tss) was determined by primer extension analysis to be 52 nucleotides (nt) upstream from the methionine start codon. A putative TATA box was found 24 nt upstream from the tss. Two putative polyadenylation signals (AATAAA) were identified 209 and 267 nt downstream from the stop codon. The gene organization predicts a mature transcript of approximately 1.1 kb, which is the size observed on Northern blots (Wong *et al.*, 1991).

The identification of potential regulatory elements was performed by computer analysis on 2 kb of 5'-flanking region. Two regions similar to the consensus mammalian binding site for the transcription factor Pit-1/GHF-1 are found at -51 to -62 nt and -118 to -128 nt from the tss. An estrogen response element (ERE) was not found within the 2 kb 5'-flanking region of the turkey Prl gene. This suggests that transcription of the turkey Prl gene may be regulated by Pit-1/GHF-1, and not by the estrogen receptor.

Introduction

Prolactin is a member of a structurally similar and an evolutionarily related peptide hormone family that includes growth hormone (GH) and placental lactogen (PL) (Miller and Eberhardt, 1983). An identical number of exons and similar splice sites in Prl, GH and PL genes among the known mammalian genes provide additional evidence that these three genes evolved from a common precursor. Prl and GH are produced by distinct cell types, lactotrophs and somatotrophs in the anterior pituitary, respectively. These cell types share a common cell lineage and are descended from somatomammotrophs, which transiently coexpress both hormones prior to the final differentiation step (Hoeffler *et al.*, 1985).

A cDNA encoding turkey Prl (tPrl) has been isolated and the deduced amino acid sequence predicts that tPrl consists of a 30 amino acid signal peptide followed by a 199 amino acid mature Prl and shows 90% homology to chicken Prl and 54-78% homology to other mammalian Prls (Wong *et al.*, 1991).

Prolactin in the turkey appears to play a role in the induction of incubation behavior in laying hens. A rise in circulating Prl levels leads to the early onset of incubation behavior, ovarian regression and the cessation of egg laying (reviewed by El Halawani and Rozenboim, 1993). Changes in plasma Prl levels are associated with the progression through the stages of the reproductive cycle (El Halawani *et al.*, 1988) and are correlated with changes in the steady state levels of pituitary Prl mRNA (Wong *et al.*, 1991). Prl mRNA is highest during the incubation phase, suggesting that an increase in Prl gene expression and/or Prl mRNA stability occurs during this phase.

Therefore, to investigate the molecular mechanism governing expression of the Prl gene in birds, the turkey Prl gene was isolated and the nucleotide sequence was analyzed.

Materials and Methods

Genomic DNA was isolated from the liver, and a genomic library was constructed in the genomic cloning vector Lambda GEM-12 (Promega, Madison, WI). The library was screened with a turkey Prl cDNA (Wong *et al.*, 1991), and two positive recombinant phage clones (λ6c and λ7) containing the complete turkey Prl gene, including more than 2 kb upstream of the methionine start codon, were isolated. The clones were mapped with restriction enzymes and fragments containing exons were subcloned into phagemid vector pTZ18R (Pharmacia, Piscataway, NJ). A nested set of deletions was constructed using an exonuclease III/mung bean nuclease kit (Stratagene, LaJolla, CA) and sequenced using the Sequenase version 2.0 kit (USB., Cleveland, OH). Sequence analysis software from the Genetics Computer Group (Madison, WI) and Lasergene (DNASTAR, Madison, WI) were used to align overlapping clones and process sequence data.

The transcription start site of the turkey Prl gene was determined by primer extension analysis. A [^{32}P]-end labeled synthetic oligonucleotide (approximately 5-10 ng), complementary to nt 18 to 35 of exon 1 (5'-TGCTCATGGTAGGGATTC-3'; tPrl-1a) of the turkey Prl gene was mixed with 1-2 μg of total pituitary RNA in 10 μl with DEPC-treated water, and heated at 95°C for 2 min, followed by incubation at 22-25°C for 3 min, then on ice for 15 min. Reverse transcriptase (RT) reactions were carried out in 50 μl reactions containing 50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol, 0.5 mM dNTPs, 20 units RNase inhibitor (Promega, Madison, WI) and 200 units of AMV reverse transcriptase (Promega, Madison, WI) at 37°C for 1 h. RT products were ethanol-precipitated at -20°C, and loaded onto polyacrylamide sequencing gels. As a marker, a DNA sequencing reaction was also performed on a plasmid containing the 5'-flanking region of the turkey Prl gene using the same oligonucleotide primer.

Results

Screening of the genomic library with the turkey Prl cDNA probe resulted in the isolation of two positive clones (λ6c and λ7). λ6c contained the entire Prl gene and 1 kb of 5'-flanking region of the gene, and λ7 contained at least exon 1 and 4 kb of 5'-flanking region.

The sequences of exons and part of the introns are shown in Fig. 2-1, and the gene structure with restriction enzyme sites in Fig. 2-2. The turkey Prl gene consists of five exons and four introns and spans approximately 6.7 kb. Exon 1 contain 52 nt of untranslated sequence and the start of the signal peptide up to the first nt of the tenth amino acid (aa) of the signal peptide. Exon 2 encodes the remainder of the signal peptide and the first 40 aa of the mature peptide. Exon 3 and 4 contain aa residues 41 to 76 and 77 to 139 of mature Prl, respectively. Exon 5 encodes aa residues 137 to 199. Two putative polyadenylation signals (AATAAA) were also found 209 and 276 nt downstream of the stop codon. This gene organization predicts a mature transcript of approximately 1.1 kb, which is the size observed on Northern blots (Wong *et al.*, 1991). A single nt polymorphism was found between the gene sequence and a previously reported cDNA sequence by Wong *et al.* (1991). The nt^{495} in the cDNA is a G (encoding Arg^{126}), while it is a T (encoding Leu^{126}) in the gene sequence. The exon-intron boundary sequences were compared (Table 2-1), and these boundaries of the tPrl gene were found to be in agreement with the widely accepted consensus splice donor/acceptor sequences (Breathnach and Chambon, 1981). The distribution of exons in the turkey Prl gene is nearly identical to the rat and human Prl genes (Table 2-1).

Primer extension analysis was conducted using an oligonucleotide primer that was complementary to nt 15 to 31 upstream of the first ATG start codon. A major transcription

start site was mapped 52 nt upstream of the methionine start codon (Figure 2-3). A putative TATA box was found 24 nt upstream from this tss (Figure 2-1).

Three regions with similar sequence to mammalian footprints I, II and III are found at -62 to -51 nt (5'-TGAATGTATGCA-3'), -99 to -86 nt (5'-GATGTTTGTAATTA-3'), and -128 to -118 nt (5'-TGAATATGAAT-3'), respectively (Table 2-2). Further analysis failed to identify an estrogen response element (ERE; 5'-GTCANNNTGACC-3') within 2 kb of 5'-flanking region of the turkey Prl gene.

Discussion

The turkey Prl gene shows a similar gene organization to the mammalian Prl genes, except that the mammalian genes are larger, approximately 10 kb in size (Chien and Thompson, 1980; Cooke and Baxter, 1982; Gubbins *et al.,* 1980; Maurer *et al.,* 1981; Truong *et al.,* 1984). The distribution of the exons is almost identical for the turkey, rat and human Prl genes, showing a conserved feature of the gene among these species (Table 2-1). The transcription start site of the turkey Prl gene was determined by primer extension analysis. A major tss was mapped 52 nt upstream of the initiator methionine codon, and a putative TATA box, TATAAA, is found 24 nt (-29 to -24) upstream of the tss. The distance between the tss and the methionine codon is similar to the rat and human Prl genes, 54/51 nt (Maurer *et al.*, 1981; Cooke and Baxter, 1982) and 57 nt (Truong *et al.*, 1984), respectively. The sizes of exon 1 vary due to the variable tss sites among the species. The size difference in exon 2 is due to a two-aa shorter signal peptide, for human Prl, and a shorter signal and mature peptide by one and two aa residues, respectively, for rat Prl. Interestingly, 145 nt upstream of the tss is a stretch of 29 adenine nucleotides in the turkey Prl gene. The function of this poly A tract is unknown.

A single nucleotide difference between the gene and cDNA was identified. The sequence of the cDNA was reexamined and confirmed to encode Arg^{126}. The samples used for the construction of the cDNA and the genomic DNA library were from different turkey hens. Thus, it is uncertain whether the nucleotide substitution resulted from a mutation during cloning or represents a prolactin isoform. Of note a partial turkey Prl cDNA reported by Karatzas *et al.* (1990) is identical to our genomic DNA sequence at Leu^{126}.

The sequence of the 2 kb 5'-flanking region of the turkey Prl gene was searched by computer for potential regulatory elements. Two sequences at -62 to -51 nt (5'-TGAATGTATGCA-3') and -128 to -118 nt (5'-TGAATATGAAT-3') were found to be highly homologous to the mammalian consensus sequence for binding of the transcription factor Pit-1/GHF-1. In the rat Prl gene, four transcription factor binding sites 250 nt upstream of the tss have been identified by DNase I footprinting (Table 2-2; Gutierrez-Hartmann *et al.*, 1987), and at least two regions (FP1; -37 to -63 and FP3; -142 to -170) represent the binding sites for Pit-1/GHF-1 (Nelson *et al.*, 1988; Fox *et al.*, 1990). A cDNA encoding the turkey Pit-1/GHF-1 has been cloned and shown to encode a protein with 94-95% aa homology in the DNA binding domain to rat Pit-1/GHF-1 (Wong *et al.*, 1992). Thus, it is likely that the turkey Prl proximal region codes for at least two Pit-1 binding sites, however, this needs to be experimentally verified.

A second region which shows high sequence identity in the turkey Prl gene and mammalian Prl genes was found at -99 to -86 nt (5'-GATGTTTGTAAT-3') of the turkey Prl gene. This region is 91% identical (11/12 bp) to a 12-bp consensus sequence of the mammalian Prl genes (5'-G/AATGTTTGAAAT-3') (Table 2-2), which is also highly conserved among all members of the GH gene family (Truong *et al.*, 1984; Gutierrez-Hartmann *et al.*, 1987). This sequence may not be involved in directing tissue-specific

expression, however, it may serve a basic transcription function for these genes. Precise function of the sequence is unknown.

In the rat Prl promoter, an ERE has been identified -1587 to -1563 nt of the tss (5'-GCATTTTTGTCACTATGTCCTAGAG-3') and shown to confer estrogen responsiveness (Maurer and Notides, 1987). Computer analysis failed to identify the consensus ERE or similar sequences within 2 kb of the 5'-flanking region of the turkey Prl gene, suggesting that transcription of the turkey Prl gene may not be controlled by the estrogen receptor. It is still possible that an ERE is located further upstream, however, the lack of an ERE is consistent with the observation that cultured turkey pituitary cells did not secrete Prl in response to estrogen (Knapp *et al.*, 1988), whereas rat pituitary cells did respond to estrogen (Lieberman *et al.*, 1978).

In the rat Prl promoter (-1831 to -1530 nt), there is a distal enhancer region which contains four Pit-1 binding sites adjacent to the ERE. More than 98% of the promoter activity is reported to be controlled by this enhancer region (Nelson *et al.*, 1988). Computer analysis of the turkey Prl gene failed to identify these distal Pit-1 binding elements. Only the two putative Pit-1 binding sites were identified within 150 bp upstream of the tss. Thus, it appears that a regulatory motif different from mammals exists for the turkey Prl gene.

Figure 2-1. The sequences of exons and part of the flanking introns of the turkey prolactin gene. Deduced amino acid sequences are shown in italics. A putative TATA box in exon 1 and two polyA signals in exon 5 are underlined. The two putative Pit-1/GHF-1 binding sites are double-underlined. The major transcription start site 52 nt upstream from the methionine start codon is shown in bold faced type and designated as +1. (The EMBL, GenBank and DDBJ Nucleotide Sequence Database accession numbers for exon 1 to 5 are UO5953 to UO5957, respectively).

Exon 1

-1980 atcccctgaatcatagaatcatagggtttggaaggcacatcctggagatcactgagtcca
-1920 aagacctctgctaatgcaggttacctatagtaggttgtacaggaaactgcccaggaagat
-1860 tttgagtatctccagaggacactcatcaatctctctgggcagcttgttccactgctctgt
-1800 caccctaaaagtaaagtttttcctaatgttcatatggaacttcctgtgttacagtttatc
-1740 cccattgctccttgttctgtcacttggcaccaccaaaaacagcctggccccattctcatc
-1680 ctttagatatctataagcattgatgagatccactcaatcttctccaggttgtgtgacccc
-1620 aggtctctgatcctttcctcagaaggaagatgctccaggcccagtcatatttgtggtctc
-1560 ccactgaactctttccagtagttccctgattttcttgaagtgaggagcccagaactggca
-1500 cagtactcaagacatggcctcatcagggcagagtagaggggggaaagtcacctcccttaac
-1440 ctgatgactacattccttttaatgcatctcaagatactactggccttgttggccgcaagg
-1380 gcacactgctagcccacggtcaacctgttgtctaccatggacatctaggtcttctcagca
-1320 gagctcctttccagcaggtcagcccccagcctgtactaacgcaagcagtaaaaaaggtct
-1260 acctttttttctccttcatagaatcacagaattgtaggggtaggagggaacctcggagatc
-1200 atctagtccaaccactctgccaaatcatcacagtaacacaggaaagtgtctggtgggttt
-1140 tgtttattattgcttcaaaaagcacagccacagttacgaaataatgggagattcaggatt
-1080 atacacatacctgttccacatgtacagaacaagttgtctagaggcaagaaaattcaggaa
-1020 cactgtataccttattcattatgatcatctaatttagaaggtcttttcgtggataaatgc
-960 atctgagaaacagatgagagattacgcatttgctaacatattcgtgcagatgaacctcac
-900 acaacaagaaaacagggccaacctgctgaagctaggttgcagattaccacagacacatta
-840 gatcaggaatcagattccactgattacgacagcatatactgtgattatggtggacatgca
-780 catcttttacgcaaagaattttcatatatagaaaatgatttcatggttcggaagctttta
-720 aaataatgctgatttaattacaaaatgtttatgattaaacagtaagcatacaaattcttc
-660 ctctttgttgttacaaattattacttttttaatgacaactgtccctgtttctcaacttat
-600 ctcatccttagtaccagttatatcattatctgttggtaaataatatccttttttagctgta
-540 tggagacaaacacacactacgtataataatgacctgtctttccagaagcctccattcaca
-480 ttctctggatcaacttcagtacaattcctattctttctcttactgtagaaattgtattat
-420 ttcctttccagaaatagctagaattggagggtgaagagacaaggaagaaacagaagatat
-360 ctgcagggatgaacaacattttataaacatagaggagaacaatctcagaactgacaactg
-300 gaccggacctttcaaggatcagtggcatttgcaactaattcagtgcaaaattttggcgtt
-240 ctcttcatccagccatactcagcatcccacaactgaaatttttaatgaaattcccactca
-180 cagttaaaaaaaaaaaaaaaaaaaaaaaaaaaaagaacccaaaagcaagtattgaatatg
-120 aatgtggaagagaggcaatttgatgtttgtaattaccgaggtaaactccacaacctgctg
-60 aatgtatgcaaactggaccccggatggtgtatataaatctgacatgcagaaagtaagagc
1 AGGTATTGAGACTTCTTTCTGGTAGAGCAAGTCATCACAGAGAATCCCTACCATGAGCAA
MetSerAs *-28*
61 CACAGGGGCTTCATTGAAAGgtaagacttttgctattccctgtctgataacttctatgtt
nThrGlyAlaSerLeuLysG *-21*
121 taggttttgattgaattaagaagaagctggagggtaacaattctagaaactaagtttt

Exon 2

1 gcatgctttcgtcaatatgttcccattttggtttgttccaaacctgaacagGTTTGTTGC
lyLeuLeuL *-18*
61 TGGCGGTTCTTCTGGTATCCAACATGCTTCTGACCAAGGAAGGAGTGACCTCCTTGCCAA
euAlaValLeuLeuValSerAsnMetLeuLeuThrLysGluGlyValThrSerLeuProI *3*
121 TCTGCTCCAGTGGATCTGTCAACTGCCAAGTTTCCCTTGGGGAGCTTTTTGATCGGGCAG
leCysSerSerGlySerValAsnCysGlnValSerLeuGlyGluLeuPheAspArgAlaV *23*
181 TTAGACTTTCACACTACATACACTTCCTCTCTTCAGAAATTTTCAATGAATTTgtaagta
alArgLeuSerHisTyrIleHisPheLeuSerSerGluIlePheAsnGluPhe *40*
241 ctttctattttcttgggagtgagaacatgcaaaatccacttcagtgtttttgagcacaa
301 acatattaatatatgaaac

Exon 3

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  1  taaaaaaaacctctccttgatgatctgcagGATGAACGCTATGCTCAGGGTCGGGGTTTC
                                   AspGluArgTyrAlaGlnGlyArgGlyPhe   50
 61  ATTACAAAAGCTGTTAATGGCTGCCACACTTCCTCCTTAACCACTCCTGAAGATAAGGAG
     IleThrLysAlaValAsnGlyCysHisThrSerSerLeuThrThrProGluAspLysGlu   70
121  CAAACTCAGCAGATTCATgtaagtcctacatctaatgccaatgactgtgagacggagtgt
     GlnThrGlnGlnIleHis                                            76
181  gggaaaaaaaaa
```

Exon 4

```
  1  acctttttcttaacatggatgaccagCACGAAGAGCTACTGAATTTGATACTGGGAGTGCT
                               HisGluGluLeuLeuAsnLeuIleLeuGlyValLe   88
 61  GCGTTCCTGGAATGATCCCCTGATCCATCTGGCCTCTGAAGTGCAAAGAATCAAAGAAGC
     uArgSerTrpAsnAspProLeuIleHisLeuAlaSerGluValGlnArgIleLysGluAl  108
121  TCCAGATACCATCCTCTGGAAGGCTGTAGAGATTGAGGAGCAAAACAAGAGGCTTTTAGA
     aProAspThrIleLeuTrpLysAlaValGluIleGluGluGlnAsnLysArgArgLeuGl  128
181  AGGAATGGAGAAAATCGTTGGGCGGgtaagtattaggtccttacagtccttccagcttat
     uGlyMetGluLysIleValGlyArg                                     136
241  tggtgcaaagaacca
```

Exon 5

```
  1  ggtggaaattcaatcaaaatctgcaagaggatatatagaacactatgcagaacagaggga
 61  gccataacaggaaacactttacaagctgtacactattaactagcgttcctcaaggccaat
121  atttcttaattctctgttctacacccagtcagattgattatcatctcttacagtacgatt
181  atgtctctctagATTCATTCTGGCGATGCTGGAAATGAAGTTTTCTCTCAGTGGGACGGC
                 IleHisSerGlyAspAlaGlyAsnGluValPheSerGlnTrpAspGly  152
241  CTTCCATCCCTGCAACTCGCTGATGAGGACTCCAGACTCTTTGCTTTTTACAACCTGCTG
     LeuProSerLeuGlnLeuAlaAspGluAspSerArgLeuPheAlaPheTyrAsnLeuLeu  172
301  CATTGCCTCCGCAGAGATTCCCACAAAATCGACAACTATCTTAAAGTTTTGAAGTGCCGC
     HisCysLeuArgArgAspSerHisLysIleAspAsnTyrLeuLysValLeuLysCysArg  192
361  CTAATCCATGATAACAATTGCtaagtacctctgggctgcataactcaccgcaaccattca
     LeuIleHisAspAsnAsnCys                                         199
421  ccgtggtgttcttgttgctttgccacttttccttgcgaactttatgaaaagccacatcgt
481  acggcaccaggatcatcagtaactttcaggcatgcttgtgtgaattctggctgcaactct
541  cagtgccatttatcttagtgtttaaaatgctgcaagtcactcgtgtaacaagaataaact
601  cctctgcctaatttcctcacttagtagcatttcactgataagttgatcgtgtagatacaa
661  ataaaaatattattaaattcaaaatcacataacgtcaagtggtatccaaaa
```

Figure 2-2. Organization of the turkey Prl gene. Exons are shown as boxes. Coding regions are shown as black boxes and non-coding regions as open boxes. Approximate locations of restriction enzyme sites are shown as letters, X, H, P, E, and S representing restriction enzymes Xba I, Hind III, Pst I, EcoRI and Sph I, respectively.

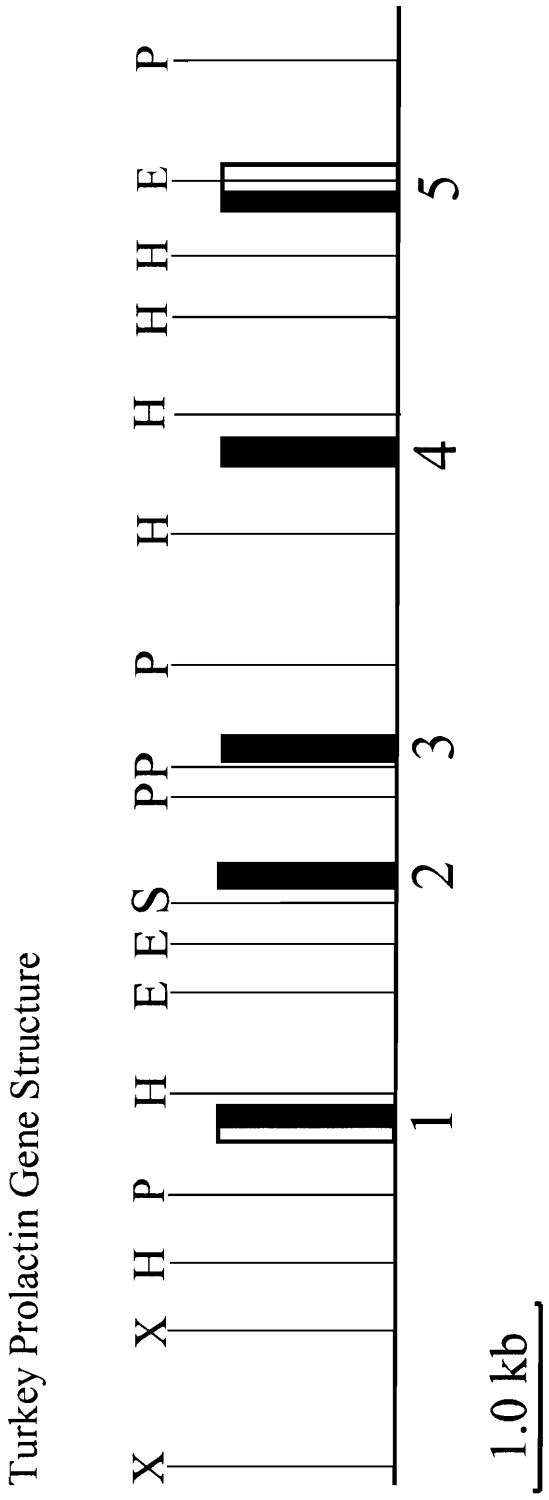

Turkey Prolactin Gene Structure
X
X
H
P
H
E
E
S
P
P
P
H
H
H
H
E
P
1
2
3
4
5
1.0 kb

Figure 2-3. Primer extension analysis of turkey Prl mRNA. Lane X is the primer extension product. Lanes A, T, G, C represent a sequence ladder generated from a plasmid containing the 5'-flanking region of the turkey Prl gene using the same primer extension oligonucleotide as a primer. The major transcription start site is mapped 52 nt upstream of the methionine start codon.

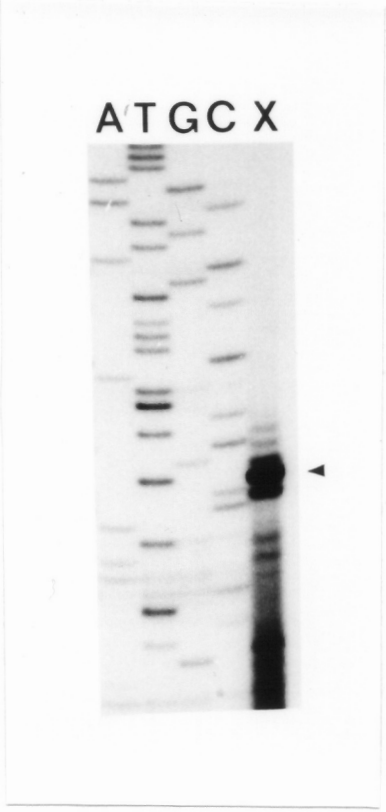

A T G C X

Table 2-1. Exon distribution and splicing sites in the rat, human and turkey Prl genes.

Table 2-1.

exon	exon size (bp)	Exon 3'- junction	5' intron 3'	Exon 5' junction	exon
1	hPrl 84 rPrl 82 (79) tPrl 80	.....tggaaag .....cggaaag .....attgaaag	GTATGTG.....AAGCAG GTATGTG.....TCTTTAG GTAAGAC.....GAACAG	ggtccctc..... cag*ggaca.. gtttgttgc.....	2
2	hPrl 176 rPrl 173 tPrl 182	..... gcgaattc .....attgaattt .....atgaattt	GTAAGTA.....TCTTTAG GTAAGTA.....TTTCTAG GTAAGTA.....TCTGCAG	gataaacg..... gataaaca..... gatgaacg.....	3
3	hPrl 108 rPrl 108 tPrl 108	.....tcagccag ....aaagtccct .....cagattcat	GTGAGTC.....CCTGCAG GTGAGTC.....GGATTAG GTAAGTC.....TGACCAG	caaaaaga..... ccggaagt..... cacgaaga....	4
4	hPrl 180 rPrl 180 tPrl 180	.....tcagccag .....ttagccag .....tgggcgg	GTGAGCA.....TGATTAG GTGAGCA.....TTATTAG GTAAGTA.....TCTCTAG	gttcatcct..... gcctatcct..... attcattctg....	5

* An alternative splicing site in the rat Prl gene.

Table 2-2. Comparison of DNase I protected sites of the rat Prl promoter region by GH_3 cell extracts (Gutierrez-Hartmann *et al.*, 1987) with the human and bovine Prl genes. The consensus sequence and the turkey Prl gene are shown for comparison.

Table 2-2.

	Footprint I			Footprint II				Footprint III	
rPrl	-67	CCTGATTATATATATATTCA	-48	-131	GATGTTTAAAAT	-120	-163	CCTGAAAATGAATAAGAA	-146
hPrl	-63	CCTGAATCATTATATTCA	-46	-129	GATGTTTGAAAT	-118	-160	CCTGAATATGAATAAGAA	-143
bPrl	-62	CCTGTTAAATATATTCA	-45	-128	AATGTTTGAAAT	-117	-160	CCTGAATATGAATAAGAAA	-142
Consensus		CCTGA(T/A)TANTATATTCA			(G/A)ATGTTTGAAAT			CCTGAA(T/A)ATGAATAAGAA	
tPrl	-62	TGAATGTATGCA	-51	-99	GATGTTTGTAATTA	-86	-128	TGAATATGAAT	-118

References Cited

Breathnach, R., and P. Chambon. 1981. Organization and expression of eucaryotic split genes coding for proteins. Annu. Rev. Biochem. 50:349-383.

Chien, Y-H, and E. B. Thompson. 1980. Genomic organization of rat prolactin and growth hormone genes. Proc. Natl. Acad. Sci. USA. 8:4583-4587.

Cooke, N. E., and J. D. Baxter. 1982. Structural analysis of the prolactin gene suggests a separate origin for its 5' end. Nature 297:603-606.

El Halawani, M. E., S. Fehrer, B. M. Hargis and T. E. Porter. 1988. Incubation behavior in the domestic turkey: physiological correlates. CRC, Critical Review in Poultry Biology. 1:285-314.

El Halawani, M. E. and I. Rozenboim. 1993. The ontogeny and control of incubation behavior in turkeys. Poult. Sci. 72:906-911.

Fox, S. R., M. T. C. Jong, J. Casanova, Z-S. Ye, F. Stanley, and H. H. Samuels. 1990. The homeodomain protein, Pit-1/GHF-1, is capable of binding to and activating cell specific elements of both growth hormone and prolactin gene promoters. Mol. Endocrinol. 4:1069-1080.

Gubbins, E. J., R. A. Maurer, M. Lagrimini, C. R. Erwin, and J. E. Donelson. 1980. Structure of the rat prolactin gene. J. Biol. Chem. 255:8655-8662

Gutierrez-Hartmann, A., S. Siddiqui, and S. Loukin. 1987. Selective transcription and DNase I protection of the rat prolactin gene by GH_3 pituitary cell-free extracts. Proc. Natl. Acad. Sci. USA. 84:5211-5215

Hoeffler, J. P., F. R. Boockfor, and L. S. Frawley. 1985. Ontogeny of prolactin cells in neonatal rats: initial prolactin secretors also growth hormone. Endocrinol. 117:187-195.

Karatzas, C. N., D. Zadworny, and U. Kuhnlein. 1990. Nucleotide sequence of turkey prolactin. Nucleic Acids Res. 18:3071

Knapp, T. R., S. C. Fehrer, J. L. Silsby, T. E. Porter, E. J. Behnke and M. E. El Halawani. 1988. Gonadal steroid modulation of basal and vasoactive intestinal peptide-stimulated prolactin release by turkey anterior pituitary cells. Gen. Comp. Endocrinol. 72:226-236.

Lieberman, M. E., R. A. Maurer and J. Gorski. 1978. Estrogen control of prolactin synthesis in vitro. Proc. Natl. Acad. Sci. USA. 75:5946-5949.

Maurer, R. A., and A. C. Notides. 1987. Identification of an estrogen-responsive element from the 5'-flanking region of the rat prolactin gene. Mol. Cell. Biol. 7:4247-4254.

Maurer, R. A., C. R. Erwin, and J. E. Donelson. 1981. Analysis of 5' flanking sequences and intron-exon boundaries of the rat prolactin gene. J. Biol. Chem. 256:10524-10528.

Miller, W. L., and N. L. Eberhardt. 1983. Structure and evolution of the growth hormone gene family. Endocrine Rev. 4:97-130.

Nelson, C., V. R. Albert, H. P. Elsholtz, L. I.-W. Lu and M. G. Rosenfeld. 1988. Activation of cell specific expression of rat growth hormone and prolactin gene by a common transcription factor. Science 239:1400-1405.

Truong, A. T., C. Duez, A. Belayew, A. Renard, R. Pictet, G. I. Bell, and J. A. Martial. 1984. Isolation and characterization of the human prolactin gene. EMBO J. 3:429-437.

Wong, E. A., N. H. Ferrin, J. L. Silsby, and M. E. El Halawani. 1991. Cloning of a turkey prolactin cDNA: Expression of prolactin mRNA throughout the reproductive cycle of the domestic turkey (Meleagris gallopavo). Gen. Comp. Endocrinol. 83:18-26.

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

CHAPTER III

Mapping Regulatory Elements of the Turkey Prolactin Gene using a Rat Pituitary Cell Line, GH_3, and Primary Turkey Pituitary Cells

Abstract

In order to identify regulatory elements in the upstream region of the turkey prolactin (Prl) gene, a rat pituitary-derived tumor cell line, GH_3 and primary turkey pituitary cells were transiently transfected with reporter gene constructs containing the 5'-flanking region of the turkey Prl gene. In the GH_3 system, relative to the construct containing 2.0 kb upstream from the tss (-2.0), promoter activity was 2.4-fold higher in a -1.3 kb construct, while the activity was lower in a -1.0 kb construct by 30%. Elimination of the putative Pit-1 binding site located between -128 to -118 nt significantly lowered promoter activity, and elimination of another Pit-1 binding site (-62 to-51 nt) resulted in the loss of promoter activity. These results indicate that negative-acting elements may be present between -2.0 and -1.3 kb and a positive-acting element is present between -1.3 and -1.0 kb of the turkey Prl gene. The decrease in promoter activity resulting from elimination of one or two Pit-1 binding sites strongly suggest that the Pit-1 transcription factor plays an essential role for turkey Prl gene activation.

Dispersed primary turkey pituitary cell cultures were prepared for transient transfection to test the regulatory elements in a native system. Despite repeated trials, no consistent reporter gene expression was obtained from the cells transfected with the Prl promoter-reporter gene constructs, while relatively high reporter gene expression was consistently recorded by an avian sarcoma virus-long terminal repeat (ASV-LTR)-driven construct. Northern blot analysis of the dispersed primary pituitary cells with Prl, Pit-1 and β-actin cDNA probes showed that Prl mRNA peaked at 72 h and slightly declined at 96 h and 120 h, demonstrating the viability of the cells and activation of the Prl gene in culture. Pit-1 mRNA was not detected at any time points throughout the 120 h incubation. High Prl levels in the media of vasoactive intestinal peptide-stimulated pituitary cells again indicated that the dispersed cells in culture were viable and responsive to known Prl-secretagogues.

Introduction

Changes in circulating prolactin (Prl) levels in turkey hens are correlated with different stages in the reproductive cycle. The onset of frequent nest visits by laying hens is often associated with an increase in circulating Prl levels, suggesting that Prl plays an important role in the induction of incubation behavior (El Halawani *et al.*, 1988). A dramatic rise in circulating Prl levels leads to ovarian regression, the cessation of egg laying and the early onset of incubation behavior (Reviewed by Hall *et al.*, 1986; El Halawani *et al.*, 1988; Sharp, 1989; El Halawani and Rozenboim, 1993). The increase in circulating Prl levels in incubating hens is due to a combination of factors: 1) increased size of the anterior pituitary, 2) increase in the number of Prl-producing lactotrophs, 3) enhanced stimulation of Prl secretion due to an increased number of vasoactive intestinal peptide (VIP)-immunoreactive neurons in the hypothalamus (Mauro *et al.*, 1989), and 4) changes in the steady state levels of pituitary Prl mRNA, resulting from an increased Prl gene expression and/or Prl transcript stability (Wong *et al*, 1991).

Precise interactions between the 5'-flanking region of genes and transcription factors are important for tissue-specific and hormone-responsive gene expression. Transient transfection of rat pituitary-derived tumor cell lines with reporter gene constructs containing the rat Prl gene promoter have been used to locate the proximal promoter region within 250 nt of the transcription start site (tss) and the distal enhancer region between -1500 and -1800 bp. Both regions are crucial for tissue-specific expression of the Prl gene (Nelson *et al.*, 1986; 1988; Crenshaw *et al.*, 1989). A transcription factor, Pit-1/GHF-1, has been shown to be required for the rat Prl gene through interactions at several binding sites clustered at the proximal promoter and the distal enhancer regions of the Prl gene (Bodner *et al.*, 1988; Ingraham *et al.*, 1988). Reconstitution of Prl promoter activity was achieved by co-transfection of non-pituitary cell lines with a Pit-1/GHF-1 expression vector

and the Prl promoter-reporter gene construct (Ingraham *et al.*, 1988; Rajnarayan *et al.*, 1995).

Computer analysis of 2 kb of 5'-flanking region of the turkey Prl gene revealed two regions (-51 to -62 nt and -118 to -128 nt) similar to mammalian Pit-1/GHF-1 binding sites (Kurima *et al.*, 1995). A cDNA encoding the turkey Pit-1/GHF-1 has been cloned and shown to encode a protein with 94-95% aa identity in the DNA binding domains to rat Pit-1/GHF-1 (Wong *et al.*, 1992), suggesting that transcription of the turkey Prl gene may be regulated by Pit-1/GHF-1.

In order to identify regulatory elements for turkey Prl gene expression, a rat pituitary-derived tumor cell line, GH_3 and primary turkey pituitary cells were transiently transfected with reporter gene constructs containing different lengths of the 5'-flanking region of the turkey Prl gene.

Materials and Methods

Cell culture

GH_3 cells were maintained in Ham's F-12 medium supplemented with 15% horse serum, 2.5% fetal calf serum, and antibiotic-antimycotic (0.1 units/ml penicillin, 100 ng/ml streptomycin, and 0.25 ng/ml amphotericin) in a humidified 5% CO_2/95% air incubator at 37°C. 24 h prior to transfection, GH_3 cells were dispersed with trypsin-EDTA solution, and 0.5-1 x 10^6 cells in 3 ml of growth medium were plated in a 35 mm dish.

For the primary turkey pituitary cell culture, actively laying hens or hens showing incubation behavior were electrocuted on site at the Virginia Tech Turkey Center. Whole pituitary glands were immediately removed and incubated in ice-cold Ca^{2+} and Mg^{2+} free-Hank's basic saline solution (CMF-HBSS) during transportation to the laboratory. The posterior pituitary and associated tissues were removed, and the anterior pituitary was

sliced into smaller pieces with a scalpel blade. Tissues were then suspended in CMF-HBSS containing 0.3% collagenase IV (Worthington, Freehold, NJ) and 3% BSA (Sigma), and incubated at 37°C for 45 min under mild agitation. Tissue fragments were periodically sheared through a sterile, siliconized, flame-polished Pasteur pipette (approx. ID = 1/32") to aid enzyme digestion. After 45 min, the sample was transferred to a conical tube and centrifuged at 400 xg for 1 min, and the supernatant was removed by aspiration. The pellet was washed with CMF-HBSS and centrifuged again. The supernatant was removed and the pellet was suspended in CMF-HBSS containing 1.25% pancreatin (Sigma) and incubated at 37°C for 45 min under mild agitation. Dispersed cells were harvested by centrifugation at 400 xg for 1 min and washed with a DMEM incubation medium supplemented with 10% fetal calf serum, 3.7 mg/ml $NaHCO_3$, 1% non-essential amino acids (Sigma) and 25 mM HEPES several times to remove pancreatin particles. Cell number was counted on a hemacytometer, and cell viability was determined by the trypan blue dye exclusion method. 0.25 -1 x 10^6 cells were plated on 4x6 multi-well plates or 35 mm culture dishes and preincubated in a humidified 5% CO_2/95% air incubator (39-40°C) for 24 to 72 h before liposome-mediated transfection.

Northern blot analysis and Prl RIA.

The dispersed cells were plated at 2 million cells/35 mm dish, and total RNA was collected every 24 hours for 5 days using TRI REAGENT (Molecular Research Center, Cincinnati, OH). Half the collected total RNA for each sample was loaded on 1% agarose gels containing 2.2 M formaldehyde. RNA sizes were estimated based on the migration of RNA ladders (0.24-9.5 kb, Gibco-BRL). RNA was transferred to MAGNA nylon membranes (MSI, Westborough, MA) by capillary action and hybridized with a turkey Prl cDNA probe labeled by random priming (Promega). After autoradiography, the membrane was boiled for 30 min in 0.015M NaCl, 0.0015 M sodium citrate, 1% SDS to remove the

Prl probe and rehybridized with a turkey Pit-1/GHF-1 cDNA probe. The membrane was rehybridized with a chicken β-actin cDNA probe after the Pit-1/GHF-1 probe was removed.

Viability of the dispersed cells in culture was further tested based on Prl secretion responsiveness to chicken vasoactive intestinal peptide (VIP). 2.5 x 10^5 dispersed cells were plated on 4x6 multi-well plates, and incubated in the presence or absence of 100 nM chicken VIP for five days. 1 ml of incubation media was collected and assayed for Prl by Dr. J. A. Proudman (ARS, USDA) (Opel and Proudman, 1988).

Plasmid constructs

The upstream region of the turkey Prl gene was amplified using polymerase chain reaction (PCR). An oligonucleotide primer Prl-1c (5'-TCGACGCGTAGAAGGTCTTTTCGTGG-3': +21 to +42) was paired with either Prl-1J, (5'-TCGACTCGTCTTACTACCAGTTCTCG-3': -2052 to -2030), Prl-1d (5'-CGCTCGAGTGTGATGACTTGCTCTAC-3': -986 to -976), Prl-1l (5'-AGCGGTACCAGCCATACTCAGC-3': -232 to -218), Prl-1k (5'-GTCGGTACCAGAACCCAAAAGC-3': -147 to -135), Prl-1t (5'-GTCGGTACCATGTGGAAGAGAGG-3': -119 to -104), Prl-1m (5'-TAAGGTACCAACTGGACCCCGG-3': -49 to -38) to amplify regions between -2052 to +42, -986 to +42, -232 to +42, -147 to +42, -119 to +42 and -49 to +42 of the tPrl gene, respectively. These PCR products were cloned into a luciferase reporter vector, pGL2-basic and pGL3-basic (Promega, Madison, WI), and named pp2052pGL, pp986pGL, pp232pGL, pp147pGL, pp119pGL, and pp49pGL, respectively. Other promoter constructs were generated by the mung bean/exonuclease III deletion kit (Stratagene) from pp986pGL or by the use of convenient restriction enzyme sites for pp1316pGL and pp355pGL from pp2052pGL. The avian sarcoma virus-long terminal repeat (ASV-LTR) fragment (1.3 kb) from pBR322/ASV-2LTR/tk A (Folger *et al.*, 1982),

was inserted into the multiple cloning site of pGL2-basic and pGL3-basic vectors. A cytomegalovirus (CMV)-β-galactosidase control plasmid was obtained from Dr. R. N. Day (University of Virginia) and was used to normalize the variation in transfection efficiency among the samples.

Transient transfection by lipofectAMINE

Prior to transfection, cells were washed with Opti-MEM (Gibco-BRL, Gaithersburg, MD), and the testing plasmid was incubated in 200 μl of Opti-MEM plus lipofectAMINE (Gibco-BRL) for 15-25 min at 22-25°C to form a complex. For GH_3 cells, 3 μg of DNA and 15 μg of lipofectAMINE were used, and the DNA-lipofectAMINE complexes in 1 ml of Opti-MEM were added to the cells and incubated for 5 hours. Transfection was terminated by diluting the transfection media with 3 ml of the growth media. After 24 h, the cells were washed with PBS, and 200 μl of Reporter Lysis Buffer (Promega) was added and incubated for 15 min at 22-25°C before harvesting. For primary cells, DNA-lipofectAMINE complexes in 1 ml of Opti-MEM were added to the cells and incubated for various times. The transfection was terminated by diluting the transfection media with 3 ml of the growth medium. After 24-72 h, the cells were washed with PBS, and 200 μl of Reporter Lysis Buffer was added and incubated for 15 min at 22-25°C before harvesting. Cell lysates were subjected to three quick freeze-thaw cycles. The samples were centrifuged at 4,000 xg for 2 min and the supernatants were collected for assaying luciferase and β-galactosidase activities.

Luciferase and β -galactosidase assays

For the luciferase assay, 50 μl of each cell lysate was diluted with 300 μl of ATP buffer (5 mM ATP, 25 mM glycylglycine [pH 7.8], 15 mM $MgSO_4$). The reaction was initiated by injecting 100 μl of luciferin buffer (1 mM luciferin, 25 mM glycylglycine, 15

mM $MgSO_4$), and light emission was measured for 25 seconds using a Lumat LB9501/16 luminometer (Berthold, Bad Wildbad, Germany).

For the β-galactosidase assay, 25 µl of cell lysate was mixed with 250 µl of β-galactosidase assay buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol, 2 mM o-nitrophenyl-β-D-galactopyranoside) and incubated at 37°C for 30 to 60 min until the yellow color developed. The reaction was terminated by adding 500 µl of 1M Na_2CO_3, and the absorbance was measured at 420 nm by spectrophotometry.

To correct for variation in transfection efficiency, luciferase activity for each sample was divided by β-galactosidase activity.

Results

Transient transfection of GH_3 cells with the turkey Prl promoter-reporter gene showed stimulation of the turkey Prl promoter (Fig. 3-1). Relative to the pp2052pGL2 construct, promoter activity of pp1316pGL2 construct was 2.4-fold higher. Promoter activities of the constructs containing -986 bp through -229 bp showed approximately 30% of the activity of pp2052pGL2. Elimination of one putative Pit-1/GHF-1 binding site (pp119pGL2) significantly lowered promoter activity. Elimination of both Pit-1/GHF-1 binding sites (pp49pGL2) showed a minimal promoter activity, but it was not statistically different from the Prl promoterless vector.

The protocol for isolating primary turkey pituitary cells consistently yielded 3-5 million cells per pituitary with greater than 95% viability based on the trypan blue dye exclusion test. The primary pituitary cells were transiently transfected under different conditions. The duration of preincubation (24-72 h), the recovery time (24-48 h), and the DNA concentrations (3-10 µg) under a constant DNA: lipofectAMINE ratio of 1:5 were tested. Despite high luciferase activity by the ASV-LTR promoter-luciferase construct, no

consistent results were obtained from the cells transfected with Prl promoter-luciferase constructs (Table 3-1).

In order to test the ability of dispersed pituitary cells to initiate normal gene activation after enzymatic cell dispersion, Northern blot analysis of Prl, Pit-1/GHF-1 and β-actin mRNAs was performed (Fig. 3-2). Northern blot analysis of total RNA collected from cultured cells every 24 hours for 5 days showed that Prl mRNA was present and peaked at 72 h in culture, and then slightly declined for the next 48 h. Pit-1 mRNA was not detected in any of the samples during the culture period. β-actin mRNA was not detected at 24 and 48 h, but was abundant at 72, 96, and 120 h.

Viability of lactotrophs was further tested by monitoring Prl secretion following VIP stimulation. VIP is a potent Prl releasing factor in avian species (Macnamee *et al.*, 1986; Opel and Proudman, 1988), and VIP-stimulated Prl secretion from dispersed turkey pituitary cells has been reported (Proudman and Opel, 1988). Prl concentrations in the incubation media collected after 5 days in culture were 16.55 μg/ml in the presence of VIP and 0.372 μg/ml without VIP.

Discussion

The presence of regulatory elements located in the 5'-flanking region of the turkey Prl gene was studied using transient transfection of a rat pituitary tumor cell line GH_3 and primary turkey pituitary cells with the turkey Prl gene promoter-luciferase constructs. Transfection of GH_3 cells resulted in expression of the reporter gene (Fig. 3-1), whereas the primary pituitary cells failed to show consistent luciferase expression (Table 3-1).

Promoter sequences of the turkey and rat Prl genes share few similarities, with the exception of three regions within 200 bp upstream of the tss. These three regions are protected in a DNase I footprinting analysis using GH_3 cell nuclear extracts (designated as

FPI, FPII and FPIII by Gutierrez-Hartmann *et al.*, 1987)(Table 2-2) and highly conserved in the human and bovine Prl promoters. Two regions, FPI and FPIII, have later been shown to be binding sites for the transcription factor Pit-1/GHF-1. In the current experiments, elimination of these putative Pit-1/GHF-1 binding sites in the turkey Prl promoter resulted in the loss of promoter activity in transiently transfected GH_3 cells. These results likely demonstrate that rat Pit-1/GHF-1 binds to the putative Pit-1/GHF-1 binding sites in the turkey Prl promoter for gene activation. Since turkey and rat Pit-1/GHF-1 share 94-95% amino acid identity in the DNA binding POU domain (Wong *et al.*, 1992), turkey Pit-1/GHF-1 likely binds to the turkey Prl gene sequences that correspond to FPI and FPIII. Pit-1/GHF-1-dependent activation of the Prl gene has been shown in rat, human, bovine, and even in a distantly related vertebrate, salmon (Bodner *et al.*, 1988; Camper *et al.*, 1990; Elsholtz *et al.*, 1992; Ingraham *et al.*, 1988; Peers *et al.*, 1990). The present data supports the hypothesis that Pit-1/GHF-1-dependent activation of the Prl gene is phylogenetically conserved in avian species. Direct evidence requires reconstitution of promoter activity of the turkey Prl gene by turkey Pit-1/GHF-1 expression in non-pituitary cell lines.

A positive-acting element was located in the -1.3 to -1.0 kb upstream region, as elimination of this region resulted in a decrease in promoter activity by 90%. In addition, the results indicated that negative elements may be present in the -2.0 to -1.3 kb region. In rat, the -1.5 to -1.8 kb upstream region contains a distal enhancer region with four Pit-1/GHF-1 binding sites and an estrogen response element (ERE) (Maurer and Notides, 1987; Nelson *et al.*, 1986; 1988). More than 98% of gene promoter activity appears to be located in this distal enhancer region (Nelson *et al.*, 1988). Computer analysis failed to identify Pit-1 binding elements or an ERE between 2.0 kb and 150 bp upstream of the tss of the turkey Prl gene. Thus, the nature of the enhancer-like activity between -1.3 and -1.0

kb appears to be Pit-1/GHF-1 independent, however this remains to be experimentally verified.

Dispersed primary turkey pituitary cell cultures were prepared to test the regulatory elements in a native system. The pituitary cell dispersion protocol with two enzymes in the present study has been used to study pituitary hormone secretagogues (Opel and Proudman, 1988). 3-5 million cells per pituitary with greater than 95% viability obtained in the present study is comparable to previous studies. Prl secretion in the incubation media was responsive to VIP, showing that the cultured cells included physiologically normal lactotrophs.

Using the Prl promoter-driven luciferase constructs, various liposome-mediated transfection conditions were tested to determine an effective condition for lactotrophs. In the current study, a range of 3 to 10 μg of DNA was complexed with lipofectAMINE at a fixed DNA:LipofectAMINE ratio of 1:5, and various combinations of preincubation times (0-72h), transfection durations (2-6 h) and recovery times (24-72h) were tested. Despite repeated transfection trials, luciferase activities of the Prl promoter construct-transfected cells were either slightly above or at basal levels observed for promoterless pGL-basic vectors, and were not consistent between or within a trial. Under identical conditions, the control luciferase construct with the ASV-LTR promoter resulted in substantial luciferase activity, indicating that pituitary primary cells were transfected and expressed luciferase under the tested conditions.

Based on a previously reported optimal condition for primary turkey pituitary cells with LipofectAMINE, 5 μg of DNA with 30 μg lipofectAMINE effectively transfected more than 50% of the pituitary cells (Sun *et al.*, 1995). In their optimization studies, a CMV promoter-driven β-galactosidase construct was used, and the transfected cells were visualized by staining with X-gal after a 2 day recovery period. However, the condition

determined by transfecting the heterogeneous population of the primary pituitary cells with a CMV promoter-driven β-galactosidase is not necessarily the optimal condition for the lactotroph population. On the other hand, more than 50% of the monolayer cells were found positive based on X-gal staining (Sun *et al.*, 1995) and more that 50% of the cells in the pituitary are presumably lactotrophs in the incubating hen (Ramesh *et al.*, 1994). Thus, it is highly likely that some lactotrophs were transfected, unless lactotrophs are particularly resistant to liposome-mediated transfection. Successful transient transfection of primary pituitary cells with pituitary hormone promoters have been reported in rat and trout. Maurer (1992) used lipofectin (Gibco-BRL) with rat Prl promoter/luciferase constructs to transfect primary rat pituitary cells, while a calcium-phosphate method was successfully used for rat luteinizing hormone α-subunit promoter/luciferase constructs (Attarddi, 1992). Trout pituitary cells were effectively transfected by electroporation (Elsholtz *et al.*, 1992).

The ability of cells to initiate normal gene activation after enzymatic cell dispersion was tested by Northern blot analysis of total RNA collected from cultured cells every 24 h for 5 days (Fig. 3-2). The steady state levels of Prl mRNA were highest at 72 h, and slightly declined thereafter, whereas Pit-1/GHF-1 mRNA was not detectable on Northern blots. The increased Prl mRNA levels demonstrate that the cells are viable and capable of activating the Prl gene. Of note, two million cells were plated on a 35 mm dish in this experiment, twice as much as the transfection preparation. Relatively smaller amounts of total RNA were recovered from the cells collected at 24 and 48 h, and β-actin mRNA was undetectable at 24 and 48 h. These could be artificial, but it may reflect the fact that the cells require at least 72 h recovery time to initiate normal cellular activity after enzymatic cell dispersion. Undetectable Pit-1/GHF-1 mRNA levels may simply reflect the fact that only a small amount of tPit-1 is necessary to support Prl gene activation. It is possible, however,

that the lack of Prl promoter stimulation in transfection trials was due to insufficient amounts of Pit-1 protein in the cultured cells. High Prl concentrations in the incubation media in the presence of VIP and the abundance of Prl mRNA likely represent continuous Prl gene activation and Prl synthesis. Thus, based on available information, the failure to detect luciferase activities in transfected pituitary cells is due to either poor transfection efficiency and/or a detrimental effect on Prl gene activation caused by the transfection procedure used in the current study.

In summary, based on the GH_3 cell system, negative-acting elements may be present between -2.0 and -1.3 kb and a positive-acting element is present between -1.3 and -1.0 kb of the turkey Prl gene. Elimination of one or two Pit-1/GHF-1 binding sites decreased promoter activity, suggesting that the Pit-1/GHF-1 transcription factor plays an important role for turkey Prl gene expression. Despite repeated attempts to transfect primary pituitary cells with the turkey Prl promoter-luciferase constructs, no consistent reporter gene expression was obtained.

Figure 3-1. Transient transfection of GH_3 cells with the turkey Prl promoter-luciferase constructs. The approximate size of the 5'-flanking region is shown on the left. The two boxes represent the locations of putative Pit-1 binding sites. Mean values of 6 replica ± S.D. relative to the value from the pp2052pGL2 construct are shown on the right.

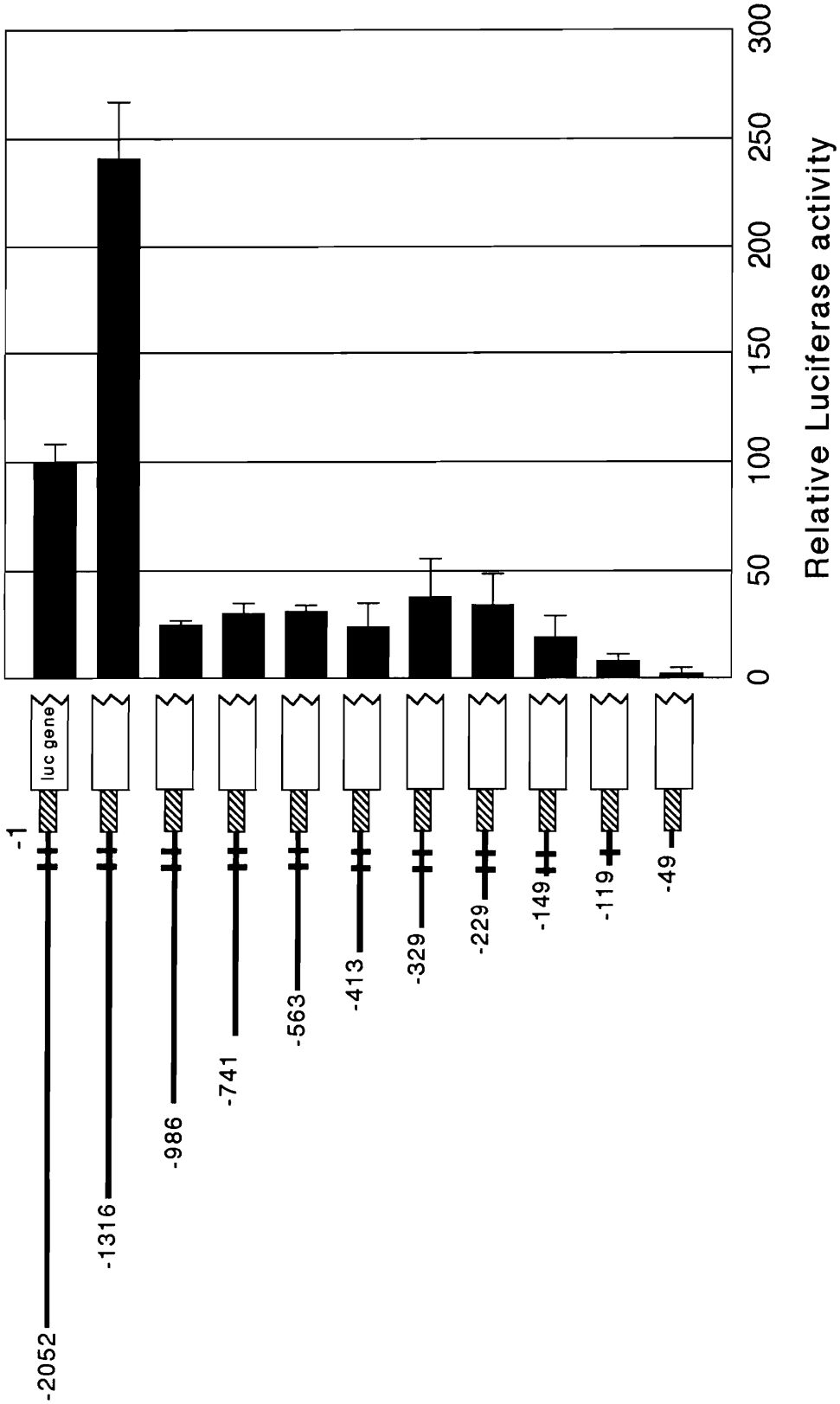

Relative Luciferase activity
0
50
100
150
200
250
300
luc gene
-1
-2052
-1316
-986
-741
-563
-413
-329
-229
-149
-119
-49

Figure 3-2. Northern blots of pituitary prolactin, tPit-1/GHF-1 and β-actin . The dispersed pituitary cells were plated at 2 million cells/35 mm dish, and total RNA was collected at 24, 48, 72, 96 and 120 hours after plating. Blot was first hybridized with a turkey prolactin cDNA probe. The same blot was stripped and rehybridized with a turkey Pit-1/GHF-1 and a chicken β-actin cDNA probe. Total RNA from laying hens was used as the positive control.

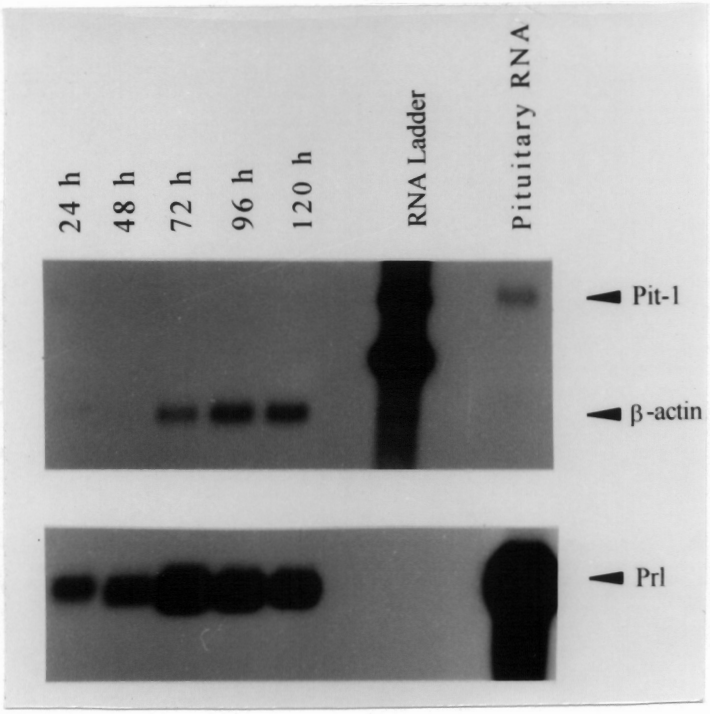

24 h
48 h
72 h
96 h
120 h
RNA Ladder
Pituitary RNA
Pit-1
β-actin
Prl

Table 3-1. Transfection of primary turkey pituitary cells.

Trial #	Incubation (h)	Transfection condition	Recovery Period	Results	Notes
1 (5/15)	48 h	*3µg:15µg #5 h ^0.25-1	48 h	1x10^5 RLU for ASV-pGL2. No activity for pp1316pGL2.	Cell #/dish did not affect the results
2 (5/23)	48 h	*3µg:15µg #2.5 or 5 h ^0.25	24 or 48 h	5x10^5 RLU for ASV-pGL2 at 48h, Greater for 2.5h than 5h transfection for ASV-pGL2	Required 48 h to express high RLU for ASV-pGL2
3 (6/7)	24 h	*3µg:15µg #2.5 or 5 h ^0.25 w/VIP(100nM)	48 or 72 h	No RLU for Prl-Luc constructs. >1x10^5 RLU for ASV-Luc. >1x10^3 RLU for SV40-Luc. No VIP effects on Prl-Luc.	ASVpromoter is stronger than SV40 promoter.
4 (6/7)	0 h	*3µg:15µg #2.5 or 5 h ^0.25	24 or 48 h	No RLU for Prl-Luc constructs.	
5 (9/18)	48 h	*3µg:15µg #5 h ^0.25 or 0. 5	24, 48 or 72h	No RLU for Prl-Luc constructs. >1x10^5 RLU for ASV-Luc throughout.	New Prl-Luc constructs has the 1st Met codon for Luc at the same position for tPrl.
6 (9/26)	24 h	*3µg:7.5 or 15 µg #1, 2 or 4 h ^0.25	48 h	No RLU for Prl-Luc constructs.	
7 (9/30)	48 h	5 µg:$CaCl_2$ method #5h ^1.0	48 h	No RLU for Prl-Luc constructs	
8 (10/6)	24 h	*1.5µg:7.5µg #5h	36 h	1-2x10^5 RLU for ASV-Luc, No RLU for Prl-Luc constructs.	
9 (10/11)	24 h	*3µg:15µg #5h ^1.0 or 0.5	36 h	2-3x10^3 RLU for ASV-Luc, No RLU for Prl-Luc constructs.	

*DNA:Lipo/million cells
#Transfection duration (h)
^ million cells/dish

Table 3-1. Transfection of primary turkey pituitary cells.

Trial #	Incubation (h)	Transfection condition	Recovery Period	Results	Notes
10 (10/18)	24 h	*3µg:15µg #5 h ^0.25, 0.5 or 1.0	48 h	$3x10^4$ RLU for ASV-Luc, no RLU for Prl-Luc constructs, no RLU for rPrl-Luc constructs.	
11 (11/2)	48 h	*3µg:15µg #5 h	48 h	$3x10^4$ RLU for ASV-Luc, $2\text{-}3x10^3$ RLU for Prl-Luc constructs.	pGL3 basic vector was used for the first time
12 (11/12)	24 h	*3µg:15µg #5 h	48 h	no RLU for Prl-Luc constructs	
13 (11/16)	43 h	*3µg:10,15,20 µg #4, 6, or 8 h	48 h	no RLU for Prl-Luc constructs	
14 (11/28)	72 h	*3µg:15µg #5 h	48 h	no RLU for Prl-Luc constructs	
15 (12/8)	72 h	*3µg:15µg #5 h	48 h	$3x10^4$ RLU for ASV-Luc, $2\text{-}3x10^3$ RLU for Prl-Luc constructs.	No consistent results between duplicates.
16 (12/14)	72 h	*3, 6 or 9µg:15µg #5 h	24 or 48 h	$1.5x10^5$ RLU for ASV-Luc, $4x10^3$ RLU for one Prl-Luc construct.	No consistent results between duplicates.

*DNA:Lipo/million cells
#Transfection duration (h)
^ million cells/dish

References Cited

Attarddi, B. 1992. Transfection of primary pituitary cell cultures using calcium phosphate. Focus 13:84-87.

Bodner, M., J. -L. Castrillo, L. E. Theill, T. Deerinck, M. Ellisman, and M. Karin. 1988. The pituitary specific transcription factor GHF-1 is a homeobox-containing protein. Cell 55:515-518.

Camper, S. A, Y. A. S. Yao, and F. M. Rottman. 1985. Hormonal regulation of the bovine prolactin promoter in rat pituitary tumor cells. J. Biol. Chem. 260:12246-12251.

Crenshaw, E. B., III., K. Kalla, D. M. Simmons, L. W. Swanson, and M. G. Rosenfeld. 1989. Cell-specific expression of the prolactin gene in transgenic mice is controlled by synergic interactions between promoter and enhancer elements. Genes Dev. 3:959-972.

El Halawani, M. E., S. Fehrer, B. M. Hargis and T. E. Porter. 1988. Incubation behavior in the domestic turkey: physiological correlates. CRC, Critical Review in Poultry Biology. 1:285-314.

El Halawani, M. E. and I. Rozenboim. 1993. The ontogeny and control of incubation behavior in turkeys. Poult. Sci. 72:906-911.

Elsholtz, H. P., S. Majumdar-Sonnylal, F. Xiong, Z. Gong, C. L. Hew. 1992. Phylogenetic specificity of prolactin gene expression with conserved Pit-1 function. Mol. Endocrinol. 6:515-522.

Folger, K. R., E. A. Wong, G. Wahl, and M. R. Capecchi. 1982. Patterns of integration of DNA microinjected into cultured mammalian cells : Evidence for homologous recombination between injected plasmid DNA molecules. Mol. Cell. Biol. 2:1372-1387.

Fox, S. R., M. T. C. Jong, J. Casanova, Z-S. Ye, F. Stanley, and H. H. Samuels. 1990. The homeodomain protein, Pit-1/GHF-1, is capable of binding to and activating cell specific elements of both growth hormone and prolactin gene promoters. Mol. Endocrinol. 4:1069-1080.

Gutierrez-Hartmann, A., S. Siddiqui, and S. Loukin. 1987. Selective transcription and DNase I protection of the rat prolactin gene by GH_3 pituitary cell-free extracts. Proc. Natl. Acad. Sci. USA. 84:5211-5215.

Hall, T. R., S. Harvey and A. Chadwick. 1986. Control of prolactin secretion in birds: A review. Gen. Comp. Endocrinol. 62:171-184.

Ingraham, H. A., R. Chen, H. J. Mangalam, H. P. Elsholtz, S. F. Flynn, C. R. Lin, D. M. Simmons, L. Swanson, and M. G. Rosenfeld. 1988. A tissue-specific factor containing a homeodomain specifies a pituitary phenotype. Cell 55:519-529.

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

Macnamee, M. C., P. J. Sharp, R. W. Lea, R. J. Sterling and S. Harvey. 1986. Evidence that vasoactive intestinal peptide is a physiological prolactin-releasing factor in the bantam hen. Gen. Comp. Endocrinol. 62:470-478.

Maurer, R. A., and A. C. Notides. 1987. Identification of an estrogen-responsive element from the 5'-flanking region of the rat prolactin gene. Mol. Cell. Biol. 7:4247-4254.

Maurer, R. A. 1992. Cationic liposome-mediated transfection of primary cultures of rat pituitary cells. Focus 11:25-27.

Mauro, L. J., R. P. Elde, O. M. Youngren, R. E. Phillips and M. E. El Halawani. 1989. Alterations in hypothalamic vasoactive intestinal peptide-like immunoreactivity are associated with reproduction and prolactin release in the female turkey. Endocrinol. 125:1795-1804.

Nelson, C., E. B. Crenshaw, III, R. Franco, S. A. Lira, V. R. Albert, R. M. Evans, and M. G. Rosenfeld. 1986. Discrete cis-active genomic sequences dictate the pituitary cell type-specific expression of rat prolactin and growth hormone gene. Nature 322:557-562.

Nelson, C., V. R. Albert, H. P. Elsholtz, L. I.-W. Lu and M. G. Rosenfeld. 1988. Activation of cell specific expression of rat growth hormone and prolactin gene by a common transcription factor. Science 239:1400-1405.

Opel, H. and J. A. Proudman. 1988. Stimulation of prolactin release in turkeys by vasoactive intestinal peptide. Proc. Soc. Exp. Biol. Med. 187:455-460.

Peers, B., M. Voz, P. Monget, M. Mathy-Hartert, M. Berwaer, A. Belayew and J. A. Martial. 1990. Regulatory elements controlling pituitary-specific expression of the human prolactin gene. Mol. Cell. Biol. 10:4690-470

Proudman, J. A. and H. Opel. 1988. Stimulation of prolactin secretion from turkey anterior pituitary cells in culture. Proc. Soc. Exp. Biol. Med. 187:448-454.

Rajnarayan. S., M. Chiono, L. M. Alexander, and A. Gutierrez-Hartmann. 1995. Reconstitution of protein kinase A regulation of the rat prolactin promoter in HeLa nonpituitary cells: identification of both GHF-1/Pit-1-dependent and -independent mechanisms. Mol. Endocrinol. 9:502-512.

Ramesh, R., J. A. Proudman, and W. J. Kuenzel. 1994. Changes in pituitary somatotrophs and lactotrophs associated with incubation behavior in turkey hens Poult. Sci. 73(suppl.)1:82.

Sharp, P. J. 1989. Physiology of egg production. *In* "Recent advances in turkey science". (C. Nixey and T. C. Gray, Eds). Battetworths, England.

Sun , S., E. A. Wong, J. A. Proudman, B. Wentworth, and M. E. El Halawani. 1995. Prolactin antisense DNA and RNA blocks cVIP-stimulated prolactin secretion from cultured turkey pituitary cells. Poult. Sci. 74 (suppl.)1:157.

Wong, E. A., N. H. Ferrin, J. L. Silsby, and M. E. El Halawani. 1991. Cloning of a turkey prolactin cDNA: Expression of prolactin mRNA throughout the reproductive cycle of the domestic turkey (Meleagris gallapavo). Gen. Comp. Endocrinol. 83:18-26.

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

CHAPTER IV

Isolation of Turkey Pit-1 cDNA Isoforms and the Effect of Each tPit-1 Isoform on Prolactin Gene Expression

Abstract

Three turkey Pit-1 cDNAs variable at the 5' end were identified by reverse transcriptase-polymerase chain reaction (RT-PCR) and 5'rapid amplification of cDNA ends (RACE). The ability of each of the three turkey Pit-1 isoforms to regulate Prl gene transcription was examined.

The newly identified turkey Pit-1 cDNAs, tPit-1* and tPit-1β*, use the transcription start sites (tss) of exon 1, while the previously reported turkey Pit-1 cDNA, referred to as tPit-1W*, uses the tss of exon 2, thus, it lacks exon 1 sequences. Relative to tPit-1*, tPit-1β* has an additional 28 amino acid at the N-terminal domain due to the use of an alternative splicing site 84 nt upstream from the splicing site of exon 2 used for tPit-1*.

In order to determine the role of each tPit-1 isoform in prolactin (Prl) gene regulation, mouse Ltk- cells were co-transfected with an expression vector for each tPit-1 isoform and turkey Prl promoter-luciferase gene constructs. Relative to the control expression vector, luciferase activity was increased by approximately 50 to 70 % by tPit-1* in luciferase gene constructs containing -2054 bp and -355 bp of the turkey Prl gene promoter, demonstrating that tPit-1* is able to promote transcription using the turkey Prl gene promoter. tPit-1* was also able to activate the rat Prl gene promoter by 2- to 3-fold. Both tPit-1W* and tPit-1β* increased luciferase activities in a construct containing -355 bp of the turkey Prl promoter, but to a lesser extent than tPit-1*. Clearly defined differential trans-activation effects of these tPit-1 isoforms were not observed in mouse Ltk- cells.

Introduction

Studies of the regulatory mechanisms that govern prolactin (Prl) gene expression in rats have shown that the transcription factor Pit-1/GHF-1 (hereafter referred to as Pit-1) interacts with a specific DNA sequence motif located in both the Prl and growth hormone (GH) promoters and directs tissue-specific expression of these genes (Bodner *et al.*, 1988; Fox *et al.*, 1989; Gutierrez-Hartmann *et al.,* 1987; Ingraham *et al.*, 1988; Nelson *et al.*, 1988). Pit-1 has since been shown to activate several pituitary specific genes, including the thyrotropin stimulating hormone (TSH) β-subunit gene (Mason *et al.*, 1992), the growth hormone releasing factor (GRF) receptor gene (Lin *et al.*, 1993), and its own gene (Chen *et al.*, 1990; McCormick *et al.*, 1990). Pit-1-dependent Prl gene activation has also been shown in human (Peers *et al.*, 1990), cow (Wolf *et al.*, 1990), and even in a distantly related vertebrate, salmon (Elsholtz *et al.*, 1992). Direct evidence that Pit-1 regulates Prl gene transcription has not been demonstrated in birds.

In rats and mice, a number of Pit-1 isoforms have been identified. For example, alternative translation initiation of Pit-1 mRNA generates a Pit-1 protein lacking the first 26 amino acids at the N-terminus (Voss *et al.*, 1991). Pit-1β isoform has a 26 amino acid insertion due to alternative splicing at exon 2 of the gene (Konzak and Moore, 1992: Morris *et al.*, 1992; Theill *et al.*, 1992), and has been shown to activate the GH promoter, but not the Prl promoter (Morris *et al.*, 1992; Rajnarayan *et al.*, 1995; Theill *et al.*, 1992). Pit-1T isoform isolated from a mouse thyrotroph-derived tumor cell line, TtT-97, contains a 14 amino acid insertion also due to alternative splicing at exon 2 (Haugen *et al.*, 1993). Pit-1T appears to preferentially trans-activate the TSHβ gene (Haugen *et al.*, 1994). Another Pit-1 isoform, referred to as Pit-1ΔIV, which has a 55 amino acid deletion in the POU-specific domain due to alternative splicing of 165 bp sequence corresponding to exon IV of the Pit-1 gene, has been isolated from the rat anterior pituitary and transplanted GH_3 cells

(Voss *et al.*, 1993; Day and Day, 1994). Pit-1ΔIV isoform stimulated transcription from the GH promoter, but not the Prl promoter, reflecting the fact that the transplanted GH_3 cells do not produce Prl but continue to produce GH. Differential expression of isoforms with different trans-activation efficiencies may be one mechanism to control temporal- and spatial-specific expression of Pit-1-dependent genes.

A previously reported turkey Pit-1 cDNA encodes a 327 amino acid protein with 94% amino acid homology in the POU-domain to rat Pit-1 (Wong *et al.*, 1992). Despite the high homology in the DNA binding POU-domain to rat Pit-1, this original turkey Pit-1 contains two unique insertions of 38 and 7 amino acids in the N-terminus, and the 5'end of the cDNA contains no sequence similar to exon 1 found in the rat Pit-1 cDNA. Based on the recently isolated turkey Pit-1 gene (Kurima *et al.*, 1996), the 38 amino acid region is encoded by an extra exon, designated as exon 2a, located between exon 2 and exon 3 of the gene, and the 7 amino acid insertion is encoded by exon 3. The original turkey Pit-1 also contains a short sequence similar to the 26 amino acid sequence inserted in a rat Pit-1 isoform, Pit-1β. The 5' end of the turkey Pit-1 cDNA was originally isolated by 5'rapid amplification of cDNA ends (RACE) using an exon 2-specific oligonucleotide primer. In addition to this original Pit-1 cDNA, an unique cDNA in which the 33 nt sequence encoding the first 11 amino acid of the Pit-1 was replaced with 54 nt sequence similar to exon 1 of the rat Pit-1 gene was isolated, suggesting that Pit-1 isoform(s) may also exist in turkeys.

In the present study, the putative exon 1 sequence was used to isolate tPit-1 cDNAs containing exon 1. To determine the role of each tPit-1 isoform in Prl gene regulation, tPit-1 isoform cDNAs were cloned into expression vectors, and each tPit-1 isoform expression vector and Prl promoter-luciferase construct were co-transfected into mouse Ltk- cells.

Materials and Methods

Two sense and antisense oligonucleotide primers, (5'-AGAGTGCCTGCCCGTTTC-3'; tPit-S30) and (5'-GGAGACAACACTGGTGCATGG-3'; tPit-AS29), respectively, corresponding to the putative exon 1 region from the previous 5'RACE cDNA were synthesized. Approximate locations of the primers are shown in Fig. 4-2. tPit-AS29 primer was then used to isolate sequences further upstream of Pit-1 cDNA by 5'RACE, while the tPit-S30 primer was used to confirm tPit-1 isoform(s) by RT-PCR.

Reverse transcriptase reactions of turkey pituitary RNA.

Total RNA from the anterior pituitaries of laying or incubating turkey hens was extracted using TRI REAGENT (Molecular Research Center, Cincinnati, OH). 1 to 2 μg of total RNA was mixed with 10 ng of either tPit-AS29, an exon 3-specific (5'-CCTGCGGAATTCCTGCTT-3'; tPit-AS18) or an exon 6-specific antisense oligonucleotide (5'-AACAGGAACCCACAGCTA-3'; tPit-AS6) in 10 μl with DEPC-treated water, and heated at 95°C for 2 min, followed by incubation at 22-25°C for 3 min, then on ice for 15 min. Reverse transcriptase (RT) reactions were carried out in 50 μl reactions containing 50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol, 0.5 mM dNTPs, 20 units RNase inhibitor (Promega, Madison, WI), and 500 units Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (USB, Cleveland, OH) for 60 min at 37°C. The RT products were purified by chromatography through Sephadex G-50 spin columns and ethanol precipitated in the presence of 1/10 volume of 3 M sodium acetate.

Isolation of the 5' end of tPit-1 isoform(s).

RT products with tPit-AS29 primer were used as templates in 5'RACE to isolate sequences further upstream of exon 1. Terminal transferase reactions were first performed in a final volume of 50 μl, containing the RT products, 100 nM dATP, 15 units terminal deoxynucleotidyl transferase (Promega, Madison, WI) in 1 x terminal transferase buffer

(100 mM cacodylate buffer, pH 6.8, 1 mM CoCl, 0.1 mM dithiothreitol) at 37°C for 7 min. The reactions were terminated by incubating at 65°C for 5 min. PCR was performed using 1 to 2 µl of the terminal transferase reaction mix (50 µl) as a template. 50 ng of an adapter primer with T_{17} (5'-GGCCACGCGTCGACTAGTACT$_{17}$-3'), 50 ng of an oligonucleotide adapter primer without T_{17}, (5'-GGCCACGCGTCGACTAGTAC-3'), 100 ng of tPit-AS29 were mixed in a final volume of 50 µl with 1 x PCR buffer (10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100) containing 250 µM dNTPs, 1 mM $MgCl_2$, 1.25 units Taq DNA polymerase (Promega). PCR was subjected to 30 PCR cycles (95°C for 60s, 56°C for 60s, 72°C for 60s) using a Precision GTC-2 thermalcycler (Chicago, IL). The adapter primers encode a *Sal* I site for cloning purposes. The RT-PCR products were digested with *Sal* I, and cloned into the phagemid pTZ18R (Pharmacia, Piscataway, NJ) which had been digested with *Hin*cII and *Sal* I. A plasmid containing the longest cDNA insert was sequenced by the dideoxynucleotide chain-termination method using Sequenase kits from U.S. Biochemical (Cleveland, OH). The longest cDNA included a putative methionine start codon and an additional 170 nt upstream from the methionine codon. A sense oligonucleotide primer (5'- CTAGTGGTATAAATACCTGCACACG-3'; tPit-S33) corresponding to the most 5' end of the 5'RACE cDNA was synthesized for subsequent PCR amplification.

Confirmation of tPit-1 isoform(s).

RT products with an exon 3-specific (tPit-AS18) or an exon 6-specific (tPit-AS6) antisense primer were used as templates for subsequent PCR to confirm tPit-1 isoform(s). The presence of a tPit-1W* transcript was confirmed by RT-PCR using the oligonucleotide primer (5'-CTGATCTACACACTACTG-3'; tPit-S21) corresponding to 102 to 119 nt upstream from the putative methionine start codon in exon 2, paired with an exon 2a (5'-GCAATGTGCTGACACCAT-3'; tPit-AS17)- or an exon 3 (tPit-AS18)-specific antisense

primer. Pit-1 isoform(s) containing exon 1 was confirmed by RT-PCR using an exon 1 (tPit-S30)-specific primer, paired with either an exon 2a (tPit-AS17)- or an exon 3 (tPit-AS18)-specific primer. Amplified DNA fragments were electrophoresed on 1.0% agarose gels for analysis. Fragments representing partial tPit-1 cDNAs containing exon 1 were excised from the gel and purified for subsequent sequencing. Southern blot analysis of the PCR products was also performed using a tPit-1W* cDNA as a probe.

The presence of 3' end variables among the tPit-1 cDNAs was tested by RT-PCR. using an exon 1 (tPit-S30)-specific or an exon 2 (tPit-S21)-specific sense primer paired with an exon 2a (tPit-AS17)-, an exon 3 (tPit-AS18)-, an exon 4 (5'-AGCCAGTGCTTCACCAAC-3'; tPit-AS10)-, an exon 5 (5'-GCAGGAGAACTAGATACG-3'; tPit-AS12)-, or an exon 6 (tPit-AS6)-specific antisense primer. In addition, the presence of tPit-1 mRNAs lacking exon 4 was examined using the combination of an exon 3 (5'-GAGCCTGTCGACATGGAT-3'; tPit-S9)-specific sense and an exon 5 (tPit-AS12)-specific antisense oligonucleotide primers.

Expression vector for each Pit-1 cDNA isoform.

Megaprimer PCR technique was employed to synthesize a complete tPit-1* and tPit-1β* cDNAs. For tPit-1*, tPit-S33 primer was paired with tPit-AS29 primer to amplify the 5'RACE plasmid, and the amplified fragments were mixed with an equal amount of the RT-PCR products amplified with tPit-S30 and tPit-AS18 primers. Approximately 1 μg of each fragment was mixed with 20 nM of tPit-S33 and tPit-AS18 primers, and a megaprimer PCR was carried out in a final volume of 50 μl with 30 cycles (95°C for 60s, 56°C for 60s, 72°C for 60s). Since there is no variation among the turkey Pit-1 isoforms at the 3' end of the cDNA, a tPit-1W* cDNA in a pTZ18R phagemid was digested with *Sal* I and *Sma* I to remove the 5'end of tPit-1W* cDNA, and replaced with mega-amplified fragments digested with *Sal* I. For Pit-1β*, both sense and antisense oligonucleotide

primers, (5'-GTCTCCACAGTCCCGTCTGTTTTGTC-3'; tPit-S31) and (5'-AGACGGGACTGTGGAGACAACACTGG-3'; tPit-AS32), respectively, corresponding to the junction between exon 1 and exon 2 were synthesized. tPit-S33 primer was paired with tPit-AS32 primer to amplify the 5'RACE plasmid, and the amplified fragments were mixed with an equal amount of the RT-PCR products amplified with tPit-S31 and tPit-AS18 primers. Megaprimer PCR and cloning of tPit-1β* was carried out as above.

For an expression vector construction, the avian sarcoma virus-long terminal repeat (ASV-LTR) fragment (1.3 kb) from pBR322/ASV-2LTR/tk A (Folger *et al.*, 1982) and a simian virus (SV40) splice and polyadenylation signal fragment (871bp) from pMSG (Pharmacia) were subcloned into phagemid pTZ19R (Pharmacia), and designated as PAS. Each tPit-1 isoform cDNA was cloned into the multiple cloning sites of the PAS expression vector. A SV40-driven rat Pit-1 expression vector was obtained from Dr. H. P. Elsholtz (University of Toronto), and used as a positive control for the transfection/luciferase protocols.

Prolactin promoter-luciferase gene constructs.

The luciferase reporter constructs containing various lengths of the turkey Prl promoter have been described in Chapter III. A luciferase gene construct containing the 3.0 kb rat Prl gene promoter was obtained from Dr. H. P. Elsholtz (University of Toronto). A cytomegalovirus (CMV)-β-galactosidase control plasmid was obtained from Dr. R. N. Day (University of Virginia) and was used to normalize for variation in transfection efficiency among the samples.

Transfection protocol

Mouse Ltk- cells were routinely grown in DMEM supplemented with 10% fetal calf serum and antibiotic-antimycotic (0.1 units/ml penicillin, 100 ng/ml streptomycin, 0.25 ng/ml amphotericin) in a humidified 5% CO_2/95% air incubator at 37°C. 0.5 million cells were plated 24 h prior to transfection. 25 μg of lipofectAMINE (Gibco-BRL) was mixed

with 1 μg of each Pit-1 expression vector, 4 μg of tPrl promoter-luciferase construct, and 0.125 μg of CMV-β-galactosidase plasmid in 200 μl of Opti-MEM (Gibco-BRL), and incubated for at least 20-30 min at 22-25°C. The cells were rinsed with pre-warmed Opti-MEM and transfected with 1 ml of DNA-lipofectAMINE complex in Opti-MEM for 5 h at 37°C in a CO_2 incubator. The transfection was terminated by diluting the transfection medium with 3 ml of DMEM supplemented with 10% FCS. After 43 to 48 h, cells were rinsed with PBS and lysed with 200 μl Reporter Lysis Buffer (Promega) for 15 min at 22-25°C. The cell lysates were subjected to three freeze-thaw cycles and centrifuged at 4,000 xg for 2 min and the supernatant was collected for luciferase and β-galactosidase assays. Luciferase and β-galactosidase activities were analyzed as described in Chapter III.

Results

Isolation of three Pit-1 isoform cDNAs.

Three tPit-1 cDNAs variable at the 5' ends were identified from the anterior pituitary of turkey hens. 5'RACE using tPit-AS29 primer isolated a RT-PCR product containing a putative methionine start codon and an additional 170 nt upstream from the methionine codon. The deduced amino acid sequence was 75% identical to the rat Pit-1 N-terminus, which is encoded by exon 1.

RT-PCR using tPit-S30 primer paired with either an exon 2a (tPit-AS17) or an exon 3 (tPit-AS18)-specific primer amplified two distinct bands in each PCR (Fig. 4-3). The DNA sequences of the two amplified RT-PCR products showed that these two tPit-1 partial cDNAs differed at their exon 2 splicing sites (Fig. 4-2; 4-4) and are comparable to the rat Pit-1 and Pit-1β (Fig. 1-4). Because the two tPit-1 cDNAs were comparable to rat Pit-1 and Pit-1β, and yet both contain the 114 nt insert encoded by exon 2a of the turkey Pit-1 gene, they were denoted with an asterisk (*) to differentiate them from comparable rat Pit-1s. Relative to tPit-1* cDNA, tPit-1β* cDNA contains an additional 84 nt at the exon 1

and exon 2 junction, indicating that tPit-1β* is generated due to the use of an alternative splicing site 84 nt upstream from the splicing site of exon 2 used for tPit-1* (Fig.4-2 and 4-4). Of note, the origin of a faint band, approximately 400 bp in the exon1/exon2a lane, is unknown.

The presence of the previously reported turkey Pit-1 cDNA was confirmed by RT-PCR. Using the oligonucleotide primer, tPit-S21, corresponding to 119 nt upstream from the putative methionine start codon in exon 2, paired with exon 2a (tPit-AS17)- or an exon 3 (tPit-AS18)-specific antisense primers, RT-PCR amplified the predicted bands of 414 bp and 563 bp, respectively (Fig. 4-3). Because this original Pit-1 cDNAs contains the 114 nt insert encoded by exon 2a of the turkey Pit-1 gene, this was also denoted with an asterisk (*) and designated as tPit-1W*. The data indicated the absence of Pit-1 isoform lacking the sequences encoded by exon 2a.

RT-PCR using exon 1-specific (tPit-S30) sense primer paired with an exon 2a, 3, 4, 5, or 6-specific antisense primers amplified the predicted sizes of the PCR products for tPit-1* and tPit-1 β* : 224 and 308 bp for exon1/exon 2a, 373 and 457 bp for exon1/exon3, 508 and 592 for exon1/exon4, 685 and 769 bp for exon1/exon5, and 1029bp for exon1/exon6. No PCR products indicating isoforms variable at the 3' end of Pit-1 cDNAs were detected by ethidium bromide staining (Fig. 4-3). RT-PCR using an exon 3 (tPit-S9)-specific sense and an exon 5 (tPit-AS12)-specific antisense primers showed no Pit-1 cDNA lacking the sequences encoded by exon 4 (data not shown). It was concluded that all three isoforms, tPit-1*, tPit-1β* and tPit-1W*, share the identical 3'end encoded by exons 2a, 3, 4, 5, and 6 of the turkey Pit-1 gene.

tPit-1 isoforms for Prl gene regulation in Ltk- cells.

In order to determine the ability of each of the three tPit-1 isoforms to regulate Prl gene expression, mouse Ltk- cells were co-transfected with an ASV-LTR-driven expression vector for each of the three turkey Pit-1 isoforms and turkey Prl promoter-

luciferase gene constructs (Fig. 4-5 and 4-6). Relative to the control expression vector, tPit-1* increased luciferase activities approximately 20 to 70% for turkey Prl promoter-luciferase constructs. However, statistically significant increases by tPit-1* were detected only for the construct containing -2054 and -355 bp ($P < 0.05$). Both tPit-1β* and tPit-1W* increased luciferase activities from the Prl promoter constructs, but to a lesser extent than tPit-1*. Statistically significant trans-activation effects by tPit-1β* and tPit-1W* isoforms were detected only for the construct containing -355 bp of the turkey Prl promoter.

When the ability of tPit-1* to regulate the turkey or rat Prl promoters was compared, tPit-1* showed a 2- to 3-fold activation of the rat Prl promoter (-3.0 kb), while it showed only a 1.7-fold activation of the turkey Prl promoter (-2054 bp) (Fig.4-6). In contrast, rat Pit-1 activated the rat Prl promoter more than 30-fold and the turkey Prl promoter 2- to 3-fold (Fig.4-7). Of note, the tPit-1* expression was ASV-LTR-driven, while the rat Pit-1 expression was driven by a SV40-promoter.

Discussion

Three turkey Pit-1 cDNAs variable at the 5'end, referred to as tPit-1*, tPit-1β* and tPit-1W*, were identified from the anterior pituitaries of turkey hens by reverse transcriptase-polymerase chain reaction (RT-PCR) and 5'rapid amplification of cDNA ends (RACE) (Fig.4-2). Predicted molecular weights of each isoform are 37.5, 40.4 and 36.8 kDa for tPit-1*, tPit-1β* and tPit-1W*, respectively. Three tPit-1 cDNAs were amplified by RT-PCR from the same total RNA preparation, thus, three tPit-1 isoforms are likely co-expressed.

Based on the recently isolated turkey Pit-1 gene (Kurima *et al.*, 1996), tPit-1* and tPit-1β* use the transcription start site (tss) of exon 1, while tPit-1W* uses the tss of exon

2, thus, it lacks exon 1. The use of exon 2 tss has not been documented among the reported Pit-1 isoforms in mammals. Confirmation of the tPit-1W* isoform was made by RT-PCR using the primer (tPit-S21) corresponding to the region between 119 to 102 nt upstream from the putative methionine start codon in exon 2 paired with an exon 2a- or an exon 3-specific antisense primers (Fig. 4-3).

Detailed nucleotide sequence analysis at the exon-intron junctions of exon 1 and exon 2 predicted that the 3'end sequence of exon 1 is 5'-CAG↓GTACCT-3', and the 5'end sequence of exon 2 is 5'-CAGCAG↓GAC-3' for tPit-1* and 5'-TTACCAG↓TC-3' for tPit-1β*, where the downward arrow indicates the splice site used. The splicing sites for both tPit-1* and tPit-1β* are in agreement with the "GT-AG" rule (Breathnach and Chambon, 1981). Based on RT-PCR of tPit-1* and tPit-1β* isoforms, the partial cDNA encoding tPit-1* was amplified 5- to 10-fold more than tPit-1β* (Fig. 4-3). Therefore, preferential use of the tPit-1* splicing site likely generates more tPit-1* mRNA than tPit-1β* mRNA.

In addition, the 5'end sequence of exon 2 for tPit-1*, 5'-CAGCAG↓GAC-3', shows a possible alternative splicing site 3 nt upstream from the predicted splicing site (5'-CAG↓CAGGAC-3') (Fig.4-4). This could give rise a tPit-1 that is 27 amino acids shorter than tPit-1β* isoform, rather than 28 amino acids shorter in tPit-1*. However, a tPit-1 cDNA that splices at a point 3 nt upstream has not been identified.

Involvement of Pit-1 on Prl gene regulation has been shown to be phylogenetically conserved in mammals and in even a distantly related vertebrate, salmon (Elsholtz *et al.*, 1992). The role of Pit-1 in Prl gene regulation has not been determined in birds. In the 5'-flanking region of the turkey Prl gene, two regions with high sequence similarity with a rat Pit-1 binding motif were identified (Kurima *et al.*, 1995), and the DNA binding POU-specific domain and POU homeodomain in turkey Pit-1 showed 94-95% amino acid

identity to the corresponding rat Pit-1 POU domains (Wong *et al.*, 1992). Transient transfection of a rat pituitary tumor cell line GH_3 with turkey Prl promoter-reporter gene constructs have shown that elimination of the Pit-1 binding sites resulted in the lack of promoter activity (Fig. 3-3), suggesting that Pit-1 is involved in Prl gene expression in turkeys.

In the present studies, cDNAs encoding three tPit-1 isoforms were cloned in the ASV-LTR promoter-driven expression vector, and the abilities of each tPit-1 to promote transcription from the turkey Prl promoter were tested in a heterologous cell line, mouse Ltk-. Relative to the control expression vector, tPit-1* increased the Prl promoter activities approximately 20 to 70% in the Prl promoter-luciferase constructs containing -2052, -1320, -986, -355 and -149 bp (Fig.4-5 and 4-6). However, a statistically significant increase by tPit-1* was detected only in the constructs containing -2052 and -355 bp of the turkey Prl promoter. tPit-1W* and tPit-1β* also increased luciferase activities in the construct containing -355 bp of the turkey Prl promoters, but to a lesser extent than tPit-1*.

Surprisingly, a statistically significant increase in trans-activation of the Prl gene promoter by turkey Pit-1s was observed only in the construct containing -355 bp of the Prl gene. However, unlike the GH_3 cell system (Fig. 3-1), the longer the length of the Prl promoter, the less absolute luciferase activity in the Ltk- cell system, indicating the presence of negative-acting elements in the turkey Prl promoter that restrict Prl promoter stimulation in Ltk- cells. Thus, it is possible that negative-acting elements may counteract the stimulatory effect of Pit-1 on the turkey Prl promoter acting through the two Pit-1 binding sites located within -130 bp of the Prl promoter. Because of the relatively weak trans-activation effect of turkey Pit-1 on the turkey Prl promoter, the locations of Pit-1 responsive elements in the turkey Prl promoter and differential trans-activation effects of three Pit-1 isoforms were not clearly defined in the Ltk- cell system.

Reconstitution of Prl gene activation by Pit-1 has been demonstrated in rat using Ltk- (Lew *et al.*, 1994), as well as several other nonpituitary cell lines (Fox *et al.*, 1990; Ingraham *et al.*, 1988; Morris *et al.*, 1992; Theill *et al.*, 1992). Rat Pit-1 has been shown to activate the rat Prl promoter from 10- to 2,000-fold. Experimental factors such as the viral promoter controlling Pit-1 expression, the length or arrangement of the Prl promoter, the reporter genes, the cell lines, the transfection methods, the baselines set for calculations and the cell culture conditions probably contributed to these variations. In the present experiment, Ltk- cells were co-transfected with a SV40-rat Pit-1 expression vector and a -3.0 kb rat Prl promoter-luciferase reporter gene construct, and transfected cells were harvested 48 h later. Rat Pit-1 increased both rat and turkey Prl promoter activities by 30-fold and 3-fold, respectively (Fig. 4-7). In contrast, tPit-1* increased rat and turkey Prl promoter activities by approximately 3-fold and only 20 to 70%, respectively (Fig. 4-5 and 4-6). The difference in trans-activation efficiency between rat and turkey Pit-1 could be simply due to different amounts of Pit-1 expressed under the different viral promoters used. Alternatively, tissue- or species-specific effects of the heterologous cell line used in the present experiment could be a factor for the weak trans-activation effect of turkey Pit-1. Reconstitution of optimal Prl promoter activation by turkey Pit-1 may require turkey cell lines or pituitary cell lines. However, it is possible that the relatively weak trans-activation by turkey Pit-1 could be due to the structural difference between rat and turkey Pit-1. All three turkey Pit-1 isoforms contain the extra 38 amino acid and 7 amino acid insertions encoded by exon 2a or exon 3, respectively, at the N-terminus. The N-terminal domain (residues 8-80) of rat Pit-1 has been shown to be critical for trans-activation function of Pit-1 (Ingraham *et al.*, 1990), and differential Prl promoter stimulation by several Pit-1 isoforms variable at the N-terminus have been reported. A chimeric DNA binding protein with the N-terminal domain (residues 8-80) of Pit-1 was able to mediate dopaminergic

inhibition of Prl gene activation, showing that the N-terminal domain of Pit-1 can mediate hormonal regulation of Prl gene expression, independent of the DNA binding POU domain (Lew and Elsholtz, 1995). Therefore, the presence of the extra domains at the N-terminus in turkey Pit-1 is likely to have an impact on the trans-activation function.

The Pit-1-induced increases in promoter activity was significantly lower in the turkey Prl promoter than the rat Prl promoter, showing relatively weak Pit-1-regulated promoter activity in the turkey Prl gene. This could be, in part, due to the number of Pit-1 binding sites. In the 5'-flanking region of the rat Prl gene there are eight Pit-1 binding sites clustered at both the proximal promoter and the distal enhancer regions, whereas computer analysis of the 5'-flanking region of the turkey Prl gene revealed only two proximal Pit-1 binding sites (Kurima *et al.*, 1995). In fact, decreased promoter activity by reduced Pit-1 binding sites has been observed in the rat Prl promoter (Ingraham *et al.*, 1988).

The presence of the distal enhancer region with multiple Pit-1 binding sites in the rat Prl gene may reflect the fact that Pit-1 is the dominant factor to determine the rate of Prl gene expression in rat. The sequence analysis of the bovine and human Prl 5'-flanking regions demonstrated that the rat Prl enhancer region between -1737 to -1549 is 80% identical to the region between -1184 to -994 of the bovine Prl gene and highly homologous to the region between -1.4 and -1.2 kb of the human Prl gene (Wolf *et al.*, 1990; Peers *et al.*, 1990). The rat Prl distal enhancer region confers more than 98% of the promoter activity (Nelson *et al.*, 1988), and mutations or deletion of the Pit-1 binding sites resulted in decreased basal and hormone-stimulated Prl promoter activity (Day and Maurer, 1989; Day *et al.*, 1990; Nelson *et al.*, 1986; Nowokowski and Maurer, 1994). In contrast, based on the GH_3 cell system, a positive-acting element is present between -1.3 and -1.0 kb of the turkey Prl gene, but no sequence similar to the consensus Pit-1 binding sequence is found. The difference in the Pit-1 binding site arrangement between the turkey

and mammalian Prl promoters likely reflects variable Pit-1-dependent Prl gene regulatory mechanism between these species. Prl secretion in mammals is mainly under inhibitory hypothalamic control, while it is stimulatory in avian species. Dopamine (DA) is the most potent inhibitor of Prl secretion (Ben-Jonathan, 1985). It has been shown that dopaminergic inhibition of Prl secretion is associated with decreases in Prl synthesis and Prl gene expression (DeCamilli *et al.*, 1979; Maurer, 1980; 1981) and that Pit-1 mediates the dopaminergic inhibition of Prl gene expression (Lew and Elsholtz, 1995). These data suggest that the arrangement of Pit-1 binding sites in the Prl promoters can be the molecular basis of the differential Prl gene regulatory mechanisms between mammalian and avian species.

The role of Pit-1 in Prl gene activation in birds has not been clearly defined. However, based on the GH_3 cell system, elimination of the Pit-1 binding sites in the turkey Prl promoter resulted in the lack of promoter activity, indicating that Pit-1 is involved in, at least, tissue-specific Prl gene expression. Whether Pit-1 is able to mediate hormone-responsive Prl gene activation acting through the Pit-1 binding sites remains to be elucidated.

In summary, three tPit-1 isoforms variable at the 5'end were identified in turkeys. Alternative splicing of tPit-1 transcripts and alternative transcription start sites likely generate these three isoforms. Turkey Pit-1 appear to activate transcription of the turkey Prl gene. Use of turkey cell lines or pituitary cell lines may more definitively address the role that Pit-1 plays in turkey Prl gene activation.

Figure 4-1. Nucleotide and predicted amino acid sequence for the turkey Pit-1 gene. For exons 1 and 2, numbering is based on the methionine start codon (+1), and potential Pit-1 binding sites are underlined and TATA boxes are double underlined. The nucleotide sequence data can be found in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession numbers U62732 (exon 1) and U18923-18928 (exon 2, 2a, 3, 4, 5 and 6, respectively).

Exon 1 and 5′ flanking region

```
-505  gacttttgattgagctctcagccaggttgtcttccacacaattgtatggatgagattccg
-445  ggggcaggtgttaatgtggaaacctacttagtattaaacgtacctgtctgatggctaccg
-385  actttggagacaaagtttttatatatttttttctggtcattgttctttacaaaagacct
-325  aattgaatatatttgtcaaatcatgtccctggtttccctcttcttctccccctgctaatt
-265  ggacatgtattgtgtttgtgtgggaaggaaggaggctctatagcaggacagcaatattgc
-205  tgggaaaagaacattaaaatggataaagggattttctagtggtataaatacctgcacacg
-145  acctccctgttctctggagtccttctgtatatgtatatacaagtgcaaatactggaatag
-85   agttggagctgccattaatcgctcaggacctgatcagttagaaaacagggaagggtggct
-25   ctttgcctttcacctgtttgagggcATGACTTGCCAAGCATTTGCTTCATCCGACAATTT
                                M  T  S  Q  A  F  A  S  S  D  N  F
 36  TGTACCCTTGAATTCTGACTCTTCTCCCTCCCTGCCTCTGATAATGCATCACAGCGCAGC
      V  P  L  N  S  D  S  S  P  S  L  P  L  I  M  H  H  S  A  A
 96  AGAGTGCCTGCCCGTTTCTAACCATGCGACCAGTGTTGTCTCCACAGgtacttttaaaa
       E  C  L  P  V  S  N  H  A  T  S  V  V  S  T
156  ataatattgctctctataacgctcatttgtagtgcttttagatgatatgtgtgcaacgag
216  ttttacaatatttaaag
```

Exon 2 and 5' flanking region

```
-918  agaaattgcattaaataatgccattctggtggaaacagataaagaaaacttgtgcaaaac
-858  agtagaaacagaagaattttgaataatgacataacgtacatgtaaaaatttattaaacag
-798  cgtgcaataatcctgtattcttaaataaggatgagtatacttctgttcaagaagtatact
-738  gtatcatatagctaatattctgcggtatcctctgcagttaagttgatattatttaattca
-678  aatagcatcttcctccctaccttgtattaaatctttattagtcagttgaaaatgggaatt
-618  tttaagcataaaaattgtattgattatggattatgaaataataatagaaaaacaaacacc
-558  tacaagtaaactcctagatattatttcaacagaaaagttgtgttgctcaacatgcatggg
-498  tattattaattcaatctctcttttggatgaacttacagttgtacaggtatgactgttcca
-438  gtagcatttgtatgcgtaaatgtgtaaatggcattttacagttctgatcactgttctgaa
-378  aaacagatacttacccctaattaatcatattgttctgaaaaactagaatgtgtgctctaa
-318  agttcagcttttcacgttcacaaatatgaatttacaatattactctttacaacagttgtt
-258  agagggtactgtacgtttctgctgcctacttacacaaggaaaacaaccatgtaccttgca
-198  ttgaacaaaaaattgcctgattcttcaggcactgggaggttactgagcaaagacctgaaa
-138  tacctatctcttcatcttactgatctacacactactgacttttgcttcccagagattgcc
-78   agatggttatatcctagagttaaaaatatttgcaggagaactagatacgaggatactgtc
-18   tacctctgaactgaaaggATGTATCTTGAATCCTCATGCGTTTTCTTACCAGTCCCGTCT
                           M  Y  L  E  S  S  C  V  F  L  P  V  P  S
 43  GTTTTGTCTTTGATACAAACTCCTAAATGTTCCCATCTCCATTTTGCCATGATGACTTCG
      V  L  S  L  I  Q  T  P  K  C  S  H  L  H  F  A  M  M  T  S
103  GGAAATGTGTCAGCAGGACTTCACTACTCTGTGCCCTCCTGTCACTATGGAAATCAAACC
      G  N  V  S  A  G  L  H  Y  S  V  P  S  C  H  Y  G  N  Q  T
163  TCTACCTATGGAGTGATGACAGgtaagaggtagcctcatctgaatgagctattagcataa
       S  T  Y  G  V  M  T
223  tgagtttggtcagctttcctttagaggaataaattcaaaagagttgcataaagacagaac
283  tggagttaaggcgctacaaaagaagttagcaaagcaaggggcagtg
```

Exon 2a

```
1    tccccaggtaaaatccataggtgttaaacaatagatattaaaaacaggtattgctcagaa
61   gtttattttgttttgttccaaaataaatgcatcttcatctcactcctttctttcctttaa
121  tatgcttcttttgctctagGCATCAAGCCTGCAACTCCAGAGATGCTATCAGCAAGTCTC
                          G  I  K  P  A  T  P  E  M  L  S  A  S  L
181  TCCCAGAGTCGTATCTTACAGACATGCAGCATGCCACATCCCAATGTAGTAAATGGTGTC
      S  Q  S  R  I  L  Q  T  C  S  M  P  H  P  N  V  V  N  G  V
241  AGCACATTGCAAAgcaagtctttctcttttcttgtattttctcttattccttgggaaaa
      S  T  L  Q
301  tctcttttgtggtttccctgtgtgaaatgtataatacatttcaatggggtagaacttcta
361  atactaaagacagctaccagagcagtaaagaaaacaagtagaagaggaaagtggaggggt
421  tgctaatgaagctctgggttctgctgctaatgtttgtgtcatttag
```

Exon 3

```
1    ggatgagaaagtagatgttgttctgcttttgtatagggggacaagagaaagactgggtct
61   tctggaagatccttaccctctctcttgagtagcttttcacacgtgttacaaaaaaaatat
121  tacaagctttggctatgagattacaaaggtacttttttatagtgttgcaaaataactat
181  atttagttttcttttgggttctgctcagGTAGCCTGACTCCTTGCCTTTATAAGTTCCCT
                                  S  S  L  T  P  C  L  Y  K  F  P
241  GAGCATGCCCTGAGTGCCAGCTCTTGTGCCCTGGGCCACAGCTTCACACCAATGCACCAG
      E  H  A  L  S  A  S  S  C  A  L  G  H  S  F  T  P  M  H  Q
301  ACCCTCCTCAGTGATGATCCCACAGCTGCAGACTTCAAGCAGGAATTCCGCAGGAAAAGC
      T  L  L  S  D  D  P  T  A  A  D  F  K  Q  E  F  R  R  K  S
361  AAGTCTGTGGAAGAGCCTGTCGACATGGATTCCCCTGAAATCAGGGAACTGGAGAAATTC
      K  S  V  E  E  P  V  D  M  D  S  P  E  I  R  E  L  E  K  F
421  GCTAATGAATTCAAACTGCGCAGAATTAAACTAGgtatgtgcttttagccagtcagcaaa
      A  N  E  F  K  L  R  R  I  K  L
481  atttccaaggagatccctattatttttaattattttatggtctgggccaagcacttgtct
541  catcataactttaatcctgctggtctgactaactttctctattcctagaaaacttgcaaa
601  gaattaataaatttcagcttttagcatcttagtgtgccaaaggtaactttaaatattcct
661  tcttt
```

Exon 4

```
1    ttgaagtgtgaccttatcagtgctgagtgcatacaccacaggaacaccaggttcctaaga
61   gtccacaatgaaggactcagtggctcaagaacagtcactgtctgtagaaagatccagagt
121  gacttttcctcttaattttgacagGTTATACACAAACCAACGTTGGTGAAGCACTGGCT
                              G  Y  T  Q  T  N  V  G  E  A  L  A
181  GCTGTGCACGGCTCTGAATTCAGTCAAACTACCATTTGCCGCTTTGAGAACTCGCAGCTA
      A  V  H  G  S  E  F  S  Q  T  T  I  C  R  F  E  N  S  Q  L
241  AGCTTTAAGAATGCATGCAAACTGAAATCAATACTGTCCAAGTGGCTGGAAGAAGCAGAA
      S  F  K  N  A  C  K  L  K  S  I  L  S  K  W  L  E  E  A  E
301  CAAGTGGGAGgtaaagaattgaaatcctctacgtatttactcaagtagatcacaatccta
      Q  V  G
361  taaatactttgaaggactaatatctgccttccataaactgagagtttt
```

Exon 5

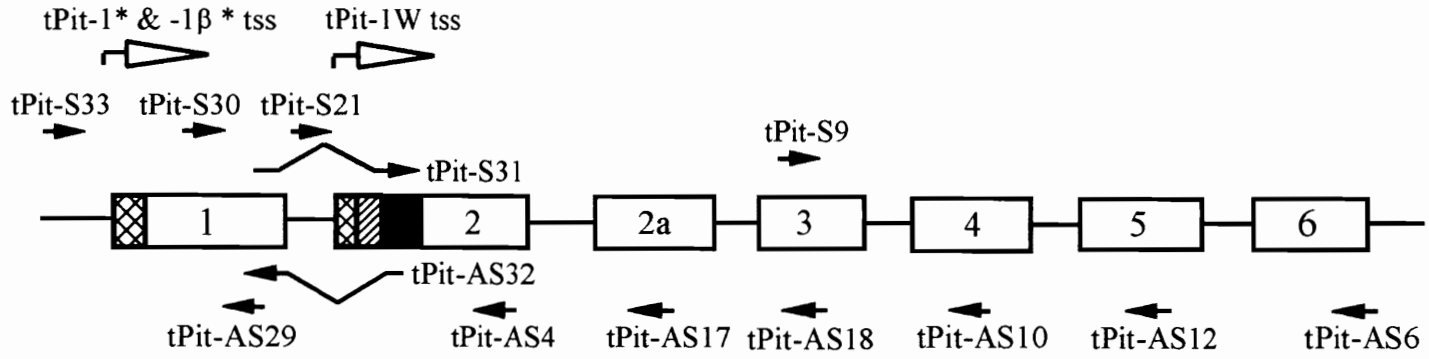
```
1    gtatgtaaaatcagtggtgtgacacgtctccccatgtacgtgggcatagaagcagagcca
61   gtgctattgcagctttaccactggcatgctgtttttgcttataaccattttcagctggag
121  ttaaaatgaccaaggctcttaaagaaaaaagtatagctctgtggtgcactctccttattc
181  agtagtattatctgttacactttcttctgtcttcaaacaaaggcaaacttttttttctag
241  tgatgactgcaaacagtttccatcccctgtggaagttaactgctcttttggttttgtttt
301  gtttttaatacagCTTTATACAATGAAAAAGTTGGCGTGAATGAGAGGAAGAGGAAGCGC
                    A  L  Y  N  E  K  V  G  V  N  E  R  K  R  K  R
361  AGAACCACCATAAGgttatacatatttatggggttacatgttagaaatgcttctagctaa
      R  T  T  I  S
421  gaagtttcattttgggggttgaaatcaaagtgttgttttctatttgtgaatagttaatg
481  aaggataaactattttcaaacatttcaagtgaaaaataaaaac
```

Exon 6

```
1     ctttgtctcttatccagTATTGCTGCCAAAGAAGCCCTGGAGAGGCACTTTGGAGAACAA
                          I  A  A  K  E  A  L  E  R  H  F  G  E  Q
61    AGCAAACCTTCTTCTCAGGAAATCATGAGGATGGCTGAGGGGCTTAATCTTGAGAAGGAA
       S  K  P  S  S  Q  E  I  M  R  M  A  E  G  L  N  L  E  K  E
121   GTTGTGAGAGTTTGGTTTTGCAACAGAAGACAAAGGGAAAAAAAGAGTGAAGACAAGTCTG
       V  V  R  V  W  F  C  N  R  R  Q  R  E  K  R  V  K  T  S  L
181   CATCAGAACGCCTTTAGCTCTATTATCAAGGAGCACCACGAGTGCCGGTAAagcttttc
       H  Q  N  A  F  S  S  I  I  K  E  H  H  E  C  R  *
241   atgtgtagctgtgggttcctgttttgctttattttaagtgctttgttagcttttcatgt
301   gtagctgtgggttcctgttttgctttattttaagtgctttgttgtttaaaaaaaacaaaa
361   caaaacaaaacaaaaaaaaaatggtaaaacacttgattcctttacaagctagccagcttc
421   agatgcaatttggtatagaaggcctgatttcattgtaaaatattgggggaaaaaatgcta
481   ttgcatatatctgaacttttgaaggcctaacttcaaaggaacctattaggcatcaaactc
541   aaccagaaatgctttagaaatagcaaaaacatgt
```

Figure 4-2. Structure of the turkey Pit-1 gene and three turkey Pit-1 isoforms. Exons are indicated by open boxes, and introns by lines. Exons and introns are not drawn to scale. Non-translated regions are indicated as cross-hatched boxes, while the 84 bp region encoded in tPit-1W* and tPit-1β* isoforms are indicated as solid boxes. The first 11 amino acids unique in tPit-1W* are indicated as a hatched box. Approximate locations of the oligonucleotide primers used are shown.

Structure of the turkey Pit-1 gene and tPit-1 isoforms



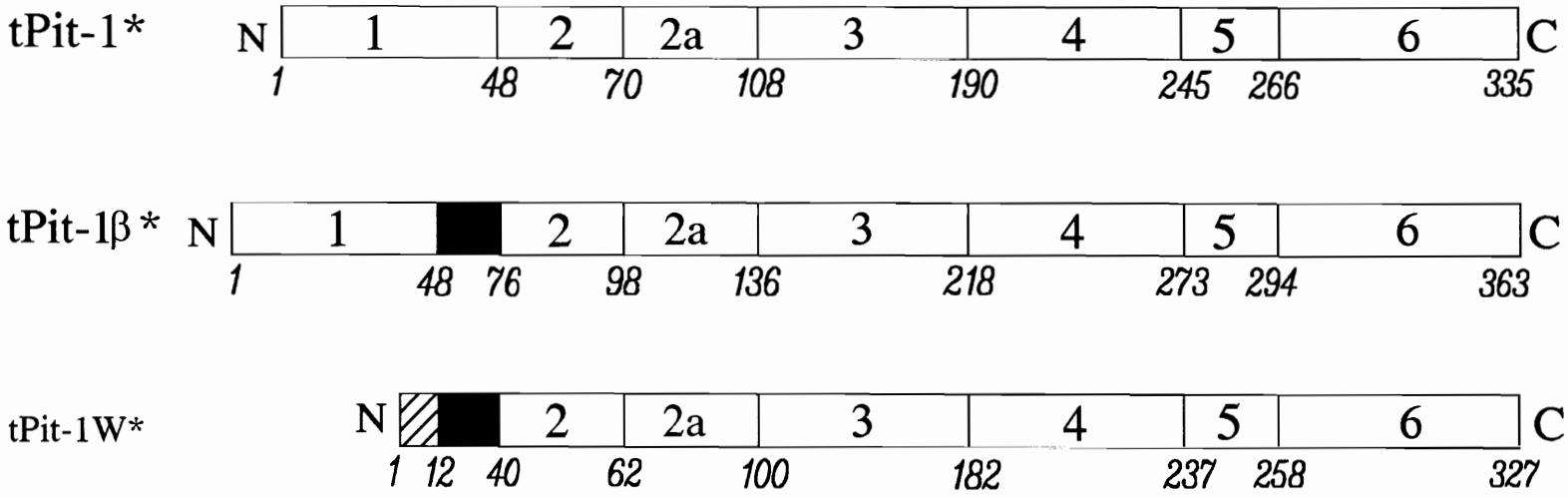


Figure 4-3. Agarose gel analysis of RT-PCR for the presence of each Pit-1 isoform. Combinations of primers are indicated for each lane. ϕX174 DNA-HaeIII digest (New England Biolabs, Beverly, MA) was used as the size marker. The sizes of the five bands from the top are 1,353, 1,078, 872, 603, and 300 bp.

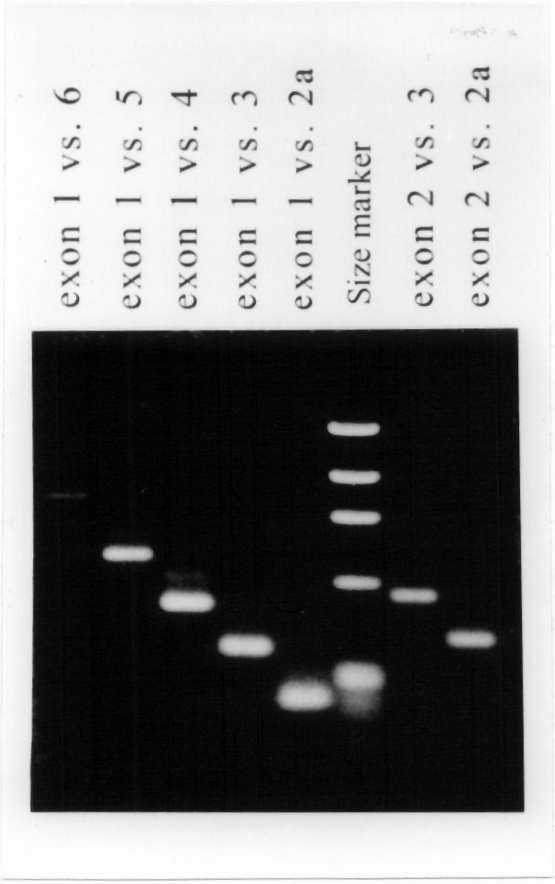

exon 1 vs. 6
exon 1 vs. 5
exon 1 vs. 4
exon 1 vs. 3
exon 1 vs. 2a
Size marker
exon 2 vs. 3
exon 2 vs. 2a

Figure 4-4. Nucleotide and predicted amino acid sequences for tPit-1*, tPit-1β* and tPit-1W*. The 28 amino acid insertion found in tPit-1β* and tPit-1W* is underlined.

tPit-1*/1β*

```
ATGACTTGCCAAGCATTTGCTTCATCCGACAATTTTGTACCCTTGAATTCTGACTCTTCT
 M  T  C  Q  A  F  A  S  S  D  N  F  V  P  L  N  S  D  S  S

CCCTCCCTGCCTCTGATAATGCATCACAGCGCAGCAGAGTGCCTGCCCGTTTCTAACCAT
 P  S  L  P  L  I  M  H  H  S  A  A  E  C  L  P  V  S  N  H

GCGACCAGTGTTGTCTCCACAGTCCCGTCTGTTTTGTCTTTGATACAAACTCCTAAATGT
 A  T  S  V  V  S  T  V  P  S  V  L  S  L  I  Q  T  P  K  C

TCCCATCTCCATTTTGCCATGATGACTTCGGGAAATGTGTCAGCAGGACTTCAC...
 S  H  L  H  F  A  M  M  T  S  G  N  V  S  A  G  L  H
```

tPit-1W*

```
ATGTATCTTGAATCCTCATGCGTTTTCTTACCAGTCCCGTCTGTTTTGTCTTTGATACAA
 M  Y  L  E  S  S  C  V  F  L  P  V  P  S  V  L  S  L  I  Q

ACTCCTAAATGTTCCCATCTCCATTTTGCCATGATGACTTCGGGAAATGTGTCAGCAGGA
 T  P  K  C  S  H  L  H  F  A  M  M  T  S  G  N  V  S  A  G

CTTCAC...
L  H
```

Figure 4-5. Effects of tPit-1 isoforms on the turkey Prl promoter.
Mouse Ltk- cells were co-transfected with luciferase constructs containing different lengths of the turkey Prl promoter and each of the three tPit-1 isoform expression vectors. Values represent the mean of three replica ± S.E. in three separate experiments. Statistically significant increases from the control value are indicated by * ($p<.05$).

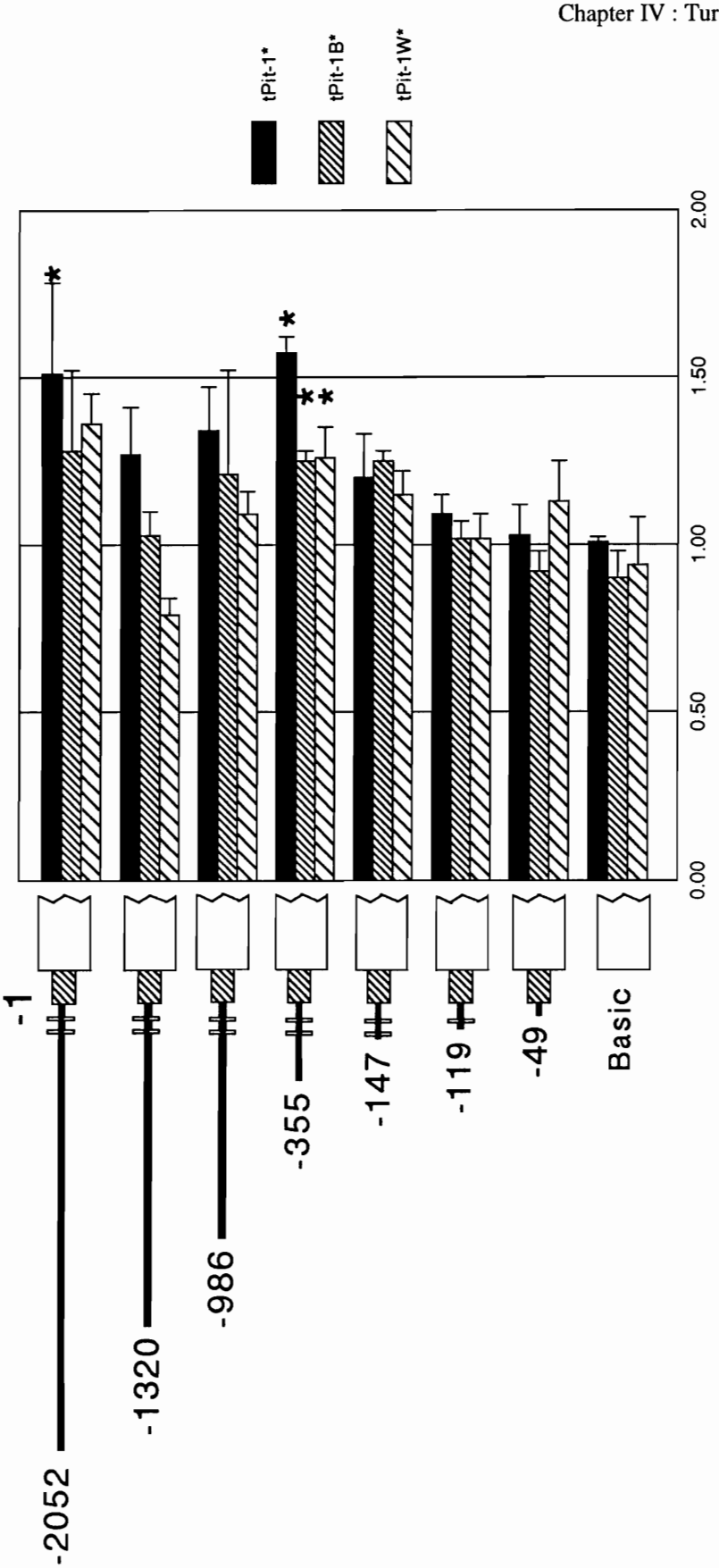

tPit-1*
tPit-1B*
tPit-1W*
0.00
0.50
1.00
1.50
2.00
*
*
**
-1
-2052
-1320
-986
-355
-147
-119
-49
Basic

Figure 4-6. Effects of tPit-1* on the turkey or rat Prl promoter.
Mouse Ltk- cells were co-transfected with a luciferase construct containing either 2.0 kb of the turkey Prl promoter or 3.0 kb of the rat Prl promoter and a an ASVLTR-tPit-1* expression vector. Values represent the mean of four replica ± S.E. in two separate experiments.

Fig. 4-6. Effect of tPit-1* on the Turkey or Rat Prl Promoter

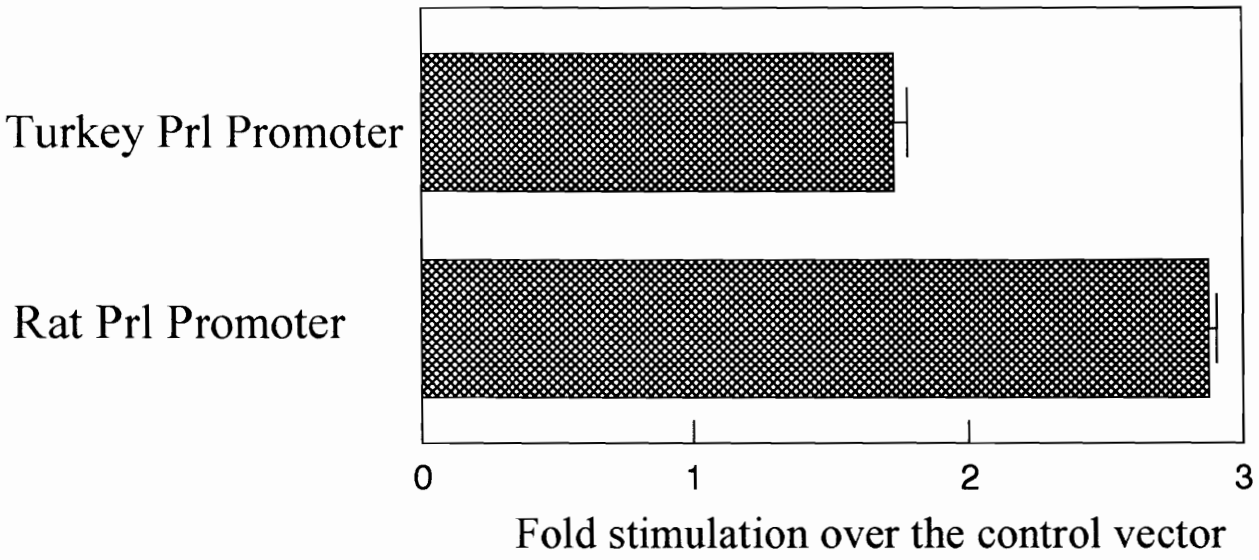
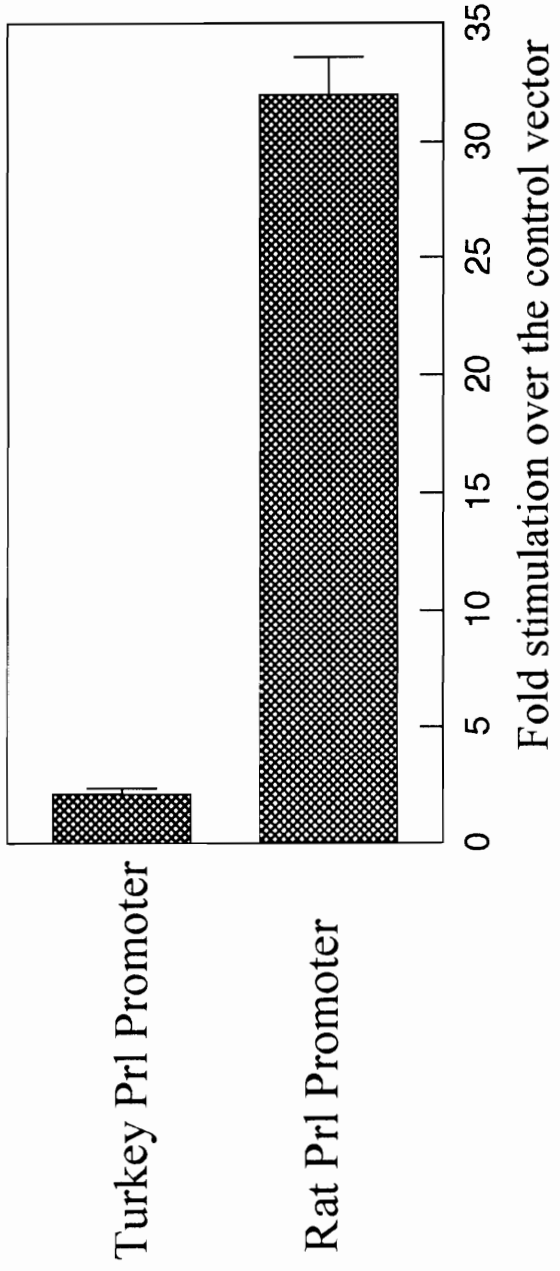


Figure 4-7. Effects of rat Pit-1 on the turkey or rat Prl promoter.
Mouse Ltk- cells were co-transfected with a luciferase construct containing either 2.0 kb of the turkey Prl promoter or 3.0 kb of the rat Prl promoter and a SV40 rat Pit-1 expression vector. Values represent the mean of four to six replica ± S.E. in two separate experiments.

Fig. 4-7. Effect of Rat Pit-1 on the Turkey or Rat Prl Promoter



References Cited

Ben-Jonathan, N. 1985. Dopamine: a prolactin-inhibiting hormone. Endocrine Rev. 6:564-589.

Bodner, M., J. -L. Castrillo, L. E. Theill, T. Deerinck, M. Ellisman, and M. Karin. 1988. The pituitary specific transcription factor GHF-1 is a homeobox-containing protein. Cell 55:515-518.

Breathnach, R., and P. Chambon. 1981. Organization and expression of eucaryotic split genes coding for proteins. Annu. Rev. Biochem. 50:349-383.

Chen, R., H. A. Ingraham, M. N. Treacy, V. R. Albert, L. Wilson, and M. G. Rosenfeld. 1990. Autoregulation of Pit-1 gene expression mediated by two cis-active promoter elements. Nature 364:583-586.

Day, R. N., and K. H. Day. 1994. An alternatively spliced form of Pit-1 represses prolactin gene expression. Mol. Endocrinol. 8:374-381

Day, R. N., S. Koike, M. Sakai, M. Muramatsu, and R. A. Maurer. 1990. Both Pit-1 and the estrogen receptor are required for estrogen responsiveness of the rat prolactin gene. Mol. Endocrinol. 4:1964-1971.

Day, R. N., and R. A. Maurer. 1989. The distal enhancer region of the rat prolactin gene contains elements conferring response to multiple hormones. Mol. Endocrinol. 3:3-9

De Camilli, P., and D. Macconi. 1979. Dopamine inhibits adenylate cyclase in human prolactin-secreting pituitary adenomas. Nature 278:252-254.

Elsholtz, H. P., S. Majumdar-Sannylal, F. Xiong, Z. Gong, and C. L. Hew. 1992. Phylogenetic specificity of prolactin gene expression with conservation of Pit-1 function. Mol. Endocrinol. 6:515-522.

Folger, K. R., E. A. Wong, G. Wahl, and M. R. Capecchi. 1982. Patterns of integration of DNA microinjected into cultured mammalian cells : Evidence for homologous recombination between injected plasmid DNA molecules. Mol. Cell. Biol. 2:1372-1387.

Fox, S. R., M. T. C. Jong, J. Casanova, Z-S. Ye, F. Stanley, and H. H. Samuels. 1990. The homeodomain protein, Pit-1/GHF-1, is capable of binding to and activating cell specific elements of both growth hormone and prolactin gene promoters. Mol. Endocrinol. 4:1069-1080

Gutierrez-Hartmann, A., S. Siddiqui, and S. Loukin. 1987. Selective transcription and DNase I protection of the rat prolactin gene by GH_3 pituitary cell-free extracts. Proc. Natl. Acad. Sci. USA. 84:5211-5215.

Haugen, B. R, W. M. Wood, D. F. Gordon, and E. C. Ridgway. 1993. A thyrotrope-specific variant of Pit-1 transactivates the thyrotropin β promoter. J. Biol. Chem. 268:20818-20824.

Haugen, B. R, D. F. Gordon, A. R. Nelson, W. M. Wood, and E. C. Ridgway. 1994. The combination of Pit-1 and Pit-1T have synergistic stimulatory effect on the thyrotropin β-subunit promoter but not the growth hormone or prolactin promoters. Mol. Endocrinol. 8:1574-1582.

Ingraham, H. A., R. Chen, H. J. Mangalam, H. P. Elsholtz, S. F. Flynn, C. R. Lin, D. M. Simmons, L. Swanson, and M. G. Rosenfeld. 1988. A tissue-specific factor containing a homeodomain specifies a pituitary phenotype. Cell 55:519-529.

Ingraham, H. A., S. E. Flynn, J. W. Voss, V. R. Albert, M. S. Kapiloff, L. Wilson, and M. G. Rosenfeld. 1990. The POU-specific domain of Pit-1 is essential for sequence-specific, high affinity DNA binding and DNA-dependent Pit-1-Pit-1 interactions. Cell 61:1021-1033.

Konzak, K. E., and D. D. Moore. 1992. Functional isoforms of Pit-1 generated by alternative messenger RNA splicing. Mol. Endocrinol. 6:241-247.

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

Kurima, K., L. Sharova, K. L. Weatherly, and E. A. Wong. 1996. Organization of the turkey Pit-1 gene: synthesis of Pit-1 mRNA variants by alternative splicing and transcription initiation. (Submitted)

Lew, A. M., H. Yao and H. P. Elsholtz. 1994. Giα2- and Goα-mediated signaling in the Pit-1-dependent inhibition of the prolactin gene promoter. J. Biol. Chem. 269:12007-12013.

Lew, A. M., and H. P. Elsholtz. 1995. A dopamine-responsive domain in the N-terminal sequence of Pit-1. J. Biol. Chem. 270:7154-7160.

Lin, S-C., C. R. Lin, I. Gukovsky, A. J. Lusis, P. E. Sawchenko, and M. G. Rosenfeld. 1993. Molecular basis of the little mouse phenotype and implications for cell type-specific growth. Nature 364:208-213.

Mason, M. E., K. E. Friend, J. Copper, and M. A. Shupnik. 1992. Pit-1/GHF-1 binds to TRH-sensitive regions of the rat thyrotropin β gene. Biochem. 32:8932-8938.

Maurer, R. A. 1980. Dopaminergic inhibition of prolactin synthesis and prolactin messenger RNA accumulation in cultured pituitary cells. J. Biol. Chem. 255:8092-8097.

Maurer, R. A. 1981. Transcriptional regulation of the prolactin gene by ergocryptine and cyclic AMP. Nature 294:94-97.

McCormick, A., H. Brady, L. E. Theill, and M. Karin. 1990. Regulation of the pituitary-specific homeobox gene GHF1 by cell-autonomous and environmental cues. Nature 345: 829-832.

Morris, A. E., B. Kloss, R. E. McChesney, C. Bancroft, and L. A. Chasin. 1992. An alternatively spliced Pit-1 isoform altered in its ability to trans-activate. Nucleic Acids Res. 20:1355-1361

Nelson, C., E. B. Crenshaw, III, R. Franco, S. A. Lira, V. R. Albert, R. M. Evans, and M. G. Rosenfeld. 1986. Discrete cis-active genomic sequences dictate the pituitary cell type-specific expression of rat prolactin and growth hormone gene. Nature 322:557-562.

Nelson, C., V. R. Albert, H. P. Elsholtz, L. I.-W. Lu and M. G. Rosenfeld. 1988. Activation of cell specific expression of rat growth hormone and prolactin gene by a common transcription factor. Science 239:1400-1405.

Nowakowski, B. E., and R. A. Maurer. 1994. Multiple pit-1-binding sites facilitate estrogen responsiveness of the prolactin gene . Mol. Endocrinol. 8:1742-1749.

Peers, B., M. Voz, P. Monget, M. Mathy-Hartert, M. Berwaer, A. Belayew and J. A. Martial. 1990. Regulatory elements controlling pituitary-specific expression of the human prolactin gene. Mol. Cell. Biol. 10:4690-470

Rajnarayan. S., M. Chiono, L. M. Alexander, and A. Gutierrez-Hartmann. 1995. Reconstitution of protein kinase A regulation of the rat prolactin promoter in HeLa nonpituitary cells: identification of both GHF-1/Pit-1-dependent and -independent mechanisms. Mol. Endocrinol. 9:502-512.

Theill, L. E., K. Hattori, D. Lazzaro, J-L, Castrillo, and M. Karin. 1992. Differential splicing of the GHF-1 primary transcript gives rise to two functionally distinct homeodomain proteins. EMBO J. 11:2261-2269.

Voss, J. W., T. -P. Yao, and M. G. Rosenfeld. 1991. Alternative translation initiation site usage results in two structurally distinct forms of Pit-1. J. Biol. Chem. 266: 12832-12835.

Voss, J. W., L. Wilson, S. J. Rhodes, and M. G. Rosenfeld. 1993. An alternative Pit-1 RNA splicing product reveals modular binding and nonmodular transcriptional activities of the POU-specific domain. Mol. Endocrinol. 7:1551-1560.

Wolf, J. B., V. A. David, A. H. Deutch. 1990. Identification of a distal regulatory element in the 5'-flanking region of the bovine Prl gene. Nucleic Acids Res. 18:4905-4912.

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

Summary and Implications

Prolactin is involved in the induction and maintenance of incubation behavior in birds. Relatively poor reproductive performance by turkey hens compared with chickens is partially due to the early cessation of egg production associated with the Prl-induced onset of incubation behavior. This rise in circulating Prl levels in incubating hens is due to a combination of factors including changes in Prl gene expression and/or Prl transcript stability. Understanding the regulatory mechanism(s) of turkey Prl gene expression provides fundamental information to manipulate Prl production for better reproductive performance in turkey hens.

The sequence of the turkey Prl gene and its 5'-flanking region revealed nearly identical exon distribution to the rat and human Prl genes. In the 5'-flanking region of the turkey Prl gene, two regions similar to the binding sequence for the transcription factor Pit-1/GHF-1 were identified, suggesting that Pit-1/GHF-1 is involved in the regulation of turkey Prl gene expression. The results from the GH_3 cell transfection experiments with turkey Prl promoter-reporter gene constructs indicated that Pit-1/GHF-1 is involved in the regulation of Prl gene expression.

In order to directly examine the role of Pit-1/GHF-1 in turkey Prl gene expression, mouse Ltk- cells were co-transfected with an expression vector for each of the three turkey Pit-1 isoforms and Prl promoter-luciferase reporter gene constructs. Turkey Pit-1s appeared to activate turkey Prl promoter, but clearly defined differential trans-activation effects of three Pit-1 isoforms were not observed in mouse Ltk- cells. Relatively weak trans-activation effects of turkey Pit-1s and the weak activity of the turkey Prl promoter compared with of rat Pit-1 and the rat Prl promoter were noted. The lack of a Pit-1-dependent distal enhancer region in the turkey Prl promoter may suggest that the Pit-1-regulated mechanism determining the rate of Prl gene expression in turkeys is markedly different from the rat Prl gene. Since elimination of the Pit-1 binding sites resulted in the

lack of the promoter activity, it is likely that Pit-1 is involved in, at least, the tissue-specific Prl gene expression in turkeys. Whether turkey Pit-1 is able to mediate hormone-responsive Prl gene activation acting through the Pit-1 binding sites remains to be examined.

Vita

Kiyoto Kurima was born to Seiten and Sadako Kurima on December 11, 1964 in Miyako island of Okinawa. He completed his B.S. in Animal Science at the University of the Ryukyus in 1987. Under the guidance of Dr. Wayne Bacon, he completed his M.S. in Poultry Science at the Ohio State University in 1991. He started his Ph. D. program in Genetics under Dr. Eric Wong in the Department of Animal and Poultry Sciences at Virginia Polytechnic Institute and State University in 1992, and completed in 1996.

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