The Mapping of Transcription Factor Binding Sites in the Turkey Prolactin Gene

by

Lisa C. Gazzillo

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APPROVED_____
Dr. Eric A. Wong, Chairman

_____ _____
Dr. C.L. Rutherford Dr. D.M. Denbow

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Abstract

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The cessation of egg-laying during the incubation period of the turkey hen is a source of major economic loss to the turkey industry. In August of 2000 there were approximately 2.7 million turkey breeder hens in the United States. Since the value of one fertile turkey egg is $0.62, the loss of only one egg per hen per year would cost the industry $1.7 million. A number of management procedures have been implemented to control egg production and prevent incubation. However, these methods are labor intensive.

The anterior pituitary hormone prolactin (PRL) is involved in the onset of incubation in the turkey hen. Levels of circulating PRL and PRL mRNA are 10X greater in photostimulated hens than in photorefractory hens, 20X greater in laying hens, and 100X greater in incubating hens. It would be useful to determine the molecular mechansims controlling regulation of the turkey (t) PRL gene. This information could be used to modulate the release of PRL and thereby prevent the induction of the incubation period in turkey hens.

Approximately 2 kilobases (kb) of the tPRL 5'-flanking region were examined by the electrophoretic mobility shift assay (EMSA) using nuclear extracts from turkey

pituitaries and liver. Within this 2 kb fragment, only three regions of the tPRL gene were identified that participate in tissue- and sequence-specific DNA-protein interactions with nuclear extracts from turkey pituitaries. These are the regions from nucleotides (nt) -41 to -73, -105 to -137, and -175 to –199, named tprl-1, tprl-2 and tprl-3, respectively. Three shifted bands were observed using tprl-1 and tprl-2 while two shifted bands were seen using tprl-3.

Competition EMSAs done on these three regions showed that in the presence of unlabeled, excess, specific competitor DNA, the proteins bound to competitor DNA and no shifted bands were observed. If the competitor was a nonspecific DNA sequence, then there was no effect on the shifted bands. When using labeled tprl-2 and unlabeled tprl-1 as competitor DNA, no shifted bands were observed. However, when using labeled tprl-1 and unlabeled tprl-2 as competitor DNA, only one of three shifted bands was eliminated. These data indicate that tprl-1 and tprl-2 bind both common and specific pituitary nuclear proteins and have different affinities for pituitary nuclear proteins. A supershift EMSA involving the addition of rabbit-anti-rat Pit-1 indicated that tPit-1 is a common pituitary nuclear protein that is bound to tprl-1 and tprl-2. However, this interaction may not occur in the turkey in vivo. The mapping of transcription factor binding sites in the tPRL 5'-flanking region is the first step toward the identification and isolation of factors that bind to and regulate transcription of the PRL gene.

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I. LITERATURE REVIEW

Development of the Anterior Pituitary

In mammals, the five phenotypically distinct cell types of the anterior pituitary, somatotrophs, lactotrophs, thyrotrophs, corticotrophs and gonadotrophs, each secrete one or more hormones unique to that cell type. Somatotrophs secrete growth hormone (GH), lactotrophs secrete prolactin (PRL), thyrotrophs secrete thyroid-stimulating hormone-ß-subunit (TSH- ß), corticotrophs secrete proopiomelanocortin (POMC) and gonadotrophs secrete leuteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Chatelain et al., 1979; Wantabe and Daikoku, 1979; Cooke et al., 1981; Hoeffler et al., 1985; Ingraham et al., 1988; Mangalam et al., 1989). In chickens, immunofluorescence studies show that FSH and LH reside in distinct gonadotroph cells (Proudman et al., 1998).

PRL-producing lactotrophs and GH-producing somatotrophs are the last two cell types to differentiate during anterior pituitary development (Mangalam et al., 1989). Of all the hormones produced by the anterior pituitary gland, GH and PRL are the most closely related from an evolutionary perspective (Chatelain et al., 1979; Wantabe and Daikoku, 1979; Hoeffler et al., 1985). Lactotrophs are the most abundant cell type of the anterior pituitary and use the most energy alloted to the gland. This may be because PRL, unlike other anterior pituitary hormones, does not have just one function but can regulate a diverse group of cellular processes (Ben-Jonathan et al., 1989). In mammals, cell type is determined by tissue-specific factors that control the activation of genes during development (Walker et al., 1983; Staudt et al., 1986; Courtois et al., 1987; Costa

et al., 1988; Ingraham et al., 1988; Singh et al., 1988). The presence of five cell types that each secrete a different hormone make the anterior pituitary gland an excellent model system in which to study cell differentiation (Ingraham et al., 1988). This thesis will focus on PRL, the hormone secreted by the lactotroph cells of the anterior pituitary.

The Anterior Pituitary Hormone PRL

The structure and organization of PRL is highly conserved among vertebrates. In most mammals, fish and birds PRL is a five exon gene with similar exon/intron boundaries (Elsholtz et al., 1992). The cDNA encoding tPRL has 90% nt identity to chicken (c) PRL and 54-78% identity to PRL of various mammals (Fig. I-1) (Wong et al., 1991). Avian and mammalian PRLs are highly diversified from fish PRL, sharing only 30-35% sequence homology (Chang et al., 1992).

All PRLs examined to date are 197 to 199 amino acids long with the exception of fish, whose PRLs are shorter at the amino terminus by 12 amino acid residues (Rentier-Delrue et al., 1989; Bole-Feysot et al., 1998). Many PRLs undergo post-translational modifications such as glycosylation, phosphorylation, and proteolytic cleavage (Walker, 1994; Sinha, 1995). Two isoforms of tPRL, with molecular weights of 24 and 27 kDa, are thought to correspond to the glycosylated and nonglycosylated forms of the protein. Studies show that approximately 70% of PRL is glycosylated in incubating turkey hens, 60% is glycosylated in laying hens, and 38% is glycosylated in out-of-production birds. Since the percentage of PRL glycosylated varies with the turkey's reproductive status, it is hypothesized that this particular post-translational modification may be involved in the induction of incubation behavior (Bedecarrats et al., 1999a, 1999b, 1999c). Analysis of

PRL secondary structure predicts that it consists of over 50% α-helices, with the rest of the protein folding into unorganized loops (Bole-Feysot et al., 1998).

PRL, GH and placental lactogen (PL) make up the GH family of proteins, which are related by function and structure (Niall et al., 1971; Miller and Eberhardt, 1983; Bole-Feysot et al., 1998). PRL is present in the pituitary gland of most vertebrates and has a diverse group of functions in a wide variety of species. The original 85 functions of the PRL peptide hormone have grown to over 300 (Bole-Feysot et al., 1998). In mammals, PRL plays an important role in lactogenesis, growth and regulation of the immune system, nurturing of the young, and metabolism (Shiu and Friesen, 1980; Ben-Jonathan et al., 1989; Gala et al., 1990). In fish, PRL has been implicated in osmoregulation (Clarke and Bern, 1980; Nicoll et al., 1981; Hirano et al., 1986). In birds, PRL is involved in the onset of the approximately 28 day incubation period (El Halawani et al., 1988; Wong et al., 1991; Tong et al., 1997). Recently, multiple PRL receptor isoforms have been cloned and sequenced in mammalian and avian species (Boutin et al., 1988; Tanaka et al., 1992; Chen and Horseman, 1994; Ohkubo et al., 1998; Pitts et al., 1999). It is hypothesized that the multiple PRL receptor isoforms may also have a role in regulating PRL's varied actions.

Lactotrophs of the pituitary gland are not the only cells that produce the PRL hormone. PRL has also been found in the brain, thymus, spleen, lymphocytes, epithelial cells and tumors, and other mammalian cells and tissues (Ben-Jonathan et al., 1996; Nagy and Berczi, 1991; Bole-Feysot et al., 1998). Recently, PRL neurons were localized to hypothalmic and extrahypothalmic regions of the turkey hen. The presence of PRL in

hypothalmic nuclei further implicate PRL in food intake and parenting and reproductive activities (Ramesh et al., 2000).

Neuroendocrine Regulation of tPRL

The removal of the pituitary of a rat from the influence of the hypothalamus by pituitary transplant or by placement of a lesion in the hypothalamus results in an increase in PRL levels (Chen et al., 1970). In mammals, the hypothalamus has been shown to have an inhibitory effect on PRL secretion. Dopamine (DA) released from the hypothalamus binds to D2 DA receptors on lactotrophs in the anterior pituitary and exerts an inhibitory effect (MacLeod et al., 1970).

In birds, removal of the anterior pituitary from the influence of the hypothalamus results in the complete inhibition of PRL release (Chadwick et al., 1978). The principal avian PRL releasing factor, vasoactive intestinal peptide (VIP), originates from a neuronal complex of the hypothalamus. Therefore, removal of the anterior pituitary means VIP is no longer present to stimulate PRL release from lactotrophs of the anterior pituitary (Chaishea et al., 1997). In avian species, dopamine has a dual role and can either stimulate or inhibit the release of PRL. In birds, a D1 DA receptor is present in the hypothalamus and a D2 DA receptor is present in the anterior pituitary. When dopamine binds to D1 DA receptors VIP is released and PRL is secreted. However, when DA binds to D2 DA receptors in the anterior pituitary the release of PRL is inhibited (Youngren et al., 1996). Administration of a high dose of DA into the third ventricle of the brain decreases PRL levels, while a low dose increases the secretion of PRL. This data suggests that low doses of DA bind to the D1 DA receptor and increase PRL release,

while high doses of DA can “leak” into the pituitary and bind to inhibitory D2 DA receptors (Youngren et al., 1996).

VIP acts as a PRL releasing factor in the turkey both in vitro and in vivo (Opel and Proudman, 1988; Schnell et al., 1999). Turkey pituitary cells superfused with VIP showed a dose-related increase in PRL secretion. The lowest dose of VIP tested resulted in a 12-fold increase in PRL levels of cultured turkey pituitary cells (Opel and Proudman, 1988). Both the level and half life of PRL mRNA increased following treatment with VIP, consistent with the hypothesis that VIP acts at the transcriptional and post-transcriptional level to modulate the gene (Tong et al., 1998). Immunofluorescence studies have localized VIP to the hypothalamus of the Japanese quail (Mikami et al., 1984), chicken (Macnamee et al., 1986) and turkey hen (Chaiseha and El-Halawani, 1999). The removal of the avian pituitary from the control of the hypothalamus and the influence of VIP completely inhibits PRL release (Chadwick et al., 1978). Active immunization of turkey hens with chicken VIP also reduces serum PRL levels and prevents incubation (Bedecarrats et al., 1999b).

Serotonin (5-HT) has been shown to increase levels of circulating PRL in avian species. Immunizing birds against VIP blocks the 5-HT stimulated release of PRL, indicating that VIP is an important mediator in this pathway (Youngren et al., 1998). The opioid peptide dynorphin can also stimulate the release of PRL, but this is prevented when 5-HT receptors are blocked (Youngren et al., 1999). Dopamine is a third hormone that influences the 5-HT stimulation of PRL, and experiments have demonstrated that DA, D1 DA receptors and 5-HT must all be present for PRL secretion to occur (Youngren et al., 1998). Thus, dynorphin, 5-HT and DA will stimulate PRL secretion

under the condition that there is an intact VIPergic system. This evidence further suggests that the common pathway of *k*-opioid, serotonergic, dopaminergic and VIPergic receptors are arranged in that functional order (El-Halawani et al., 1995; Youngren et al., 1996; Youngren et al., 1999).

The Role of PRL in Incubation Behavior

In turkey hens, the start of the incubation period is marked by a significant increase in PRL serum and mRNA levels (Dawson and Goldsmith, 1982; El-Halawani et al., 1988; Wong et al., 1991; Bedecarrats et al., 1997; Karatz et al., 1997; Tong et al., 1997). The domestic turkey hen begins laying after an increase in daylight from 8 to 14 hours known as photostimulation (Etches et al., 1979; Siopes et al., 1984; El-Halawani et al., 1988). During the approximately 28 day period between the end of lay and the hatching of the clutch an incubating turkey hen almost never leaves the nest and participates in nesting activity (Burke et al., 1981; El-Halawani et al., 1988). Levels of PRL mRNA are 10X greater in photostimulated hens than in photorefractory hens, 20X greater in laying hens and 100X greater in incubating hens (Wong et al., 1991). The higher serum and PRL mRNA levels are a result of both an increased rate of transcription and an increase in PRL mRNA stability (Tong et al., 1997). The fluctuation of PRL levels in the turkey hen can be further explained by the redistribution of anterior pituitary cell types that occurs during the incubation period. In the incubating turkey hen, the caudal lobe of the anterior pituitary undergoes a change from somatotrophic to lactotrophic cells (Ramesh et al., 1996). In cells that are making this transition, named

mammosomatotrophs, PRL mRNA and GH are colocalized in the same cell (Ramesh et al., 1998).

The onset of incubation in turkey hens involves much more than increased levels of circulating PRL. Incubating turkey hens also experience a drop in levels of circulating gonadotropin and ovarian steroids, altered brain neurotransmitter activity, nest protection instincts, a disinterest in food, the appearance of a "brood" patch, and ovarian regression (Burke et al., 1981; Lea et al., 1981; Siopes and Burke, 1984; Porter et al., 1987; El-Halawani et al., 1988).

The turkey industry has attempted a number of management procedures to prevent the incubation period. However, these methods are often labor intensive. One way to prevent incubation is to deprive turkey hens of their nests. Circulating PRL levels in incubating hens drop to the levels of laying hens after 48 hours of nest deprivation and cage confinement, although they return to their former levels after only 8 hours back at the nest (El-Halawani et al., 1980). Incubation behavior in turkeys can also be prevented by injection with antibodies raised against tPRL (passive immunization) or by injection with a tPRL gene construct that elicits in the animal an antibody response (active immunization) (Crisostomo et al., 1997; Crisostomo et al., 1998). Alternatively, incubating hens can be injected with parachlorophenylalanine (PCPA), a drug that decreases circulating PRL levels by blocking synthesis of the PRL releasing factor serotonin (El-Halawani et al., 1980; El-Halawani et al., 1988).

Molecular Regulation of the PRL Gene

Tissue-specific regulation of the PRL gene may be somewhat conserved among vertebrates. In mice, expression of the PRL gene was shown by DNase I footprinting analysis to be controlled by a proximal enhancer near the TATA box and a distal enhancer -1.8 to -1.5 kilobases away from the transcription start site. Both regions contain multiple binding elements for the anterior pituitary transcription factor mouse (m) Pit-1 (Nelson et al., 1986; Crenshaw et al., 1989; Elsholtz et al., 1992).

DNase I footprinting showed that the rat (r) PRL gene also contains two regulatory regions, a distal enhancer (-1831 to -1530) and proximal region (-422 to -36). In vitro transcriptional analysis further showed that the distal segment of the rPRL gene accounts for 99% of the transcriptional activity (Nelson et al., 1986; Cao et al., 1987; Guitierrez-Hartmann et al., 1987; Lufkin and Bancroft, 1987; Nelson et al., 1988; Ingraham et al., 1990a). These two rat regulatory regions contain numerous sites to which Pit-1 can bind, and the distal enhancer contains an estrogen response element (ERE) that binds the estrogen receptor (Fig. I-2). Mutation of even one Pit-1 binding site on the rPRL promoter reduces rPRL gene expression by 80 to 90% (Nelson et al., 1988). Computer analysis of the 5'-regulatory region of the rPRL gene with the 5'-regulatory regions of the bovine (b) and human (h) PRL genes show that among these species the structures of the distal and proximal enhancers are highly conserved. Sequence similarities among Pit-1 binding sites on the rPRL and bPRL genes or rPRL and hPRL genes range from 80 to 88% (Peers et al., 1990; Wolf et al., 1990; Elsholtz et al., 1992).

A significant divergence between the 5'-regulatory regions of the rPRL and salmon (s) PRL genes suggests that there may be differences in the regulation of mammalian versus teleost PRL. However, computer searches show that despite these differences consensus sequences for mammalian Pit-1 binding to PRL and GH are present in the 5'-regulatory region of the salmon genes (Chen et al., 1990; Elsholtz et al., 1992).

Turkey PRL is a 6.7 kb gene with five exons and a transcriptional start site 51 to 53 bases upstream of the translation start site. The 5'-flanking region of tPRL contains two putative tPit-1 binding sites (Kurima et al., 1995). Unlike the rPRL 5'-flanking region, the tPRL 5'-flanking region does not contain an ERE (Fig. I-2). The lack of an ERE is consistent with the findings that cultured turkey pituitary cells do not secrete PRL in response to estrogen (Knapp et al., 1988).

The pituitary-specific transcription factor Pit-1 belongs to the POU domain family of proteins (Herr et al., 1988; Herr and Cleary, 1995). In general, POU domain proteins appear to be involved in tissue-specific regulation of gene expression as well as tissue development (Rosenfeld et al., 1991; Ruvken and Finney, 1991; Verijzer and van der Vliet, 1993; Herr and Cleary, 1995). Pit-1 was found to be the common transcription factor that binds to multiple sites within the enhancer element of the rPRL and rGH genes (Nelson et al., 1988; Mangalam et al., 1989). A consensus sequence, (T/A)TATNCAT, has been suggested for Pit-1 binding to mammalian PRL and GH genes. A consensus sequence for Pit-1 binding to teleost and avian PRL and GH has also been proposed, (T/A)NCTNCAT (Nelson et al., 1988; Ohkubo et al,. 1996). The difference between the teleost/avian and mammalian Pit-1 binding sites may be related to the differences in the

structures of mammalian and avian/teleost Pit-1 proteins. Teleost/avian Pit-1 have 40-50 additional amino acids in the amino terminal domain when compared with mammalian Pit-1 (Ono and Takayama, 1992; Wong et al., 1992; Yamada et al., 1993; Ohkubo et al., 1996).

The structure of the POU domain family of proteins is unique because it contains two separate domains that function together to bind DNA. The POU-homeodomain (POU_{HD}), a 75 amino acid N-terminal region, and the POU-specific domain (POU_S), a 60 amino acid carboxy terminal region, are highly conserved. The two regions are joined by a flexible, more variable linker segment that can be from 15 to 56 amino acids in length (Thiell et al., 1989; Greenstein et al., 1994; Klemm et al., 1994; Herr and Cleary, 1995). Both the POU_{HD} and POU_S domains are needed for sequence-specific, high affinity DNA binding and transcriptional activation (Ingraham et al., 1990b). The subdomain/linker structure of the POU domain family of proteins allows the group to be very flexible in its binding to DNA. This flexibility of the POU domain proteins, in terms of their ability to form homo- or heterodimers on the DNA and adopt alternate conformations, aids in their versatility as transcription factors (Jacobsen et al., 1997).

DNase I footprinting analysis and in vitro transcriptional activation studies have shown that PRL gene expression is controlled by a number of factors besides the anterior pituitary transcription factor Pit-1. In mammals, these factors include cyclic AMP (cAMP), thyrotropin releasing hormone (TRH), epidermal growth factor (EGF), calcium, estrogen and phorbol ester (Ben-Jonathan et al., 1989; Day and Maurer, 1989; Keech et al., 1992). These factors may have one or more than one response element in the 5'-flanking region of the PRL gene. For example, both the distal (-1831 to -1530) and

proximal (-422 to -36) enhancer regions of the rPRL gene contain elements that confer responsiveness to cAMP, TRH and EGF. However, only the distal region of rPRL contains an ERE, while the proximal region contains an element that confers responsiveness to phorbol ester (Day and Maurer, 1989).

While some of these factors, like estrogen through the estrogen receptor, increase PRL levels by binding directly to the gene promoter, others cooperate with a second factor to regulate gene transcription. In most cells cAMP interacts with the nuclear transcription factor CREB; however, in lactotrophs cAMP requires Pit-1 to activate gene transcription (Keech et al., 1992). Other factors mediated by Pit-1 are calcium, estrogen and TRH (Ben-Jonathan et al., 1989; Day et al., 1990; Hoggard et al., 1991; Yan and Bancroft, 1991; Zhang et al., 1993). For example, Pit-1 is the factor responsible for transducing calcium and TRH signals to the PRL gene promoter, resulting in an increase in PRL gene expression (Yan and Bancroft, 1991).

The factors that regulate the tPRL gene are unknown. Our lab previously reported that although tPit-1 can activate tPRL in vitro, tPit-1 and tPRL are not colocalized in the same cell. Thus, Pit-1 does not appear to play a role in the regulation of PRL gene expression in the domestic turkey (Weatherly et al., 1997).

Electrophoretic Mobility Shift Assays (EMSAs) Identify DNA-Protein Complexes

The regulation of gene expression involves the binding of protein/transcription factors to the regulatory region of a gene. DNA-protein interactions can be identified by an electrophoretic mobility shift assay (EMSA). The DNA sequence of interest (a 30-50 nt oligonucleotide) is radiolabeled and incubated with nuclear extract from animal tissue.

The protocol adapted for the extraction of nuclear proteins from small amount of animal tissue is quick, efficient and inexpensive. The procedure first breaks apart the cell membrane without disrupting the nuclear membrane, allowing for the extraction of transcription factors that may be involved in regulation of the PRL gene (Roy et al., 1991). DNA-protein complexes are allowed to form and the reaction is run on a non-denaturing, polyacrylamide gel. If no protein-DNA complexes form all the unbound, radiolabeled DNA is found at the bottom of the gel. DNA-protein complexes are large and run more slowly through the gel than DNA without bound protein. DNA-protein complexes appear as retarded bands nearer to the top of the gel (Ausubel et al., 1989; Rezvin et al., 1989; Guille and Kneale, 1994).

Comparison of PRL Amino Acid Sequences

```
                                        -30                                  -1
Turkey:                                 MSNTGASLKG  LLLAVLLVSN  MLLTKEGVTS
Chicken:                                ---R------  -F--------  T---------
Human:                                  -NIK-SPW--  S.LL------  .--LCQS-AP
Cow:                                    -DSK-S-Q--  SR-LL---VS  N--LCQ--V-
Rat:                                    -NSQVSAR-A  GT-LL-MM--  .--FCQN-QT

            1                                                          50
Turkey:     LPICSSGSVN  CQVSLGELFD  RAVRLSHYIH  FLSSEIFNEF  DERYAQGRGF
Chicken:    ----PI----  ----------  ---K------  Y---------  ----------
Human:      ----PG-AAR  ---T-RD---  ---V------  N----M-S--  -K--TH----
Cow:        T-V-PN-PG-  -----RD---  ---MV-----  D----M----  -K-----K--
Rat:        --V--G-..D  --TP-P----  -V-M------  T-YTDM-I--  -KQ-V-D-E-

            51                                                        100
Turkey:     ITKAVNGCHT  SSLTTPEDKE  QTQQIHHEEL  LNLILGVLRS  WNDPLIHLAS
Chicken:    ----------  ----------  -A------D-  ---VV-----  ----------
Human:      ----I-S---  ---A------  -A--MNQKDF  -S--VSI---  --E--Y--VT
Cow:        --M-L-S---  ---P------  -A--T---V-  MS----L---  -----Y--VT
Rat:        -A--I-D-P-  ---A------  -A-KVPP-V-  -----SLVH-  -----FQ-IT

            101                                                       150
Turkey:     EVQRIKEAPD  TILWKAVEIE  EQNKRRLEGM  EKIVGRIHSG  DAGNEVFSQW
Chicken:    ----------  ----------  -----L----  ------V---  -----IY-H-
Human:      --RGMQ---E  A--S------  --T--L----  -L--SQV-PE  TKE--IYPV-
Cow:        --RGM-G---  A--SR-I---  -E---L----  -M-F-QVIP-  AKET-PYPV-
Rat:        GLGG-H----  A-ISR-K---  -----L---I  ---ISQAYPE  AK---IYLV-

            151                                                       199
Turkey:     DGLPSLQLAD  EDSRLFAFYN  LLHCLRRDSH  KIDNYLKVLK  CRLIHDNNC
Chicken:    ----------  ----------  ----------  ----------  ------S--
Human:      S------M--  -E---S-Y--  ----------  -------L--  --I--N---
Cow:        S------TK-  --A-YS----  ---------S  ---T---L-N  --I-YN---
Rat:        SQ-----GV-  -E-KDL----  NIR-------  -V-----F-R  -QIV-K---
```

Figure I-1 – Comparison of amino acid sequences for mammalian and avian PRLs. Amino acid sequences for three mammalian and two avian PRLs are shown. Dashes indicate amino acids that are identical to tPRL. Periods indicate amino acids left blank to optimize the alignment.

Comparison of Rat PRL and Turkey PRL 5'-Flanking Regions

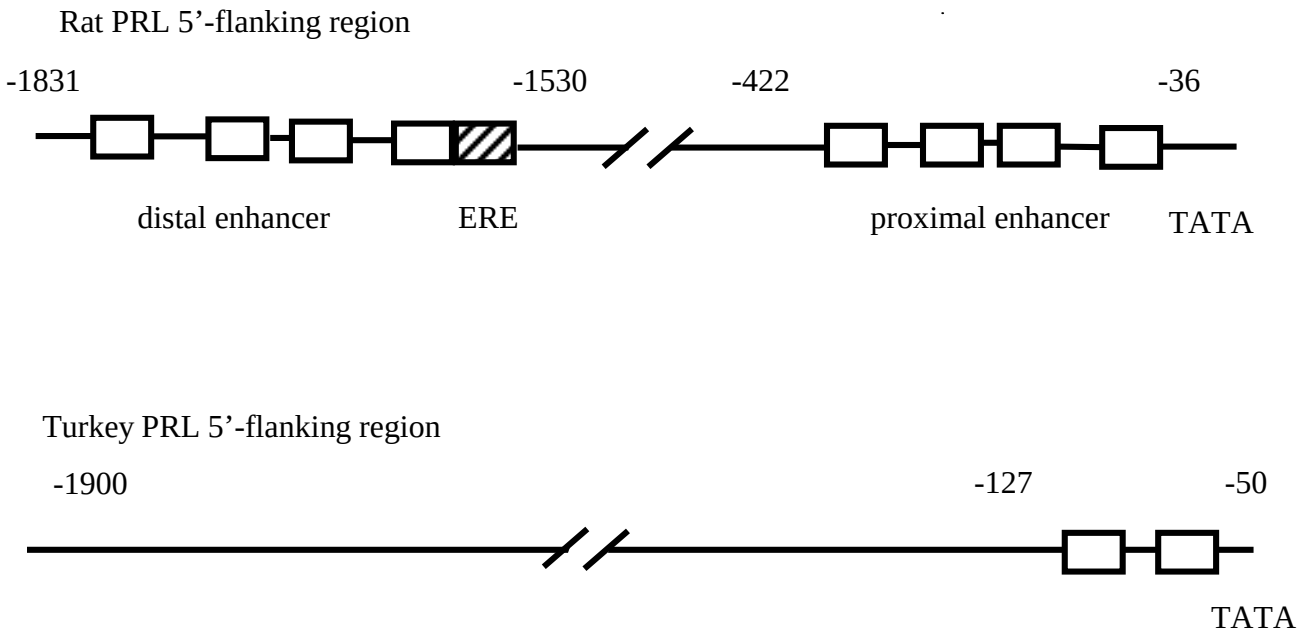

Figure I-2 – Comparison of rat and turkey 5'-flanking regions. White boxes represent Pit-1 binding sites in the rat 5'-flanking region and putative Pit-1 binding sites in the turkey 5'-flanking region. ERE stands for estrogen response element, and the TATA box is indicated (Nelson et al., 1988).

II. OBJECTIVES

The anterior pituitary hormone PRL is involved in the onset of the incubation period in the turkey hen. Transcription factors that bind to and regulate the tPRL gene remain to be determined. The objective of this research is to map the 5'-flanking regions of the tPRL gene that may be involved in gene regulation. The specific objectives of this thesis are:

1. To perform gel shift assays covering 2 kb of the 5'-flanking region of the tPRL gene with turkey pituitary and liver nuclear extracts
2. To determine the sequence specificity of these DNA/protein interactions using competition assays
3. To determine if any of these DNA-protein complexes include tPit-1 using a supershift assay

III. MATERIALS AND METHODS

Experimental Animals

The animals used in the following experiment were Nicholas Large White turkeys (Nicholas Breeding Farms, Sonoma, CA) that were kept at the Virginia Tech Turkey Center. The birds were grown on 8 hours of light:16 hours of darkness. To induce the onset of the laying period, birds were photostimulated at 28-30 weeks of age with 16 hours light:8 hours darkness.

Nuclear Extraction from Tissue

Nuclear extraction was performed on pituitary and liver tissue samples from laying turkey hens (adapted from Roy et al., 1991). Following electrocution, pituitaries were surgically removed and stored at -80º C. Pituitaries were weighed and approximately 0.6g of pituitary and 0.6g of previously frozen liver tissue were transferred to a Dounce tissue grinder. After adding 1.2 mL of NE1 buffer (250 mM sucrose, 15 mM Tris-HCl pH 7.9, 140 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 1 mM dithiothreitol, 0.4 mM Phenylmethylsulphonyl fluoride [PMSF], 25 mM KCl, 2 mM $MgCl_2$) the tissue samples were homogenized with 10 strokes of the Dounce tissue grinder. Large chunks were eliminated from the homogenate with filtration through cheesecloth, 0.5% of Nonidet P-40 was added and the homogenization was repeated. The lysate was transferred to a microfuge tube and centrifuged at 1,000xg for 8 minutes at 4º C. The supernatant was discarded and the pellet (cell nuclei) washed once with 1.5 mL of NE1 buffer. Nuclei were lysed by adding .45 mL NE2 buffer (NE1

buffer containing 350 mM KCl) and incubating the sample at 4º C for five minutes. One-tenth the packed cell volume of 4M KCl was added and the extract was again homogenized by 20 strokes in the Dounce tissue grinder. The homogenate was transferred to a microcentrifuge tube and centrifuged for 5 minutes at 12,000xg to eliminate larger cell debris. The supernatant was collected and centrifuged at 180,000xg (56,000 rpm) for 90 minutes in a Beckman TLA 100.3 rotor to remove small organelles.

The high-speed supernatant was concentrated and .2 mL of DNase I buffer (50 mM KCl, 4mM $MgCl_2$, 20mM K_3PO_4 pH 7.4, 1mM ß-mercaptoethanol, 20% glycerol) was exchanged using a Microcon YH-3 spin column for 90 minutes (Millipore, Bedford, MA).

A commercial protein assay (Bio-rad Laboratories, Hecules, CA) based on the method of Bradford (1976) was used to determine the concentration of protein in the pituitary and liver nuclear cell extracts. Bovine serum albumin was used as a protein standard. Protein yields varied but 0.6 g of tissue typically yielded 2 mg of protein.

Electrophoretic Mobility Gel Shift Assay (EMSA)

The tPRL promoter region (Kurima et al., 1995) was divided into 30-50 nt segments and the corresponding oligonucleotides were synthesized in the sense and antisense directions (MWG Biotech). Oligonucleotides containing a confirmed Pit-1 binding site on rPRL (nt -37 to -68, Maurer et al., 1981) were also synthesized for use in a preliminary EMSA experiment (adapted from Fried and Crothers, 1981).

To anneal complementary strands, sense and antisense oligonucleotides were added in a 1:1 ratio along with 1X React 2 buffer (50 mM Tris-HCl pH 8.0, 10 mM

$MgCl_2$, 50 mM NaCl; Life Technologies, Rockville, MD) and sterile water. The reaction was heated to 95ºC for 5 minutes and then slowly cooled to room temperature. Annealed oligonucleotides were stored at -80ºC until used.

Double-stranded oligonucleotides were end-labeled using 100 µCi of [gamma-^{32}P]-adenosine triphosphate with a specific activity of 4,500 Ci/mmol and T4 polynucleotide kinase in 1X kinase buffer (70 mM Tris-HCl pH 7.6, 5 mM DTT, 10 mM $MgCl_2$, New England Biolabs, Beverly, MA). Following a 45 minute incubation at 37ºC, radioactively-labeled oligonucleotides were passed through two 1 mL G-25-80 (Sigma) spin columns to remove any unincorporated [gamma-^{32}P]-adenosine triphophate. The end-labeled oligonucleotides were stored approximately 2 hours at 4°C prior to use in EMSAs and were always used the day of labeling.

A binding reaction was assembled containing 1 X EMS buffer (8 mM HEPES pH 7.8, 60 mM KCl, 2 mM EDTA, 4 mM spermidine, 100 g/ml BSA, 0.03% Nonidet P-40, 0.5% Ficoll), 1 mM DTT, 10% glycerol, 0.5 µg poly dI:dC, 1 µg denatured salmon sperm DNA and 20 µg cell nuclear extract. The reaction was preincubated for 15 minutes at 4ºC and centrifuged for 30 seconds at 12,000xg. Approximately 5 ng of end-labeled oligonucleotides were added to the binding reaction and incubated for 20 minutes at 25ºC after which the sample was loaded onto the gel. Six percent polyacrylamide gels (19:1, acrylamide:bisacrylamide) were prewarmed by running for 60 minutes at 120 V prior to the addition of samples. Samples were run in .1% bromphenol blue at 120 V for approximately 90 minutes in 0.5X TBE running buffer. Gels were washed three times for 15 minutes in 100 ml of dH_2O and once with 50 ml of Gel-Dry Drying Solution (Novex, San Diego, CA). Gels were wrapped in cellophane, placed in a drying frame (Novex,

San Diego, CA) and dried overnight. The following day, gels were removed from the gel drying frame and exposed to Kodak x-ray film (Eastman Kodak Co., Rochester, NY). The gels were typically exposed to the film for 1.5 to 3.5 hours.

A competition EMSA was performed with the following modifications. Competitor DNA was annealed as described previously. Nuclear extract was incubated in the absence of labeled DNA for 15 minutes at 4ºC . Competitor DNA was added and the reaction was incubated for 10 minutes at 25ºC. Following the addition of 5 ng of end-labeled oligonucleotides the reaction proceeded as previously described. Either 10-fold, 25-fold or 50-fold more specific or nonspecific unlabeled competitor DNA than radiolabeled DNA was used. The nonspecific competitor DNA used in the competition EMSA experiment was a 33 nt unrelated sequence AGTGAACTGATGAGTCCGTGAGGACGAAACAGC.

When a supershift EMSA was performed, 1 µg of rabbit-anti-rat Pit-1 antisera (gift from Dr. Simon J. Rhodes, Indiana University-Purdue University, Indianapolis, IN) was incubated with nuclear extract in the absence of labeled DNA for 30 minutes at 4ºC . Following the 30 minute incubation, 5 ng of end-labeled oligonucleotides were added and the reaction continued as described previously. As a control, another reaction was done in which 1 µg of the rabbit preimmune sera was added in place of post-immunization antisera.

IV. RESULTS

Mapping DNA-Protein Interactions in the 5'-Flanking Region of the PRL Gene

To optimize appropriate binding conditions for the EMSAs, 0.1-5 µg of rPit-1 protein (gift from Dr. Richard Day, University of Virginia, Charlottesville, VA), partially purified using phosphocellulose columns, was used in a preliminary assay. In Fig. IV-1, two shifted bands were observed (lanes 2-5, bands a and b) when a radiolabeled oligonucleotide containing a rPit-1 binding site of the rPRL gene (-37 to -68, Maurer et al., 1981) was incubated with increasing amounts of partially purified rPit-1. This result demonstrates that partially purified rPit-1 binds to the oligonucleotides under the conditions utilized. A shift was not observed in the absence of rPit-1 (lane 6) or when 5 µg of liver extract was used instead of rPit-1 (lane 1).

The sequence of the 5'-flanking region of tPRL is shown in Fig. IV-2 (Kurima et al., 1995). The series of EMSAs of the tPRL 5'-flanking region was initiated with two complementary oligonucleotides containing the putative tPit-1 binding sites (33-mers; nt -41 to -73 and -105 to -137, Fig. IV-3). The series continued in 50 nt increments from nt -175 to -1,981, with the last oligonucleotide 57 nt in length. The EMSA series was also done with 20 µg of liver nuclear extract instead of pituitary nuclear extract to distinguish between common DNA binding proteins and those that are specific to pituitary tissue.

Results of the first series of 12 EMSA fragments can be seen in Fig. IV-3a (pituitary, exposed 1.5 h), IV-3b (pituitary, exposed 3 h) and IV-3c (liver), representing nt -41 to -673. Lanes 11 and 12 (Fig. IV-3a, IV-3b and IV-3c) span the two putative tPit-1 binding sites, or the regions from nt -105 to -137 and -41 to -73, respectively. These

two regions showed three shifted bands (Fig. IV-3a and IV-3b, lanes 11 and 12, bands a, b and c) when pituitary nuclear extracts were used, which were absent with liver nuclear extracts (Fig. IV-3c, lanes 11 and 12). Two different exposures of the first set of EMSA fragments with pituitary nuclear extract are shown (Fig. IV-3a and IV-3). The lighter exposure of the first set of EMSA fragments in Fig. IV-3a more clearly depicts the shifted bands in lanes 1-10 than does the darker exposure of the first set seen in Fig. IV-3b. These results show that the shifted bands in lanes 1-10 of Fig. IV-3a (pituitary) are similar to the shifted bands in lanes 1-10 of Fig. IV-3c (liver). On the other hand, the shifted bands in lanes 11 and 12 are more easily seen in Fig. IV-3b than Fig. IV-3a. Also shown in Fig. IV-3a, IV-3b and IV-3c is the difference in the distance migrated between the 50 nt unshifted oligonucleotides used in lanes 1-10 and the 33 nt unshifted oligonucleotides used in lanes 11 and 12. The larger, 50 nt oligonucleotides did not migrate through the gel as quickly as the 33 nt oligonucleotides. One 49 nt oligonucleotide was inadvertently used to represent the region from nt -375 to -423. To verify these results, this first series of EMSA fragments was repeated once with the same pituitary and liver nuclear extracts and once with different pituitary and liver nuclear extracts. A similar pattern of shifted bands was observed in all cases (data not shown).

The second set of EMSA fragments can be seen in Fig. IV-4a (pituitary) and IV-4b (liver), representing the region from nt -674 to -1373. The distance migrated by the unshifted 50 nt oligonucleotides is indicated by an arrow. The second series of EMSA fragments shows that this segment of the 5'-flanking region of the tPRL gene binds both pituitary and liver nuclear extracts in a similar manner. Thus, shifted bands in the second EMSA series likely represent common transcription factor/protein binding.

The final series of EMSA fragments are shown in Fig. IV-5a (pituitary) and IV-5b (liver), which represent shifts in the region from nt -1374 to -1981. The distance migrated by the unshifted 50 nt oligonucleotides in lanes 2 through 12 is indicated by an arrow on the right hand side of the figure. The arrow on the left of the figure shows the migration of the 57 nt oligonucleotide used in lane 1. Again, the third series shows that this segment of the 5'-flanking region binds both pituitary and liver nuclear extracts in a similar manner. Consequently, shifted bands in the final EMSA series also likely represent common transcription factor/protein binding. Since common transcription factor/protein binding was not a focus of this thesis, the second and third EMSA series were not examined further.

The first series of EMSAs showed that nt -175 to -224 contained four shifted bands when incubated with nuclear extracts from turkey pituitaries or liver (Fig. IV-3a, IV-3b and IV-3c, white arrowheads). To determine if a shifted band unique to pituitary would appear with shorter fragments the region from nt -175 to -224 was divided into two parts (nt -175 to -199 and -200 to -224) for an EMSA. No shifted bands were seen using the region from nt -200 to -224 and pituitary or liver nuclear extracts (data not shown). When using the region from nt -175 to -199, named tprl-3, gel-shifted bands were present with pituitary but not liver tissue (Fig. IV-6, lanes 1 (liver) and 5 (pituitary), white arrowheads). The region from nt -138 to -174 was not used as an EMSA fragment because it contains only adenine nucleotides (see Fig. IV-2).

The oligonucleotide containing the region from nt -105 to -137, named tprl-2, resulted in three tissue-specific shifted bands as seen previously (Fig. IV-6, lane 6, bands a, b and c). tprl-2 and liver nuclear extract did not result in any gel-shifted bands (Fig.

IV-6, lane 2). No shifted bands were seen with the oligonucleotide from nt -74 to -104 and either pituitary or liver nuclear extracts (Fig. IV-6, lanes 7 and 3). Gel-shifted bands were again observed with the oligonucleotide containing the region from nt -41 to -73, named tprl-1, and pituitary (Fig. IV-6, lane 8, bands a, b and c) but not liver (Fig. IV-6, lane 4) nuclear extract. In short, in the region from -41 to -199 three fragments showed pituitary-specific shifted bands, suggesting that these regions are involved in pituitary-specific regulation of the tPRL gene.

Sequence Specificity of DNA-Protein Interactions using tprl-1, tprl-2 and tprl-3

To determine the sequence specificity of the DNA-protein interactions EMSAs were performed using tprl-1, tprl-2, tprl-3 and various kinds of unlabeled competitor DNA. The competition EMSA done with tprl-1 is shown in Fig. IV-7. Lane 1 showed the expected three shifted bands (labeled with a, b and c) when no competitor was added. Lanes 2-4 showed the effect of adding 10-, 20- or 50-fold more unlabeled competitor (tprl-1) than radiolabeled DNA. As expected, adding excess, unlabeled tprl-1 competitor DNA eliminated the shifted bands observed when no competitor was present (compare with lane 1). Lanes 5-7 showed that increasing amounts of the oligonucleotide containing the other putative tPit-1 site, tprl-2, successfully competed out band c but not bands a and b. This data indicates that band c may include a nuclear protein that binds to both tprl-1 and tprl-2 oligonucleotides. Lanes 8-10 of Fig. IV-7 showed that adding increasing amounts of nonspecific competitor DNA did not compete with the radiolabeled DNA for pituitary nuclear proteins and cannot eliminate the shifted bands observed even at 50-fold excess.

The competition EMSA was repeated with labeled tprl-2 and pituitary nuclear extract and the results are shown in Fig. IV-8. In the absence of competitor DNA (lane 1) the three expected shifted bands (labeled with a, b and c) were seen. Lanes 2-4 demonstrate that the specific unlabeled, tprl-2 competitor DNA successfully competed out the previously observed shifted bands (lane 1). In Fig. IV-8, lanes 5-7 showed that increasing amounts of unlabeled tprl-1 competitor DNA eliminated all shifted bands (a, b and c). In addition, at 10X competitor concentrations, tprl-1 in lanes 5-7 competed out the previously observed gel-shifted bands a, b and c more efficiently than the specific competitor tprl-2 (Fig. IV-8, lanes 2-4). This data again indicates that the same nuclear proteins may be binding to tprl-1 and tprl-2 and that the proteins may bind to tprl-1 with higher affinity. As seen in lanes 8-10 of Fig. IV-8, adding increasing amounts of nonspecific competitor DNA again had no effect on the observed gel-shifted bands (a, b and c).

Fig. IV-9 shows the results of a competition EMSA with radioactively labeled tprl-3. In lane 1 no competitor DNA was used and the expected two shifted bands (labeled a and b) were seen. Lanes 2-4 show that increasing amounts of unlabeled, specific tprl-3 competitor DNA eliminated the previously observed gel-shifted bands. When increasing amounts of the nonspecific competitor was added to the reaction the shifted bands a and b remained (lanes 5, 6 and 7).

Identification of a tPit-1/DNA Interaction using a Supershift Assay

Since tprl-1 and tprl-2 both contain putative tPit-1 binding sites, the hypothesis that Pit-1 was contained in one of the three protein-DNA complexes previously observed

(Fig. IV-4b, lanes 11 and 12, bands a, b and c) was investigated using a supershift EMSA. Radiolabeled tprl-1 and tprl-2 were used in an assay with nuclear extract from turkey pituitaries plus 1 µg of rabbit-anti-rat Pit-1 antisera. In Fig. IV-10, lanes 1 and 5 show the results of incubating tprl-2 and tprl-1 oligonucleotides, respectively, in the absence of pituitary nuclear extract. Lanes 2 (tprl-2) and 6 (tprl-1) show the expected three shifted bands (a, b and c) that resulted from the addition of only pituitary nuclear extract. In Fig. IV-10, lanes 4 (tprl-2) and 8 (tprl-1) show that band c could be eliminated by the addition of rabbit-anti-rat Pit-1, implying that the missing band contains a complex of DNA and tPit-1. Band c in Fig. IV-10 is the same band that disappeared during the EMSA with radiolabeled tprl-1, pituitary nuclear extract and unlabeled tprl-2 competitor DNA (Fig. IV-7, lanes 2-4, band c). Thus, proteins in the shifted band c must be able to bind to both tprl-1 and tprl-2. This data further suggests that the missing band contains a complex of DNA and tPit-1, a protein which is thought to bind to sites in both tprl-1 and tprl-2. When 1 µg of rabbit preimmune sera was added to the binding reaction (Fig. IV-10, lanes 3 and 7) there was no change in the binding of nuclear extract to either oligonucleotide, confirming that immunization of the rabbit with rPit-1 induced the synthesis of an antibody that bound to the protein in band c.

V. DISCUSSION

The structure and organization of the PRL gene is conserved among vertebrates. This conservation of PRL organization and structure suggests that among vertebrates gene regulation may also be conserved. However, unlike the rat and mouse PRL gene promoters, which contain both proximal and distal enhancer regions, computer analysis of the 5'-flanking region of the tPRL gene shows only two putative tPit-1 binding sites in the proximal segment of the gene (Kurima et al., 1995). This computer analysis is consistent with the results of EMSAs performed on the 5'-flanking region of tPRL, which showed DNA-protein complexes in the proximal region of the gene exclusively. Three shifted bands (Fig. IV-3a and IV-3b, bands a, b and c) were seen using oligonucleotides containing the regions from nt -41 to -73 (tprl-1) and -105 to -137 (tprl-2) with pituitary nuclear extracts that were not seen with nuclear extracts from liver (Fig. IV-3c). Two pituitary-specific shifted bands (Fig. IV-6, bands a and b) were also seen using the oligonucleotide containing the region from nt -175 to -199 (tprl-3).

Competition EMSAs were used to show that these tissue-specific bands were also sequence-specific. Excess unlabeled competitor DNA specific for nuclear proteins present in the pituitary extract successfully competed out shifted bands previously observed with tprl-1, tprl-2 and tprl-3 (Fig. IV-7, IV-8 and IV-9, lanes 2-4). With the nonspecific DNA competitor, the shifted bands remained (Fig. IV-7 and IV-8, lanes 8-10, and Fig. IV-9, lanes 5-7). In yellowtail fish, competition assays using a Pit-1 binding site in the yellowtail GH promoter region and pituitary nuclear extract showed shifted bands that disappear when competitor specific for the Pit-1 binding site is added (Ohkubo et al.,

1996). In rats, DNase I competition analysis showed that the rPit-1 binding sites in the proximal and distal enhancer regions were sequence-specific and that they competed for binding to PRL enhancer elements with different affinities (Nelson et al., 1988).

In this thesis, additional competition studies were done with radiolabeled tprl-1, excess unlabeled tprl-2 competitor and nuclear extract from pituitaries. tprl-2 competitor DNA successfully competed out only band c (Fig. IV-7, lanes 5-7). When the experiment was reversed, with tprl-2 radiolabeled and tprl-1 acting as unlabeled competitor DNA, all the previously observed shifted bands were competed out (Fig. IV-8, lanes 5-7). This data suggests that the same nuclear proteins may be binding to tprl-1 and tprl-2 and that the proteins may bind to tprl-1 with higher affinity. In particular, it suggests that band c contains a nuclear protein that binds to both regions of the tPRL gene. Since both tprl-1 and tprl-2 contain putative tPit-1 binding sites, the common protein may be tPit-1.

The hypothesis that tPit-1 is the common protein forming one of the three DNA-protein complexes seen with both tprl-1 and tprl-2 was tested using a supershift EMSA. When rabbit-anti-rat Pit-1 was added to the binding reactions pituitary nuclear extract and tprl-1 or tprl-2, band c was no longer present (Fig. IV-10, lanes 8 and 4, respectively). This is the same band that was missing in the competition assay with radiolabeled tprl-1, pituitary nuclear extract and tprl-2 competitor DNA. In one of the binding reactions, rabbit preimmune sera was included as a control to ensure that the sera was not the cause of any change in the observed DNA-protein complexes. It is possible that the nuclear protein bound to tprl-1 and tprl-2 interacts instead with rabbit-anti-rat-Pit 1, and the larger protein complex is unable to re-bind the DNA. This would explain the absence of a more

typical supershift, in which the protein-DNA complex binds antibody and migrates at an even slower rate than the DNA-protein complex alone. For example, in a supershift EMSA with an oligonucleotide containing a putative Pit-1 binding site in the cPRL gene and nuclear extract from GH3 cells, the Pit-1-DNA complex observed during a regular EMSA migrated at a slower rate when rabbit-anti-rat Pit-1 was added (Ohkubo et al., 2000).

These data imply that rabbit-anti-rat Pit-1 binds to the protein in band c, and that this protein is tPit-1. If this is the case, tprl-1 and tprl-2 bind tPit-1 as well as other nuclear proteins. The enhancer regions of various mammalian, teleost and avian PRL promoters contain multiple Pit-1 binding sites that bind Pit-1 and work cooperatively to regulate transcriptional activation of the PRL gene (Nelson et al., 1986; Crenshaw et al., 1989; Peers et al., 1990; Wolf et al., 1990; Elsholtz et al., 1992). In turkeys, although Pit-1 activates the PRL gene in vitro, Pit-1 may not be involved in the activation of PRL gene transcription in vivo (Weatherly et al., 1997).

The apparent interaction between tPit-1 and the tPRL 5'-flanking region may be due to the way in which the nuclear extracts were prepared. The nuclear extracts used in these EMSA experiments contained a mixture of proteins from all cell types of the anterior pituitary, including lactotrophs and somatotrophs. Immunofluorescence studies show that although lactotrophs either do not contain tPit-1 at all or at levels high enough to be detected by immunofluorescence, GH-producing somatotrophs do contain tPit-1 (Ramesh et al., 1998). Since tPit-1 and tPRL are not coexpressed in lactotrophs, tPit-1 likely does not activate tPRL in vivo. Thus, the in vitro studies with tPit-1 and PRL may not reflect what is taking place in the turkey in vivo.

If tPit-1 is not a transcription factor involved in the in vivo activation of the PRL gene, then the transcription factors involved remain to be determined. In mammals, VIP is involved in the secretion of PRL but may not be the main regulator because dopamine acts as a tonic inhibitor of PRL secretion (Tong et al., 1998). In birds, the prolactin releasing factor VIP has a tonic stimulatory effect on PRL secretion and may prove to be the main regulator of the avian PRL gene (Opel and Proudman, 1988; Tong et al., 1998; Schnell et al., 1999). Avian dopamine, serotonin and the opioid peptide dynorphin are all PRL releasing factors that rely on an intact VIPergic system to regulate transcription of the PRL gene (Youngren et al., 1999). Cyclic AMP (cAMP), thyrotropin releasing hormone (TRH), epidermal growth factor (EGF), calcium, estrogen and phorbol ester are also factors that are involved in mammalian PRL gene regulation (Ben-Jonathan et al., 1989; Day and Maurer, 1989; Keech et al., 1992). While some factors, like estrogen through the estrogen receptor, increase PRL levels by binding directly to the gene promoter, others cooperate with a second factor to regulate gene transcription.

In mammals, regulation of PRL gene transcription by cAMP, calcium, estrogen and TRH is mediated by Pit-1 (Ben-Jonathan et al., 1989; Day et al., 1990; Hoggard et al., 1991; Yan and Bancroft, 1991; Zhang et al., 1993). For example, Pit-1 is the factor responsible for transducing calcium and TRH signals to the PRL promoter, resulting in an increase in PRL gene expression (Yan and Bancroft, 1991). Pit-1 is either a direct or indirect target of the signaling cascade initiated by cAMP to up-regulate PRL gene transcription (Keech et al., 1992). The response of the PRL gene to bound estrogen receptor is mediated by the binding of Pit-1 to the PRL ERE (Day et al., 1990). In turkeys, these factors must rely on proteins other than tPit-1 to mediate transcription of

the PRL gene. In addition, most mammalian PRL promoters contain one or more EREs that computer analysis shows are absent from the 5'-flanking region of tPRL. Thus, estrogen may be involved in mammalian PRL but not tPRL gene regulation.

Future experiments will focus on the identification and purification of non-tPit-1 proteins that bind to and regulate expression of the tPRL gene. The research can proceed in a number of different directions now that specific regions involved in regulating the tPRL gene have been identified. DNA-binding proteins can be isolated by affinity chromatography, run on an SDS PAGE gel and analyzed by amino acid sequencing. The genes encoding the proteins can then be amplified by RT-PCR based on the amino acid sequence, cloned into vectors and sequenced. Full length cDNAs of the binding proteins can be obtained by using the RT-PCR products to screen a turkey pituitary cDNA library.

In conclusion, this research maps transcription factor binding sites that may be important in the regulation of the tPRL gene. Three segments of the 5'-flanking region of the tPRL gene participated in tissue-specific, sequence-specific shifts with nuclear proteins from the pituitaries of laying turkey hens. The regions have different affinities for the pituitary nuclear extract. In turkeys, PRL and Pit-1 may not be colocalized in the same cell, so the apparent tPit-1-tPRL interaction in vitro may not reflect the situation in vivo. Understanding the molecular mechanisms controlling the regulation of tPRL gene expression may be useful to prevent incubation behavior and the cessation of egg-laying. Future work will focus on the isolation and identification of transcription factors that bind to and regulate the production of tPRL.

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EMSA using a Radiolabeled Oligonucleotide Containing a Rat Pit-1 Binding Site of the Rat PRL Gene

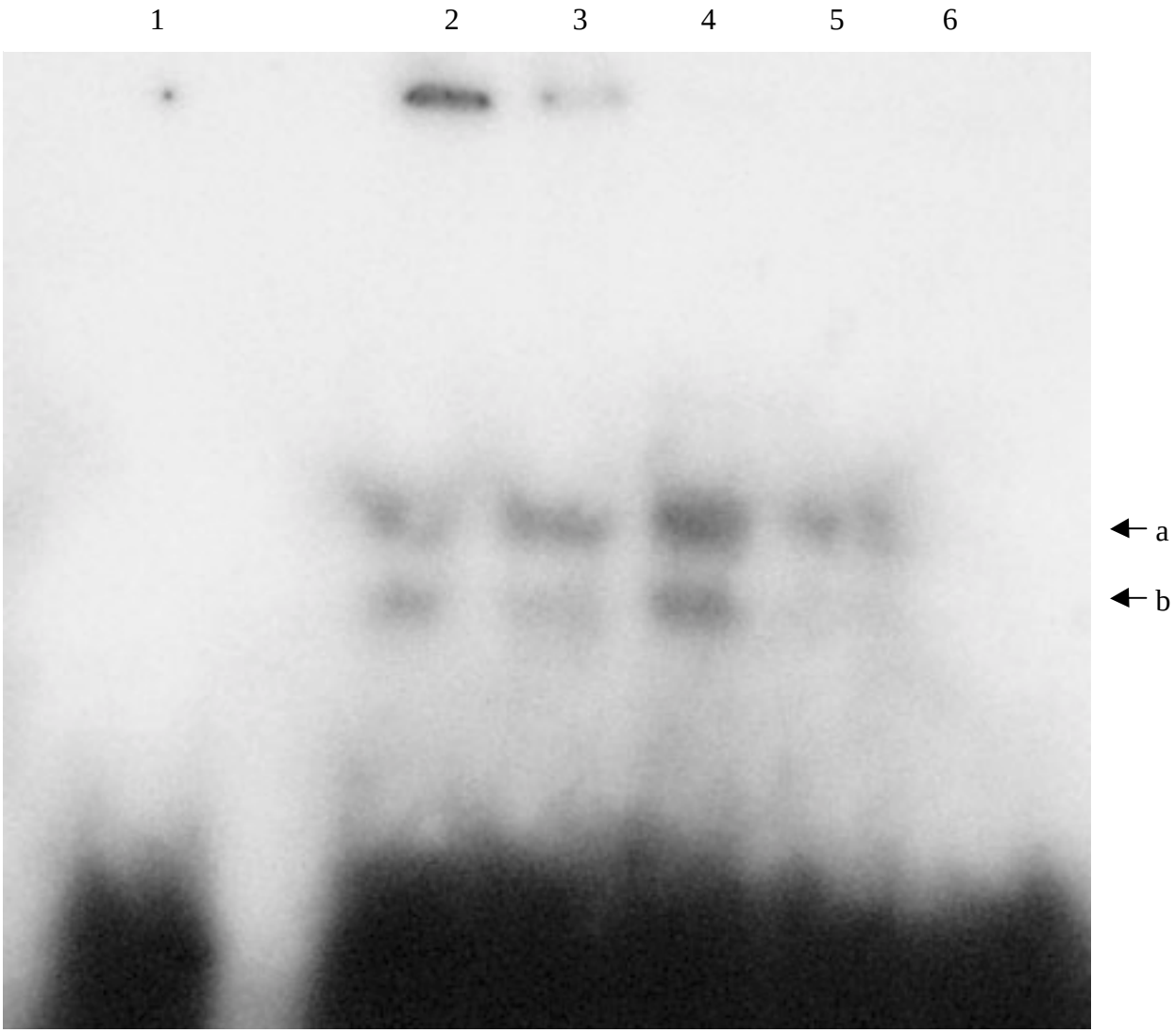

Figure IV-1 – EMSA using a radiolabeled oligonucleotide containing a rat Pit-1 (rPit-1) binding site of the rat PRL (rPRL) gene and increasing amounts of partially purified rPit-1 or liver nuclear extract. Arrows a and b indicate rPRL-rPit-1 complexes. The exposure time was 1 hour and 30 minutes. Lane 1: 5 μg liver, Lane 2: 5 μg rPit-1, Lane 3: 3 μg rPit-1, Lane 4: 2 μg rPit-1, Lane 5: 0.1 μg rPit-1, Lane 6: 0 μg rPit-1

The 5'-Flanking Region of the Turkey PRL Gene

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tcccctgaatc atagaatcat agggtttgga aggcacatcc tggagatcac tgagtccaa -1921
agacctctgct aatgcaggtt acctatagta ggttgtacag gaaactgccc aggaagatt -1861
ttgagtatctc cagaggacac tcatcaatct ctctgggcag cttgttccac tgctctgtc -1801
accctaaaagt aaagtttttc ctaatgttca tatggaactt cctgtgttac agtttatcc -1741
ccattgctcct tgttctgtca cttggcacca ccaaaaacag cctggcccca ttctcatcc -1681
ctttagatatc tataagcatt gatgagatcc actcaatctt ctccaggttg tgtgacccc -1621
aggtctctgat cctttcctca gaaggaagat gctccaggcc cagtcatatt tgtggtctc -1561
ccactgaactc tttccagtag ttccctgatt ttcttgaagt gaggagccca gaactggcg -1501
acagtactcaa gacatggcct catcagggca gagtagaggg ggaaagtcac ctcccttaa -1441
cctgatgacta cattccttt aatgcatctc aagatactac tggccttgtt ggccgcaag -1381
ggcacactgct agcccacggt caacctgttg tctaccagga catctaggtc ttctcagca -1321
gagctcctttc cagcaggtca gcccccagcc tgtactaacg caagcagtaa aaaaggtct -1261
acctttttct ccttcataga atcacagaat tgtaggggta ggagggaacc tccggagat -1201
catctagtcca accactctgc caaatcatca cagtaacaca ggaaagtgtc tggtgggtt -1141
ttgtttattat tgcttcaaaa agcacagcca cagttacgaa ataatgggag attcaggat -1081
tatacacatac ctgttccaca tgtacagaac aagttgtcta gaggcaagaa aattcatta -1021
acactgtatac cttattcatt atgatcatct aatttagaag gtcttttcgt ggataaatg  -961
catctgagaaa cagatgagag attacgcatt tgctaacata ttcgtgcaga tgaacctca  -901
cacaacaagaa aacagggcca acctgctgaa gctaggttgc agattaccac agacacatt  -841
agatcaggaat cagattccac tgattacgac agcatatact gtgattatgg tggacatgc  -781
acatcttttac gcaaagaatt ttcatatata gaaaatgatt tcatggttcg gaagctttt  -721
aaaataatgct gatttaatta caaaatgttt atgattaaac agtaagcata caaattctt  -661
cctctttgttg ttacaaatta ttacttttt aatgacaact gtccctgttt ctcaactta  -601
tctcatcctta gtaccagtta tatcattatc tgttggtaaa taatatcctt tttagctgt  -541
atggagacaaa cacacactac gtataataat gacctgtctt tccagaagcc tccattcac  -481
attctctggat caacttcagt acaattccta ttctttctct tactgtagaa attgtatta  -421
tttcctttcca gaaatagcta gaattggagg gtgaagagac aaggaagaaa cagaagata  -361
tctgcagggat gaacaacatt ttataaacat agaggagaac aatctcagaa ctgacaact  -301
ggaccggacct ttcaaggatc agtggcattt gcaactaatt cagtgcaaaa ttttggcgt  -241
tctcttcatcc agccatactc agcatcccac aactgaaatt tttaatgaaa ttcccactc  -181
acagttaaaaa aaaaaaaaaa aaaaaaaaaa aaaagaaccc aaaagcaagt attgaatat  -121
gaatgtggaag agaggcaatt tgatgtttgt aattaccgag gtaaactcca caacctgct   -61
gaatgtatgca aactggaccc cggatggtgt atataaatct gacatgcaga aagtaagag    -1
CAGGTATTGAG ACTTCTTTCT GGTAGAGCAA GTCATCACAG AGAATCCCTA CCATGAGCA    60
ACACAGGGGCT TCATTGAAAG gtaagacttt tgctattccc tgtctgataa cttctatgt   120
ttaggttttga ttgaattaag aagaagctgg agggtaacaa ttctagaaac taagttttg   180
```

Figure IV-2 – The tPRL promoter gene sequence, exon 1. TATA box is underlined, two potential tPit-1 binding sites are in bold type, the transcription start site is designated as +1, and the first exon is capitalized (Kurima et al., 1995)

EMSA of the Turkey PRL 5'-Flanking Region (nt -41 to -673) using Pituitary Nuclear Extracts

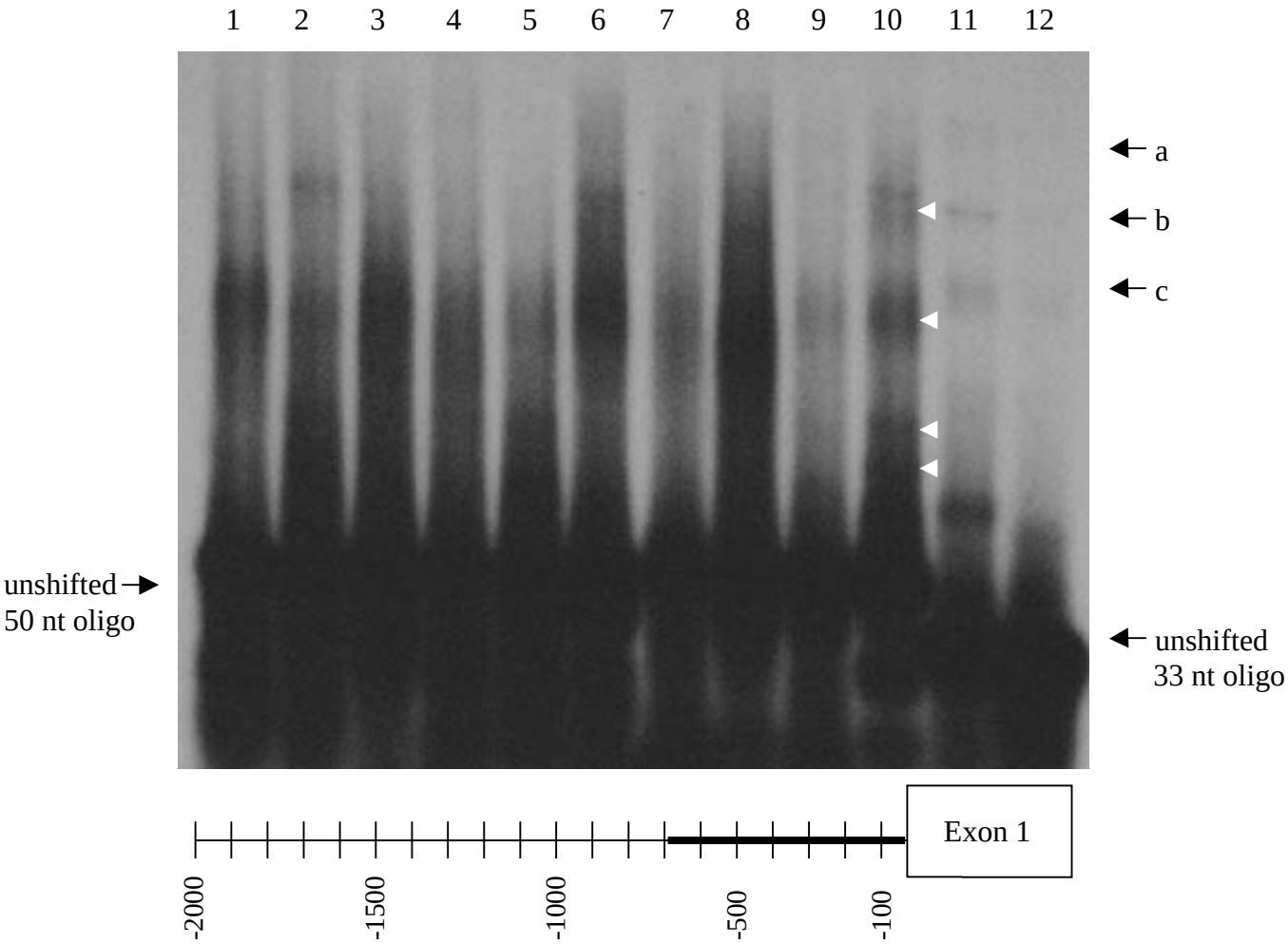

Figure IV-3a - The first series of EMSAs, representing nt -41 to -673. Each oligonucleotide was incubated with 20 µg of turkey pituitary nuclear extract. White arrowheads and arrows a, b and c indicate tPRL-nuclear protein complexes. The exposure time was 2 hours and 30 minutes. Lane 1: -673 to -624, Lane 2: -623 to -574, Lane 3: -573 to -524, Lane 4: -523 to -474, Lane 5: -473 to -424, Lane 6: -423 to -375, Lane 7: -374 to -325, Lane 8: -324 to -275, Lane 9: -274 to -225, Lane 10: -224 to -175, Lane 11: -137 to -105, Lane 12: -73 to -41

EMSA of the Turkey PRL 5'-Flanking Region (nt -41 to -673) using Pituitary Nuclear Extracts

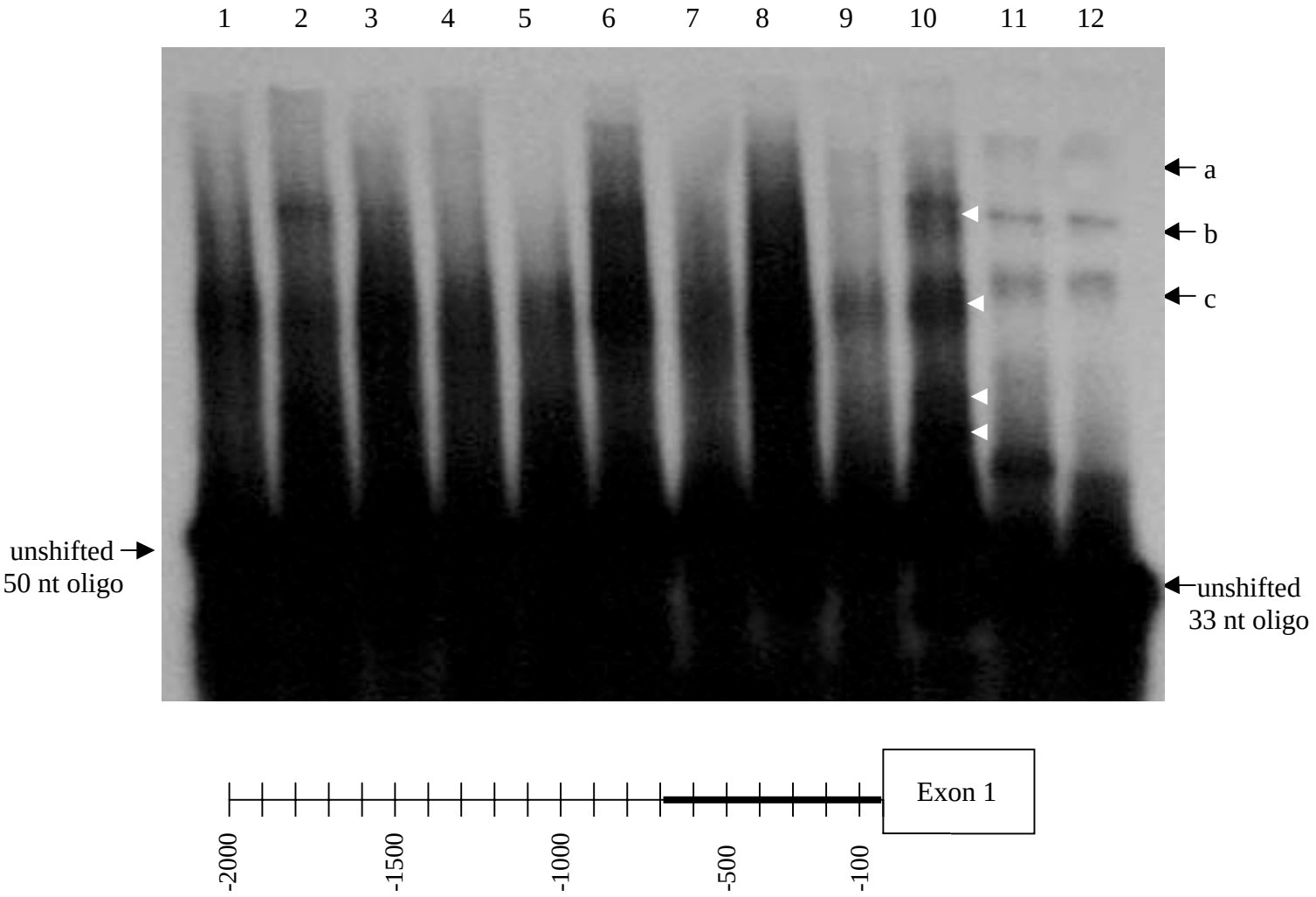

Figure IV-3b - The first series of EMSAs, representing nt -41 to -673. Each oligonucleotide was incubated with 20 µg of turkey pituitary nuclear extract. White arrowheads and arrows a, b and c indicate tPRL-nuclear protein complexes. The exposure time was 3 hours and 30 minutes. Lane 1: -673 to -624, Lane 2: -623 to -574, Lane 3: -573 to -524, Lane 4: -523 to -474, Lane 5: -473 to -424, Lane 6: -423 to -375, Lane 7: -374 to -325, Lane 8: -324 to -275, Lane 9: -274 to -225, Lane 10: -224 to -175, Lane 11: -137 to -105, Lane 12: -73 to -41

EMSA of the Turkey PRL 5'-Flanking Region (nt -41 to -673) using Liver Nuclear Extracts

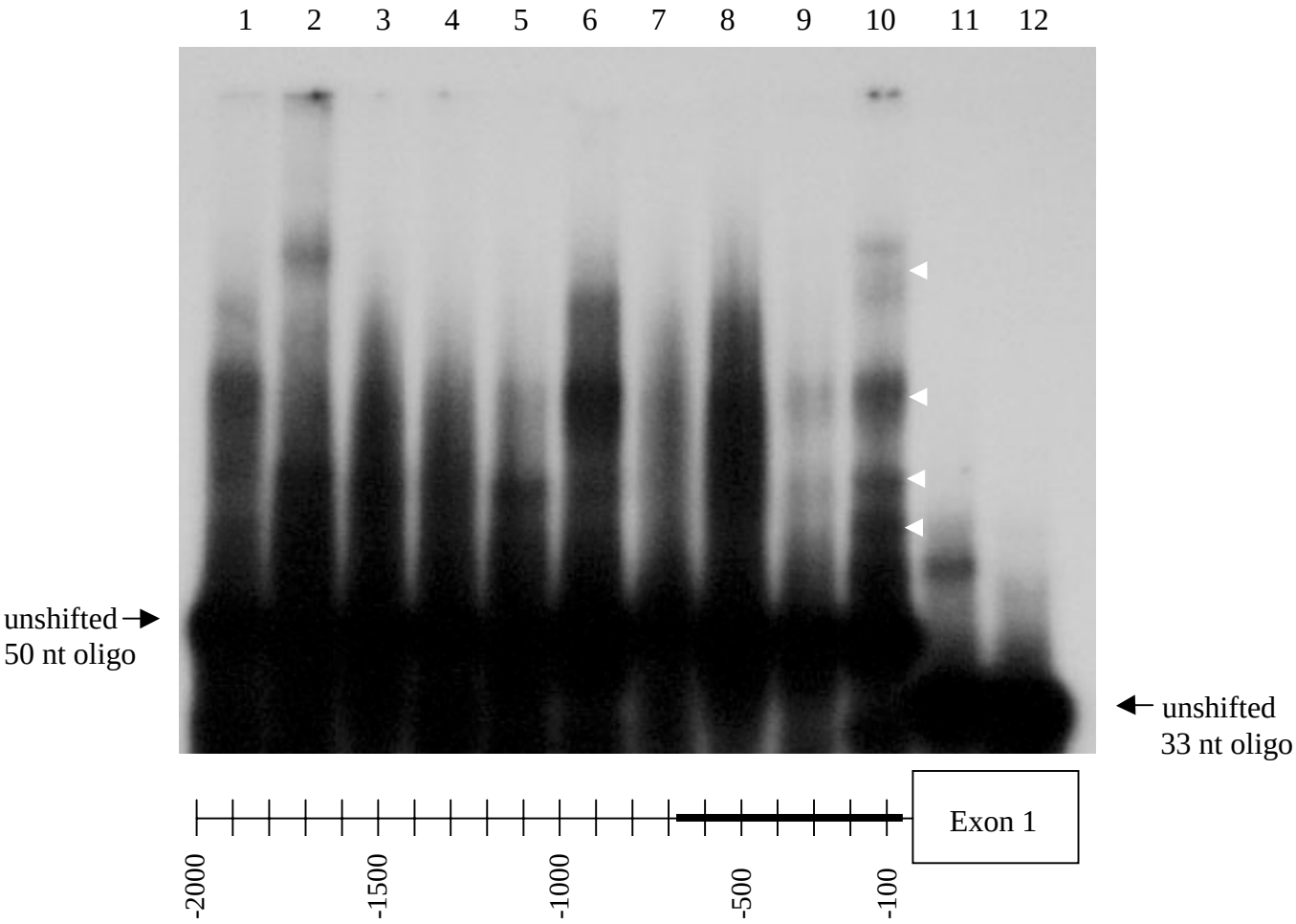

Figure IV-3c - The first series of EMSAs, representing nt -41 to -673. Each oligonucleotide was incubated with 20 µg of turkey liver nuclear extract. White arrowheads indicate tPRL-nuclear protein complexes. The exposure time was 2 hours and 30 minutes. Lane 1: -673 to -624, Lane 2: -623 to -574, Lane 3: -573 to -524, Lane 4: -523 to -474, Lane 5: -473 to -424, Lane 6: -423 to -375, Lane 7: -374 to -325, Lane 8: -324 to -275, Lane 9: -274 to -225, Lane 10: -224 to -175, Lane 11: -137 to -105, Lane 12: -73 to -41

EMSA of the Turkey PRL 5'-Flanking Region (nt -674 to -1373) using Pituitary Nuclear Extract

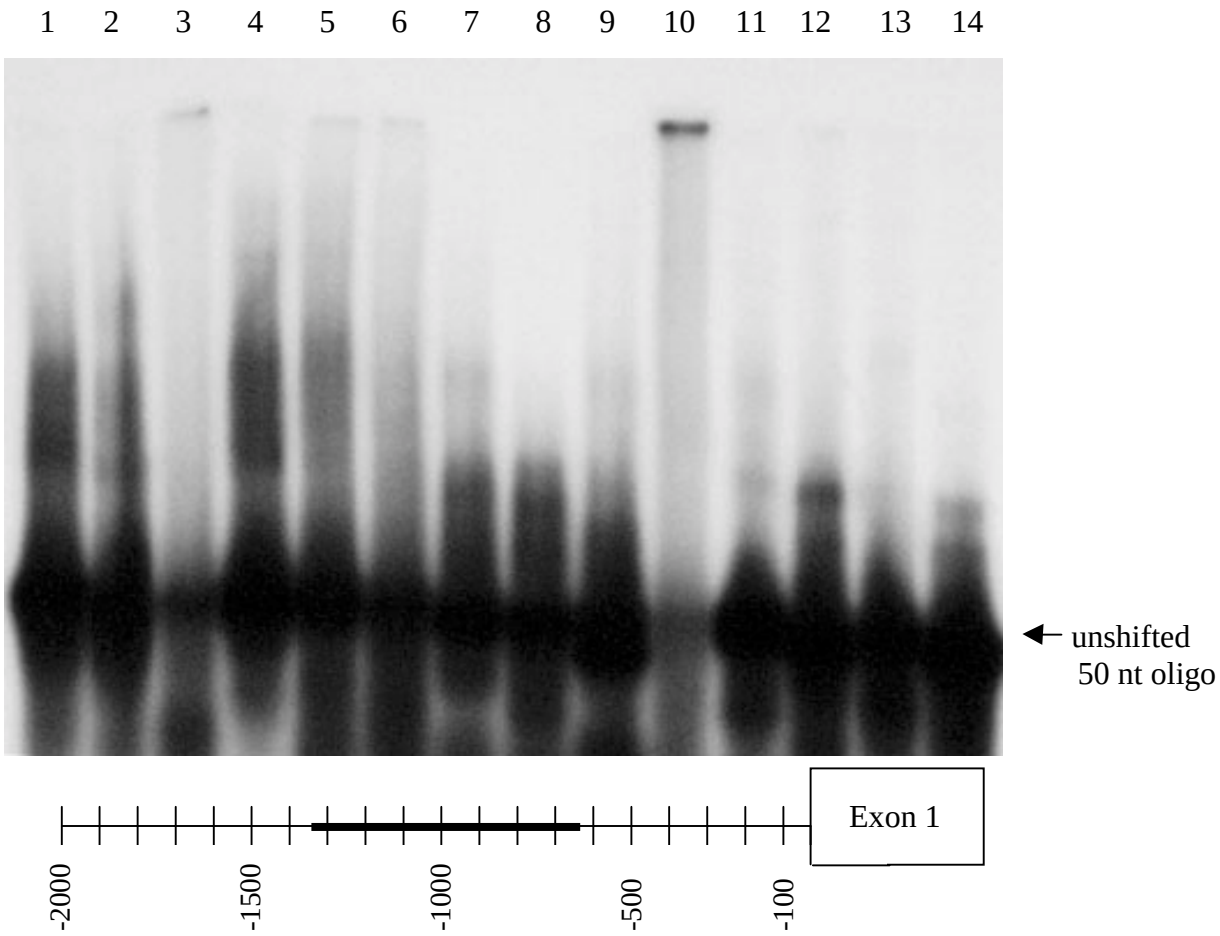

Figure IV-4a – The second series of EMSAs, representing nt -674 to -1373. Each oligonucleotide was incubated with 20 µg of turkey pituitary nuclear extract. The exposure time was 1 hour and 40 minutes. Lane 1: -1373 to -1324, Lane 2: -1323 to -1274, Lane 3: -1273 to -1224, Lane 4: -1223 to -1174, Lane 5: -1173 to -1124, Lane 6: -1123 to -1074, Lane 7: -1073 to -1024, Lane 8: -1023 to -974, Lane 9: -973 to -924, Lane 10: -923 to -874, Lane 11: -873 to -824, Lane 12: -823 to -774, Lane 13: -773 to -724, Lane 14: -723 to -674

EMSA of the Turkey PRL 5'-Flanking Region (nt -674 to -1373) using Liver Nuclear Extract

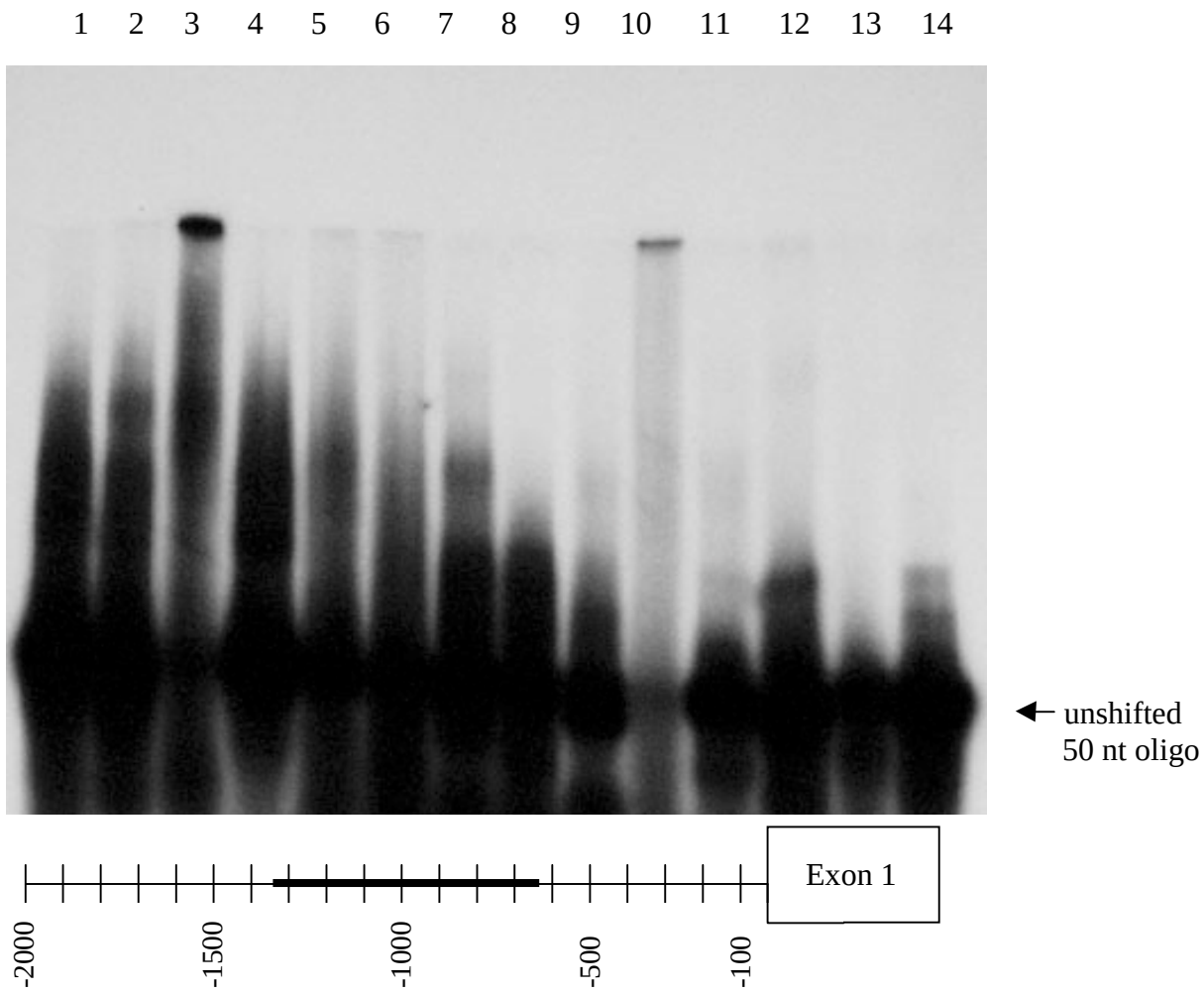

Figure IV-4b – The second series of EMSAs, representing nt -674 to -1373. Each oligonucleotide was incubated with 20 µg of turkey liver nuclear extract. The exposure time was 1 hour and 40 minutes. Lane 1: -1373 to 1324, Lane 2: -1323 to -1274, Lane 3: -1273 to -1224, Lane 4: -1223 to -1174, Lane 5: -1173 to -1124, Lane 6: -1123 to -1074, Lane 7: -1073 to -1024, Lane 8: -1023 to -974, Lane 9: -973 to -924, Lane 10: -923 to -874, Lane 11: -873 to -824, Lane 12: -823 to -774, Lane 13: -773 to -724, Lane 14: -723 to -674

EMSA of the Turkey PRL 5'-Flanking Region (nt -1374 to -1981) using Pituitary Nuclear Extract

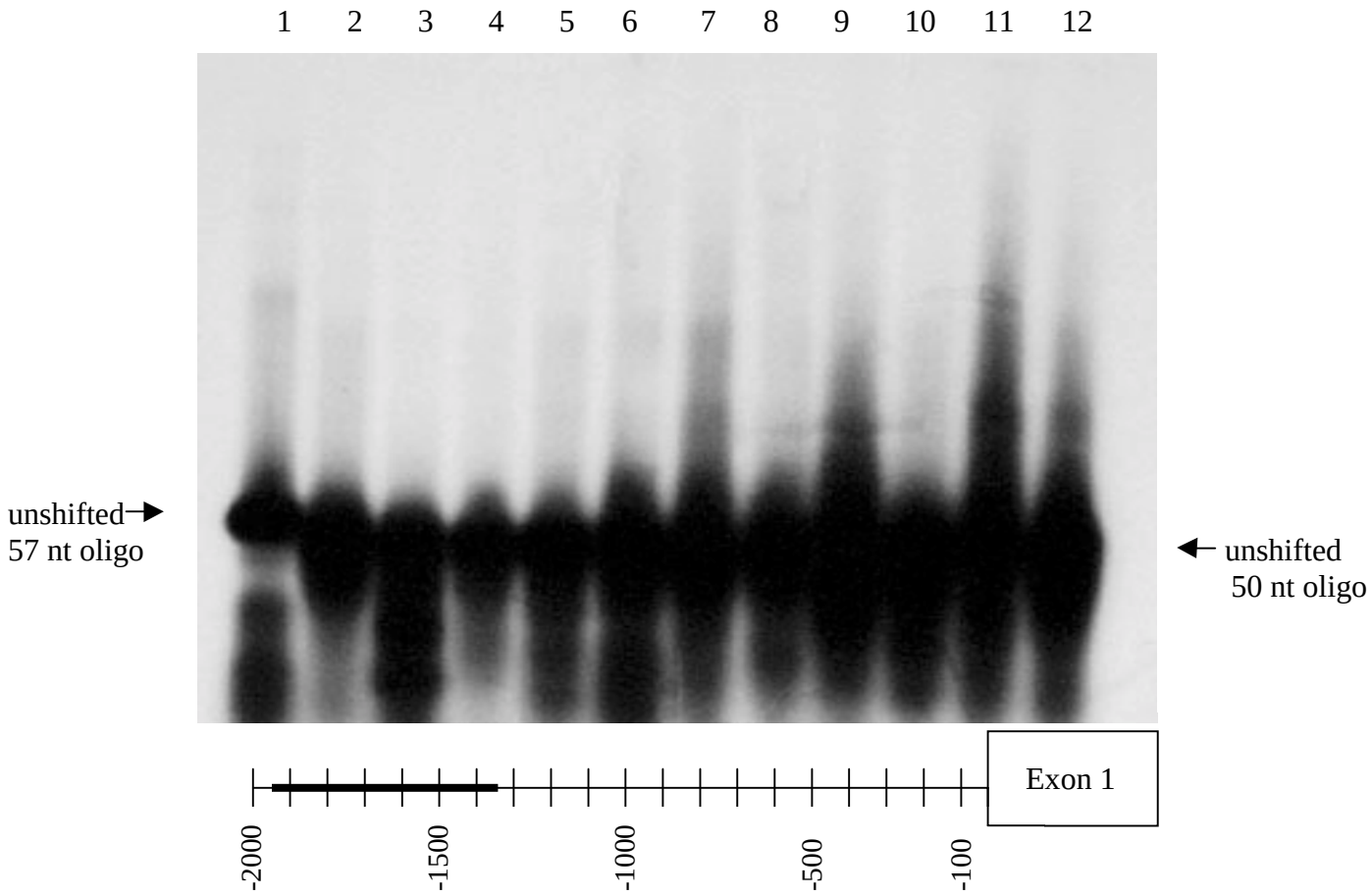

Figure IV-5a – The third series of EMSAs, representing nt -1374 to -1981. Each oligonucleotide was incubated with 20 µg of turkey pituitary nuclear extract. The exposure time was 1 hour and 10 minutes. Lane 1: -1981 to -1924, Lane 2: -1923 to -1874, Lane 3: -1873 to -1824, Lane 4: -1823 to -1774, Lane 5: -1773 to -1724, Lane 6: -1723 to -1674, Lane 7: -1673 to -1624, Lane 8: -1623 to -1574, Lane 9: -1573 to -1524, Lane 10: -1523 to -1474, Lane 11: -1473 to -1424, Lane 12: -1423 to -1374

EMSA of the Turkey PRL 5'-Flanking Region (nt -1374 to -1981) using Liver Nuclear Extract

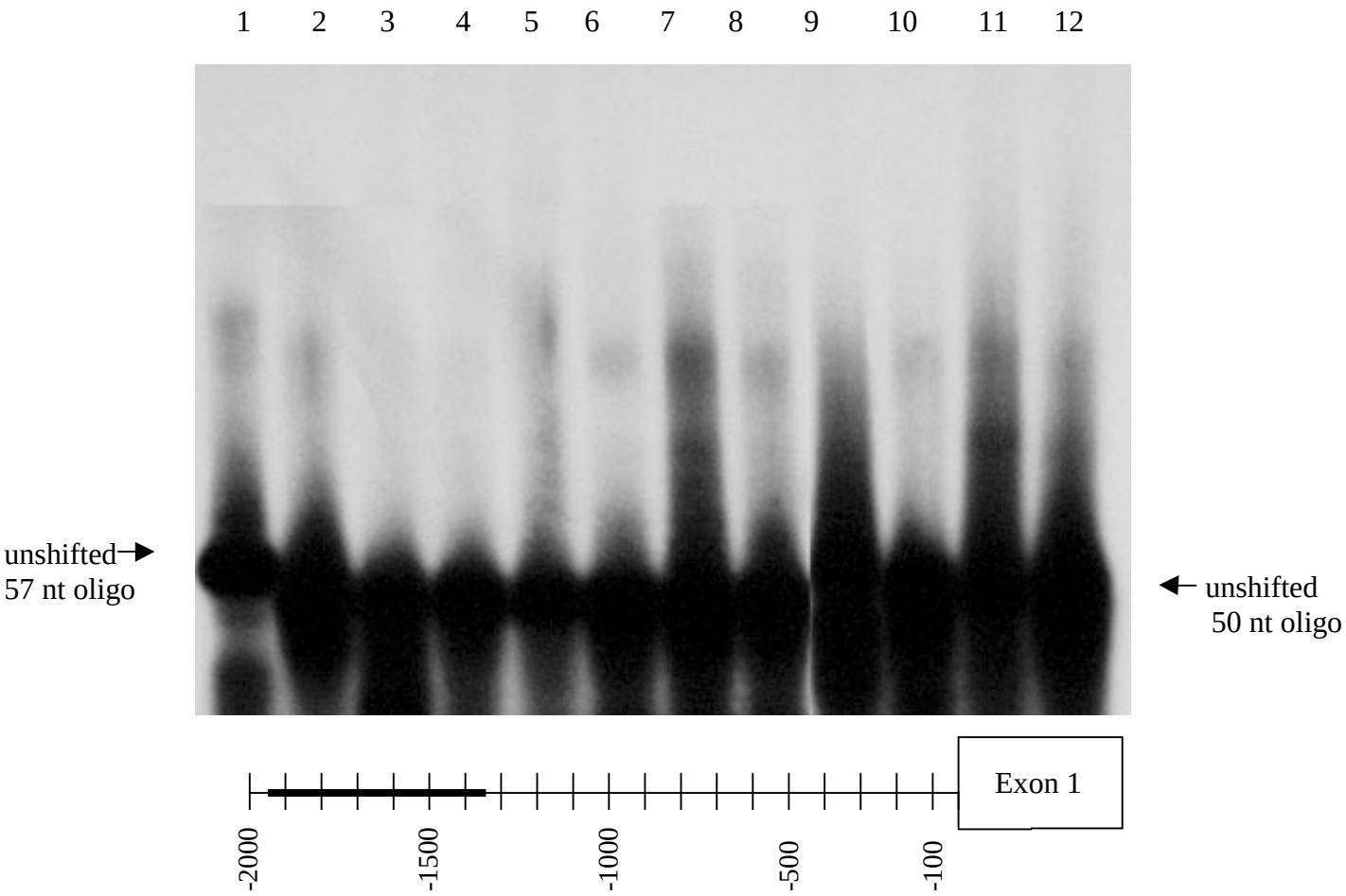

Figure IV-5b – The third series of EMSAs, representing nt -1374 to -1981. Each oligonucleotide was incubated with 20 µg of turkey liver nuclear extract. The exposure time was 1 hour and 10 minutes. Lane 1: -1981 to -1924, Lane 2: -1923 to -1874, Lane 3: -1873 to -1824, Lane 4: -1823 to -1774, Lane 5: -1773 to -1724, Lane 6: -1723 to -1674, Lane 7: -1673 to -1624, Lane 8: -1623 to -1574, Lane 9: -1573 to -1524, Lane 10: -1523 to -1474, Lane 11: -1473 to -1424, Lane 12: -1423 to -1374

EMSA of the Turkey PRL 5'-Flanking Region (nt -41 to -199) using Pituitary and Liver Nuclear Extracts

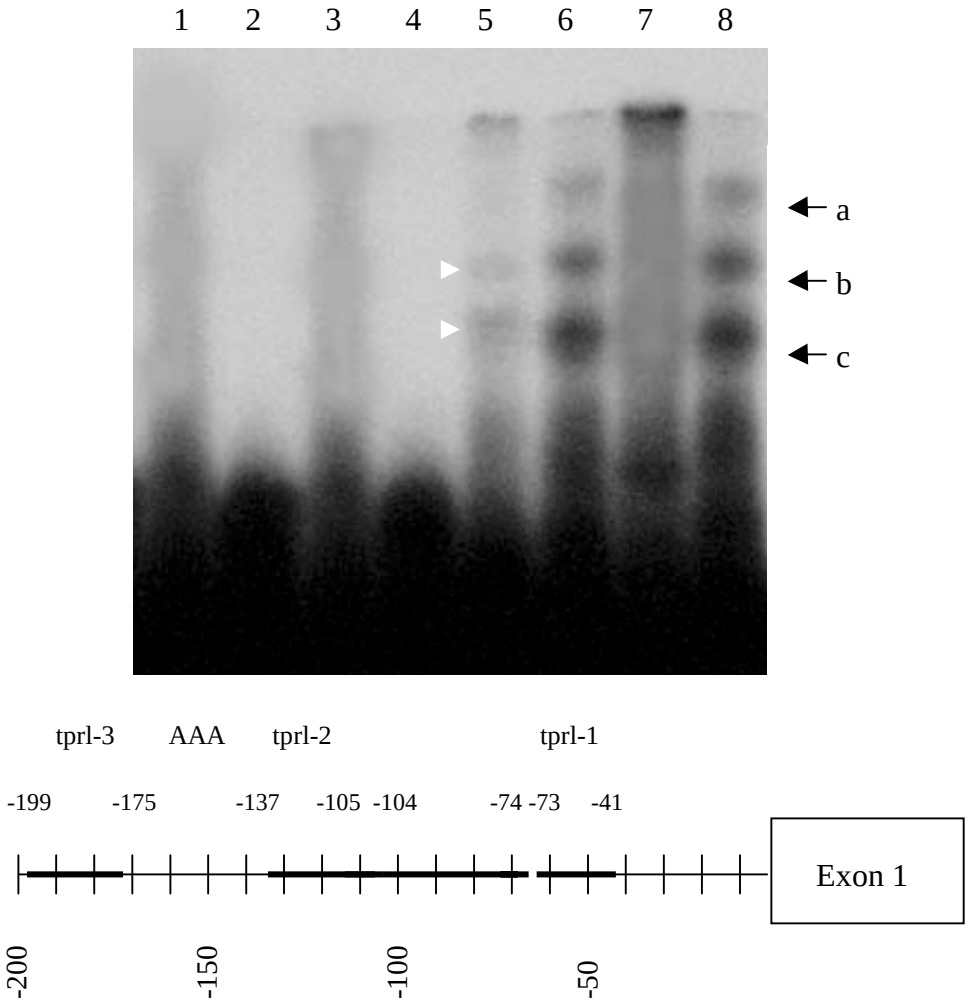

Figure IV-6 – EMSA using oligonucleotides from nt -41 to -199. White arrowheads and arrows a, b, and c indicate tPRL-nuclear protein complexes. The exposure time was 2 hours. Lanes 1-4: 20 µg liver nuclear extract, Lanes 5-8: 20 µg pituitary nuclear extract. Lanes 1 and 5: -175 to -199 (tprl-3), Lanes 2 and 6: -105 to -137 (tprl-2), Lanes 3 and 7: -74 to -104, Lanes 4 and 8: -41 to -73 (tprl-1)

Competition EMSA using tprl-1 and Pituitary Nuclear Extract

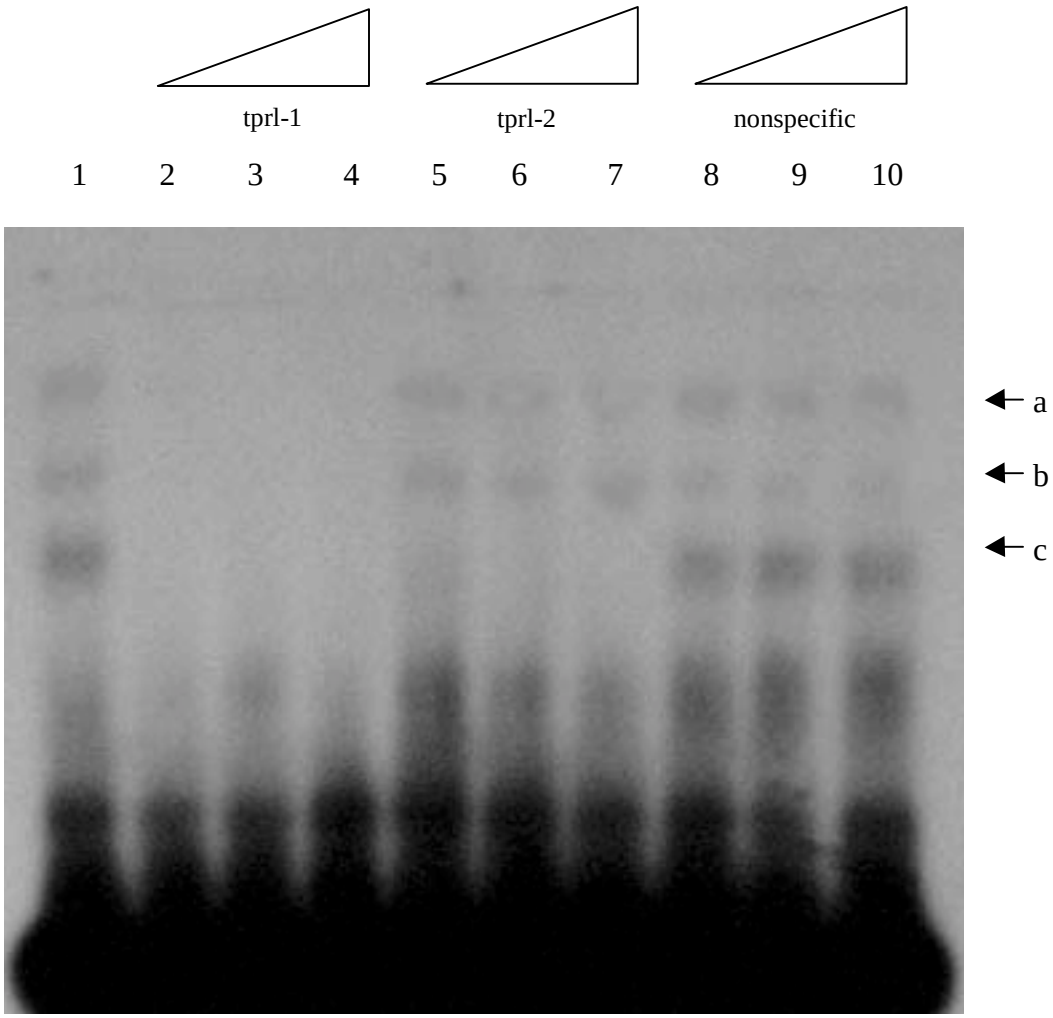

Figure IV-7 – Competition EMSA performed using labeled tprl-1, 20 µg nuclear extract from turkey pituitaries and varying amounts of unlabeled competitor DNA. Arrows a, b and c indicate tPRL-nuclear protein complexes. The exposure time was 3 hours. Lane 1: no competitor, Lanes 2-4: 10X, 25X and 50X tprl-1 competitor, Lanes 5-7: 10X, 25X and 50X tprl-2 competitor, Lanes 8-10: 10X, 25X and 50X nonspecific competitor

Competition EMSA using tprl-2 and Pituitary Nuclear Extract

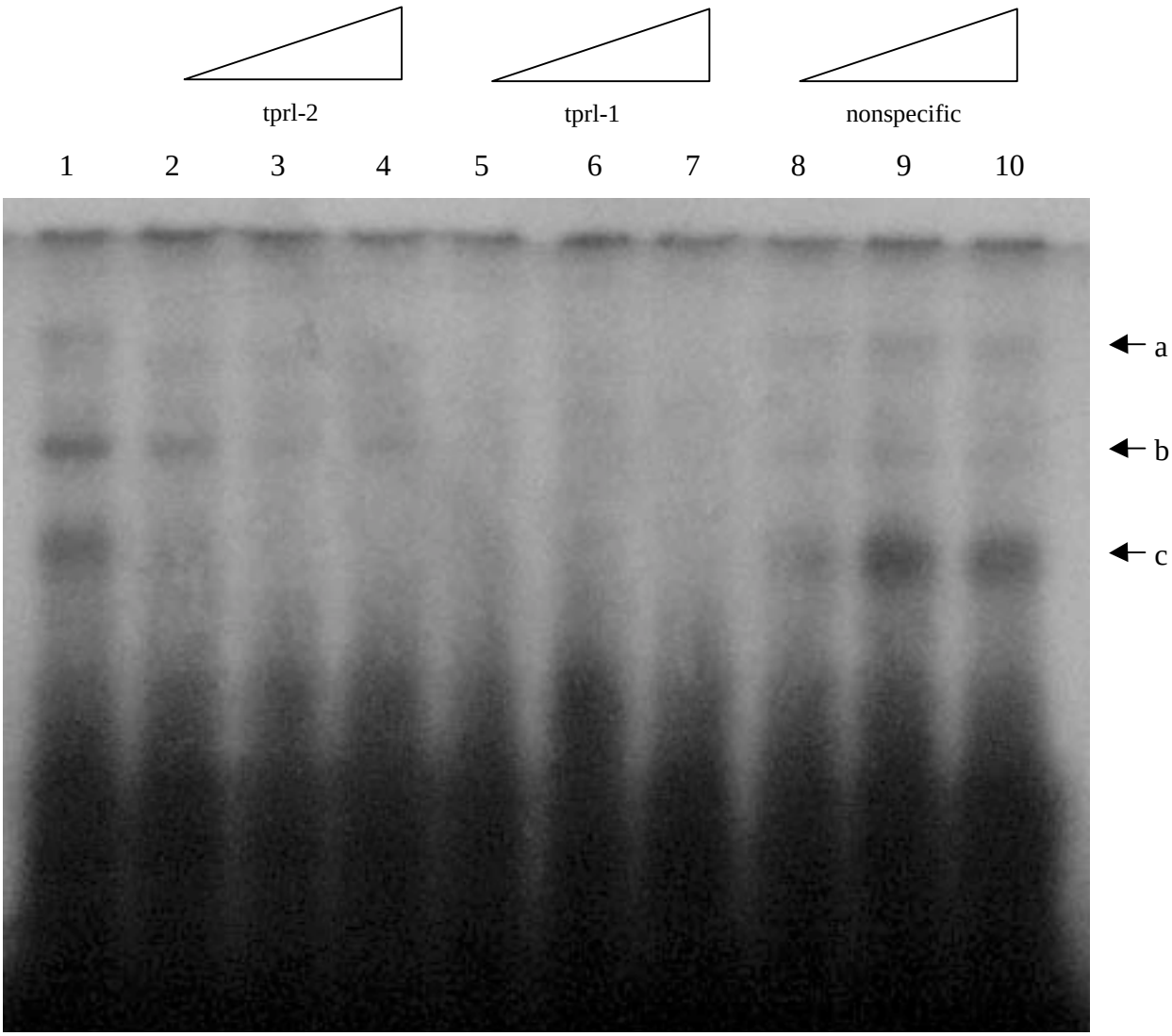

Figure IV-8 – Competition EMSA performed using labeled tprl-2, 20 µg nuclear extract from turkey pituitaries and varying amounts of unlabeled competitor DNA. Arrows a, b and c indicate tPRL-nuclear protein complexes. The exposure time was 24 hours. Lane 1: no competitor, Lanes 2-4: 10X, 25X and 50X tprl-2 competitor, Lanes 5-7: 10X, 25X and 50X tprl-1 competitor, Lanes 8-10: 10X, 25X and 50X nonspecific competitor

Competition EMSA using tprl-3 and Pituitary Nuclear Extract

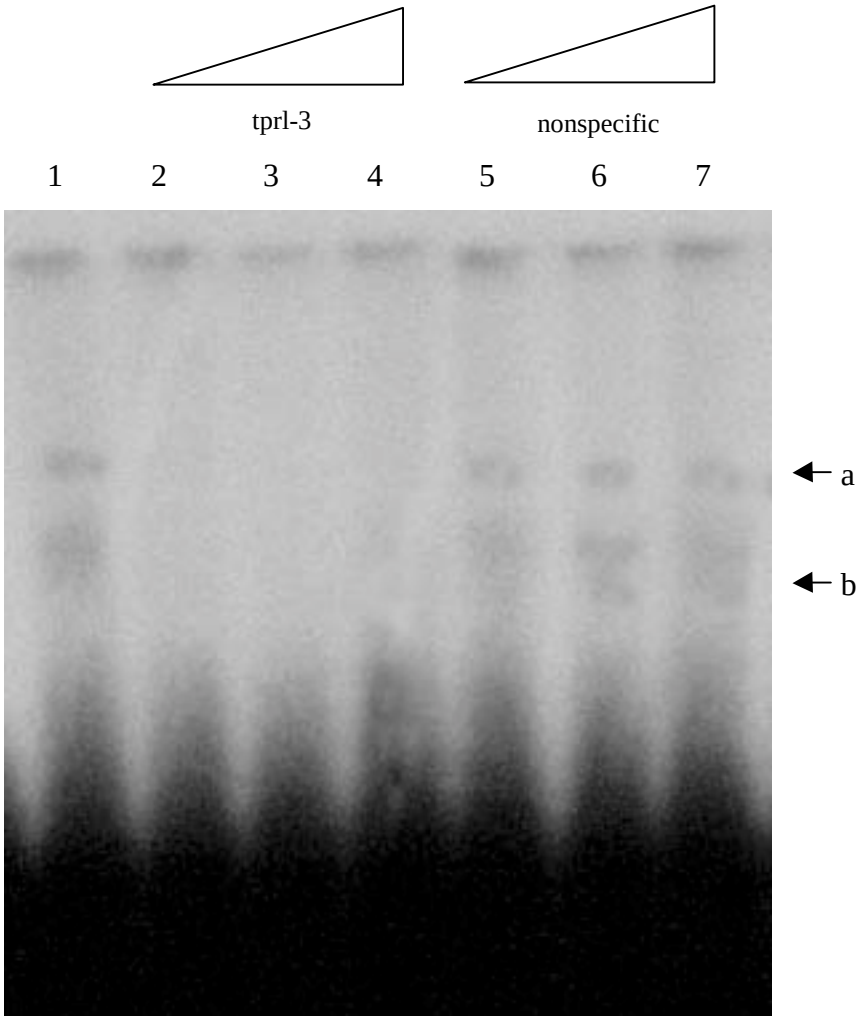

Figure IV-9 – EMSA performed using labeled tprl-3, 20 µg nuclear extract from turkey pituitaries and varying amounts of unlabeled competitor DNA. Arrows a and b indicate tPRL-nuclear protein complexes. The exposure time was 2 hours. Lane 1: no competitor, Lanes 2-4: 10X, 25X and 50X tprl-3, Lanes 5-7: 10X, 25X and 50X nonspecific competitor

Supershift EMSA using tprl-1, tprl-2 and Pituitary Nuclear Extract

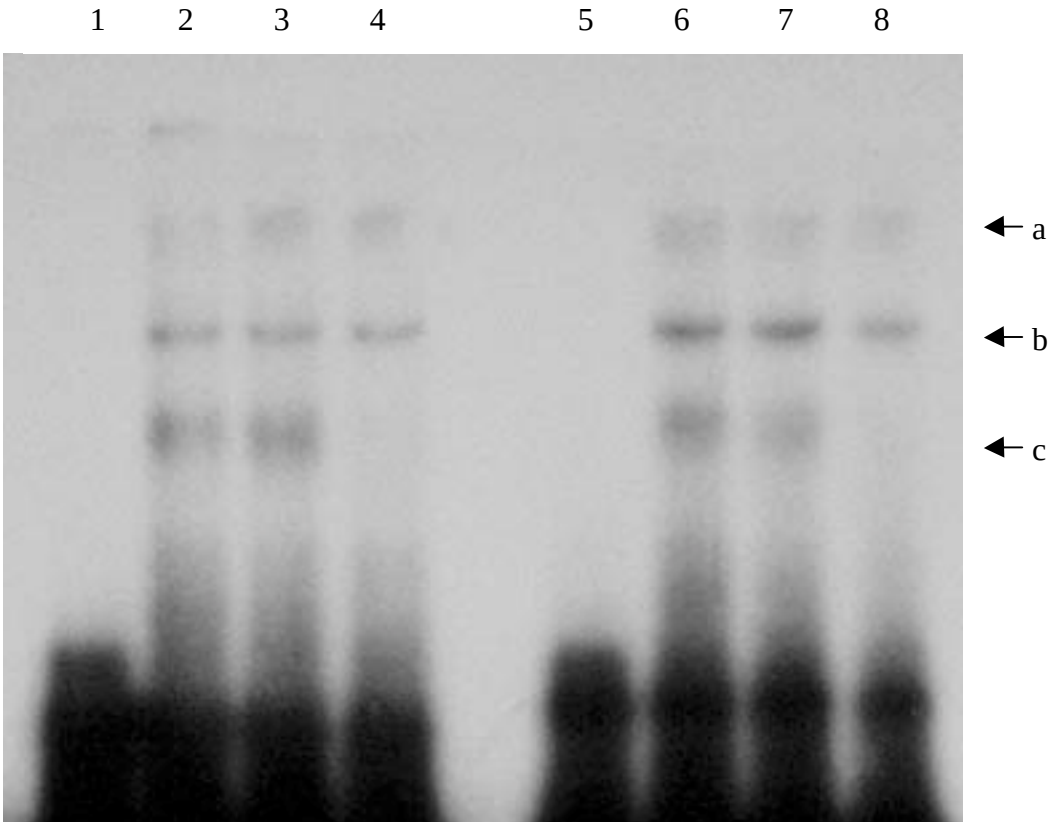

Figure IV-10 – Supershift EMSA with pituitary nuclear extract, rabbit-anti-rat Pit-1 and radiolabeled tprl-1 and tprl-2. Lanes 1-4: radiolabeled tprl-2, Lanes 5-8: radiolabeled tprl-1. Arrows a, b and c indicate tPRL-nuclear protein complexes. The exposure time was 3 hours. Lanes 1 and 5: no pituitary nuclear extract, Lanes 2 and 6: 20 µg pituitary nuclear extract only, Lanes 3 and 7: 20 µg pituitary nuclear extract plus 1 µg rabbit preimmune sera, Lanes 4 and 8: 20 µg pituitary nuclear extract plus 1 µg rabbit-anti-rat Pit-1

VITA

Lisa C. Gazzillo was born in Livingston, NJ, the daughter of Dr. Frank Gazzillo, M.D. and Mary Ellen Kautz. She graduated with a Bachelor of Arts degree in biology from the University of Virginia in May, 1998. She began work with Dr. Eric A. Wong on a Master of Science degree in Animal and Poultry Sciences at Virginia Tech in August, 1998 and finished in November, 2000.