2009

Transcriptional regulation of the porcine type II GnRH receptor gene

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by

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Thesis (M.S.)

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University of Nebraska-Lincoln, 2009

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CHAPTER I

INTRODUCTION

The classical mammalian form of gonadotropin releasing hormone (GnRH), GnRH I, is a hypothalamic neuropeptide that functions as a key regulator of reproduction. The interaction between GnRH I and its receptor, GnRH receptor I (GnRHR I), stimulates gonadotropin secretion from the anterior pituitary gland. The gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), act on the female and male gonads to stimulate either steroidogenesis and ovulation or gametogenesis, respectively. The steroid hormones produced in the gonads act through a feedback pathway to regulate the production of both GnRH I from the hypothalamus and gonadotropins from the anterior pituitary.

Another form of GnRH, GnRH II, has recently been discovered. This isoform differs from GnRH I by 3 amino acids and is highly conserved from bony fish to man (Neill, 2001). Like GnRH I, the GnRH II ligand has been implicated in reproduction. In the rhesus monkey, GnRH II can induce LH and FSH production (Densmore and Urbanski, 2003). Furthermore, administration of GnRH II to mice and musk shrews rescued reproductive behavior in nutritionally deprived females, demonstrating that GnRH II is correlated with energy balance and reproduction in females (Kauffman et al., 2004; 2006). In male reproduction, GnRH II is involved in steroidogenesis as GnRH II agonist treatment of murine Leydig cells increased testosterone production and

expression of genes encoding the steroidogenic enzymes, steroidogenic acute regulatory protein (StAR), 17-α-hydroxylase and 3β-hydroxysteroid dehydrogenase (3β-HSD; Lin et al., 2008). Also, boars immunized against GnRH II had decreased gonadotropin secretion and the Leydig cells had reduced ability to respond to LH challenges, indicative of a role in both gonadotropin release and Leydig cell function (Bowen et al., 2006).

The novel receptor specific to GnRH II, GnRHR II, has 40% homology to GnRHR I yet differs by the presence of an intracellular cytoplasmic tail (Neill, 2002). Expression of GnRHR II has been demonstrated in mammals, however, translation of a functional protein is controversial. For instance, the human GnRHR II coding sequence contains a frame shift mutation and premature stop codon, therefore, it may require post-transcriptional modifications to produce a functional protein. On the other hand, the GnRHR II coding sequence in old world monkeys, musk shrews and swine contain no frame shifts or premature stop codons. Although the function of GnRHR II remains to be elucidated, ubiquitous expression of GnRHR II suggests it may have a key cellular function. While controversial, GnRH II may act through GnRHR II to regulate cell proliferation in human tumors. In endometrial and ovarian cancer cell lines with a targeted GnRHR I deletion, GnRH II maintained the ability to stimulate anti-proliferation of cells (Gründker et al., 2004). In contrast, siRNA targeted reduction of GnRHR II did not alter anti-proliferative effects by GnRH II in prostate cancer cells (Montagnani et al., 2009). Perhaps, GnRH II regulates cell proliferation through both GnRHR I and II utilizing different mechanisms. For instance, the presence of a C-terminal, intracellular cytoplasmic tail on GnRHR II, but not GnRHR I, could result in differential receptor

desensitization and downregulation via interactions of the tail with intracellular proteins such as β-arrestin.

By characterizing the porcine GnRHR II gene promoter, we hope to gain the potential for improved reproductive performance of sows during periods of under-nutrition and control of cell proliferation in cancer cells. First, GnRH II has the potential to increase estrus behavior when sows are in a negative energy plane such as during the post-partum period. The potential cost benefit to a swine producer that can increase pregnancy rates from an average of 85 to 90% of sows bred at first service immediately following weaning could be dramatic. In other words, a producer could breed only 111 instead of 118 sows each farrowing group to get 100 pregnant females. At an estimated $12.77 (Richards, 2009) in feed cost per sow from farrowing to weaning, a swine operation with 20 farrowing groups would save $1,787.80 annually. Second, GnRH II is a potent inhibitor of cell proliferation in reproductive cancers, representing a critical aspect of human medicine. Consider potential lives saved if the GnRH II ligand-receptor system could be manipulated to treat genital and prostate cancers which combined were responsible for an estimated 57,820 deaths in the United States in 2008 (American Cancer Society, 2008).

To determine the importance of the GnRH II ligand-receptor system, the pig represents an ideal model because it produces both the GnRH II ligand and its receptor. Recently, our laboratory isolated 3029 bp of the 5’ untranslated region for the porcine GnRHR II gene promoter. Functional analysis indicated that the GnRHR II promoter is unique because it was active in all cell lines tested, whereas the GnRHR I promoter was

functional only in the gonadotrope-derived cell line, αT3-1 (Figure 1.1). Since GnRHR II may be involved in a critical cellular function, the aim of this experiment is to elucidate the transcription factors and corresponding binding sites contributing to basal activity of the porcine GnRHR II gene promoter in swine testis (ST) cells.

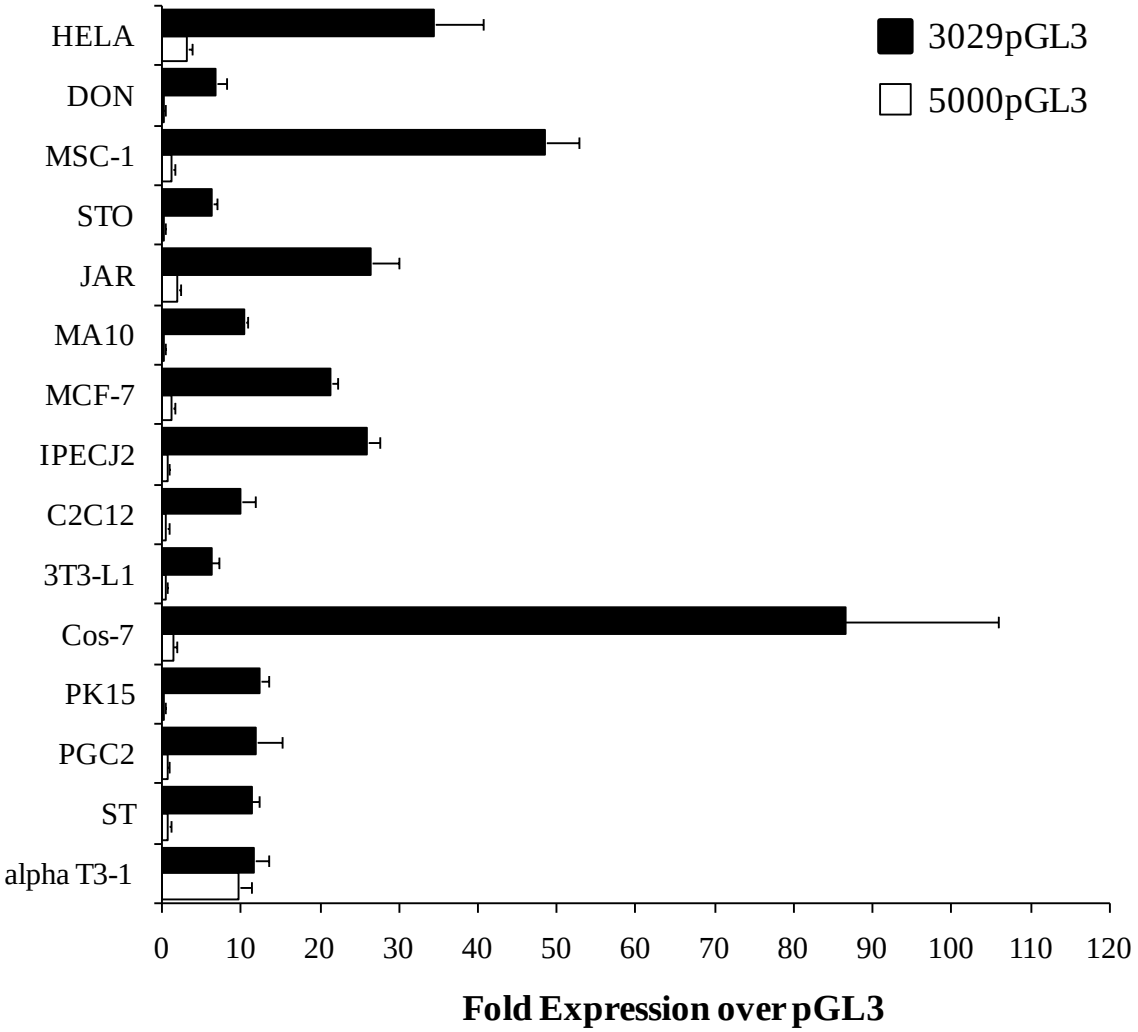

Figure 1.1. Comparison of functional activity for the porcine GnRH I (white) and II (black) receptor gene promoters. Luciferase vectors containing either the GnRHR I (5000 bp) or GnRHR II (3029 bp) proximal promoters were constructed and transiently transfected into cell lines. Transfected cell lines were: HELA, human cervical cancer; DON, Chinese hampster lung; MSC-1, mouse Sertoli cell; STO, mouse embryonic fibroblast; JAR, human placenta; MA10, mouse Leydig cell; MCF-7, human breast cancer; IPECJ2, piglet intestinal epithelium; C2C12, mouse muscle myoblast; 3T3-L1, mouse adiposite fibroblast; Cos-7, green monkey kidney; PK15, porcine kidney; PGC2, porcine granulosa cell; ST, swine testis; and alphaT3-1, murine gonadotrope. Cells were harvested and assayed for luciferase and β-galactosidase (β-gal) activity. Luciferase values were divided by β-gal values in order to correct for transfection efficiency. Values represent the mean ± SEM of at least three transfections with three different vector preparations.

CHAPTER II

LITERATURE REVIEW

Isoforms of Gonadotropin-Releasing Hormone

Gonadotropin-Releasing Hormone I

Structure. The classical mammalian decapeptide GnRH I exhibits the following amino acid structure: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Table 2.1; Matsuo et al., 1971). Across vertebrate and protochordate species, a large degree of homology between different forms of GnRH I suggests that the conserved regions are important to functionality of the hormone (Millar et al., 2005). Specifically, the length of the decapeptide as well as amino and carboxyl termini of GnRH I are highly conserved; therefore, these regions likely contribute to receptor binding and activation (Millar et al., 2005). While the amino acid in position eight is the most variable, it is important for ligand binding to the mammalian GnRHR I (Karten and Rivier, 1986; Sealfon et al., 1997). In mammalian GnRH I, the Arg^8 along with amino acids 5-7 induce a β-II type turn which provides specificity for ligand binding to GnRHR I and contributes to increased biological activity (Karten and Rivier, 1986; Sealfon et al., 1997). Whereas the mammalian GnRH I peptide is highly folded, the non-mammalian form lacking Arg^8 exhibits an extended tertiary structure (Guarnieri and Weinstein, 1996; Maliekal et al., 1997; Millar et al., 2004). The extended form of GnRH I is less biologically active in mammals, demonstrating that the tertiary structure of GnRH I is a key factor in species-

TABLE 2.1. COMPARISION OF GnRH ISOFORMS.

GnRH I	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
GnRH II	pGlu-His-Trp-Ser-**His**-Gly-**Trp**-**Tyr**-Pro-Gly-NH_2
GnRH III	pGlu-His-Trp-Ser-**His**-**Asp**-**Trp**-**Lys**-Pro-Gly-NH_2

* Amino acids in bold are different from the classic mammalian decapeptide, GnRH-I.

specific biological activity (Flanagan et al., 1994; Fromme et al., 2001).

Role in Reproduction. Gonadotropin-releasing hormone I plays a critical role in reproductive function (Schally et al., 1971). The GnRH I decapeptide, synthesized and released from the hypothalamus in a pulsatile manner, travels through the hypothalamo-hypophyseal portal system to the anterior pituitary gland (Figure 2.1). Here, GnRH I binds to its receptor on gonadotropes eliciting up-regulation of the genes encoding: the common α-subunit, the specific luteinizing hormone (LH) and follicle stimulating hormone (FSH) β-subunits, and the GnRHR I itself (Hamernik and Nett, 1988; Gharib et al., 1990; Sealfon and Millar, 1995). The gonadotropins, LH and FSH, are heterodimers comprised of the α-subunit and either the LH or FSH β-subunit, respectively (Conn and Crowley, 1994). Furthermore, GnRH I binding to its cognate receptor leads to secretion of FSH and LH into the bloodstream (Figure 2.1; Clayton and Catt, 1981; Clarke et al., 1983).

In the female, gonadotropins released from the anterior pituitary gland are key regulators of female cyclicity. Upon binding to its receptor in granulosa cells, FSH regulates oogenesis, as well as follicle recruitment and maturation. The developing follicle, in turn, secretes the steroid hormone, estradiol-17-β (Conn and Crowley, 1994). Luteinizing hormone is responsible for inducing ovulation of the dominant follicle(s) and subsequent formation of the corpus luteum that produces progesterone. The gonadal steroid hormones modulate GnRH I release from the hypothalamus and gonadotropin

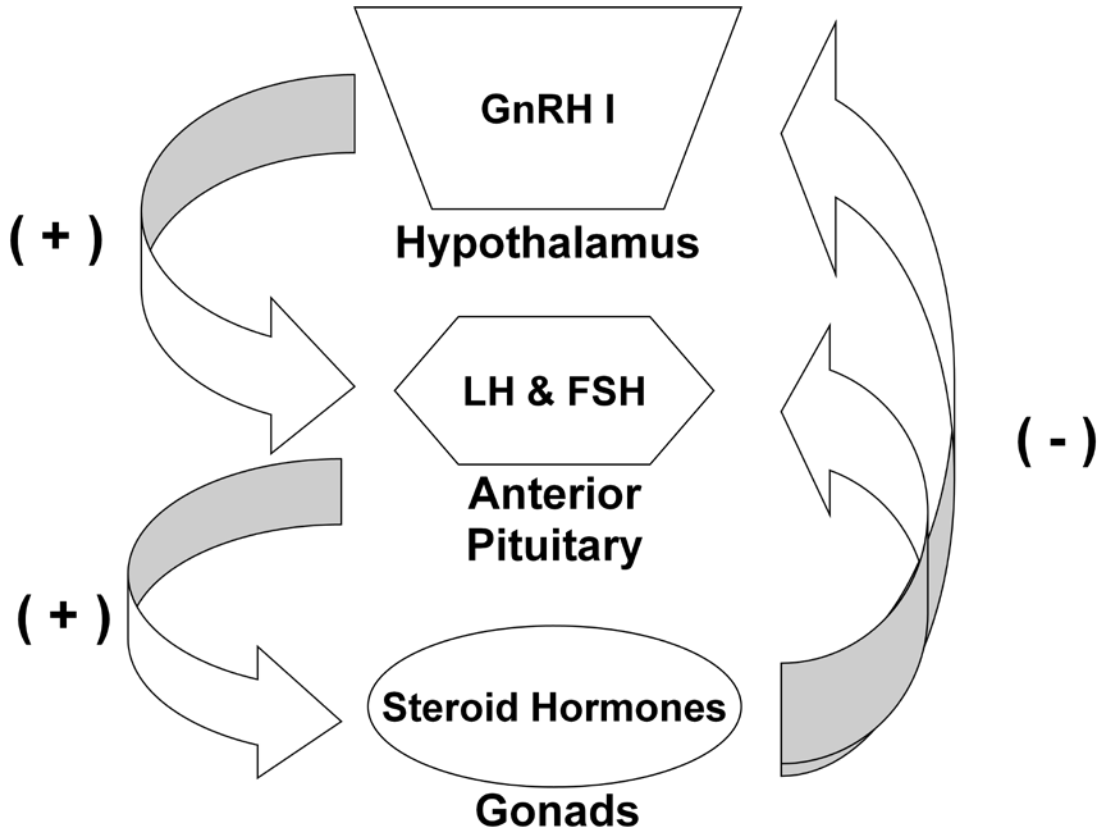

Figure 2.1. Schematic of the reproductive axis. Gonadotropin-releasing hormone I (GnRH I) produced in the hypothalamus binds its receptor, GnRHR I, on gonadotrope cells within the anterior pituitary gland. Subsequently, gonadotropes release the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). At the gonads, FSH and LH stimulate the release of steroid hormones. Through negative feedback loops, the steroid hormones regulate GnRH I and gonadotropin production and secretion.

secretion at the anterior pituitary gland through negative and positive feedback loops (Figure 2.1). Similar to the female, FSH and LH in the male are necessary for reproduction. Follicle-stimulating hormone is responsible for spermatogenesis within the seminiferous tubules in the testis. Testosterone production in the Leydig cells of the testis is stimulated by LH. Like estrogen and progesterone in the female, testosterone acts on the hypothalamus and anterior pituitary gland to control GnRH and gonadotropin release, respectively.

Gonadotropin-Releasing Hormone II

Structure. Discovered in the chicken, GnRH II is completely conserved from bony fish to humans (Figure 2.2) suggesting it has a critical function from its lack of evolutionary change (Millar and King, 1987). The GnRH II decapeptide differs from GnRH I by three amino acids: pGlu-His-Trp-Ser-**His**-Gly-**Trp**-**Tyr**-Pro-Gly-NH_2 (Table 2.1; White et al., 1998). The GnRH I and II ligands are also very similar in tertiary structure but differ in the rigidity of protein conformation. Like GnRH I, the tertiary structure of GnRH II is a β-II' turn, yet, in GnRH II it appears to be preconfigured and not require conformational changes to bind GnRHR II (Pfleger et al., 2002). This is evident as any natural dextrorotary–amino acid[6] substitution does not enhance the binding affinity of GnRH II to GnRHR I from chicken, catfish, *Xenopus* II, or bullfrog GnRHR III (Pfleger et al., 2002). Thus, while the tertiary structure of GnRH I is configured upon binding to its receptor to enhance binding, the preconfigured GnRH II

	GnRH I		GnRH II		GnRH III	
Animal	**L**	**R**	**L**	**R**	**L**	**R**
Fish	+	+	+	-	+	+
Amphibians	+	+	+	+	+	+
Reptiles	+	+	+	+	-	-
Birds	+	+	+	?	?	-
Pig, Monkey	+	+	+	+	-	-
Cow,Sheep,Chimpanzee	+	+	+	-	-	-
Human	+	+	+	?	-	-
Mouse	+	+	?	-	?	-

Figure 2.2. Occurrence of GnRH I, GnRHR I, GnRH II, GnRHR II, GnRH III and GnRHR III across species. "+" = Present, "-" = Not Present, "?" = Unknown to date. The GnRH I ligand and receptor are conserved across species, whereas GnRH III and GnRHR III appear only in fish and amphibians. According to cDNA sequence, the GnRHR II protein is absent or non-functional in some vertebrates (fish, cow, sheep, chimpanzee and mouse), whereas amphibians, reptiles, pigs and old world monkeys presumably produce functional GnRHR II. Controversy remains whether humans produce a functional GnRHR II. Adapted from Millar (2003).

does not undergo significant conformation changes to confer specificity of binding when interacting with G-protein coupled receptors (GPCRs).

Role in Reproduction. In many vertebrates, GnRH II has been linked to reproductive behavior. As GnRH II is localized in areas associated with GnRH I-stimulated sexual arousal in rodents (Moss, 1979; Muske, 1993; Rissman et al., 1997), it has been suggested that GnRH II is involved in sexual behavior (Millar et al., 2003). Both GnRH II and a GnRH II analogue stimulated reproductive behavior in ring doves (King and Millar, 1995, 1997a,b) and song sparrows (Maney et al., 1997). In nutritionally compromised, ovariectomized female mice, GnRH II rescued sexual behavior (Kauffman et al., 2004). Similarly, sexual behavior was restored in nutrient restricted, female musk shrews upon administration of GnRH II (Kauffman et al., 2006). Female marmoset monkeys that were treated with GnRH II, independent of estradiol, displayed increased proceptive mating behaviors (Barnett et al., 2006).

In cell lines, GnRH II has been shown to modulate expression of reproductive genes in a similar manner to GnRH I. Like GnRH I, GnRH II down-regulated LH and FSH receptors and decreased hCG-induced progesterone production in human granulosa-luteal cells (Kang et al., 2001). The gonadotropins, LH and FSH, decrease GnRH II expression in immortalized ovarian surface epithelium and ovarian cancer cells, indicating that GnRH II may be involved in gonadotropin production (Choi et al., 2006). While the role of GnRH II in the human placenta is undetermined, there are high-affinity binding sites for GnRH II in the human placenta (Siler-Khodr et al., 2001). In addition,

GnRH II is secreted in a pulsatile manner from isolated implants of human placenta (Siler-Khodr et al., 2001). Taken together with the expression of GnRH II and GnRHR II in human myometrium and leiomyomas (Parker et al., 2007), GnRH II could be involved in normal uterine and placental function within the uterus.

In males, GnRH II may be involved in steroidogenesis. For instance, GnRH II agonists increased testosterone production and expression of genes for the steroidogenic enzymes, steroidogenic acute regulatory (StAR) protein, 17-alpha-hydroxylase and 3β-hydroxysteroid dehydrogenase (3β-HSD), in murine Leydig cells in both a dose and time dependent manner (Lin et al., 2008). In azoospermic men, increased GnRH II expression is correlated with increased intra-testicular testosterone (Lin et al., 2008). Additionally, boars immunized against GnRH II had decreased gonadotropin secretion and reduced ability of their Leydig cells to respond to LH challenges, indicating GnRH II has some role in gonadotropin release and Leydig cell function (Bowen et al., 2006).

GnRH II in Cancer and Apoptosis. While both GnRH I and II have an anti-proliferative effect on cancer cells, it appears that GnRH II is a more potent inhibitor (Emons et al., 2003). In healthy, human uterine tissue as well as endometrial fibroids both GnRH II and its cognate receptor are expressed (Parker et al., 2007). The GnRHR II is expressed in endometrial (Hec-1A) and ovarian (SK-OV-3, OVCAR-3) cancer cell lines (Grundker et al., 2002). Native GnRH II reduced cell proliferation of GnRH II positive, but GnRHR I negative, ovarian cancer cells (SK-OV-3) in a dose and time dependent manner (Grundker et al., 2002). Both LH and FSH given prior to GnRH II

treatment inhibited the anti-proliferative effects of GnRH II on ovarian cancer cells (Choi et al., 2006). Similar to other reproductive cancers, GnRH II caused anti-proliferation in the prostate cancer cell lines, LNCaP and PC3 (Darby, 2007). Apoptosis was increased significantly (1.6-fold) in response to GnRH II in ovarian cancer cells (OVCAR-3; Kim et al., 2004). Like GnRH I, GnRH II also induces apoptosis through caspase-3 (effector), -8 (inducer) and -9 (effector) in human granulosa cells (Hong et al., 2008). Interestingly, in the GnRHR I expressing cell line, HEK293, GnRH II was more effective than GnRH I at inhibiting proliferation and activating apoptosis (López de Maturana et al., 2008). Thus, it appears that GnRH II, like GnRH I, is involved in inhibiting cell proliferation and inducing apoptosis in reproductive cell lines derived from tumors as well as healthy tissue.

Gonadotropin-Releasing Hormone III

Structure. Differing from GnRH I by 4 amino acids, pGlu-His-Trp-Ser-**His-Asp-Trp-Lys**-Pro-Gly-NH_2 (Table 2.1), a third GnRH decapeptide, GnRH III, was isolated in fish (Figure 2.2; Yu et al., 1997). While the GnRH III ligand is absent in most mammals, it is expressed in the hypothalamus of rodents (Hinley et al., 2002) and sparrows (Figure 2.2; Bentley et al., 2004). Of utmost importance, there is speculation that GnRH III is the putative FSH releasing factor (FSHRF) in vertebrates. Early studies indicated that exogenous GnRH III selectively stimulated the release of FSH in rodents (Yu et al., 1997) and cattle (Dees et al., 2001) during the luteal phase of the estrous cycle. However, it remains debatable as additional studies in rodents demonstrated GnRH III did not

selectively stimulate FSH production in vitro (Lovas et al., 1998; Montaner et al., 2001; Kovacs et al., 2004) or in vivo (Kovacs et al., 2004). Similarly, treatment of cattle with GnRH III resulted in both FSH and LH production (Am Stalden, 2004).

Role in Reproduction, Cancer and Apoptosis. The GnRH III ligand, like the GnRH I and II ligands, appears to be involved in both reproduction and anti-proliferation of cancer cells. Immunization of mice against GnRH III resulted in a 32% decrease in spermatogenic activity within the seminiferous tubules (Khan et al., 2007). In a similar fashion to GnRH II immunized pigs, boars immunized against GnRH III displayed decreased gonadotropin secretion compared to controls as well as reduced ability of their Leydig cells to respond to LH challenges (Bowen et al., 2006). Further, the GnRH III ligand also exhibited an anti-proliferative effect on breast (Lovaset et al., 1998; Palyi et al., 1999), prostate (Lovaset et al., 1998; Palyi et al., 1999) and endometrial (Palyi et al., 1999) cancer cell lines. While GnRH III does not exist in most mammals, including humans and livestock species, it also has potential to be used in reproduction and/or as a therapeutic cancer treatment.

Addional forms of Gonadotropin-Releasing Hormone

In the fish, at least 24 different isoforms of GnRH and 5 GnRHRs have been identified (Moncaut et al., 2005). While the function of many GnRH and GnRHR isoforms remains to be determined, some have been indicated to have a role in reproduction. In goldfish, increased expression of GnRH I during ovulation and increased

GnRH II during spawning indicate GnRH I could be involved in the LH surge, whereas GnRH II may have a role in spawning (Canosa et al., 2008). Also, lamprey GnRH III, the putative FSHRF, stimulated steroidogenesis and gametogenesis in lampreys (Deragon and Sower, 1994). Due to the existence of multiple GnRH ligand and receptor isoforms in fish, they offer great insight into the evolution of GnRH isoforms.

Isoforms of Gonadotropin-Releasing Hormone Receptor

G-Protein Coupled Receptors.

The receptors grouped into the six families of GPCRs share some basic structural characteristics, as determined from the crystal structure of rhodopsin. These structural characteristics include 7 transmembrane (TM) domains as well as 3 extracellular (EC) and intracellular (IC) domains (Palczewski et al., 2000). The 6 families of GPCR are: rhodopsin (A), secretin (B), metabotropic glutamate (C), fungal pheromone α- and P-factor (D), fungal pheromone A- and M-factor (E), and *Dictyostelium* species cAMP (F; Kolakowski, 1994). Based on evolutionary homology and functional similarity, GPCRs can also be grouped into three superfamilies consisting of either GPCR family A alone, a grouping of B, E and F GPCR families as well as a grouping of C, D and H GPCR families (Graul and Sadee, 1997). The secretin receptor is the model for the B family of GPCRs because it was the first isolated (Ishihara et al., 1991). Following the discovery of over 30 members of the B family of GPCRs, this superfamily of receptors is now classified into three subfamilies based on a phylogenetic tree: classic hormone receptors

(B1), LNB-TM7 proteins (B2) and Methuselah-like proteins (B3; Harmar, 2001). Both GnRHR I and IIs are classified in the secretin receptor family within the B1 subfamily.

Gonadotropin-Releasing Hormone Receptor I

Structure. The GnRHR I, a 342 amino acid peptide (Figure 2.3, 2.4), was first cloned from an immortalized murine gonadotrope-derived cell line (αT3-1 cells; Tsutsumi et al., 1992). Three exons and 2 introns comprise the molecular structure of GnRHR I (Kang et al., 2003). This receptor has also been cloned in the human (Kakar et al., 1992), marmoset (Bryne et al., 1999), pig (Weesner et al., 1994), sheep (Brooks et al., 1993) and rat (Eidne et al., 1992). In mammalian species, there is 85% homology among GnRHR I amino acid sequences (Cheung and Leung, 2000). The mammalian GnRHR I is a 7-TM GPCR (Figure 2.3), consisting predominantly of hydrophobic residues in the TM domains and hydrophilic residues in the loop regions (Millar et al., 2008). The 3 extracellular loop regions are involved in ligand binding, the intracellular domains interact with G-proteins and other signaling factors, and the TM regions are thought to contribute to conformational changes involved in receptor activation (Millar et al., 2005). Amino acids in extracellular loop 3 (ECL3) are important for ligand selectivity of GnRH isoforms to GnRHR I (Millar et al., 2008). The amino acid in position 8 of GnRH I, arginine, interacts with amino acids in the ECL3 domain of GnRHR I, consequently inducing GnRH I into the β-II' type turn conformation and achieving a high specificity of ligand binding (Stewart et al., 2008). The Arg^{8} of GnRH I interacts with the Asp^{302}

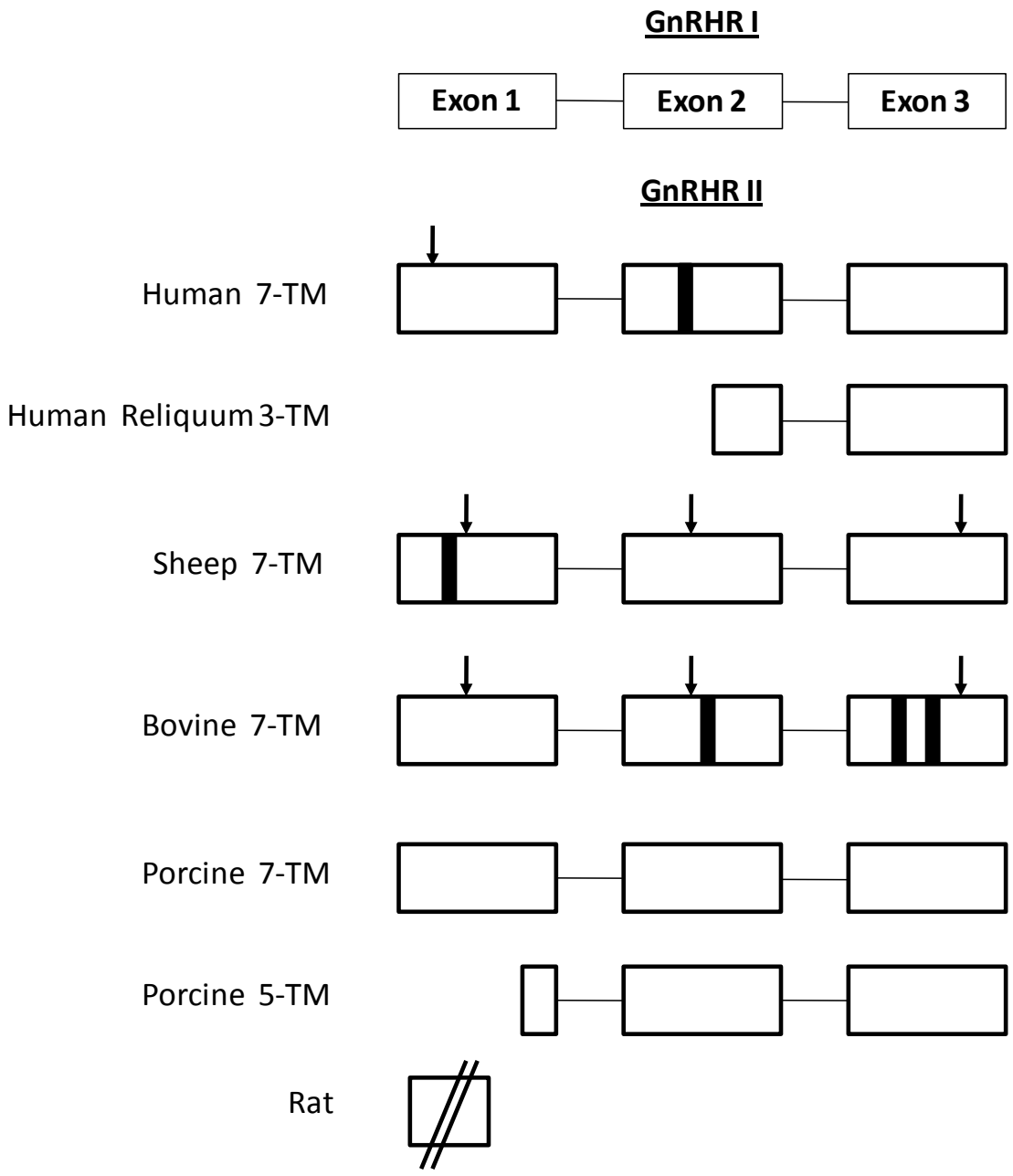

Figure 2.3. Schematic diagram representing exons coding for GnRHR I and II isoforms in the human, sheep, cow, pig and rat. Three exons code for the classic 7-transmembrane (TM) isoform of GnRHR I and II. In the human, sheep and cow premature stop codons (black boxes) and frame shift mutations exist (arrows). A human structural isoform (human GnRHR II reliquum) was artificially created by using an alternative start site in Exon 2 resulting in a 3-TM receptor. Alternative splicing within Exon 1 results in a 5-TM structural isoform of the porcine GnRHR II. Lastly, the rat only has a remnant of the GnRHR II gene.

within the ECL3 domain (Millar et al., 2004). Unlike most GPCRs, GnRHR I lacks a C-terminal intracellular cytoplasmic tail (Figure 2.4; Kakar et al., 1992). Thus, GnRHR I fails to interact with β-arrestin or undergo ligand induced phosphorylation of serine and threonine amino acids at the C-teminus, a characteristic normally associated with GPCR desensitization and downregulation (McArdle et al., 2002). In GnRHR I, the mechanism of receptor desensitization may involve several potential phosphorylation sites within the intracellular loops including sites for protein kinase A (Ser^{74}, Thr^{84}, and Thr^{265}), PKC (Thr^{40}, Thr^{42}, Thr^{51}, Ser^{74}, Thr^{84}, Ser^{118}, Thr^{265}, and Thr^{274}), and calmodulin-dependent kinase 2 (Thr^{265}; Kakar et al., 2002; Shacham et al., 2005). Others have suggested that an inhibitory protein binds to intracellular loop 2 (ICL2) near Ser^{153} to terminate the GnRHR I signaling pathway in a phosphorylation-independent manner (Shacham et al., 2005).

Signaling Pathways. The GnRHR I undergoes conformational changes upon ligand binding, activating multiple signaling pathways. The GnRHR I can activate the $G_{\alpha q/11}$, $G_{\alpha s}$ or $G_{\alpha i}$ subunits, depending on specificity of GnRH ligand interaction and the tissue type the interaction occurs within (Lu et al., 2005). Upon ligand binding, the conformational change of GnRHR I causes dissociation of the G-protein alpha subunit (G_{α}) from the beta (G_{β}) and gamma (G_{γ}) subunits of the G-protein trimer complex attached to the intracellular loops and C-terminus of GnRHR I (Koelle, 1997; Millar et al., 2004). Activation of the G_{α} is mediated by the exchange of a guanosine diphosphate (GDP), docked to G_{α}, for guanosine triphosphate (GTP). Inactivation of the G_{α} subunit occurs through hydrolysis of the attached GTP to GDP followed by reformation of the trimer

with G_β and G_γ (Koelle, 1997; Millar et al., 2004). Activation of the G_α subunit leads to the downstream activation of either protein kinase C (PKC), protein kinase A (PKA) or phosphodiesterases.

Protein Kinase C. When GnRH I binds to GnRHR I in gonadotrope cells, the PKC pathway (Figure 2.5) is predominantly activated (Stojilkovic and Catt, 1995). Dissociation of the $G_{\alpha q/11}$ subunit leads to activation of phospholipase C β (PLCβ; Millar et al., 2004). Phospholipase C β hydrolyzes phosphotidylinositol 4,5-bisphosphate (PIP_2) into the second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG; Figure 2.5). Inositol triphosphate increases intracellular calcium by stimulating its release from the endoplasmic reticulum. Within the plasma membrane, DAG opens voltage-gated calcium channels to increase available calcium within the cytoplasm, in addition to activating PKC (Lin and Conn, 1998).

Protein kinase C isoforms are serine/threonine kinases that, together with PKA and protein kinase G (PKG), make up the AGC family of protein kinases (Nishizuka, 1992; Steinberg, 2008). While all PKC isoforms contain a conserved catalytic domain that interacts with ATP/substrate, the isoforms are discerned by unique regulatory regions within the amino-terminus that maintain the enzyme in an inactive form (Steinberg, 2008). Isoforms of PKC are divided into three classes: conventional (α, βI, βII and γ; Nishizuka, 1992; Dutil et al., 1998), novel (δ, ε, η and θ; Gschwendt, 1999), and atypical (ζ and ι/λ; Zhou et al., 1994). The conventional PKC category of kinases requires calcium, DAG and activation of a phospholipid such as phosphotidycholine (Nishizuka,

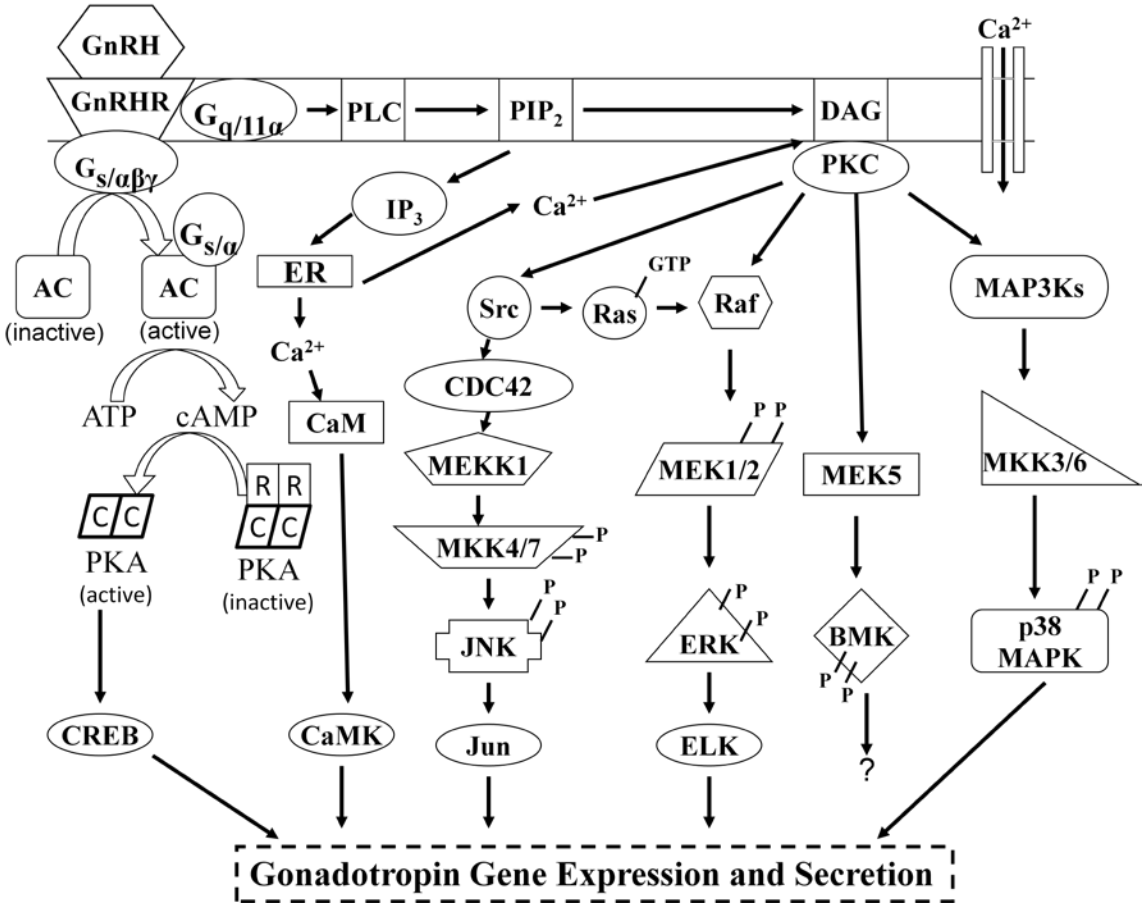

Figure 2.5. Schematic of cell signaling pathways activated following GnRH I binding to GnRHR I. The interaction between GnRH I and its receptor activates $G_{s/\alpha\beta\gamma}$ causing dissociation of $G_{s/\alpha}$ that activates adenylate cyclase (AC). Adenylate cyclase converts ATP to cAMP which subsequently activates protein kinase A (PKA) and cAMP response element binding protein (CREB). Alternatively, $G_{q/11\alpha}$ stimulates phospholipase C (PLC) which converts phosphoinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Calcium is released from extracellular and intracellular (endoplasmic reticulum; ER) stores by DAG and IP_3 stimulation. Increased calcium binds calmodulin (CaM) and activated calmodulin kinase (CaMK). Protein kinase C (PKC) is stimulated by Ca^{2+} and DAG. Multiple mitogen activated protein kinase (MAPK) pathways are activated by PKC including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 MAPK, and big MAPK (BMK) resulting in the recruitment of transcription factors (i.e., Jun and ELK). All of these events are involved in gonadotropin gene expression and secretion. Adapted from Naor et al. (2000).

1992; Dutil et al., 1998). Novel PKCs respond to DAG, but not calcium (Gschwendt, 1999), whereas the atypical PKCs depend on phosphoionositides for activation (Zhou et al., 1994). Calcium and DAG activate conventional PKCs by binding to their regulatory domain (Steinberg, 2008). In gonadotrope-derived αT3-1 cells, treatment with GnRH I agonists led to increased expression of the PKCγ and PKCϵ isoforms, specifically (Harris et al., 1997). Following activation, PKC isoforms initiated mitogen activated protein kinase (MAPK) pathways (Naor, 2000; Lin and Conn, 1998).

Mitogen activated protein kinases. The MAPKs are key factors in most mammalian cell signaling pathways, including those associated with cell survival/apoptosis, differentiation, gene expression and mitosis (Pearson et al., 2001). The first MAPKs were identified in yeast; specifically, the microtubule-associated protein-2 (MAP-2) kinase, later named MAPK, was shown to phosphorylate/activate the S6 kinase in Xenopus laevis (Sturgill et al., 1988). From yeast to mammals, the MAPK family of kinases are highly conserved and include: extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38 MAPK, and big MAPK (BMK), also known as ERK5 (Graves et al., 1995). The MAPKs are activated by serine/threonine or tyrosine phosphorylation as evident by inactivation of MAPKs by either serine/threonine (Ahn et al., 1990, 1991) or tyrosine phosphatases (Anderson et al., 1990). In the MAPK cascade, an extracellular stimulus leads to phosphorylation of MAPK kinase kinase (MAPKKK), MAPKKK phosphorylates serine/threonine residues on MAPK kinase

(MAPKK) and finally MAPKK activates MAPK by phosphorylation of serine and tyrosine residues (Seger et al., 1992; Posada et al., 1993).

In the GnRHR I signaling pathway, multiple MAPKs are activated including ERK, JNK, p38 MAPK and BMK (Figure 2.5; Naor, 2000; Cheung and Wong, 2008), however, kinase activation is dependent on cell line. In the GnRH I-induced ERK signaling cascade, β-arrestin is hypothesized to act as a scaffolding protein (Pierce et al., 2001). Two different mechanisms of ERK activation have been shown in αT3-1 cells. Protein kinase C either activates the signaling protein Raf-1 directly or stimulates indirectly to Raf-1 through the proteins, Src and Ras, followed by sequential activation of MAPK/ERK kinase (MEK) and ERK (Figure 2.5; Harris et al., 2002). However, ERK is not involved in GnRH signaling in all cell lines. One example is the GGH3 cell line, consisting of rat somatomammotrope-derived cells (GH3) that were stably transfected with GnRHR I. In the GGH3 cell line, an inhibitor of ERK increased GnRH I-induced activity of the GnRHR I promoter (Linn and Conn, 1999), whereas treatment of αT3-1 cells with an ERK inhibitor decreased GnRH I-stimulated GnRHR I activity (Norwitz et al., 1999; White et al., 1999). Similarly, the mechanism of JNK activation involves the contiguous stimulation of PKC, Src, CDC42 and MEKK1 (Levi et al., 1998). Similar to αT3-1 cells, GnRH I activates both the ERK and JNK signaling cascades in LβT-2 cells (Harris et al., 2002).

After activation, MAPKs translocate to the nucleus to activate transcription of gonadotropic genes (common α-subunit, FSH and LHβ-subunits or GnRHR I; Bonfil et al., 2004). Regulation of LHβ-subunit gene synthesis by GnRH I was shown to involve

both ERK and JNK signaling cascades in the gonadotrope-derived LβT-2 cell line (Harris et al., 2002). Though debatable, it was suggested that ERK, but not JNK, is responsible for alpha-subunit transcription in αT3-1 cells (Harris et al., 2003). All 3 kinases, ERK, JNK and p38 MAPK, are involved in FSHβ-subunit transcription (Bonfil et al., 2004). While signaling through PLC is the predominant pathway for GnRHR I signaling (Naor et al., 2007), alternative pathways, phospholipase A_2 (PLA_2), D (PLD), and PKA, can be activated when GnRH ligands bind GnRHR I in the pituitary.

Mechanisms exist to extend the length of cellular activation induced by GnRH binding with its receptor. The GnRHR I-activated PLA_2 and/or PLD enzymes remain activated in gonadotropes for 1-2 minutes following GnRH I treatment, considerably longer than the activation period of PLC (Chamson-Reig et al., 2003). The PLD hydrolyzes the foremost membrane protein, phosphatidylcholine, to form phosphatidic acid (PA) that can be converted by PA-phosphohydrolase to DAG (Naor, 2009). The increased amount and cytoplasmic presence of DAG results in prolonged activation of PKC isoforms. Hydrolysis by PLA_2 results in the production of many long chain fatty acids including arachidonic, linoleic, linolenic and oleic acids (Naor, 2009). Without the aid of the second messengers, DAG or Ca^{2+}, these long chain fatty acids can activate PKC in response to GnRH I binding to GnRHR I (Chamson-Reig et al., 2003). Furthermore, the lipoxygenase products resulting from arachidonic acid (AA) activation have been linked to gonadotropin synthesis and release (Ben-Menahem et al., 1992; Dan-Cohen et al., 1992; Shraga-Levine et al., 1996). Therefore, DAG, AA and subsequent

PKC isoforms are activated for a longer period by PLD and PLA_2 compared to PLC (Naor, 1990; Stojikovic et al., 1994; Shacham et al., 2001; Naor, 2009).

Lipid Rafts. The location of GnRHR I within the plasma membrane provides the foundation for GnRH I-induced cell signaling. Constitutive localization of GnRHR I in low-density microdomains of the αT3-1 plasma membrane (lipid rafts) is necessary for GnRHR I signaling to ERK (Navratil et al., 2003). In addition to GnRHR I, the signaling proteins, $G_{\alpha q}$, c-raf, and calmodulin are present in the low-density lipid rafts (Bliss et al., 2002). Lipid rafts consisting of cholesterol and sphingolipids serve as scaffolding for the assembly of cell signaling proteins (Brown et al., 1998). These regions are in the liquid ordered (l_o) phase, where acyl chains of lipids reside in an extended, tightly packed gel phase, while retaining the lateral mobility of a liquid phase (Ipsen et al., 1987). The unique environment of the l_o phase triggers signaling proteins to alter binding affinities and interact differently with proteins within the lipid raft compared to the environment outside of the region (Bliss et al., 2007). Lipid raft localization of GnRHR I and its signaling proteins represents a possible mechanism of receptor regulation as it lacks the c-terminal intracellular cytoplasmic tail of other GPCRs.

Protein Kinase A. Upon GnRH I binding to GnRHR I, the PKA signaling pathway can be stimulated in gonadotrope cells within the anterior pituitary gland (Figure 2.5). First, the α-subunit of the $G_{\alpha s}$-protein complex activates adenylate cyclase (AC), which then converts ATP to cyclic AMP (cAMP). Cyclic AMP binds to PKA, a

holoenzyme comprised of 2 regulatory and 2 catalytic subunits (Figure 2.4). The catalytic subunits dissociate and activate other proteins through phosphorylation including cAMP response element binding protein (Figure 2.5; CREB). Studies have demonstrated differential regulation of the GnRH I-induced PKA signaling pathway between primary pituitary cell cultures and gonadotrope-derived cell lines. Basal activity of the human GnRHR I gene promoter increases in response to the AC activator, forskolin, in gonadotrope-derived αT3-1 cells (Cheng and Leung, 2001). In addition, forskolin decreased basal GnRHR I promoter activity in this cell line, indicating that activators of the PKA signaling pathway may alter gonadotrope responsiveness to GnRH I (Alarid and Mellon, 1995). Whether or not the interaction of GnRH I with GnRHR I stimulates the PKA signaling pathway in αT3-1 cells remains controversial as White and associates discovered that forskolin did not mimic GnRH stimulation of the GnRHR I promoter (White et al., 1999). Interestingly, forskolin treatment blocked GnRH I-induced activation of the GnRHR I promoter in αT3-1 cells (Ellsworth et al., 2001). In contrast, studies in the LβT2 gonadotrope cell line suggest that GnRH I-activated PKC leads to an increase in cAMP through PKC-sensitive isoforms of AC, AC5 and 7 (Lariviere et al., 2007). Indeed, GnRH I was shown to increase cAMP in LβT2 cells (Lariviere et al., 2007). Furthermore, cross-talk between GnRH I and pituitary adenylyl cyclase-activating polypeptide (PACAP) exists as GnRH I-activated PKC phosphorylates PACAP-specific receptor type 1 (PAC1-R; Counis et al., 2007). While various studies in gonadotrope cell lines have implied that the PKA pathway is activated by the interaction of GnRH I with GnRHR I, a debate still remains since contrasting evidence has been presented in

multiple cell lines using different stimulation methods. In primary pituitary cells, there appears to be interplay between the PKC and PKA pathways. Tsujii and associates demonstrated that the PKA pathway is activated upon GnRH I binding to GnRHR I in primary pituitary cultures (Tsujii et al., 1995). Moreover, activation of the GnRH-induced, PKC signaling pathway influences the production of RI, RII and C subunits of PKA (Garrel et al., 1995).

In addition to conventional pathways associated with the G_s and G_q subunits, the interaction between GnRH I and GnRHR I also results in $G_{i1/2/3}$ subunit activation (Knollman and Conn, 2008). While activation of $G_{\alpha s}$ results in increased cAMP concentrations, $G_{\alpha i}$ inhibits intracellular cAMP. Thus, this pathway may either occur as a mutation in unhealthy cells or represent a method of down-regulation of the GnRHR I by ablating the PKA or PKC signaling pathways. Interestingly, coupling of the receptor to G_i has been demonstrated in ovarian carcinomas (Gründker et al., 2000; Gründker et al., 2001; Imai et al., 2006), uterine leiomyosarcomas (Imai et al., 2006), uterine endometrial carcinomas (Imai et al., 1997; Gründker et al 2001), and human prostate cancer cells (Limonta et al., 1999). While the mechanism underlying GnRHR I activation of $G_{\alpha i}$ remains to be elucidated, this pathway may be involved in the anti-proliferative effects of GnRH I in cancer cell lines (Gründker et al 2001; Everest et al., 2001).

Tissue Expression. The GnRHR I is expressed within a variety of reproductive tissues. Of functional importance is the expression of GnRHR I in gonadotrope cells of the anterior pituitary gland (Childs et al., 1987). Studies in gonadotrope-derived cell lines

expressing GnRHR I, αT3-1 (Alarid and Mello, 1995) and LβT2 (Turgeon et al., 1996), have provided valuable insight into the functional role of GnRHR I. While the importance is not yet understood, GnRHR I is also expressed in the hypothalamus (Wilson et al., 2006). Ongoing studies in hypothalamic cell lines, such as TE-671, that express GnRHR I may reveal the function of GnRHR I within the hypothalamus (Li et al., 1996). In addition, expression of the GnRHR I was identified within the syncytiotrophoblast and cytotrophoblast layers of the placenta (Lin et al., 1995). Studies in multiple placental cell lines, a choriocarcinoma cell line (JEG-3), immortalized extravillous trophoblasts (IEVT) and first trimester cytotrophoblast cells in primary culture, suggest that the GnRH I system regulates hCG in the placenta (Lin et al., 1995; Cheng et al., 2000a; Cheng et al., 2001b). Likewise in the ovary, GnRH I may act in an autocrine and/or paracrine manner through GnRHR I to regulate reproduction (Cheng et al., 2002a). Expression of GnRHR I in the ovary has been specifically demonstrated in multiple cell types including ovarian eplithlium (OSE; Kang et al., 2000), granulosa (Latouche et al., 1989; Billig et al., 1994) and luteal cells (Popkin et al., 1983). The GnRHR I expressed in the ovary is involved in gene expression (Richards, 1994), follicular atresia/apoptosis (Billig et al., 1994), oocyte maturation, ovulation (Hillensjo and LeMaire, 1980) and steroidogenesis (Hsueh and Jones, 1981). Furthermore, GnRHR I has been isolated in mouse and rat testicular germ cells (Bull et al., 2000) and adult rat testes (Hseuh and Jones, 1981). Specifically, GnRHR I was identified in Leydig cells within the interstitial space of the testes (Clayton et al., 1980; Lefebvre et al., 1980; Sharpe and Fraser, 1980). The GnRH I system may even have a role in early

development as it is expressed in oocytes (Dekel et al., 1988) and embryos (Raga et al., 1999; Casan et al., 1999).

Besides healthy reproductive tissues, GnRHR I expression occurs in cancerous reproductive tissues. In human breast (Casan et al., 1998), prostate (Dondi et al., 1994), uterine endometrium (Casan et al., 1998; Raga et al., 1998) and myometrium (Chegini et al., 1996), GnRHR I is present. Moreover, the GnRHR I has been linked to anti-proliferation in reproductive cancer cell lines such as those derived from the ovary (OVCAR-3, SKOV-3; Kim et al., 2006) and prostate (PC3; Montagnan et al., 2009). Thus, the presence of GnRHR I in reproductive tumors represents a potential target for cancer therapies.

Transcriptional Regulation. In many mammalian species including mouse (Albarracin et al., 1994), rat (Pincas et al., 1998), sheep (Campion et al., 1996), pig (Jiang et al., 2001) and human (Kakar et al., 1997), the 5' flanking sequence for the GnRHR I gene has been isolated and transcriptional regulation has been well characterized. Transcriptional regulation of the GnRHR I gene differs between species and tissue types as evident in Figure 2.6. In the mouse, a tripartite basal enhancer consisting of binding sites for activator protein-1 (AP-1) at -336/-330 bp, steroidogenic factor-1 (SF-1) at -244/-236 bp and an element originally termed GnRHR activating sequence (GRAS) at -391/-380 bp is attributable for activation of the GnRHR I receptor gene promoter in the pituitary (Figure 2.6; Duval, 1997; White et al., 1999). Studies utilizing transgenic mice demonstrated that 1900 bp of the murine GnRHR I promoter drives expression of

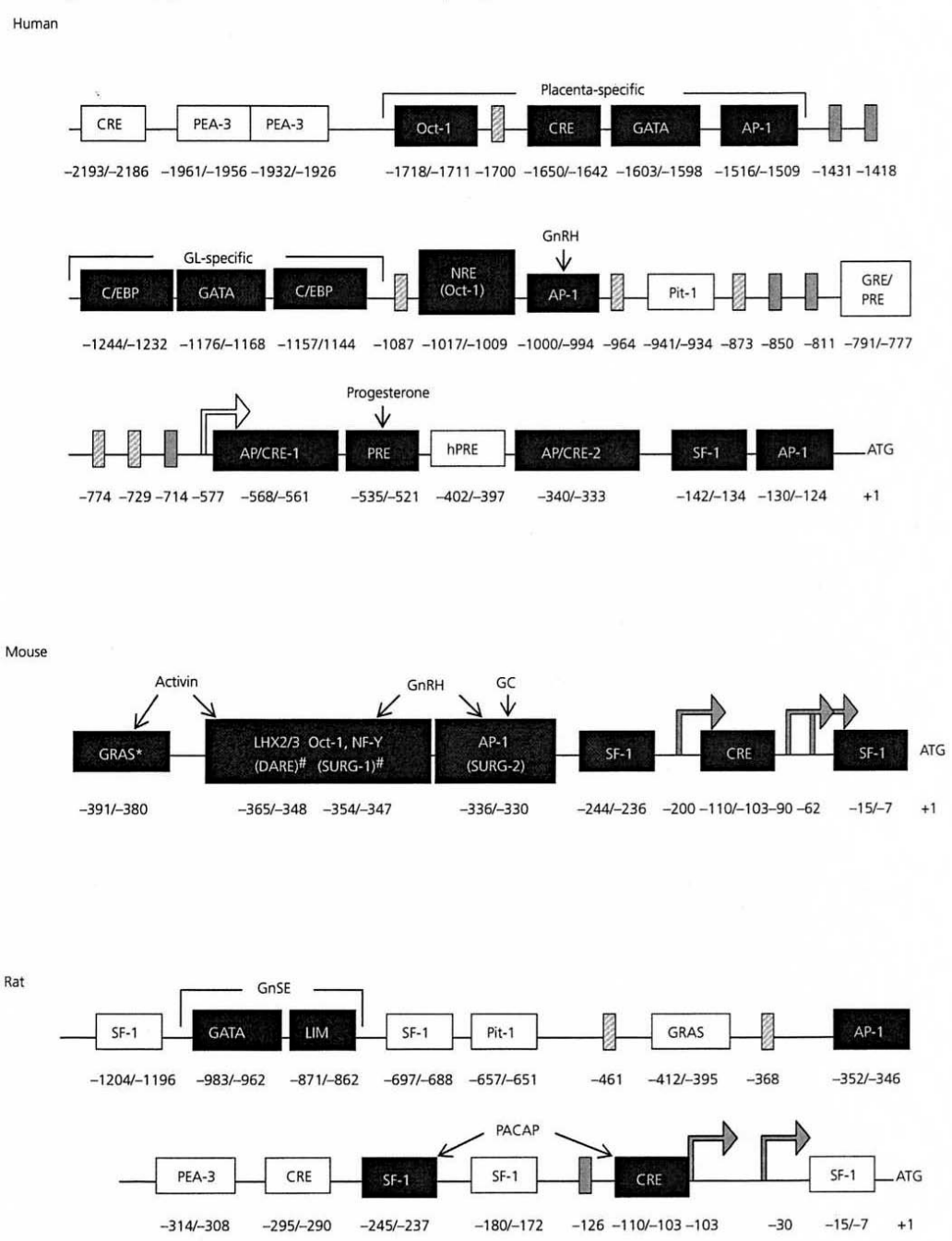

Figure 2.6. Schematic representation of transcription factor binding sites conferring basal and hormonal regulation of the GnRHR I gene promoter in the human, mouse and rat. Transcriptional start sites are represented by arrows and translational start sites are indicated with an 'ATG'. The shaded and striped boxes represent TATA and CCAAT boxes, respectively. Black boxes indicate elements that have been functionally characterized, whereas white boxes identify putative binding sites. From Hapgood et al. (2005).

reporter genes in the pituitary, brain and testis, but not the ovary, suggesting tissue-specific promoter utilization (McCue et al., 1997). With over 80% homology to the mouse GnRHR I promoter, the rat promoter also contains an SF-1 element contributing to gonadotrope-specific promoter activation (Figure 2.6; Pincas et al., 1998, 2001). However, located within -115/-735 bp of the rat promoter is a GnRHR I-specific enhancer (GnSE), consisting of binding sites for GATA-related and LIM homeodomain-related factors, that interacts with SF-1 (-245 bp) to confer gonadotrope-specific promoter activation (Figure 2.6; Pincas et al., 1998, 2001). Furthermore, the rat GnRHR I gene promoter also contains an AP-1 element (–352/–346 bp), although, unlike the mouse promoter, it is responsible only for basal promoter activity (Figure 2.6; Hapgood et al., 2005).

Although transcriptional regulation of the human GnRHR I gene is much more complex than that of mice and rats, there is some conservation of elements. Unfortunately, a human gonadotrope-derived cell line does not exist, thus, studies of gonadotrope-specific human GnRHR I gene promoter activity are limited to models derived from other species, mainly murine gonadotrope-derived αT3-1 and LβT2 cells. The 5’ flanking region of the human GnRHR I gene, like the mouse and rat, contains SF-1 elements (Figure 2.6). In α-T3 cells, the SF-1 element located at -140/-134 bp is involved in mediating gonadotrope-specific promoter activity (Ngan et al., 2000). Interestingly, Oct-1 is required for basal promoter activity at an element located at -1718 bp, whereas an Oct-1 binding site (-1017 bp) within the negative regulatory element (NRE) is a repressor in placental (JEG-3), ovarian (OVCAR-3) and gonadotrope-derived

(αT3-1) cells (Figure 2.6; Cheng et al., 2001b, 2002b). Taken together, studies indicate tissue-specific transcriptional regulation of the human GnRHR I gene. For instance, a GATA element (-1176/-1168 bp) and two putative CCAAT/enhancer binding protein (C/EBP) motifs (-1244/-1232 and -1157/-1144 bp) are attributable for cell-specific GnRHR I expression in human ovarian granulosa-luteal cells (SVOG-4o and SVOG-4m) (Figure 2.6; Cheng et al., 2002a). However, placental-specific expression is due to GATA (-1603/-1598 bp) and cAMP response elements (CRE; -1650/-1642 bp; Cheng et al., 2001b).

Regulation of GnRHR I gene expression within the pituitary can also be influenced by hormones and growth factors. In αT3-1 cells, 2 regions of the mouse GnRHR I gene promoter, designated sequence underlying responsiveness to GnRH-1 (SURG-1) and SURG-2 which contain either Oct-1 and NF-Y or AP-1, respectively, are responsible for the GnRH I-induced increase in GnRHR I expression (Figure 2.6; Norwitz et al., 1999). White and coworkers confirmed the role of AP-1 in GnRH I regulation of the mouse GnRHR I promoter, but not the Oct-1 or NF-Y elements (White et al., 1999; Ellsworth et al., 2001). As described previously, GGH_3 cells utilize the PKA signaling pathway to activate a CRE located within the mouse GnRHR I promoter in response to GnRH I (Maya-Nunez et al., 1999). Alternatively, an AP-1 element is activated by the PKC signaling pathway in αT3-1 cells (White et al., 1999). Taken together, GnRH responsiveness is dependent on cell type. Progesterone also regulates GnRHR I gene expression in sheep pituitary (Laws et al., 1990) and αT3-1 cells (Cheng et al., 2001c). Specifically, a glucocorticoid response element (GRE)/progesterone

response element (PRE) at -535/-521 bp of the human GnRHR I gene promoter mediated down-regulation of GnRHR I by progesterone in αT3-1 cells (Cheng et al., 2001c). In GGH_3 cells, an AP-1 site (-336 bp) on the mouse GnRHR I gene promoter interacts with ligand-bound glucocorticoid receptors to increase GnRHR I expression (Figure 2.6; Maya-Nunez et al., 2003). In addition, the growth factors, activin and inhibin, regulate transcription of the GnRHR I gene. Specifically, an AP-1 site within GRAS and Smad binding sites contribute to activin responsiveness of the mouse GnRHR I gene promoter in αT3-1 cells (Figure 2.6; Norwitz et al., 2002a, 2002b; Ellsworth et al., 2003).

Transcriptional regulation of the porcine GnRHR I gene promoter has been elucidated utilizing gonadotrope-derived αT3-1 cells. Like other species, SF-1 binding sites, located at -315/-307 and -179/-171 bp, partially comprise the gonadotrope-specific promoter (Cederberg et al., unpublished data). In addition, located at -1760/-1537 bp within the porcine GnRHR I gene promoter is a critical enhancer region termed swine upstream promoter enhancing region (SUPER; Cederberg et al., unpublished data), which contains another SF-1 site (Smith et al., unpublished data). Interestingly, there is also divergent regulation of porcine GnRHR I gene expression across swine lines. The control/white crossbred line of swine utilizes a p65/p52 heterodimer of NF-κB subunits (-1690 bp) within SUPER for basal activity of the porcine GnRHR I gene promoter (McDonald et al., unpublished data). On the other hand, the Meishan swine line incorporates a GATA-4 binding site (-1690 bp) within SUPER, a complex containing a Sp1-like protein interacting with the p65/p52 subunits of NF-κB (-1235 bp), and an

additional GATA-4 binding site (-845 bp) for basal activity of the GnRHR I gene promoter in αT3-1 cells (Smith et al., unpublished data).

Gonadotropin-Releasing Hormone Receptor II

Structure. Similar to GnRHR I, the GnRHR II gene contains 3 exons and 2 introns and the 379 amino acid GnRHR II protein is comprised of 7 TM domains (Figure 2.3, 2.4; Neill, 2002). The primate GnRHR II amino acid sequence has 40% homology to that of the human GnRHR I (Millar et al., 2001). In addition, the primate GnRHR II amino acid sequence is closer in identity to non-mammalian, vertebrate GnRHR II (55%) than to mammalian GnRHR I (40%; Neill et al., 2001). According to the cDNA sequence, coding for a functional GnRHR II is present in fish, amphibians, birds, pigs, old world monkeys and musk shrews (Millar et al., 2005). While the human GnRHR II cDNA sequence exhibits a frame shift mutation near the N-terminus (AA^{9}) and a premature stop codon (AA^{181}), theories on post-transcriptional modification predict a functional peptide exists (Neill, 2004). The GnRHR II contains 2 extracellular N-linked glycosylation sites that may contribute to ligand interaction (Neill, 2002). Unlike the GnRHR I, the GnRHR II does not have the Asp/Asn microdomains in TM domains 2 and 7 that contribute to receptor activation (Flanagan et al., 1999). Serine and threonine sites on the carboxy-terminal, intracellular, cytoplasmic tail of GnRHR II are possible phosphorylation sites implicated in receptor desensitization of non-mammalian GnRHR IIs (Blomenrohr et al., 1998).

Signaling Pathway. Upon ligand binding, the GPCR, the GnRHR II, shares some similar signaling properties with GnRHR I (Figure 2.7), however, the presence of a C-terminal, intracellular, cytoplasmic tail on GnRHR II (Figure 2.3) suggests there is divergent regulation of signaling. Specifically, the C-terminal tail represents a site of phosphorylation potentially altering G-protein association. Similar to GnRHR I, activation of GnRHR II by ligand binding results in dissociation of activated $G_{\alpha q}$ (Figure 2.7; Tensen et al., 1997). Treatment of monkey kidney cells (Cos-1) containing GnRHR IIs with GnRH II resulted in increased inositol phosphate (IP), suggesting activation of PLC to cleave PIP_2 and a resultant increase in IP_3 and DAG levels (Neill et al., 2001). In another monkey kidney cell line (Cos-7), GnRH II binding to GnRHR II was a potent activator of ERK2 within the PKC signaling pathway, but GnRH I could also activate ERK2 through GnRHR II (Millar et al., 2001). Stimulation of GnRHR II by the GnRH II ligand, but not GnRH I, resulted in the phosphorylation of p38 MAPK (Millar et al., 2001). While both GnRH I and II activated p38 MAPK through GnRHR II, the time course for p38 MAPK activation by GnRH II was more prolonged than GnRH I (Millar et al., 2001). In ovarian cancer cells (OVCAR-3 and SKOV-3), the anti-proliferative effects of GnRH II are mediated by ERK1/2 and p38 MAPK (Kim et al., 2004; 2005). Following homologous ligand binding, the receptors differentially activate JNK and c-Src. Stimulation of GnRHR I by GnRH I results in activation of these proteins, whereas GnRH II binding to GnRHR II fails to activate these proteins (Figure 2.7; Pawson et al., 2003). Thus, the divergence in signaling pathways is likely contributable to both the interaction of proteins at the c-terminal tail of GnRHR II and the availability of signaling

Binding and Coupling	Marmoset GnRHR II	Human GnRHR I
Binding		
GnRH I	0.02	1.00
GnRH II	1.00	0.10
GnRH III	0.10	0.10
Antagonist 135-18	Full Agonist	Full Antagonist
Coupling		
Gq/11	+	+
Ca2+	+	+
PKC	+	+
ERK1/2	+(transient)	+(protracted)
p38	+	+
JNK	nil	nil
c-Src	nil	+
Receptor Internalization	rapid	slow
Receptor Desensitization	+	nil

Figure 2.7. Binding and coupling of GnRH ligands and signaling proteins with the marmoset GnRHR II compared to human GnRHR I. GnRH I and II bind their respective receptors with high affinity whereas GnRH II binds GnRHR I with 10-fold higher affinity than GnRH I binds to GnRHR II. Agonists to GnRHR II are antagonists to GnRHR I. Both GnRHR I and II activate the the $G_{q/11}$/ Ca^{2+}/protein kinase (PKC) pathways and p38 mitogen activated protein kinase, but not c-Jun N-terminal kinase (JNK). However, activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and mammalian homologue r-Src of Rous sarcoma virus (c-Src), in addition to receptor internalization and desensitization, are divergent between GnRH/GnRHR systems. Adapted from Millar (2003).

intermediates or inhibitors within different cell types.

Tissue Expression. The GnRHR II differs from GnRHR I in tissue expression pattern. While GnRHR I is specifically expressed in reproductive tissues, primarily within gonadotropes of the anterior pituitary gland, GnRHR II is ubiquitously expressed (Millar et al., 2001). Expression of GnRHR II was displayed in all human tissues tested including the adrenal, brain, breast, heart, kidney, pituitary, stomach, small intestine, large intestine, skeletal muscle, thymus, sperm, spleen, lung, liver, pancreas, thyroid, placenta, uterus, prostate, ovary, and testis (Neill et al., 2001; von Biljon et al; 2002). Additionally, the GnRHR II is expressed in rhesus monkey hypothalamus (Latimer et al., 2000) as well as normal human ovarian surface epithelia (Choi et al., 2001). A comparison of relative human GnRHR II expression levels indicated that GnRHR II is expressed in higher amounts in the kidney (30-fold), bone marrow (4-fold) and prostate (4-fold) compared to the brain (White et al., 1998). The GnRHR II is also expressed in cancer cell lines including those derived from human prostate (PC3; Montangani et al., 2009), endometrium (HEK-1a), and ovary (EFO-21, OVCAR-3, SK-OV-3; Grundker et al., 2002). The ubiquitous expression of GnRHR II in reproductive and non-reproductive tissues suggests GnRHR II has a broad cellular function, whereas the primary role of GnRHR I is reproduction.

Ligand Interaction. The GnRH II ligand binds with high affinity to its receptor, GnRHR II. Comparing the affinities of GnRH II and GnRH I for GnRHR II, the GnRH II

ligand binds to GnRHR II with a 24-fold higher binding affinity than GnRH I (Millar et al., 2001). However, GnRH II binds with 10-fold higher affinity to GnRHR I than the GnRH I ligand binds to GnRHR II (Figure 2.7; Neill et al., 2004). Thus, GnRH II can elicit its effects through GnRHR I, in addition to GnRHR II. Much controversy exists regarding whether GnRH II interacts with GnRHR I or II when functioning in reproduction, anti-proliferation and/or apoptosis. In a human ovarian cancer cell line (SK-OV-3) that expresses GnRHR II but not GnRHR I, GnRH II has anti-proliferative effects (Völker et al., 2002). On the other hand, knockdown of GnRHR I by siRNA reversed the anti-proliferative effects of GnRH I and II on ovarian cancer cells (SKOV-3 and OVCAR-3; Kim et al., 2006). As GnRH II appears to stimulate anti-proliferation of cancer cells through both GnRHR I and II, further studies are necessary to determine if the specific mechanisms of anti-proliferation are dependent on the receptor utilized.

Structural Isoforms. According to the GnRHR II cDNA sequence in swine, a classic 7-TM and unique 5-TM isoform could be produced. There is precedence for functional 5-TM GPCRs as 5-TM mutants of the chemokine receptors, CCR5 and CXCR4, have been described previously (Figure 2.8; Ling et al., 1999). These mutant 5-TM receptors were produced by deletion of the EC1, TM1, IC1 and TM2 domains (Ling et al., 1999). Although artificially created, the 5-TM receptors were functional as evident by their ability to mediate chemokine-stimulated chemotaxis and undergo agonist-induced internalization and desensitization (Ling et al., 1999). Similar to the functional 5-TM chemokine receptors, alternative splicing of the porcine GnRHR II creates a 5-TM

receptor that is missing TM domains 1 and 2 (Figure 2.8; Neill et al., 2004). Preliminary studies in our laboratory indicate that the 5- and 7-TM isoforms of the porcine GnRHR II are equally abundant in the ST cell line and localize to the same region of the cytoplasm (Wiarda et al., unpublished data).

While some investigators postulate that the cDNA sequence predicts that only a human GnRHR II mRNA transcript can be produced and not a peptide, other studies point to the presence of a human GnRHR II peptide. During post-transcriptional modifications and translation, mechanisms of mRNA editing may correct the frame-shift mutations and premature stop codon. Transcripts in which the stop codon is removed through alternative splicing have been noted (Millar et al., 2004). The non-sense codon, UGA, can also code for the amino acid, selenocysteine. In prokaryotes, a stem-loop structure making up a selenocysteine insertion site is present immediately 3' of the UGA (Heider et al., 1992). The selenocysteine insertion sequence (SECIS) is present in the 3' untranslated region in eukaryotes (Berry et al., 1991) and can function at distances of up to 5000 nucleotides (Berry et al., 1993). The SECIS consists of an AAA sequence in or near the apical loop, GA nucleotides on the 3' side of the stem, and an AUGA sequence on the 5' side of the stem (Nasim et al., 2000). Thus, a SECIS motif in the 3' untranslated region of the gene encoding for GnRHR II and a specific tRNA, like the $tRNA^{Sec}$(SelC) in bacteria, may allow the stop codon to become a selenocysteine residue (Low et al., 1996; Nasim et al., 2000; Millar et al., 2004).

Alternatively, frame-shift mutations may be overcome by corrective shifts in the reading frame. While rare in mammalian genes, programmed, corrective frame shifts

have been documented, primarily in genes of retroviral origin (Namy et al., 2004). Neill and associates hypothesized another scenario in which an in-frame, non-AUG start codon is utilized downstream of the frame shift mutation (Neill et al., 2004). The human GnRHR II sequence contains a nominal valine codon (GUG) that meets the Kozak criteria to be a non-AUG start codon, appropriate surrounding amino acids (-3ggaGUGg+4; Kozak et al., 1997) and an obligatory hairpin loop immediately following the GUG codon (Kozak et al., 1999). The result of the corrective frame shift would be a 7-TM GPCR that was shortened by 22 amino acids on the N-terminus (Neill et al., 2004). Additionally, it is postulated that the human GnRHR II may not function as a traditional 7-TM receptor, but as a 5- or 6-TM receptor. Provided that the putative stop codon results in a selenocysteine residue, alternative splicing in Exon 1 could result in a 5-TM human GnRHR II receptor lacking TM regions I and II (Neill et al., 2004). Furthermore, translation of 2 peptide fragments may result in a 6-TM human GnRHR II. A peptide fragment that begins with the non-AUG start codon at position 23 described previously would contain TM domains I-IV and extend to the stop codon at position 181 (Neill et al., 2004). Reinitiation of gene translation, previously described in mammals (Kozak et al., 2001), would result in a peptide fragment containing the TM domains V and VI (Neill et al., 2004). Through non-covalent interactions, the 2 peptide fragments could result in the formation of a 6-TM receptor. This hypothesis is validated by studies demonstrating there is a tendency for fragments to form heterodimers (Schoneberg et al., 1995; Gudermann et al., 1997).

Oligodimerization of G-protein Coupled Receptors. Association of GPCRs in oligodimers has been well documented and may be a mechanism necessary for function of the human GnRHR II. Bioluminesence and fluorescence resonance energy transfers indicated that receptors in close proximity have the propensity to associate with each other (Gurevich and Gurevich, 2008). Additionally, the functional importance of GPCR oligomerization has been described for the δ- and μ-opioid receptors in neuroblastoma cells (Gomes et al., 2000), the metabotropic glutamate type 1α and adenosine A_1 receptors in cortical neurons (Ciruela et al., 2001) as well as the angiotensin AT_1 and bradykinin B_2 receptors in smooth muscle cells (Abdalla et al., 2000). Interestingly, inhibition of either the angiotensin II type 1 or the beta-adrenergic receptor with a selective antagonist in mouse cardiomyocytes results in disruption of signaling through both receptors, indicating the importance of dimerization in signaling (Barki-Harrington et al., 2003).

Experiments utilizing a human GnRHR II peptide translated from a start site past the frame shift and stop codon (termed reliquum; Figure 2.3) suggested that GnRHR I and II may interact. Although the human GnRHR II gene is widely expressed, a frame shift mutation and premature stop codon presumably prevent the synthesis of a full-length GnRHR II protein. Pawson and associates created a human GnRHR II reliquum that contained the ICL 3, TM domain 6 and 7, ECL 3 and carboxyl terminal tail (Pawson et al., 2005). Transient transfection of constructs containing the human GnRHR II reliquum and GnRHR I resulted in selective disruption of GnRHR I expression and signaling (Pawson et al., 2005). Furthermore, targeted inhibition of de novo protein synthesis

(cycloheximide) or proteinase-mediated degradation (leupeptin and phenylmethylsulfonyl fluoride) failed to disrupt the inhibitory effects of the GnRHR II reliquum on GnRHR I (Pawson et al., 2005). This suggested that GnRHR I and II interaction occurs within the nucleus, endoplasmic reticulum, or Golgi apparatus (Pawson et al., 2005). Thus, it is hypothesized that the human GnRHR II peptide functions through oligodimerization either by interactions of fragments to form a 6-TM receptor or through specific interaction of the GnRHR II peptide fragments with GnRHR I.

Putative Transcription Factors within the GnRHR II Promoter. Early growth response factor 1 (Egr-1) is a 553 amino acid zinc-finger transcription factor. The sequence is proline, serine and threonine rich and contains 3 tandem repeats by the carboxyl terminus that each form a $cysteine_2$-$histidine_2$ zinc finger. The zinc-finger binding domain of Egr-1 binds to the consensus sequence, 5'-CGCCCCCGC-3'. The Egr-1 transcription factor is implicated in many functions including cell division, differentiation, apoptosis and stress response. Early studies showed that Egr-1 is involved in normal development of the skeleton (Sukhatme, 1990). Also, Egr-1 is involved in neuronal cell differentiation, possibly through its up-regulation of thyroid hormone (Pignatelli et al., 2003). In breast and prostate cancer, the in vivo role of Egr-1 in inducing tumor angiogenesis, growth and metastasis has been shown (de Mestre et al., 2005). The Egr-1 protein specifically acts through regulation of heparanase, a heparin sulfate-degrading enzyme, gene expression (de Mestre et al., 2005). Heparanase degrades the extracellular matrix, causing cell invasion that promotes tumor metastasis,

angiogenesis and inflammation (de Mestre et al., 2005). In neuroblastoma, human melanoma, and prostate cells, Egr-1 significantly increased apoptosis (Pignatelli et al., 2003). A major role of Egr-1 is regulation of inflammatory response genes. For instance, Egr-1 knockout mice had reduced expression of genes associated with inflammation, whereas over-expression of Egr-1 exacerbated the inflammatory response (Tureyen et al., 2008). In addition, Egr-1 activates many promoters of genes with a wide range of functions including but not limited to: heparanase (de Mestre et al., 2005), thymidine kinase (Molnar et al., 1994), tumor necrosis factor (TNF; Kramer et al., 1994), synapsins (Thiel et al., 1994), acetylcholinesterase (Li et al., 1993), telomerase (Akutagawa et al., 2007; 2008), cdk inhibitor p57 (Figliola et al., 2008), and dopamine beta-hydroxylase (Cheng et al., 2008).

The Egr-1 transcription factor can interact with other binding factors either through competition or "cross-talk" between binding sites. Of primary importance, the Egr-1 consensus element contains a consensus binding site for the Sp1 transcription factor (5'-CGCCCC-3'). Both Sp1 and another family member, specificity protein 3 (Sp3), may compete with Egr-1 for binding, dependent on the concentrations of Sp1, Sp3 and Egr-1 within a particular cell. Interestingly, Egr-1 forms a tripartite binding complex with SF-1 and Sp1, specifically a GnRH responsive element, on the rat LHβ-subunit gene promoter (Kaiser et al., 2000). Additionally, Egr-1 modulates regulation of tyrosine hydroxylase expression via "cross-talk" between an Egr-1 and AP-1 site as evident by decreased luciferase promoter activity when the distance between the sites is increased within reporter constructs (Nakashima et al., 2003). Pertinent to our studies, Egr-1,

shown to be widely expressed and involved in many cellular functions, has not been implicated in any specific functions within ST cells.

Specificity protein 1 is a ubiquitously expressed transcription factor containing 785 amino acids. Like Egr-1, Sp1 has a DNA binding domain comprised of 3 cysteine$_2$-histidine$_2$ zinc fingers, a characteristic of this subfamily of transcription factors. The Sp1 consensus site is 5'-(G/T)GGGCGG (G/A)(G/A)(C/T)-3', a GC box element. The Sp1 protein contains a trans-activation domain consisting of 2 glutamine-rich sub-domains to activate transcription. These domains have been shown to bind to TATA binding protein (TBP) and TBF associated factor 4 (TAF4) stabilizing the transcription initiation complex (Wang et al., 2007). Furthermore, Sp1 can bind a single site, form homo-oligomers with its self to activate multiple Sp1 sites, or act as a super-activator which does not bind DNA directly but instead interacts with a DNA-bound Sp1 protein (Wiestra, 2008). In addition, Sp1 has 2 domains involved in synergistic activation; the highly-charged, domain C and the c-terminal, domain D. For example, a "buttonhead element" within domain C acts synergistically with sterol-regulatory element binding proteins (Yieh et al., 1995; Athanikar et al., 1997). Transcription factors that act synergistically with Sp1 include but are not limited to: c-Jun/c-Fos, NF-κβ, p53, p73α, p73β, GATA-1, HNF-4, AP-2, Oct-1, and NeuroD1 (Wiestra, 2008). The inhibitory region of Sp1 is at the N-terminus. Specifically, the inhibitory domain interacts with co-repressors, such as silencing mediator of retinoid and thyroid receptors (SMRT), nuclear receptor corepressor (NCoR), and BCL6 corepressor (BCoR) to inhibit Sp1 from binding to DNA (Lee et al., 2005). Consistent with the ubiquitous presence of Sp1, it is involved

in negative or positive regulation of genes involved in almost every cellular process including cell growth and survival, angiogenesis, metastasis, apoptosis, anti-angiogenesis, cell adhesion, DNA mismatch repair, and metabolism. While Sp1 has been implicated in a wide range of cellular functions, Sp1 binds to the porcine lactoferrin gene promoter in ST cells (Wang et al., 1998).

Another Sp family member, Sp3, is slightly larger than Sp1 (781 amino acids), yet is also ubiquitously expressed and contains a DNA binding domain comprised of 3 $cysteine_2$-$histidine_2$ zinc fingers. The consensus binding site for Sp3 is the same as Sp1 and Sp3 also has the same glutamine-rich sub-domains in its binding domain, the "buttonhead element" in domain C and the synergistic activity of domain D (Pascal and Tjian 1991). While 90% homologous in sequence to Sp1, Sp3 differs structurally as it has an inhibitory domain in front of the zinc-finger domain (Dennig et al. 1996; Suske 1999; Li et al., 2004). Uniquely, Sp3 can form 2 shorter proteins, not as a result of alternative RNA splicing, but from different translational initiations (Kennett et al., 1997). These isoforms are expressed alongside the long form; however, they lack the trans-activation domain A and appear to be responsible for the repressive actions of Sp3 only (Kennett et al., 1997, 2002). Unlike Sp1, Sp3 is unable to act synergistically on promoters containing multiple Sp1/3 sites (Yu et al., 2003). The relative levels of Sp1 and 3 can regulate some gene promoters. For example, a high ratio of Sp3 to Sp1 protein represses the human secretin receptor gene, whereas the inverse relationship increases gene expression in pancreas-derived PANC-1 cells (Pang et al., 2004). The main function for Sp3 appears to be repression of Sp1-activated genes, but it can also activate gene expression. As an

example, a gene involved in cell growth, dihydrofolate, is specifically activated by Sp1 but repressed by Sp3 (Birnbaum et al., 1995). On the other hand, Sp3 activates the mouse growth hormone receptor gene, as well as the human α_2(I) collagen and c-Met genes (Zhang et al., 2003). Also, Sp3 enhanced Sp1-activated gene expression of the growth hormone receptor and c-Met genes, demonstrating that Sp3 may act synergistically with Sp1 or through super-activation of Sp1 (Zhang et al., 2003). Though Sp3 may inhibit Egr-1 from binding to an overlapping Egr-1/Sp3 site (Al-Sarraj et al., 2005), to date, Sp3 has not been indicated to interact synergistically with Egr-1. As expected, Sp3 has been identified in ST cells, although its specific function remains to be determined.

The mammalian nuclear factor (NF)-κB complex consists of the following subunits: p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2). The proteins NF-κB1 and NF-κB2 begin as the larger subunit p105 and p100 and are processed by an ubiquitin/proteasome pathway to the mature p50 and p52 proteins. Within the family, the proteins are characterized by a 300 amino acid Rel homology domain (RHD) near the N-terminus. This conserved domain binds to DNA, associates in dimers and interacts with the inhibitor protein, IκB, in an inactivated state (Hayden and Ghosh, 2004). In unstimulated cells, NF-κB exists in homo- and heterodimers with IκB within the cytoplasm (Hong et al., 2003). Upon activation of the NF-κB signaling cascade, IκB is phosphorylated and degraded allowing NF-κB subunits to locate to the nucleus, bind to its specific element and regulate transcription (Chen et al., 1995). The most abundant form of NF-κB is a heterodimer of the p65 and p50 subunits (Hong et al., 2003). Involved in the immune system, NF-κB regulates gene expression of pro-

inflammatory cytokines [including TNF-a, interleukin-1 (IL-1), and IL-6 (Tomita et al., 2001)], chemokines [IL-8 and macrophage inflammatory protein-1a (MIP-1a; Delgado et al., 2001)], and adhesion molecules [vascular cell adhesion molecule-1 (VCAM-1; Binion et al., 2009) and intracellular cell adhesion molecule-1 (ICAM-1; Tomita et al., 2001)]. Outside of the immune system, NF-κB has a role in early embryonic development including development of bone, the central nervous system, mammary glands and skin (Hayden and Ghosh, 2004). Additionally, testis-related genes, androgen receptor, urokinase, proenkephalin and TNF-α, were regulated by NF-κB (Delfino, 1998). The NF-κB transcription factor has also been implicated in tumor cell growth. In pituitary adenomas, targeted pharmacological or molecular inhibition of NF-κB resulted in decreased viability (Vender et al., 2008). Within the pituitary, NF-κB has been implicated in gene regulation of GnRHR I (McDonald et al., unpublished data) in gonadotropes, interleukin-1 receptor in somatotropes (Parnet et al., 2004) and thyrostimulin in corticotropes (Suzuki et al., 2009).

CHAPTER III

MATERIALS AND METHODS

Plasmid Preparation

Reporter Vector. Initially, primers (Table 3.1; Integrated Life Technologies Corp., Carlsbad,CA) were used to isolate an 829 and 2612 bp region of the porcine GnRHR II gene promoter from the CHORI-242 porcine BAC library clones (BACPAC Resources Central, Children's Hospital Oakland Research Institute, Oakland, CA). Both 829 and 2612 bp sections of the GnRHR II gene promoter were first sub-cloned into separate pSK or pBSK basic vectors (Stratagene, La Jolla, CA) using *EcoRVI* and *SpeI* restriction endonuclease sites. The 829pBSK vector was digested with *SpeI* and *KpnI* while the 2612pBSK vector was cut with *SpeI* and *XhoI*. A 3-way ligation was performed to ligate the 829 and 2612 bp products into the pGL3 basic vector (Figure 3.1; Promega Corp., Madison, WI) between the *KpnI* and *XhoI* sites. Therefore, the test vector consisted of 3029 bp of GnRHR gene promoter fused to the cDNA encoding luciferase. The Rous Sarcoma Virus promoter fused to the cDNA encoding β-galactosidase (RSV-βgal, Stratagene, La Jolla, CA.) was used as a control to normalize the transfections.

Promoter Deletions. Vectors containing progressively less 5' flanking sequence within the context of the 3029 bp promoter (3029pGL3) were constructed utilizing

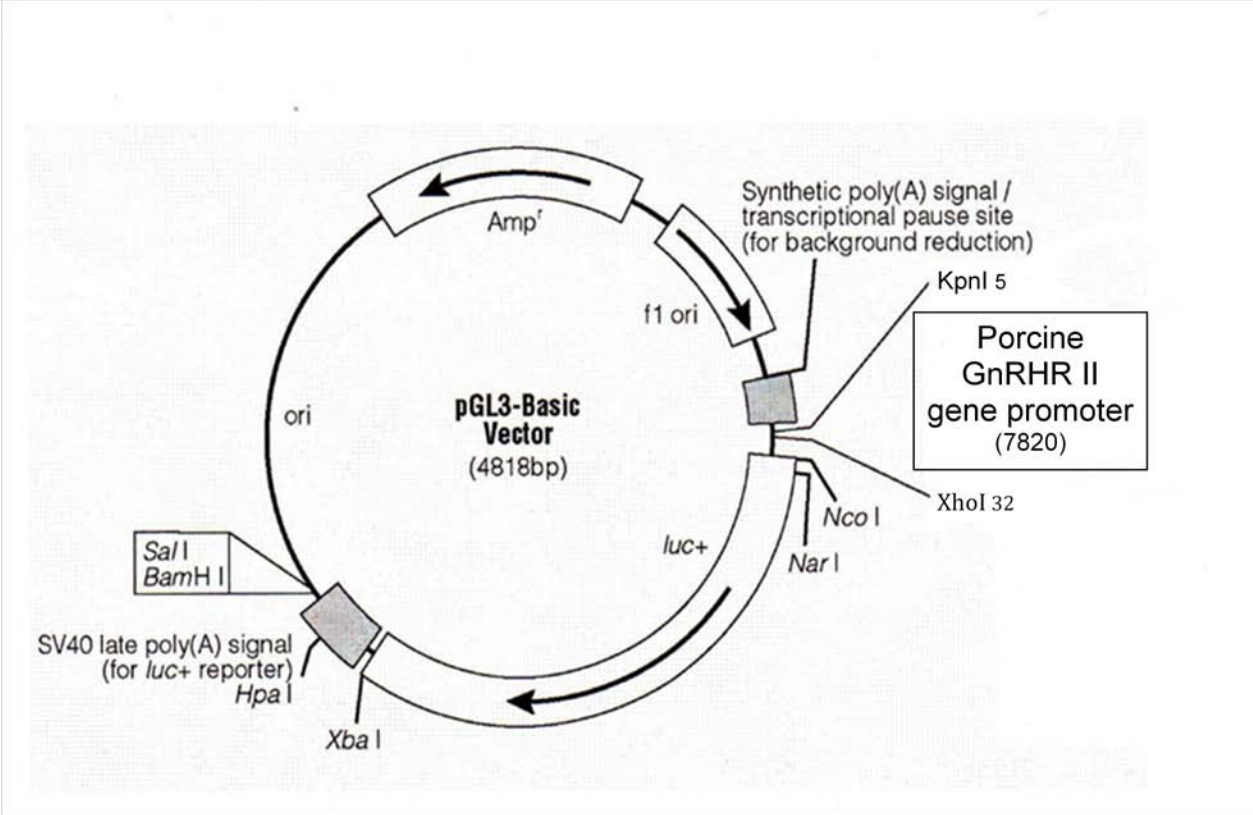

Figure 3.1. The full-length porcine GnRHR II gene promoter was sub-cloned into the pGL3-basic vector by *Kpn*I and *Xho*I restriction endonuclease recognition sequences.

TABLE 3.1. PRIMERS USED TO GENERATE THE 3029bp
PORCINE GnRHR II GENE PROMOTER

Deletion Vector	Primer	Sequence
829pGL3	Forward	5’-GGT GAC ATG GAA GGG AAA GG-3’
	Reverse	5’-TAG ATG CAC AGC TCG TTT GG-3’
1612GL3	Forward	5’-AGC AGG CAG TGA GGG ACA -3’
	Reverse	5’-TTG ACC CTC TTA ACC GCA AG-3’

restriction endonuclease digestion or PCR. Deletions were prepared from the 5' end of the porcine GnRHR II promoter (-3029) utilizing either restriction enzymes [2355pGL3 (SpeI), 788pGL3 (ApaI), 402pGL3 (PvuII), and 186pGL3 (PstI)] or primers [1696pGL3, 707pGL3, 579pGL3, 488pGL3, 357pGL3, and 280pGL3; Table 3.2]. PCR products were inserted into pGL3 by digesting 3029pGL3 with *KpnI* and *XhoI*, polishing the digested vector with shrimp alkaline phosphatase (SAP) and performing a blunt-end ligation.

Block Replacement Mutations. Primers were designed to replace a specific element within the porcine GnRHR II gene promoter with a restriction enzyme site (Table 3.3). Polymerase chain reaction was used to replicate both the sense and anti-sense strands of the DNA using specific primers (Table 3.3; Integrated DNA Technologies, Coralville, IA) for the region to be mutated. The PCR reaction consisted of 10X AccuPrime *Pfx* reaction mix (50 mM Tris-HCL (pH 8.0), 50 mM KCL, 1 mM DTT, 0.1 mM EDTA, 50% (v/v) glycerol, 1 mM $MgSO_4$ and 0.3 mM dNTPs; Invitrogen Life Technologies Corp., Carlsbad, CA), 10 μM primers, 50 ng/μl DNA template, AccuPrime *Pfx* DNA polymerase (Invitrogen Life Technologies Corp.) and Millipore water to bring the final volume to 50 μl. The PCR cycling conditions to generate the mutation in each strand were as follows: Step 1 - 94°C for 2 min; Step 2 - 94°C for 15 sec; Step 3 - 59°C for 30 sec; Step 4 - 68°C for 2 min; Step 5 - repeat step 2 through 4, 35 times; and Step 6 - 68°C for 10 min.

TABLE 3.2. PRIMERS USED TO GENERATE 5’ DELETIONS OF THE PORCINE GnRHR II GENE PROMOTER

Deletion Vector	Primer	Sequence
1696pGL3	Forward	5’-CTG AAC CTG CAC CAC TGA AA-3’
707pGL3	Forward	5’-CCT CCC CCA TTC CTG TTC-3’
579pGL3	Forward	5’-GCC CCT AGT CAC GCC TAC C-3’
488pGL3	Forward	5’-GCG GAG TTT CGG AAC TAG AA-3’
357pGL3	Forward	5’-TTA AAC ATT CGA GGC CAA GG-3’
280pGL3	Forward	5’-GGC CGC TAG GAT AAG GTT TC-3’
All of Above	Reverse	5’-GGT GAC ATG GAA GGG AAA GG-3’

The sequences generated from the above PCR reactions were utilized in a second PCR reaction to design the template DNA. The second PCR reaction used the same PCR cycling conditions as previously described. After completion of the second PCR reaction, the products were purified using the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA). To replace specific transcription factor binding sites, block replacement mutation vectors contained: a *BamHI* restriction endonuclease site in place of the overlapping Egr-1 / Sp1/3 motif element at -1596/-586 bp (μEgr-1Sp1/3pGL3); an *HindIII* site replacing the Sp1 element at -581/-574 bp (μSp1/3pGL3); and a *BglII* site replacing the NF-κB element located at -498/-492 bp (μNF-κBpGL3) within the porcine GnRHR II gene promoter.

The PCR reaction was purified with the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA). Briefly, 5 volumes of buffer PB1 was added to 1 volume of the PCR sample, mixed and placed in a QIAquick spin column within the 2-ml collection tube that was provided. Next, the sample was centrifuged using a Biofuge Pico microcentrifuge (Kendro Lab Products, Hanau, Germany) at 16,000 x *g* for 1 min. The flow-through was discarded and the spin column was placed back into the same collection tube. Next, the column was washed with 750 μl of buffer PE and centrifuged at 16,000 x *g* for 1 min. The flow-through was discarded and the spin column was placed back in the same tube and centrifuged for 1 min. The QIAquick column was placed in a clean 1.5-ml microcentrifuge tube and DNA was eluted from the QIAquick column upon addition of 50 μl of buffer EB and centrifugation at 16,000 x *g* for 1 min. After PCR purification, vectors containing the full-length GnRHR gene promoter and PCR products were

digested with the *AvrII* restriction endonuclease to ligate the PCR product (insert) into the digested vector containing the full-length GnRHR gene promoter (template). Following the restriction digest, the insert and template were gel extracted and quantitated. To set up ligations, insert to template ratios were 1:0 (control), 1:1, 1:5 or 1:10. The ligation reaction containing T4 ligase (5 U/µl, Fermentas, Glen Burnie, MD) and T4 ligase buffer (Fermentas) was incubated overnight at 15°C.

Gel Extraction. Plasmid fragments were extracted from agarose gels with the Perfectprep® Gel Cleanup Kit (Eppendorf, Westbury, NY). The excised gel slice was weighed and a maximum of 400 mg placed into a 2-ml microcentrifuge tube. Next, 3 volumes of binding buffer were added for every volume of each gel slice (1 mg of weight equaled 1 µl of volume). Gel slices were incubated at 50°C for 5 to 10 min in a heat block and vortexed every 2 to 3 min. After the gel slice was completely dissolved, an equal volume of isopropanol was added to the original gel slice volume and mixed by inversion. The sample (up to 800 µl) was placed in a spin column with a 2-ml collection tube and centrifuged using a Biofuge Pico (Kendro Lab Products) at 6,000 to 10,000 x *g* for 1 min. Next, the filtrate was discarded and the spin column was replaced in the same collection tube. If the sample was larger than 800 µl, the sample was reloaded and centrifuged again. Diluted wash buffer was prepared by adding 600 µl of 100% ethanol to 150 µl of concentrated wash buffer. Next, 750 µl of diluted wash buffer was added to each spin column and columns were centrifuged for 1 min at 6,000 to 10,000 x *g*. The filtrate was discarded and the spin column was replaced in the same collection tube. The

spin column in the collection tube was centrifuged for 1 additional min at 6,000 to 10,000 x *g* to remove any residual wash buffer. The spin column was then placed in a new 2-ml collection tube, 30 µl of elution buffer was added to the center of the spin column and it was centrifuged for 1 min at 6,000 to 10,000 x *g*. The plasmid fragments were quantitated with a Pharmacia GeneQuant spectrophotometer (Pfizer, New York, NY) using A_{260} and A_{280} values.

Transformation. Plasmids were transformed utilizing Rb-Cl competent DH5α cells (Invitrogen Life Technologies Corp.). A total of 5 µl of each plasmid ligation reaction was added to 50 µl of DH5α cells, gently mixed and incubated on ice for a total of 30 min. The reaction was then heat shocked for 30 sec at 42°C on a heat block and incubated on ice for 2 min. Next, 200 µl of LB media (Sigma Chemical Co., St. Louis, MO) was added to each reaction and placed in a 37°C shaking incubator for 1 h. Plasmids were plated (50-200 µl) on LB agar plates (Sigma Chemical Co., St. Louis, MO) containing 50 µg/ml ampicillin and incubated inverted at 37°C overnight. Approximately 16 h later, colonies were counted, evaluated, stored at 4°C for up to 1 mo and utilized in alkaline lysis mini plasmid preparations.

Alkaline Lysis Mini Plasmid Preparation. Plasmid DNA was extracted from Rb-Cl competent DH5α cells (Invitrogen Life Technologies Corp.) for screening purposes. A single colony containing an ampicillin resistant plasmid in RbCl cells was inoculated overnight and grown in 2 ml of LB media (Sigma Chemical Co.) containing

0.05 mg/ml ampicillin shaking at 37°C. Cells were pelleted by centrifuging 1 ml of culture at 16,000 x *g* for 1 min with a Biofuge Pico microcentrifuge (Kendro Lab Products). The supernatant was removed and the cell pellet was resuspended in 100 µl of GTE [50 mM glucose, 25 mM Tris-Cl, 10 mM ethylenediaminetetraacetic acid (EDTA)] and incubated at room temperature for 5 min. Next, 200 µl of NaOH/SDS (0.2 N NaOH, 1% (wt/vol) sodium dodecyl sulfate) was added to each sample, mixed and incubated on ice for 5 min. Following the incubation, 150 µl of 5 M potassium acetate (29.5 ml glacial acetic acid, potassium hydroxide (KOH) pellets, pH 4.8) was added to each sample, vortexed for 2 s, and incubated on ice for an additional 5 min. The samples were centrifuged at 16,000 x *g* for 3 min and the supernatant was placed in a clean 1.5-ml microcentrifuge tube. The supernatant was mixed with 800 µl of 100% ethanol and incubated at room temperature for 2 min. The samples were then centrifuged at 16,000 x *g* for 1 min to pellet plasmid DNA. The supernatant was then removed and the pellet was washed with 1 ml of 70% ethanol. The samples were centrifuged for 1 additional min at 16,000 x *g*, the supernatant was removed again, and the pellet dried at room temperature. Once dried, the pellet was resuspended in 1X tris-ethylenediaminetetraacetic acid (TE, pH 8.0). Following resuspension, 1 µl of RNase A was added to each sample and samples were incubated at 37°C for 30 min. Plasmids were later screened by restriction digests for confirmation prior to being utilized in a support protocol.

Midi Plasmid Purification. Plasmids were prepared for transfection using a Plasmid Purification Midi Kit (Qiagen, Inc.) and confirmed with restriction enzyme

digests. An overnight culture, shaking at 37°C, was inoculated with 100 ml of LB broth containing 0.05 mg/ml ampicillin and a bacterial stock containing the vector of interest. The bacterial cells were harvested by centrifugation at 6,000 x *g* for 15 min at 4°C using a Sorvall RC2-B centrifuge (Du Pont Co., Newton, CT). The bacterial pellet was resuspended in 4 ml of buffer P1 and completely vortexed until no cell clumps remained. Next, 4 ml of lysis buffer P2 was added followed by a 5 min incubation at room temperature. Then, 4 ml of pre-chilled precipitation buffer P3 was added to each sample and incubated on ice for 15 min. Following incubation on ice, samples were centrifuged (20,000 x *g*) for 30 min at 4°C using a Sorvall RC2-B centrifuge (Du Pont Co.).

Each resin column was equilibrated prior to the addition of sample (cell lysate) with 4 ml buffer QBT. After each sample was added to the columns, they were washed twice with 10 ml of buffer QC. Next, the plasmid DNA was eluted upon the addition of 5 ml of buffer QF to each column. The plasmid DNA was precipitated by the addition of 3.5 ml of room temperature isopropanol and centrifuged (15,000 x *g*) for 30 min at 4°C using a Sorvall RC2-B centrifuge (Du Pont Co.) The supernatant was decanted and pellets were washed with 2 ml of room temperature 70% ethanol and centrifuged (15,000 x *g*) for 10 min at 4°C using a Sorvall RC2-B centrifuge (Du Pont Co.). Next, the pellets were air dried for 5-10 min and resuspended in approximately 250 µl of Millipore water. The purified plasmids were quantitated utilizing a Lambda EZ 150 spectrophotometer (Perkin Elmer, Boston, MA) and screened using restriction enzymes. To confirm that vectors contained the insert, they were sequenced prior to use in transient transfection assays. Finally, vectors were stored at -20°C until use.

Cell Culture

Swine Testis (ST) cells (ST; ATCC cat.# CRL-1746; S.J. Wessman) were maintained on 150-mm cell culture plates (Corning Inc., Corning, NY) in 20 ml of ST media. The media was comprised of high glucose Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Herndon, VA) with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (Gibco, Grand Island, NY). The cultures of the ST cells were maintained at 37°C in a humidified 5% CO_2 in air environment.

Transient Transfections

Day 1. At approximately 85% confluency**,** ST cells were typsinized, counted, and plated for transfection utilizing a liposome-mediated protocol. The media was aspirated from the cells and they were washed with 10 ml of 1X phosphate-buffered saline (PBS) 2 times. Cells were trypinized by adding 3 ml of 1X trypsin-EDTA (Mediatech Inc., Herdon, VA) and incubated for 5 min at 37°C in a humidified 5% CO_2 in air environment. After the cells detached from the plate, 7 ml of ST media was added to disperse cell clumps and cells were transferred to a 50-ml conical tube. A 100 µl sample of cells was diluted in 900 µl of 1X PBS and 10 µl of the dilution was loaded onto a hemacytometer to perform cell counts. Approximately 2 x 10^5 ST cells were plated in a 6-well culture plate (Corning Inc., Corning, NY) to achieve 50% confluency at transfection. Each well contained 2 ml of ST media.

Day 2. A 1.5-ml microcentrifuge tube for each test vector (3 wells) contained the total of 291 µl of serum-free DMEM and 9 µl of Fugene6 (Roche Diagnostics Corp., Indianapolis, IN). Each test vector was transfected in triplicate and 0.9 µg/well of test vector and 0.1 µg/well of the control vector (RSV-βgal) was added to each 1.5-ml microcentrifuge tube. The reaction was incubated at room temperature for approximately 45 min. Following incubation, 96 µl of the reaction (DMEM, Fugene6 and vectors) was added to each well.

Day 3. Transfected ST cells were harvested approximately 24 h later. The media was aspirated from the wells and they were rinsed twice with 1 ml of 1X PBS. Following the rinses, 200 µl of Lysis Buffer (Galacto-Light kit; Applied Biosystems, Bedford, MA), which contained 1 M dithiothreitol (DTT), was added to each well. The 6-well plates were incubated and shaken at 4°C for at least 10 min. The cell lysates were harvested by pipetting and washing the plates with the buffer. Next, the cell lysates and Lysis Buffer were transferred to a 0.5-ml microcentrifuge tube and centrifuged at 4°C for 2 min at 16,000 x *g* using a Spectrofuge 16M microcentrifuge (E&K Scientific, Campbell, CA). Cell lysates (20 µ1) were transferred in duplicate into a white, 96-well Microflux2 plate (Thermo Labsystems, Franklin, MA) for luciferase and β-galactosidase assays. The β-galactosidase assay was incubated at room temp for 30-60 min prior to analyzing its activity using the Wallac Victor^2 instrument (Perkin Elmer, Waltham, Massachusetts). The plates were analyzed for luciferase and β-galactosidase activity utilizing the Wallac Victor^2 instrument (Perkin Elmer). Luciferase values were divided by β-galactosidase

values to adjust for transfection efficiency. Transient transfections utilized 3 different plasmid preparations and were completed a minimum of 3 times.

Protein Extraction

A Nuclear and Cytoplasmic Extraction Reagent Kit (NE-PER®; Pierce Biotechnology, Rockford, IL) was utilized to obtain nuclear protein extracts from ST cells for electrophoretic mobility shift assays (EMSAs). Four 150 mm plates of ST cells at 85% confluency were rinsed once with 10 ml of ice cold 1X PBS. Next, cells were removed from the plates with 10 ml of TNE buffer (10 mM Tris-Cl, 140 mM sodium chloride (NaCl), and 1 mM EDTA, pH of 8.0). Once cells were removed from the plates, they were centrifuged for 5 min at 500 x *g* and 4°C using a Beckman TJ-6 centrifuge (Beckman, Palo Alto, CA).

The supernatant was completely removed from each cell pellet. The dried cell pellet was resuspended in ice-cold CER I reagent (Pierce Biotechnology), by vortexing for 15 s, and incubated on ice for 10 min. 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) and Protease Inhibitor Cocktail [100X stock containing 104 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 80 µM aprotinin, 2 mM leupeptin, 4 mM bestatin, 150 µM pepstatin A and 140 µM E-64; Sigma Chemical Co., St. Louis, MO] were added with the CER I reagent. Next, ice-cold CER II reagent (Pierce Biotechnology) was added to the cells, vortexed vigorously for 5 s and incubated

on ice for 1 min. Lysed cells were then centrifuged using a Spectrafuge 16M microcentrifuge at 16,000 x *g* (E&K Scientific, Campbell, CA) for 5 min at 4°C. The supernatant was then transferred to a clean pre-chilled 1.5-ml microcentrifuge tube and stored at -80°C. The insoluble pellet was resuspended in ice-cold NER reagent (Pierce Biotechnology) containing protease and phosphatase inhibitors. The pellet was vortexed for 15 s and then incubated on ice for a total of 40 min, vortexing for 15 s every 10 min. Lastly, the lysed nuclei were centrifuged at 16,000 x *g* for 10 min at 4°C using a Spectrafuge 16M microcentrifuge (E&K Scientific). The supernatant (nuclear extract) was then immediately transferred to a clean, pre-chilled 1.5-ml microcentrifuge tube.

The total protein concentration of nuclear extracts was measured using the BCA™ Assay Kit (Pierce Biotechnology). Nine standards were used for the standard curve and compared to unknown samples. A microplate (Sarstedt, Inc., Newton, NC) was used and 25 µl of each standard and unknown was loaded onto the plate in triplicate. A working reagent was prepared by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B (50:1, Reagent A:B). The working reagent (200 µl) was added to each well and mixed thoroughly on a plate shaker for 30 s. Next, the plate was covered and incubated at 37°C for 30 min. Following incubation, the plate was cooled to room temperature and light absorbance was measured on a Spectra Max 250 plate reader (Molecular Devices, Sunnyvale, CA) at 562 nm. After the nuclear extracts were quantitated, they were stored at -80°C in 100 µl aliquots until use.

Electrophoretic Mobility Shift Assays

Oligonucleotides were annealed prior to use in EMSAs by adding 1 µl of the antisense DNA (50 µM), 1 µl of the sense DNA (50 µM), 1 µl of 10X NET buffer (1 M NaCl, 10 mM EDTA, and 100 mM Tris-Cl, pH 7.5) and Millipore water to bring to a final volume of 10 µl. The mixture was heated to 95°C for 10 min to denature the oligonucleotides, incubated at 37°C for 30 min to anneal the oligonucleotides and incubated at 25°C for an additional 30 min. Oligonucleotides had a final concentration of 5 µM and were stored at -20°C.

T4 polynucleotide kinase (PNK; Fermentas, Inc., Hanover, MD) was used to end label the oligonucleotides with [γ-^{32}P]ATP. One µl of the annealed oligonucleotide, 1 µl of 10X T4 PNK Buffer A, 4 µl of Millipore water, 1 µl of T4 PNK, and 3 µl of [γ-^{32}P]ATP were incubated together for 30 min in a 37°C water bath. After incubation, 35 µl of Millipore water was added to each reaction. MicroSpin™ G-25 columns (Amersham Biosciences Corp., Piscataway, NJ) were equilibrated by centrifuging at 3,000 x *g* for 1 min using a Mikroliter microcentrifuge (Hettich AG, Bach, Switzerland). Each reaction was added to an equilibrated MicroSpin™ G-25 column (Amersham Biosciences Corp.) and centrifuged for 2 min at 3,000 x *g* using a Mikroliter microcentrifuge (Hettich AG). Next, 5 µl of 10X NET buffer was added to each reaction. To measure percent incorporation of radioactivity on DNA, 4 ml of scintillation fluid was added to 1 µl of each reaction in a scintillation vial and counted using a 1900TR liquid scintillation counter (Packard Instrument Co., Meriden, CT).

Electrophoretic mobility shift assays utilized a 5% polyacrylamide gel that consisted of 6.25 ml of 38% acrylamide, 2% bisacrylamide; 5 ml of 10X TGE buffer (0.25 M tris base, 1.9 M glycine, 10 mM EDTA, pH 8.3); 150 µl of 10% ammonium persulfate, 50 µl of TEMED and Millipore water which was added to bring the final volume to 50 ml. After polymerization, the gel was pre-run at 100 V for at least 30 min in 1X TGE buffer (25 mM tris base, 190 mM glycine, and 1mM EDTA, pH 8.3). A master mix was prepared consisting of 4 µl of 2X Dignam D buffer [20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M potassium chloride (KCl), 0.2 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride (PMSF; added fresh), 0.5 mM DTT (added fresh)], 1 µl of 20 mM DTT, 2 µg poly(dI·dC) (Amersham Biosciences) and 5 µg of ST nuclear extracts. The total volume was brought up to 18 µl with Millipore water and incubated on ice for 15 min. Following incubation, 1 µl of either homologous, heterologous, or consensus oligonucleotide competitors were added. Consensus oligonucleotides (Table 3.4; Integrated DNA Technologies) that were used in EMSAs included Egr-1, Sp1, Sp3 and NF-κB. Assays which utilized unlabeled competitor oligonucleotides were added with 1 µl of radiolabeled probe and the reaction was incubated at room temperature for 20 min. To determine which proteins comprised specific binding complexes, 1 µg of a rabbit polyclonal antibody directed against p50 subunit of NF-κB (catalog no. sc-114X), Egr-1 (catalog no. sc-110X), Sp2 (catalog no. sc-643x) or 4 (catalog no. sc-13019x; Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit polyclonal antibody directed against the p65 (catalog no. PC137; Calbiochem, La Jolla, CA) or p52 subunits of NF-κB (catalog no. 06-413; Upstate, Charlottesville, VA), rabbit polyclonal Sp1 (catalog no. 07-645) or 3

TABLE 3.4. OLIGONUCLEOTIDES CONTAINING CONSENSUS TRANSCRIPTION FACTOR BINDING SITES USED AS COMPETITORS IN ELECTROPHORECTIC MOBILITY SHIFT ASSAYS[a]

Transcription Factor[b]	Binding Sequence
AP-2	5'-GAT CGA ACT GAC CGC CCG CGG CCC GT-3'
Egr-1	5'-GGA TCC AGC GGG GGC GAG CGG GGG CGA -3'
NF-κB	5'-AGT TGA GGG GAC TTT CCC AGG C -3'
Sp1	5'-AAA CGA TCG GGG CGG GGC GGC GAG-3'

[a] The complement strand was annealed for each consensus oligonucleotide prior to use in EMSAs.

[b] AP-2 = Activator Protein 2; Egr-1= early growth response 1; Sp1 = specificity protein 1; NF-κB = nuclear factor- κB; NF-Y = nuclear factor-Y

antibodies (catalog no. 07-107; Upstate, Lake Placid, NY) or an equal mass of rabbit IgG (Santa Cruz Biotechnology) was used. Prior to addition of the probe labeled with [γ-^{32}P]ATP, the master mix was incubated with Sp antibodies for 2 h at 4°C, or Egr-1 and NF-κB antibodies for 30 min at room temperature. After addition of the radiolabeled probe, the reactions were incubated at room temperature for 20 min. Reaction samples were then loaded on the acrylamide gel and subjected to electrophoresis at 40 mA for approximately 1.5 hours. Next, the acrylamide gel was transferred to blotting paper (3 mm; Whatman, Maidstone, England), dried on a Fisherbiotech Gel Dryer Model FB-GD-45 (Fisher Scientific, Pittsburgh, PA) and exposed to Blue Sensitive Radiographic Film (Marsh Bio Products, Inc., Rochester, NY) for 20-24 h at -80°C. The film was later developed using an SRX-101A medical film developer (Konica Corp., Wayne, NJ).

Statistical Analysis

Data were analyzed using the analysis of variance (ANOVA) procedure of the Statistical Analysis System software (version 8.2, SAS Institute Inc, Cary, NC). Means for luciferase activity of test vectors were compared to each other using Tukey's honest significant difference test. All transfections were performed a minimum of 3 times using different plasmid preparations. Additionally, each plasmid was transfected in triplicate wells within each transfection.

CHAPTER IV

Transcriptional Regulation of the Type II Porcine GnRH Receptor Gene

Abstract

Unlike the central role that the native form of gonadotropin-releasing hormone (GnRH I) plays in the reproductive axis, the second mammalian isoform (GnRH II) has specifically been implicated in the interaction between female energy balance and reproductive function. In addition, GnRH II has an anti-proliferative/apoptotic role in cancer cell lines, similar to GnRH I. A specific receptor for GnRH II, GnRHR II, was isolated in mammals, although there is inconsistency in the literature with regard to whether a functional protein is produced. Since the porcine GnRHR II gene codes for a putative functional protein, we wanted to examine how GnRHR II gene expression is controlled. To study transcriptional regulation of this novel isoform, our laboratory isolated 3029 bp of 5' flanking sequence for the porcine GnRHR II gene. Transient transfections with luciferase reporter vectors containing the GnRHR II promoter (3029pGL3) indicated that this promoter, unlike that of GnRHR I, is active in cell lines derived from both reproductive and non-reproductive tissues. Reporter plasmids containing progressive 5' deletions of the GnRHR II promoter indicated that the regions from -707/-402 bp contained elements critical to promoter activity in swine testis (ST) cells. Electrophoretic mobility shift assays (EMSAs) performed with 30-bp radiolabeled oligonucleotides spanning the -707/-488 bp region and ST nuclear extracts, revealed

specific binding complexes for probes corresponding to the -512/-489, -590/-570 and -605/-580 bp regions of proximal promoter. Antibody addition to EMSAs indicated that the p65 and p52, but not p50, subunits of NF-κB comprised the specific complex binding to the oligonucleotide containing the -512/-489 bp promoter region. Use of vectors harboring a block replacement mutation of the NF-κB binding site located at -502/-492 bp of the promoter in transient transfections of ST cells resulted in a 26% decrease in luciferase activity ($P < 0.05$). Next, addition of antibodies directed against the Sp1 and 3 transcription factors, but not Sp2 or 4, resulted in supershifts of the specific DNA:protein complexes for the radiolabeled oligonucleotide corresponding to -590 to -570 bp of GnRHR II promoter. Transient transfection of vectors containing a mutation of the Sp1/3 site (-579/-574 bp) within 3029pGL3 resulted in a 61% decrease in promoter activity ($P < 0.05$). Finally, additional EMSAs revealed that the specific DNA:protein complexes within the -605/-580 bp region shifted upon the addition of Egr1, Sp1 or Sp3 antibodies. Vectors containing a conversion of the overlapping Egr1/Sp1/3 site (-596/-586 bp) to a restriction enzyme site within the context of the 3029 bp promoter resulted in a 56% decrease in promoter activity ($P < 0.05$) following transient transfections into ST cells. Thus, NF-κB, Sp1/3 and overlapping Egr1/Sp1/3 binding sites are critical to expression of the porcine GnRHR II gene in ST cells.

Introduction

To date over 20 isoforms of GnRH and 5 forms of GnRHR have been identified in vertebrates (Moncaut et al., 2005). The classical mammalian form (GnRH I) is a

hypothalamic neuropeptide that functions as a key regulator of reproduction. The interaction between GnRH I and its receptor (GnRHR I) stimulates gonadotropin synthesis and secretion from the anterior pituitary gland. The gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), act on the gonads to stimulate either steroidogenesis and ovulation or gametogenesis, respectively.

In addition to GnRH I, a GnRH II decapeptide, first discovered in chickens (Miyamoto et al., 1984), was identified in humans, primates, ruminants and swine (Millar, 2003). Similar to GnRH I, GnRH II has been implicated in reproduction. In males, GnRH II is involved in steroidogenesis, as GnRH II agonists increased testosterone production and expression of genes encoding steroidogenic acute regulatory protein (StAR), 17-α-hydroxylase and 3β-hydroxysteroid dehydrogenase (HSD) in murine Leydig cells (Lin et al., 2008). In azoospermic men, increased GnRH II expression is correlated with increased intratesticular testosterone concentrations (Lin et al., 2008). Additionally, boars immunized against GnRH II had decreased levels of gonadotropin secretion and reduced ability of Leydig cells to respond to LH challenges, indicating GnRH II has some role in gonadotropin release and Leydig cell function (Bowen et al., 2006). Furthermore, GnRH II has also been implicated in female reproduction since it can induce LH and FSH production in the rhesus monkey (Densmore and Urbanski, 2003) and downregulate LH and FSH receptors in human granulosa-luteal cells (Kang et al., 2001). The GnRH II ligand also has a role in the interaction between reproduction and energy balance. Administration of GnRH II rescued mating behavior in nutrient deprived female mice (Kauffman et al., 2004) and musk

shrews (Kauffman et al., 2006) while increasing mating behavior in female marmoset monkeys fed a normal diet (Barnett et al., 2006).

Although controversial, GnRH II may act through GnRHR II to regulate cell proliferation and apoptosis in human tumors. The GnRHR II is expressed in endometrial (Hec-1A), ovarian (SK-OV-3, OVCAR-3; Gründker et al., 2002) and prostate (PC3; Montangani et al., 2009) cancer cell lines. In GnRH II positive, GnRHR I negative, ovarian cancer cells (SK-OV-3) GnRH II reduced cell proliferation (Gründker et al., 2002). In contrast, siRNA targeted reduction of GnRHR II did not alter the anti-proliferative effects of GnRH II on prostate cancer cells (PC3; Montagnani et al., 2009). Furthermore, apoptosis was increased significantly (1.6-fold) in response to GnRH II in ovarian cancer cells (OVCAR-3), likely by induction of caspase-3, -8 and -9 (Hong et al., 2008).

While the GnRHR II gene has been isolated in musk shrews, old world monkeys, cattle, sheep, pigs and humans, the complete gene for the receptor is not present in rats and mice (Millar et al., 2001; Neill et al., 2001). The amino acid sequence for the marmoset GnRHR II has approximately 40% identity to the human GnRHR I, but the most striking difference is the intracellular cytoplasmic c-terminal tail specific to GnRHR II (Millar et al., 2001). In the human, GnRHR II mRNA has been isolated; however, similar to cattle and sheep (Morgan et al., 2006), a full-length GnRHR II protein may not exist due to a premature stop codon and a frame shift mutation (Neill, 2002). Instead, it has been suggested that the human GnRHR II is a unique 5- or 6-TM G-protein coupled receptor (GPCR) utilizing a non-AUG start codon to overcome the frame shift mutation

and a selenocysteine incorporated at the UGA codon instead of a stop codon (Neill, 2004). Preliminary studies indicated that GnRHR I and II share similar cell signaling pathways. Both GPCRs activate the $G_{q/11}$ protein to increase ionositol phosphate, Ca^{2+} and diacylglycerol levels stimulating protein kinase C (PKC) activity (Millar et al., 2003). Due to the presence of an intracellular cytoplasmic tail, the marmoset GnRHR II undergoes rapid internalization and desensitization relative to GnRHR I (Millar et al., 2003). While GnRH II has a high affinity to its cognate receptor (GnRHR II), GnRH II binds GnRHR I with one-tenth the affinity of GnRH I (Millar et al., 2003).

Ubiquitous expression of GnRHR II across tissues (adrenal, brain, breast, heart, kidney, pituitary, stomach, small intestine, large intestine, skeletal muscle, thymus, sperm, spleen, lung, liver, pancreas, thyroid, placenta, uterus, prostate, ovary and testis) in humans and marmoset monkeys suggests a key cellular function of GnRHR II (Millar et al., 2001; Faurholm et al., 2001; von Biljon et al., 2002). As expected based on gene expression studies, the marmoset GnRHR II promoter is functional in multiple tissues including: kidney (COS-7), prostate (PC-3), cervix (HeLa-S3), testis (TM3 and TM4), hypothalamus (GT1-7), pituitary (LβT2) and neurons (ND7/23; Faurholm et al., 2007). Moreover, promoter regions containing transcriptional activators were identified, including a -766/-665 bp enhancer region containing elements contributing to promoter activity (Faurholm et al., 2007). Since the porcine GnRHR II cDNA sequence codes for a putative functional protein, we chose to examine transcriptional regulation of this gene.

We isolated 3029 bp of 5’ flanking region for the porcine GnRHR II gene (Genbank No. FJ872917) and inserted the promoter into a luciferase reporter vector

(3029pGL3). Transient transfections of this construct indicated that the porcine GnRHR II gene promoter was active in all reproductive and non-reproductive cell lines tested (Wiarda et al., unpublished data). Of the available immortalized, porcine cell lines, 3029pGL3 displayed robust activity in ST cells and, given the proposed role of GnRH II in reproductive function of the testis, this cell line represented an appropriate in vitro model to study transcriptional regulation of the porcine GnRHR II gene. Thus, the aim of this study was to elucidate the transcription factors and corresponding binding sites contributing to basal activity of the porcine GnRHR II gene promoter in ST cells.

Materials and Methods

Materials. The antibody specific to Sp1 (catalog no. 07-645) was purchased from Millipore (Temecula, CA) and the antibody specific to the p65 subunit of NF-κB (catalog no. PC137) was purchased from Calbiochem (La Jolla, CA). The antibodies directed against Sp3 (catalog no. 07-107) and the p52 subunit of NF-κB (catalog no. 06-413) were purchased from Upstate (Lake Placid, NY). The specific antibodies for Egr-1 (catalog no. sc-110X), Sp2 (catalog no. sc-643X), Sp4 (catalog no. sc-13019X) and the p50 subunit of NF-κB (catalog no. sc-114X) were all obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For experiments using EMSAs, oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

Plasmids. Using specific primers (Table 3.1), we isolated the porcine GnRHR II gene promoter from the CHORI-242 porcine BAC library clones (BACPAC Resources Central, Children's Hospital Oakland Research Institute, Oakland, CA). The porcine

GnRHR II gene promoter (-3029 bp) was sub-cloned into the pGL3 basic reporter vector (3029pGL3; Promega Corp., Madison, WI). Progressive 5' deletions of the porcine GnRHR II promoter were produced utilizing restriction enzymes [2355pGL3 (SpeI), 788pGL3 (ApaI), 402pGL3 (PvuII), and 186pGL3 (PstI)] or primers [1696pGL3, 707pGL3, 579pGL3, 488pGL3, 357pGL3, and 280pGL3; Table 3.2]. Plasmids harboring block replacement mutations within the context of the full-length, 3029 bp GnRHR II gene promoter were constructed by replacing individual elements with restriction enzyme sites as follows: a HindIII site replacing the Sp1/3 binding site at -581/-574 bp (μSp1/3pGL3), a BamH1 site substituted for the overlapping Egr-1/Sp1/3 element at -592/-587bp (μEgr-1/Sp1/3pGL3), and a BglII site replacing the NF-κB site at -512/-489 (μNF-κBpGL3). Briefly, overlap extension PCR mutagenesis was performed through 2 rounds of PCR in order to specifically mutate the binding element of interest. The first round of PCR utilized primers to replace the binding site of interest with a restriction site, and the second round used product from the first round as template to anneal and replicate the mutated element and flanking sequence. To verify that the correct mutations had been introduced, vectors were sequenced at the University of Nebraska-Lincoln Genomics Core Facility. The vector used as a control for transfection efficiency in all experiments contained the Rous sarcoma virus promoter fused to the cDNA encoding β-galactosidase (RSV-β-gal, Stratagene, La Jolla, CA). A Plasmid Midi Kit (Qiagen, Valencia, CA) was used to isolate transfection quality DNA.

Cell Culture and Transient Transfections. Cultures of ST cells were maintained at 37°C in a humidified 5% CO2 in air atmosphere. The ST cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate (Gibco, Grand Island, NY). Transient transfections were carried out using a liposome-mediated protocol (Fugene6, Roche Diagnostics Corp., Indianapolis, IN) according to manufacturer's instructions. Briefly, 2 x 10^5 cells were plated in 6-well tissue culture dishes the day prior to 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 RSV-β-gal control vector, were used per well. Approximately 20-24 h post-transfection, cells were washed twice with ice-cold 1X PBS and harvested with 200 μl of lysis buffer [Tropix Lysis Buffer, Applied Biosciences, Bedford, MA, containing 1 mM dithiothreitol (DTT)]. Lysates were cleared by centrifugation at 16,000 x *g* for 2 min at 4°C. Lysates (20 μl) were immediately analyzed according to manufacturer's instructions for both luciferase (Promega Corp.) and β-gal (Tropix, Applied Biosystems, Bedford, MA) activity using a Wallac Victor^2 microplate reader (PerkinElmer Life Sciences, Boston, MA). Luciferase values were divided by β-gal values to adjust for transfection efficiency.

Electrophoretic Mobility Shift Assays. Nuclear protein extracts were obtained from approximately 2.8 x 10^8 ST cells using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology, Rockford, IL). The nuclear extracts were

treated with protease (catalog no. P8340; Sigma Chemical Co., St. Louis, MO) and phosphatase (catalog no. 524625; Calbiochem, La Jolla, CA) inhibitor cocktail solutions to prevent enzymatic degradation of proteins. Protein concentrations were determined using a bicinchoninic acid (BCA) Protein Assay (Pierce). Oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Fermentas Inc., Hanover, MD) and purified using sephadex G-25 spin columns (Amersham Biosciences Corp., Piscataway, NJ). Nuclear extracts (5 μg) were incubated in 18 μl reactions containing 2X Dignam D buffer [20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF (added fresh), and 0.5 mM DTT (added fresh)], 20 mM DTT, and 2 μg of poly(dI•dC; Amersham Biosciences) for 15 minutes on ice. Prior to addition of the radiolabeled probe, the master mix was incubated with Sp1 (2 μg), 2 (1 μg), 3 (2 μg) or 4 (1 μg) antibodies for 2 h at 4°C, NF-κB antibodies (1 μg) for 30 min at room temperature, Egr-1 antibodies (1 μg) for 15 minutes at room temperature or an equal mass of rabbit IgG. Following incubation, radiolabeled probe (100,000 cpm) and 50-fold molar excess of either homologous or heterologous unlabeled competitor was added. Where indicated, 50-fold molar excess of unlabeled oligonucleotides containing consensus binding sequences for the Sp family, Egr-1 or NF-κB transcription factors were also added. The final reactions were incubated at 25°C for 20 min before bound probe was separated from free probe at 40 mA for 1.5 h on a 5% polyacrylamide gel that had been pre-run at 100 V for 1 h in 1X TGE [25 mM tris base, 190 mM glycine and 1 mM EDTA, pH 8.3]. Gels were transferred to blotting paper, dried, and exposed to Blue Sensitive Radiograph Film

(Marsh Bio Products Inc., Rochester, NY) for 20-24 h at -80°C before being developed in a SRX-101A medical film developer (Konica Corp., Wayne, NJ).

Statistical Analysis. Data were analyzed using the analysis of variance (ANOVA) procedure of the Statistical Analysis System software (version 8.2, SAS Institute Inc, Cary, NC). Means for luciferase activity of test vectors were compared using Tukey's honest significant difference test. All transfections were performed a minimum of 3 times using 3 different plasmid preparations. Additionally, each plasmid was transfected in triplicate wells within each transfection.

Results

Deletional analysis of the porcine GnRHR II gene promoter in ST cells revealed regions containing putative activators and repressors. Transient transfection of luciferase reporter plasmids containing progressively less 5' flanking sequence for the 3029 bp GnRHR II promoter (-2355, -1696, -788, -707, -579, -488, -402, -357, -280 and -186 bp) were performed in ST cells. The 3029 bp promoter exhibited 8-fold higher luciferase activity than promoterless control (Figure 4.1; $P < 0.05$). Reduction of the -3029 promoter to 1696 bp of 5' flanking region did not impact promoter activity (Figure 4.1; $P > 0.05$). In contrast, deletion of the region from -1696 to -788 bp and -788 to -707 bp resulted in significant increases in promoter activity (Figure 4.1). The robust activity of the -788 and -707 bp promoters, 14.5- and 19–fold over promoterless control, respectively, suggests a putative repressor(s) exists within the -1696/-707 bp region. Significant decreases in luciferase activity occurred upon further deletion of the promoter

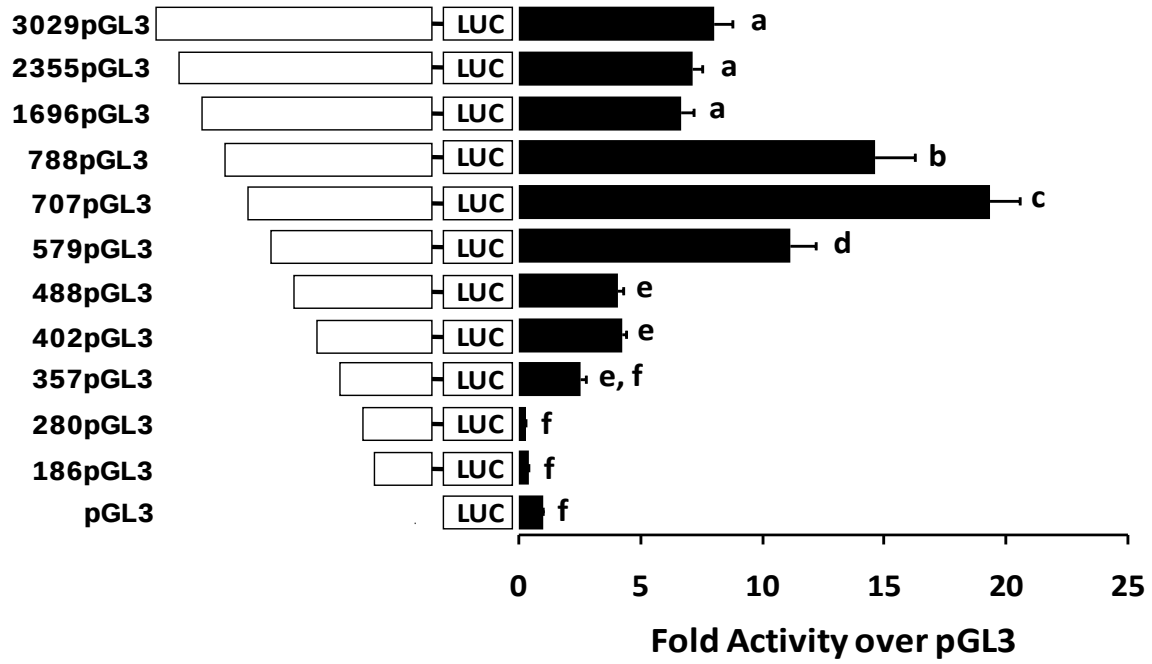

Figure 4.1. Critical 5' flanking regions conferring basal activity of the porcine GnRHR II gene promoter in ST cells. Luciferase (LUC) reporter vectors containing progressively less 5' flanking region of the 3029 bp porcine GnRHR II gene promoter (3029pGL3) or promoterless control (pGL3) were transiently transfected into ST cells. Cells were cotransfected with RSV-β-galactosidase (β-gal) and after 24 h of transfection, cells were harvested and lysates assayed for LUC and β-gal activity. To control for transfection efficiency, LUC values were divided by β-gal values and results are expressed as fold activity over pGL3. Values represent the mean ± SEM of triplicate measurements from at least three replicates containing different plasmid preparations. Unique letters indicate means that are significantly different ($P < 0.05$).

to -579 (11-fold) and -488 bp (4-fold) of 5' UTR (Figure 4.1). The overall loss in promoter activity (79%) upon shortening the 5' flanking region of the GnRHR II gene from -707 (19-fold) to -488 bp (4-fold) suggests putative elements contributing to promoter activation are within the -707/-488 bp region. Although the reporter vector harboring 357 bp of proximal promoter did not differ from the promoterless control ($P >$ 0.05), an 80% reduction in promoter activity from 357 (2.5-fold) to 280 bp (0.5-fold) indicates that this region potentially contains putative elements required for basal activation of the porcine GnRHR II promoter in ST cells (Figure 4.1). Finally, when the promoter was reduced to 280 or 186 bp, promoter activity was completely abolished (Figure 4.1). Thus, we have identified 2 regions contributing to basal activity of the porcine GnRHR II gene promoter in ST cells: a putative repressor located at -1696/-707 bp and a putative activator isolated between the -707/-488 bp upstream of the translational start site.

Binding of the p65 and p52 subunits of NF-κB modulate basal activity of the porcine GnRHR II gene promoter in ST cells. Electrophoretic mobility shift assays using 30 bp radiolabeled oligonucleotides spanning the -707/-488 bp region of the porcine GnRHR II gene promoter revealed that nuclear proteins from ST cells bound to 3 specific regions, -605/-580, -590/-570 and -512/-489 bp relative to the translational start site. First, we examined the specific complex binding to the radiolabeled oligonucleotide spanning the -512/-489 bp region of the promoter (Figure 4.2). Sequence analysis of the -512/-489 bp region revealed a putative overlapping AP-2/NF-κB site located at -502/-492

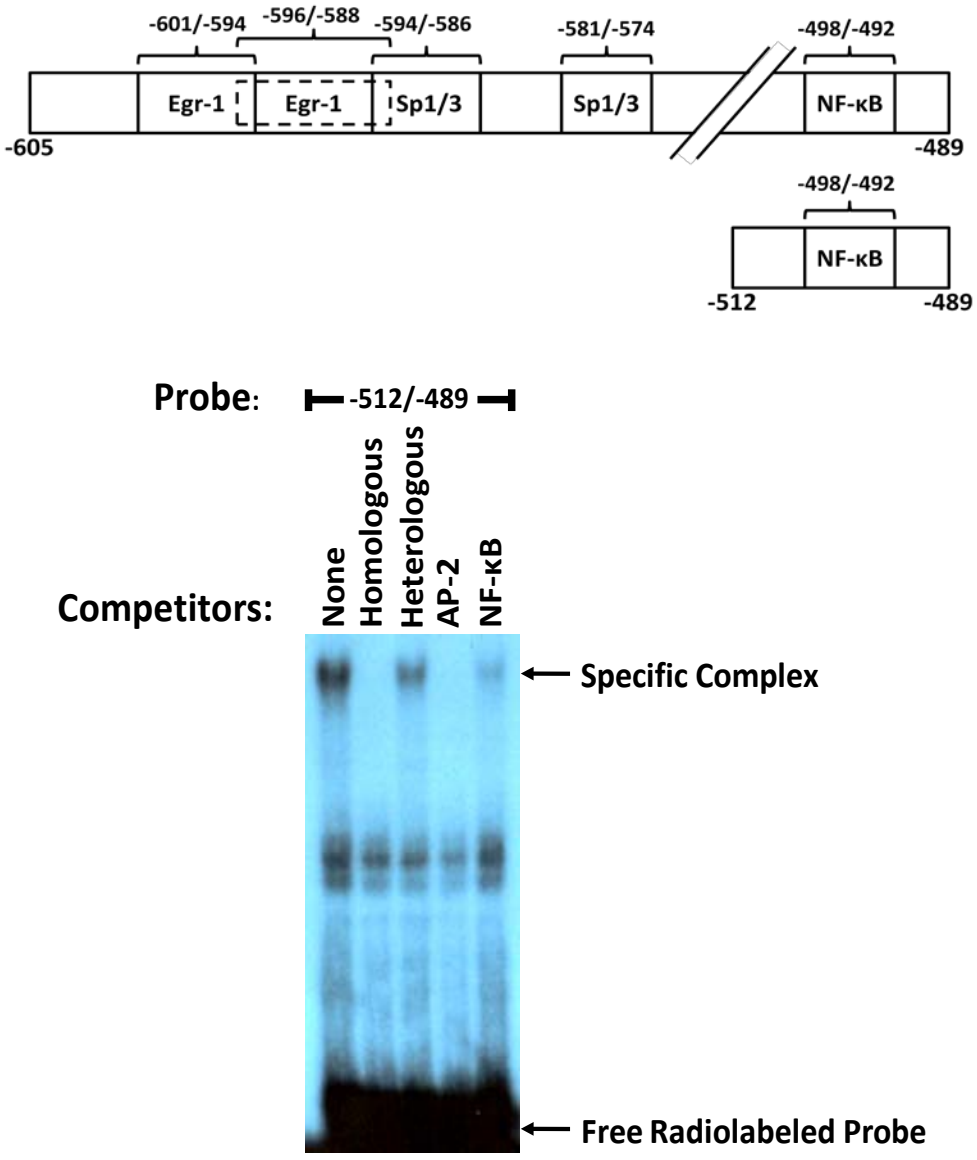

Figure 4.2. Electrophoretic mobility shift assays revealing putative AP-2 and NF-κB elements within the -512/-489 bp region of 5' flanking region for the porcine GnRHR II gene. EMSAs utilizing radiolabeled oligonucleotides representing the -512/-489 region of the porcine GnRHR II gene promoter and ST nuclear extracts revealed the formation of a specific binding complex. The DNA-protein complex was tested for specificity through competition with 50-fold molar excess of unlabeled homologous or heterologous DNA. Furthermore, addition of 50-fold molar excess of unlabeled oligonucleotides containing consensus binding sequences for either AP-2 or NF-κB abrogated the DNA-protein complex. Binding reactions and subsequent electrophoresis were performed as described in Materials and Methods.

bp of the porcine GnRHR II gene promoter. The utilization of oligonucleotides containing consensus sequences for AP-2 or NF-κB as unlabeled competitors in EMSAs resulted in ablation of the specific binding complex (Figure 4.2). Addition of antibodies directed against the p50, p52 and p65 subunits of NF-κB, as well as AP-2α (data not shown), demonstrated that the p65 and p52 subunits of NF-κB comprised the specific complex (Figure 4.3). To examine functional relevance of the NF-κB binding site, luciferase reporter vectors harboring a block replacement mutation of NF-κB was transiently transfected into ST cells. Block replacement mutation of the NF-κB site (μNF-κBpGL3) resulted in a 26% reduction in promoter activity ($P < 0.05$; Figure 4.4), confirming the contribution of the NF-κB motif to basal activation of the porcine GnRHR II gene promoter in ST cells.

A Sp1/3 consensus site located at -581/-574 bp contributes to basal activity of the porcine GnRHR II gene promoter in ST cells. Next, we evaluated the specific DNA-protein complexes identified in EMASs to be interacting with the radiolabeled oligonucleotide corresponding to -590/-570 bp of the porcine GnRHR II promoter (Figure 4.5). Sequence analysis of this region revealed a putative Sp1/3 element located at -581/-574 bp. Next, the addition of oligonucleotides containing consensus sites for the Sp family of transcription factors resulted in the ablation of both specific binding complexes (Figure 4.5). Inclusion of antibodies directed against Sp1 and 3, but not Sp2 or 4, in EMSAs with radiolabeled oligonucleotides spanning -590/-570 bp of the porcine GnRHR II promoter resulted in supershifts of the DNA-protein complexes (Figure 4.6),

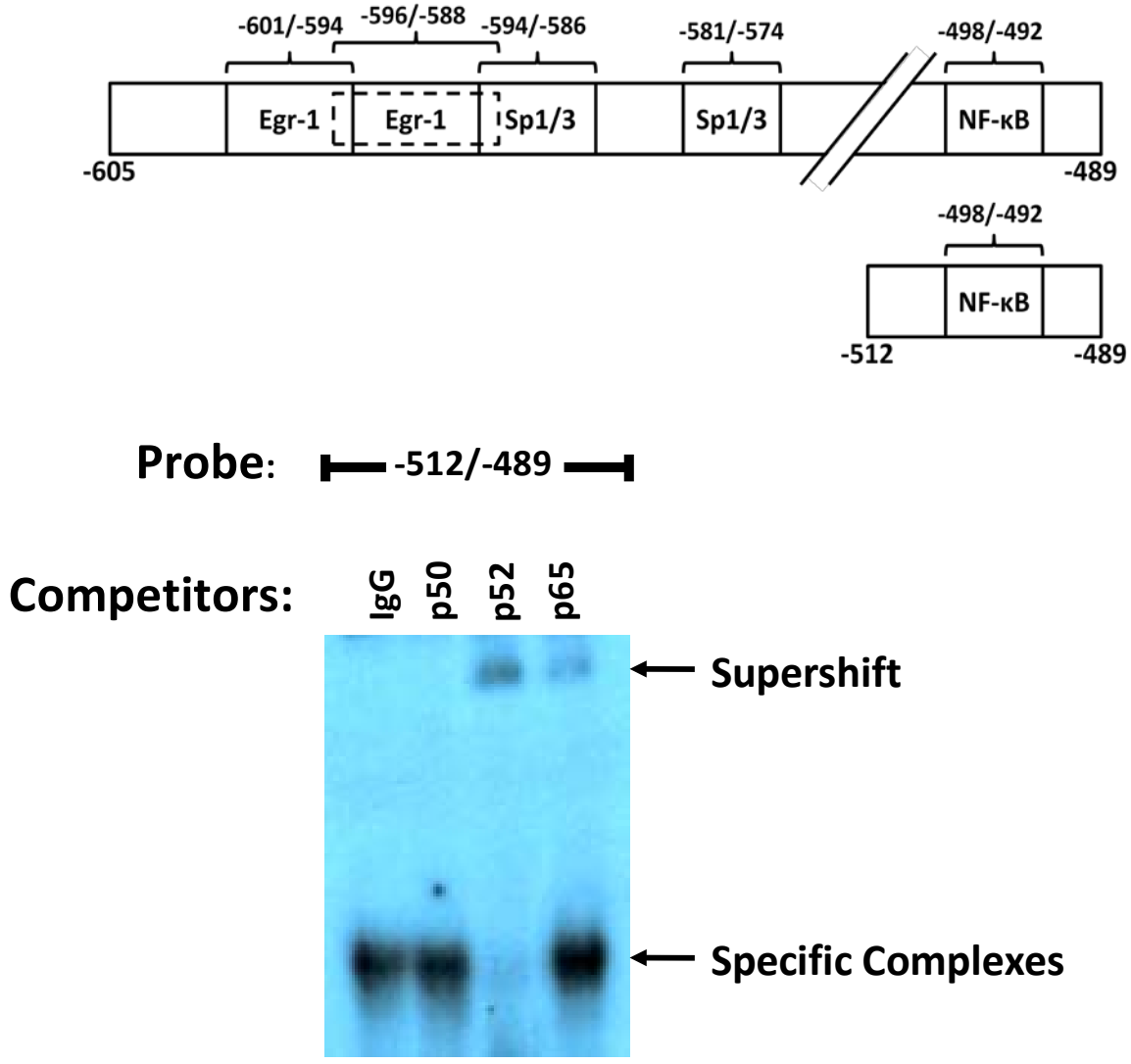

Figure 4.3. The p65 and p52 subunits of NF-κB interact with an element located at -498/-492 bp of 5' flanking sequence for the porcine GnRHR II gene. Nuclear extracts from ST cells were incubated with a radiolabeled oligonucleotide spanning the -512/-489 bp region of the porcine GnRHR II gene promoter. Antibodies directed against either the p50, p52, or p65 subunits of the NF-κB (1 μg) or an equal mass of rabbit IgG were added. Binding reactions and subsequent electrophoresis were performed as described in Materials and Methods.

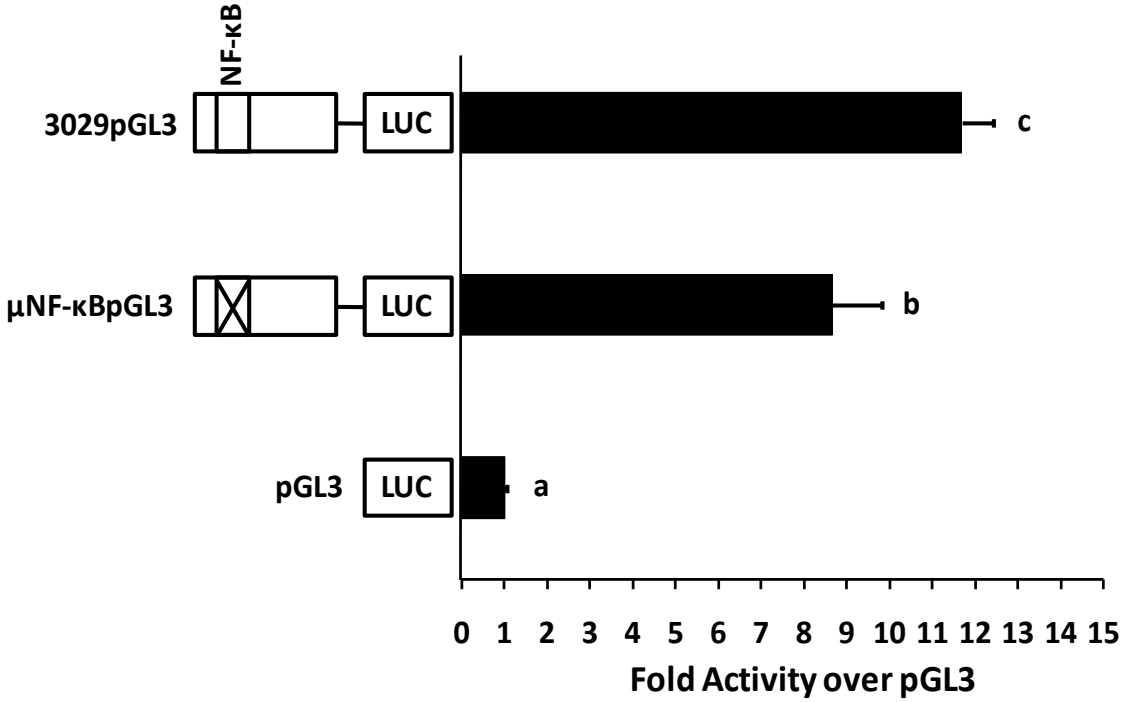

Figure 4.4 Functional significance of the NF-κB binding site within the porcine GnRHR II gene promoter in ST cells. Luciferase (LUC) vectors containing either the native, 3029 bp porcine GnRHR II gene promoter (3029pGL3), a block replacement mutation of the NF-κB binding site located at -498/492 (μNF-κBpGL3) or promoterless control (pGL3) were transiently transfected into ST cells. Cells were cotransfected with RSV-β-galactosidase (β-gal), and after 24 hours of transfection, cells were harvested and cell 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 activity over pGL3. Values represent the mean ± SEM of triplicate measurements from at least three replicates containing different plasmid preparations. Unique letters indicate means that are significantly different from one another ($P < 0.05$).

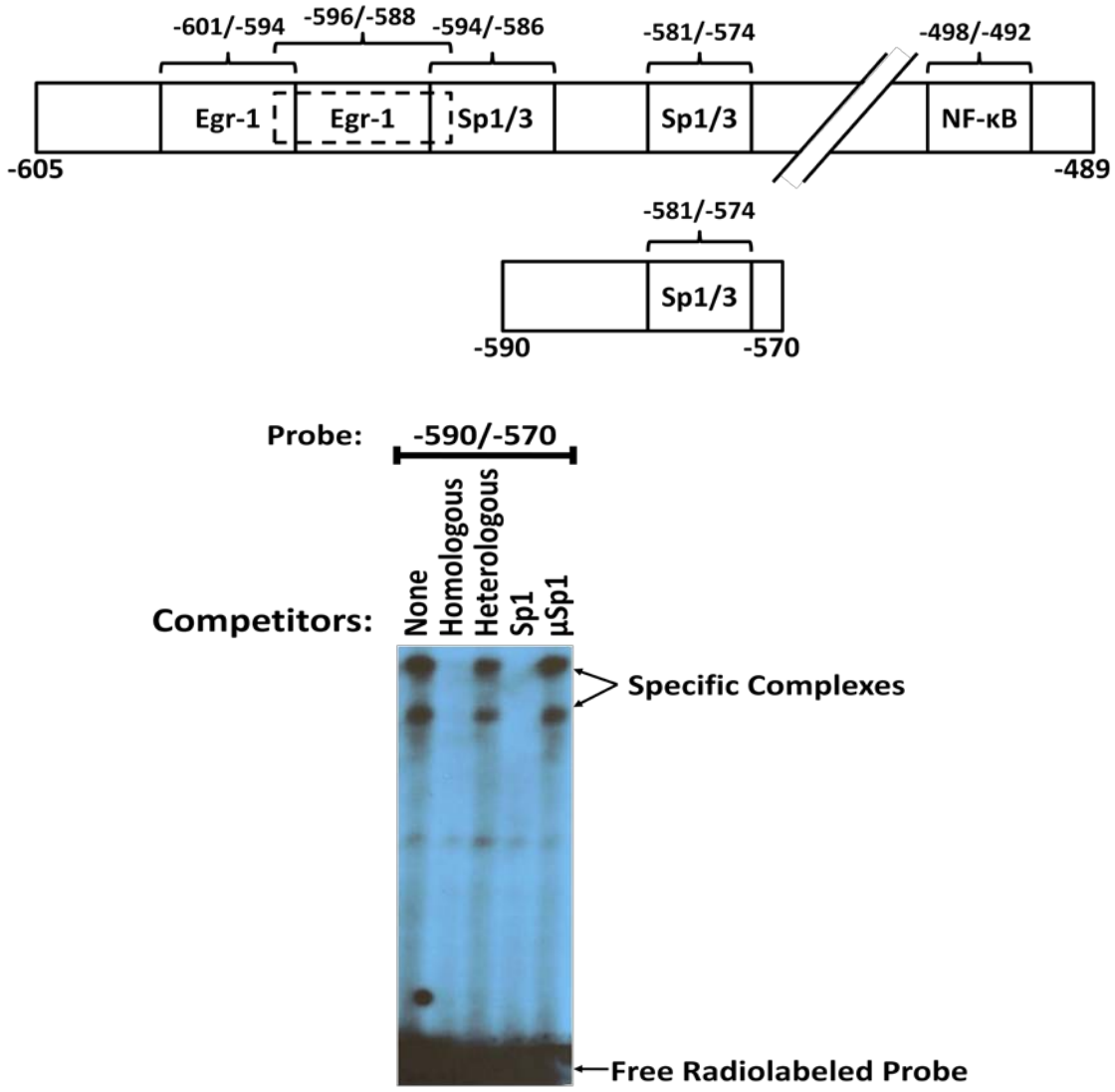

Figure 4.5. Electrophoretic mobility shift assay revealing a Sp1/3 element within the -590/-570 bp region of the porcine GnRHR II gene promoter. Incubation of ST cell nuclear extracts with radiolabeled oligonucleotides representing the -590/-570 bp region of 5' flanking region for the porcine GnRHR II gene revealed the formation of specific binding complexes. The DNA-protein complex was tested for specificity through competition with 50-fold molar excess of unlabeled homologous or heterologous DNA. Furthermore, addition of 50-fold molar excess of unlabeled oligonucleotides containing consensus binding sequences for Sp1 abrogated the specific binding complexes. Addition of an oligonucleotide containing a mutation of the Sp1/3 site within the -590/-570 oligonucleotide failed to ablate the specific DNA-protein complex. Binding reactions and subsequent electrophoresis were performed as described in Materials and Methods.

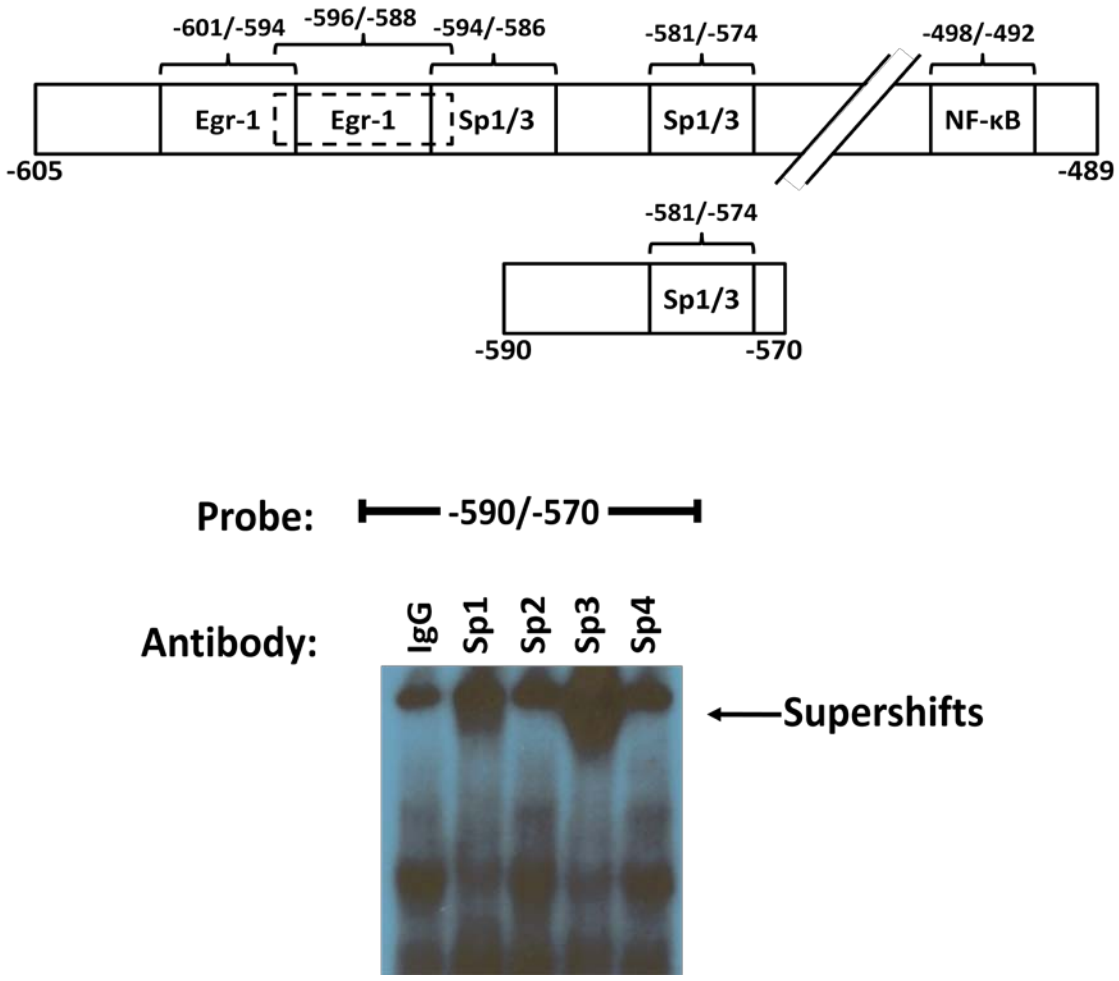

Figure 4.6. The Sp1/3 transcription factors are members of the specific complex binding to the oligonucleotide spanning the -590/-570 bp region of the porcine GnRHR II gene promoter. In EMSAs, a radiolabeled oligonucleotide representing the -590/-570 bp region of the porcine GnRHR II promoter was incubated with nuclear extracts from ST cells. Antibodies directed against either the Sp1, 2, 3 or 4 transcription factors (1 μg) or an equal mass of rabbit IgG were added. Binding reactions and subsequent electrophoresis were performed as described in Materials and Methods.

confirming Sp1 and 3 as components of the specific binding complexes. Next, the contribution of this Sp1/3 binding site to functional activity of the porcine GnRHR II gene promoter was determined. Transient transfections in ST cells with luciferase reporter vectors containing a block replacement mutation of the Sp1/3 site (μSp1/3pGL3) resulted in a 61% reduction of luciferase activity compared to the 3029 bp porcine GnRHR II promoter, demonstrating the importance of this site to basal activity in ST cells (Figure 4.7). While the Sp1/3 site is attributable in part for activation of basal GnRHR II gene expression in ST cells, luciferase activity of the μSp1/3pGL3 reporter vector was still greater than promoterless control ($P < 0.05$; Figure 4.7). This suggests that additional transcription factors are contributing to basal activity of the porcine GnRHR II gene promoter in ST cells.

Basal activity of the porcine GnRHR II promoter in ST cells is attributable in part to overlapping Egr-1 and Sp1/3 elements located at -596 to -586 bp. Finally, EMSAs performed by incubating ST nuclear extracts with the radiolabeled oligonucleotide spanning -605/-580 bp of proximal GnRHR II promoter revealed 4 specific binding complexes (Figure 4.8). Further, sequence analysis identified 2 putative Egr-1 (-601/-594 and -596/-588 bp) and 1 Sp1/3 (-594/-586 bp) consensus sites within this region. Ablation of 3 DNA-protein complexes occurred when unlabeled oligonucleotides containing consensus sites for either the Sp family of transcription factors or Egr-1 were added (Figure 4.8). Next, antibodies directed against Sp1, 2, 3, and 4 as well as Egr-1 were added. These EMSAs indicated that Sp1, Sp3 and Egr-1 each

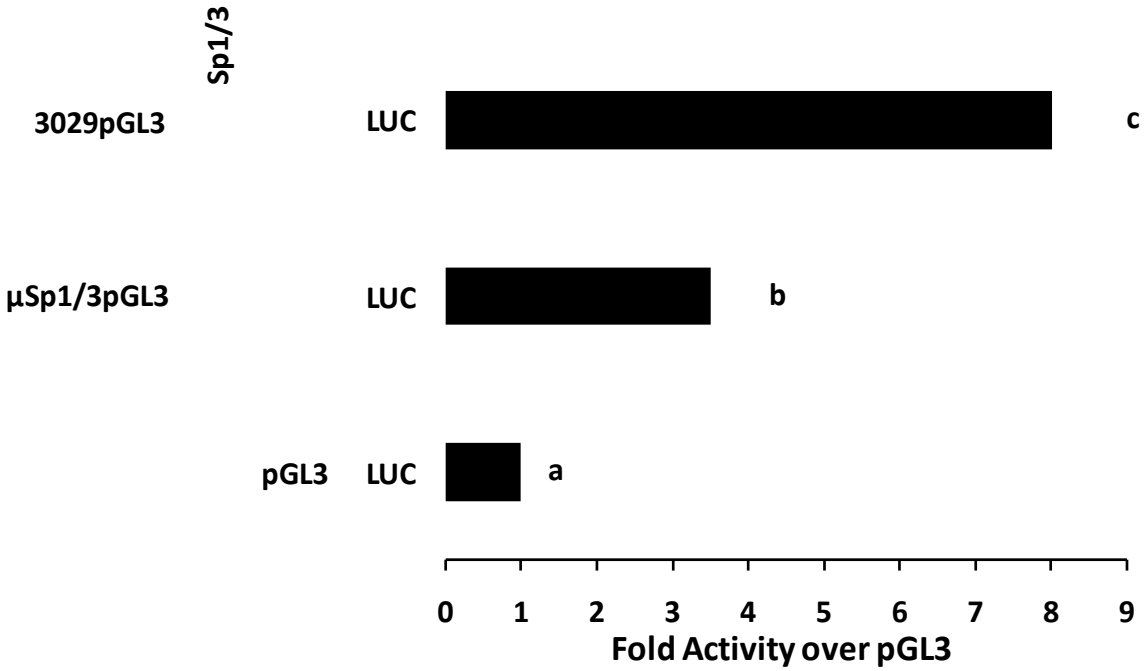

Figure 4.7. Specific contribution of the Sp1/3 element to basal activity of the porcine GnRHR II gene promoter in ST cells. Luciferase (LUC) reporter vectors containing either the native, 3029 bp porcine GnRHR II gene promoter (3029pGL3), a block replacement mutation of the Sp1/3 binding site located at -581/-574 bp within the 3029 bp promoter (μSp1/3pGL3), or promoterless control (pGL3) were transiently transfected into ST cells. Cells were cotransfected with RSV-β-galactosidase (β-gal), and after 24 h of transfection, cells were harvested and lysates 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 a fold activity over pGL3. Values represent the mean ± SEM of triplicate measurements from at least three replicates containing different plasmid preparations. Unique letters indicate means that are significantly different ($P < 0.05$).

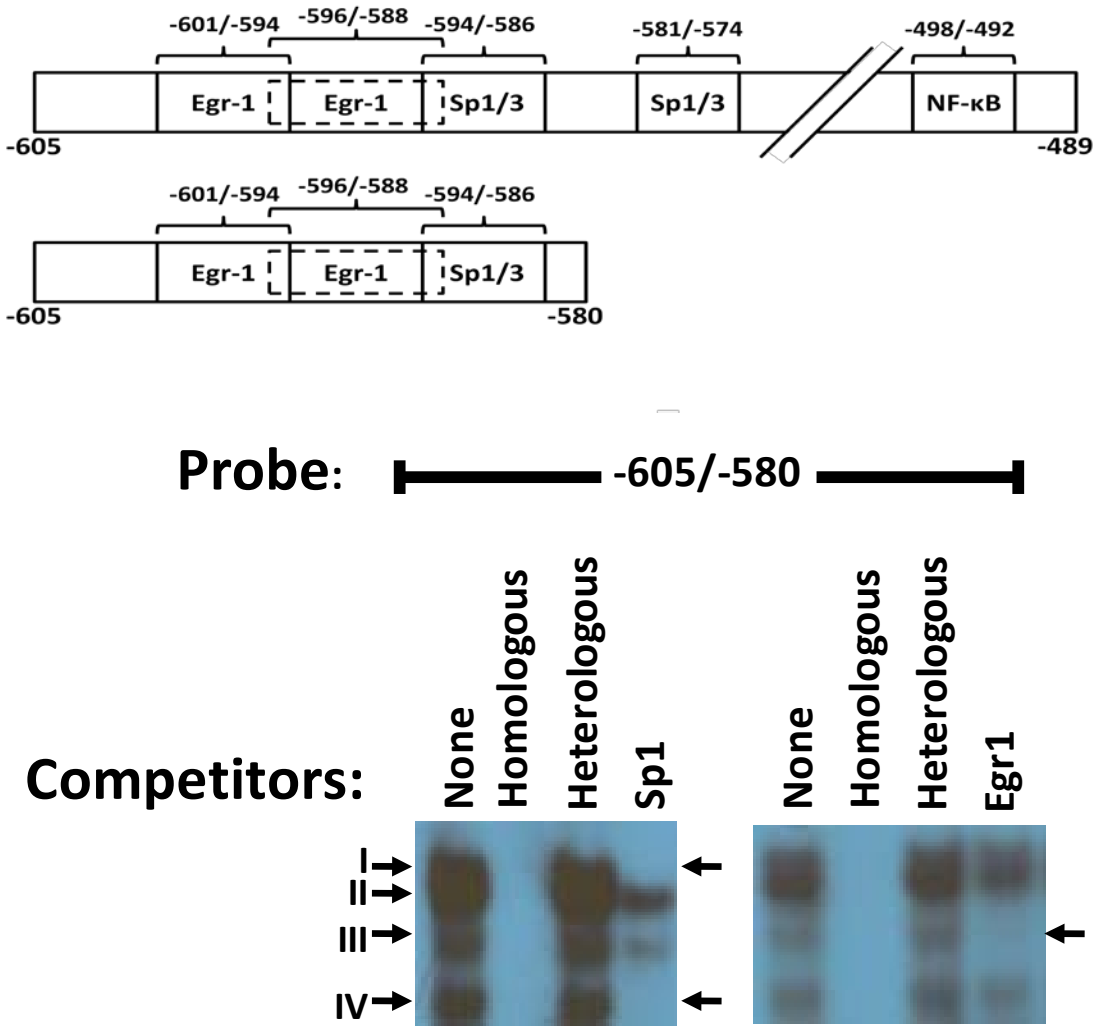

Figure 4.8. Electrophoretic mobility shift assays revealing overlapping Egr-1 and Sp1 elements within the -605/-580 bp region of the porcine GnRHR II gene promoter. Nuclear extracts from ST cells were incubated with radiolabeled oligonucleotides spanning -605/-580 bp of GnRHR II promoter. EMSAs revealed the formation of 4 specific DNA-protein binding complexes. Specificity of the specific binding complexes was determined through competition with 50-fold molar excess of unlabeled homologous or heterologous DNA. Also, addition of 50-fold molar excess of unlabeled oligonucleotides containing consensus binding sequences for either Egr-1 or the Sp family of transcription factors abrogated separate specific binding complexes as indicated by right-handed arrows. Binding reactions and subsequent electrophoresis were performed as described in Materials and Methods.

comprised separate specific binding complexes (Figure 4.9). A luciferase reporter vector containing a block replacement mutation of the overlapping Egr-1 and Sp1/3 binding elements (μEgr-1/Sp1/3pGL3) located at -596/-586 bp of proximal promoter was transiently transfected into ST cells to determine the specific contribution of this binding site to basal activity of the porcine GnRHR II gene promoter. The vector containing the block replacement mutation (μEgr-1/Sp1/3pGL3) reduced luciferase activity by 56% compared to the 3029 bp native porcine GnRHR II promoter ($P < 0.05$; Figure 4.10). Since the block replacement mutation of the overlapping Egr-1/Sp1/3 site did not reduce luciferase activity to levels of the promoterless control (Figure 4.10), additional binding sites are likely contributing to GnRHR II gene expression.

Interestingly, additional EMSAs suggested that Egr-1 may interact with either overlapping Egr-1 site, but only in the presence of a downstream Sp1/3 site. Utilizing a radiolabeled oligonucleotide containing the sequence from -605 to -591 bp of the porcine GnRHR II promoter that encompasses only 1 Egr-1 site (-601/-594 bp) and ST cell nuclear extracts, EMSAs revealed no specific binding complexes (Figure 4.11). However, EMSAs performed with a radiolabeled oligonucleotide containing a mutation of this Egr-1 site (-601/-594 bp) within the -605/-580 bp region (containing both putative Egr-1 elements) resulted in the presence of only 2 specific DNA-protein complexes (Figure 4.12) compared to the 4 specific complexes formed when the -605/-580 bp oligonucleotide was incubated with ST nuclear extracts (Figure 4.8). In addition, EMSAs utilizing an unlabeled oligonucleotide containing consensus sequences for the Sp family of transcription factors resulted in ablation of the 2 specific DNA-complexes, indicating

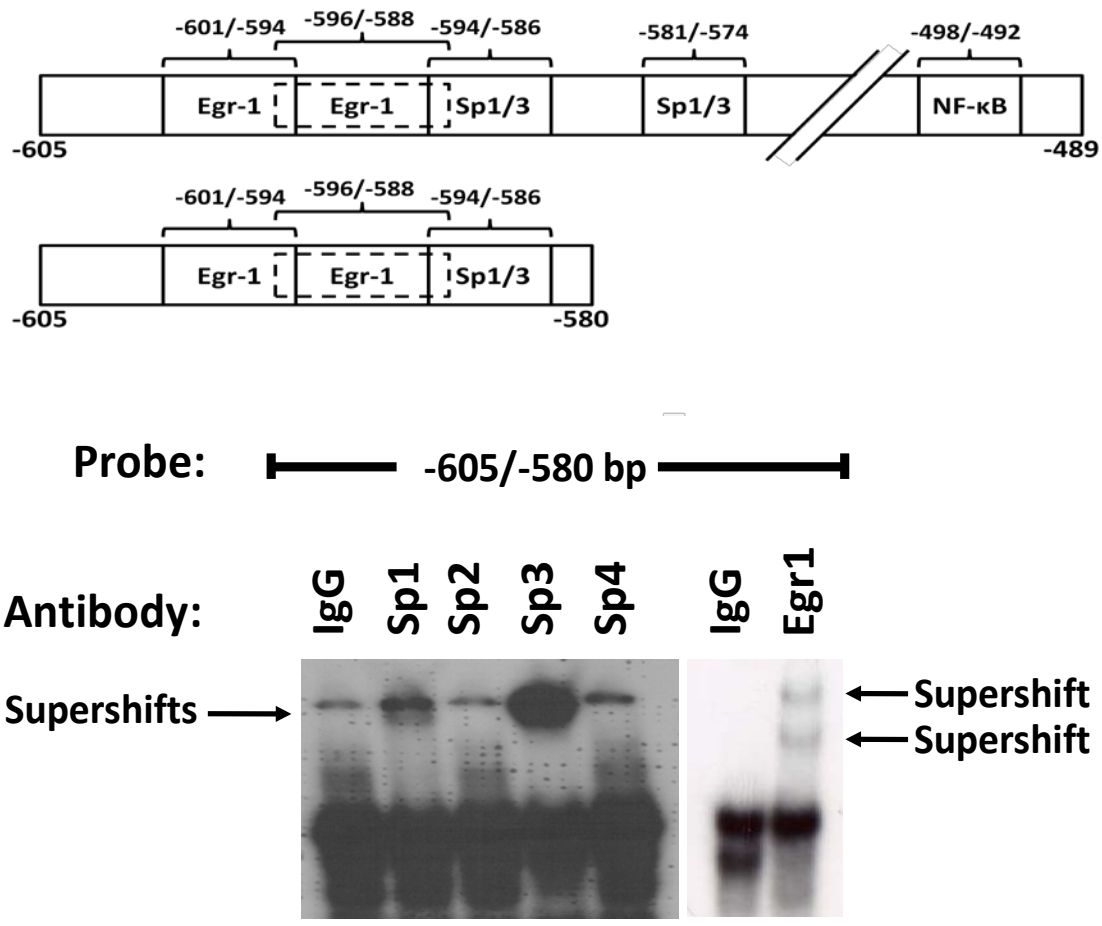

Figure 4.9. The Egr-1, Sp1 and Sp3 transcription factors comprise specific DNA-protein complexes binding to the oligonucleotide spanning the -605/-580 bp region of the porcine GnRHR II gene promoter. Nuclear extracts from ST cells were incubated with a radiolabeled oligonucleotide representing the -605/-580 bp region of the porcine GnRHR II gene promoter. Antibodies directed against either Sp1, 2, 3 and 4 as well as Egr-1 transcription factors (1 μg) or an equal mass of rabbit IgG were added. Binding reactions and subsequent electrophoresis were performed as described in Materials and Methods.

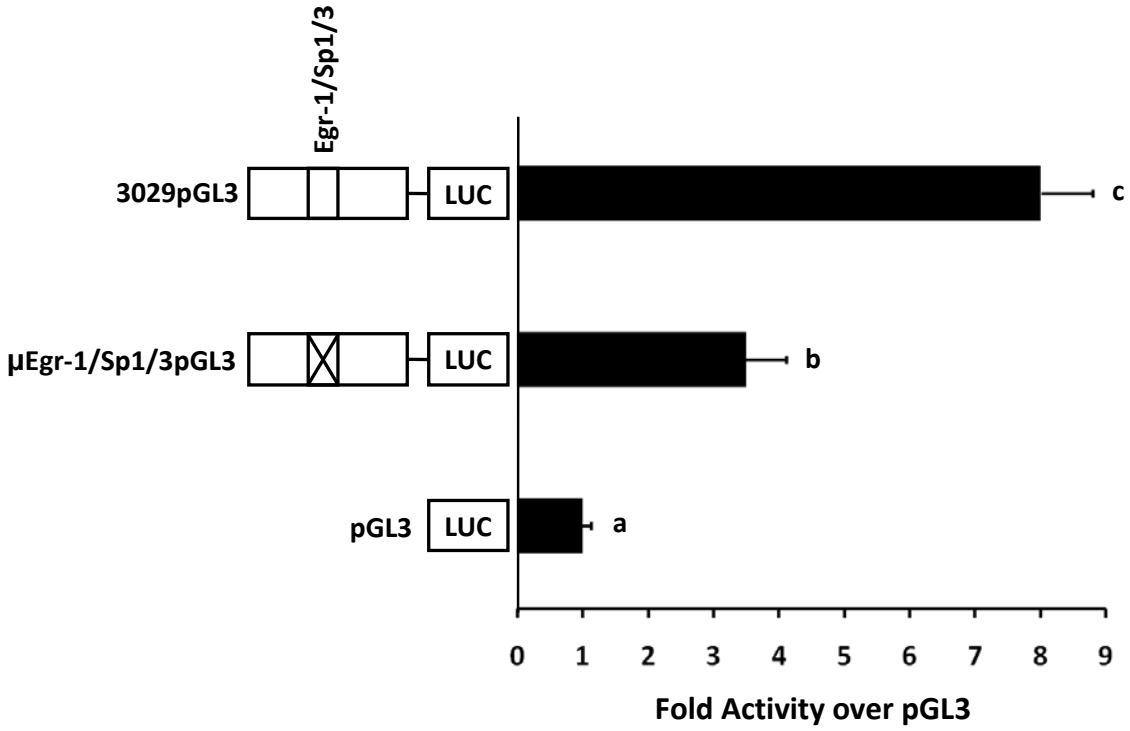

Figure 4.10. Specific contribution of overlapping Egr-1/Sp1/3 binding site to basal activity of the porcine GnRHR II gene promoter in ST cells. Luciferase (LUC) vectors containing either the native, 3029 bp porcine GnRHR II gene promoter (3029pGL3), a block replacement mutation of the overlapping Egr-1/Sp1/3 binding site located at -595/-586 bp within the 3029 bp promoter (μEgr-1/Sp1/3pGL3) or promoterless control (pGL3) were transiently transfected into ST cells. Cells were cotransfected with RSV-β-galactosidase (β-gal) and after 24 h of transfection, cells were harvested and lysates 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 activity over pGL3. Values represent the mean ± SEM of triplicate measurements from at least three replicates containing different plasmid preparations. Unique letters indicate means that are significantly different ($P < 0.05$).

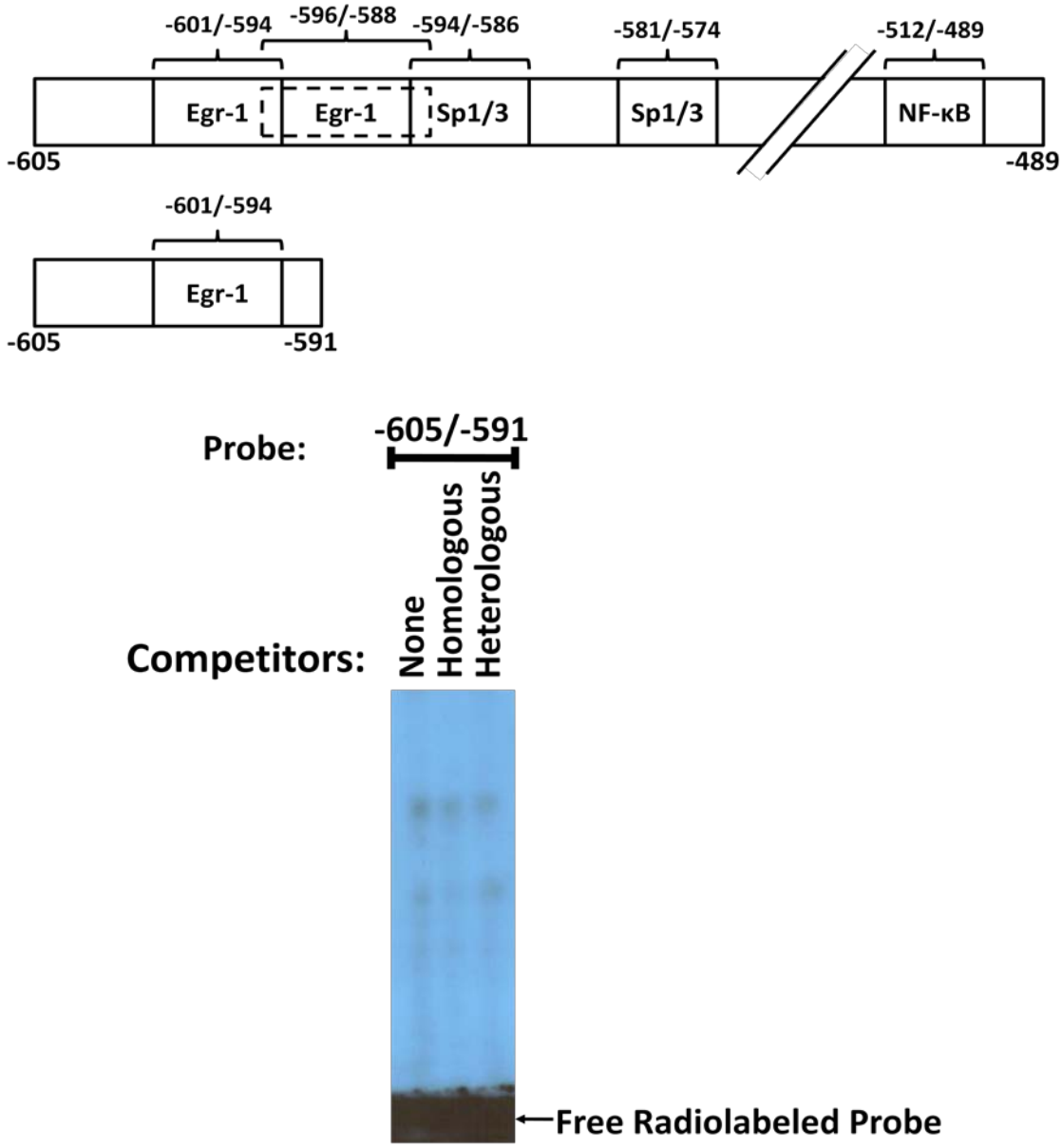

Figure 4.11. Electrophoretic mobility shift assay utilizing an oligonucleotide containing only one Egr-1 site (-601/-594 bp) indicated that Egr-1 may rely on interactions with downstream Sp1/3 elements for DNA binding. Radiolabeled oligonucleotides representing the -605/-591 bp region of the porcine GnRHR II gene promoter failed to form specific DNA-protein complexes with nuclear extracts from ST cells. The DNA-protein complex was tested for specificity through competition with 50-fold molar excess of unlabeled homologous and heterologous DNA. Binding reactions and subsequent electrophoresis were performed as described in Materials and Methods.

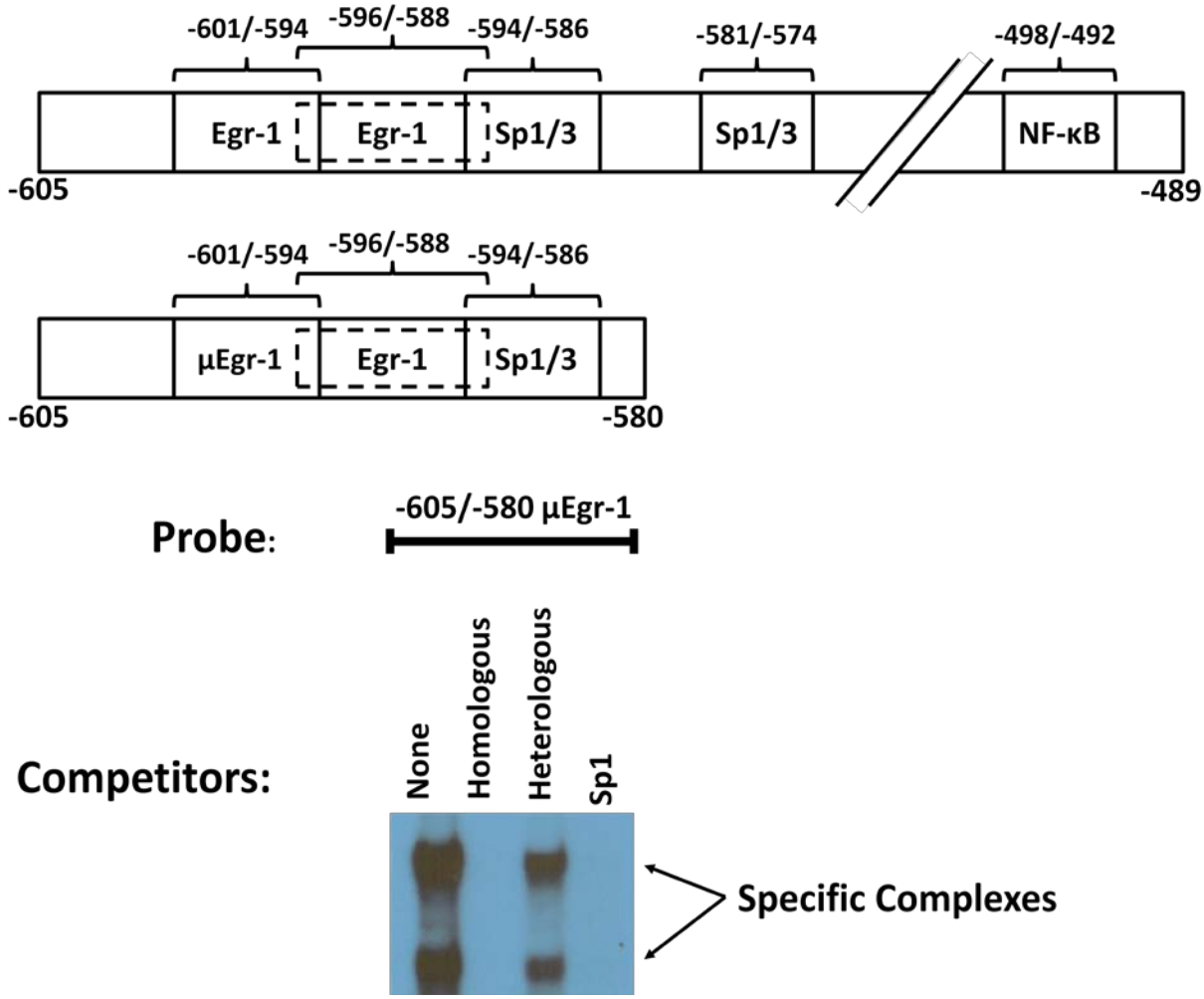

Figure 4.12. Mutation of the Egr-1 site located at -606/-594 bp indicated Egr-1 may interact with Sp1/3 to bind to the -601/-594 bp region of the porcine GnRHR II gene promoter. Radiolabeled oligonucleotides (-605/-580μEgr-1) containing a mutated Egr-1 site (-606/-594 bp) within the -605/-580 bp of the porcine GnRHR II gene promoter revealed specific DNA-protein complexes. The specific binding complexes were tested for specificity through competition with 50-fold molar excess of unlabeled homologous or heterologous DNA. Furthermore, addition of 50-fold molar excess of unlabeled oligonucleotides containing consensus binding sequences for Sp1 abrogated some of the DNA-protein complexes. Binding reactions and subsequent electrophoresis were performed as described in Materials and Methods.

the importance of the Egr-1 site located at -601/-594 bp of the porcine GnRHR II promoter (Figure 4.12). Furthermore, EMSAs utilizing a radiolabeled oligonucleotide containing proximal promoter sequence for both the overlapping Egr-1/Sp1/3 motif (-596/-588 bp) and downstream Sp1/3 binding site (-581/-574 bp) resulted in formation of 4 specific complexes (Figure 4.13). These binding complexes corresponded to the 4 specific DNA-protein complexes formed in EMSAs using the -605/-570 oligonucleotide (Figure 4.8). Likewise, addition of unlabeled oligonucleotides containing consensus binding sequences for either Egr-1 or the Sp family of transcription factors abrogated the specific binding complexes (Figure 4.13). We hypothesize that the interaction of Egr-1 at the overlapping Egr-1/Sp1/3 can occur within this oligonucleotide because it contains the downstream Sp1/3 binding element. Thus, our data suggests that Egr-1 may bind to either overlapping Egr-1 element if a downstream Sp1/3 binding site is present indicating a potential interaction between the Egr-1 and Sp1/3 transcription factors.

A high degree of conservation exists within a 108 bp region of 5' flanking sequence for the GnRHR II gene between the marmoset monkey and pig. Utilizing the Basic Local Alignment Search Tool (BLAST; NCBI, U.S.A.), the -766/-658 bp region of the marmoset GnRHR II gene promoter (GenBank No. AY676461.1), previously shown to contain elements contributing to enhanced promoter activity (Faurholm et al., 2007), has approximately 90% identity to the -670/-652 bp region of the porcine GnRHR II gene promoter (GenBank No. FJ872917.1; Figure 4.14). Comparison of elements within the specified regions of proximal promoter indicated conserved

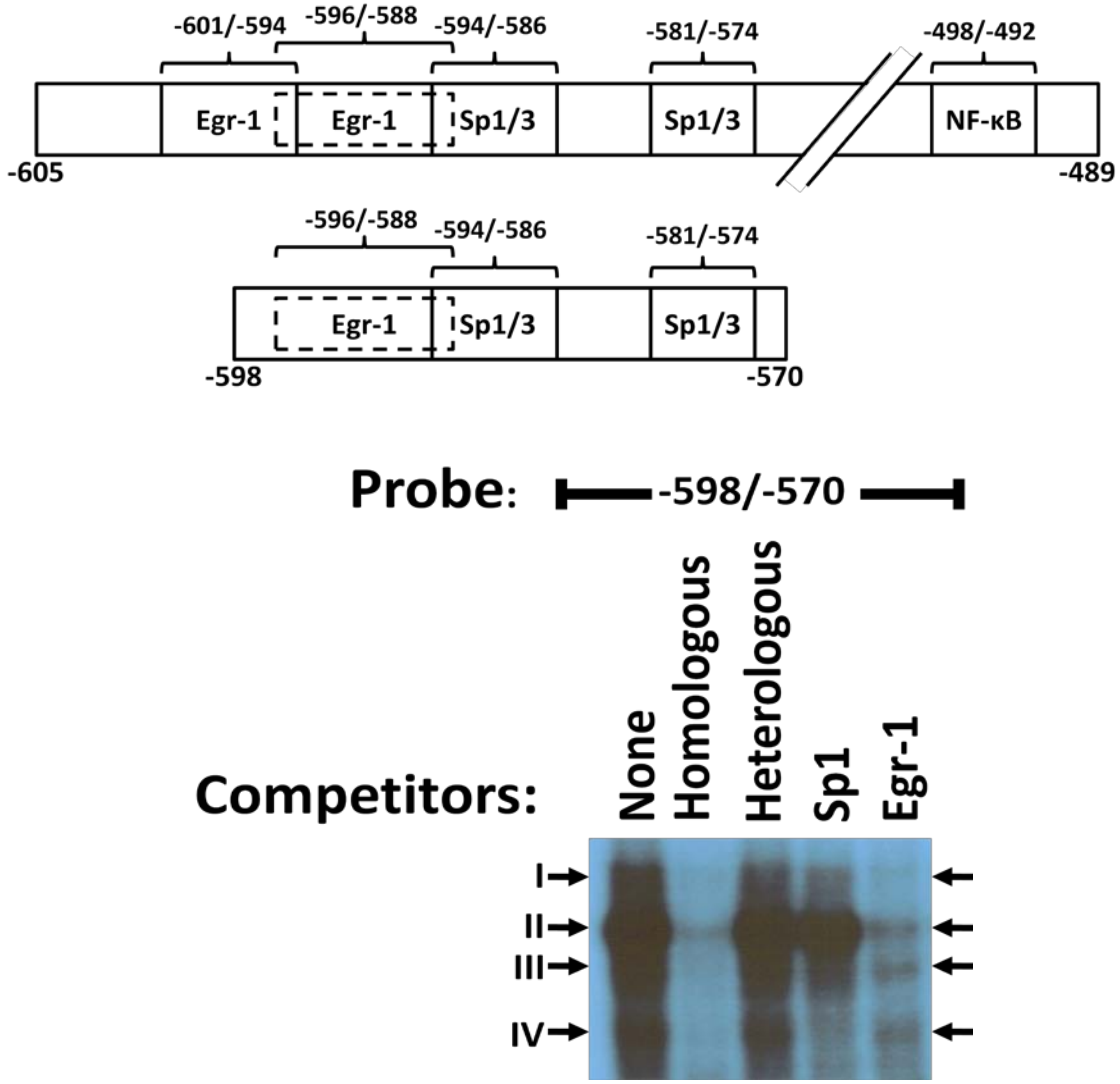

Figure 4.13. Electrophoretic mobility shift assay indicating that the overlapping Egr-1/Sp1/3 motif located at -596/-588 bp may interact with the downstream Sp1/3 binding site. Incubation of ST cell nuclear extracts with radiolabeled oligonucleotides containing the sequence encoding the -598/-570 bp region of the porcine GnRHR II gene promoter unveiled the formation of specific DNA-protein complexes. The DNA-protein complex was tested for specificity through competition with 50-fold molar excess of unlabeled homologous and heterologous DNA. Furthermore, the addition of 50-fold molar excess unlabeled oligonucleotides containing the consensus binding sequence for Sp1 or Egr-1 challenged the DNA-protein complex by competition for binding with ST cell nuclear extracts. Binding reactions and electrophoresis were performed as described in Materials and Methods.

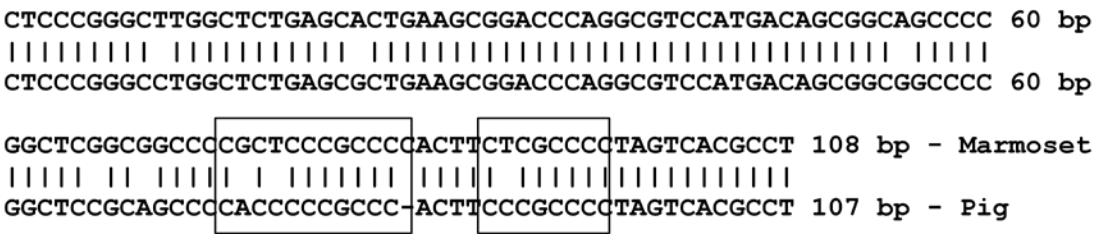

Figure 4.14. Alignment of specific 5' flanking regions of the marmoset (-766/-658 bp) and swine (-670/-562 bp) GnRHR II genes. The -766/-658 bp region of the marmoset gene promoter, indicated previously to contain elements that enhance promoter activity (Faurholm et al., 2007), has approximately 90% identity to the -670/-562 bp region of the porcine GnRHR II gene promoter. Boxes surround conserved putative elements for Egr-1 and Sp1/3 within the marmoset GnRHR II promoter corresponding to functional binding sites within the porcine GnRHR II promoter.

elements for Egr-1 and Sp1/3 corresponding to functional Egr-1 and Sp1/3 binding sites isolated within the porcine promoter in our study (Figure 4.14). Thus, our data provides critical evidence that these elements regulate GnRHR II gene expression across species.

Discussion

To date, no transcription factors have been reported to confer functional expression of GnRHR II gene in mammals. Herein, we have discovered that NF-κB, Sp1/3 and Egr-1/Sp1/3 binding sites contribute to activation of the GnRHR II promoter, represented by our working model (Figure 4.15). To evaluate the functionality of the porcine GnRHR II gene promoter within the context of reproduction, we developed an in vitro cell culture system utilizing the ST cell line. It is notable that we discovered a region that appeared to contain a repressor element as deletion of the region between -1696/-788 bp resulted in a dramatic increase in luciferase activity. Of primary importance, enhanced promoter activity was achieved when the region between -707/-488 bp was removed, indicating that the -707/-488 bp region contains key elements contributing to promoter activation. Upon examination of the -707/-488 bp region, we identified NF-κB (-498/-492 bp), Sp1/3 (-581/-574 bp) and overlapping Egr-1/Sp1/3 (-596/-580 bp) elements contributed to promoter activation.

From bony fish to man, the amino acid sequence for GnRH II is highly conserved (Neill et al., 2001). This information coupled with ubiquitous tissue expression of genes for both the GnRH II ligand and receptor implies that the GnRH II/GnRHR II system likely has an important biological role. Consistent with this theory, the marmoset GnRHR

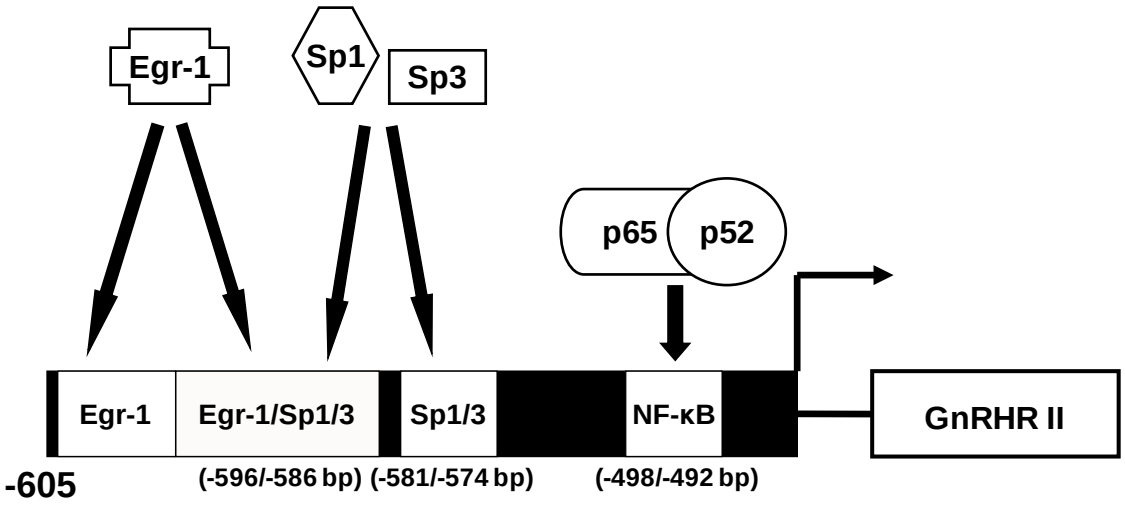

Figure 4.15. Working model for activation of the porcine GnRHR II promoter. A heterodimer of NF-κB activates the promoter at -498/-492 bp. Either Sp1 or Sp3 may bind to a Sp1/3 site located at -581/-574 bp of the promoter also conferring expression of the porcine GnRHR II gene. An overlapping Egr-1/Sp1/3 site (-596/-586 bp) is partially attributable for activation of the porcine GnRHR II promoter. Also, an upstream overlapping Egr-1 element may also be involved in promoter activation.

II promoter was active in all reproductive and non-reproductive cell lines tested and contains a conserved region (-766/-658 bp) demonstrated to enhance promoter activity (Faurholm et al., 2007). Comparative sequence analysis of this significant region between the marmoset (-766/-658 bp; Faurholm et al., 2007) and porcine (-670/-562 bp) GnRHR II promoters indicated approximately 90% homology. In addition, putative Egr-1 and the Sp family of transcription factors elements are conserved between the porcine and marmoset promoters within this region. Therefore, this region likely contains elements involved in regulating the expression of GnRHR II in reproductive tissues and tumors.

Consistent with some homology (40%) between the amino acid sequence of GnRHR I and II, a degree of similarity exists between promoters for GnRHR I and II in the pig. Like the porcine GnRHR I promoter, we discovered a NF-κB binding site in the porcine GnRHR II gene promoter. Specifically, previous studies reported that a p65/p52 heterodimer of NF-κB binds to the -1689/-1684 bp region of 5' flanking sequence for the porcine GnRHR I gene, contributing to gonadotrope-specific promoter activation (Smith et al., unpublished data). Likewise in this study, we report a p65/p52 heterodimer of NF-κB associates with an element at -498/-492 bp of the GnRHR II promoter to activate gene expression in the ST cell line. In contrast, the -1760/-1667 bp of proximal promoter, termed swine upstream promoter enhancing region (SUPER), is critical to activity of the porcine GnRHR I promoter in gonadotrope-derived αT3-1 cells (Cederberg et al., unpublished data), whereas our data from the current study indicated a region closer to the translational start site (-707/-488 bp) is the most significant contributor to activation of the porcine GnRHR II promoter in ST cells. While the 5' flanking sequence for the

porcine GnRHR II gene contains functional overlapping Egr-1/Sp1/3 or Sp1/3 elements, the porcine GnRHR I promoter utilizes 3 SF-1 binding sites for functional activity in αT3-1 cells (McDonald et al., unpublished data; Smith et al., unpublished data).

Identification of the NF-κB binding site is the first description of a functional element within the GnRHR II gene promoter. Although NF-κB usually exists as a heterodimer comprised of p65 (RelA), p50/p105, p52/p100, RelB and/or c-rel, it can also form homodimers of p50, p52 or p65 (Ganchi et al., 1993; Hayden and Ghosh, 2004). Inactive NF-κB subunits are located in the cytoplasm, inhibited by attachment to IκB proteins. Upon phosphorylation and subsequent degradation of the IκB proteins, the bond between IκB and the NF-κB proteins is ablated, rendering the NF-κB subunits active. Activation of the NF-κB subunits, p50 and p52, require an additional step. Phosphorylated IκBs are polyubiquinated, resulting in, not only their own degradation, but processing of the precursor p105 and p100 subunits to p50 and p52, respectively (Karin and Ben-Neriah, 2000). Following transcriptional activation, NF-κB subunits translocate to the nucleus and subsequently activate or inhibit transcription of other genes. Taken together, these data illustrate the potential mechanisms employed by the NF-κB subunits to activate GnRHR II gene expression in ST cells.

From EMSA experiments in our study, we determined that the p65 and p52 subunits of NF-κB were binding to the site located at -498/-492 bp of the porcine GnRHR II gene promoter. While heterodimers of NF-κB subunits may interact with other transcription factors such as AP-1, Sp1 and C/EBP to activate gene expression (Stein and Baldwin, 1993; Oeth et al., 1997), p50/p50 and p52/p52 homodimers, lacking identifiable

transactivation domains, rarely activate transcription. Instead, the p50 and p52 subunits typically form a heterodimer with p65 or RelB that contain transactivation domains (Hayden and Ghosh, 2004). However, it has been reported that p52/p52 homodimers can transactivate with the unique IκB protein, Bcl-3 (Bours et al., 1993). Although p65/p50 heterodimers are most common, heterodimers of p65 and p52 NF-κB subunits can also activate gene expression. The NF-κB transcription factors are typically known for their role in regulating genes involved in the immune response (Hayden et al., 2006). For example, the p65/p52 heterodimer of NF-κB activates human pro-interleukin-1β gene expression (Goto et al., 1999). Similarly, we discovered that a p65/p52 heterodimer of NF-κB contributes to basal activation of the porcine GnRHR II gene promoter in ST cells.

In addition to NF-κB, identification of a Sp1/3 binding site contributing to basal activity of the porcine GnRHR II gene promoter in ST cells is novel. The Sp family of transcription factors (Sp1-9) contain a highly conserved DNA binding domain comprised of three $Cysteine_2$-$Histidine_2$ zinc fingers. While Sp1 has a wide variety of binding sites comprised of the 5'-(G/T)GGGCGG (G/A)(G/A)(C/T)-3' (GC box element), it also shares binding sites with Sp3 (Li et al., 2004). Within the 5' flanking sequence for the porcine GnRHR II gene, functional Sp1/3 sites containing the 5'-CCGCCCC-3' consensus sequence are located at -581/-574 and -592/-586 bp. Consistent with similar studies (Zhao and Ennion, 2006; Kumar and Butler, 1997), we found that both Sp1 and 3 transcription factors can bind to this consensus site. While Sp1 has been widely demonstrated to activate gene expression (Li et al., 2004), studies have suggested that

Sp3 functions as either an activator (Liang et al., 1996) or repressor (Suttamanatwong et al., 2009). Whether Sp3 acts as an activator or repressor may depend on the presence of single or multiple Sp3 binding sites within the proximal promoter, respectively (Dennig et al., 1996). The Sp1/3 site may function as a single element contributing to basal promoter activation (Kumar et al., 1997) or as a coactivator required for other transcription factors to bind to the promoter (Majello et al., 1994, 1995). As Sp1 contains a transactivation domain, it can form protein complexes with other transcription factors prior to DNA binding the promoter.

An overlapping Egr-1/Sp1/3 site is located 4 bp upstream of the Sp1/3 site. The Egr-1/Sp1 motif is commonly found in promoters such as those for acetylcholinesterase (Mutero et al., 1995), colony-stimulating factor-1 (Harrington et al., 1993), murine thrombospondin-1 (Shingu and Bornstein, 1994), transforming growth factor-β1 (Kim et al., 1989) and tumor necrosis factor (Kramer et al., 1994) genes, as well as the Egr-1 gene promoter itself (Cao et al., 1993). Previous reports suggested that Egr-1 and Sp1 compete for binding at Egr-1/Sp1 motifs and that Egr-1 can function as the repressor by inhibiting Sp1 from binding to the promoter (Gannon and Kinsella, 2008). In contrast, Egr-1 can compete with Sp1 to activate the platelet-derived growth factor-A (*PDGF-A*) and -B (*PDGF-B*) gene promoters (Khachigian et al., 1996). Although our EMSA experiments demonstrated that Egr-1, Sp1 and 3 can bind to the site located at -596/-586 bp, it is likely that only 1 transcription factor can bind to these recognition sequences at a time, as no Egr-1/Sp1 or Egr-1/Sp3 hetero-oligomer has been described to date. Instead, the close proximity of the Sp1/3 element to the upstream overlapping Egr-1/Sp1/3 motif suggests

transactivation by combinations of either Sp1 and Egr-1 or Sp1 and Sp1 binding to these 2 elements. Reports have indicated that the central cysteine (C) on the DNA sequence of Sp1 sites (GGGCGGG) must be at located at least 10 bp apart for 2 adjacent Sp1 proteins to bind (Gidoni et al., 1995). Consistent with this, the central cysteine within the Sp1 sites located at -581/-574 and -594/-586 bp of the porcine GnRHR II gene promoter are separated by 10 bp. The close proximity of the Sp1/3 and overlapping Egr-1/Sp1/3 elements is presumably a mechanism for divergent regulation of the porcine GnRHR II gene promoter in different cellular environments. For instance, previous studies have shown that estrogen can alter the phosphorylation of Egr-1 by an inducible casein kinase II, resulting in decreased affinity of Egr-1 to bind with Sp1 and subsequent stimulation of macrophage colony-stimulating factor (M-CSF) gene expression (Srivastava et al., 1998). Therefore, post-transcriptional regulation of the transcription factors, Egr-1, Sp1 and Sp3, may alter the preference of these proteins to bind to the Egr-1/Sp1/3 motif and modulate GnRHR II gene expression.

Consistent with ubiquitous expression patterns, Egr-1 and Sp1 are involved in many cellular functions including apoptosis, cell growth, development and stress response. It is important to note that Sp1 and Egr-1 have been implicated previously in the regulation of genes involved in reproduction and associated with cancer cell growth, as this is the proposed role of GnRHR II. First, the association of Egr-1 and Sp1 in reproduction is demonstrated by the presence of a tripartite GnRH response element in the rat LH-β gene promoter containing Egr-1, Sp1 and steroidogenic factor-1 (SF-1) binding sites (Kaiser et al., 2000). It appears that the presence or absence of SF-1

facilitates the association of either Egr-1 or a binding complex consisting of both Sp1 and Egr-1 to the gene promoter (Kaiser et al., 2000). Furthermore, Egr-1 mediates the effects of insulin at the GnRH promoter (Divall et al., 2007) whereas Egr-1, Sp1 and 3 regulate gene expression of the Dmrt1 gene in Sertoli cells (Lei and Heckert, 2002). In addition to involvement in reproduction, the Egr-1 and Sp1 transcription factors also regulate gene expression associated with cancer. For instance, Egr-1 is overexpressed in prostate cancer cells (Ogishima et al., 2005). On the other hand, the Egr-1 gene itself has been implicated as a cancer suppressor gene since exogenous Egr-1 expression results in reduced cell growth and tumorigenicity (Liu et al., 1996). Likewise, Sp1 has been associated with tumor metastasis, acting as a regulator of the matrix metalloproteinases (MMPs; Qin et al., 1999). Together with Egr-1 and Sp1 elements, a NF-κB binding site regulates the tissue factor pathway inhibitor-2 (TFPI-2) gene, associated with cell migration and tumor invasion, in human choriocarcinoma cells (JEG-3; Hubé et al., 2003).

In summary, activity of the porcine GnRHR II promoter in ST cells is largely conferred by NF-κB (-498/-492), Sp1/3 (-581/-574) and overlapping Egr-1/Sp1/3 (-596/-586) elements. A heterodimer of the p65/p52 subunits interacts at the NF-κB element. The Egr-1/Sp1/Sp3 motif and Sp1/3 element contribute to expression of the porcine GnRHR II gene, presumably through exchange of Egr-1, Sp1 and Sp3 in response to different cellular environments. To our knowledge, this represents the first description of functional transcription factor binding sites contributing to basal activity of the GnRHR II gene promoter. In addition, our data suggests that the -1696/-788 bp region contains a putative repressor(s) of gene expression, which will be further explored. Thus, the

elucidation of transcription factors and binding elements contributing to basal expression of the porcine GnRHR II gene promoter will not only enhance reproductive efficiency in swine, but also unveil new mechanisms to discover the importance of the novel GnRH II receptor system in humans.

Implications

The GnRH II ligand-receptor system may potentially benefit livestock production as well as provide a therapy for human reproductive problems and cancer. First, GnRH II may act through GnRHR II to increase mating behavior in females with a lower energy status. As a result, swine producers could administer post-weaning sows GnRH II to improve pregnancy rates first service following weaning when sows are in a negative energy balance. Further, increase of steroidogenic enzymes and intra-testicular testosterone upon GnRH II treatmen, suggests that manipulation of this system may increase testosterone production in males, thereby improving reproductive performance. Lastly, mechanisms underlying GnRH II binding to its receptor have been targeted as a potential therapy for endometrial, ovarian and prostate cancers with the potential to save human lives.

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