Regulation of Pituitary Genes by the Transcription Factor, Pit-1, in the Domestic Turkey

(A Turkey is NOT a Feathered Rat)

by

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Abstract

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The transcription factor, Pit-1, is involved in the transcriptional regulation of the mammalian prolactin (Prl), growth hormone (GH) and thyroid stimulating hormone β-subunit genes (TSHβ) as well as its own gene. The role of Pit-1 in avian species is unknown.

Three turkey (t) Pit-1 isoforms have been identified that arise from alternative transcription initiation and alternative splicing. Splicing of exon 1 to an alternative acceptor splice site in exon 2 results in a 28 amino acid insertion in tPit-1β* relative to tPit-1*. Both isoforms initiate transcription at exon 1. A tPit-1 transcript unique to the turkey has been identified and arises following transcription initiation upstream of the alternative acceptor splice site in exon 2. Western blot analysis of pituitary extracts has revealed two isoforms of 37 and 40 kDa.

The ability of Pit-1 to transactivate the Prl, GH, and Pit-1 promoters was determined with cotransfection assays. The tPrl, tGH, tPit-1 and rat (r) Prl promoters were cloned upstream of the luciferase gene in a reporter construct. Turkey Pit-1 isoforms and rPit-1 were expressed under the control of the Avian Sarcoma Virus Long

Terminal Repeat (ASVLTR) promoter. Cotransfection analyses in mouse L cells indicate that tPit-1* activates the tPrl, tGH, tPit-1 and rPrl promoters 4.6-, 3.8-, 1.7-, and 29.0-fold, respectively. Similar results were observed when cotransfection assays were performed in a turkey pituitary-derived cell line and in primary turkey pituitary cells. These results indicate that tPit-1 is not a strong activator of the tPrl, tGH, or tPit-1 genes, whereas Pit-1 does activate these genes in mammals. A point mutation at amino acid position 176 (ser $\Rightarrow$ leu) in the POU-homeodomain results in a mutant tPit-1 that shows decreased activity on all promoters tested. Turkey Pit-1* (ser-176) activates the rPrl promoter 14-fold lower than the wild type tPit-1* (leu-176).

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I. LITERATURE REVIEW

Anterior Pituitary Development in Mammals

Development of the anterior pituitary in mammals gives rise to five distinct cell types, which derive from a common cell lineage. These cell types, somatotrophs, lactotrophs, thyrotrophs, corticotrophs and gonadotrophs, are differentiated by the hormones that they secrete. Thyrotrophs secrete thyroid stimulating hormone, corticotrophs secrete proopiomelanocortin (POMC) and gonadotrophs secrete leutinizing hormone (LH) and follicle stimulating hormone (FSH). Somatotrophs and lactotrophs secrete the closely related growth hormone (GH) and prolactin (PRL) hormones, respectively. Prior to the development of lactotrophs, somatotroph type cells, termed mammosomatotrophs, produce both GH and PRL hormones (Chatelain et al., 1979; Watanabe and Daikoku, 1979; Cooke et al., 1981; Hoeffler et al., 1985; Ingraham et al., 1988; Mangalam et al., 1989). These mammosomatotrophs account for 5-15% of the total cell population present in the developing anterior pituitary gland (Frawley et al., 1985; Frawley, 1989; Frawley and Boockfor, 1991).

Role of Prolactin in Vertebrates

Prolactin is a 23 kDa, single chain polypeptide. In mammals, prolactin plays a major role in mammogenesis and lactogenesis (DeVlaming, 1979). Prolactin is also involved in reproduction, growth and development and regulation of the immune system (Gala et al., 1991). In teleosts, prolactin plays a role in osmoregulation. Specifically, prolactin allows the adapation from sea water to fresh water in some fish.

In birds, prolactin plays a role in incubation behavior and has been studied extensively in the domestic turkey. The domestic turkey is photostimulatory, requiring a shift in daily lighting from 8 hours to 16 hours for approximately 3 weeks, in order to begin the egg laying cycle. After the egg laying cycle, the turkey hen displays incubation behavior. Incubation behavior is defined as increased nesting or nest visits and is associated with ovarian regression and cessation of egg laying. El Halawani et al. (1988) have shown that the onset, as well as maintenance, of incubation behavior in the turkey is correlated with an increase in circulating plasma prolactin levels. Additionally, northern blot analysis has shown that prolactin mRNA levels increase 20-fold in incubating birds relative to quiescent birds (Wong et al., 1992) (Figure 1-1). When ovine prolactin is injected into turkey hens near peak egg production, the hens begin to exhibit incubation behavior (Hargis et al., 1987).

Incubation behavior in turkeys can be interrupted by changing housing conditions. Placing broody turkeys, or turkeys exhibiting incubation behavior, in wire cages to prevent nesting lowers prolactin levels to that of a laying hen within 48 hours. When these birds are returned to nesting conditions, prolactin levels revert to high incubation stage levels within 8 hours (El Halawani, 1980). Following hatch, circulating prolactin levels in the turkey hen decline (Burke, 1981). These studies suggest that prolactin plays a role in the induction of incubation behavior in the turkey hen.

Role of Growth Hormone in Vertebrates

Growth hormone is a 20-22 kDa protein which regulates growth rate in mammals. The development of transgenic mice, which over-express the human GH gene, grew twice as large as litter mates (Palmiter et al., 1983). A recombinant GH injection given to pigs

resulted in increased growth rate (Campbell et al., 1989). Growth hormone also regulates the metabolism of nitrogen, lipids, carbohydrates and minerals in vertebrates (Davidson et al., 1987). Recombinant GH injection increased milk production in dairy cows and decreased fat deposition in pigs (Bauman et al., 1985; Campbell et al., 1989). Growth hormone also plays an important role in development of the immune system in mammals (Hooghe-Peters and Hooghe, 1995; Koojman et al., 1996).

In teleosts, growth hormone plays two important roles. Growth hormone regulates growth rate and is important for adaptation from fresh water to sea water (Sakamoto et al., 1991; Yada et al., 1991). The seawater adaptation and growth promoting activities are independent of one another in teleosts and are regulated by two distinct GH genes (Bolton et al., 1987; Collie et al., 1989).

Growth promotion is not the predominant role of GH in avian species. Injected GH shows no effect on the growth rate of adult chickens or turkeys (Burke et al., 1987; Cogburn et al., 1989; Proudman et al., 1994). When GH was injected into incubating embryos at various stages, however, body weight was increased in both chickens and turkeys (Hargis et al., 1989; Maruyama et al., 1996).

Growth hormone plays a larger role in adipose deposition in birds. Recombinant GH injected into adult chickens decreased abdominal fat pads by 60% (Radecki et al., 1997). When injected into 4 week old chickens, GH infusions increased fat deposition (Moellers and Loghurn, 1995). When 18 week old turkeys were administered recombinant GH, a 70% decrease in the abdominal fat pad was observed (Bacon et al., 1995). These results suggest there is not a significant correlation between GH and growth

rate in older birds. Fat deposition, however, is greatly decreased by GH administration in older birds.

Endocrine Control of Prolactin and Growth Hormone Secretion in Vertebrates

The neuroendocrine regulation of prolactin secretion is under inhibitory control by the hypothalamus in mammals. When the pituitary is removed from hypothalamic regulation, prolactin is secreted from the anterior pituitary (Chen et al., 1970). In addition, administration of dopamine inhibits prolactin secretion from a pituitary that has been removed from hypothalamic control, suggesting that dopamine is the hypothalamic factor which regulates the release of prolactin (MacLeod and Lehmeyer, 1974). In contrast, prolactin is under stimulatory hypothalamic control in the chum salmon (Suzuki et al., 1987).

Avian prolactin secretion is not under dominant inhibitory control by the hypothalamus as is the case in mammals. When the pituitary is removed from hypothalamic control in turkeys, prolactin is not secreted (Chen et al., 1970). Instead, stimulatory factors are necessary to cause prolactin secretion from the pituitary in avian species.

Vasoactive Intestinal Peptide (VIP), synthesized in the hypothalamus, is the most potent factor that causes a stimulatory release of prolactin from the avian pituitary. When injected into ovariectomized turkey hens (Opel and Proudman, 1988), porcine VIP caused an increase in plasma prolactin levels. Also, when pituitary cells in culture are exposed to VIP, prolactin secretion is increased (Proudman and Opel, 1988; El Halawani, 1990).

Serotonin (5-HT) has been shown to have a positive stimulatory effect on VIP release from the hypothalamus and thus prolactin secretion in turkeys. Administration of

p-chlorophenylalanine (PCPA), a 5-HT inhibitor, to incubating turkeys with high prolactin levels resulted in decreased levels of circulating prolactin. After administration of 5-hydroxytryptophan (5-HTP), a 5-HT precursor, plasma prolactin levels are restored to normal incubating levels (El Halawani, 1980). 5-HT was not capable of stimulating prolactin secretion from an isolated pituitary (Fehrer et al, 1985), demonstrating that 5-HT itself is not capable of increasing prolactin secretion directly, but must work through some other intermediate like VIP to increase prolactin secretion.

Organization of the Prolactin and Growth Hormone Genes

The GH and PRL genes are thought to have originated from a common ancestral gene that duplicated over 350 million years ago (Barsh et al., 1983; Miller and Eberhardt, 1983). Because prolactin and growth hormone are evolutionarily related, the expression of the PRL and GH genes may be regulated by a common mechanism.

The gene structure of prolactin is highly conserved between birds and mammals. The turkey prolactin gene consists of 5 exons and spans 6.7 kb (Kurima et al., 1995; Kurima dissertation, 1996). The turkey amino acid sequence is greater than 90% similar to chicken and 54-79% similar to mammals (Wong et al., 1991). The mammalian prolactin promoter region contains positive and negative regulatory elements, which bind hormones and transcription factors that regulate prolactin gene expression (Cao, 1987; Ingraham, 1988; Nelson, 1988).

The GH gene structure in mammals, fish and birds is conserved. The mammalian GH gene consists of 5 exons (for example, Seeburg et al., 1977; Barta et al., 1981). Two GH genes have been identified in salmonid fish but only one form exists in evolutionarily lower species (Ber and Daniel, 1992). This observation is significant because GH plays

two distinct roles in salmonid fish: growth promotion and seawater adaptation. Teleost species, which contain GH genes consisting of 5 exons as in mammals, are the common carp (Chiou et al., 1990), grass carp (Zhu et al., 1992) and the silver carp (Hong et al., 1993). The fish species which contain GH genes with an extra exon (total of six) are the Atlantic salmon (Johansen et al., 1989; Male et al., 1992), chinook salmon (Du et al., 1993), rainbow trout (Agellon et al., 1988), tilapia (Ber and Daniel, 1992) and flounder (Tanaka et al., 1995).

Analysis of the growth hormone gene in birds is limited to the chicken. The gene in the chicken consists of 5 exons and spans approximately 3.5 kb. Introns of the cGH gene are significantly larger than mammalian GH. The 5' flanking region of the cGH gene shows minimal homology to the mammalian GH 5' flanking region (Tanaka et al., 1992).

Pit-1, a Transcription Factor Regulating the Prolactin and Growth Hormone Genes

Functional Domains of Pit-1

The tissue-specific transcription factor, Pit-1, regulates the expression of genes in the anterior pituitary. In addition to regulating the prolactin (Ingraham, 1988; Fox, 1990; Haugen, 1993; Nelson et al., 1988; Mangalam et al., 1989) and growth hormone genes (Nelson et al., 1988), Pit-1 activates thyroid stimulating hormone β-subunit (Steinfelder et al., 1991; Haugen et al., 1993) , Growth Hormone Releasing Factor Receptor, GRFR, (Lin et al., 1993) and its own gene (Chen 1990; McCormick, 1990) in mammals.

Pit-1 shares two regions of homology with other transcription factors, referred to as POU domains. The POU domains were first identified in the proteins Pit-1/GHF-1, Oct-1, Oct-2, and unc-86. Pit-1, Oct-1 and Oct-2 are involved with transcriptional regulation while unc-86 plays a larger role in cell differentiation and cell lineage

determination (Herr et al., 1988). Oct-1 is ubiquitiously expressed and has been classified as both a transcription factor as well as an adenovirus DNA replication factor. Unlike Oct-1, Oct-2 is expressed specifically in B-cells where it is able to initiate *in vitro* transcription of immunoglobulin genes (Scheidereit et al., 1987; Lebowitz et al., 1988). The unc-86 gene product is involved with cell lineage and differentiation, as deletion of the unc-86 gene results in altered lineage and a lack of differentiation in one neural cell type (Herr et al., 1988).

The Pit-1 protein consists of three functional domains: transactivation, POU-homeo and POU-specific. The N-terminal region of the Pit-1 protein (71 amino acids in the rat) contains the transactivation domain. This region is highly variable among species (Lew and Elsholtz 1991; Elsholtz et al., 1992; Wong et al., 1992), and is necessary for the trans-activation abilities of Pit-1 (Theill et al., 1989; Ingraham et al., 1990).

The POU-domain consists of a POU-specific and a POU-homeodomain. The POU-specific domain is located N-terminally to the POU-homeodomain and is well conserved among the family of POU-domain transcription factors (Theill et al., 1993; Ingraham et al., 1990). Among members of the POU family, the POU-specific and the POU-homeodomains are always found in close proximity. It is believed that these two domains co-evolved (Herr et al., 1995). The POU-specific domain is separated from the POU-homeodomain by a highly flexible, 15 amino acid linker in the Pit-1 gene and is the shortest known linker among the POU-domain proteins (Herr et al., 1995; Jacobsen et al., 1997).

Located within the POU-specific and POU-homeodomains are a set of amino acids conserved throughout Pit-1s from various species. The POU-specific domain contains a

set of 28 invariant amino acids and the POU-homeodomain contains 22 invariant amino acids (Scholer, 1991). Conservation of these particular sets of amino acids suggest that they are functionally important for Pit-1 activity.

Mutations in Pit-1 Result in a Dwarf Phenotype

Mutations in the mouse and human Pit-1 genes cause altered phenotypes. The dwarf Jackson strain of mice contains an insertion or inversion in the Pit-1 gene which results in the dwarf phenotype. Pit-1 RNA and protein are not detected in the Jackson dwarf mouse due to structural changes of the gene. The Snell dwarf strain of mice has deficiencies in GH, Prl and TSH production. This strain does not contain a DNA insertion, instead, a single point mutation in the POU-homeodomain changes a cysteine to a tryptophan. This indicates that a single amino acid change in the POU-homeodomain can abolish Pit-1 function by eliminating Pit-1 binding to the responsive element (Li et al., 1990).

In humans, a mutation in the POU-homeodomain which changes arginine to tryptophan at codon 271 causes Combined Pituitary Hormone Deficiency (CPHD). This amino acid change in the POU-homeodomain alters the charge of the POU domain. However, this mutant is still capable of binding to a Pit-1 binding site, suggesting that arg-271 is necessary for transactivation (Radovick, 1992; Jacobsen et al., 1997).

Pit-1 Binding to its Responsive Element

Binding of Pit-1 to its DNA responsive element is different than that of traditional homeodomain proteins. Normally, only the homeodomain comes in contact with the DNA sequence (Muller et al., 1988). In rat Pit-1, both the POU-homeodomain and the POU-specific domains interact with the DNA response element (Aurora and Herr, 1992;

Verrijzer et al., 1992). The POU-homeodomain alone is sufficient for binding to DNA but it is the POU-specific domain which is necessary to increase the specificity of this binding (Ingraham et al., 1990). Both the POU-specific and the POU-homeodomain contain a helix-turn-helix motif which is a highly conserved structure among DNA binding domains (Herr et al., 1995). This is to be expected since both domains of the Pit-1 protein bind the target DNA element. Ingraham et al. (1990) showed that tPit-1 exists as a monomer in solution but can form dimers on certain DNA elements.

Binding of the POU-domain proteins is similar. Oct-1 and Oct-2 are known to bind to the same consensus octameric sequence 5'-ATTTGCAT-3' (Herr et al., 1988; Herr et al., 1995). Pit-1 can also bind this octameric sequence, however, it is not the preferred binding site sequence in vitro (Elsholtz et al., 1990; Aurora and Herr, 1992). Instead, Pit-1 binds to the consensus sequence 5'-(A/T)TATNCAT-3' (Nelson et al., 1988; Mangalam et al., 1989; Herr et al., 1995).

A short sequence of the chicken GH gene, 5'-CATCTGCATTTATGCAAGGAGGG-3', is predicted to be a binding site for the transcription factor Pit-1 based on homology with the antisense strand of the Pit-1 binding site in the rat GH gene (Tanaka et al., 1992). Based on the similarities between fish and bird 5' flanking sequences, Ohkubo et al. (1996) have proposed a modified teleost/avian Pit-1 binding site (5'-T/A NCTNCAT-3').

Xray analysis indicates that Pit-1 binds to the DNA response element in a manner different from that seen with the highly related Oct-1 protein. When bound as a monomer, the POU-homeodomain and the POU-specific domain bind to opposite sides of the DNA in Oct-1 (Klemm et al., 1994). Pit-1, however, binds to perpendicular faces of the DNA

molecule. This occurs because the POU-specific domain of Pit-1 alters its orientation and spacing. The dimerization of Pit-1 is characterized by the binding of one POU-homeodomain to the DNA responsive element and the POU-specific domain to the POU-homeodomain of a second Pit-1 molecule. The POU-specific domain of the second Pit-1 molecule then binds to the DNA responsive element. During the formation of this dimer, the last four residues of the homeodomain of the second Pit-1 molecule are denatured possibly allowing the formation of the dimer complex.

In addition to binding to the DNA response element as a monomer or a homodimer, Pit-1 can also bind to the DNA response element as a complex heterodimer. Voss et al., (1991), reported that the POU domains of Pit-1 and Oct-1 can synergisticaly activate the prolactin promoter through the formation of a heteromeric complex. Unlike Pit-1 dimerization, Oct-1 dimerization occurs through interactions between POU-specific domains only (Ingraham et al., 1990).

Rat Pit-1 Gene, Isoforms and Gene Activation

The mammalian Pit-1 gene is highly conserved from humans to rodents as evidenced by 90% identity at the DNA level and 96% identity at the amino acid level (Tatsumi et al., 1992; Theill and Karin, 1993). The rat Pit-1 gene contains six exons with a single transcription initiation site (Figure 1-2). The POU-specific domain is located between exons 3 and 4 and the conserved POU-homeodomain is in exons 5 and 6 (Theill, 1992). Pit-1 appears as 31 and 33 kDa bands by western blot analysis. The 31 and 33 kD forms of Pit-1 arise from a single RNA transcript by alternative translation initiation (Voss et al., 1991). The 31 kDa form lacks the N terminal 27 amino acids. Both forms of

Pit-1 activate the rat prolactin promoter to a similar extent in transient co-transfection studies (Nelson et al., 1988; Mangalam et al., 1989; Voss et al., 1991).

Three isoforms of rat Pit-1, which arise by alternative splicing, have been identified (Figure 1-2). The Pit-1β (also known as Pit-1a, GHF2) isoform is an RNA splice variant which contains an additional 26 amino acids within the 5' transactivation domain (Diamond and Gutierrez-Hartmann, 1996). Pit-1β encodes a protein of 35.8 kDa and is 7 times less abundant than Pit-1. Pit-1β is capable of activating the GH promoter, but does not significantly activate the Prl or Pit-1 promoters. This indicates that the 26 amino acid insertion in the activation domain reduces the ability of Pit-1 to activate the Prl promoter (Morris et al., 1992; Konzac and Moore, 1992; Theill et al., 1992).

Pit-1T, found only in thyrotrophs, is an alternatively spliced form which encodes an additional 14 amino acids at the 5' end of exon 2. This isoform stimulates the TSHβ promoter, but has no effect on the GH promoter. A combination of Pit-1 and Pit-1T does not stimulate the Prl or GH promoters but does synergistically stimulate the TSHβ promoter in a thyrotrope derived cell line (Haugen et al., 1993; Haugen et al., 1994).

The third isoform identified, Pit-1Δ4, is created by alternative splicing which deletes the 170 bp encoding exon 4. This exon contains most of the POU-specific domain (Day and Day, 1994). Pit-1Δ4 inhibits prolactin promoter activation in cotransfection assays. Since the POU-specific domain increases the specificity of DNA binding and promotes protein/protein interactions, it is hypothesized that this alternative form of Pit-1 has lost the ability to interact properly with DNA and protein molecules to promote prolactin activation (Day and Day, 1994).

The mammalian Pit-1 gene is autoregulated. The rat Pit-1 5' flanking sequence contains two putative Pit-1 binding sites one 5' and one 3' of the TATA box. The binding sites upstream and downstream of the TATA box are 5'-ATGTATAAA-3' and 5'-ATGTATATA-3' for rat Pit-1. Chen et al. (1990) have shown that Pit-1 will bind to both sites with high affinity. In cotransfection assays, mutation of the upstream rat Pit-1 binding site decreases rPit-1 promoter activation by more than half. When the downstream rPit-1 binding site is mutated, rPit-1 promoter activation is increased by more than 4-fold. The upstream Pit-1 binding site may act as a positive regulator of Pit-1 production, while the downstream binding site acts to lower the stimulatory effects either by decreased transcription initiation or attenuation of nascent transcripts (Chen et al., 1990).

Turkey Pit-1 Gene, Isoforms and Activation Abilities

The turkey Pit-1 gene consists of seven exons and spans approximately 12 kb in length (Kurima et al., 1998). The gene sequence is 94% homologous to the rat Pit-1 gene in the POU-domains (Wong et al., 1992). The turkey Pit-1 gene contains an exon, located between exons 2 and 3, which encodes 38 amino acids not found in mammals. This exon is designated as exon 2a. The 5'-flanking regions upstream of exon 1 of the rat and turkey Pit-1 genes are highly homologous (Figure 1-3). Both rat Pit-1 and turkey Pit-1* 5'-flanking sequences contain identical TATA box sequences. In addition, both rat and turkey 5'-flanking sequences contain two putative Pit-1 binding sites one upstream and one downstream of the TATA box. The binding site upstream of the TATA box is ATGTATAAA and ATGGATAAA for rat and turkey, respectively. Only one nucleotide (underlined) is different between these two Pit-1 binding sites. The Pit-1 binding site

downstream of the TATA box is ATGTATATA for both rat and turkey Pit-1. Since tPit-1 contains similar Pit-1 binding sites, it is hypothesized that tPit-1 is also under similar autoregulatory control.

Three tPit-1 cDNA isoforms have been identified, (tPit-1*, tPit-1β* and tPit-1W*) all of which contain the extra 38 amino acids encoded in exon 2a and thus are denoted with an asterisk to distinguish them from comparable mammalian Pit-1 isoforms (Figure 1-4). The tPit-1* isoform initiates transcription at the 5' end of exon 1. RNase protection assays showed that tPit-1* is the most abundant isoform in the turkey pituitary (Kurima et al., 1998). The tPit-1* isoform activates the rat prolactin promoters 2- to 3-fold and the turkey prolactin promoter 1.7-fold over a cDNA-less expression vector. Turkey Pit-1 activated the rat prolactin promoter 2- to 3-fold while rat Pit-1 stimulated the rat prolactin promoter more than 30-fold. The tPit-1β* isoform is generated by alternative splicing from the end of exon 1 into the region of exon 2 similar to the alternative splice site used in the synthesis of the rat Pit-1β isoform. The tPit-1W* isoform is generated by transcription initiation upstream of the splice site used for the tPit-1β* isoform and thus lacks exon 1 entirely. Turkey Pit-1W* activates the turkey prolactin promoter to levels equal to tPit-1β*.

The N-terminus of tPit-1 more closely resembles the amino-termini found in the fish gilthead seabream and chum salmon than that in mammals. This suggests that birds are more closely related to fish than mammals. Additionally, there are 2 insertions, consisting of 33/38 and 26 amino acids, within the N-terminus which are conserved between birds and fish (Figure 1-5). The 26 amino acid subdomain, or portions of this

subdomain, are also found in alternatively spliced forms of rat and human Pit-1 (Vila, et al., 1995).

II. OBJECTIVES

The turkey Pit-1 gene and three cDNA variants have been cloned. The goal of this research was to examine expression of Pit-1 in the turkey pituitary and to investigate the ability of the tPit-1 isoforms to transactivate pituitary gene promoters. The specific objectives for this thesis were:

1. to examine tPit-1 isoform expression in the turkey pituitary by western blot
2. to measure the ability of tPit-1 isoforms to activate the tPrl and tGH promoters and to autoactivate its own promoter in cotransfection studies

III. MATERIALS AND METHODS

Experimental Animals

Nicholas Large White turkeys (Nicholas Turkey Breeding Farms, Sonoma, CA) were housed at the Virginia Tech Turkey Center. At 30 weeks of age, turkey hens were photostimulated (shifted from 8L:16D to 16L:8D). Hens started laying eggs 2 to 3 weeks after photostimulation.

Western Blot Analysis

Western blot analysis was performed to identify isoforms of Pit-1 in turkey pituitary. Adult birds were killed by electrocution. Pituitaries were removed immediately and placed in tubes and frozen on dry ice or liquid nitrogen, and stored at -80°C until used. Pituitaries were thawed and weighed. Approximately 0.2g of pituitary and 0.2 g of previously frozen liver tissue were homogenized with a dounce homogenizer in 4 mL NE1 buffer (25 mM sucrose, 15 mM Tris-HCl pH 7.9, 140 mM NaCl, 2 mM EDTA, 0.5 mM spermine, 1 mM DTT, 0.4 mM Phenylmethanesulfonyl fluoride (PMSF), 25 mM KCl, 2 mM $MgCl_2$). The homogenate was filtered through cheese cloth and Nonidet P40 was added to a concentration of 0.5%. After a second homogenization step in a dounce homogenizer, the lysate was centrifuged for 8 minutes at 1000xg at 4°C to pellet nuclei. Nuclei were washed once with NE1 buffer and lysed with 1 packed cell volume of NE2 Buffer (NE1 buffer + 350mM KCl) for 5 minutes at 4°C. 1/10 packed cell volume of 4 mM KCl was added and the nuclear cell extract homogenized again. Cellular debris was removed by centrifugation at 12,000xg for 90 minutes. The supernatant was further centrifuged at 180,000xg for 90 minutes to remove small organelles. The high-speed supernatant was dialyzed for 1 hour at 4°C against DNase I buffer (50 mM KCl, 4

mM $MgCl_2$, 20 mM KPO_4 pH 7.4, 1 mM β-mercaptoethanol, 20% glycerol). Lysates were stored at -80°C, if not used immediately.

Concentration of protein in the liver and pituitary cell extract was determined using the Protein Assay Dye Reagent Concentrate detection kit (BioRad Labs, Hercules, CA) with BSA as a standard curve. Protein yields were 66.8 μg of protein/0.2 g liver tissue and 48.4 μg of protein/0.2 g pituitary tissue.

Proteins were separated on the basis of molecular weight by SDS polyacrylamide gel electrophoresis. A separating gel (0.1% SDS, 0.37 M Tris-HCl pH 8.8, 12% bis:acrylamide, 0.05% ammonium persulfate (APS), 0.05% TEMED) was prepared. The gel was overlayed with water and allowed to polymerize for 1 to 2 hours. Following polymerization, a stacking gel (0.12 M Tris-HCl pH 6.8, 0.1% SDS, 4% bis:acrylamide, 0.05% APS, 0.1% TEMED) was prepared. The comb was positioned in the stacking gel.

To each sample, 20 μl of sample buffer (0.06 M Tris-HCl pH 6.8, 9.8% glycerol, 2% SDS, 0.7 M β-mercaptoethanol, 0.01% bromophenol blue) (adapted from method of R. Day, Univ. of VA, Charlottesville, VA) was added. Samples were boiled 5 minutes and loaded into the wells of the stacking gel. The gel was run at 36 volts for 20 hours in tank buffer (0.03 M Tris, 0.004 M SDS, 0.19 M Glycine). A prestained low range protein standard (20.9-101 kDa; Biorad Labs) was boiled and loaded on the gel.

Following gel electrophoresis, the acrylamide gel was removed from the electrophoresis equipment and the stacking gel was discarded. Proteins in the separating gel were electroblotted to Immobilon P membrane® (Millipore, Bedford, MA) in transfer buffer (0.01 M Tris, 0.08 M glycine, 20% methanol) for 20 hours at 36 volts in a Hoefer

(San Francisco, CA) transfer tank. Following electroblotting, the membrane was marked to identify location of wells and dried at room temperature for 2 hours on absorbent paper.

The membrane was blocked in blocking buffer (10% nonfat dry milk, 0.05% Tween-20, in PBS) for 30 minutes at room temperature with gentle rocking. The membrane was incubated for 1 hour at room temperature with polyclonal rabbit-anti-rat Pit-1 antisera, generous gifts from R. Day (Univ. of VA, Charlottesville, VA) and S. Rhodes (IUPUI, Indianapolis, IN) diluted 1:2000, in blocking buffer. Following primary antibody incubation, the membrane was washed 2 times in PBS and then incubated for 30 minutes with a horseradish peroxidase conjugated goat anti-rabbit antiserum for 30 minutes at room temperature. The secondary antibody was diluted 1:10,000 in blocking buffer. Following secondary antibody incubation, the membrane was immediately washed in PBS for approximately 24 hours.

Chemiluminescent reagent (Du Pont NEN, Wilmington, DE) was diluted (1:1) with distilled water. The membrane was rinsed for 1 minute in diluted chemiluminescent reagent and immediately blotted with a kimwipe to remove excess reagent. The membrane was wrapped in Saranwrap and exposed to Kodak X-AR5 x-ray film (Eastman Kodak Co., Rochester, NY).

Expression Vector and Reporter Gene Constructs

Construction of Turkey Pit-1 Expression Vectors:

Total RNA was isolated from three turkey pituitaries using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH). 2 μg of total RNA was mixed with 10 ng of oligo AS6 (5'- AACAGGAACCCACAGCTA-3') and boiled for 2 minutes. The reaction was allowed to cool to room temperature for 3 minutes and then placed on ice for 15

minutes. Reverse transcriptase reaction mix (1x first strand buffer, 0.75 mM dNTPs, 1mM DTT, 500 U MMLV-Reverse Transcriptase (Gibco-BRL, Gaithersburg, MD), 20 U RNasin (Promega, Madison, WI)) was added and incubated for 1 hour at 37°C. The cDNA was centrifuged through a Sephadex G-50 column at 1,200xg for 2 minutes, ethanol precipitated for 3 hours at -20°C and pelleted at 52,000xg for 15 minutes. The cDNA was resuspended in a total volume of 20 µl TE and 1 µl cDNA was used for PCR in *Taq* DNA polymerase reactions with primers tPit-AS44 (5'-GCTTTACCGGCACTCGTGGTG-3') and tPit-S45 (5'-GCTGCCATTAATCGCTCAG-3') for the tPit-1* and tPit-1β* isoforms and with primers tPit-AS44 and tPit-S5 (5'-GCAGGAGAACTAGATACG-3') for the tPit-1W* isoform. PCR conditions were 1 cycle of 95°C, 3 min., 55°C, 1 min., 72°C, 2 min.; 30 cycles of 95°C 1 min., 55°C, 1 min., 72°C, 1.5 min.; and 1 cycle of 95°C, 1 min., 55°C, 1 min., 72°C, 5 min. The mixture of tPit-1*, tPit-1β* and tPit-1W* PCR products were precipitated with ethanol and cloned into the pGEM-T Easy vector (Promega, Madison, WI). cDNAs were sequenced and subcloned into plasmid pLTR/SV40. This expression vector consists of the avian sarcoma virus long terminal repeat (ASVLTR) and a simian virus 40 (SV40) splice site and polyadenylation signal.

Sequencing of previously reported tPit-1 cDNA constructs revealed a single point mutation, which changed a leucine to a serine at position 176 (Kurima, 1996). Based on consensus sequences from other species, leucine at amino acid position 176 is considered the wild type form of tPit-1. Thus, wild type tPit-1 expression vectors are indicated as tPit-1(leu-176) and the mutant form is indicated as tPit-1(ser-176).

Although expression vectors for tPit-1β* and tPit-1W* were constructed, data are not reported for these constructs because they were found to have other mutations within the cDNA sequence.

Construction of a Rat Pit-1 Expression Vector:

An SV40 rat Pit-1 expression vector was obtained from Dr. H. P. Elsholtz (University of Toronto), to be used as a positive control. The rat Pit-1 cDNA was excised from this plasmid and cloned into the expression vector pLTR/SV40 used for turkey Pit-1 expression vectors.

Construction of Rat and Turkey Prolactin-Luciferase Reporter Vectors:

3.0 kb of the rat prolactin promoter was obtained from H. Elsholtz and cloned into the pGL3 luciferase reporter vector (Promega). 2.0 kb of the 5' region of the tPrl gene has been previously cloned into pGL3 (Kurima, 1996).

Construction of a Turkey Growth Hormone-Luciferase Reporter Vector:

Approximately 900 bp of the tGH promoter was isolated using the Genome Walker Kit (Clontech, Palo Alto, CA) and cloned into plasmid pGEM-T Easy. The tGH 5'-flanking region was amplified with primers tGH-1 (5'-CGCTCGAGCCAGGAGAGCTGCTCGGGTG) and Reverse Primer (5'-CAGGAAACAGCTATGAC-3'). This PCR product was digested with *Mlu* I and *Xho* I. An internal *Mlu* I site on the 5' end and an *Xho I* site in primer tGH-1 (underlined) were used for cloning into the *Mlu* I and *Xho* I sites of the pGL3 luciferase reporter vector.

Construction of a Turkey Pit-1-Luciferase Reporter Vector:

489 bp of the turkey Pit-1 exon 1 5'-flanking region (Kurima et al., 1998) was subcloned into the plasmid pALTER (Promega, Madison, WI). The tPit-1 fragment was

amplified from the pALTER plasmid, using the primers tPit-S36 (5'-TCGACGCGTCAGCCAGGTTGTCTTCC-3') and tPit-AS39x (5'-CGCTCGAGGCCCTCAAACAGGTGAAAG-3'). The fragment was digested at the *Mlu* I and *Xho* I sites which are located within the primer sequences (underlined) and cloned into the *Mlu* I / *Xho* I sites of the pGL3 reporter vector.

Construction of turkey Pit-1-Luciferase Reporter Vector with Mutated Pit-1 Binding Sites

Two putative Pit-1 binding sites were present in the tPit-1 promoter, one upstream and one downstream of the transcription start site (Kurima et al., 1998). Chen et al. (1990) have shown that the upstream site acts as a positive regulatory element while the dowstream site acts as a negative regulatory element.

The downstream Pit-1 binding site in the tPit-1 promoter was mutated by the overlap extension PCR mutation method (Figure 3-1). Primers with mutations located in the center, were used to create fragments that contained changes from the original tPit-1 fragments. PCR was performed using primers tPit AS41 (5'-CACTTGTATATACBTATACAGAAGGAC-3') and T7 primer (5'-TAATACGACTCACTATAGGG-3') as well as tPit-S43 (5'-GTCCTTCTGTATABGTATATACAAGTG-3') and Reverse primer (5'-CAGGAAACAGCTATGAC-3'). D represents nucleotides G, A or T while B represents nucleotides G, T or C. PCR products with mutations were combined, denatured, annealed and reamplified with T7 and Reverse primer to synthesize the entire fragment.

The upstream Pit-1 binding site in the tPit-1 promoter was also mutated by a similar strategy using the oligo combinations tPit-AS40 (5'-

AATCCCTTTATCDBTTTAATGTCTT-3') and T7 primer as well as tPit-S42 (5'-AAGAACATTAAAAVHGATAAAGGGATT-3') and Reverse primer. D represents nucleotides G, A or T; B represents nucleotides G, T or C; V represents nucleotides G, C or A; H represents nucleotides A, C or T.

CMV* b-*galactosidase control vector:

A Cytomegalovirus β-gal control plasmid was obtained from Dr. R. N. Day (Univ. of Virginia) to normalize for differenced in transfection efficiency.

Transfection of Cell Lines

Mouse L cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotic-antimycotic (90 IU/ml penicillin, 90 μg/ml streptomycin, 0.9 μg/ml amphotericin) in a 37°C incubator with 5% CO_2. The turkey pituitary-derived cell line, obtained from D. Foster (Univ. MN), was maintained in supplemented DMEM (10% FCS, 9 μg/ml insulin, 0.02 M Hepes pH 7.0).

Approximately 24 hours prior to transfection, 0.5 million cells were plated in 35mm culture dishes. 25 μg lipofectamine (Life Technologies) was incubated with 1 μg Pit-1 expression vector, 4 μg promoter-luciferase reporter construct and 0.125 μg CMV-β-galactosidase plasmid in 200 μl OptiMEM (Life Technologies) for 30 minutes at 22-25°C. Cells were rinsed with OptiMEM and transfected with 1 ml of lipofectamine DNA complex (200 μl DNA lipofectamine complex plus 800 μl OptiMEM) for 5 hours in a 37°C incubator with 5% CO_2. Transfection was terminated with the addition of 3 ml DMEM supplemented with 10% FCS. Cells were incubated for a 43 hour recovery period before collection. Cells were washed once with PBS and incubated for 15 minutes in 1X

Reporter Lysis Buffer (Promega) at 22-25°C. Cells were scraped with rubber-tipped plate scrapers, transferred to microfuge tubes and subjected to three rapid freeze-thaw cycles (with dry ice) and centrifuged for 2 minutes at 4,000xg. The supernatant was transferred to a new microfuge tube and used for luciferase and β-galactosidase assays. Between assays, samples were stored on ice.

To determine luciferase activity, 50 µl of each cell lysate was added to 300 µl of ATP buffer (5 mM ATP, 25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$). Tubes were placed in a Lumat LB9501 Luminometer (Berthold, Bad Wilbad, Germany), and the reaction was initiated by injection of 100µl luciferin (1 mM luciferin, 25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$). Light emission was measured for 10 seconds. Each sample was assayed twice. An average luciferase value was calculated.

To determine β-galactosidase activity, 25 µl of cell lysate was diluted with 25 µl 1X reporter lysis buffer (Promega). 250 µl of β-galactosidase assay buffer (82.3 mM $NaPO_4$ pH 7.5, 1.9 mM $MgCl_2$, 88.1 mM β-mercaptoethanol, 13.3 mM O-Nitrophenyl-β-D-galactopyranoside) were added and allowed to incubate for 15 minutes at 37°C. Reactions were terminated by the addition of 500 µl of 1M Na_2CO_3, and absorbance was measured at 420nm. Each sample was assayed twice. An average β-galactosidase activity was calculated. Results are expressed as fold stimulation over a cDNA-less expression vector.

To correct for variations in transfection efficiencies, the average luciferase activity was divided by the average β-galactosidase activity for each sample.

Primary Pituitary Cell Collection

A total of three pituitaries were extracted from reproductively active turkey hens killed by electrocution. Pituitaries were stored in 1 ml of ice cold CMF-HBSS (Ca^{2+} and Mg^{2+} free-Hank's basic saline solution) for transport back to the laboratory. Pituitaries were removed from CMF-HBSS in a laminar flow hood and chopped into small pieces with a sterile razor blade. Pituitary pieces were suspended in 3 ml of sterile CMF-HBSS containing 3% BSA and 3% collagenase and incubated in a siliconized flask at 37°C for 45 minutes with continuous rocking. At 10-15 minute intervals, the pituitary pieces were gently dispersed with a narrow bore siliconized pasteur pipette. The cell suspension was transferred to a conical tube and centrifuged at 400xg for 1 minute. The supernatant was removed and the pellet washed once with CMF-HBSS. The cell pellet was resuspended in 3 mL of sterile CMF-HBSS containing 1.25% pancreatin and 10 μg/mL DNaseI and incubated at 37°C with continuous rocking for 1 hour. Again, cells were dispersed every 10-15 minutes with a siliconized pipette and then filtered through autoclaved 60 μm Spectra/mesh (Spectrum, Houston, TX) to remove remaining pituitary pieces. Cells were centrifuged at 400xg for 1 minute and the cell pellet was washed twice with media (DMEM supplemented with 1% non-essential amino acids, 25 mM Hepes pH 7, 10% FCS, 90 IU/ml penicillin, 90 μg/ml streptomycin, 0.9 μg/ml amphotericin) to remove any remaining pancreatin (Proudman and Opel, 1988). Cells were counted with a hemacytometer and cell viability was determined by the trypan blue dye exclusion method. Typical percent viability was approximately 95%. Cells were plated in medium and allowed to recover for approximately 3-4 days before replating for transfection. 150,000 cells were plated in 35 mm dishes and transfected 2 days later as described in the previous section, "Transfection of Cell Lines".

IV. RESULTS

Identification of tPit-1 Isoforms in the Turkey Pituitary by Western Blot

The presence of tPit-1 in pituitary extracts was assayed by western blot analysis. A polyclonal rabbit-anti-rat Pit-1 antisera detected two predominant bands at 37 and 40 kDa in turkey pituitary and no Pit-1 protein in turkey liver (Figure 4-1). Based on nucleotide sequences, the predicted protein molecular weights for tPit-1*, tPit-1β* and tPit-1W* are 38, 40, and 37 kDa, respectively. To try to verify the assignment of these bands to particular variants, mouse L cells were transiently transfected with expression vectors for the three different tPit-1 variants. However, no tPit-1 protein could be detected on western blots in cell extracts from these transfected cells (data not shown). Thus, assignment of these bands to specific variants remains to be determined.

tPit-1 Activation of the tPrl, tGH, and rPrl Promoters

In order to test the ability of tPit-1 to activate the tPrl, tGH and rPrl promoters, mouse L cells were transiently cotransfected with either tPit-1* or rPit-1 (Figure 4-2). Relative to a Pit-1 cDNA-less expression vector, tPit-1* activated the tPrl, tGH and rPrl promoters 4.5-, 3.8-, and 29-fold (bars 1, 3 and 5), while rPit-1 activated the same promoters 6.8-, 6.9-, and 29.4-fold (bars 2, 4 and 6), respectively. These results indicate that tPit-1* is capable of activating the tPrl, tGH and rPrl promoters to a level equivalent to that of rat Pit-1.

It is possible that mouse L cells do not provide a cofactor needed for tPit-1* transactivation of the tPrl promoter and activation levels are therefore lower than those for rPrl activation. Therefore, primary turkey pituitary cells were used to test the ability of tPit-1* and rPit-1 to activate the tPrl, tGH and rPrl promoters (Figure 4-3). Turkey Pit-

1* activated the tPrl, tGH and rPrl promoters 1.5-, 2.0- and 1.9-fold (bars 1, 3, and 5), while rPit-1 activated these same promoters 1.4-,4.8- and 2.7-fold (bars 2, 4 and 6), respectively. These results indicate that primary turkey pituitary cells do not contain a turkey specific factor necessary for enhanced turkey promoter activation. Both tPit-1 and rPit-1 activation of the rPrl promoter are decreased by approximately 15-fold in primary turkey pituitary cells over the same transfection in mouse L cells. Presumably, the absence of a mammalian-specific factor in the primary turkey pituitary cells is responsible for this decrease in activation.

A set of invariant amino acids in the POU-specific domain of Pit-1 have been identified (Scholer, 1991). This domain is necessary for increasing binding specificity to the DNA responsive element to activate transcription. One cloned tPit-1 cDNA contains one amino acid change, leu-176 to ser-176, within this set of invariant amino acids.

To test whether the amino acid change in the POU-specific domain played an important role in the transactivation ability of Pit-1 on tPrl, tGH and rPrl promoters, cotransfections were performed in mouse L cells (Figure 4-4). Compared to a cDNA-less expression vector, wild type tPit-1*(leu-176) activated the tPrl promoter 3.3-fold (bar 1) while mutant tPit-1*(ser-176) activated the tPrl promoter 1.2-fold (bar 2). Rat Pit-1 activated the tPrl promoter 4.6-fold (bar 3). Compared to a cDNA-less expression vector, wild type tPit-1*(leu-176) activated the tGH promoter 3.7-fold (bar 4), while mutant tPit-1*(ser-176) activated the tGH promoter 1.1-fold (bar 5). Rat Pit-1 activated the tGH promoter 4.7-fold (bar 6). Compared to a cDNA-less expression vector, wild type tPit-1*(leu-176) activated the rPrl promoter 23.3-fold (bar 7), while mutant tPit-1*(ser-176) activated the rPrl promoter only 1.6-fold (bar 8). Rat Pit-1 activated the rPrl promoter

22.6-fold (bar 9). These results indicate that a single point mutation in the POU-specific domain of tPit-1 is responsible for decreased activation of the tPrl, tGH and rPrl promoters.

tPit-1 Activation of the tPit-1 Promoter

Turkey Pit-1 is predicted to have two promoters for gene activation, one upstream of exon 1 and one upstream of exon 2. The promoter upstream of exon 1 contains two putative Pit-1 binding sites, one upstream and one downstream of the transcription start site. These sites may autoregulate the tPit-1 gene in a manner similar to that reported for rPit-1. In the rat Pit-1 gene, the upstream Pit-1 site acts as a positive regulator while the downstream site acts as a negative regulator (Chen et al., 1990). To test autoregulation, the promoter upstream of exon 1 for tPit-1 was cloned into the pGL3 luciferase-reporter vector.

In mammals, mutation of the downstream Pit-1 binding site in the exon 1 promoter from 5'-ATGTATATA-3' to 5'-ATGGATATA-3' resulted in an increase in Pit-1 activation (Chen et al., 1990). It was hypothesized that a comparable mutation of the tPit-1 downstream binding site would result in increased activation of the tPit-1 exon 1 promoter. Therefore, the downstream Pit-1 binding site was mutated by the overlap extension PCR mutation method from 5'-ATGTATATA-3' to 5'-ATGGATATA-3'.

Cotransfection experiments were performed in mouse L cells with the tPit-1 promoter, the tPit-1 promoter containing a mutated Pit-1 binding site and the rPrl promoter (Figure 4-5). Wild type tPit-1*(leu-176) activated the tPit-1 promoter 1.7-fold (bar 1), while rPit-1 activated the tPit-1 promoter 1.9-fold (bar 2). The tPit-1 promoter with a mutation in the downstream Pit-1 binding site was activated 1.9- and 1.9-fold by

tPit-1* and rPit-1, respectively (bars 3 and 4). Again, rPrl was activated 34.6- and 36.1-fold by tPit-1* and rPit-1* (bars 5 and 6). These results suggest that tPit-1 is not regulated in the same manner as rPit-1 since tPit-1 expression was not dramatically increased when the downstream Pit-1 binding site was mutated, as seen with the rPit promoter.

In order to eliminate the possibility that a turkey specific cofactor, necessary for turkey Pit-1 activation, was lacking in the mouse L cells, experiments were repeated in primary turkey pituitary cells (Figure 4-6). Turkey Pit-1* and rPit-1 activated the tPit-1 promoter 1.7- and 2.5-fold, respectively (bars 1 and 2). The tPit-1 promoter with a mutation in the downstream Pit-1 binding site was activated 1.2-fold by tPit-1* (bar 3) and 2.9-fold by rPit-1 (bar 4). The rPrl promoter was activated 1.9- and 2.7-fold by tPit-1* and rPit-1, respectively (bars 5 and 6). These results indicate that tPit-1 is a weak activator of the tPit-1 promoter even after mutation of the putative negative downstream Pit-1 binding site.

V. DISCUSSION

The pituitary of the domestic turkey contains Pit-1 isoforms as determined by western blot analysis. Proteins of 37 and 40 kDa are detected in turkey pituitary extracts, however, computer analysis predicts three tPit-1 isoforms of 37, 38 and 40 kDa. Based on the close proximity of the 37 and 40 kDa tPit-1 isoforms in the acrylamide gel, it is possible that the 38 kDa protein may not have separated from the 37 kDa protein. Since it is also possible that proteins are migrating anomalously, it is not possible to identify the isoforms present on this gel based solely on predicted protein size. Attempts to identify the tPit-1 isoforms by expressing the individual isoforms in mouse L cells were unsuccessful due to low protein expression in those cells. Future experiments, involving immunoprecipitation of [^{35}S] labeled tPit-1 isoforms synthesized in cultured cells may help identify the isoforms present in turkey pituitary extracts.

Another reason for the identification of only 2 bands on western blot may be the antisera used for tPit-1 detection. The antiserum was produced in a rabbit against rPit-1. It is possible that the antibodies were produced only against the variable N-terminal region of the rPit-1 protein, because the POU-specific and the POU-homeodomains are highly conserved between species. Since all differences among tPit-1 isoforms occur in the N-terminal region, it is possible that the antibody could be directed towards exon 1, which is lacking in the tPit-1W* isoform. This could account for the lack of identification of a third band by western blot analysis. Although the antibodies produced are polyclonal, the antibody has not been tested, and it is possible that the tPit-1W* isoform is not detected by western blot analysis.

Turkey Pit-1*(leu-176) activates the rPrl promoter 29-fold in mouse L cell cotransfection assays. This fold stimulation is equal to the stimulation of the rPrl promoter by rPit-1, indicating that tPit-1*(leu-176) is a functional Pit-1 isoform. In addition, the presence of the 38 amino acids encoded in tPit-1, exon 2a, does not appear to affect the activity of tPit-1 compared to rPit-1.

Both tPit-1*(leu-176) and rPit-1 are reduced in their ability to activate the tPrl promoter. Turkey Pit-1*(leu-176) and rPit-1 activate the tPrl promoter only 4.8- and 6.8-fold for tPit-1*(leu-176) and rPit-1, respectively. It is possible that the tPrl promoter is activated less than the rPrl promoter because only two putative Pit-1 binding sites are present in the tPrl promoter whereas there are eight known Pit-1 binding sites in the rPrl promoter. Therefore, the higher fold stimulation seen with rPrl may just be an additive effect of the total amount of Pit-1 protein that is able to bind to the promoter region. Similar studies indicate that multiple Pit-1 binding sites in the rPrl promoter are responsible for increased activation of the Prl promoter by Pit-1 in transfection assays (Nelson et al., 1988).

Turkey Pit-1 is not a strong activator of the tGH promoter. Compared to a cDNA-less expression vector, tPit-1*(leu-176) activated the tGH promoter 3.8-fold while rPit-1 activated the tGH promoter 6.9-fold in mouse L cell cotransfections. When repeated in primary turkey pituitary cells, tPit-1 and rPit-1 activated the tGH promoter 2.0- and 4.8-fold, respectively.

Turkey Pit-1* is not a strong autoactivator of its own promoter as in mammals. The tPit-1*(leu-176) isoform activates the tPit-1 and the tPit-1 mutated promoter 1.7- and 1.9-fold in mouse L cell cotransfections and 1.7-, and 1.2-fold in primary turkey pituitary

cell cotransfections. Since this is a cotransfection experiment, and cells are removed from control of other organs, it is possible that mouse L cells do not provide and the primary cells lack, a turkey specific cofactor necessary for tPit-1 activation.

It is important to determine the activation ability of tPit-1 isoforms on the Prl and GH promoters as well as its own promoter. Rat Pit-1β is capable of activating the GH promoter but does not significantly activate the Prl or Pit-1 promoters. Turkey Pit-1β* may have a similar function in activating tPrl and tGH. In addition, tPit-1W* is unique to the turkey and cotransfection analysis will determine the activation ability of this isoform on the tPrl and tGH promoters as well as its own promoter.

A single amino acid mutation (leu-176 to ser-176) in a conserved region of the POU-homeodomain reduces the activity of tPit-1*. Scholer et al. (1991) identified 28 invariant amino acids present in the POU-specific domain. Mutation of the amino acid leu-176 to ser-176, in the tPit-1 POU-specific domain, results in a 15-fold decrease in stimulation of rPrl in mouse L cells. The POU-specific domain is responsible for increasing the specificity and affinity of binding to the DNA responsive element (Ingraham et al., 1990). Mutation of this invariant amino acid in the POU-specific domain may therefore decrease the affinity of tPit-1* binding to the rPrl promoter.

It is not clear whether this mutation in the POU-specific domain was a naturally occurring mutation in the turkey tested or whether the mutation was an artifact of a reverse transcriptase error in producing cDNA. It is possible that this mutation creates a dominant-negative form of tPit-1. When binding to a responsive element, tPit-1 binds as a dimer. If this dominant mutant binds with wild type Pit-1 on a responsive element, it is possible that the dominant negative mutant can squelch the activation of wild type tPit-1.

To verify this possibility, cotransfections would need to be repeated with wild type tPit-1(leu-176), mutant tPit-1(ser-176) and a combination of both forms together.

In a related study not included in this thesis, preliminary immunohistochemistry data suggests that Pit-1 may not be important for Prl activation in turkeys. Immunofluorescence of turkey pituitary sections with antibodies directed against tPit-1 and turkey prolactin have been performed in collaboration with R. Ramachandran (USDA, Univ. MD). Cells which are prolactin positive do not costain for Pit-1 protein in the nucleus. This suggests that Pit-1 and prolactin are not present in the same cells, and therefore Pit-1 likely does not play a role in the regulation of the prolactin gene in turkeys. In contrast, cells in the caudal lobe of the turkey pituitary, consisting mainly of somatotrophs, are co-stained for both GH in the cytoplasm and Pit-1 in the nucleus. This result indicates that Pit-1 likely plays a role in regulating GH gene expression.

Regulation of prolactin in the turkey must be accomplished by factors other than Pit-1. Although tPit-1 can activate the turkey and rat Prl promoters in cotransfection assays, the immunohistochemistry data clearly shows that tPit-1 and tPrl are not expressed in the same cell. It is possible that a turkey specific repressor of tPit-1 expression exists in lactotrophs while somatotrophs contain an inducer of tPit-1 expression. Since tPit-1 does not activate prolactin expression in the turkey, other factors, specifically VIP, may regulate prolactin synthesis independently of Pit-1.

Future experiments should focus on positively identifying Pit-1 binding sites in the Prl, Pit-1 and GH promoters. DNase footprinting must be performed to identify whether tPit-1 is binding to these promoters. Also, after the downstream Pit-1 binding site is mutated, DNase footprinting can be used to verify that binding is eliminated.

In conclusion, this thesis is a preliminary characterization of turkey pituitary gene regulation. Emphasis was placed on the ability of the transcription factor, Pit-1, to regulate the prolactin promoter, growth hormone promoter and its own promoter through cotransfection experiments with various cell types. Turkey Pit-1 activates the rPrl promoters to levels equivalent of rPit-1. A point mutation in the POU-specific domain of tPit-1 (leu-176 to ser-176) reduces the ability of tPit-1 to activate the rPrl, tPrl and tGH promoters. Finally, immunohistochemistry results not present in this thesis indicate that tPit-1 does not regulate tPrl, but may regulate tGH. Based on this evidence, future experiments should focus on further characterization of the specific role of Pit-1 in the domestic turkey.

VI. References

Agellon, L.B., Davies, S.L., Chen, T.T., and Powers, D.A. (1988). Structure of a fish (rainbow trout) growth hormone gene and its evolutionary implications. Proc. Natl. Acad. Sci. USA. 85: 5136-5140.

Aurora, R., and Herr, W. (1992). Segments of the POU domain influence one another's DNA-binding specificity. Mol. Cell. Biol. 12:455-467.

Bacon, W.L., Long, D.W., and Vasilatos-Younken, R. (1995). Responses to exogenous pulsatile turkey growth hormone by growing 8-week-old female turkeys. Comp. Biochem. Physiol. 111B:471-482.

Barsh, G.S., Seeburg, P.H., and Gelinas, R.E. (1993). The human growth hormone gene family: structure and evolution of the chromosomal locus. Nucleic Acids Res. 11:3993-3958.

Barta, A., Richards, R.I., Baxter, J.D., and Shine, J. (1981). Primary structure and evolution of rat growth hormone gene. Proc. Natl. Acad. Sci. U.S.A. 78:4867-4871.

Bauman, D.E., Eppard, P.J., DeGeeter, M.J., and Lanza, G.M. (1985). Responses of high-producing dairy cows to long-term treatment with pituitary somatotropin and recombinant somatotropin. J. Dairy Sci. 68:1352-1362.

Ber, R., and Daniel, V. (1992). Structure and sequence of the growth hormone-encoding gene from *Tilapia nilotica*. Gene 113:245-250.

Bolton, J.P., Collie, N.L., Kawauchi, H., and Hirano, T. (1987). Osmoregulatory actions of growth hormone in rainbow trout (*Salmo gaidneri*). J. Endocrinol. 112:63-68.

Burke, W.H., Dennison, P.T., Silsby, J.L., and El Halawani, M.E. (1981). Serum prolactin levels of turkey hens in relation to reproductive function. Adv. Physiol. Sci. 33:109.

Burke, W.H., Moore, J.A., Ogez, J.R., and Builder S.E. (1987). The properties of recombinant chicken growth hormone and its effects on growth, body composition, feed efficiency, and other factors in broiler chickens. Endocrinology 127:651-658.

Cao, Z., Barron, E.A., Carrillo, A.J., and Sharp, Z.D. (1987). Reconstitution of cell-type-specific transcription of the rat prolactin gene in vitro. Mol. Cell. Biol. 7:3402-3408.

Campbell, R.G., Steele, N.C., Caperna, T.J., and McMurtry, J.P. (1989). Interaction between sex and exogenous growth hormone administration on the performance, body composition and protein and fat accretion of growing pigs. J. Anim. Sci. 67:177-186.

Chatelain, A., Dupouy, J.P., and Dubois, M.P. (1979). Ontogenesis of cells producing polypeptide hormones (ACTH, MSH, LPH, GH, Prolactin) in the fetal hypophysis of the rat: Influence of the hypothalamus. Cell Tissue Res. 196:409-427.

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

Chen, R., Ingraham, H.A., Treacy, M.N., Albert, V.R., Wilson, L., and Rosenfeld, M.G. (1990). Autoregulation of Pit-1 gene expression mediated by two *cis*-active promoter elements. Nature 346:583-586.

Chiou, C.-S., Chen, H.-T., and Chang, W.-C. (1990). The complete nucleotide sequence of the growth-hormone gene from the common carp (*Cyprinus carpio*). Biochim. Biophys. Acta 1087:91-94

Cogburn, L.A., Liou, S.S., Rand, A.L., and McMurtry, J.P. (1989). Growth, metabolic and endocrine response of broiler cockerels given a daily subcutaneous injection of natural or biosynthetic chicken growth hormone. Amer. Inst. of Nutr. 119:1213-1222.

Collie, N.L., Bolton, J.P., Kawauchi, H., and Hirano, T. (1989). Survival of salmonids in seawater and the time-frame of growth hormone action. Fish Physiol. Biochem. 7:315-321.

Cooke, N.E., Coit, D., Weiner, R.I., Baxter, J.D., and Martial, J.A. (1981). Human prolactin: Structural analysis and evolutionary comparisons. J. Biol. Chem. 256-4007-4016.

Davidson, M.B. (1987). Effect of growth hormone on carbohydrate and lipid metabolism. Endocrinol. Rev. 8:115-131.

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

DeVlaming, V.L. (1979). Actions of prolactin among the vertebrates. In: Barrington EJW (ed) Hormones and Evolution, Academic Press, New York. pp 561-642.

Diamond, S.E., Guiterrez-Hartmann, A. (1996). A 26-amino acid insertion domain defines a functional transcription switch motif in Pit-1β. J. Biol. Chem. 271: 28925-28932.

Du, S.J., Devlin, R.H., and Hew, C.L. (1993). Genomic structure of growth hormone genes in chinook salmon (*Oncorhynchus tshawytscha*): presence of two functional genes, GH-I and GH-II and a male specific pseudogene, GH-Ψ. DNA Cell Biol. 12:739-751.

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

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

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

Elsholtz, H.P., Albert, V.R., Treacy, M.N., and Rosenfeld, M.G. (1990). A two-base change in a POU factor-binding site switches pituitary-specific to lymphoid-specific gene expression. Genes Dev. 4:43-51.

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

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

Frawley, L.S., Boockfor, F.R., and Hoeffler, J.P. (1985). Identification by plaque assays of a pituitary cell type that secretes both growth hormone and prolactin. Endocrinol. 116:734-737.

Frawley, L.S. (1989). Mammosomatotrophs: current status and possible functions. Trends Endocrinol. Metab. 1:31-34.

Frawley, L.S., and Boockfor, F.R., (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.

Hargis, B.M., El Halawani, M.E., and Porter, T.E. (1987). Ovarian regression and incubation behavior induced by ovine prolactin (oPrl) in Nicholas turkey hens. Poult. Sci. 66 (Suppl. 1):111.

Hargis, P.S., Pardue, S.L., Lee, A.M., and Sandel, G.W. (1989). *In ovo* growth hormone alters growth and adipose tissue development of chickens. Growth Dev. Aging 53:93-99.

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

Haugen, B.R., Gordon, D.F., Nelson, A.R., Wood, W.M., and Ridgeway, E.C. (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.

Herr, W., Sturm, R.A., Clerc, R.G., Corcoran, L.M., Baltimore, D., Sharp, P.A., Ingraham, H.A., Rosenfeld, M.G., Finney, M., and Ruvkin, G. (1988). The POU domain: A large conserved region in the mammalian pit-1, oct-1, oct-2 and *C. elegans* unc-86 gene products. Genes Dev. 2:1513-1516.

Herr, W. and Cleary, M.A. (1995). The POU domain: versatility in transcriptional regulation by a flexible two-in-one DNA-binding domain. Genes Dev. 9:1679-693.

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

Hooghe-Peters, E.L., and Hooghe, R. (1995). Growth hormone, prolactin, and IGF-I as lymphohemopoetic cytokines. R.G. Landes/Springer-Verlag, Austin. pp 1-256.

Hong, Y., and Schartl, M. (1993). Sequence of the growth hormone (GH) gene from the silver carp (*Hypophthalmichthys molitrix*) and evolution of GH genes in vertebrates. Biochim. Biophys. Acta. 1174:285-288.

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

Ingraham, H.A., Albert, V.R., Chen, R., Crenshaw, E.B. III, Elsholtz, H.P., He, X., Kapiloff, M.S., Mangalam, H.J., Swanson, L.W., Treacy, M.N., and Rosenfeld, M.G. (1990). A family of POU-domain and Pit-1 tissue specific transcription

factors in pituitary and neuroendocrine development. Annu. Rev. Physiol. 52:773-791.

Jacobson, E.M., Li, P., Leon-del-Rio, A., Rosenfeld, M.G., and Aggarwal, A.K. (1997). Structure of Pit-1 POU domain bound to DNA as a dimer: unexpected arrangement and flexibility. Genes Dev. 11:198-212.

Johansen, B., Johansen, O.C., and Valla, S. (1989). The complete nucleotide sequence of the growth-hormone gene from Atlantic salmon (*Salmo salar*). Gene 77:317-324.

Klemm, J.D., Rould, M.A., Aurora, R., Herr, W., and Pabo, C.O. (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 Moore, D.D. (1992). Functional isoforms of Pit-1 generated by alternative messenger RNA splicing. Mol. Endocrinol. 6:241-247.

Kooijman, R., Hooghe, R., and Hooghe-Peters E.L. (1996). Prolactin, growth hormone and insulin-like growth factor-I in the immune system. Adv. Immunol. 63:377-453.

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

Kurima, K. (1996). Transcriptional regulation of the prolactin gene in turkeys. Ph.D. dissertation, Virginia Tech.

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

LeBowitz, J.H., Kobayashi, T., Staudt, L., Baltimore, D., and Sharp, PA. (1988). Octamer-binding proteins from B or HeLa cells stimulate transcription of the immunoglobulin heavy-chain promoter in vitro. Genes Dev. 2:1227-1237.

Lew, A.M., and Elsholtz, H.P. (1991). Cloning of the human cDNA for transcription factor Pit-1. Nucleic Acids Res. 19:6329.

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

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

Male, R., Nerland, A.H., Lorens, J.B., Telle, W., Lossius, I., and Totland, G.K. (1992). The complete nucleotide sequence of the Atlantic salmon growth hormone I gene. Biochim. Biophys. Acta 1130:345-348.

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

Maruyama, K., Kanemaki, N., and Proudman, J.A. (1996). Effects of growth hormone injection to embryos on growth and myosin heavy chain isoforms in growing turkeys (Meleagris gallopavo). Comp. Biochem. Physiol. 113:315-321.

McCormick, A., Brady, H., Theill, L.E., and Karin, M. (1990). Regulation of the pituitary-specific homeobox gene GHF-1 by cell-autonomous and environmental cues. Nature 345:829-832.

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

Moellers, R.F., and Cogburn, L.A. (1995). Chronic infusion of chicken growth hormone increases body fat content of young broiler chickens. Comp. Biochem. Physiol. 110A:47-56.

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

Muller, M., Affolter, M., Leupin, W., Otting, G., Wuthrich, K., and Gehring, W.J. (1988). Isolation and sequence specific DNA binding of the *Antennapedia* homeodomain. EMBO J. 7:4299-4304.

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

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

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

Palmiter, R.D., Norstedt, G., Gelinas, R.E., Hammer, R.E., and Brinster, R.L., (1983). Metallothionein-human GH fusion genes stimulate growth of mice. Science 222:809-814.

Proudman, J.A., and Opel, H. (1988). Stimulation of prolactin secretion from turkey anterior pituitary cells in culture. Proc. Soc. Exp. Biol. Med. 187:445-454.

Proudman, J.A., McGuinnes, M.C., Krishnan, K.A., and Cogburn, L.A. (1994). Endocrine and metabolic responses of intact and hypophysectomized turkey poults given a daily injection of chicken growth hormone. Comp. Biochem. Physiol. 190C:47-56.

Radecki, S.V., McCann-Levorse, L., Agarwal, S.K., Burnside, J., Proudman, J.A., and Scanes, C.G. (1997). Chronic administration of growth hormone (GH) to adult chickens exerts marked effects on circulating concentrations of insulin-like growth factor-I (IGF-I), IGF binding proteins, hepatic GH regulated gene I and hepatic GH receptor mRNA. Endocrine 6:117-124.

Radovick, S., Nations, M., Du, Y, Berg, L.A., Weintraub, B.D., and Wondisford, F.E. (1992). A mutation in the POU-homeodomain of Pit-1 responsible for combined pituitary hormone deficiency. Science 257:1118-1121.

Sakamoto, T., Iwata, M., and Hirano, T. (1991). Kinetic studies of growth hormone and prolactin during adaptation of coho salmon, *Oncorhynchus isutch*, to different salinities. Gen. Comp. Endocrinol. 82:184-191.

Scheidereit, C., Heguay, A., and Roeder, R.G. (1987). Identification and purification of a human lymphoid-specific octamer binding protein (OTF-1) that activates transcription of an immunoglobulin promoter in vitro. Cell 51:783-793.

Scholer, H.R. (1991). Octamania: The POU factors in murine development. Trends Genet. 7:323-329.

Seeburg, P.H., Shine, J., Martial, J.A., Baxter, J.D., and Goodman, H.M. (1977). Nucleotide sequence and amplification in bacteria of structural gene for rat growth hormone. Nature 270:486-494.

Steinfelder, H.J., Hauser, P., Nakayama, Y., Radovick, S., McClaskey, J.H., Taylor, T., Weintraub, B.D., and Wondisford, F.E. (1991). Thyrotropin-releasing hormone regulation of human TSHβ expression: Role of a pituitary-specific transcription factor (Pit-1/GHF-1) and potential interaction with a thyroid hormone-inhibitory element. Proc. Natl. Acad. Sci. U.S.A. 88:3130-3134.

Suzuki, R., Kishida, M., Ogesawara, T., Haregawa, S., and Hirano, T. (1987). Prolactin

and growth hormone secretion during long-term incubation of the pituitary *pars distalis* of mature chum salmon, *Oncorhynchus keta*. Gen. Comp. Endocrinol. 68:76-81.

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

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

Tatsumi, K., Notomi, T., Amino, N., and Miyai, K. (1992). Nucleotide sequence of the complementary DNA for human Pit-1/GHF1. Biochim. Biophys. Acta 1129:231-234.

Theill, L.E., Castrillo, J., Wu, D., and Karin, M. (1989). Dissection of functional domains of the pituitary-specific transcription factor GHF-1. Nature 342:945-948.

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

Theill, L.E., and Karin, M. (1993). Transcriptional control of GH expression and anterior pituitary development. Endocrinol. Rev. 14:670-689.

Verrijzer, C.P., Alkema, M.J., Van Weperen, W.W., Van Leeuwen, H.C., Strating, M.J.J, and Van der Vliet, P.C. (1992). The DNA binding specificity of the bipartite POU domain and its subdomains. EMBO J. 11:4993-5003.

Vila, V., Jimenez, O., Guell, A., Vallejo, D., De La Hoya, M., Burgos, A., Etxabe, J., Martinez-Barbera, J.P., and Castrillo, J.L. (1995). The pituitary transcription factor GHF-1/Pit-1: an evolutionary overview. Netherlands J. Zool. 45:229-234.

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

Watanabe, Y.G., and Daikoku, S. (1979). An immunohistochemical study on the cytogenesis of adenohypophysial cells in fetal rats. Dev. Biol. 68:557-660.

Wong, E.A., Ferrin, N.H., Silsby, J.L., and El Halawani, M.E.. (1991). Cloning of a turkey prolactin cDNA: Expression of prolactin mRNA throughout the

reproductive cycle of the domestic turkey (*Meleagris gallopavo*). Gen. Comp. Endocrinol. 83:18-26.

Wong, E.A., Silsby, J.L., and El Halawani, M.E. (1992). Complementary DNA cloning and expression of Pit-1/GHF-1 from the domestic turkey. DNA Cell Biol. 11:651-658.

Yada, T., Takahashi, K., and Hirano, T. (1991). Seasonal changes in seawater adaptability and plasma levels of prolactin and growth hormone in landlocked sockeye salmon (*Oncorhynchus nerka*) and amago salmon (*O. rhodurus*). Gen. Comp. Endocrinol. 82:33-44.

Zhu, Z., He, L., and Chen, T.T. (1992). Primary-structural and evolutionary analyses of the growth-hormone gene form grass carp (*Ctenopharyngodon idellus*). Euro. J. Biochem. 207:643-648.

Figure 1-1: Circulating plasma prolactin levels (ng/ml) in the domestic turkey during the various stages of the reproductive cycle: quiescent (Q), photostimulated (PS), laying (L), incubating (I) and photorefractory (PR). Birds are photostimulated by shifting from 8 hours of light to 16 hours of light per day. Northern blot results show pituitary Prl mRNA abundance at different reproductive stages.

Figure 1-1:

Profile of Circulating Prolactin in the Turkey Hen

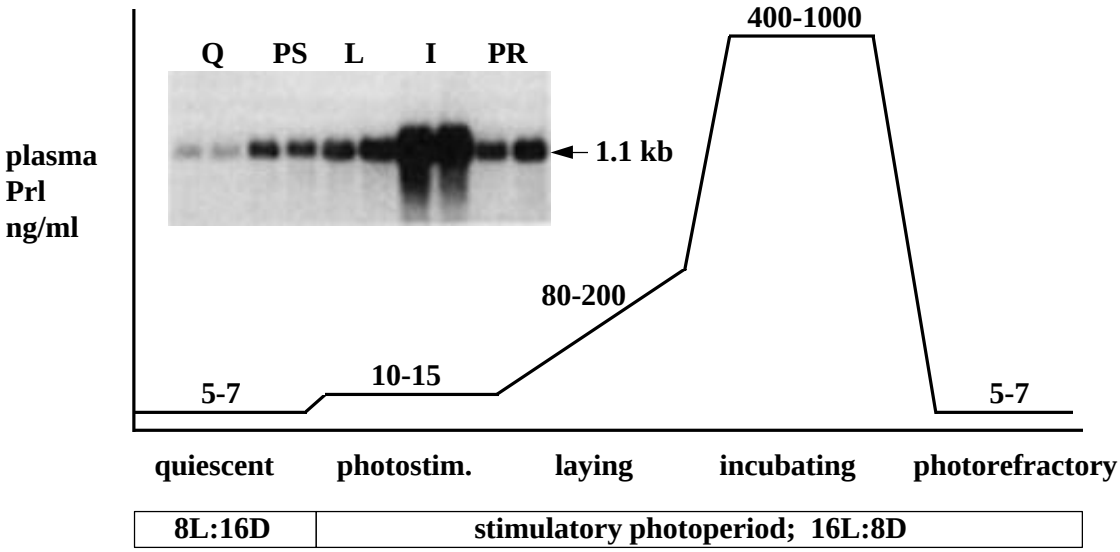


Figure 1-2: The Rat Pit-1 gene structure and various isoforms. Exons are denoted by boxes and intron by lines. The transcription start site is indicated by a downward arrow. Size of introns are shown in kilobases. Exons and introns are not drawn to scale.

Figure 1-2:

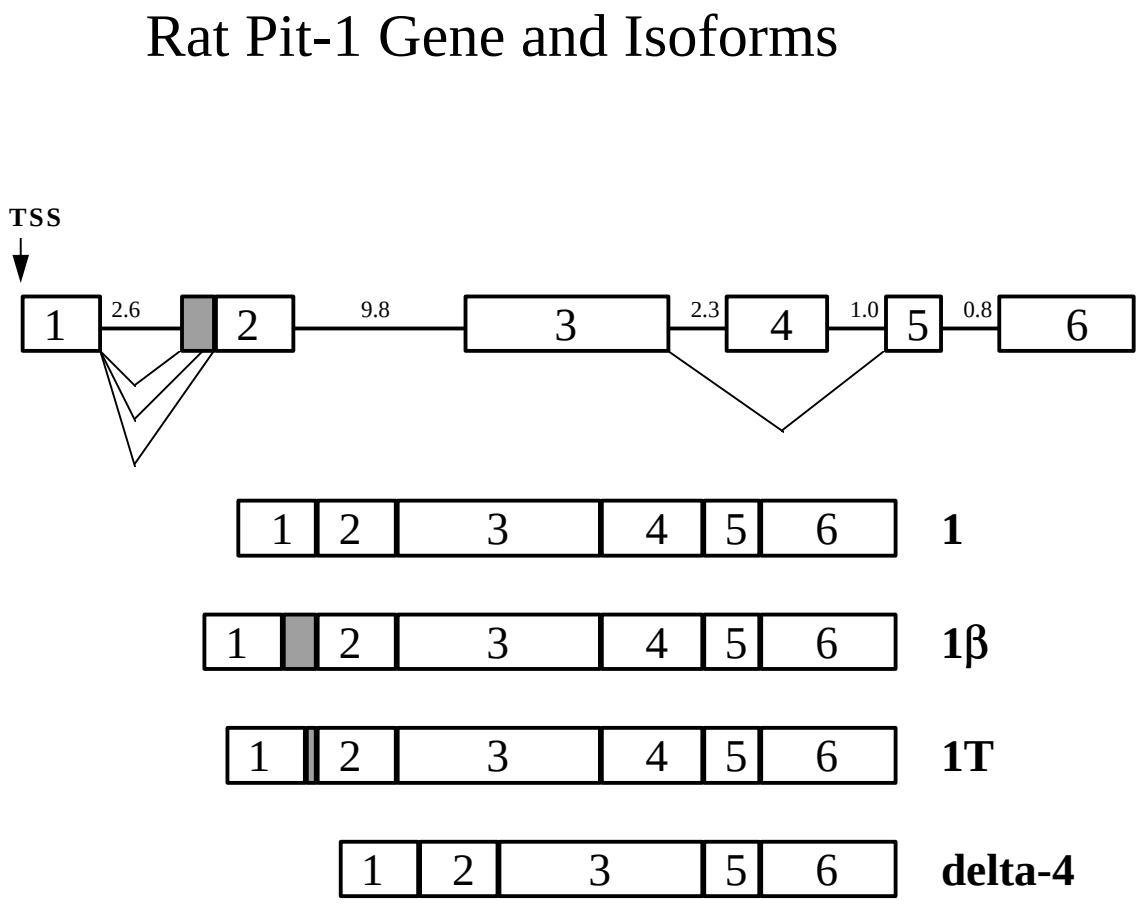

Rat Pit-1 Gene and Isoforms
TSS
1
2.6
2
9.8
3
2.3
4
1.0
5
0.8
6
1 2 3 4 5 6
1
1 2 3 4 5 6
1β
1 2 3 4 5 6
1T
1 2 3 5 6
delta-4

Figure 1-3: Comparison of the nucleotide sequences of the 5'-flanking regions for rat (r) and turkey (t) Pit-1 exons 1 and 2. The nucleotide sequences for exon 1 were aligned using the Martinez-Needleman-Wunsch DNA alignment program in Lasergene software (DNASTAR, Madison, WI). Putative Pit-1 binding sites are underlined and a TATA-box is double underlined. The proposed transcription start sites for rat (Chen et al., 1990; McCormick et al., 1990) and turkey are indicated by arrows. Translation start codons are marked in bold type.

Figure 1-3:

Comparison of the 5' flanking region of exon 1 for rat and turkey Pit-1

```
r   GGGAAAAAAACTATTAACATGTATAAATGGATTTCCTCAGA-GTATAAATAC-TA
t   GGGAAAAGAAC-ATTAAAATGGATAAAGGGATTTTCT-AGTGGTATAAATACCT-
              ↓  ↓↓
r   GC-CTCGACCCTCC-TGGTGCCTCAGAGCCG-CC--CTG---ATGTATATA----
t   GCACACGACC-TCCCTGTT-C-TCTG-GA-GTCCTTCTGTATATGTATATACAAG
                 ↑↑
r   TGCAA-TAGGGGCCGTGAATCG-GCCCTTTGATACAGTAATATAATAAAAGC---
t   TGCAAATA----CTG-GAATAGAG---TTGGAG-CTGCCAT-TAATC---GCTCA

r   GGAC-TGGCAAGCGGTGC-GTCTTAGTTCTCTACTCTCTTGTGGGA**ATG**AGTTGC
t   GGACCTG--A-----T-CAGT-T-AGAAAAC-A-------G-GG-AAGG-GTGGC

r   -CAA--CCTTTCACCTC---G-G--C-TG
t   TCTTTGCCTTTCACCTGTTTGAGGGC**ATG**
```

Figure 1-4: Turkey Pit-1 gene and isoforms. Exons are indicated as boxes and introns as lines. Transcription start sites (TSS) are indicated by downward arrows. Exon 2a, indicated in a cross-hatched pattern, is not present in mammalian species. All tPit-1 isoforms contain exon 2a and are designated with an asterisk (*) to distinguish them from mammalian isoforms. Location of POU-specific and POU-homeodomains are indicated. Exons and introns are not drawn to scale.

Figure 1-4:

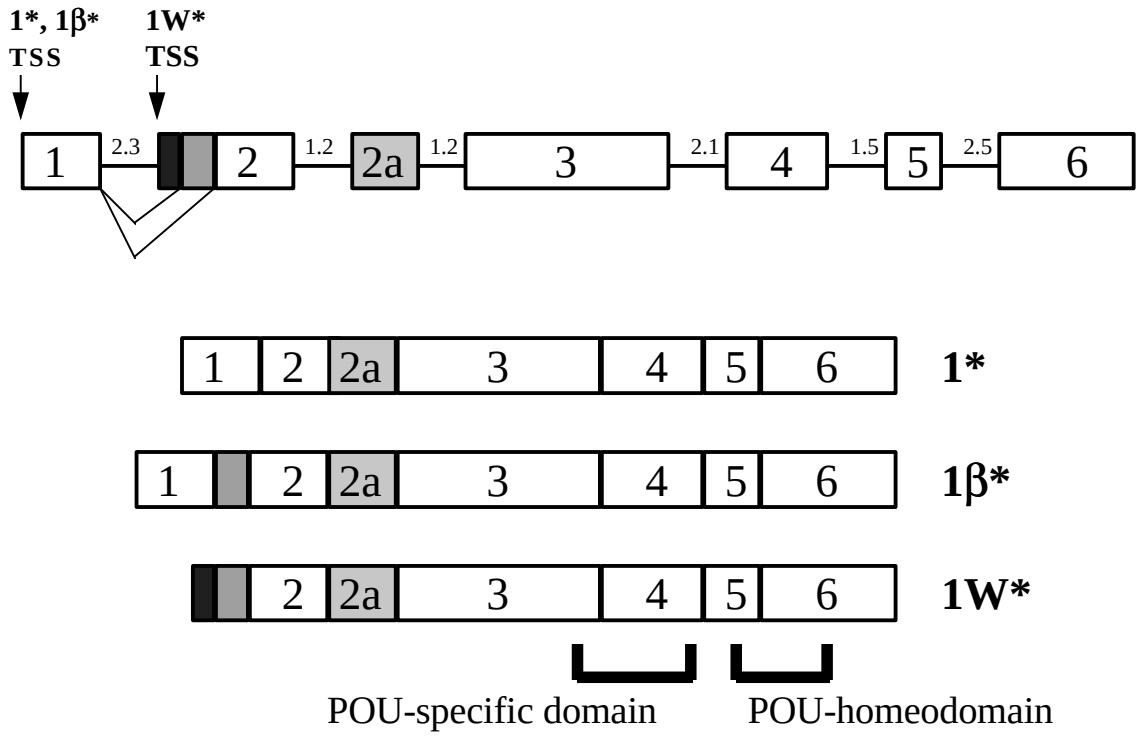

Turkey Pit-1 Gene and Isoforms
1*, 1β*
TSS
1W*
TSS
1
2.3
2
1.2
2a
1.2
3
2.1
4
1.5
5
2.5
6
1
2
2a
3
4
5
6
1*
1
2
2a
3
4
5
6
1β*
2
2a
3
4
5
6
1W*
POU-specific domain
POU-homeodomain

Figure 1-5: Comparison of the amino termini of rat, turkey and salmon Pit-1 isoforms.

Figure 1-5:

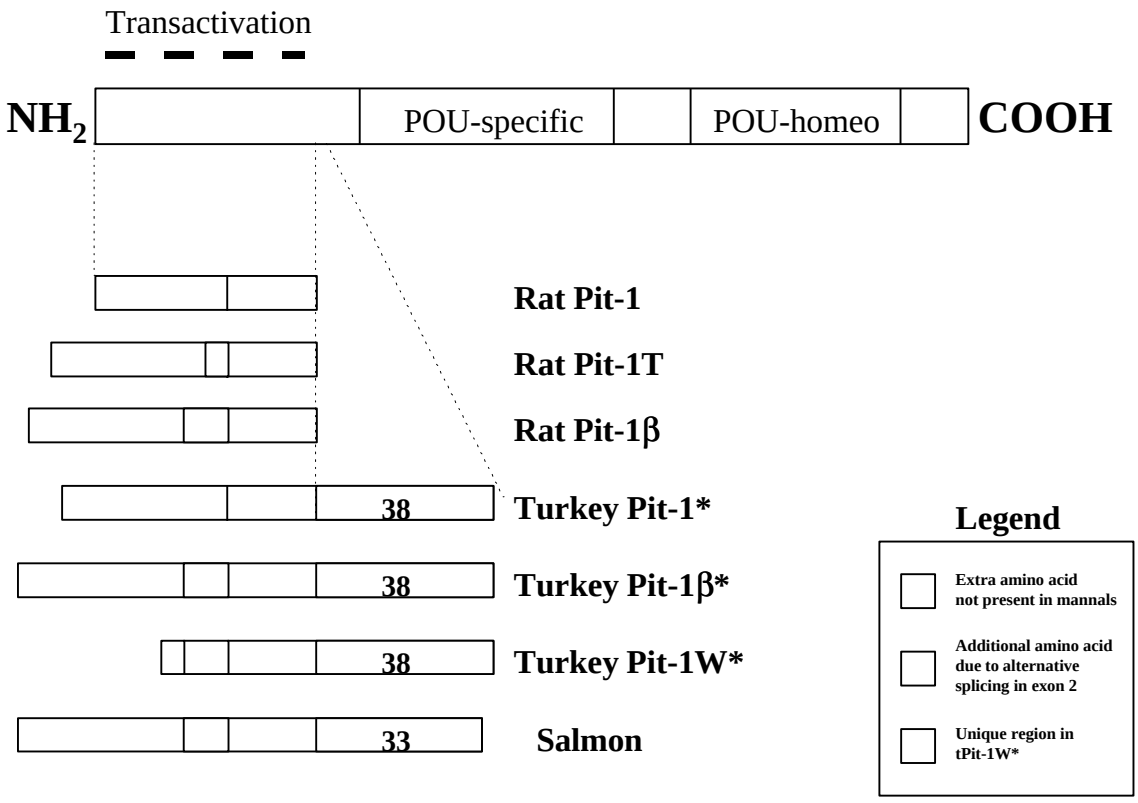

Transactivation
NH2
POU-specific
POU-homeo
COOH
Rat Pit-1
Rat Pit-1T
Rat Pit-1β
38
Turkey Pit-1*
38
Turkey Pit-1β*
38
Turkey Pit-1W*
33
Salmon
Legend
Extra amino acid not present in mannals
Additional amino acid due to alternative splicing in exon 2
Unique region in tPit-1W*

Figure 3-1: Strategy for producing tPit-1 Promoter Luciferase Reporter Vectors with a Mutation in the Downstream Pit-1 Binding Site. PCR reactions using primers T7 and AS41 or primers S43 and Reverse were performed. The two PCR products were mixed, denatured and annealed. PCR was performed again, using the flanking primers T7 and Reverse. The same strategy was used to mutate the upstream Pit-1 binding site with primers AS40 and T7 and S42 and Reverse.

Figure 3-1:

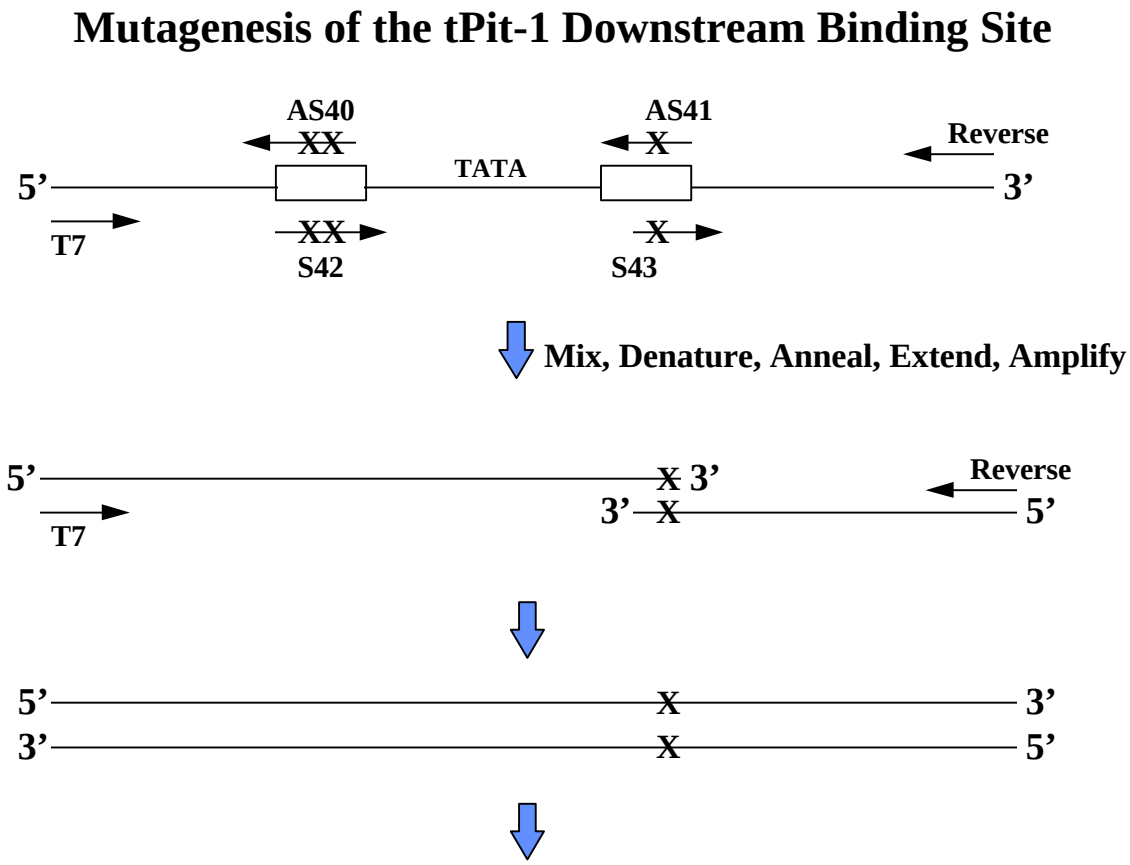

Mutagenesis of the tPit-1 Downstream Binding Site
AS40
XX
AS41
X
Reverse
5'
TATA
3'
T7
XX
S42
X
S43
Mix, Denature, Anneal, Extend, Amplify
5'
X 3'
Reverse
3'
X
5'
T7
5'
X
3'
3'
X
5'
Clone into pGL3 Luciferase Reporter Vector

Figure 4-1: Western blot of turkey liver and pituitary extracts. 240 μg pituitary and 120 μg liver extract were loaded per lane. A polyclonal rabbit anti rat Pit-1 antiserum was used as the primary antibody and a horseradish peroxidase conjugated goat anti rabbit antiserum was used as the secondary antibody.

Figure 4-1: Western Blot of Turkey Liver and Pituitary Extracts

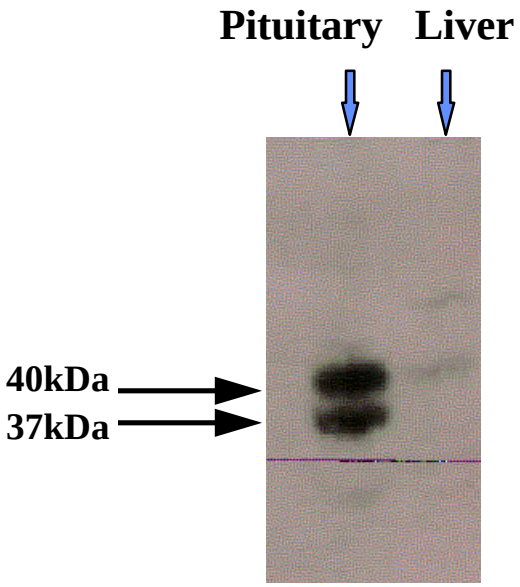


Figure 4-2: Activation of tPrl, tGH and rPrl promoters by tPit-1* and rPit in mouse L cells. Results are expressed as fold stimulation over a cDNA-less expression vector. Data represents averaged results from 2 experiments performed in duplicate (n=4).

Figure 4-2:

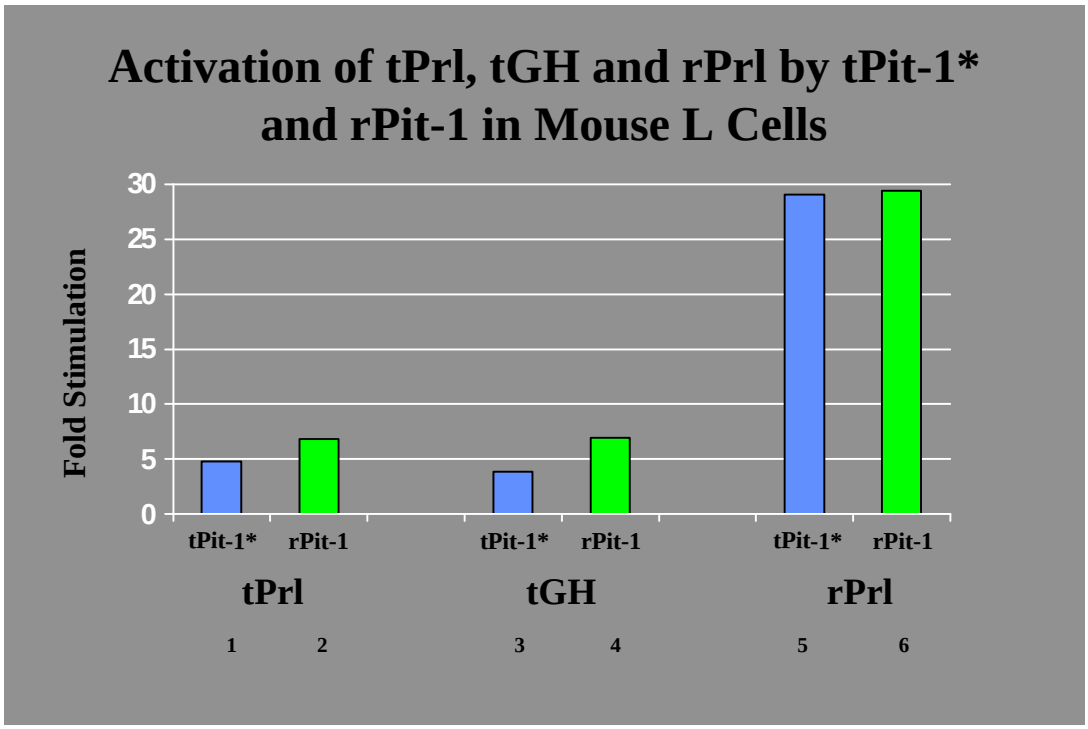

Activation of tPrl, tGH and rPrl by tPit-1* and rPit-1 in Mouse L Cells
Fold Stimulation
30
25
20
15
10
5
0
tPit-1*
rPit-1
tPit-1*
rPit-1
tPit-1*
rPit-1
tPrl
tGH
rPrl
1
2
3
4
5
6

Figure 4-3: Activation of tPrl, tGH and rPrl promoters by tPit-1* and rPit-1 in primary turkey pituitary cells. Results are expressed as fold stimulation over a cDNA-less expression vector. Data represent results from one experiment performed in duplicate (n=2).

Figure 4-3:

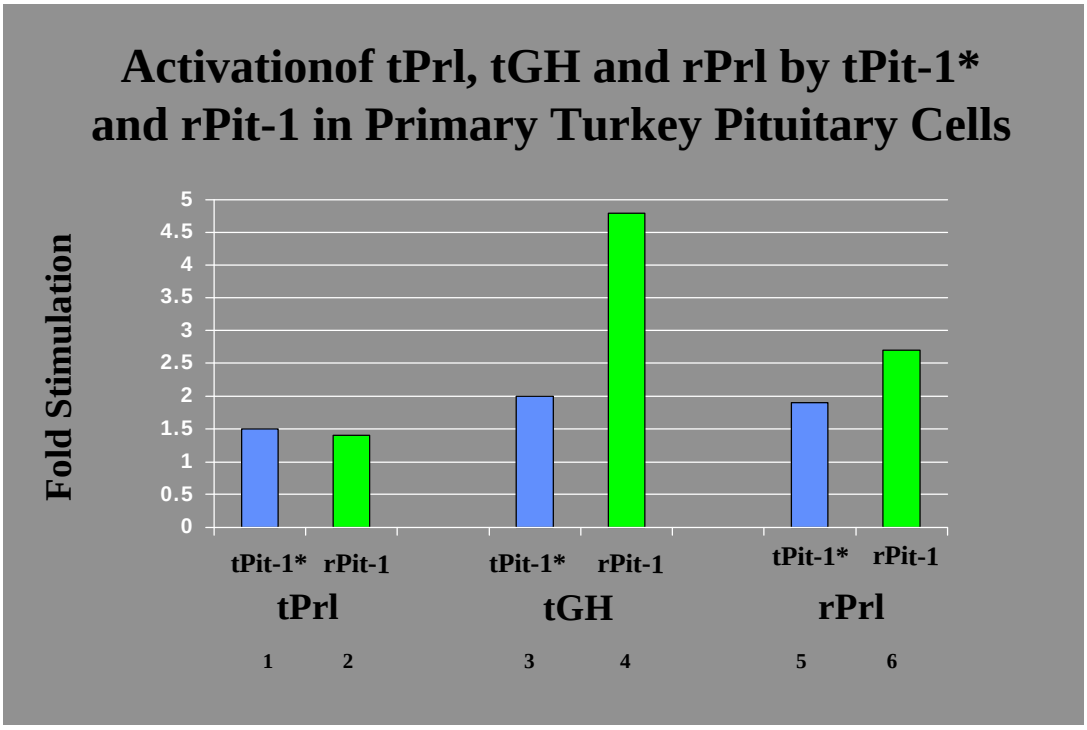

Activationof tPrl, tGH and rPrl by tPit-1* and rPit-1 in Primary Turkey Pituitary Cells
Fold Stimulation
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
tPit-1*
rPit-1
tPrl
tPit-1*
rPit-1
tGH
tPit-1*
rPit-1
rPrl
1
2
3
4
5
6

Figure 4-4: Activation of tPrl, tGH and rPrl promoters by tPit-1*(leu-176), tPit-1*(ser-176) and rPit-1 in mouse L cells. Results are expressed as fold stimulation over a cDNA-less expression vector. Data represent results from one experiment performed in duplicate (n=2).

Figure 4-4:

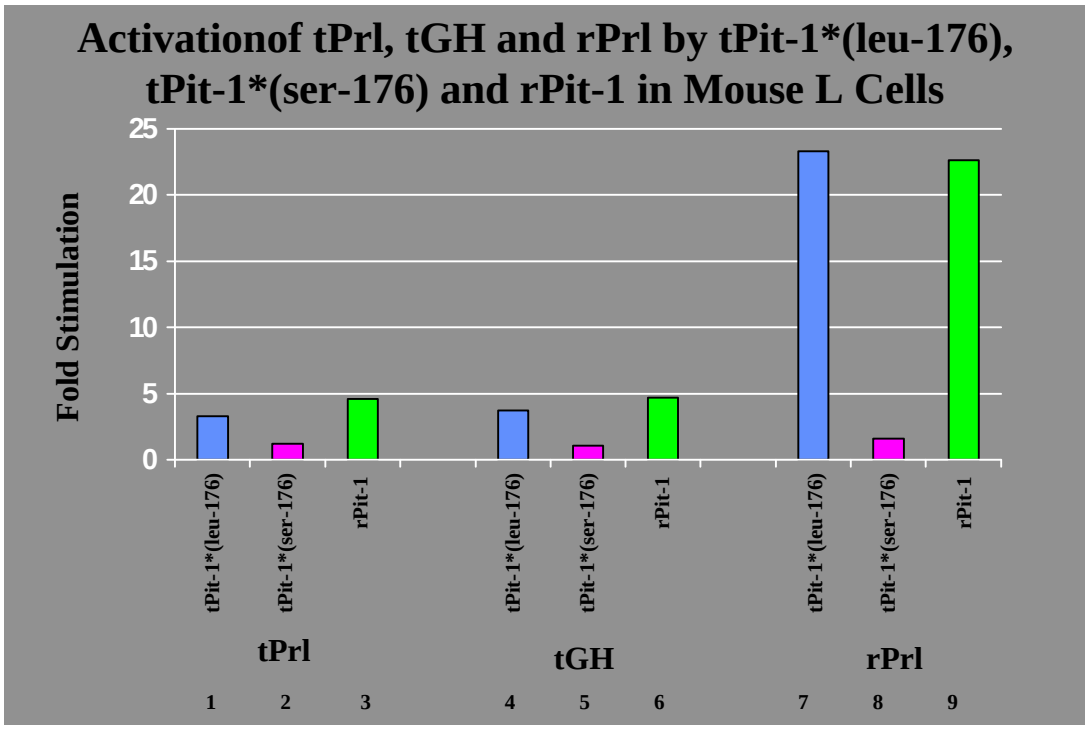

Activationof tPrl, tGH and rPrl by tPit-1*(leu-176), tPit-1*(ser-176) and rPit-1 in Mouse L Cells
Fold Stimulation
25
20
15
10
5
0
tPit-1*(leu-176)
tPit-1*(ser-176)
rPit-1
tPit-1*(leu-176)
tPit-1*(ser-176)
rPit-1
tPit-1*(leu-176)
tPit-1*(ser-176)
rPit-1
tPrl
tGH
rPrl
1
2
3
4
5
6
7
8
9

Figure 4-5: Autoactivation of the tPit-1, tPit-1 with a mutated binding site, and rPrl promoters by tPit-1*(leu-176) and rPit-1 in mouse L cells. Results are reported as fold stimulations over a cDNA-less expression vector. Data represent results from one experiment performed in duplicate (n=2).

Figure 4-5:

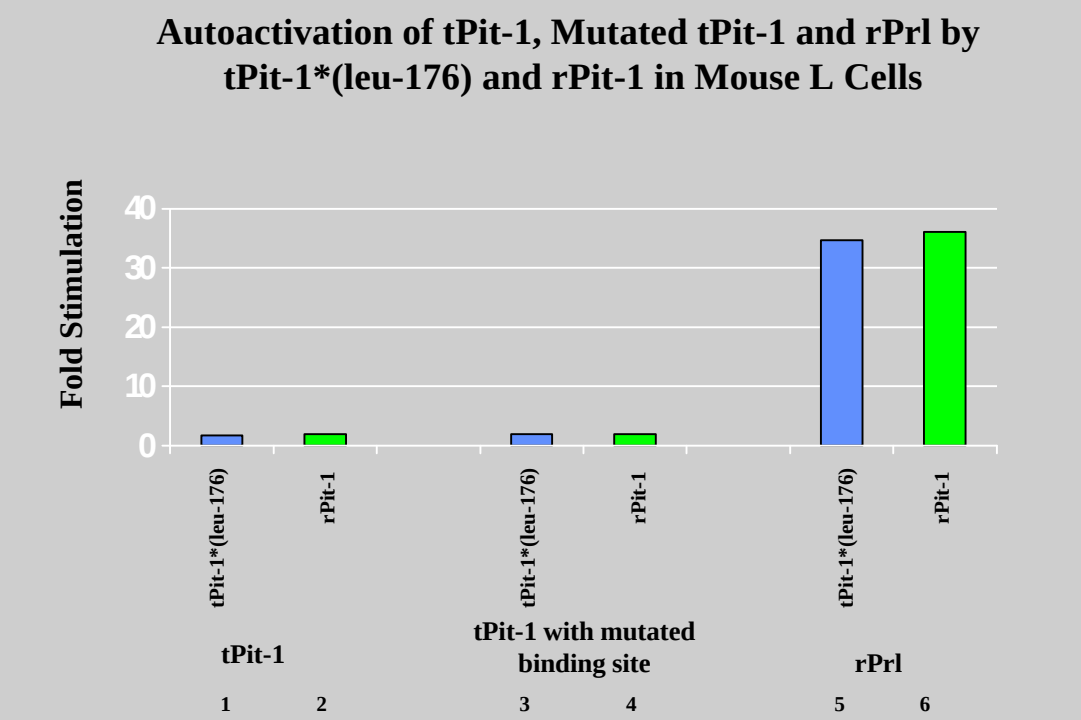
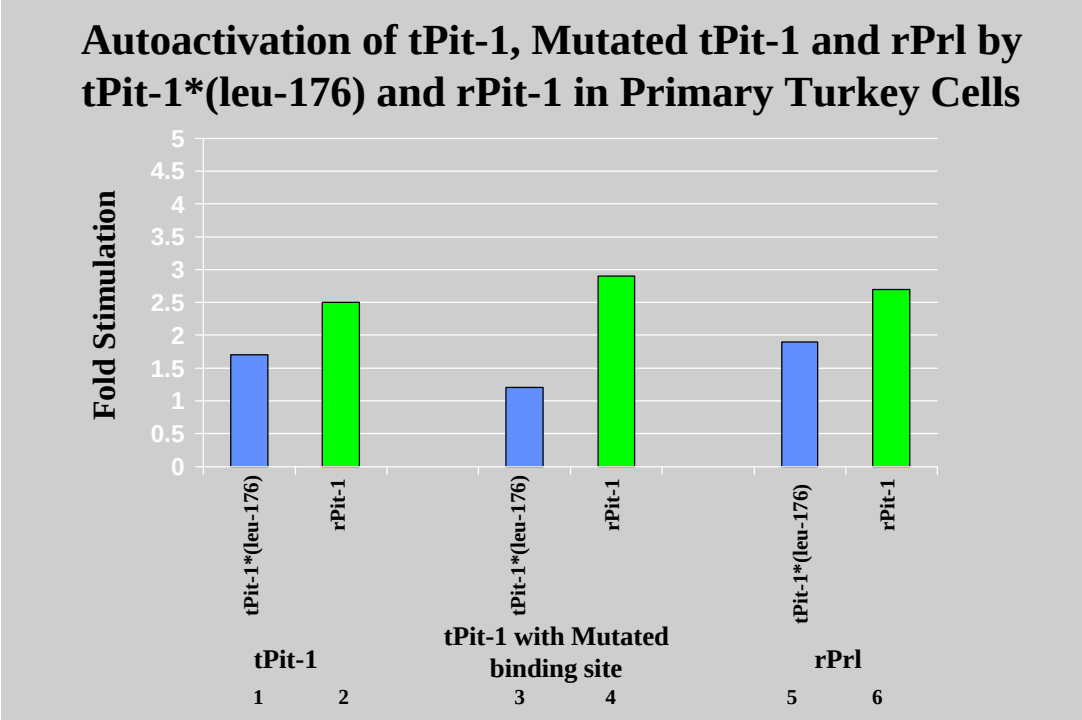

Autoactivation of tPit-1, Mutated tPit-1 and rPrl by tPit-1*(leu-176) and rPit-1 in Mouse L Cells
Fold Stimulation
40
30
20
10
0
tPit-1*(leu-176)
rPit-1
tPit-1*(leu-176)
rPit-1
tPit-1*(leu-176)
rPit-1
tPit-1
tPit-1 with mutated binding site
rPrl
1
2
3
4
5
6

Figure 4-6: Autoactivation of tPit-1, tPit-1 with a mutated binding site, and rPrl promoters by tPit-1*(leu-176) and rPit-1 in primary turkey pituitary cells. Results are reported as fold stimulation over a cDNA-less expression vector. Data represent results of one experiment performed in duplicate (n=2).

Figure 4-6:


Autoactivation of tPit-1, Mutated tPit-1 and rPrl by tPit-1*(leu-176) and rPit-1 in Primary Turkey Cells
Fold Stimulation
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
tPit-1*(leu-176)
rPit-1
tPit-1*(leu-176)
rPit-1
tPit-1*(leu-176)
rPit-1
tPit-1
tPit-1 with Mutated binding site
rPrl
1
2
3
4
5
6

VITA

Kristy L. Weatherly, daughter of Karen and Allan Blair, was born January 15, 1973, in Norfolk, VA. She graduated from Virginia Tech with a Bachelor of Science degree in Animal Science in May, 1995. Under the guidance of Dr. Eric A. Wong, she began work towards a Master of Science degree at Virginia Tech in the Department of Animal and Poultry Sciences in August, 1995 and completed in April, 1998.