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# Transcriptional Regulation of The Porcine GnRH Receptor Gene by Glucocorticoids

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# TRANSCRIPTIONAL REGULATION OF THE PORCINE GnRH RECEPTOR GENE BY GLUCOCORTICOIDS

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Binding of GnRH to its receptor (GnRHR) stimulates the synthesis and secretion of the gonadotropins, as well as up-regulation of GnRHR. Thus, the interaction between GnRH and GnRHR represents a central point for regulation of reproduction. Glucocorticoids alter reproduction by reducing GnRH responsiveness of gonadotropes within the anterior pituitary gland, potentially via transcriptional regulation of the GnRHR gene. Investigation into this mechanism, however, revealed that the murine GnRHR gene was stimulated by glucocorticoids. To determine the effect of glucocorticoids on porcine GnRHR gene expression, gonadotrope-derived αT3-1 cells were transiently transfected with a vector containing 5118 bp of 5' flanking sequence for the porcine GnRHR gene fused to luciferase for 12 h and treated with increasing concentrations of the glucocorticoid agonist, dexamethasone (0, 1, 10, 100 and 1,000 nM) for an additional 12 h prior to harvest. Maximal induction of luciferase activity was detected at 100 nM of dexamethasone (2-fold over vehicle; $P < 0.05$). Deletion from 274 to 323 bp of proximal promoter eliminated glucocorticoid responsiveness, suggestive of a glucocorticoid response element (GRE). Electrophoretic mobility shift assays (EMSAs) using a radiolabeled oligonucleotide spanning -290/-270 bp of proximal promoter

revealed increased binding of nuclear extracts from αT3-1 cells treated with 100 nM dexamethasone compared to vehicle. Mass spectrometry analysis of isolated proteins from a pull-down using a biotinylated oligonucleotide (-290/-270 bp) identified PARP-1 as the binding component. EMSAs with either GR or PARP-1 antibodies resulted in a supershift of the specific binding complex, whereas addition of both antibodies abolished the supershift. Inhibition of p38 and ERK1/2 mitogen-activated protein kinase (MAPK) pathways decreased dexamethasone-induced promoter activity ($P < 0.05$), indicating their involvement in glucocorticoid stimulation of the promoter. Thus, our working model for glucocorticoid responsiveness of the porcine GnRHR gene suggests that binding of glucocorticoid to its receptor (GR), triggers GR phosophorylation by p38 and ERK1/2 MAPK pathways, resulting in the recruitment of PARP-1 by phosphorylated, ligand-bound GR to a GRE located within -290/-270 bp of the porcine GnRHR promoter.

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# CHAPTER I

## INTRODUCTION

The decapeptide gonadotropin-releasing hormone (GnRH) is a key factor that mediates the function of the hypothalamic-pituitary-gonadal axis in mammals. Generated by hypothalamic neurons, GnRH is released in an intermittent manner, traveling to gonadotropes in the anterior pituitary gland via the hypothalamo-hypophyseal portal system (Fink, 1988). Gonadotropin-releasing hormone binds to high-affinity receptors on gonadotrope cells resulting in the biosynthesis and secretion of both follicle stimulating hormone (FSH) and luteinizing hormone (LH), which stimulate steroid synthesis and gametogenesis in the gonads (Clayton and Catt, 1981; Clarke et al., 1983; Mason et al., 1986). Upon binding to its receptor, GnRH regulates expression of at least 4 gonadotropic genes including those encoding: the common α-glycoprotein subunit, the specific LHβ- and FSHβ-subunits that combine to produce LH or FSH (Hamernik and Nett, 1988; Gharib et al., 1990), and the GnRH receptor (GnRHR) itself (Sealfon and Millar, 1995). Transcriptional regulation of GnRHR is mediated by GnRH via protein kinase A (PKA) and C (PKC) activation of multiple mitogen-activated protein kinase (MAPK) pathways including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 MAPK, and ERK5, also known as big MAPK1 (BMK1; Sundaresan et al., 1996; Roberson et al., 1999; Noar et al., 2000; Liu et al., 2003; Bonfil et al., 2004). Thus, the interaction between GnRH and its receptor represents a central point for regulation of reproductive function in mammals.

Many studies have examined responsiveness of the GnRHR gene to hormones including GnRH (White et al., 1999; Norwitz et al., 1999; Ellsworth et al., 2003; Liu et al., 2003), estradiol-17β (Gregg et al., 1990; Laws et al., 1990b; Wu et al., 1994; Duval et al., 2000), progesterone (Laws et al., 1990a; Cheng et al., 2001a), testosterone (Kaiser et al., 1993; Curtin et al., 2001; Zapatero-Caballero et al., 2003) activin (Fernandez-Vazquez et al., 1996; Pernasetti et al., 2001; Norwitz et al., 2002), and inhibin (Braden et al., 1990; Gregg et al., 1991; Wu et al., 1994). Several reports indicated that GnRH regulates GnRHR numbers and mRNA levels in the pituitary gland from the rat (Pieper et al., 1982; Clayton, 1982; Kaiser et al., 1993; Bauer-Dantoin et al., 1995), sheep (Turzillo et al., 1994), and cow (Vizcarra et al., 1997). In contrast, others reported no change in amounts of GnRHR mRNA after GnRH treatment in the gonadotrope-derived αT3-1 cell line (Tsutsumi et al., 1993, 1995; Alarid and Mellon, 1995). Besides regulation of the level of GnRHR mRNA, GnRH can both up- and down-regulate receptor numbers in the pituitary (McArdle et al., 1987; Uemura et al., 1992; Bauer-Dantoin et al., 1993; Conn et al., 1995). Norwitz et al. (1999) determined two elements involved in GnRH responsiveness, sequence underlying responsiveness to GnRH-1 and 2 (SURG-1 and SURG-2, respectively) and Kam et al. (2005) reported that nuclear factor Y (NF-Y) and octamer transcription factor-1 (Oct-1) bind to the SURG-1 element to increase basal and GnRH-stimulated expression of the mouse GnRHR gene. White et al. (1999) isolated an activation protein-1 (AP-1) element within SURG-2 that conferred GnRH responsiveness of the GnRHR promoter. Moreover, these investigators identified that GnRH regulation of the GnRHR gene was mediated via activation of an AP-1 element by PKC (White et al., 1999) and the JNK pathway (Ellsworth et al., 2003). In addition, the GnRHR

activating sequence (GRAS), important to basal promoter activity, also mediated activin responsiveness of the mouse GnRHR gene (Duval et al., 1999). The binding of Smad3 and 4 proteins to GRAS (Duval et al., 2000) and AP-1 complexes to an overlapping AP-1 element regulated activin responsiveness (Ellsworth et al., 2003). Further studies demonstrated that binding of the LIM homeodomain proteins, LHX2 and 3, to a downstream activin regulatory element (DARE) is also necessary for activin responsiveness (Cherrington et al., 2005; Cherrington et al., 2006).

Reproductive function is suppressed under various stress conditions including infection, malnutrition, restraint, strenuous exercise, and surgical trauma (Collu et al., 1984; Rabin et al., 1988; Rivest and Rivier, 1995). Hormones that comprise components of the HPA axis, such as CRH, arginine vasopressin, ACTH, and glucocorticoids have all been shown to inhibit GnRH/gonadotropin secretion at the hypothalamic and/or pituitary levels. CRH inhibits GnRH release in hypophyseal portal blood (Rabin et al., 1988) or GnRH pulse generator activity (Williams et al., 1990). Arginine vasopressin and ACTH are also reported to inhibit LH secretion by decreasing responsiveness of the pituitary to GnRH as well as decreasing GnRH release (Dobson and Smith, 2000; Cates et al., 1999; Mann et al., 1985).

Inhibitory effects of glucocorticoids on gonadotropin secretion have been well documented in a variety of species, ranging from rodents to ruminants and primates, including humans. Chronic or acute glucocorticoid treatment is reported to suppress LH secretion in normal (nonstressed) animals or subjects (Baldwin, 1979; Saketos et al., 1993; Breen et al., 2004). Thus, exogenous glucocorticoids injected into normal subjects do inhibit LH secretion. The importance of the report by Matsuwaki et al. (2006) is that it

elucidated the role of endogenous glucocorticoids released in response to stimuli induced by stressors. The investigators found that glucocorticoids are protective rather than inhibitory to LH secretion under stressful conditions. Their findings imply that there may be substantial differences in the observed actions of exogenous glucocorticoids administered into normal (nonstressed) subjects and endogenous glucocorticoids released in stressed subjects. Previous reports by the same authors showed that glucocorticoids counteract the suppressive effects of TNF-α, which mimics infectious stress, on both the pulsatile (Matsuwaki et al., 2003) and surge (Matsuwaki et al., 2004) secretion of LH in rats. In support of their findings, two other reports demonstrated that endogenous glucocorticoids did not mediate endotoxin-induced inhibition of pulsatile LH secretion in rats (Watanobe and Habu, 2003) and sheep (Debus et al., 2002). They show that lipopolysaccharide suppresses LH pulses even in adrenalectomized rats and in sheep treated with metyrapone, a glucocorticoid synthesis inhibitor.

Glucocorticoids can also act at both the hypothalamus and anterior pituitary gland to regulate gonadotropin secretion (Brann and Mahesh, 1991; Tilbrook et al., 2000). Recent investigations into stress-related influences on reproductive function implicated glucocorticoids in the physiological regulation of GnRH and its receptors. For example, glucocorticoids acted directly at the hypothalamus to suppress GnRH synthesis (Chandran et al., 1994) and reduced the activity of the GnRH pulse-generating center (Dubey and Plant, 1985). Similarly, in the anterior pituitary gland, glucocorticoids decreased GnRH responsiveness of gonadotrope cells (Kamel and Kubajak, 1987; Baldwin et al., 1991). In contrast to inhibition of GnRH secretion from the hypothalamus and reduced responsiveness of gonadotropes to GnRH, glucocorticoids also increased

GnRHR mRNA and protein levels. In male rats, the glucocorticoid agonist, dexamethasone, acted directly on gonadotrope cells to modulate a GnRH-induced increase in GnRHR numbers, as well as gonadotropin gene expression and secretion (Rosen et al, 1991). In addition, dexamethasone treatment increased endogenous GnRHR mRNA levels in the gonadotrope-derived LβT2 cell line (Turgeon et al., 1996) and activity of the murine GnRHR promoter in pituitary adenoma-derived $GGH_3$ (Maya-Núñez and Conn, 2003) and LβT2 (McGillivray et al., 2007) cells. Maya-Núñez and Conn (2003) identified the glucocorticoid response element (GRE) as an AP-1 binding site located between 255 and 331 bp upstream of the transcriptional start site that bound the transcription factor, c-Jun, suggesting that ligand-bound glucocorticoid receptors interact directly or indirectly with c-Jun to regulate GnRHR transcription. Our laboratory has isolated the porcine GnRHR gene promoter and identified elements conferring basal activity in αT3-1 cells. The objectives of this study are to determine glucocorticoid responsiveness of the porcine GnRHR gene, to isolate the glucocorticoid response element(s) located within the porcine GnRHR promoter and to examine the molecular mechanisms underlying glucocorticoid stimulation of GnRHR gene expression in αT3-1 cells.

# CHAPTER II

# LITERATURE REVIEW

## Characterization of Gonadotropin-Releasing Hormone

### Function of Gonadotropin-releasing hormone

Gonadotropin-releasing hormone (GnRH) is a hypothalamic decapeptide that plays a critical role in regulation of reproduction. It was first isolated from mammalian hypothalami (Schally et al., 1971; Matsuo et al., 1971; Baba et al., 1971), and is important for normal mammalian sexual maturation as well as reproductive function and pregnancy (Bauer-Dantoin et al., 1993; Kaiser et al., 1997; Zapatero-Caballero et al., 2003; Granger et al., 2004). GnRH is secreted from hypothalamic neurons in a pulsatile manner every 30-120 minutes (Millar et al., 2004), travels through a portal system to the anterior pituitary gland, and stimulates one of five cell types, gonadotropes, in the anterior pituitary gland (Conn and Crowley, 1994; Carmel et al., 1976; Levine et al., 1982). GnRH binds to its cognate receptor on the plasma membrane of gonadotrope cells to stimulate the biosynthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH; Figure 2.1). Upon binding to is receptor, GnRH upregulates at least gonadotropic genes including: the common α-glycoprotein subunit, specific LH and FSH β-subunits and the GnRHR itself (Gharib et al., 1990; Sealfon and Millar, 1995). The common α-subunit then interacts with either LH or FSH β-subunits to produce LH and

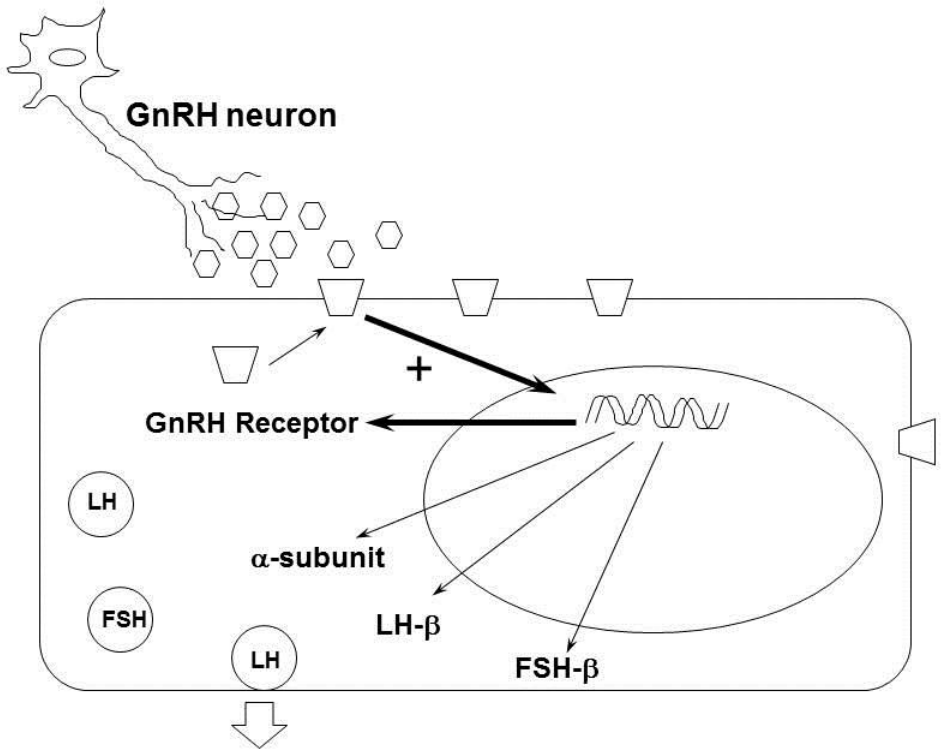



Figure 2.1. Diagram of GnRH action on a gonadotrope cell. GnRH is secreted by neurons in the hypothalamus and acts on gonadotrope cells of the anterior pituitary gland. Binding of GnRH to its cognate receptor on the plasma membrane of the cell causes the upregulation of genes encoding the common glycoprotein hormone α-subunit and specific FSHβ- and LHβ-subunit genes, as well as the GnRHR itself.

FSH, respectively (Clarke et al., 1983). Following synthesis and secretion of these gonadotropins, they act on the gonads to function in reproduction. LH causes ovulation in the female and promotes testosterone production in the male (Velardo, 1960; Veyssiere et al., 1977). FSH recruits follicle development in the female and stimulates spermatogenesis in the male (Velardo, 1960; Weissenberg et al., 1982). In addition, gonadotropins induced the production of androgens, progesterone and estradiol-17β. These steroid hormones can negatively feedback at the level of the anterior pituitary and the hypothalamus to regulate GnRH and gonadotropin secretion (Nakai et al., 1978; McNeilly et al., 2003). Therefore, the interaction between GnRH and its receptor represents a central point for regulation of reproductive function in mammals.

In addition to its role in regulating reproduction, GnRH can also have diverse functions in neuroendocrine, paracrine, autocrine and neurotransmitter/neuromodulatory roles. Neuroendocrine functions have been shown in growth hormone release in certain fish species, paracrine roles in placenta and gonads, autocrine actions in GnRH neurons, immune cells, breast and prostatic cancer cells, and neurotransmitter/neuromodulatory roles in the central and peripheral nervous system (Emons and Schally, 1994; Sherwood, 1987; Millar and King, 1987; Millar and King 1988; Jennes and Conn, 1994; Millar et al., 2004; Millar, 2005; Kochman, 2012). It has been reported that GnRH peptides may originally function in cellular communication in sexual reproduction of simple organisms. Later, they were recruited to nerve cells to translate external and internal signals into activation of reproduction, initially by acting directly on germ cells, and subsequently via pituitary gonadotrope activation (Millar, 2005; Millar et al., 1987; Hsueh and Schaeffer, 1985; Jennes and Conn, 1994)

**Structure of Gonadotropin-Releasing Hormone**

Mammalian GnRH was first isolated from the ovine, bovine and porcine hypothalamus (Kochman, 1966; Kochman 1969; Kochman and Domański, 1969), but its characterization and primary structure were not known until Andrew V. Schally and Roger Guillemin demonstrated them in 1971 (Matsuo et al., 1971; Burgus et al., 1972). During subsequent years of intensive studies, 30 structurally different forms of GnRH have been identified (Table 2.1). In vertebrates, 15 structural variants of the GnRH molecule have been reported and 15 variants were found in invertebrates (Millar et al., 2004; Roch et al., 2011).

The mammalian GnRH peptide consists of 10 amino acids which is pGlu-His-Trp-Ser-Tyr-Leu-Arg-Pro-Gly-$NH_2$ (Schally et al., 1971). The N-terminal (pGlu-His-Trp-Ser) and C-terminal (Pro-Gly-$NH_2$) sequences have been highly conserved for approximately 600 million years of evolution (Millar et al., 2004; Millar, 2005), with the exception of two cases of conservative Tyr substitutions (Table 2.1). These highly conservative features are critically important for receptor binding and activation and variations in positions 5, 7 and 8 are important for ligand selectivity (Millar et al., 2004; Kochman, 2012). However, position 8 is the most variable amino acid and this variation suggests that virtually any residue is tolerated in the position. Therefore, this residue may play an important function in ligand-selectivity of the different GnRH receptors (Millar, 2005). It has been reported that these conserved $NH_2$- and COOH-terminal groups are closely apposed when mammalian GnRH binds its receptor, due to a β-II type turn involving residues 5-8 (Figure 2.2; Sealfon et al., 1997; Karten and Rivier, 1986). This conformational change results from intramolecular interactions with side chain of Arg in

TABLE 2.1 AMINO ACID SEQUENCES OF NATURALLY OCCURRING GnRH STRUCTURAL VARIANTS SPANNING APPROXIMATELY 500 MILLION YEAR OF EVOLUTION

| | | Amino acid sequence | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| 1 | Mammal* | **pGlu** | **His** | **Trp** | **Ser** | Tyr | Gly | **Leu** | **Arg** | **Pro** | **Gly** | **$NH_2$** |
| 2 | Chicken I | **pGlu** | **His** | **Trp** | **Ser** | Tyr | Gly | **Leu** | **Gln** | **Pro** | **Gly** | **$NH_2$** |
| 3 | Guinea Pig | **pGlu** | ***Tyr*** | **Tyr** | **Ser** | Tyr | Gly | **Val** | **Arg** | **Pro** | **Gly** | **$NH_2$** |
| 4 | Frog | **pGlu** | **His** | **Trp** | **Ser** | Tyr | Gly | **Leu** | **Trp** | **Pro** | **Gly** | **$NH_2$** |
| 5 | Seabream | **pGlu** | **His** | **Trp** | **Ser** | Tyr | Gly | **Leu** | **Ser** | **Pro** | **Gly** | **$NH_2$** |
| 6 | Salmon*** | **pGlu** | **His** | **Trp** | **Ser** | Tyr | Gly | **Trp** | **Leu** | **Pro** | **Gly** | **$NH_2$** |
| 7 | Medaka | **pGlu** | **His** | **Trp** | **Ser** | Phe | Gly | **Leu** | **Ser** | **Pro** | **Gly** | **$NH_2$** |
| 8 | Catfish | **pGlu** | **His** | **Trp** | **Ser** | His | Gly | **Leu** | **Asn** | **Pro** | **Gly** | **$NH_2$** |
| 9 | Whitefish | **pGlu** | **His** | **Trp** | **Ser** | Tyr | Gly | **Met** | **Asn** | **Pro** | **Gly** | **$NH_2$** |
| 10 | Herring | **pGlu** | **His** | **Trp** | **Ser** | His | Gly | **Leu** | **Ser** | **Pro** | **Gly** | **$NH_2$** |
| 11 | Dogfish | **pGlu** | **His** | **Trp** | **Ser** | His | Gly | **Trp** | **Leu** | **Pro** | **Gly** | **$NH_2$** |
| 12 | Lamprey II | **pGlu** | **His** | **Trp** | **Ser** | His | Gly | **Trp** | **Phe** | **Pro** | **Gly** | **$NH_2$** |
| 13 | Chicken II** | **pGlu** | **His** | **Trp** | **Ser** | His | Gly | **Trp** | **Tyr** | **Pro** | **Gly** | **$NH_2$** |
| 14 | Lamprey III | **pGlu** | **His** | **Trp** | **Ser** | His | Gly | **Trp** | **Lys** | **Pro** | **Gly** | **$NH_2$** |
| 15 | Lamprey I | **pGlu** | **His** | ***Tyr*** | **Ser** | Leu | Glu | **Trp** | **Lys** | **Pro** | **Gly** | **$NH_2$** |
| 16 | Chelyosoma I | **pGlu** | **His** | **Trp** | **Ser** | Asp | Tyr | **Phe** | **Lys** | **Pro** | **Gly** | **$NH_2$** |
| 17 | Chelyosoma II | **pGlu** | **His** | **Trp** | **Ser** | Leu | Cys | **His** | **Ala** | **Pro** | **Gly** | **$NH_2$** |
| 18 | Ciona I | **pGlu** | **His** | **Trp** | **Ser** | Tyr | Ala | **Leu** | **Ser** | **Pro** | **Gly** | **$NH_2$** |
| 19 | Ciona II | **pGlu** | **His** | **Trp** | **Ser** | Leu | Ala | **Leu** | **Ser** | **Pro** | **Gly** | **$NH_2$** |
| 20 | Ciona III | **pGlu** | **His** | **Trp** | **Ser** | Asn | Gln | **Leu** | **Thr** | **Pro** | **Gly** | **$NH_2$** |
| 21 | Ciona IV | **pGlu** | **His** | **Trp** | **Ser** | Tyr | Glu | **Phe** | **Met** | **Pro** | **Gly** | **$NH_2$** |
| 22 | Ciona V | **pGlu** | **His** | **Trp** | **Ser** | Tyr | Glu | **Tyr** | **Met** | **Pro** | **Gly** | **$NH_2$** |
| 23 | Ciona VI | **pGlu** | **His** | **Trp** | **Ser** | Lys | Gly | **Tyr** | **Ser** | **Pro** | **Gly** | **$NH_2$** |
| 24 | Ciona VII | **pGlu** | **His** | **Trp** | **Ser** | Asn | Lys | **Leu** | **Ala** | **Pro** | **Gly** | **$NH_2$** |

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | Octopus | **pGlu** | Asn | Tyr | His | Phe | Ser | Asn | **Gly** | Trp | His | Pro | **Gly** | **$NH_2$** |
| 26 | Sea Urchin | **pGlu** | Val | His | His | Arg | Phe | Ser | **Gly** | Trp | Arg | Pro | **Gly** | **$NH_2$** |
| 27 | Aplysia | **pGlu** | Asn | Tyr | His | Phe | Ser | Asn | **Gly** | Trp | Tyr | Ala | **-** | **$NH_2$** |
| 28 | Limpet | **pGlu** | His | Tyr | His | Phe | Ser | Asn | **Gly** | Trp | Lys | Ser | **-** | **$NH_2$** |
| 29 | Marine worm | **pGlu** | Ala | Tyr | His | Phe | Ser | His | **Gly** | Trp | Phe | Pro | **-** | **$NH_2$** |
| 30 | Leech | **pGlu** | Ser | Ile | His | Phe | Ser | Asn | Ser | Trp | Gln | Pro | **-** | **$NH_2$** |

* mammal - mammalian GnRH; mGnRH:GnRH I

** chicken II - cGnRH II : GnRH II

*** salmon GnRH - sGnRH : GnRH III

† The encircled amino acid residues (on the left and on the right) show the conserved $NH_2$- and COOH-terminal residues that play important functional roles. Non-conserved residues are either unimportant or convey ligand selectivity for a particular GnRH receptor. The GnRHs are named according to the species in which they were first discovered, and they may be represented in more than one species. Adapted from Kochman (2012).

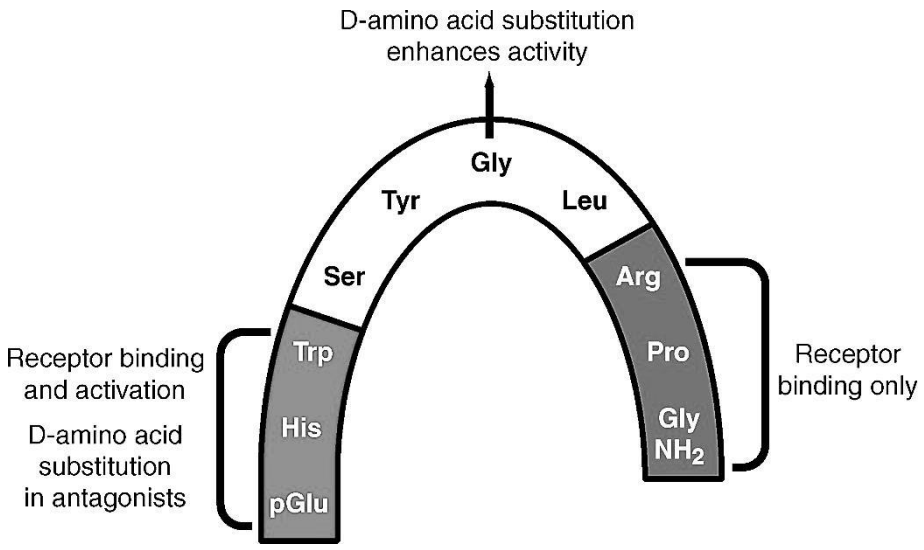



Figure 2.2. Schematic presentation of mammalian GnRH in the folded conformation in which it is bound to the GnRH receptor. The molecule is bent around the flexible glycine in position six. Substitution with D-amino acids in this position stabilizes the folded conformation, increases binding affinity, and decreases metabolic clearance. This feature is incorporated in all agonist and antagonist analogues. The $NH_2$ and COOH termini are involved in receptor binding. The $NH_2$ terminus alone is involved in receptor activation and substitutions in this region produce antagonists. Adapted from Millar (2005)

position 8 (Milton et al., 1983; Guarnieri and Weinstein, 1996). In addition, substitution of Arg caused a more extended form with a loss of a specific tertiary structure and a low binding affinity for its receptor (Maliekal et al., 1997). These extended forms, however, showed high activity in non-mammalian GnRH receptors (Sealfon et al., 1997; Illing et al., 1999; Tensen et al., 1997), whereas they exhibited low activity at mammalian receptors (Flanagan et al., 1994; Fromme et al., 2001).

## Characterization of Gonadotropin-Releasing Hormone Receptor

### Amino Acid Sequences of Gonadotropin-Releasing Hormone Receptor

The amino acid sequence of the GnRH receptor (GnRHR) was established first for the mouse receptor cloned from the murine gonadotrope-derived cell line, αT3-1, utilizing a PCR-based homology cloning strategy (Windle et al., 1990; Sealfon et al., 1990; Tsutsumi et al, 1992) and was subsequently confirmed using Xenopus oocyte and mammalian cell line expression cloning (Reinhart et al., 1992; Perrin et al., 1993). This sequence provided the basis for cloning of additional homologous pituitary cDNAs in other mammalian species including the human (Kakar et al., 1992; Chi et al., 1993), rat (Eidne et al., 1992; Kaiser et al., 1992; Perrin et al., 1993), sheep (Brooks et al., 1993; Illing et al., 1993), cow (Kakar et al., 1993) and pig (Weesner and Matteri, 1994). An alignment of the cloned GnRHR sequences is shown in Figure 2.3. This sequence alignment shows more than 85% conserved homology overall and is nearly identical within the putative transmembrane domains (Sealfon et al., 1997; Cheng and Leung, 2000). Also, this alignment revealed that the cow, sheep, pig and human receptors have

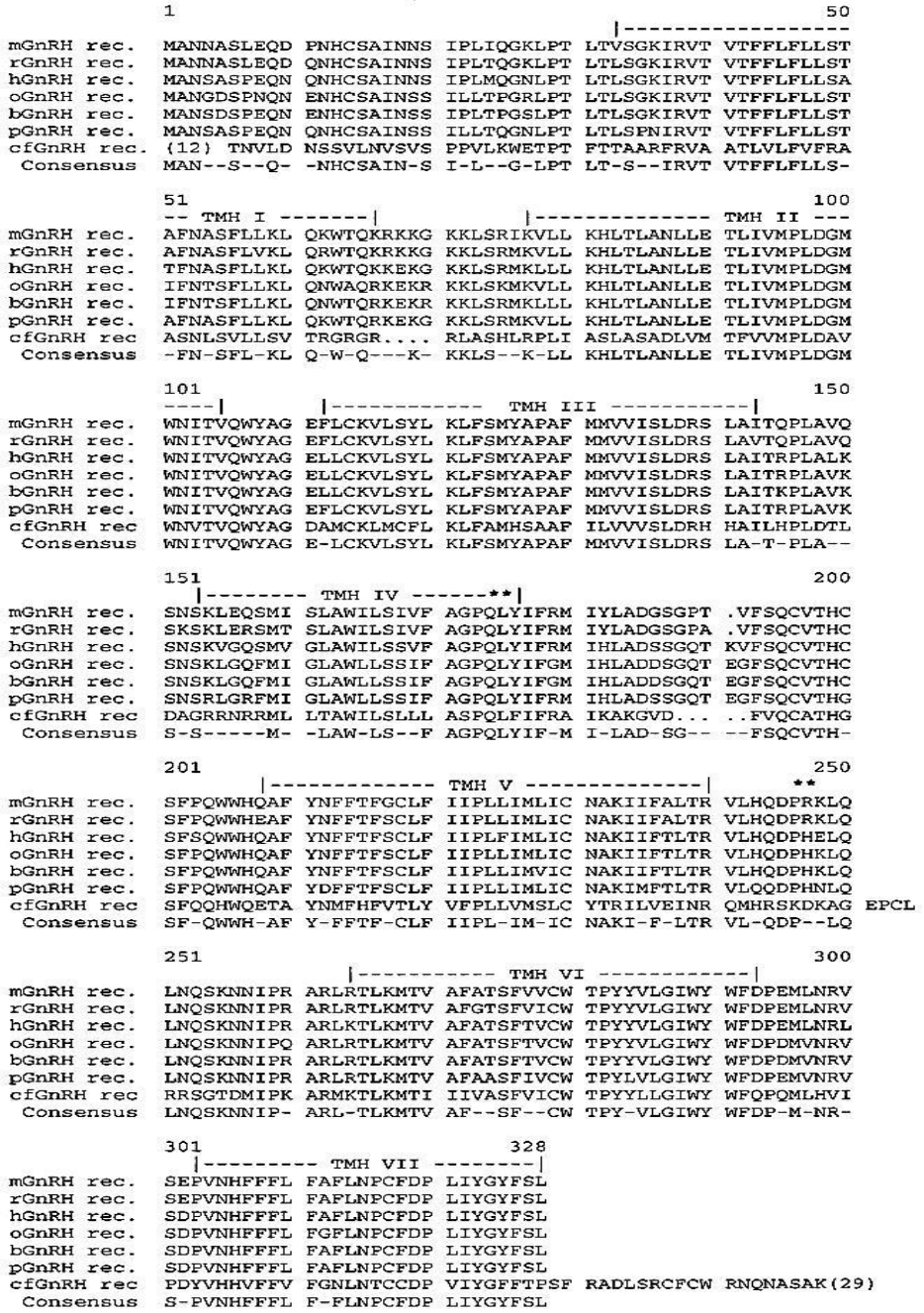



Figure 2.3. Sequence alignment of cloned GnRH receptors. The putative transmembrane domains are indicated. m, Murine; r, rat; h, human; o, ovine; b, bovine; p, pig; cf, catfish. The consensus sequence reflects the mammalian sequences only. The junctions between exons in the murine and human receptors are indicated by **. Adapted from Sealfon (1997).

one more amino acid compared to the mouse and rat receptors (327 amino acid residues), due to the absence of one residue in the second extracellular domain (Kochman, 2012; Cheng and Leung, 2000; Millar, 2005). Unlike the mammalian receptors, non-mammalian receptors have only 58 – 68% identity among each other and those with the greatest homology to mammalian pituitary receptors have at most 42 – 47% amino acid identity (Millar, 2005; Millar et al., 2004; Kochman, 2012).

**Structure of Gonadotropin-Releasing Hormone Receptor**

The GnRHR is a member of the rhodopsin-like, G protein-coupled receptor (GPCR) family which contains seven transmembrane (TM) domains (Stojilkovic et al., 1994; Sealfon et al., 1997; Cui et al., 2000). The $NH_2$-terminal group is followed by seven putative, α-helical TM domains connected by three extracellular loop and three intracellular loop domains (Figure 2.4; Kakar et al., 2002; Neill, 2002; Millar, 2005). The extracellular and superficial regions of the TMs are usually important for conformational change during receptor activation following ligand binding, whereas the intracellular domains are involved in interacting with G-proteins and other proteins associated with intracellular transduction (Millar, 2005; Kakar et al., 2002).

Unlike other members of the GPCR family, GnRHRs do not have an intracellular C-terminal domain which is required for desensitization and internalization of many GPCRs (Conn et al., 1995; Kakar et al., 1992; Tsutsumi et al., 1992; Stojilkovic et al., 1994). In most GPCRs, the C-terminus contains several phosphorylation sites that are involved in ligand binding and play an important role in receptor desensitization

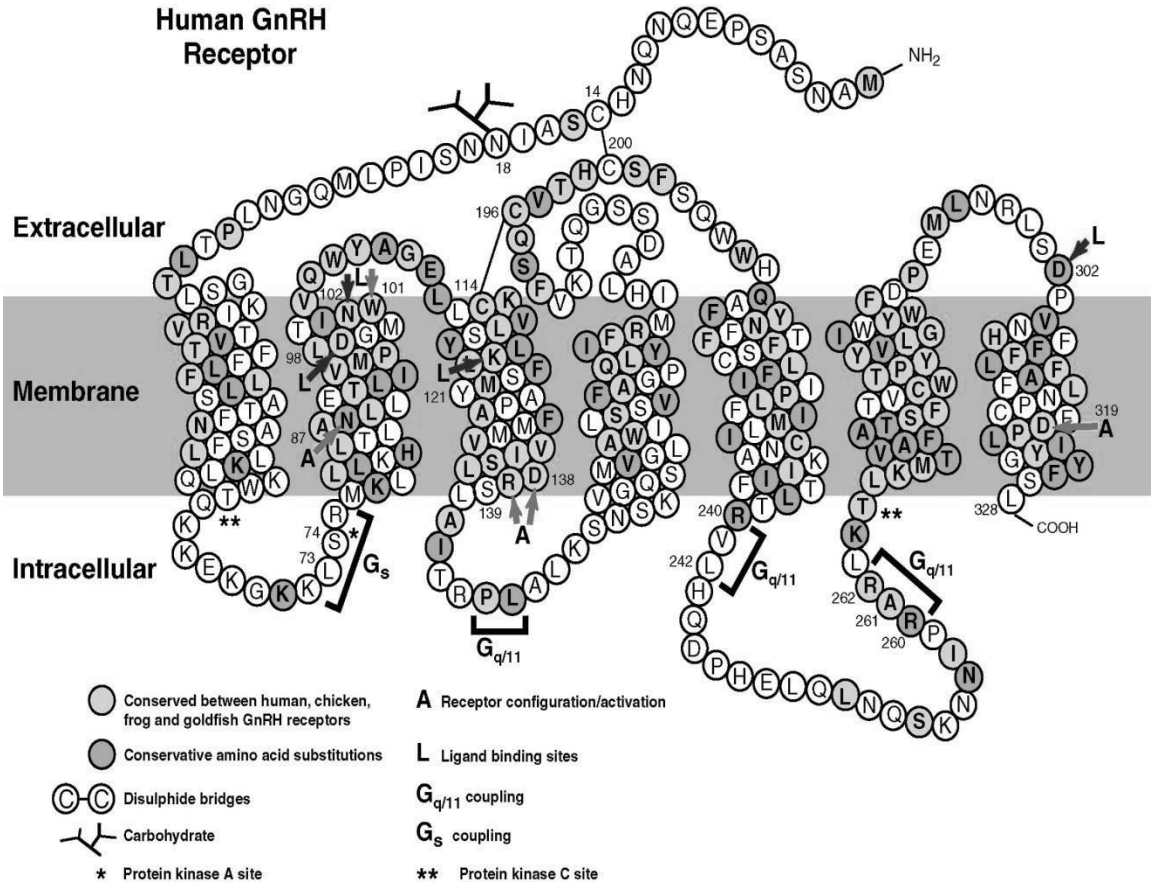



Figure 2.4. Two-dimensional representation of the human GnRH receptor showing amino acids conserved between cloned vertebrate GnRH receptors (yellow) and conservative substitutions (blue). Putative ligand binding sites and residues important in receptor configuration, activation and G-protein coupling are indicated. Glycosylation, phosphorylation and disulphide bridge sites are also shown. Adapted from Millar (2005).

and internalization (Leeb-Lundberg et al., 1987; Sibley et al., 1987). For example, removal of the C-terminal domain decreased the rate of internalization of thyrotropin-releasing hormone receptor (Nussenzveig et al., 1993), diminished agonist-induced internalization of gastrin-releasing peptide (Benya et al., 1993), lost internalization and endocytosis of the angiotensin type I receptor (Hunyady et al., 1994; Thomas et al., 1995) and delayed desensitization and sequestration of the LH, α-1B-adrenergic and β-adrenergic receptors (Lattion et al., 1994; Strader et al., 1987; Zhu et al., 1993). In αT3 cells, GnRH stimulated the accumulation of [$^3$H]-thymidine for at least 90 min (Davidson et al., 1994b) and increased the levels of inositol triphosphate ($IP_3$) for at least 5 min (Anderson et al., 1995; McArdle et al., 1996; Merelli et al., 1992), suggesting that the GnRHR does not undergo rapid desensitization in αT3 cells due to the lack of C-terminal tail and the phosphorylation sites necessary for agonist-dependent desensitization (Kakar et al., 2004).

In addition, the GnRHR contains five potential phosphorylation sites in the intracellular loops. Willars et al. (1998) reported that these sites are not involved in agonist-dependent phosphorylation, suggesting the phosphorylation sites present in the intracellular loops may not be important for desensitization of the receptor. The GnRHR also possesses potential protein kinase C (PKC), phospholipase C (PLC) and cAMP-dependent protein kinase A (PKA) phosphorylation sites in the intracellular loops. One PKC phosphorylation site is located in the first intracellular loop, whereas three potential PLC sites and one potential PKA site are present in the third intracellular loop (Kakar et al., 2004). Cassina et al. (1999) demonstrated that both PKC and PKA phosphorylated synthetic peptides for the three intracellular loops in vitro. Consistent with this, they

showed that treatment of rat pituitary cell cultures in combination with phorbol 12-myristate 13-acetate (PMA) and cholera toxin reduced LH secretion, whereas individual treatment of PMA and cholera toxin had no effect on LH secretion, suggesting that more than one signaling pathway is required to induce GnRHR desensitization. Mutation of all the potential PKC phosphorylation sites ($Thr^{238}$, $Ser^{253}$, and $Thr^{264}$) in the third intracellular loop to Ala abolished the binding affinity of the receptor and reduced $IP_3$ production (Lin et al., 1998; Ulloa-Aguirre et al., 1998), whereas mutation of only $Ser^{253}$ and $Thr^{264}$ to Ala did not alter ligand binding or $IP_3$ production. However, ligand binding affinity (Ulloa-Aguirre et al., 1998) and receptor-mediated signal transduction (Lin et al., 1998) were decreased when the C-terminal domain of the third intracellular loop was deleted. Collectively, this evidence indicated that residues in the C-terminal domain of the third intracellular loop, including the PKC phosphorylation sites, are important for the structural integrity, expression, and signal transduction of the GnRHR.

**Tissue Expression of Gonadotropin-Releasing Hormone Receptor**

In general, GnRHRs are expressed only on the plasma membrane of gonadotropes to control reproductive function (Clayton and Catt, 1981; Childs and Unabia, 1997). Rodents are an exception with in that GnRHRs are also present in the gonads (Kakar et al., 1992). However, other tissues bind [$^{125}$I]-labled GnRH agonists, suggestive of GnRHR expression. Early studies described that the binding affinities of GnRH to extrapituitary tissues measured using radioreceptor assays were significantly lower than those of the receptor on gonadotropes of the anterior pituitary (Bramley et al., 1986; Eidne et al., 1985; Emons et al., 1989; Vincze et al., 1991). These include specific nuclei

in the central nervous system (Jennes et al., 1997), human placenta (Wolfahrt et al., 1998), somatotropes (Leibow et al., 1991), and various tumors of the pituitary and pancreas (Emons et al., 1998). In addition, the discovery of more potent GnRH analogues allowed the identification of high affinity binding sites in various hormone-responsive human tumors (breast and prostate) as well as cells derived from breast, prostate, liver, uterine endometrium, and ovarian tumors (Chen et al., 1992; Emons et al., 1993; Emons and Schally, 1994; Irmer et al., 1995; Segal-Abramson et al., 1992; Yano et al., 1994).

In the ovary, granulosa-luteal cells express GnRHR mRNAs and mRNA levels increase with follicular growth and development (Peng et al., 1994). GnRHR binding has been demonstrated in luteinized granulosa cells, late antral follicles and developing corpora lutea, but not in primordial, early antral and preovulatory follicles (Brus et al., 1997; Choi et al., 2006). This stage-specific expression of GnRHR in human granulosa and luteal cells suggests a role for GnRH in the regulation of ovarian physiology (Cheung and Wong, 2008). GnRHR protein and mRNA have also been found in human ovarian tumors, ovarian cancer cell lines and their tissue of origin, ovarian surface epithelium (Choi et al., 2001; Emons et al., 1989). Interestingly, GnRHR levels in ovarian carcinomas are increased in advanced (stages III and IV) compared to early (stages I and II) stages (Chien et al., 2004).

In addition to the ovary, GnRHR has been found in testicular germ cells of the rat and mouse (Bull et al., 2000) and receptor binding studies with GnRH agonists revealed the presence of GnRHR on Leydig cells of human testicular tissue (Clayton et al., 1980; Lefebvre et al., 1980). Later, the mRNA sequence of these testicular receptors was

determined to be identical to those on gonadotrope cells of the anterior pituitary (Botte et al., 1998). However, GnRHR mRNA levels were decreased by direct binding of GnRH analogs to testicular GnRHRs (Botte et al., 1999), suggesting that specific GnRHRs on Leydig cells were important in the physiological regulation of testicular function and the anti-fertility effects of GnRH agonists within the testis (Lefebvre et al., 1980).

The human placenta also had specific binding sites for GnRH that interacted with GnRH agonists and antagonists (Iwashita et al., 1986) and were localized to cytotrophoblast and syncytiotrophoblast cell layers of the placenta (Casan et al., 1999; Boyle et al., 1998). This placenta GnRHR was structurally and biochemically identical to pituitary GnRHRs, although GnRH binding affinity was lower (Escher et al., 1988; Bramley et al., 1992). During pregnancy, GnRHRs are dynamically dispersed (Cheng et al., 2001) and temporal expression of GnRHRs in placental cells has been observed at different weeks of gestation, along with chorionic gonadotropin secretion level (Lin et al., 1995).

Although GnRHRs have been isolated in breast tissue (Casan et al., 1998), its expression was questioned due to a small sample size (Kottler et al., 1997). However, others immunolocalized the GnRHR to the cytoplasm of human breast cancer cell lines and tumor biopsy specimens (Kottler et al., 1997; Moriya et al., 2001; Eidne et al., 1987).

## Signal Transduction Mechanisms of the Mammalian GnRH Receptor

### Receptor Activation

GnRH activation of signaling pathways is initiated by a change in receptor conformation and propagated by intracellular pathways within the cell (Kenakin, 1993). For GPCRs, the active conformation is related to a ternary complex consisting of hormone, receptor and G-protein (De Lean et al., 1980). This ternary complex develops an initial binding step common to both agonists and antagonists, followed by a transition step, exclusive to agonists, which leads to formation of the ternary complex. The model also allows for spontaneous formation of a receptor-G-protein complex, which has a higher affinity for agonists and is stabilized via binding of agonists. When GTP binds to the G-protein, the receptor returns to the low affinity conformation and the complex dissociates (De Lean et al., 1980). There are a number of different GnRHR active conformations that are selective for GnRH analogues and intracellular signaling pathways (Millar and Pawson, 2004).

### G-Protein Coupling

GPCRs transmit their signals primarily via GTP-binding proteins (G-proteins), which are heterotrimeric proteins composed of an α subunit (Gα) that binds guanine nucleotides, and a dimer that consists of a β and γ subunit (Gβγ). Upon stimulation, Gα dissociates from the Gβγ dimer rendering to its active GTP-bound form to influence effector molecules. The βγ dimer remains attached to the plasma membrane and can initiate several signaling events (Kraus et al., 2001). G-proteins can be classified into the

four following groups: $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$. $G\alpha_s$ mainly activates adenylate cyclase, which induces the production of high levels of the second messenger cAMP. Unlike $G\alpha_s$, the $G\alpha_i$ protein has inhibitory effects on adenylate cyclase (Birnbaumer, 1992). $G\alpha_{q/11}$ principally exerts its action by activating membrane-associated phospholipase C (Hsieh and Martin, 1992), whereas $G\alpha_{12/13}$ primarily operates by stimulation of protein tyrosine kinases (PTKs; Jiang et al., 1998).

The nature of G-protein coupled signaling initiated by the GnRHR depends largely on the cellular context (Figure 2.5). For example, it has been demonstrated that the human GnRHR couples to $G\alpha_{q/11}$ in heterologous Chinese hamster ovary-K1 and COS-7 cells (Grosse et al., 2000) but to $G\alpha_s$ in the placenta (Cheng et al., 2000). In contrast, others reported that the receptor couples selectively to $G\alpha_i$ in some reproductive tract tumors and cell lines derived from them (Emons et al., 1998; Grundker et al., 2001; Limonta et al., 1999; Imai et al, 1996). Interestingly, the rodent GnRHR couples to multiple G-proteins in LβT2 (Liu et al., 2002), $GGH_3$ (Stanislaus et al., 1998), and GT1-7 (Krsmanovic et al., 2003) cells. In GT1-7 neurons, elevated GnRH analogue concentrations induced a ligand-dependent switch of G-protein coupling from $G\alpha_s$ to $G\alpha_i$, which inhibits episodic GnRH release (Krsmanovic et al., 2003). Such negative feedback action serves as an autocrine mechanism for pulsatile GnRH secretion that is essential for the maintenance of normal gonadotropin release profiles and gonadal function (Cheng and Leung, 2005).

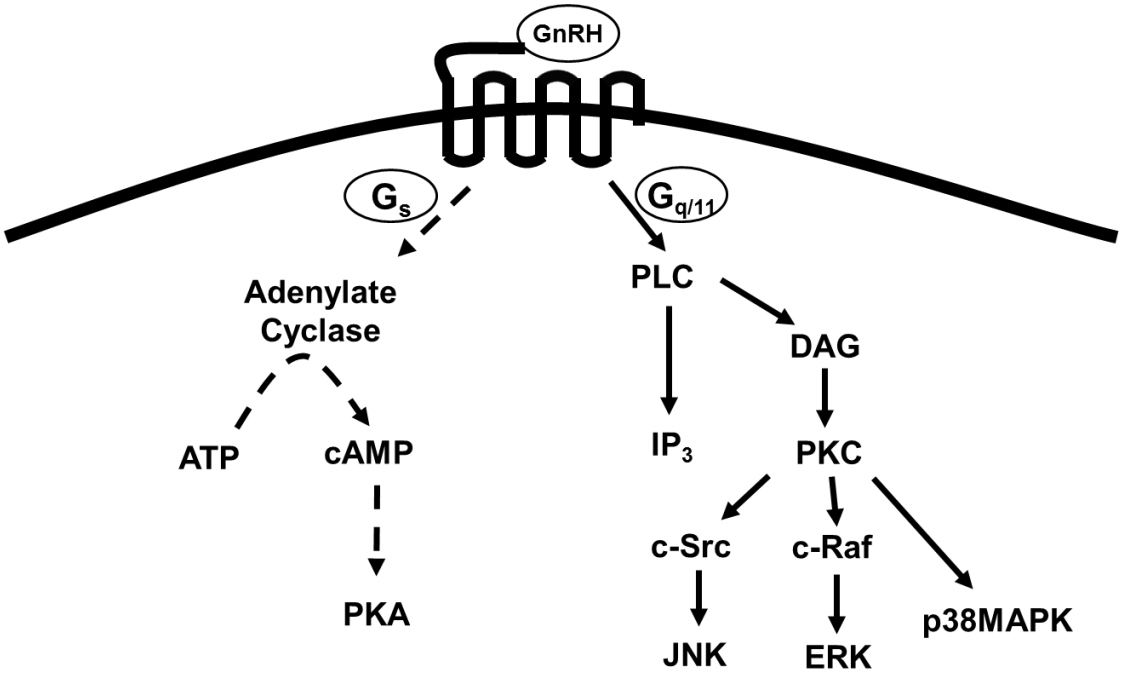



Figure 2.5. Signal transduction cascades activated by GnRH within gonadotrope cells. Upon binding of GnRH to GnRHR, intracellular signaling pathways are triggered via PKA and/or PKC. Following activation of PKC, a variety of MAPK pathways including JNK, ERK, or p38 MAPK can be activated.

**Activation of Protein Kinase A or Protein Kinase C**

**Protein Kinase A.** Regulation of the GnRHR by GnRH occurs via two major signal transduction pathways, activation of protein kinase A (PKA) or protein kinase C (PKC). In the PKA pathway, GnRHR is coupled to the $G\alpha_s$ protein (Figure 2.5). Upon binding of GnRH to its receptor, the receptor is activated by a conformational change allowing dissociation of the $G\alpha_s$ protein from Gβ and Gγ protein subunits. $G\alpha_s$ consequently activates adenylate cyclase, which converts adenosine triphosphate (ATP) to cAMP (Figure 2.5). Following production of increased amounts of cAMP, cAMP activates PKA (Figure 2.5), resulting in phosphorylation of a variety of other downstream signaling cascade proteins within the cell (Naor et al., 2000; Millar et al., 2004).

In rat pituitary cell cultures, GnRH stimulated cAMP production (Hawes et al., 1993). Furthermore, GnRH agonist-induced release of cAMP occurred in somatolactotropes over-expressing the rat GnRHR (GH3; Kuphal et al, 1994), baculovirus-insect cells (sf9; Delahaye et al. 1997), monkey kidney COS-7 cells over-expressing the human GnRHR (Arora et al., 1998) and Chinese hamster ovary (CHO; Nelson et al., 1999) cells. In αT3-1 cells, treatment with 8-bromo-cAMP and forskolin stimulated GnRHR mRNA levels (Sadie et al., 2003). Additional studies indicated that GnRH utilizes the PKA signaling pathway to stimulate its target cells. For instance, treatment of both pituitary and αT3-1 cells with pituitary adenylate cyclase-activating polypeptide (PACAP) resulted in increased α-subunit mRNA levels (Tsujii et al., 1995). However, White et al. (1999) reported that forskolin treatment completely blocked GnRH-stimulated GnRHR promoter activity. Similarly, Norwitz et al. (1999) found that the major signaling pathway in αT3-1cells that confers GnRH responsiveness of the

mouse and human GnRHR gene promoters is the PKC, and not the PKA pathway. These results indicates that, although GnRHR may not be directly regulated via the PKA signaling pathway, the receptor can be regulated in response to other signaling molecules that activate the PKA pathway.

**Protein Kinase C.** In αT3-1 cells, binding of GnRH to its heptahelical receptor leads to the stimulation of $G\alpha_{q/11}$ protein (Shah and Milligan, 1994) and activation of phospholipase Cβ1 (PLCβ1; Naor, 1990) followed by enhanced phosphoinositide turnover to inositol 1,4,5-triphosphate ($IP_3$), production of diacylglycerol (DAG), and activation of various PKC subspecies (Harries et al., 1997). Pituitary cells particularly express PKCδ and PKCε, but also atypical PKCs, which are DAG- and $Ca^{2+}$-independent (Harries et al., 1997). Generation of $IP_3$ leads to an initial rapid rise in intracellular $Ca^{2+}$ concentration resulting from release of intracellular $Ca^{2+}$ stores. DAG leads to activation of PKC isozymes and contributes to a more sustained rise in intracellular $Ca^{2+}$ through L-type voltage gated channels (Benard et al., 2001).

$Ca^{2+}$ is an important intracellular messenger that regulates diverse physiological processes. For example, treatment of pituitary cells with the $Ca^{2+}$ ionophore ionomycin resulted in a significant stimulation of GnRH secretion (Stojilkovic et al., 1994; Stojilkovic et al., 1995; Trueta et al., 1999). GnRH induces immediate intracellular $Ca^{2+}$ mobilization followed by extracellular $Ca^{2+}$ influx. These GnRH-induced $Ca^{2+}$ oscillations occur through mobilization of two distinct calcium pools (Stojilkovic et al., 1994; Iida et al., 1991). Extracellular $Ca^{2+}$ enters the cell through voltage-gated calcium channels in the plasma membrane, while $IP_3$ releases $Ca^{2+}$ from intracellular stores. The

release of $Ca^{2+}$ from intracellular stores is an important trigger for secretion in many cells. In the case of GnRH, both intracellular and extracellular $Ca^{2+}$ were shown to be involved in the secretion of LH and FSH (Stojilkovic et al., 1994; Naor, 1990; Stojilkovic et al., 1995). However, the $IP_3$-released calcium seems to be critical for gonadotropin secretion, whereas $Ca^{2+}$ influx through the plasma membrane is mainly required for the renewal of internal stores (Tse et al., 1997). Therefore, activation of PKC and increased $Ca^{2+}$ concentrations are among the important events that mediate GnRH action in pituitary cells. Furthermore, these events are prerequisite for activation of mitogen-activated protein kinase (MAPK) activity, in particular the extracellular signal-regulated kinase (ERK) pathway (Mulvaney and Roberson, 2000).

**Activation of Mitogen-Activated Protein Kinase**

The MAPK pathways are highly conserved signal transduction cascades that mediate cellular responses to a large variety of environmental stimuli (Bliss et al., 2010). In mammals, there are 4 predominant MAPK pathways: the ERK, jun-N-terminal kinase (JNK), p38, and ERK5/Big MAP kinase (ERK5/BMK) pathways. Activation of the pathway begins with phosphorylation of an upstream MAP kinase-kinase kinase (MAPKKK), which phosphorylates and activates an intermediate level MAP kinase kinase (MAPKK). The MAPKK in turn activates and the terminal MAP kinase (MAPK). In addition to these core kinases, numerous scaffolding and adaptor proteins play important roles in this pathway (Morrison and Davis, 2003). Activated MAPKs phosphorylate numerous substrates throughout the cell including those involved with

transcriptional machinery, chromatic components, cytoskeletal structures, and other downstream enzymes (Pearson et al., 2001; Yoon and Seger, 2006).

In pituitary-derived cells, GnRH activated all four MAPK cascades to various extents by a PKC- and tyrosine-kinase dependent mechanism (Naor et al., 2000). In αT3-1 cells, GnRH activated the JNK pathway by approximately 20-fold (Levi et al., 1998). Activation of JNK was transient, peaking at 30 min and declining thereafter. Similarly, the ERKs were also activated (~12-fold), ERK phosphorylation was detected 2 min after GnRH treatment, peaked at 7 min, and decreased to basal levels within 60 min (Benard et al., 2001). On the other hand, the remaining two MAPKs, p38 MAPK and BMK1, were only slightly activated (2 to 3-fold) in a transient manner, peaking 30 min after stimulation (Roberson et al., 1999). However, the mechanism underlying MAPK activation is significantly varied among systems. For example, in $GGH_3$ cells, ERK activity is mediated by both PKA and PKC (Han and Conn, 1999). In Caov-3 cells, GnRHR signals either through Gα or the dissociated Gβγ dimer, whereas in LβT2 cells, JNK activation by GnRH is not dependent on PKC (Yokoi et al., 2000).

Activation of ERKs by GnRH has been widely studied (Sim et al., 1993; Mitchell et al., 1994; Roberson et al., 1995). Alterations in GnRH pulse pattern affect the responsiveness of ERKs to GnRH (Haisenleder et al., 1998). Continuous exposure of αT3-1 cells to GnRH stimulated short-term (2 h) ERK activity, whereas pulsatile GnRH stimulated ERK activity for at least 8 h. Thus, GnRH pulses are required to maintain and prolong activation of ERKs. In similar manner, TPA (a PKC activator) caused a 2 h stimulation of ERKs, whereas EGF induced a much shorter effect (Reiss et al., 1997). The similar time-course obtained with GnRH and TPA indicated that PKC may be

involved in the activation of ERKs by GnRH in αT3-1 cells. PKC depletion results in marked inhibition of the GnRH response (Reiss et al., 1997) and the PKC inhibitor GF109203X abolishes GnRH-induced ERK activation (Benard et al., 2001). Therefore, ERK activation by GnRH in pituitary cells is mainly PKC-dependent (Kraus et al., 2001). Activation of ERKs by GnRH in αT3-1 cells involves two distinct signaling pathways that converge at the level of Raf1 (Benard et al., 2001). The main pathway involves direct activation of Raf1 by PKC, whereas the second, supportive pathway involves activation of Ras in a c-Src- and dynamin-dependent manner (Benard et al., 2001). Pretreatment with the PKC inhibitor GF109203X abolished GnRH-induced ERK activation, whereas the PTK inhibitor, genistein, and the c-Src inhibitor, PP1, only partially inhibited by ~40%, respectively. In addition, co-overexpression with the C-terminal Src kinase (CSK), which acts as a dominant-negative interfering mutant of c-Src, and with the dominant negative form of Ras had a partial inhibitory effect by 35% on the GnRH to ERK pathway (Benard et al., 2001). Dynamin appears important for the GnRH to ERK pathway because its dominant-negative form inhibited activation of Ras in a PKC-independent manner.

$Ca^{2+}$ is a critical mediator of the induction of gonadotropin secretion by GnRH. Removal of $Ca^{2+}$ markedly reduced ERK activation by GnRH (Reiss et al., 1997) and calcium influx through voltage-gated calcium channels (VGCCs) is absolutely required for activation of ERKs by GnRH in both αT3-1 and primary cultured pituitary cells (Mulvaney and Roberson, 2000). Treatment of ells with the PKC inhibitor staurosporine blocks the GnRH-induced VGCC signal, indicating that $Ca^{2+}$ operates downstream of PKC in the pathway that leads to ERK activation. Similar dependency on PKC and $Ca^{2+}$

was also demonstrated in LβT2 cells (Yokoi et al., 2000), indicating that ERK activation by GnRH in pituitary cells is strongly dependent upon DAG-sensitive PKC isozymes and requires $Ca^{2+}$.

The JNK signaling cascade is also the main MAPK pathway involved in GnRH signaling in αT3-1 cells. JNKs ate activated 20 to 50-fold by GnRH and by TPA in αT3-1 cells (Levi et al., 1998). Time-course of JNK activation in response to GnRH is slower than that of ERK activation, indicating that regulation of JNK activation by GnRH is different from that of ERK. Levi et al. (1998) also showed that activation of JNKs is both PKC- and PTK-dependent. However, PTK inhibitors completely abolished GnRH-induced JNK activation, but depletion of PKC or the use of PKC inhibitors prevented only ~70% of GnRH-JNK signals, indicating the existence of a PKC-independent signaling component. They found that GnRH as well as TPA also increased the activity of c-Src. Coexpression of CSK and constitutively active forms of c-Src, together with JNK1, confirmed the involvement of c-Src downstream of PKC in the GnRH-JNK1 pathway. They further demonstrated that c-Src together with the small GTPase CDC42 (and possibly Rac) and MEKK1 are the main mediators of the GnRH-JNK1 pathway. Collectively, GnRH stimulates JNKs activity by a pathway that includes sequential activation of PKC, c-Src, CDC42 (Rac), and MEKK1.

MAPKs play a role in the regulation of gene expression in various systems, either by activating nuclear transcription factors directly or by phosphorylating downstream cytoplasmic protein kinases. For example, ERK phosphorylates the ternary complex factor Elk1, a member of the Ets family of transcription factors (Marais et al., 1993). Elk1 has been shown to bind to the GnRH-responsive element located within the α-

subunit gene promoter region, and GnRH increases promoter activity in αT3-1 cells (Roberson et al., 1995). In αT3-1 cells, ERK is translocated into the nucleus within 20-30 min of GnRH stimulation and this nuclear translocation supports a role for ERKs in transcriptional regulation of gonadotropin subunit genes (Kraus et al., 2001; Roberson et al., 1995; Haisenleder et al., 1998). Moreover, the MEK inhibitor PD098059 was shown to block GnRH-stimulated mRNA accumulation of the α-subunit and FSHβ in GnRH via ERK-, PKA, and $Ca^{2+}$- dependent pathways (Lin and Conn, 1999).

JNKs were also shown in many systems to translocate into the nucleus upon stimulation to phosphorylate and activate transcription factors such as c-Jun, ATF2, Elk1, and p53. Levi et al. (1998) reported that the delayed response of JNKs observed in their experiments may indicate that JNKs are involved in a later stage of transcriptional regulation. Activation of JNKs and ERKs provides a route for activation of c-jun and c-fos, respectively to form the jun-fos dimer that might activate the activator protein-1 (AP-1) responsive element present in both LHβ and FSHβ promoter (Hirai et al., 1990). Strahl et al., (1998) also showed that GnRH induces FSHβ gene expression via activation of the AP-1 element. Although GnRH induction and basal control of the α-subunit gene appear to occur through the ERK pathway, induction of the LHβ gene is dependent on JNKs, suggesting the differential stimulation of transcription of LH subunit genes by GnRH (Kraus et al., 2001). Yokoi et al., (2000) has shown that the JNK cascade is necessary to elicit the LHβ promoter in a c-Jun-dependent mechanism in LβT2 cells. In addition, it was reported that the common α-subunit gene is regulated by c-Jun and ATF2 at the cAMP-responsive element (CRE) site and by Elk1 at the Ets site of the pituitary glycoprotein hormone basal element (PGBE) domain (Roberson et al., 1999; Heckert et

al., 1996). Therefore, it is likely that the MAPK cascades play a role in the expression of α- and β-subunits of gonadotropin genes.

## Transcriptional Regulation of GnRHR by Hormone

### Basal Expression of GnRHR Gene

The 5′ flanking regions of GnRH I genes have been characterized in the mouse (Albarracin et al., 1994), rat (Reinhart et al., 1997), human (Fan et al., 1995; Kakar, 1997) and sheep (Campion et al., 1996). The mouse and rat promoters share >80% homology over 1.9 kb. However, the rat promoter shares 55% homology with the human promoter over 2.2 kb and 63% homology with the sheep promoter over 0.9 kb (Pincas et al., 1998). There are several highly homologous regions within the proximal 500 bp of the mouse, rat, human and sheep promoters (Pincas et al., 1998). The mouse GnRHR I proximal promoter was the first to be isolated and characterized (Albarracin et al., 1994). The major transcription start site in primary pituitary tissue and αT3-1 cells is located at −62 (all numbering is relative to the translation start site) and is not associated with a consensus TATA box (Sadie et al., 2003; Albarracin et al., 1994). In addition to this site, Clay et al. (1995) identified other pituitary transcription start sites at −90 and −200 bp in αT3-1 cells. Regulation of the basal activity of the mouse promoter in αT3-1 cells is conferred by a tripartite basal enhancer, which includes binding sites for steroidogenic factor-1 (SF-1) at −244/−236, activator protein-1 (AP-1) at −336/−330, and GnRHR-activating sequence (GRAS) at −391/−380 (Duval et al., 1997). The pan-pituitary homeobox transcription factor Pitx-1 has been shown to interact with AP-1 in intact

LβT2 cells, indicating that this interaction might be important for GnRHR I gonadotroph-specific, basal promoter activity (Jeong et al., 2004). In addition, the promoter region around −360, shown to bind LHX3 homeodomain protein in vitro and in intact cells, was recently demonstrated to be important for mouse GnRHR I basal promoter activity in αT3-1 cells (McGillivray et al., 2005).

In the rat proximal GnRHR I promoter, the transcription start site in αT3-1 cells was initially found to be 103 bp upstream from the start codon, with a putative TATA box 23 bp upstream from the transcription start site (Reinhart et al., 1997). Later, five major transcription start sites in αT3-1 cells were identified, including four of them are clustered around −103, and one is located at −30, along with several minor start sites (Pincas et al., 1998). Maximal cell specific expression of the rat GnRHR I is achieved by multiple regulatory domains within 1260 bp of 5′ flanking region. A distal GnRHR-specific enhancer (GnSE), located between −1135 and −753, contains binding sites for GATA-related and LIM homeodomain-related factors, and facilitates gonadotroph-specific expression through functional interaction with an SF-1 site at −245 (Pincas et al., 1998; Pincas et al., 2001). AP-1 site in the rat promoter is also involved in basal promoter activity, but has no influence on the GnSE function (Hapgood et al., 2005). The function of the proximal rat promoter and the GnSE is supported by results obtained in transgenic mice, showing that the proximal 1.1-kb rat GnRHR I promoter is sufficient to drive gonadotroph-specific expression. Furthermore, 3.3 kb of the rat promoter was found to drive cell-specific expression of the transgene in gonadotrophs and certain areas of the brain (Granger et al., 2004).

The 5′ flanking regions of the human and sheep genes are much more complex than that of the mouse and rat genes, with the presence of multiple transcription start sites and CAP sites (Campion et al., 1996; Ngan et al., 2000). Although the sheep proximal 5′ flanking region is structurally similar to the mouse promoter, it has greater sequence homology to the human promoter (Campion et al., 1996). In contrast to the single start site identified in mouse pituitary tissue (Sadie et al, 2003), 18 transcription start sites have been identified for the human GnRHR I gene in human pituitary tissue (Kakar, 1997). These start sites are located between −1748 and −577 and are well dispersed among several TATA and CCAAT boxes. The proximal 173 bp of the human 5′ flanking region is critical for basal promoter activity in αT3-1 cells (Ngan et al., 1999). However, upon limited availability of human gonadotropic cell lines, the results in mouse cell lines may not be physiologically relevant (Hapgood et al., 2005). The mouse, rat and human promoters all contain several SF-1 sites, with at least one site in each promoter occurring in the 5′ untranslated region (UTR). For the human promoter, this site is located at −140/−134 and is primarily responsible for mediating high cell-specific expression in αT3-1 cells (Ngan et al., 1999), whereas for the mouse and rat, it is situated at −15/−7 in both species and has no function for the cell-specific expression (Sadie et al., 2003). An upstream Oct-1 binding at −1718 is also required for basal activity of the human promoter in αT3-1 cells (Cheng et al., 2001).

The transcription factor Oct-1 appears to regulate basal GnRHR I gene expression both positively and negatively, depending on the species and cell-type (Hapgood et al., 2005). For example, Oct-1 is required for basal expression of the human GnRHR I gene in several cell types, including placental, ovarian and gonadotroph cell lines, via an Oct-1

binding site at −1718 (Cheng et al., 2001). On the other hand, in placental JEG-3 cells, ovarian OVCAR-3 cells and αT3-1 cells, Oct-1 acts as a potent repressor of the human GnRHR I promoter via a negative regulatory element (NRE) at position −1017 (Cheng et al., 2002). Oct-1 is also involved in basal and GnRH-stimulated activity of the mouse GnRHR I promoter in αT3-1 cells via the SURG-1 (Sequence Underlying Responsiveness to GnRH) element (Kam et al., 2005).

The mouse CRE has been found to be essential for basal promoter activity in some pituitary cell lines, such as LβT2 cells (Hapgood et al., 2005) and $GGH_3$ somatolactotroph cells (Maya-Nunez and Conn, 1999), but the rat CRE does not appear to be involved in basal promoter activity in αT3-1 cells (Pincas et al., 2001). A CRE at position −1650 is required for placenta-specific expression of the human GnRHR I gene (Cheng et al., 2001). These findings demonstrate that CREs contribute to a cell- and/or species-specific expression of GnRHR I gene.

**Transcriptional Regulation of GnRHR by GnRH**

Homologous regulation of the GnRHR is a physiologically relevant mechanism for increasing pituitary sensitivity to GnRH during ovulation (White et al., 1999). Therefore, GnRH activation of GnRHR is a potent stimulus for increased expression of multiple genes including the gene encoding the GnRHR itself. GnRH regulates the GnRHR with short-term exposure to hormone leading to an increase in receptor expression, whereas prolonged exposure leads to receptor down-regulation (Norwitz et al., 1999). It is widely accepted that pulsatile GnRH stimulation is essential for appropriate GnRHR expression levels, at the same time avoiding receptor down-

regulation due to continuous hormonal stimulation (Liu et al., 2003). The effects of GnRH on GnRHR protein and/or mRNA levels in primary pituitary cultures and cell lines suggest a direct mechanism of GnRH on pituitary cells, with a combination of both transcriptional and post-transcriptional mechanisms regulating GnRHR expression levels (Hapgood et al., 2005). For example, in attempts to mimic the situation in vivo, rat pituitary cultures were stimulated with GnRH in a pulsatile fashion, resulting in increased GnRHR mRNA levels (Kaiser et al., 1993) via MAPK and possibly also cAMP/PKA pathways (Haisenleder et al., 1998; Borgeat et al., 1972). Different pulse frequencies were found to have different effects on GnRHR mRNA, with higher pulse frequencies causing maximal stimulation (Kaiser et al., 1997).

The expression levels of mouse GnRHR promoter–reporter constructs transfected into αT3-1 cells increase in response to 100 nM GnRH after 4–6 h of continuous stimulation (Norwitz et al., 1999). This GnRH responsiveness was identified to two regions, designated SURG-1 and SURG-2 (Norwitz et al., 1999). SURG-1 contains binding sites for nuclear factor Y (NF-Y) and Oct-1, and it was shown by chromatin immunoprecipitation assays that GnRH increased binding of these factors to SURG-1 in intact cells (Kam et al., 2005). GnRH responsiveness via SURG-2 appears to be mediated by PKC-induced activation of JNK which increases expression, activity and binding of AP-1 proteins to SURG-2 (Ellsworth et al., 2003). SURG-1 and SURG-2 can respond to GnRH independently, but the AP-1 element is critical for conferring maximal GnRH responsiveness (Norwitz et al., 1999). These findings are consistent with the results obtained in transgenic mice (Ellsworth et al., 2003). However, in the mouse promoter, responsiveness to GnRH also involves binding of Smad and AP-1 factors to

another composite element called GRAS, which occurs further upstream at position −391/−380 (Norwitz et al., 2002; Norwitz et al., 2002).

In LβT2 cells, endogenous GnRHR mRNA and protein levels are up-regulated upon long-term pulsatile GnRH stimulation (Turgeon et al., 1996; Bedecarrats et al., 2003), whereas long-term continuous stimulation down-regulates receptor levels (Bedecarrates et al., 2003). However, both continuous and pulsatile stimulation induced only a small increase in the activity of a transfected 1.2 kb mouse GnRHR promoter (Bedecarrates et al., 2003). Stanislaus et al. (1994) studied the regulation of mouse GnRHR promoter activity in the $GGH_3$ cell line, which was engineered by stably transfecting $GH_3$ rat somatolactotroph cells with rat GnRHR cDNA. They found several intracellular signaling pathways involved in mediating the up-regulation of the mouse GnRHR promoter activity by GnRH in these cells, such as PKA (Lin and Conn, 1998), PKC and the $Ca^{2+}$ signaling pathway (Lin and Conn, 1999). In contrast to the results in αT3-1 cells, the AP-1 site does not appear to be involved (White et al., 1999; Norwitz et al., 1999; Maya-Nunez and Conn, 2003). However, functional studies indicate a role for the PKA pathway and cAMP response elements (CREs) in regulating GnRHR promoter activity in mouse, rat and human. These results may indicate differences in GnRHR G-protein coupling between the cell lines. All of these promoters contain functional CREs and are up-regulated by activators of the PKA pathway in αT3-1 cells (Sadie et al., 2003; Pincas et al., 2001; Cheng and Leung, 2001). Other factors likely to be involved in mediating PKA responses are CREB (Tsutsumi et al., 1995) for the rat and SF-1 (Sadie et al., 2003; Pincas et al., 2001) for the rat and mouse promoters.

**Transcriptional Regulation of GnRHR by Steroid Hormones**

**Estradiol.** Studies in rat, sheep and cow conclude that estradiol increases the level of GnRHR mRNA and protein in pituitary consistent with a requirement for a strong, prolonged LH surge for ovulation during the preovulatory phase of the reproductive cycle (Crowder and Nett, 1984; Savoy-Moore et al., 1980). Experiments in ovariectomized transgenic mice harboring a sheep GnRHR promoter-reporter construct, as well as experiments in sheep primary pituitary cells, suggest that transcription is the predominant mechanism of estradiol up-regulation of GnRHR numbers in the pituitary (Duval et al., 2000; Wu et al., 1994; Laws et al., 1990; Gregg et al., 1990). However, estradiol stimulation of αT3-1 cells was found to down-regulate GnRHR numbers (McArdle et al., 1992), whereas estradiol stimulation of LβT2 cells had little effect on endogenous GnRHR gene expression (Turgeon et al., 1996; McArdle et al., 1992). These conflicting results highlight the apparent discrepancies that may occur when using transformed cell lines compared to primary cells that contain mixed cell populations. In addition, Turzillo et al. (1994) reported that the GnRHR I mRNA levels increase before an increase in circulating concentration of estradiol and this result leads to postulate that a decrease in progesterone, rather than an increase in estradiol, is required for up-regulation of GnRHR I numbers.

**Progesterone**. In most mammals, high levels of progesterone correlate with reduced GnRHR I protein levels in pituitary and reduced pituitary responsiveness to GnRH I during the luteal phase of the menstrual cycle and during pregnancy (Crowder and Nett, 1984; Batra and Miller, 1985; Sakurai et al., 1997). In sheep pituitary cells, GnRHR numbers were dramatically down-regulated by progesterone within 48 h (Wu et

al., 1994; Laws et al., 1990), consistent with a direct effect of progesterone on the pituitary. Progesterone was also able to prevent estradiol- and inhibin-induced increases in GnRHR I mRNA levels in these cells. Recent results with the human GnRHR I promoter in αT3-1 cells showed that progesterone administration and overexpression of progesterone receptor (PR) isoforms inhibited GnRHR I promoter activity (Cheng et al., 2001), suggesting that, at least for the human promoter, repression by progesterone occurs via direct transcriptional effects on the GnRHR I promoter in gonadotrophs (Hapgood et al., 2005). Furthermore, this negative effect was shown to occur via a glucocorticoid response element (GRE)/progesterone response element (PRE) at −535/−521, which has 75% homology to a consensus progesterone response element, and to which PR isoforms were shown to bind in vitro (Cheng et al., 2001). In the same study, a half-PRE binding site was shown to be located at −402/−397. However, this site did not show a function in the progesterone-mediated transcriptional effects. Interestingly, another putative GRE/PRE is located further upstream (Fan et al., 1995), but its function remains unknown.

**Testosterone**. In male rats, pituitary GnRHR I mRNA levels appear to be repressed by testosterone because a negative correlation exists between mRNA levels and testosterone concentrations in serum (Kaiser et al., 1993; Zapatero-Caballero et al., 2003). GnRHR I numbers in primary pituitary cultures from male rats decreased after treatment with α-dihydrotestosterone (Tibolt and Childs, 1985), consistent with in vivo results and suggesting direct actions of α-dihydrotestosterone on the pituitary. In

contrast, α-dihydrotestosterone up-regulated GnRHR I mRNA levels in LβT2 cells (Curtin et al., 2001).

**Transcriptional Regulation of GnRHR by Growth Factors and Other Regulators**

Activin and inhibin, both members of the transforming growth factor-β family of proteins, are produced by primary gonadotrophs (Roberts et al., 1992), αT3-1 (Fernandez-Vazquez et al., 1996) and LβT2 cells (Pernasetti et al., 2001), and exert autocrine/paracrine effects on pituitary cells. Activin-A stimulates the rate of synthesis of new GnRHRs in rat pituitary cell cultures (Braden and Conn, 1992), and decreases receptor numbers in sheep pituitary cultures (Gregg et al., 1991). Inhibin was found to prevent the stimulation of receptor synthesis by GnRH I in rat pituitary cultures (Braden et al., 1990), but increases GnRHR I mRNA levels (Wu et al., 1994) and receptor numbers (Gregg et al., 1991) in sheep pituitary cultures. In αT3-1 and LβT2 cells, long-term stimulation with activin-A up-regulates endogenous GnRHR I mRNA synthesis and mouse GnRHR I promoter-reporter activity (Fernandez-Vazquez et al., 1996; Pernasetti et al., 2001), and pretreatment of αT3-1 cells with activin enhances the response of the mouse GnRHR I promoter to GnRH I (Norwitz et al., 2002). Follistatin blocks the activin-mediated stimulation at both mRNA and promoter level. In addition, follistatin decreases the basal activity of the mouse GnRHR I promoter in αT3-1 and LβT2 cells, indicating that endogenous activin maintains basal GnRHR I expression levels in these cells (Norwitz et al., 2002; Fenandez-Vazquez et al., 1996; Penasetti et al., 2001). Activin responsiveness of the mouse GnRHR I promoter was mapped to the GRAS element (Duval et al., 1999) described earlier, together with a region immediately

downstream from GRAS, termed DARE (down-stream activin regulatory element) (Cherrington et al., 2005). The mouse GRAS element is a composite regulatory element for which the functional activity in αT3-1 cells depends on the proper organization and assembly of a multiprotein complex, which includes Smad, AP-1 and FoxL2 proteins (Ellsworth et al., 2003). Basal GnRHR I promoter activity, as well as responsiveness to GnRH I and to activin require binding of Smad factors to the Smad binding element, as well as binding of AP-1 to a novel AP-1 element contained within GRAS (Norwitz et al., 2002; Norwitz et al., 2002). The LIM-homeodomain protein LHX2 was shown to bind the DARE sequence in vitro (Cherrington et al., 2005). It has been postulated that activin responsiveness requires a specific configuration of multiple transcription factors on these distinct elements, to form a complex activin-responsive 'enhanceosome' (Cherrington et al., 2005). Interestingly, the sequence of the corresponding GRAS element in the rat Pincas et al., 2001; Cherrington et al., 2005), suggesting that the rat DARE sequence is nonfunctional for activin responsiveness.

Pituitary adenylate cyclase activating polypeptide (PACAP) is a hypothalamic peptide hormone that modulates pulsatile GnRH I release from the hypothalamus and responsiveness to GnRH I, as well as regulates gonadotropin subunit expression (Rawlings and Hezareh, 1996). The mouse, rat and human GnRHR I promoters have all been shown to be regulated by PACAP in αT3-1 cells (Sadie et al., 2003; Pincas et al., 2001; Cheng and Leung, 2001). For the rat and human promoters, this has been shown to involve PKA (Pincas et al., 2001; Cheng and Leung, 2001). Two promoter elements, designated PARE (PACAP response element) I and PARE II, are required for the PACAP response of the rat GnRHR I promoter. PARE I includes the SF-1 binding site at

position −245/−237, along with binding sites for additional factors, whereas PARE II contains an imperfect cAMP response element (CRE) at position −110/−103 that can bind CREB (Pincas et al., 2001). Both the SF-1 site and the imperfect CRE are conserved in relative position in the mouse GnRHR I promoter, raising the possibility that a similar mechanism could be responsible for the PACAP response of the mouse promoter.

## Stress and Reproduction

### Glucocorticoids (GC) and Glucocorticoid Receptor (GR)

GCs are steroid hormones that are synthesized and secreted from the adrenal cortex in response to stress via hypothalamic-pituitary-adrenal (HPA) axis. HPA activation leads to the release of the neurohormone corticotrophin-releasing hormone (CRH). The release of CRH causes an increase in the adrenocorticotrpic releasing hormone (ACTH) and cortisol levels resulting in the activation of the sympathetic nervous system causing an increase in glucose levels, heart rate and blood pressure (Smoak and Cidlowski, 2004). General functions of GC are the regulation of glucose, fat, and protein metabolism. GC are also important for the protection of the body against stress by regulating glucose metabolism and blood pressure (De Bosscher et al., 2001). In addition, they have an important role in the dynamic modulation of the inflammatory and immune responses. The action of GC is caused by the interruption of pro-inflammatory, cytokine-mediated signaling pathways and by causing apoptosis in certain cells of the immune system. In several immune and inflammatory diseases, which

include rheumatoid arthritis, inflammatory bowel disease and asthma, exogenous synthetic GC are prescribed. The biological actions of GC as anti-inflammatory agents exert their actions via the interaction of this hormone with its cognate receptor, which is a member of the nuclear receptor superfamily of proteins (Smoak and Cidlowski, 2004).

GCs act through the glucocorticoid receptor (GR), a member of the superfamily of nuclear receptors (Griekspoor et al., 2007). In the absence of ligand, GR resides in the cytosol as part of a chaperone-containing multi-protein complex, which maintains a high affinity for the ligand. Upon hormone binding, GR translocates to the nucleus, where it acts as a transcription factor (TF). The GR subunits homodimerize and bind DNA at glucocorticoid response elements (GREs) in the vicinity of target genes (Schoneveld et al., 2004). GRE-bound GR recruits multiple transcriptional co-activator complexes, which stimulate transcription (Jenkins et al., 2001; Schaaf and Cidlowski, 2002). These properties of GR are reflected by its modular structure (Fig). The central domain contains two zinc fingers providing a dimerization interface as well as the DNA binding domain (DBD; Beato et al., 1995). The C-terminal ligand-binding domain (LBD) is responsible for high affinity binding of GCs. The LBD overlaps with the activation domain AF2 (activation domain 2), which is exposed after a conformational change induced by ligand binding (Kumar and Thompson, 2003). The exposed AF2 mediates the interaction with co-activators. The N-terminal part of the receptor contains AF1, a ligand-independent activation function, required for transcriptional enhancement through the recruitment of co-activators, and association with basal transcription factors (Kumar and Thompson, 2003). The trans-activation function of GR cannot solely account for the numerous physiologic effects of GCs. GR also controls many cellular processes by

influencing multiple pathways in a trans-activation independent manner. In particular, GR modulates, positively or negatively, the trans-activation function of other TFs. The modulation may also function the other way around, GR transcriptional activity being potentiated or inhibited by another TF. The regulation can be either indirect, resulting from an interference with upstream signaling pathways regulating the activation of TFs, or can result from a direct mutual regulation of GR and the other TF at the promoter of the target gene (Kassel and Herrlich, 2007).

**Regulation of Transcription Activity via GR Binding to DNA**

Glucocorticoids action is mediated via glucocorticoid receptor (GR), nuclear receptor that regulates physiological events through activation or repression of target genes involved in inflammation, gluconeogenesis and adipocyte differentiation. In the absence of glucocorticoid ligand, GRs exist in the cytoplasm in a multimeric complex composed of heat shock proteins (HSPs), including hsp90, hsp56 and hsp40, p23, Src, and others (Dittmar et al., 1997; Hawle et al., 2006). Ligand binding induces a conformational change in GR that leads to its dissociation from the multimeric complex, resulting in its dimerization and then rapid translocation into the nucleus and can elicit changes in gene expression (Croxtall et al., 2000; Ford et al., 1997 Glass and Rosenfeld, 2000). Translocated ligand-bound GRs, then, induce transcriptional regulation through several different ways.

**Direct activation via DNA Binding**. GR can bind DNA directly to regulate target gene expression through glucocorticoid response element (GRE), which is specific

DNA sequences, recognizing activated GRs. If the GRE is in close proximity to the TATA-box, GR can recruit key components of the basal transcriptional machinery to the TATA-box, such as Transcription Factor IID (Ford et al., 1997), and thus directly promote gene activation. If the GRE is located at a distance of the TATA element, GR can associate with coactivators that function as bridges to promote the recruitment of the basal transcriptional machinery (Rosenfeld and Glass, 2001; Tronche et al., 2004). Furthermore, GR can also recruit chromatin-remodeling coactivators that alter the nucleosomal structure of the DNA and create a more favorable environment for gene expression. Examples of genes positively regulated by GREs include tyrosine aminotransferase, alanine aminotransferase, and phosphoenolpyruvate carboxykinase, all involved in liver gluconeogenesis (Tronche et al., 2004).

**Indirect Activation via Protein-Protein Interaction.** GR can regulate gene activity independent of DNA binding via protein-protein interactions with other transcription factors. Example of this mechanism is GR and Signal Transduction and Transcription proteins (STATs) interaction (Schindler, 1999; Zhang et al., 1997). STATs are transcription factors involved in the Janus kinase (JAK) signaling pathway. Activation of JAK signaling results in the phosphorylation and dimerization of STATs, leading to their translocation into the nucleus and subsequent interaction with their response elements in the DNA. In this case, STAT-5 physically interacts with GR. STAT-5 is directly associated with the DNA, while GR is recruited to the chromatin without direct interaction with the DNA. The GR-STAT-5 association leads to activation

of several genes, most notably IGF-1 in the liver that is required for postnatal growth (Zhang et al., 1997).

**nGRE Mechanism**. Direct DNA binding by GR can also lead to repression of genes by interactions with negative GREs (nGREs). nGREs are similar to GREs and almost always are located in close proximity to DNA-binding sites for other transcription factors necessary for gene expression. The osteocalcin gene promoter, for instance, contains an nGRE that overlaps with its TATA box, and GR association with this site prevents access from the basal transcriptional machinery (Meyer et al., 1997). For example, the human FasL gene contains an nGRE adjacent to a nuclear factor κ B (NFκB) site (Novac et al., 2006). GR-nGRE association in this site prevents NFκB binding and induces gene silencing (Ghosh and Karin, 2002). Pro-opiomelanocortin (POMC), Corticotropin-releasing hormone (CRH), prolactin and neuronal serotonin receptor are down-regulated by this interaction.

**Indirect Repression via Protein-Protein Interaction.** most of the genes repressed by GR occur via protein-protein independent of DNA binding by the receptor. This signaling mechanism includes NFκB, activator protein-1 (AP-1), and Smad3. NFκB is a ubiquitous homo/heterodimer transcription factor most widely known for its role in inflammation. The p50/p65 heterodimer is the most common combination involved in transcriptional processes (McKay and Cidlowski, 1998). GR can physically bind to p65 and repress the NFκB-mediated transcription. For example, the interaction of GR with p65 can result in the sequestering of the NFκB complex, thereby preventing NFκB from

reaching its DNA-binding site (Almawi and Melemedjian, 2002). GR can also interact with DNA-bound NFκB to inhibit the recruitment of the transcriptional machinery. GR can also suppress NFκB transactivation by interfering with the cellular machinery necessary for gene activation (Nissen and Yamamoto, 2000). Classical gene targets of NFκB that are repressed by GR include many pro-inflammatory cytokines and their receptors, such as tumor necrosis factor α (TNFα), interleukin-1β, and granulocyte monocyte colony stimulation factor. Similar to NFκB, GR can bind and repress the transcriptional activity of AP-1 (Shaulian and Karin, 2002; Schule et al., 1990). AP-1 is a homo/heterodimer transcription factor composed of Fos family or Jun family members. GR physically interacts with AP-1 and inhibits AP-1-mediated gene activation by employing similar mechanisms to the NFκB. Genes repressed by GR in an AP-1-dependent manner include collagenase, stromelysin, and other matrix metalloproteinases. In addition to NFκB and AP-1, GR physically interacts with Smad3 and greatly reduces the transcriptional activity of Smad3, which is phosphorylated by activated tumor growth factor β (TGFβ) receptor (Song et al., 1999; Li et al., 2003). TGFβ-Smad signaling is important for cell differentiation, extracellular matrix production, as well as immune and inflammatory responses.

**The HPA-HPG Link**

Many studies have been performed to link the HPA axis to the HPG axis. Recently, it was found that when rodents and non-human primates were treated with CRH, an immediate decrease in pulsatile GnRH and LH release (Smoak and Cidlowski, 2004; Xia et al., 1996; Feng et al., 1991). In humans, when the HPA axis is activated by

stress or by psychological disturbances, it results to an inhibition of the HPG axis. If stress is severe enough, then it can lead to suppression of the normal menstrual cycle, which is referred to as functional hypothalamic amenorrhea and can lead to infertility when fully established (Reifenstein, 1946). The final neuroendocrine events resulted in the suppression of the normal cycle is a decrease in the GnRH pulse generator resulting in a decrease in the hypothalamic GnRH activity and GnRHR number, leading to a decrease in the LH pulse frequency and eventually causing deficiencies in the normal menstrual cycle (Ferin, 1999). CRH may not be the only HPA neurohormone involved in the stress response. There is good evidence in the human and in animals that vasopressin of paraventricular origin is colocalized with CRH in perikarya and secretory granules and coreleased in stress (Mouri et al., 1993; Battaglia et al., 1997). Vasopressin is known to act synergistically with CRH as an ACTH secretagogue (Rivier et al., 1984).

Experiments using μ-opiate receptor antagonist indicate that an increased endogenous opioid activity may somehow also account for the decreased pulsatility of the GnRH pulse generator in patients with functional hypothalamic chronic anovulation (Ferin, 1999). For example, studies have shown that the administration of naloxone or naltrexone acutely restores normal LH pulse frequency, at least in a subgroup of these patients (Khoury et al., 1987). In animals, the acute inhibitory action of CRH on pulsatile LH release is also clearly prevented by naloxone or by an antiserum to β-endorphin (Rivest et al., 1993; Gindoff and Ferin, 1987; Petraglia et al., 1986). These human and animal studies suggest that increased endogenous opioid activity reflects enhanced central CRH release and mediates the endocrine actions of CRH on the HPG axis and support the existence of a HPA-HPG link (Ferin, 1999).

Although the classical HPA-HPG link implies that activation of HPA will cause a decline in gonadotropin secretion, recent studies suggest that a reverse outcome is possible under a defined endocrine condition (Ferin, 1999). For example, activation of HPA by IL-1 or endotoxin in the monkey during the midlate follicular phase (but not the early follicular phase or the luteal phase) results in an acute release of LH (Xiao et al. 1996). This observation evidently contrasts with the inhibitory effect of the cytokine on pulsatile LH secretion in the absence of estradiol (Feng et al., 1991). A release of LH after HPA activation can also be produced in the ovariectomized monkey replaced with mid- to late follicular phase estradiol levels (Xiao et al., 1994). This study indicates that the factor that is probably responsible for this acute stimulatory effect of HPA on LH release may be progesterone, as this effect is readily prevented by the administration of a progesterone antagonist (Xiao et al., 1994). Xiao et al. (1994) have speculated that in stress, the small but significant increase in adrenal progesterone that occurs in response to HPA activation synergizes with circulating estradiol to enhance LH secretion. This hypothesis was supported by the observation that the increase in LH is prevented by the administration of a CRH antagonist, demonstrating that HPA activation is required for this effect to occur (Xiao et al., 1994).

**Transcriptional Regulation of GnRHR by Glucocorticoid**

It is well documented that chronic or prolonged stress results in inhibition of gonadotropin secretion and inhibition of reproduction in mammals, whereas the effects of acute stress are less clear and can even stimulate reproduction (Tilbrook et al., 2000). Although the mechanisms whereby stress regulates reproduction in mammals are not well

defined, there is evidence that glucocorticoids play an important role in modulating pituitary responsive to GnRH I, as part of a feedback mechanism from adrenal to pituitary (Tilbrook et al., 2000; Breen and Karsch, 2004). Further evidence for direct actions of glucocorticoids on pituitary is provided by findings that cortisol inhibits GnRH-induced LH release from bovine and porcine primary pituitary cells (Padmanabhan et al., 1983; Li, 1994). One mechanism whereby glucocorticoids may regulate GnRH responsiveness in pituitary may be via modulating GnRHR levels. Rosen et al. (1991) showed that glucocorticoids augmented GnRH I-induced increase in GnRHR I numbers in castrated testosterone-replaced male rats. However, earlier studies in rats did not show a change in GnRHR I levels after treatment with corticosterone (Tibolt and Childs, 1985; Suter et al., 1988). In sheep, administration of cortisol led to a decrease in GnRHR protein, but did not reduce GnRHR I mRNA levels (Daley et al., 1999). These experiments suggest that varying effects of glucocorticoids on GnRHR I levels may depend on species, the cellular milieu, and the dose, type and duration of glucocorticoid administration. However, a direct positive transcriptional effect of glucocorticoids on the mouse GnRHR I promoter has been established. Glucocorticoids increased endogenous GnRHR I mRNA levels in LβT2 cells, whereas pretreatment with GnRH I further augmented this increase (Turgeon et al., 1996). Glucocorticoids can also directly up-regulate activity of the mouse GnRHR I promoter in $GGH_3$ cells (Maya-Nunez and Conn, 2003). Although the tested 1.2 kb of 5′ flank of the mouse gene does not contain a classical GRE, the glucocorticoid-responsive region of the mouse GnRHR I promoter was mapped to the AP-1 site at −336 in $GGH_3$ cells (Maya-Nunez and Conn, 2003). The results from this study suggest that liganded glucocorticoid receptor interacts directly or

indirectly with AP-1 proteins, such as c-Jun, to increase GnRHR I transcription (Maya-Nunez and Conn, 2003).

# CHAPTER III

# MATERIALS AND METHODS

## Materials

Dexamethasone and mifepristone (RU486) were from Sigma (St. Louis, MO) and Org 31710 was a generous gift from N.V. Organon (Oss, Netherlands). The antibody specific for PARP-1 (catalog no. 614301) was purchased from BioLegend (San Diego, CA), whereas the GR antibody (catalog no. sc-1004X) and normal rabbit IgG (catalog no. sc-2027) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For experiments using EMSA, DNA probe (-290/-270; sense sequence 5'-TTGTGAAAACCAGGCCATCTG-3' and antisense sequence 5'-CAGATGGCCTGGTTTTCACAA-3') and competitive oligonucleotides containing consensus binding sites for specific transcription factors were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Biotinylated DNA probe (5'biotin/TTGTGAAAACCAGGCCATCTG-3') was also synthesized by IDT for biotinylated DNA-pull down assays.

## Plasmid Preparation

A reporter vector containing 5118 bp of porcine GnRHR promoter (-5118pGL3) was constructed by PCR amplification of the 5' flanking region of the GnRHR gene from genomic DNA preparations of pigs representing a white crossbred line. The PCR product was subsequently subcloned into pBluescript SK- (Stratagene, La Jolla, CA). Promoter

inserts were ligated into a reporter vector containing the cDNA encoding luciferase (pGL3, Promega Corp., Madison, WI) at the SacI/EcoRV location of the multiple cloning site. Deletion constructs were made by progressively removing 5' flanking sequence (approximately 500 bp) via restriction enzyme digests and subsequent intramolecular ligation of the remaining vector backbone. Construction of the 100-bp deletion reporter vectors was achieved by amplifying the specified region of the promoter by PCR with a Taq DNA polymerase containing 3' to 5' exonuclease activity (Bioline, Springfield, NJ). Next, the fragments were subcloned into pBluescript SK- or pCR-Blunt II (Invitrogen, Carlsbad, CA), and then finally placed into pGL3.

**Gel Extraction**. Appropriate plasmid fragments were extracted from agarose gels with the Qiaex II Agarose Gel Extraction Kit (Qiagen, Inc.). Excised gel slices were weighed and buffer QX1 was added, followed by the addition of QIAEX II reagent. Gel slices were incubated at 55℃ for 10 min and vortexed every 2 min. Samples were centrifuged using a Biofuge Pico (Kendro Lab Products) at 16,000 x *g* for 1 min and resulting pellets were washed with 500 µl of buffer QX1, followed by 2 washes (500 µl each) with buffer PE. The pellet was allowed to air dry before it was resuspended in 20 µl of tris-chloride (Tris-Cl; pH 8.5) and incubated at 55℃ for 5 min. Samples were then centrifuged at 16,000 x *g* for 1 min and the supernatant was collected. Another 20 µl Tris-Cl (pH 8.5) was added to the pellet, with the incubation and centrifugation process repeated. Plasmid fragments were then quantitated using A260 and A280 values determined with a Pharmacia GeneQuant spectrophotometer (Pfizer, New York, NY) and allowed to ligate at 15℃ overnight.

**Transformation**. For replication purposes, plasmids were transformed into RbCl competent DH5α cells (Invitrogen Life Technologies Corp.). The plasmid ligation reaction (5 µl) was added to 50 µl of DH5α cells, gently mixed, and incubated on ice for 30 minutes. Cells were then heated to 42℃ for 30 seconds, followed by a two minute incubation on ice. The addition of 200 µl of SOB media (2% tryptone, 0.5% yeast extract, 8.55 mM sodium chloride, 250 mM potassium chloride, 2 M magnesium chloride) preceded a one hour incubation shaking at 37℃. Depending on the plasmid, 100-200 µl of cells were plated on petri dishes containing LB media (Sigma Chemical Co., St. Louis, MO) with the addition of ampicillin and incubated inverted overnight at 37℃.

## DNA Extraction

**Alkaline Lysis Mini Plasmid Preparation**. Plasmid DNA was isolated from RbCl competent DH5α cells (Invitrogen Life Technologies Corp.) for plasmid screening purposes. Two ml of LB media (Sigma Chemical Co.) and 2 µl of ampicillin were inoculated with a single colony of DH5α cells containing an ampicillin resistant plasmid and allowed to grow, shaking overnight at 37℃. Cells were pelleted via centrifugation at 16,000 x *g* for one minute with a Biofuge Pico microcentrifuge (Kendro Lab Products). Following removal of the supernatant, cell pellets were resuspended in 100 µl of GTE (100 mM glycine, 500 mM ethylenediaminetetraacetic acid [EDTA], 1 M tris) and incubated at room temperature for five minutes. Sodium hydroxide/sodium dodecyl sulfate (200 µl; NaOH/SDS solution) was added to each sample. Next, the sample was mixed gently

and allowed to incubate on ice for five minutes. Samples were incubated another five minutes following the addition of 150 µl of potassium acetate (5M) and vortexed for two seconds. Following centrifugation at 16,000 x *g*, the supernatant was placed in a clean 1.5-ml microcentrifuge tube, 800 µl of 100% ethanol was added, and tubes were incubated for two minutes at room temperature. Samples were centrifuged at 16,000 x *g* for five minutes, the ethanol was removed, and the pellet was washed with 100 µl of 70% ethanol. Following another five minutes of centrifugation at 16,000 x *g*, the pellet was allowed to dry at room temperature and resuspended in 1X tris-ethylenediaminetetraacetic acid (TE, pH 8.0). RNase A (0.5 µl) was added and each sample was incubated at 37°C or 30 minutes. All newly constructed plasmids were screened via endonuclease restriction digestion prior to use.

**Plasmid Purification**. Following alkaline lysis mini preparation and appropriate screening with restriction enzymes, 100 ml of LB (with 150 µl ampicillin) was inoculated with remaining broth culture from the alkaline lysis preparation and grown, shaking overnight at 37°C. Cells were isolated from the resulting broth culture by 4°C centrifugation at 6,000 x *g* for 15 minutes using a Sorvall RC2-B centrifuge (Du Pont Co., Newton, CT). Preparation of plasmids for transfection was performed through using the Plasmid Purification Midi Kit (Qiagen Inc.). Cell pellets were resuspended in 4 ml of buffer P1 and incubated for five minutes at room temperature, after the addition of 4 ml of lysis buffer P2. Following incubation, 4 ml of precipitation buffer P3 was added to each of the samples, and they were incubated on ice for 15 minutes. Samples were subjected to 4°C centrifugation at 20,000 x *g* for 30 minutes using a Sorvall RC2-B

centrifuge (Du Pont Co.) prior to being applied to a resin column. All columns were equilibrated with buffer QBT before application of the cell lysate. Columns were washed twice with 10 ml of buffer QC and plasmid DNA was eluted from the column with the addition of 5 ml of buffer QF. Plasmid DNA was precipitated by adding 3.5 ml of isopropanol, followed by centrifugation at 4℃, spinning at 15,000 x *g* for 30 minutes using a Sorvall RC2-B centrifuge. Pellets were washed with 2 ml of 70% ethanol and centrifuged at 4℃ for 10 minutes at 15,000 x *g* in a Sorvall RC2-B centrifuge (Du Pont Co.). After pellets had been allowed to air dry, they were resuspended in 250 µl of Millipore water. All purified plasmids were quantitated through the use of a Lambda EZ 150 spectrophotometer (Perkin Elmer, Boston, MA) and screened with restriction endonucleases prior to their use in transient transfection assays.

**Cell Culture**

αT3-1 (Dr. Pam Mellon, Salk Institute, La Jolla, CA) cells were maintained at 37 C in a humidified 5% $CO_2$ in air atmosphere. The αT3-1 cells were cultured in high-glucose DMEM (Mediatech, Herndon, VA) supplemented with 5% fetal bovine serum (FBS), 5% horse serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate (Gibco, Grand Island, NY). Cultures of αT3-1 cells were maintained on 150-mm cell culture plates (Corning Inc., Corning, NY) in 20 ml of media.

**Transient Transfections**

**Day 1**. Transient transfections were performed using a liposome-mediated protocol. On Day 1, αT3-1 cells, typically at 70% confluency, were trypsinized and

plated for transfection. The cells were washed with 10 ml of 1X phosphate-buffered saline (PBS) twice following aspiration of media. Cells were trypsinized by adding 3 ml of 1X trypsin-EDTA (Mediatech Inc., Herdon, VA), incubating at 37℃ in a 5% CO2 in air environment for five minutes. Following incubation, cells were rinsed with 7 ml of αT3-1 media, with all cell suspensions collected. A sample of cells (100 µl) was diluted in 900 µl of 1X PBS and 10 µl of this dilution loaded on a hemacytometer for cell counts. Approximately two million cells were plated onto 6-well cell culture dishes (Corning Inc.) in 2 ml of αT3-1 culture media.

**Day 2**. The plasmid used as a control for transfection efficiency was composed of the Rous Sarcoma Virus promoter fused to the cDNA encoding β-galactosidase (RSV-βgal, Stratagene, La Jolla, CA). For each test vector, 291 µl of serum-free DMEM and 9 µl of Fugene6 reagent (Roche Diagnostics Corp., Indianapolis, IN) were aliquoted into 1.5-ml microcentrifuge tubes. As each test vector was transfected in triplicate, sufficient quantities to result in 900 ng/well of test vector and 100 ng/well of RSV-βGal control vector were added to the microcentrifuge tube. The mixture was then incubated for a minimum of 15 minutes prior to adding 96 µl of the mixture (DMEM, Fugene6 and vectors) to each well.

**Day 3**. Transfected αT3-1 cells were harvested 20-24 hours after transfection. Media was aspirated from all wells, followed by two rinses with 1X PBS. Lysis buffer (200 µl; Galacto-Light kit; Applied Biosystems, Bedford, MA) containing 1 M dithiothreitol (DTT) was added to each well and the plates were incubated, shaking at

4℃ for 10 minutes. Cell lysates and buffer were collected and centrifuged at 4℃ for two minutes at 16,000 x *g* using a Spectrofuge 16M microcentrifuge (EandK Scientific, Campbell, CA). Lysates (20 μl) were transferred to a white 96-well Microflux2 plate (Thermo Labsystems, Franklin, MA). This was performed in duplicate for respective luciferase and β-galactosidase assays. Plates were analyzed for luciferase and β-galactosidase activity using the Wallac Victor2 instrument (Perkin Elmer). Transfections were performed a minimum of three times, using three different plasmid preparations.

**Protein Extraction**

Cytoplasmic and nuclear protein extracts were obtained from αT3-1 cells for their use in electrophoretic mobility shift assays (EMSAs) using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology, Rockford, IL). Four plates of αT3-1 cells at 70% confluency each were rinsed twice with 10 ml of 1X PBS before removing the cells via vigorous washing with buffer containing 10 mM Tris-Cl, 140 mM sodium chloride (NaCl), and 1 mM EDTA. Cells were then removed from the wash buffer by centrifugation at 4℃ for five minutes using a Beckman TJ-6 centrifuge at 500 x *g* (Beckman, Palo Alto, CA). The resultant cell pellet was resuspended in CER I reagent through vortexing for 15 seconds and incubated on ice for 10 minutes. Protease inhibitor cocktail (100X stock containing 104 mM AEBSF, 80 μM aprotinin, 2 mM leupeptin, 4 mM bestatin, 150 μM pepstatin A and 140 μM E-64; Sigma Chemical Co.) and Phosphatase Inhibitor Cocktail Set II (100X stock containing 200 mM imidazole, 100 mM sodium fluoride, 115 mM sodium molybdate, 100 mM sodium orthovanadate and 400 mM sodium tartrate dehydrate; CalBiochem, La Jolla, CA) were added

simultaneously with CER I reagent. CER II reagent was added to the cells, which were then vortexed and allowed to incubate on ice for one minute. Following incubation, lysed cells were centrifuged at 4℃ for five minutes using a Spectrafuge 16M microcentrifuge at 16,000 x *g* (EandK Scientific, Campbell, CA), with resulting supernatant transferred to a clean 1.5-ml microcentrifuge tube and stored at -80℃. The remaining pellet was resuspended in NER reagent with the addition of protease and phosphatase inhibitor cocktails and incubated on ice for 40 minutes with vortexing for 15 seconds every 10 minutes. Finally, lysed nuclei were centrifuged at 4℃ for 10 minutes using a Spectrafuge 16M microcentrifuge (EandK Scientific) at 16,000 x *g* before being transferred to a clean 1.5-ml microcentrifuge tube. Total protein concentration of nuclear extracts was quantitated using a BCA assay kit and the accompanying protocol (Pierce Biotechnology). Nuclear extracts were stored at -80℃ in 100 µl aliquots.

**Electrophoretic Mobility Shift Assays (EMSAs)**

All oligonucleotides used in EMSAs were annealed prior to use by adding complementary oligonucleotides (50 µM each) to 1X NET buffer (1 M NaCl, 10 mM EDTA, 100 mM Tris-Cl, pH 7.5) and Millipore water. Oligonucleotides were first denatured by heating to 95℃ for 10 minutes and then annealed by incubating at 37℃ for 30 minutes followed by a 30 minute incubation at 25℃.

Oligonucleotides were end-labeled with [γ-32P]ATP using polynucleotide kinase (PNK; Fermentas Inc., Hanover, MD) and a forward reaction protocol. Annealed oligonucleotide (1 µl) was incubated with 1 µl 10X PNK Buffer A, 4 µl water, 1 µl T4

PNK and 3 µl [γ-32P]ATP for 30 minutes at 37°C. Following incubation, 35 µl of water was added to all reactions. Reaction mixtures were then applied to equilibrated MicroSpin™ G-25 columns (Amersham Biosciences Corp., Piscataway, NJ) and centrifuged at 3,000 x *g* for two minutes using a Mikroliter microcentrifuge (Hettich AG, Bäch, Switzerland). Following the addition of 10X NET buffer (5µl) to each reaction, 1 µl from each reaction mixture was added to 4 ml of scintillation fluid and counted on a 1900TR liquid scintillation counter (Packard Instrument Co., Meriden, CT).

Electrophoretic mobility shift assays were performed by adding 2X Dignam D buffer (20 mM HEPES, 20% glycerol, 0.1 M potassium chloride, 0.2 mM EDTA, 0.5 mM DTT), 20 mM DTT, and 2 µg poly(dI•dC) (Amersham Biosciences) to 5 µg of αT3-1 nuclear extracts. Probe labeled with [γ-32P]ATP (approximately 100,000 cpm) was added, and the reaction was incubated at room temperature for 20 minutes. In those assays that included competitor oligonucleotides, the unlabeled, annealed competitor oligonucleotide was added just prior to the addition of radiolabeled probe. For supershift assays, a rabbit polyclonal antibody directed against the p65 subunit of NF-κB (Calbiochem) or an equal amount of rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with nuclear extracts and binding components for approximately two hours at 4°C, prior to the addition of probe. Polyacrylamide gels were subjected to electrophoresis in 1X TGE buffer (25 mM tris base, 190 mM glycine, 1 mM EDTA) for approximately one hour at 100V prior to the addition of binding reactions, with subsequent separation of bound from free probe by electrophoresis at 30 mA for approximately 1.5 hours. Gels were then transferred to blotting paper (3 mm; Whatman, Maidstone, England), dried and exposed to Biomax MS film (Eastman Kodak Co.,

Rochester, NY) for 20-24 hours at -80°C before being developed using an SRX-101A medical film developer (Konica Corp., Wayne, NJ).

**Western Blot**

Nuclear proteins from αT3-1 cells were extracted using the NE-PER® Nuclear and Cytoplasmic Extraction Reagent Kit per manufacturer's instructions (Pierce Biotechnology, Rockford, IL), quantitated with a BCA Protein Assay (Pierce) and stored at -80 C. Protein samples (40 μg) were boiled for 5 min in a 2X reducing loading buffer (130 mM Tris pH 6.8, 4% SDS, 0.02% Orange G, 20% glycerol, 100 mM DTT), cooled to room temperature and loaded onto an SDS polyacrylamide gel (PAGE) with a 5% stacking and 10% resolving gel. Gels were run at 40 mA for approximately 90 min and electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF, Immobilon –FL, Millipore, Billerica, MA) membrane with a semi-dry electroblotter (Panther, Owl Separation Systems, Portsmouth, NH). Briefly, PVDF membrane was pre-wetted in 100% methanol and soaked with the gel in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 0.1% SDS, 20% methanol) for 15 min. The proteins were transferred at 200 mA for 1 h. Membranes were blocked with StartingBlock™ (TBS) Buffer (Pierce) for 30 min at room temperature with agitation. Incubation of primary antibody directed against the GR (Santa Cruz Biotechnology) was performed in StartingBlock™ (TBS) Buffer (Pierce) supplemented with 0.05% Tween-20. Antibody was used at 1:1000 dilutions. Blots were incubated with primary antibody overnight at 4 C with gentle shaking. After incubation the blots were washed four times with TBS-T (20 mM Tris pH 7.6, 137 mM sodium chloride, 0.1% Tween-20). Each wash was performed for 5 min

with gentle agitation. The secondary antibody, Alexa Fluor 680 goat anti-rabbit IgG (A21076, Invitrogen, Carlsbad, CA) was diluted 1:8000 in StartingBlock™ (TBS) Buffer (Pierce) supplemented with 0.01% SDS and 0.05% Tween-20. The incubation was performed at room temperature for 1 h with gentle shaking. Blots were washed four times in TBS-T for 5 min with gentle agitation. After a final rinse with TBS, blots were scanned on the 700 channel of the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) following manufacturer's instructions.

**Biotinylated DNA and Protein Pull-down Assay**

We performed pull down assay according to a procedure described by Deng et al. (2003) with minor modifications (Figure 3.1). In brief, 5 ug of 5'-biotinylated DNA promoter probe containing GRE was mixed with 500 ug of nuclear extract from αT3-1 cells treated with vehicle or 100 nM dexamethasone and 100 ul of 4 % streptavidin-agarose beads in Pierce Biotinylated Protein Interaction Pull-Down Kit (product no. 2115, Thermo Fisher Scientific Inc., Rockford, IL). The final volume was adjusted to 500 ul with nuclear extract buffer from the pull-down kit. The mixture was incubated at room temperature for 1 h with gentle shaking and centrifuged at 5000 × g in a microcentrifuge for 30 sec. The supernatant was removed and the pellet was washed four times with 1 ml of iced PBS. The pulled down mixture, then, was resuspended in 50 ul of Laemmli sample buffer (Bio-Rad) and boiled for 5 min. 25 ul of the samples was placed in a 4-20 % gradient polyacrylamine minigel to perform SDS-PAGE.

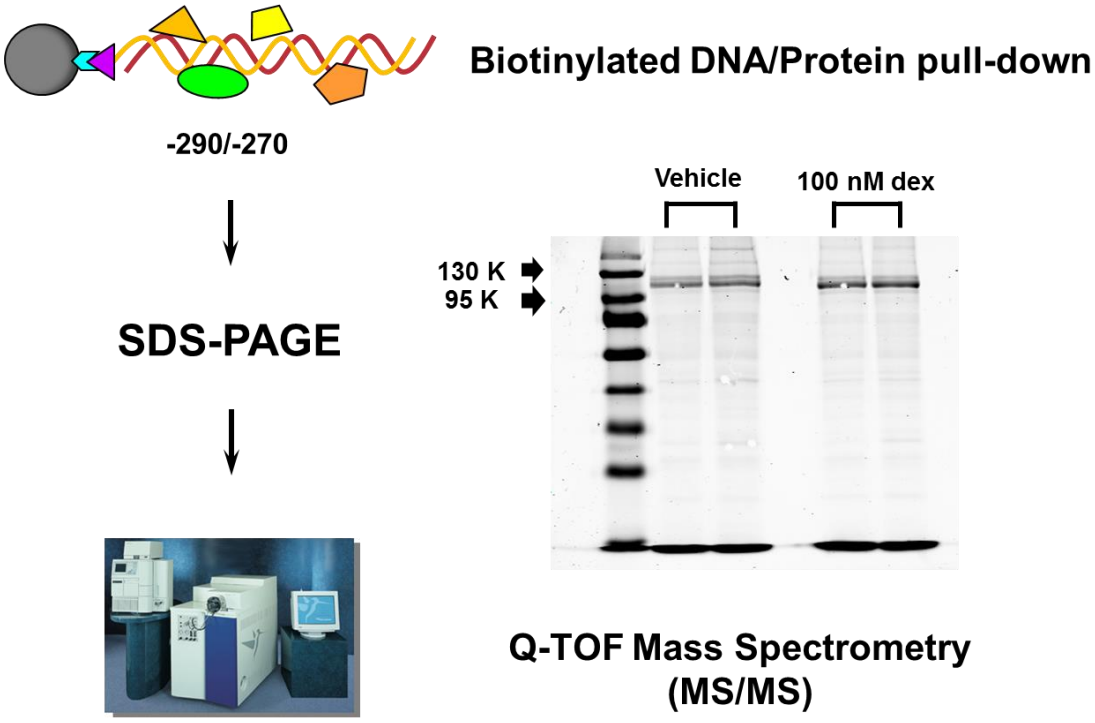



Figure 3.1. Diagram of biotinylated DNA/protein pull-down assay, SDS-PAGE, and Mass Spectrometry. In order to identify which protein binds to the 290/270 bp region during the dex treatment, I utilized a DNA pull-down assay to isolate the oligonucleotide-protein complex, and then separated proteins comprising the complex using SDS-PAGE. In the gel, protein band was captured between 95kDa and 130kDa in both treatments. Spots of interest were then excised from the SDS-PAGE gel and sent to the Nebraska Center for Mass Spectrometry for protein identification. Mass spectrometry results from both vehicle and 100 nM dexamethasone treated cells revealed Poly (ADP ribose) polymerase-1 protein.

**Mass Spectrometry**

Spots of interest were excised from the SDS-PAGE gel (Figure 3.1) and sent to the Nebraska Center for Mass Spectrometry (Lincoln, NE) for protein identification using tandem mass spectrometry (MS/MS) with a Waters Q-TOF Ultima mass spectrometer (Micromass/Waters, Milford, MA; Figure 3.1). Briefly, excised bands were digested using the method of Shevchenko et al. (1997) in which samples were washed with 100 mM ammonium bicarbonate, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, washed twice with 100 mM ammonium biocarbonate, digest *in situ* with 10 ng $\mu l^{-1}$ trypsin, and extracted with two 60 µl aliquots of 1:1 acetonitrile:water (v/v) containing 1% formic acid. The resulting peptides were separated on a C18 reversed phase column (75 µm × 15 cm; LC-Pacings, Dionex, Sunnyvale, CA), and eluted using a water + 0.1% formic acid (A)/95% acetonitrile:5% water + 0.1% formic acid (B) gradient with a 270 nl/min flow rate. The MS/MS data were processed to produce peak lists for database searching. Sequences were further searched against Matrix Science Database 20060908 (www.matrixscience.com) for peptide mass fingerprints of mouse (52735 sequences in the database) with the significance threshold set at $P < 0.05$. Molecular weight search scoring (MOWSE) used an algorithm described in Pappin et al. (1993) to determine the "rank" of the peptide compared to all matches in the database. MOWSE was also used in conjunction with the percent coverage and individual ion scores (not shown) for each amino acid in the peptide to verify homology or the identity of the protein.

**Bioinformatics and Statistical Analysis**

Analyses of sequence for transcription factor binding sites were performed with the Patch Public 1.0 program (Biobase, Wolfenbüttel, Germany). Data were analyzed using the general linear models (GLM) procedure of the Statistical Analysis System software (version 8.2, SAS Institute Inc, Cary, NC). Means for luciferase activity of test vectors were compared with control values using Dunnett's t-test, whereas means for luciferase activity among test vectors were compared using Tukey's Studentized Range test. Transfections were performed in triplicate with at least three replicates containing different plasmid preparations.

# CHAPTER IV

## Role of Poly [ADP-ribose] Polymerase-1 (PARP-1) as a Transcription Factor in Glucocorticoid Regulation of the Porcine GnRH Receptor (GnRHR) Gene.

## ABSTRACT

The binding of GnRH to its receptor results in the synthesis and secretion of the gonadotropins, as well as stimulation of the gene encoding its own receptor. Thus, the interaction between GnRH and GnRHR represents a central point for regulation of reproductive function. Glucocorticoids can alter reproduction by reducing GnRH responsiveness of gonadotropes within the anterior pituitary gland, potentially via transcriptional regulation of the GnRHR gene. Investigation of this possible mechanism, however, revealed that transcription of the murine GnRHR gene is stimulated by glucocorticoids. To determine the effect of glucocorticoids on porcine GnRHR gene expression, gonadotrope-derived αT3-1 cells were transiently transfected with a vector containing 5118 bp of 5' flanking sequence for the porcine GnRHR gene fused to luciferase for 12 h and treated with increasing concentrations of the glucocorticoid agonist, dexamethasone (0, 1, 10, 100 and 1,000 nM) for an additional 12 h prior to harvest. Maximal induction of luciferase activity was detected at 100 nM of dexamethasone (2-fold over vehicle; $P < 0.05$), and this response was blocked by the glucocorticoid antagonist, mifepristone (100 pM). Deletion from 274 to 323 bp upstream of the translational start site eliminated glucocorticoid responsiveness, suggesting the presence of a GRE(s) within this region. Electrophoretic mobility shift assays (EMSA)

using a 32P-labeled oligonucleotide spanning -290/-270 bp of proximal promoter revealed increased binding of nuclear extracts from αT3-1 cells treated with 100 nM dexamethasone compared to vehicle. Sequence analysis of this region indicated putative binding sites for PR, ER, GR, COUP-TF and GATA, as well as RXR α, β, and γ. However, competitive oligonucleotides for each of these transcription factors, including GR, were unable to compete for binding to the radiolabeled -290/-270 bp probe in EMSAs. Mass spectrometry analysis of isolated proteins from a pull-down using a biotinylated oligonucleotide (-290/-270 bp) identified PARP-1 as the key binding partner. To confirm the mass spectrometry result, we performed EMSAs with antibodies specific for either GR or PARP-1. Both the GR and PARP-1 antibodies generated a supershift of the specific binding complex. Interestingly, addition of the PARP-1 and GR antibodies together abolished the supershift. Since the competitive oligonucleotide for GR was unable to abrogate the DNA/protein complex, whereas inclusion of antibodies confirmed that GR was a member of the specific binding complex, this suggests that GR must recruit PARP-1 in order to bind the GRE. Inhibition of p38 and ERK1/2 mitogen-activated protein kinase (MAPK) pathways significantly decreased dexamethasone-induced promoter activity ($P < 0.05$), indicating the involvement of these signaling pathways in glucocorticoid stimulation of the promoter. Thus, our working model for glucocorticoid responsiveness of the porcine GnRHR gene suggests that, upon binding to its receptor, glucocorticoid triggers phosophorylation of GR by p38 and ERK1/2 MAPK pathways, resulting in the recruitment of PARP-1 by phosphorylated, ligand-bound GR to a GRE located within -290/-270 bp of the porcine GnRHR promoter.

## Introduction

The decapeptide gonadotropin-releasing hormone (GnRH) is a key factor that mediates the function of the hypothalamic-pituitary-gonadal axis in mammals. Generated by hypothalamic neurons, GnRH is released in an intermittent manner, traveling to gonadotropes in the anterior pituitary gland via the hypothalamo-hypophyseal portal system (Fink, 1988). Gonadotropin-releasing hormone binds to high-affinity receptors on gonadotrope cells resulting in the biosynthesis and secretion of both follicle stimulating hormone (FSH) and luteinizing hormone (LH), which stimulate steroid synthesis and gametogenesis in the gonads (Clayton and Catt, 1981; Clarke et al., 1983; Mason et al., 1986). Upon binding to its receptor, GnRH regulates expression of at least 4 gonadotropic genes including those encoding: the common α-glycoprotein subunit, the specific LHβ- and FSHβ-subunits that combine to produce LH or FSH (Hamernik and Nett, 1988; Gharib et al., 1990), and the GnRH receptor (GnRHR) itself (Sealfon and Millar, 1995). Transcriptional regulation of GnRHR is mediated by GnRH via protein kinase A (PKA) and C (PKC) activation of multiple mitogen-activated protein kinase (MAPK) pathways including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 MAPK, and ERK5, also known as big MAPK1 (BMK1; Sundaresan et al., 1996; Roberson et al., 1999; Noar et al., 2000; Liu et al., 2003; Bonfil et al., 2004). Thus, the interaction between GnRH and its receptor represents a central point for regulation of reproductive function in mammals.

Many studies have examined responsiveness of the GnRHR gene to hormones including GnRH (White et al., 1999; Norwitz et al., 1999; Ellsworth et al., 2001; Liu et al., 2003), estradiol-17β (Gregg et al., 1990; Laws et al., 1990; Wu et al., 1994; Duval et

al., 2000), progesterone (Laws et al., 1990; Cheng et al., 2001), testosterone (Kaiser et al., 1993; Curtin et al., 2001; Zapatero-Caballero et al., 2003) activin (Fernandez-Vazquez et al., 1996; Pernasetti et al., 2001; Norwitz et al., 2002), and inhibin (Braden et al., 1990; Gregg et al., 1991; Wu et al., 1994). Several reports indicated that GnRH regulates GnRHR numbers and mRNA levels in the pituitary gland from the rat (Pieper et al., 1982; Clayton et al., 1982; Kaiser et al., 1993; Bauer-Dantoin et al., 1995), sheep (Turzillo et al., 1994), and cow (Vizcarra et al., 1997). In contrast, others reported no change in amounts of GnRHR mRNA after GnRH treatment in the gonadotrope-derived αT3-1 cell line (Tsutsumi et al., 1993, 1995; Alarid and Mellon, 1995). Besides regulation of the level of GnRHR mRNA, GnRH can both up- and down-regulate receptor numbers in the pituitary (McArdle et al., 1987; Uemura et al., 1992; Bauer-Dantoin et al., 1993; Conn et al., 1995). Norwitz et al. (1999) determined two elements involved in GnRH responsiveness, sequence underlying responsiveness to GnRH-1 and 2 (SURG-1 and SURG-2, respectively) and Kam et al. (2005) reported that nuclear factor Y (NF-Y) and octamer transcription factor-1 (Oct-1) bind to the SURG-1 element to increase basal and GnRH-stimulated expression of the mouse GnRHR gene. White et al. (1999) isolated an activation protein-1 (AP-1) element within SURG-2 that conferred GnRH responsiveness of the GnRHR promoter. Moreover, these investigators identified that GnRH regulation of the GnRHR gene was mediated via activation of an AP-1 element by PKC (White et al., 1999) and the JNK pathway (Ellsworth et al., 2003). In addition, the GnRHR activating sequence (GRAS), important to basal promoter activity, also mediated activin responsiveness in the mouse GnRHR gene (Duval et al., 1999). The binding of Smad3 and 4 proteins to GRAS (Duval et al., 2000) and AP-1 complexes to an overlapping AP-1

element regulated activin responsiveness (Ellsworth et al., 2003). Further studies demonstrated that binding of the LIM homeodomain proteins, LHX2 and 3, to a downstream activin regulatory element (DARE) is also necessary for activin responsiveness (Cherrington et al., 2005; Cherrington et al., 2006).

Glucocorticoids can act at both the hypothalamus and anterior pituitary gland to regulate gonadotropin secretion (Brann and Mahesh, 1991; Tilbrook et al., 2000). Recent investigations into stress-related influences on reproductive function implicated glucocorticoids in the physiological regulation of GnRH and its receptors. For example, glucocorticoids acted directly at the hypothalamus to suppress GnRH synthesis (Chandran et al., 1994) and reduced the activity of the GnRH pulse-generating center (Dubey and Plant, 1985). Similarly, in the anterior pituitary gland, glucocorticoids decreased GnRH responsiveness of gonadotrope cells (Kamel and Kubajak, 1987; Baldwin et al., 1991). In contrast to inhibition of GnRH secretion from the hypothalamus and reduced responsiveness of gonadotropes to GnRH, glucocorticoids also increased GnRHR mRNA and protein levels. In male rats, the glucocorticoid agonist, dexamethasone, acted directly on gonadotrope cells to modulate a GnRH-induced increase in GnRHR numbers, as well as gonadotropin gene expression and secretion (Rosen et al, 1991). In addition, dexamethasone treatment increased endogenous GnRHR mRNA levels in the gonadotrope-derived LβT2 cell line (Turgeon et al., 1996) and activity of the murine GnRHR promoter in pituitary adenoma-derived $GGH_3$ (Maya-Núñez and Conn, 2003) and LβT2 (McGillivray et al., 2007) cells. Maya-Núñez and Conn (2003) identified the glucocorticoid response element (GRE) as an AP-1 binding site located between 255 and 331 bp upstream of the transcriptional start site that bound

the transcription factor, c-Jun, suggesting that ligand-bound glucocorticoid receptors interact directly or indirectly with c-Jun to regulate GnRHR transcription. Our laboratory has isolated the porcine GnRHR gene promoter and identified elements conferring basal activity in αT3-1 cells. The objectives of this study are to determine glucocorticoid responsiveness of the porcine GnRHR gene, to isolate the glucocorticoid response element(s) located within the porcine GnRHR promoter and to examine the molecular mechanisms underlying glucocorticoid stimulation of GnRHR gene expression in αT3-1 cells.

## Materials and Methods

**Materials.** Dexamethasone and mifepristone (RU486) were from Sigma Chemical Co. (St. Louis, MO) and Org 31710 was a generous gift from N.V. Organon (Oss, Netherlands). The antibody specific for the PARP-1 (catalog no. 614301) was purchased from BioLegend (San Diego, CA); and the specific antibody for the GR (catalog no. sc-1004X) and normal rabbit IgG (catalog no. sc-2027) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For experiments using EMSA, DNA probe (-290/-270; sense sequence 5'-TTGTGAAAACCAGGCCATCTG-3' and antisense sequence 5'-CAGATGGCCTGGTTTTCACAA-3') and competitive oligonucleotides containing consensus binding sites for transcription factor were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Biotinylated DNA probe (5'biotin/TTGTGAAAACCAGGCCATCTG-3') was also synthesized by IDT for biotinylated DNA-pull down assays.

**Plasmids.** A reporter vector containing 5118 bp of porcine GnRHR promoter (-5118pGL3) was constructed by PCR amplification of the 5' flanking region of the GnRH receptor gene from genomic DNA preparations of pigs representing a white crossbred line. The PCR product was subsequently subcloned into pBluescript SK- (Stratagene, La Jolla, CA). Promoter inserts were ligated into a reporter vector containing the cDNA encoding luciferase (pGL3; Promega Corp., Madison, WI) at the SacI/EcoRV location of the multiple cloning site. Deletion constructs were made by progressively removing 5' flanking sequence (approximately 500 bp) via restriction enzyme digests and subsequent intramolecular ligation of the remaining vector backbone. Construction of the 100-bp deletion reporter vectors was achieved by amplifying the specified region of the promoter by PCR with a Taq DNA polymerase containing 3' to 5' exonuclease activity (Bioline, Springfield, NJ). Next, the fragments were subcloned into pBluescript SK- or pCR-Blunt II (Invitrogen, Carlsbad, CA), and then finally into pGL3.

**Cell Culture and Transfections.** αT3-1 (Dr. Pam Mellon, Salk Institute, La Jolla, CA) cells were maintained at 37 C in a humidified 5% $CO_2$ in air atmosphere. The αT3-1 cells were cultured in high-glucose DMEM (Mediatech, Herndon, VA) supplemented with 5% fetal bovine serum (FBS), 5% horse serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate (Gibco, Grand Island, NY). Transient transfections were performed using a liposome-mediated protocol (Fugene6; Roche Diagnostics Corp., Indianapolis, IN) following manufacturer's instructions. The day prior transfection, cells were plated in 6-well tissue culture plates at the appropriate

density (between 2 x $10^5$ and 2 x $10^6$ cells) to result in 40% confluency on the day of transfection. Cells were transfected with a 3:1 Fugene6 to DNA ratio. A total of 1 µg of DNA, 0.9 µg of luciferase test vector and 0.1 µg of pSV-β-gal were used per well. Luciferase (Promega Corp.) and β-gal assays (Applied Biosystems, Bedford, MA) were performed following manufacturer's instructions. After 12 h transfection, cells were treated with dexamethasone or progesterone. If mentioned, antagonists were added into cells 15 min prior to agonist treatment. At 24 h post transfection, cells were washed twice with ice-cold PBS and harvested with 200 µl of lysis buffer [100 mM potassium phosphate (pH 7.8), 0.2% Triton X-100 and 1 mM dithiothreitol (DTT)]. Lysates were cleared by centrifugation at 16,000 x g for 2 min at 4 C and 20 µl of lysate was used to perform each of the assays. Luciferase and β-gal values for each sample were determined using a Wallac VICTOR$^2$ micro plate reader (PerkinElmer Life Sciences, Boston, MA). To normalize for transfection efficiency, luciferase activity was divided by β-gal values.

**Electrophoretic Mobility Shift Assays.** Nuclear protein extracts were prepared from αT3-1 cells utilizing the NE-PER® Nuclear and Cytoplasmic Extraction Reagent Kit according to manufacturer's instructions (Pierce Biotechnology, Rockford, IL). Approximately 2.8 x $10^8$ αT3-1 cells were harvested with TNE buffer [10 mM Tris (pH 8), 140 mM NaCl, 1 mM EDTA], and the extracts were treated with protease (catalog no. P8340; Sigma Chemical Co.) and phosphatase (catalog no. 524625; Calbiochem, La Jolla, CA) inhibitor cocktails to prevent enzymatic degradation of proteins. The amount of protein present in the extracts was determined using a bicinchoninic acid (BCA) assay (Pierce Biotechnology). Oligonucleotide probes were end-labeled with [γ-$^{32}$P]ATP using

T4 polynucleotide kinase (MBI Fermentas Inc., Hanover, MD) and purified using sephadex G-25 spin columns (Amersham Biosciences Corp., Piscataway, NJ). Nuclear extracts (5 μg) were incubated with 4 μl of Dignam D buffer (20 mM HEPES, 20% glycerol, 0.1 M potassium chloride, 0.2 mM EDTA, 0.5 mM DTT), 1 mM DTT, 2 μg poly(dI•dC) (Amersham Biosciences Corp.) and, where indicated, rabbit antiserum directed against GR (catalog no. sc-1004X; Santa Cruz Biotechnology) or an equal amount of rabbit IgG (catalog no. sc-2027; Santa Cruz Biotechnology) at 25 C for 30 min. Following incubation, radiolabeled probe (100,000 cpm) was added and, where indicated, 50-fold molar excess of either homologous or heterologous unlabeled competitor. Reactions were incubated for an additional 20 min at 25 C before free probe was separated from bound probe by electrophoresis for 1.5 h at 30 mA in 5 % polyacrylamide gels that were prerun for 1 h at 100 V in 1 X TGE [25 mM Tris (pH 8.3), 190 mM glycine and 1 mM EDTA]. Gels were transferred to blotting paper, dried, and exposed to Biomax MS film (Eastman Kodak Co., Rochester, NY) for 20-24 h at -80 C before being developed.

**Western Blot.** **N**uclear proteins from αT3-1 cells were extracted using the NE-PER® Nuclear and Cytoplasmic Extraction Reagent Kit per manufacturer's instructions (Pierce Biotechnology), quantitated with a BCA Protein Assay (Pierce Biotechnology) and stored at -80 C. Protein samples (40 μg) were boiled for 5 min in a 2X reducing loading buffer (130 mM Tris pH 6.8, 4% SDS, 0.02% Orange G, 20% glycerol, 100 mM DTT), cooled to room temperature and loaded onto an SDS polyacrylamide gel (PAGE) with a 5% stacking and 10% resolving gel. Gels were run at 40 mA for approximately 90

min and electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF; Immobilon –FL; Millipore, Billerica, MA) membrane with a semi-dry electroblotter (Panther; Owl Separation Systems, Portsmouth, NH). Briefly, PVDF membrane was pre-wetted in 100% methanol and soaked with the gel in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 0.1% SDS, 20% methanol) for 15 min. The proteins were transferred at 200 mA for 1 h. Membranes were blocked with StartingBlock™ (TBS) Buffer (Pierce Biotechnology) for 30 min at room temperature with agitation. Incubation of primary antibody directed against the GR (Santa Cruz Biotechnology) was performed in StartingBlock™ (TBS) Buffer (Pierce Biotechnology) supplemented with 0.05% Tween-20. Antibody was used at 1:1000 dilutions. Blots were incubated with primary antibody overnight at 4 C with gentle shaking. After incubation the blots were washed four times with TBS-T (20 mM Tris pH 7.6, 137 mM sodium chloride, 0.1% Tween-20). Each wash was performed for 5 min with gentle agitation. The secondary antibody, Alexa Fluor 680 goat anti-rabbit IgG (catalog no. A21076, Invitrogen) was diluted 1:8000 in StartingBlock™ (TBS) Buffer (Pierce Biotechnology) supplemented with 0.01% SDS and 0.05% Tween-20. The incubation was performed at room temperature for 1 h with gentle shaking. Blots were washed four times in TBS-T for 5 min with gentle agitation. After a final rinse with TBS, blots were scanned on the 700 channel of the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) following manufacturer's instructions.

**Biotinylated DNA and Protein Pull-down Assay.** We performed pull down assays according to a procedure described by Deng et al. (2003) with minor modifications. In brief, 5 ug of 5'-biotinylated DNA promoter probe containing GRE

was mixed with 500 ug of nuclear extract from αT3-1 cells treated with vehicle or 100 nM dexamethasone and 100 ul of 4 % streptavidin-agarose beads in Pierce Biotinylated Protein Interaction Pull-Down Kit (product no. 2115; Pierce Biotechnology). The final volume was adjusted to 500 ul with nuclear extract buffer from the pull-down kit. The mixture was incubated at room temperature for 1 h with gentle shaking and centrifuged at 5000 × g in a microcentrifuge for 30 sec. The supernatant was removed and the pellet was washed four times with 1 ml of iced PBS. The pulled down mixture, then, was resuspended in 50 ul of Laemmli sample buffer (Bio-Rad) and boiled for 5 min. 25 ul of the samples was placed in a 4-20 % gradient polyacrylamine minigel to perform SDS-PAGE.

**Mass Spectrometry.** Spots of interest were excised from the SDS-PAGE gel and sent to the UNL Center for Mass Spectrometry (Lincoln, NE) for protein identification using tandem mass spectrometry (MS/MS) with a Waters Q-TOF Ultima mass spectrometer (Micromass/Waters, Milford, MA). Briefly, excised bands were digested using the method of Shevchenko et al. (1997) in which samples were washed with 100 mM ammonium bicarbonate, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, washed twice with 100 mM ammonium biocarbonate, digest *in situ* with 10 ng $\mu l^{-1}$ trypsin, and extracted with two 60 µl aliquots of 1:1 acetonitrile:water (v/v) containing 1% formic acid. The resulting peptides were separated on a C18 reversed phase column (75 µm × 15 cm; LC-Pacings, Dionex, Sunnyvale, CA), and eluted using a water + 0.1% formic acid (A)/95% acetonitrile:5% water + 0.1% formic acid (B) gradient with a 270 nl/min flow rate. The MS/MS data were processed to produce peak lists for

database searching. Sequences were further searched against Matrix Science Database 20060908 (www.matrixscience.com) for peptide mass fingerprints of mouse (52735 sequences in the database) with the significance threshold set at $P < 0.05$. Molecular weight search scoring (MOWSE) used an algorithm described by Pappin et al. (1993) to determine the "rank" of the peptide compared to all matches in the database. MOWSE was also used in conjunction with the percent coverage and individual ion scores (not shown) for each amino acid in the peptide to verify homology or the identity of the protein.

**Bioinformatics and Statistical Analysis.** Analyses of sequence for transcription factor binding sites were performed with the Patch Public 1.0 program (Biobase, Wolfenbüttel, Germany). Data were analyzed using the general linear models (GLM) procedure of the Statistical Analysis System software (version 8.2, SAS Institute Inc, Cary, NC). Means for luciferase activity of test vectors were compared with control values using Dunnett's t-test, whereas means for luciferase activity among test vectors were compared using Tukey's Studentized Range test. Transfections were performed in triplicate with at least three replicates containing different plasmid preparations.

## Results

**Activity of the porcine GnRHR promoter is regulated by dexamethasone in dose-dependent manner.** To investigate glucocorticoid responsiveness of the porcine GnRHR gene, we performed transient transfections in αT3-1 cells with luciferase reporter plasmids containing the full-length porcine GnRHR promoter (-5118pGL3) followed by

treatment with increasing concentrations of the glucocorticoid agonist, dexamethasone, or 100 nM dexamethasone in combination with increasing amounts of glucocorticoid antagonist, mifepristone (RU 486; Figure 4.1). Treatment with increasing amounts of dexamethasone resulted in a dose-dependent increase in promoter activity ($P < 0.05$) and optimal promoter activity occurred after treatment with 100 nM of dexamethasone (2-fold). To confirm specificity of this response, we treated cells with 100 nM dexamethasone and increasing amounts of RU 486 (Figure 4.1). Dexamethasone-induced promoter activity was reduced to basal levels following treatment with 100 pM RU 486, suggesting that GR or PR may mediate glucocorticoid responsiveness of the porcine GnRHR gene (Figure 4.1). Although 10 pM is within the range of GR-specific inhibition for RU 486, this inhibitor can also interact with PR a dfferent binding affinities.

**Dexamethasone responsiveness of the porcine GnRHR gene is mediated by GR and GR translocation to nucleus.** To investigate whether dexamethasone responsiveness of the porcine GnRHR gene was mediated by GR or PR, we transiently transfected αT3-1 cells with luciferase reporter plasmids containing the full-length porcine GnRHR gene promoter (-5118pGL3) and treated with increasing concentrations of dexamethasone or 100 nM dexamethasone in combination with increasing amounts of the progesterone antagonist, Organon 31710 (Org31710; Figure 4.2). It has been reported that Org31710 has a similar relative binding affinity for cytoplasmic PR as RU 486, but a much lower binding affinity (over 30-fold) for GR than RU 486 (Kloosterboer et al., 1994). Similar to results shown in Figure 4.1, dexamethasone-induced luciferase activity of the GnRHR gene increased in a dose-dependent manner. However, increasing

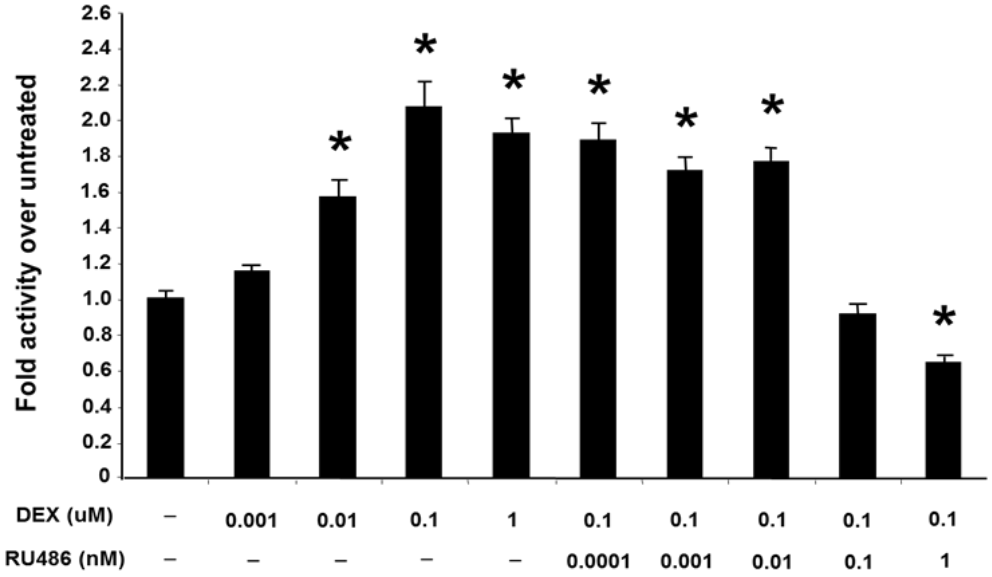



Figure 4.1. Luciferase activity of αT3-1 cells transiently transfected with porcine GnRHR promoter constructs following treatment with increasing concentrations of the glucocorticoid agonist, dexamethasone, or 100 nM dexamethasone in combination with increasing amounts of the glucocorticoid antagonist, RU486. αT3-1 cells were transfected with luciferase reporter (LUC) vectors containing the full length porcine GnRHR promoter. After 12 h, cells were treated with 0.001, 0.01, 0.1, and 1 uM dexamethasone or combinations of 0.1 uM dexamethasone and 0.0001, 0.001, 0.01, 0.1, or 1 nM RU486. RU486 was added to cells 15 min prior to dexamethasone treatment. Cells were co-transfected with RSV-β-gal and, after 24 h of transfection, cells were harvested and cellular lysates were assayed for LUC and β-gal activity. To control for transfection efficiency, LUC activity was divided by β-gal values and results are expressed as fold expression over pGL3. Optimal promoter activity occurred after treatment with 0.1 uM dexamethasone and activity was reduced to basal levels by 0.1 nM RU486.

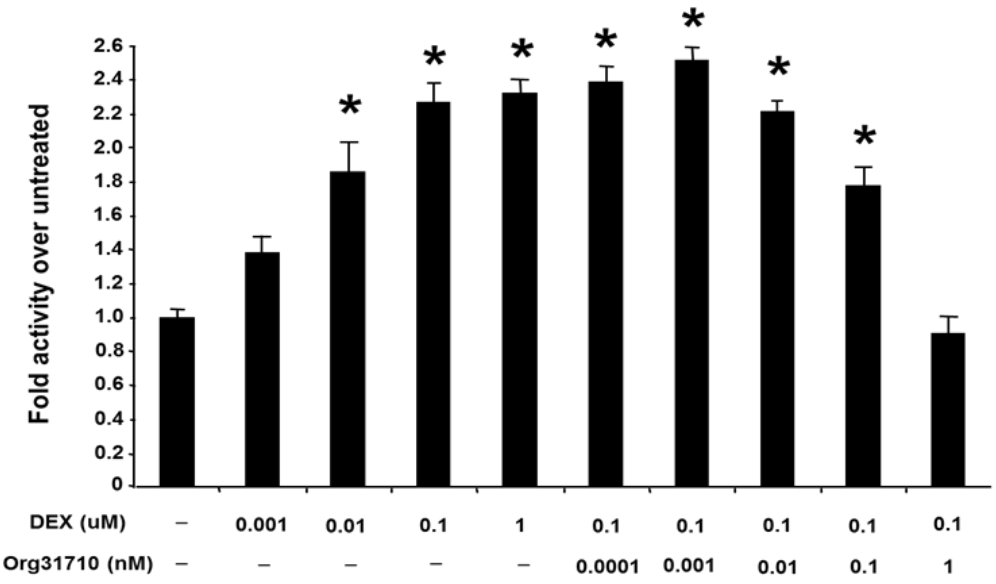



Figure 4.2. Luciferase activity of αT3-1 cells transiently transfected with porcine GnRHR promoter constructs following treatment with increasing concentrations of the glucocorticoid agonist, dexamethasone, or 100 nM dexamethasone in combination with increasing amounts of the progesterone antagonist, Org31710. αT3-1 cells were transfected with the full length porcine GnRHR promoter. After 12 h, cells were treated with 0.001, 0.01, 0.1, or 1 uM dexamethasone or combinations of 0.1 uM dexamethasone and 0.0001, 0.001, 0.01, 0.1, or 1 nM Org31710. Org31710 was added to cells 15 min prior to dexamethasone treatment. Optimal promoter activity occurred after treatment with 0.1 uM dexamethasone and activity was reduced to basal levels by 1 nM RU486. Bars with asterisks are different from controls ($P < 0.05$).

amounts of Org31710, at concentrations similar to RU 486, were unable to reduce dexamethasone-stimulated luciferase activity (Figure 4.2). Therefore, this result indicates that dexamethasone regulates the porcine GnRHR gene through binding to GR. Interestingly, 1 nM Org31710 treatment eliminated dexamethasone-induced promoter activity. However, this is due to the fact that Org 31710 has an affinity for both GR and PR (Kloosterboer et al., 1994).

Typically, GRs are located within the cytoplasm of the cell in unstimulated conditions (Dittmar et al., 1997; Hawle et al., 2006). In the presence of ligands, however, GRs are dimerized and translocated to the nucleus (Croxtall et al., 2000; Ford et al., 1997 Glass and Rosenfeld, 2000). To examine the translocation of GR in αT3-1 cells, we treated αT3-1 cells with vehicle and 100 nM dexamethasone and analyzed proteins from these cells using Western blot analysis (Figure 4.3). Nuclear extracts from αT3-1 cells treated with 100 nM dexamethasone expressed more GR protein in the nucleus compared to those treated with vehicle. This indicates GR translocation during dexamethasone exposure.

**Sequential 5' deletions of the full-length porcine reporter construct demonstrates that the 290/270 bp region of porcine GnRHR promoter is critical for dexamethasone responsiveness.** To determine the location of the glucocorticoid response element (GRE), we investigated dexamethasone responsiveness of αT3-1 cells transiently transfected with reporter vectors containing sequential deletion of 5' flanking sequences for the porcine GnRHR gene (Figure 4.4). Reduction of proximal promoter from 5000 to 320 bp had no effect on dexamethasone-stimulated luciferase activity ($P >$

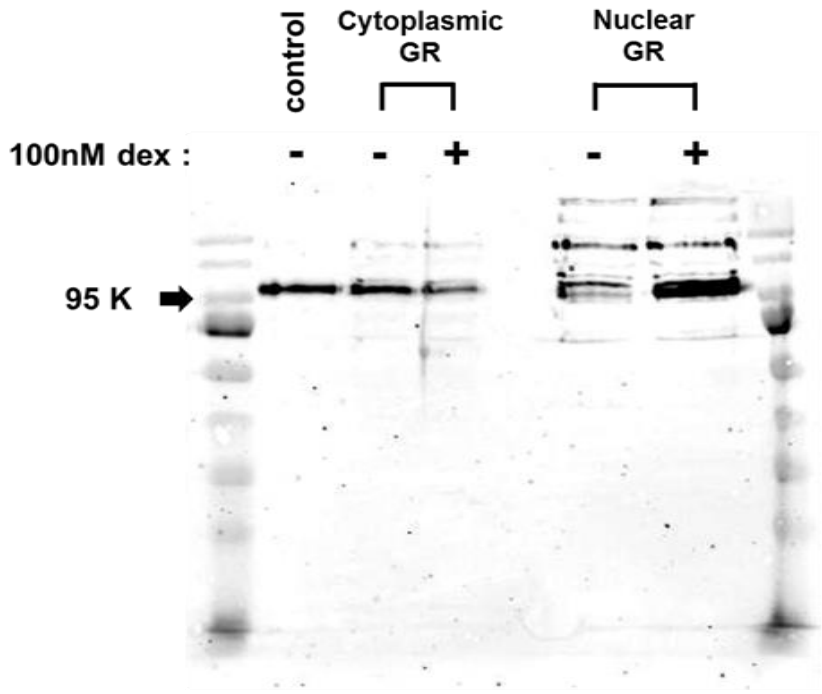



Figure 4.3. Determination of the translocation of GR following treatment with vehicle or 100 nM dexamethasone using western blot analysis. Detailed protocols are described in *Materials and Methods.* Briefly, nuclear extracts (40 μg) were boiled for 5 min in a 2X reducing loading buffer, cooled to room temperature and loaded onto an SDS polyacrylamide gel (PAGE) with a 5% stacking and 10% resolving gel. Gels were run at 40 mA for approximately 90 min and electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF, Immobilon –FL, Millipore, Billerica, MA) membrane with a semi-dry electroblotter (Panther, Owl Separation Systems, Portsmouth, NH). Incubation of primary antibody directed against the GR (Santa Cruz Biotechnology) was performed in StartingBlock™ (TBS) Buffer (Pierce) with 0.05% Tween-20 at 1:1000 dilutions. The secondary antibody, Alexa Fluor 680 goat anti-rabbit IgG (A21076, Invitrogen, Carlsbad, CA) was diluted 1:8000 in StartingBlock™ (TBS) Buffer (Pierce) supplemented with 0.01% SDS and 0.05% Tween-20. GR concentration was increased in nucleus while decreased in cytoplasm when αT3-1 cells were treated with 100nM dexamethasone. This indicates nuclear translocation of the GR in αT3-1 cells during dexsamethasone stimulation.

0.05). However, reduction of the promoter from 320 to 270 bp reduced luciferase activity to basal levels ($P < 0.05$). Additional deletion vectors within 320 and 270 bp region were constructed to more precisely locate the GRE (Figure 4.5). The vector containing the 320 to 290 bp region of the porcine promoter maintained dexamethasone-induced luciferase activity, whereas a complete loss in dexamethasone-induced luciferase activity was observed when the promoter was reduced to 270 bp ($P < 0.05$). Therefore, this result suggests that a glucocorticoid response element(s) is located within the -290/-270 bp region upstream of the translational start site for the porcine GnRHR promoter.

**EMSAs revealed that increased protein binding to the -290/-270 bp promoter region is responsible for dexamethasone-induced activity of the porcine GnRHR gene.** EMSAs were performed with αT3-1 nuclear extracts and radiolabeled probe spanning -290/-270 bp region generated from the porcine GnRHR promoter to identify binding factor(s) within the -290/-270 region. Results indicated increased specific binding complexes to the nuclear extracts from αT3-1 cells treated with 100 nM dexamethasone compared to vehicle (Figure 4.6). Sequence analysis of this region has revealed several putative transcription factor binding sites including estrogen receptor (ER), progesterone receptor (PR), glucocorticoid receptor (GR), signal transducer and activator of transcription (STAT), sma and mad related protein (SMAD), chicken ovalbumin upstream promoter transcription factor (COUTP-TF), and retinoid X receptor (RXR). To determine transcription factor(s) binding to GRE from the sequence analysis results, EMSAs were performed with αT3-1 nuclear extracts, radiolabeled probe containing -290/-270 region of the porcine GnRHR promoter, and non-labeled

oligonucleotides generated from the consensus sequence for each of the putative binding sites as a competitor. Although ER consensus oligonucleotides competed with the probe for binding to GRE, we were unable to identify protein(s) comprising the specific binding complex following addition of antibodies to EMSAs (Figure 4.6). Therefore, I mutated putative ER binding site, -279/-274 bp and investigated glucocorticoid responsiveness of the vector whether thie ER site is GRE or not (Figure 4.7). However, the mutation vector maintained dexamethasone-induced promoter activity, indicating that glucocorticoid response element is located in different site within -290/-270 bp other than putative ER binding site.

**Binding of GR and poly (ADP-ribose) polymerase-1 (PARP-1) induces dexamethasone responsiveness of the porcine GnRHR promoter.** Since we did not have any information regarding the binding protein(s), we needed to isolate the protein(s) from nuclear extracts of αT3-1 cells treated with vehicle or 100 nM dexamethasone. We performed DNA-protein pull-down assays to separate protein(s) bound to probe containing the GRE from the nuclear extracts. Purified samples were sent to the Nebraska Center for Mass Spectrometry (University of Nebraska-Lincoln, Lincoln, Nebraska) to identify the protein samples. The mass spectrometry results from both pull-downed samples showed several possible protein identifications (Figure 4.8 and 4.9). The primarly candidate protein was poly (ADP-ribose) polymerase-1 (PARP-1) in both vehicle and 100 nM dexamethasone treatment sample with the highest protein score, 1104 and 1478, respectively (Figure 4.8 and 4.9).

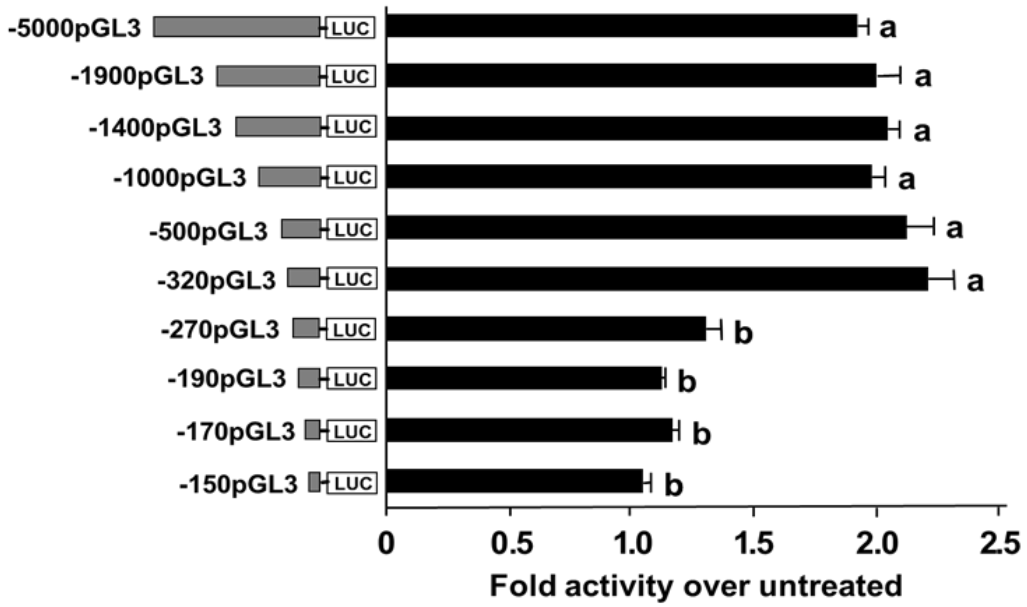



Figure 4.4. Transient transfection of αT3-1 cells with luciferase vectors containing sequential 5' deletions of the full-length porcine GnRHR promoter. αT3-1 cells were transfected with pGL3 constructs containing 5000, 1900, 1400, 1000, 500, 320, 270, 190, 170 and 150 bp of proximal promoter, or promoterless control. After 12 h, cells were treated with 100 nM dexamethasone for 12 h. Reduction of proximal promoter from 320 to 270 bp inhibited glucocorticoid-stimulated luciferase activity by 50%, indicating the presence of a glucocorticoid response element(s).

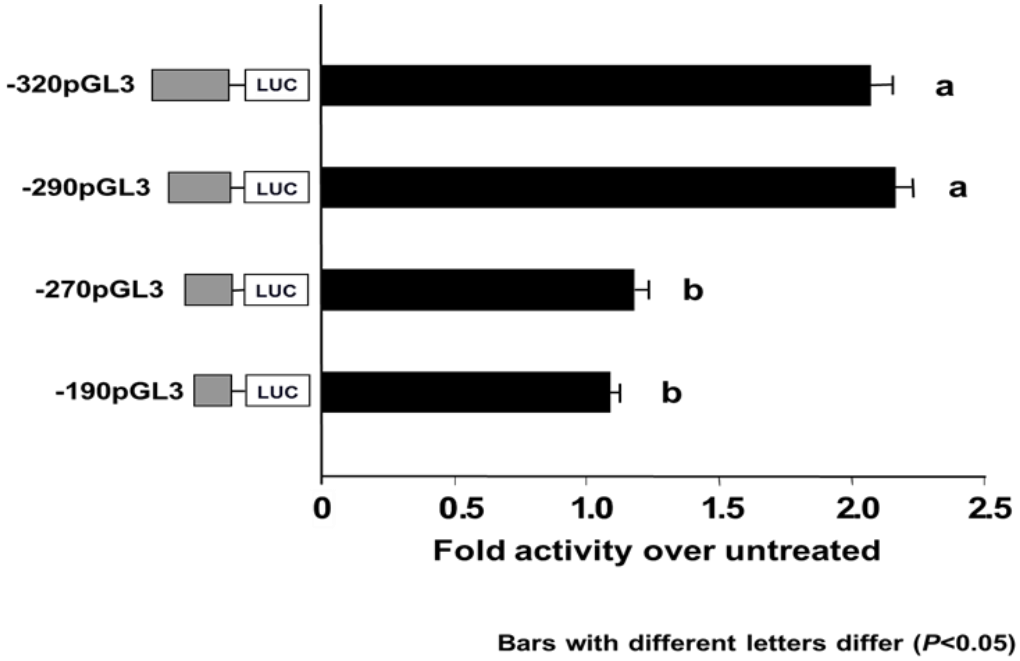



Figure 4.5. Transient transfection of αT3-1 cells with luciferase vectors containing sequential 5' deletions of the full-length porcine GnRHR promoter. Transient transfections were performed as shown in A with vectors containing 320, 290, 270, and 190 bp of 5' flanking sequence to more precisely locate the glucocorticoid response element(s). Results indicate glucocorticoid response element(s) is located between 270 and 290 bp upstream of the translational start site. Bars with different superscripts differ ($P < 0.05$).

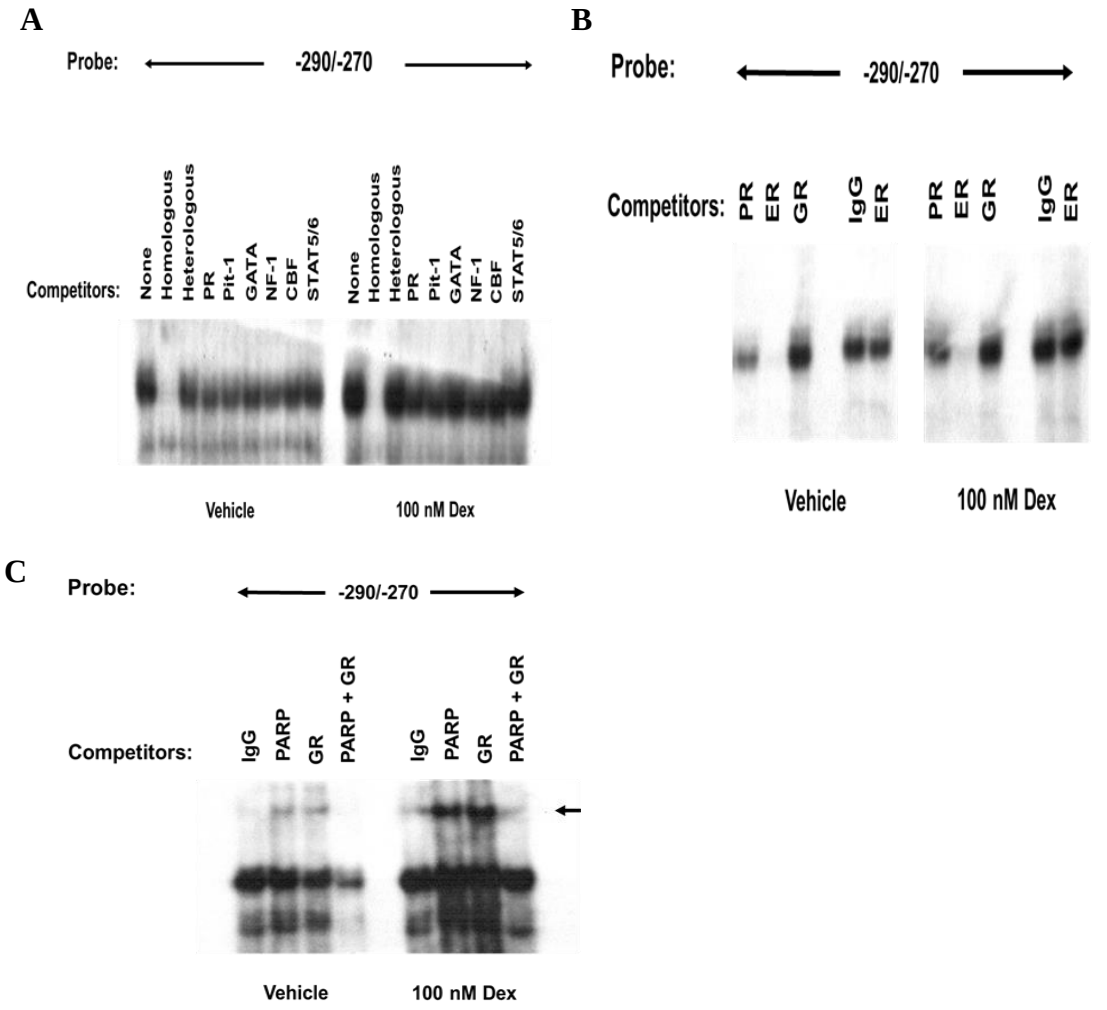



Figure 4.6. EMSA using a radiolabeled probe corresponding to the -290/-270 bp region of the porcine GnRHR promoter. A, Nuclear extracts (5 µg) from αT3-1 cells treated with vehicle or 100 nM dexamethasone were incubated with a radiolabeled probe spanning the -290/-270 region of the porcine GnRHR promoter. To determine the specificity of DNA-protein interactions, we added 50-fold molar excess of unlabeled homologous and heterologous probe or unlabeled oligonucleotides containing consensus sequences for specific transcription factor binding sites as competitors. Binding reactions were subjected to electrophoresis through polyacrylamide gels as described in *Materials and Methods*. Increased binding was detected for nuclear extracts from αT3-1 cells treated with 100 nM dexamethasone compared to those treated with vehicle (70% ethanol). B, EMSA was performed with PR, ER, and GR consensus oligonucleotides. In addition, nuclear extracts were incubated with antibodies (1 ug) directed against either ER or normal rabbit IgG before the addition of radiolabeled probe. However, the ER antibody did not alter the specific binding complex. C, EMSA was performed with antibodies directed against GR or poly (ADP-ribose) polymerase (PARP). Arrow indicates supershit complex. Both PARP and GR antibodies generated a supershift of the specific binding complex, whereas a combination of PARP and GR antibodies abolished the supershift generated from the specific binding complex.

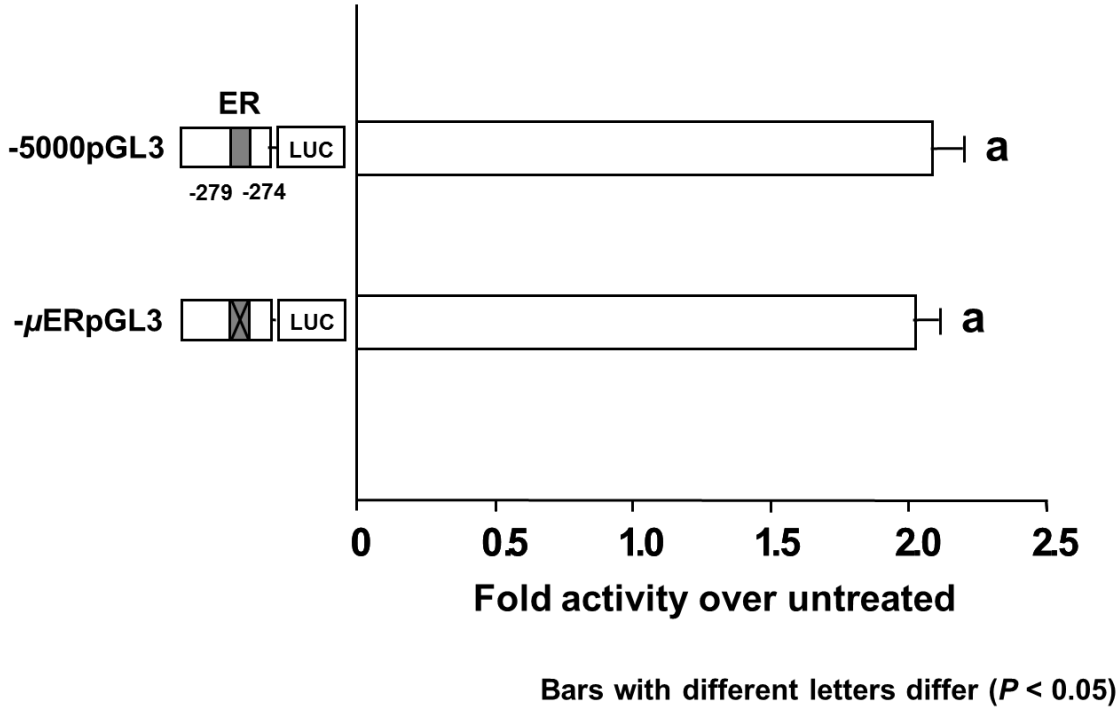



Figure 4.7. Mutation of ER binding site on the porcine GnRHR promoter. The mutation vector maintained dexamethasone-induced promoter activity, indicating that glucocorticoid response element is located in different site within -290/-270 bp other than putative ER binding site.

**A**

Database : NCBInr 20100601 (11112683 sequences; 3786819628 residues)
Taxonomy : Mus. (147782 sequences)
Timestamp : 10 Jun 2010 at 21:03:20 GMT
Protein hits:

| | |
|---|---|
| gi\|20806109 | poly [ADP-ribose] polymerase 1 [Mus musculus] |
| gi\|387397 | epidermal keratin subunit I [Mus musculus] |
| gi\|4159806 | type II keratin subunit protein [Mus musculus] |
| gi\|12843914 | unnamed protein product [Mus musculus] |
| gi\|16303309 | type II keratin 5 [Mus musculus] |
| gi\|148672085 | mCG144996 [Mus musculus] |
| gi\|13272554 | cytokeratin KRT2-6HF [Mus musculus] |
| gi\|22164776 | keratin, type II cytoskeletal 79 [Mus musculus] |
| gi\|46485130 | TPA: TPA_exp: keratin Kb40 [Mus musculus] |
| gi\|511654 | keratin type II [Mus musculus] |
| gi\|398168 | keratin 2 epidermis [Mus musculus] |
| gi\|741022 | keratin 15 |
| gi\|6754480 | keratin, type I cytoskeletal 13 [Mus musculus] |
| gi\|6730310 | Chain A, Crystal Structure Of The Arf-Gap Domain And Ankyrin Repeats Of Papbeta |
| gi\|74213106 | unnamed protein product [Mus musculus] |
| gi\|85701680 | keratin, type II cytoskeletal 2 oral [Mus musculus] |
| gi\|7106335 | keratin, type I cytoskeletal 17 [Mus musculus] |

**B**

Match to: gi|20806109; Score: 1104; poly [ADP-ribose] polymerase 1 [Mus musculus]
Nominal mass ($M_r$): 113506; Calculated pI value: 9.06
Taxonomy: Mus musculus
Sequence Coverage: 41%
Matched peptides shown in ***Bold***

| | | | | | |
|---|---|---|---|---|---|
| 1 | MAEASERLYR | VEYAKSGRAS | CKK***CSESIPK*** | DSLR***MAIMVQ*** | ***SPMFDGK***VPH |
| 51 | WYHFSCFWKV | GHSIR***QPDVE*** | ***VDGFSELR***WD | DQQKVKKTAE | AGGVAGKGQD |
| 101 | GSGGKAEK***TL*** | ***GDFLAEYAK***S | NRSMCKGCLE | KIEKGQMRLS | KKMVDPEKPQ |
| 151 | LGMIDRWYHP | TCFVKKRDEL | GFRPEYSASQ | LK***GFSLLSAE*** | ***DK***EALKKQLP |
| 201 | AIKNEGKR***KG*** | ***DEVDGTDEVA*** | ***K***KKSKKGKDK | DSSKLEKALK | AQNELIWNIK |
| 251 | DELKK***ACSTN*** | ***DLKELLIFNQ*** | ***QQVPSGESAI*** | ***LDRVADGMAF*** | ***GALLPCKECS*** |
| 301 | ***GQLVFKSDAY*** | ***YCTGDVTAWT*** | ***K***CMVKTQNPS | RKEWVTPKEF | REISYLKKLK |
| 351 | VKKQDR***IFPP*** | ***ESSAPAPLAL*** | ***PLSVTSAPTA*** | ***VNSSAPADKP*** | ***LSNMK***ILTLG |
| 401 | KLSQNKDEAK | AVIEKLGGKL | TGSANK***ASLC*** | ***ISTK***KEVEKM | SKKMEEVKAA |
| 451 | NVR***VVCEDFL*** | ***QDVSASTKSL*** | ***QELLSAHSLS*** | ***SWGAEVK***AEP | GEVVAPKGKS |
| 501 | AAPSKKSKGA | VKEEGVNKSE | KRMKLTLK***GG*** | ***AAVDPDSGLE*** | ***HSAHVLEK***GG |
| 551 | K***VFSATLGLV*** | ***DIVK***GTNSYY | K***LQLLEDDKE*** | ***SR***YWIFRSWG | R***VGTVIGSNK*** |
| 601 | LEQMPSKEDA | VEHFMKLYEE | KTGNAWHSK***N*** | ***FTKYPKKFYP*** | ***LEIDYGQDEE*** |
| 651 | ***AVK***KLTVKPG | TKSKLPKPVQ | ELVGMIFDVE | SMKK***ALVEYE*** | ***IDLQK***MPLGK |
| 701 | LSRR***QIQAAY*** | ***SILSEVQQAV*** | ***SQGSSESQIL*** | ***DLSNR***FYTLI | PHDFGMK***KPP*** |
| 751 | ***LLNNADSVQA*** | ***K***VEMLDNLLD | IEVAYSLLR***G*** | ***GSDDSSKDPI*** | ***DVNYEK***LKTD |
| 801 | IK***VVDRDSEE*** | ***AEVIRK***YVKN | THATTHNAYD | LEVIDIFKIE | REGESQRYKP |
| 851 | FRQLHNRRLL | WHGSR***TTNFA*** | ***GILSQGLRIA*** | ***PPEAPVTGYM*** | ***FGKGIYFADM*** |
| 901 | ***VSK***SANYCHT | SQGDPIGLIL | LGEVALGNMY | ELKHASHISK | LPKGKHSVKG |
| 951 | LGKTTPDPSA | SITLEGVEVP | LGTGIPSGVN | DTCLLYNEYI | VYDIAQVNLK |
| 1001 | YLLKLKFNFK | TSLW | | | |

Figure 4.8. MALDI-TOF MS identification of DNA pull-down protein extracted from αT3-1 cells treated with vehicle. A shows protein hits from mouse sequence in NCBI database (147782 sequences). Protein hits are listed in descending order of MOWSE score. B shows highest matching protein based on MOWSE score, molecular mass, and sequence coverage.

**A**

Database : NCBInr 20100601 (11112683 sequences; 3786819628 residues)
Taxonomy : Mus. (147782 sequences)
Timestamp : 10 Jun 2010 at 21:11:58 GMT

| | | |
|---|---|---|
| Protein hits: | gi\|20806109 | poly [ADP-ribose] polymerase 1 [Mus musculus] |
| | gi\|4159806 | type II keratin subunit protein [Mus musculus] |
| | gi\|12859782 | unnamed protein product [Mus musculus] |
| | gi\|387397 | epidermal keratin subunit I [Mus musculus] |
| | gi\|9910294 | keratin, type II cytoskeletal 71 [Mus musculus] |
| | gi\|741022 | keratin 15 |
| | gi\|46485130 | TPA: TPA_exp: keratin Kb40 [Mus musculus] |
| | gi\|52789 | unnamed protein product [Mus musculus] |
| | gi\|22164776 | keratin, type II cytoskeletal 79 [Mus musculus] |
| | gi\|13272554 | cytokeratin KRT2-6HF [Mus musculus] |
| | gi\|85701680 | keratin, type II cytoskeletal 2 oral [Mus musculus] |
| | gi\|398168 | keratin 2 epidermis [Mus musculus] |
| | gi\|7106335 | keratin, type I cytoskeletal 17 [Mus musculus] |

**B**

**Match to: gi|20806109; Score: 1478; poly [ADP-ribose] polymerase 1 [Mus musculus]**
**Nominal mass ($M_r$): 113506; Calculated pI value: 9.06**
Taxonomy: Mus musculus
Sequence Coverage: **43%**
Matched peptides shown in **Bold**

| | | | | | | |
|---|---|---|---|---|---|---|
| **1** | MAEASERLYR | VEYAKSGRAS | CKK**CSESIPK** | DSLR**MAIMVQ** | **SPMFDGK**VPH |
| **51** | WYHFSCFWKV | GHSIR**QPDVE** | **VDGFSELR**WD | DQQKVKKTAE | AGGVAGKGQD |
| **101** | GSGGKAEK**TL** | **GDFLAEYAK**S | NRSMCKGCLE | KIEKGQMRLS | KKMVDPEKPQ |
| **151** | LGMIDRWYHP | TCFVKKR**DEL** | **GFRPEYSASQ** | **LKGFSLLSAE** | **DK**EALKKQLP |
| **201** | AIKNEGKR**KG** | **DEVDGTDEVA** | **K**KKSKKGKDK | DSSKLEKALK | **AQNELIWNIK** |
| **251** | DELKK**ACSTN** | **DLKELLIFNQ** | **QQVPSGESAI** | **LDRVADGMAF** | **GALLPCK**ECS |
| **301** | GQLVFK**SDAY** | **YCTGDVTAWT** | **K**CMVKTQNPS | R**KEWVTPK**EF | R**EISYLKK**LK |
| **351** | VKKQDR**IFPP** | **ESSAPAPLAL** | **PLSVTSAPTA** | **VNSSAPADKP** | **LSNMK**ILTLG |
| **401** | KLSQNKDEAK | AVIEKLGGKL | TGSANK**ASLC** | **ISTK**KEVEKM | SKKMEEVKAA |
| **451** | NVR**VVCEDFL** | **QDVSASTKSL** | **QELLSAHSLS** | **SWGAEVK**AEP | GEVVAPKGKS |
| **501** | AAPSKKSKGA | VKEEGVNKSE | KRMKLTLKGG | AAVDPDSGLE | HSAHVLEKGG |
| **551** | K**VFSATLGLV** | **DIVK**GTNSYY | K**LQLLEDDKE** | **SRYWIFR**SWG | R**VGTVIGSNK** |
| **601** | LEQMPSKEDA | VEHFMKLYEE | KTGNAWHSK**N** | **FTKYPKKFYP** | **LEIDYGQDEE** |
| **651** | **AVK**KLTVKPG | TKSKLPKPVQ | ELVGMIFDVE | SMKKALVEYE | IDLQKMPLGK |
| **701** | LSR**RQIQAAY** | **SILSEVQQAV** | **SQGSSESQIL** | **DLSNR**FYTLI | PHDFGMKKPP |
| **751** | LLNNADSVQA | KVEMLDNLLD | IEVAYSLLR**G** | **GSDDSSKDPI** | **DVNYEK**LKTD |
| **801** | IKVVDR**DSEE** | **AEVIRK**YVKN | THATTHNAYD | LEVIDIFKIE | REGESQRYKP |
| **851** | FRQLHNRR**LL** | **WHGSRTTNFA** | **GILSQGLRIA** | **PPEAPVTGYM** | **FGKGIYFADM** |
| **901** | **VSKSANYCHT** | **SQGDPIGLIL** | **LGEVALGNMY** | **ELK**HASHISK | LPKGKHSVKG |
| **951** | LGKTTPDPSA | SITLEGVEVP | LGTGIPSGVN | DTCLLYNEYI | VYDIAQVNLK |
| **1001** | YLLK**LKFNFK** | TSLW | | | |

Figure 4.9. MALDI-TOF MS identification of DNA pull-down protein extracted from αT3-1 cells treated with 100 nM dexamesathone. A shows protein hits from mouse sequence in NCBI database (147782 sequences). Protein hits are listed in descending order of MOWSE score. B shows highest matching protein based on MOWSE score, molecular mass, and sequence coverage.

To confirm PARP-1 binding and demonstrate interaction between GR and PARP-1 to the -290/-270 region of the porcine GnRHR promoter, EMSAs were performed with the addition of an antiserum directed against PARP-1 and GR to the specific binding complex for a probe spanning -290/-270 region of the promoter. Addition of antibodies against GR and PARP-1 generated a supershift with nuclear extracts of αT3-1 cells treated with both vehicle or 100 nM dexamethasone (Figure 4.6). Interestingly, the combination of antibodies specific for GR and PARP-1 abrogated the specific binding complex both control and 100 nM dexamethasone treated cells. Thus, this result indicates GR and PARP-1 copoerate to regulate glucocorticoid responsiveness of the porcine GnRHR gene.

**The glucocorticoid receptor (GR) is phosphorylated via ERK1/2 and p38 MAPK signaling pathways in αT3-1 cells**. Ligand binding to GR leads to phosphorylation of GR, stimulation GR signaling and glucocorticoid responsiveness of a target cell. In order to examine which signaling pathway(s) were responsible for phosphorylation of GR underlying glucocorticoid responsiveness of the porcine GnRHR promoter, we transiently transfected αT3-1 cells with the full-length porcine GnRHR promoter and then treated with 100 nM dexamethasone in the presence or absence of SB202190, a p38 MAPK inhibitor, or U0126, a MEK1/2 inhibitor (Figure 4.10). Both inhibitors reduced dexamethasone-induced luciferase activity of the porcine GnRHR promoter. However, western blot results showed no changes of the phosphorylation of ERK and p38 MAPK during dexamethasone treatment (Figure 4.11). Interestingly, both ERK and p38 MAPK were already phosphorylated before dex treatment. This result indicates that ERK and p38 MAPK are not phosphorylated by GR signaling but they

phosphorylate GR to activate the GR translocation. This result is also consistent with Kotitschke et. al. (2009). Next, to investigate the relevance of the PKC or PKA signaling pathways in glucocorticoid responsiveness of the porcine GnRHR promoter, we transiently transfected αT3 cells with the full-length porcine GnRHR promoter and then treated αT3 cells with 100 nM dexamethasone and either a PKC inhibitor (GF109203X), a PKA inhibitor (SQ22536), or combination of both (Figure 4.12). Neither inhibitor decreased dexamethasone-stimulated luciferase activity of the promoter. Therefore, protein kinase A (PKA) and C (PKC) do not participate in GR activation and the glucocorticoid responsiveness of the porcine GnRHR gene, whereas p38 MAPK and ERK1/2 regulate glucocorticoid-induced promoter activity of the porcine GnRHR gene via GR phosphorylation.

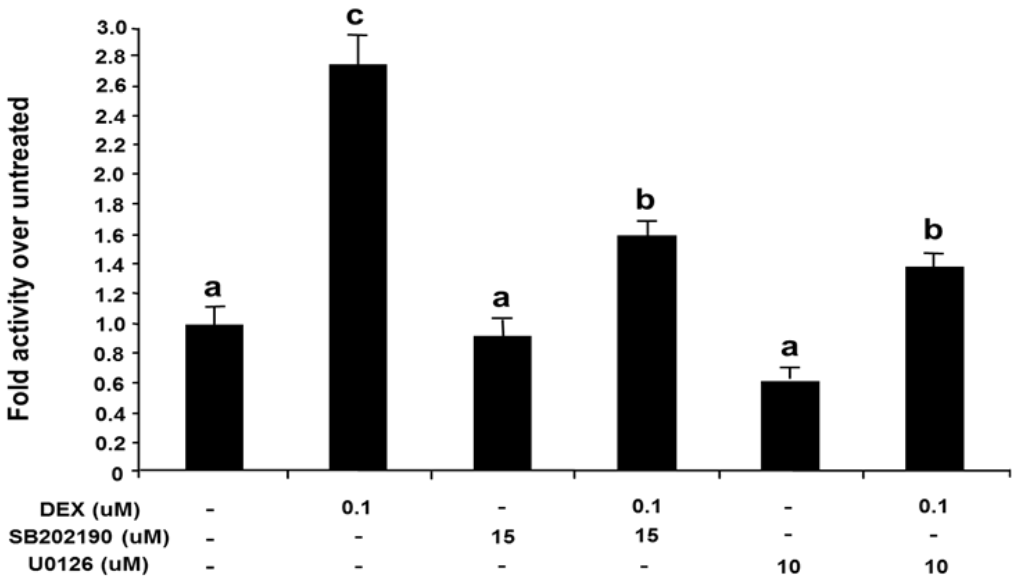



Figure 4.10. Signaling pathways underlying glucocorticoid responsiveness of the porcine GnRHR promoter. αT3-1 cells were transiently transfected with the full length porcine GnRHR promoter. After 12 h, cells were treated with either SB202190 (p38 MAPK inhibitor) or U1206 (MEK1/2 inhibitor) 15 min prior to 100 nM dexamethasone treatment. Both inhibitors decreased dexamethasone-induced promoter activity.

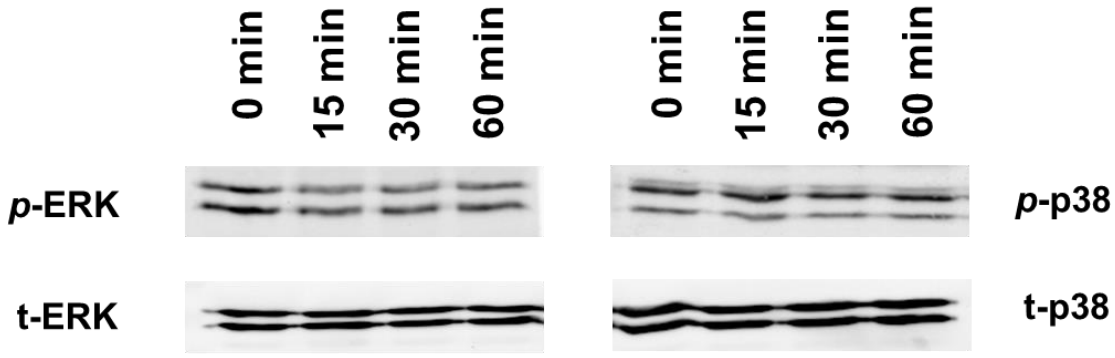



Figure 4.11. Phosphorylation of ERK and p38 MAPK during 100 nM dexamethasone treatment. Western blot results showed no changes of the phosphorylation of ERK and p38 MAPK during dexamethasone treatment. Interestingly, both ERK and p38 MAPK were already phosphorylated before dex treatment.

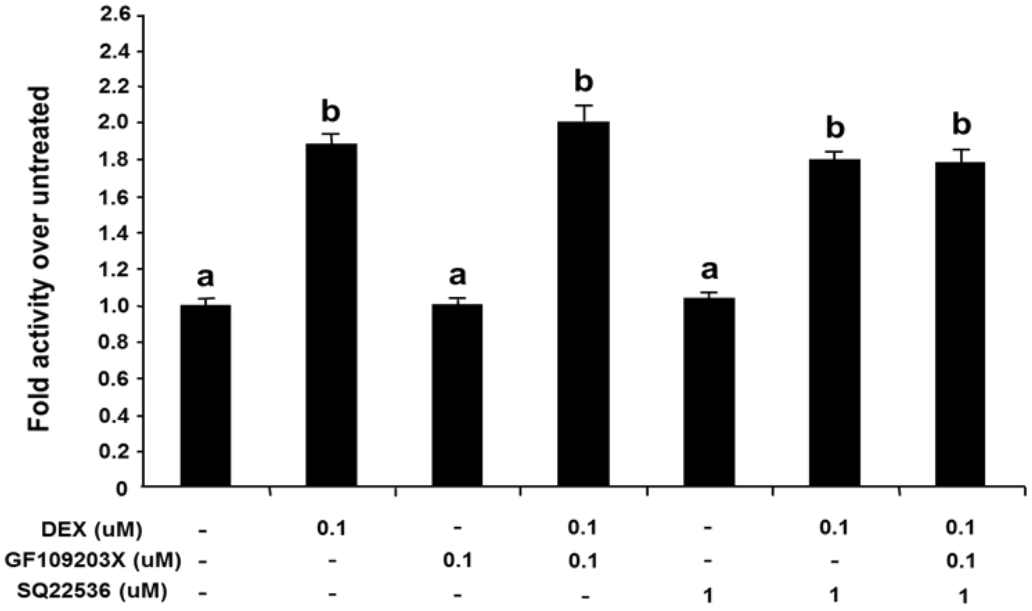



Figure 4.12. Signaling pathways underlying glucocorticoid responsiveness of the porcine GnRHR promoter. Cells were transfected and treated with either GF109203X (PKC inhibitor) or SQ22536 (PKA inhibitor) as shown in A. Both inhibitors had no effect on dexamethasone-induced promoter activity. Bars with different superscripts differ ($P < 0.05$).

## Discussion

Many studies have shown that hormones, including GnRH, estradiol, and progesterone induced responsiveness of the GnRHR gene. For example, GnRH can up- or down-regulate the numbers of its receptor in the pituitary (McArdle et al., 1987; Uemura et al., 1992), and regulate GnRHR mRNA levels in pituitary cells of the rat (Kaiser et al., 1993; Bauer-Dantoin et al., 1995), sheep (Turzillo et al., 1995), and cow (Vizcarra et al., 1997). Estradiol treatment of rat pituitary primary cultures can increase (long-term exposure) or decrease (short-term exposure) the number of GnRHRs (Menon et al., 1985; Emons et al., 1995). In the ewe, exogenous estradiol consistently increased the number of GnRHRs both in vivo (Kirkpatrick et al., 1998) and in vitro (Gregg et al., 1990; Laws et al., 1990). On the other hand, administration of progesterone decreased GnRHR mRNA levels in the ovine pituitary (Bauer-Dantoin et al., 1995) as well as primary pituitary cultures from the sheep (Wu et al., 1994). In addition to these hormones, glucocorticoids have also been implicated in the physiological regulation of GnRH and its receptor. Chandran et al. (1994) reported that glucocorticoids act directly at the hypothalamus to suppress GnRH synthesis. Dubey and Plant (1985) also demonstrated that glucocorticoids reduced the activity of the GnRH pulse-generating center within the hypothalamus. Similarly, in the pituitary, glucocorticoids decreased GnRH responsiveness of the gonadotropes. Although most studies have reported inhibitory effects of glucocorticoids on the production of the GnRHR, conflicting reports exist as others have demonstrated stimulatory effects. For example, glucocorticoids increased responsiveness of the mouse GnRHR gene (Maya-Núñez et al., 2003,

Kotitschke et al., 2009), GnRHR mRNA levels (5-fold; Turgeon et al., 1996), and gonadotropin subunit gene expression as well as gonadotropin secretion (Rosen et al., 1991). Thus, the mechanisms underlying glucocorticoid responsiveness of the GnRHR gene remain to be uncovered.

In this present study, we determined dexamethasone responsiveness of the porcine GnRHR gene using transient transfections in mouse gonadotrope-derived αT3-1 cells. Further, we investigated the signaling mechanisms underlying the dexamethasone responsiveness of the porcine GnRHR gene. Our results showed that dexamethasone activated the porcine GnRHR gene in a dose-dependent manner (Figure 4.1). Moreover, we reported that the optimal dexamethasone treatment was 100 nM and this response was mediated by GR and its translocation to the nucleus (Figure 4.2 and 4.3). Our observation that dexamethasone increased GnRHR promoter activity is consistent with previous reports. Maya-Núñez and coworkers (2003) examined glucocorticoid responsiveness of the mouse GnRHR gene promoter. In this study, dexamethasone activated the mouse GnRHR promoter in rat pituitary adenoma-derived GGH3 cells and this response was blocked by a glucocorticoid antagonist (RU 486). Progressive 5'-deletion study identified a putative glucocorticoid response element within the region -331/-255 of the mouse GnRHR gene promoter and point mutation study revealed AP-1 as the glucocorticoid response element. Further, they determined that this AP-1 element was directly associated with the c-Jun binding protein to increase dexamethasone induced gene expression of mouse GnRHR. Like Maya-Núñez et al. (2003), we also utilized RU 486 as a glucocorticoid antagonist to determine the specificity of the dexamethasone response by GR. RU 486, however, was the prototype of antiprogestins which have

potential uses for contraception and for treatment of hormone-related pathological conditions such as breast cancer, endometriosis, and leiomyomata (Attardi et al., 2004). Moreover, the RU 486 and PR complex still binds to progesterone responsive elements (PREs) at the target gene, but this does not induce gene activation due to conformational changes of the complex (Baulieu, 1991; Kloosterboer et al., 1994). Therefore, in order to confirm the specificity of the dexamethasone response mediated by not PR but GR, we utilized a new potent antiprogestagen, Org 31710 (Figure 4.2). It has been reported that Org 31710 had almost similar to RU 486 in relative binding affinity to the cytoplasmic PR, and Org 31710, however, had above 30 fold lower binding to the GR than RU 486 (Kloosterboer et al., 1994). They also suggested that in vivo, Org 31710 appear to be 10 fold more selective in antiprogestagenic effect than RU 486, which is mainly due to its low antiglucocorticoid activity. In our experiments, Org 31710 functioned well as a potent antiprogestagen with no effect on dexamethasone induced porcine GnRHR gene promoter activity via GR. Interesting result in that 1 nM Org 31710 abolished dexamethasone induced the promoter activity can be explained by its binding affinity to GR. It is because increasing amount of Org 31710 can increase binding to GR and reduce GR function even though it has 30 times less binding affinity than RU 486. This was confirmed by that 10 times more Org 31710 was required than RU 486 to remove dexamethasone stimulated promoter activity (Figure 4.1 and 4.2).

It has been well established that the GR resides in the cytoplasm in an unliganded state and the presence of glucocorticoid results in cytoplasmic liganded GR translocation to nucleus (Wikstrom et al., 1987; Picard and Yamamoto, 1987). Our western blot analysis result supported this statement, showing predominant appearance of GR protein

in the nucleus than in the cytoplasm after dexamethasone treatment (Figure 4.3). In the absence of glucocorticoid, unliganded GR exists as an oligomer associated with the chaperone complex including hsp90, hsp70, p23 and one hsp90-binding tetratricopeptide repeat (TPR) protein in the cytoplasm (Pratt et al., 2004). hsp90 binds to the GR ligand-binding domain, which leads to open the ligand binding cleft and maintains the GR in a conformation suitable for ligand-binding (Picard et al., 1990; Pratt et al., 2006). The hsp90 chaperone machinery is also required for the translocation of the ligand-GR complex into the nucleus (Pratt et al., 2006). Upon ligand-binding, the GR undergoes a conformational change and hyperphosphorylation, and subsequently move within the nucleus to their sites of action (Avenant et al., 2010; Czar et al., 1997). Translocation of ligand-GR complex is mediated by nuclear localization signal (NLS) sequences in the receptors themselves (Picard and Yamamoto, 1987) and bidirectional shuttling of receptors into and out of the nucleus occurs constantly (Guichon-Mantel et. al., 1991; Chandran and DeFranco, 1992; Madan and DeFranco, 1993; Dauvois et al., 1993). Studies of mechanisms on the nuclear localization of the cytoplasmic steroid receptors have demonstrated two different mechanisms. The most efficient mechanism is rapid with less than 5 min half-life and depends on the hsp90-FKBP52-based heterocomplex (Davies et al., 2002; Galigniana et al., 2002; Gallo et al., 2007; Harrell et al., 2004; Pilipuk et al., 2007). The alternative mechanism is hsp90 independent with about 40 to 60 min half-life, which allows the formation of degradasomes and the subsequent targeting of the receptor to proteasomal degradation (Galigniana et al., 2004). In contrast to the translocation of the receptor to the nucleus, GR cycles back very slowly to the cytoplasm with 8 to 12 hour upon steroid withdrawal (Galigniana et al., 1998).

Once the nuclear localization of ligand-GR complex occurs, the active GR can directly bind to either positive GREs that lead transcription activation or negative GREs that direct transcription repression on DNA to regulate target gene expression (Lu and Cidlowski, 2006). In addition, the active GR interacts with transcription factors to either enhance or repress the transcription of target genes by interfering with or modifying the actions of the GR partners (Lu and Cidlowski, 2006; Lu and Cidlowski, 2004). The GR can recruit a variety of proteins such as co-activators, co-repressors and chromatin remodeling complexes, depending on the cell type and promoter context to result in transactivation or transrepression (McKenna et al., 1999; Lu and Cidlowski, 2006; Avenant et al., 2010). Therefore, to localize the GRE(s) within the porcine GnRHR promoter that is responsible for glucocorticoid responsiveness via GR-mediated transcriptional activation, we performed transfection assays with reporter vector containing sequential deletion of 5' flanking sequences for the porcine GnRHR gene (Figure 4.4). These data suggested that the regions -320 to -270 are responsible for dexamethasone-induced porcine GnRHR activity and there is/are important element(s), GRE(s), within the regions. Further investigations by EMSA revealed more precise location of GRE, showing increased binding to the nuclear extracts from dexamethasone treated αT3-1 cells compared to vehicle (Figure 4.6) as well as by transfection assays (Figure 4.5). Interestingly, the result of EMSA from the binding competition based on sequence analysis showed that instead of GR, ER bound to GRE (Figure 4.6). However, supershift assay using ER antibody did not generate ER-antibody binding complex with the probe containing -290 to -270. Taken together, our findings suggested that the porcine GnRHR promoter does not have GRE, and the GR, thus, does not bind directly to

the promoter but rather via GR-protein interaction or nongenomic signaling without GR-DNA binding to regulate dexamethasone-stimulated responsiveness on the porcine GnRHR gene. In addition to GR, ER and PR, sequence analysis of the regions -290 to -270 has indicated several putative transcription factor binding sites including STAT, SMAD, COUP-TF, GATA, and Pit-1. Unfortunately, we were unable to identify protein(s) comprising the specific binding complex following addition of antibodies to EMSAs (data not shown). We assumed that it is because limitation of sequence analysis database, TRANSFAC® public 6.0 which provides data on transcription factors, their experimentally-proven binding sites, consensus binding sequences and regulated genes in eukaryotes. Thus, we directly isolated transcription factor(s) from the complex with the regions -290 to -270 of the promoter by DNA-protein pull-down assay and SDS-PAGE, and then identified the protein(s) using mass spectrometry. Results from the experiments showed a novel transcription factor, PARP-1 which interacts with GR in αT3-1 cells (Figure 4.8 and 4.9) and were confirmed with super shift assay using the specific antibody to PARP-1 (Figure 4.6). Our interesting finding of GR-PARP-1 interaction was supported by Muthumani et al. (2006). They reported that the 96-amino acid viral protein R (Vpr) of HIV-1, which functions as a vital accessory gene by regulating various cellular functions, uses the GR pathway as a recruitment vehicle for the NF-κB co-activating protein, PARP-1. They also mentioned that the interaction of Vpr with GR may lead to conformational changes within the complex to expose binding sites for the PARP-1 but dexamethasone treatment, however, is insufficient to recruit PARP-1 and interact with GR. Although their indication that the GR association with PARP-1 is a gain of function which is solely attributed to HIV-1 Vpr is different with our observation,

we believe that we are the first group to prove direct interaction of GR with PARP-1 on the porcine GnRHR gene promoter and it is cell specific event. However, it still remains to be determined to understand the details of this regulation *in vitro* and *in vivo*.

PARP is a nuclear enzyme that catalyzes the transfer of ADP-ribose units utilizing $NAD^+$ as a substrate to various nuclear proteins (de Murcia et al., 1994; Lindahl et al., 1995). PARP-1 is the most abundant and ubiquitous member of this family including PARP-1, -2, -3, v-PARP, and tankyrase (Smith et al., 1998; Ame et al., 1999; Johansson, 1999; Kickhoefer et al., 1999). PARP-1 has been implicated in several catalytic activities and biological processes, including DNA replication (Simbulan-Rosenthal et al., 1998), HIV replication (Cole et al., 1991), DNA repair (de Murcia et al., 1994), carcinogenesis (Kun, 1998; Bauer et al., 1995), Chromatin remodeling (Gottschalk et al., 2009; Ahel et al., 2009), cell proliferation (Sakamaki et al., 2009; Pagano et al., 2007), and apoptosis (Boulares et al., 1999; Alano et al., 2010). Further, several reports suggested a novel role for PARP-1 as a coactivator or repressor of transcription factors such as AP-1, SP-1, Oct-1, YY-1, and STAT-1 that mediate the stress/inflammation response (Nie et al., 1998; Oei et al., 1997; Ha et al., 2002). Consistent with these reports, in the present study, we determined that PARP-1 played a role as a coactivator showing that the anti-PARP-1 antibody did not completely abolish the specific DNA-protein complex in the supershift assay, thus indicating that other transcription factors or coregulatory proteins may contribute to the complexes with PARP-1. Therefore, we postulate that PARP-1 function as a member of the transcription machinery with GR to regulate the porcine GnRHR gene expression following dexamethasone treatment.

Although steroid hormones act as the primary signal in activating the receptor's transcriptional regulatory functions, the phosphorylation of the receptors is also a mechanism to positively or negatively modulate receptor-mediated transcriptional responses (Ismaili and Garabedian, 2004). Unliganded GR is phosphorylated and in the presence of glucocorticoid, GR becomes hyperphosphorylated by additional phosphorylation to result in translocation of ligand-binding GR to nucleus and transactivation or transrepression in gene expression (Wang et al., 2002; McKenna et al., 1999). As a first step toward the characterization of the signaling mechanisms effecting dexamethasone-induced translocation and transcriptional regulation of GR, we investigated the potential roles of MAPKs. Our findings suggested that ERK1/2 and p38 MAPK are responsible for dexamethasone induced promoter activity via GR phosphorylation (Figure 4.10 and 4.11). Kurl and Jacob (1984) reported direct evidence for GR phosphorylation showing incubation of GR from rat liver cytosol with $\gamma$ $^{32}$P-ATP and $Mg^{2+}$ resulted in transfer of the radiolabeled phosphate to the GR. Since that, many studies have been reported on ligand-dependent GR phosphorylation in vivo (Ortí et al., 1989; Dalman et al., 1988) and Kaul et al. (2002) suggested the involvement of endogenous protein kinases during the phosphorylation event of GR from rat liver cytosol. MAPKs respond to a variety of cellular stimuli such as growth factors, stress events, cytokines, and mitogens and, when they are activated, mediate diverse cellular processes including gene transcription, chromatin remodeling, apoptosis, and inflammation (Pearson et al., 2001). Glucocorticoid-mediated GR phosphorylation via MAPKs has been studied intensively in many cells and it has been suggested that this event is dependent on multiple kinases in a cell-, promoter-, and glucocorticoid-dose-

dependent manner (Ismaili et al., 2004; Chen et al., 2008; Webster et al., 1997; Kino et al., 2007). For example, JNK and ERK pathway have been involved in phosphorylation of the rat GR at Ser-246 or human GR at Ser-211 (equivalent to mouse GR Ser-234) as well as in dex-mediated transcription, showing direct role in GR phosphorylation and transcriptional regulation (Krstic et al., 1997; Rogatsky et al., 1998; Miller et al., 2007). In addition, Kotitschke et al. (2009) showed combinations of JNK, ERK, and p38 MAPK inhibitors decreased dex-induced phosphorylation of the endogenous mouse GR at Ser-234 and suggested a complex interplay between MAPKs and possibly other kinases in response to dex treatment to modulate mouse GR phosphorylation and transcription in LβT2 cells.

In conclusion, we demonstrated glucocorticoid responsiveness of the porcine GnRHR gene in αT3-1 cells and identified PARP-1 as a transcription factor responsible for this responsiveness. In addition, we determined that this transcriptional regulation was mediated by ERK1/2 and p38 MAPK pathway, phosphorylating dex-bound GR. Finally, we suggested working model for the mechanisms underlying glucocorticoid regulation of the porcine GnRHR gene (Figure 4.13) Once glucocorticoid binds to GR in the cytoplasm, ERK and p38 MAPK phosphorylate and activate GR to activate, releasing heat shock protein (HSP). Phosphorylated, ligand-bound GRs are translocated to the nucleus and recruit poly (ADP-ribose) polymerase-1 (PARP-1) to bind to the glucocorticoid response element, activating the porcine GnRHR gene.

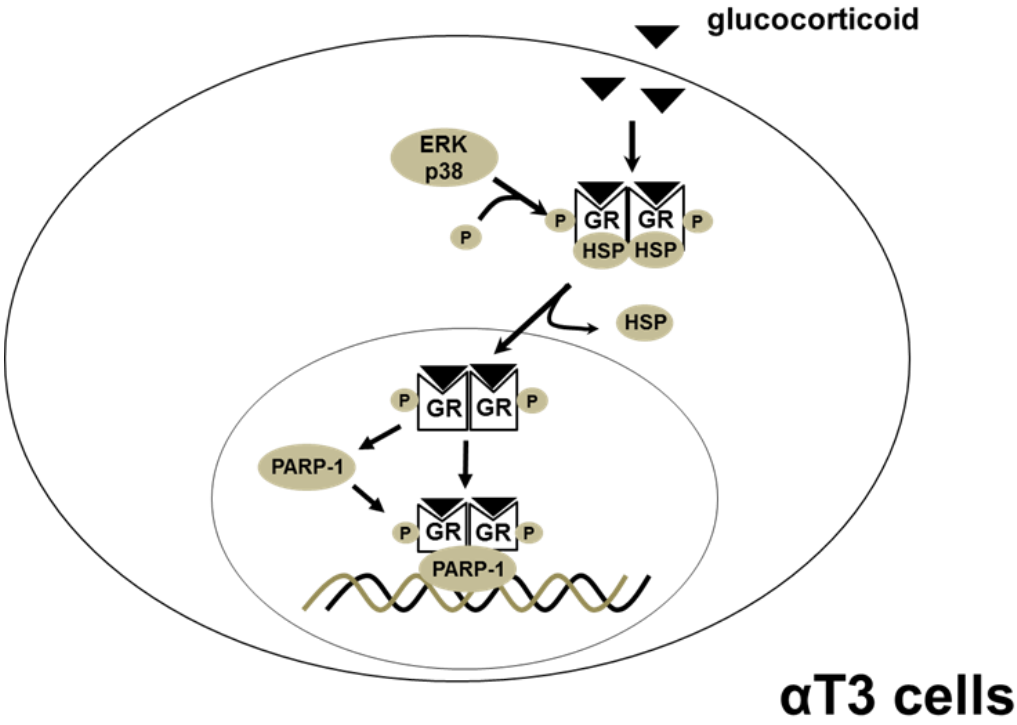
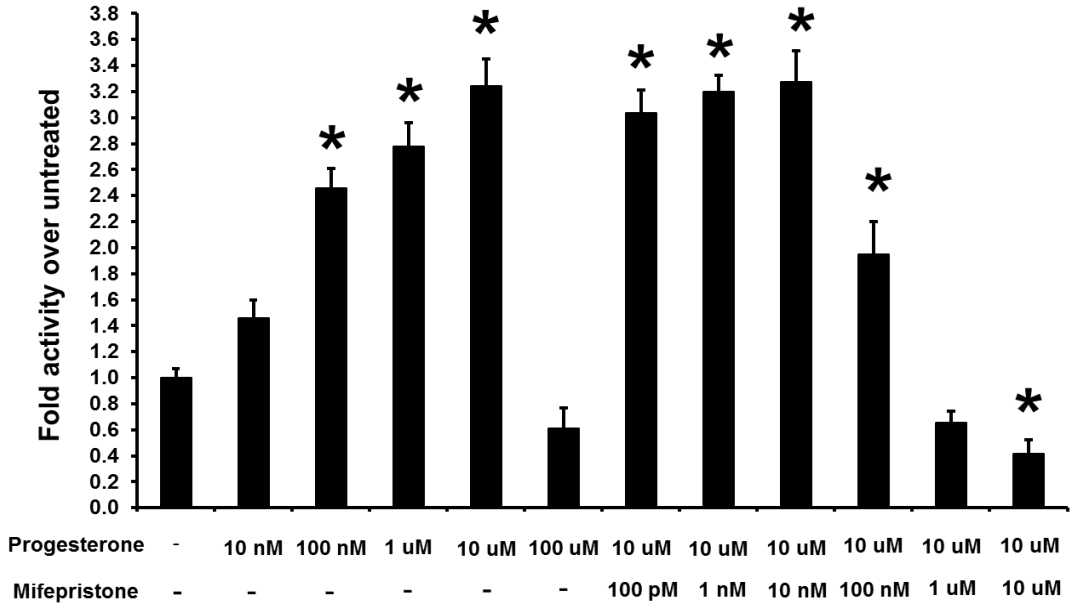


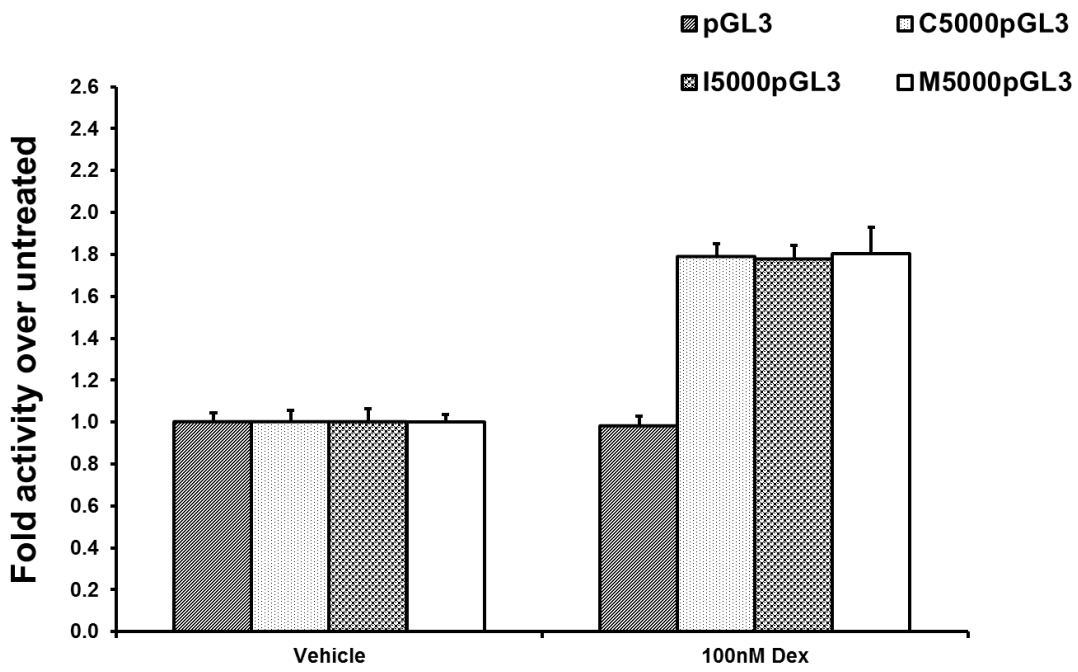

Figure 4.13. Working model for the mechanisms underlying glucocorticoid regulation of the porcine GnRHR gene. Once glucocorticoid binds to GR in the cytoplasm, ERK and p38 MAPK phosphorylate and activate GR to activate, releasing heat shock protein (HSP). Phosphorylated, ligand-bound GRs are translocated to the nucleus and recruit poly (ADP-ribose) polymerase-1 (PARP-1) to bind to the glucocorticoid response element, activating the porcine GnRHR gene.

**Appendix I**



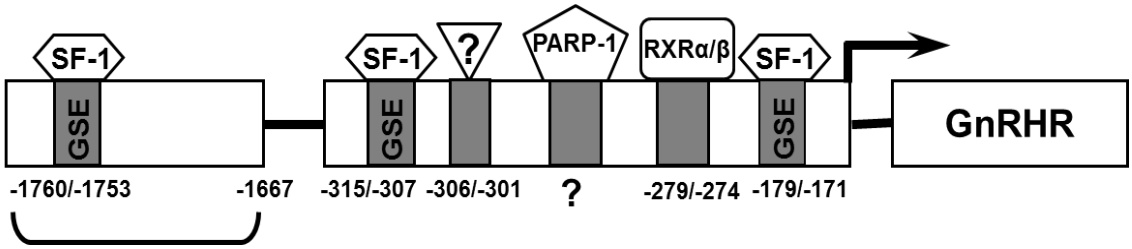

In addition to glucocorticoid responsiveness, I determined progesterone responsiveness of the porcine GnRHR gene. The promoter showed increased progesterone-induced activity in a dose dependent manner and decreased activity by mifepristone. Whereas dexamethasone-stimulated promoter activity was blocked with 100Pm mifepristone, progesterone-induced activity required 10,000 times more antagonist. This result also demonstrated further confirmation that dexamethasone responsiveness of the porcine GnRHR gene is mediated by GR, not PR.

## Appendix II




Our laboratory investigated breed differences on the porcine GnRH receptor promoters of Chinese Meishan, UNL Index, White crossbred line because each breed has different ovulation rate and litter size. Transfection study showed that Meishan promoter was most highly expressed in aT3 cells compare to other two breeds. In addition, we have identified polymorphism among the breeds. Therefore, because of these breed differences in cell specific expression, I examined breed differences on the glucocorticoids responsiveness. Reporter constucts containing either the Control, Index or Meishan GnRHR gene promoter responded equally (about 2-fold) to 100 nM dexamethasone treatment.

**Appendix III**




In this study, I determined two cell specific expression factors binding to the porcne GnRHR gene promoter. Previously, our lab found 2 SF-1 site located -1760/-1753 and -179/-171. I identified another SF-1 located between -315 and -307, and RXRα and β between -279 and -274. Mutation of SF-1 site decreased basal expression of the porcine GnRHR gene about 30%, and however, mutation of RXR α and β site completely block the promoter activity. In addition, I also determined that there is a repressor between -306 and -301. Mutation of this site increased basal expression of the receptor gene about 17-fold.

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