NOTE TO USERS

DISSERTATION

MOLECULAR MECHANISMS UNDERLYING ACTIVIN REGULATION OF THE GONADOTROPIN RELEASING HORMONE RECEPTOR GENE

Submitted by

Brian D. Cherrington

Biomedical Sciences Program

In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Colorado State University

Ft. Collins, Colorado

Fall 2005

COLORADO STATE UNIVERSITY

October 25, 2005

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY BRIAN D. CHERRINGTON ENTITLED MOLECULAR MECHANISMS UNDERLYING ACTIVIN REGULATION OF THE GONADOTROPIN RELEASING HORMONE RECEPTOR GENE BE ACCEPTED AS FULFULLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Committee on Graduate Work

Advisor

Department Head

ABSTRACT OF DISSERTATION

MOLECULAR MECHANISMS UNDERLYING ACTIVIN REGULATON OF THE MURINE GONADOTROPIN-RELEASING HORMONE RECEPTOR GENE

Activin regulation of the mouse GnRHR promoter was thought to occur solely through the GRAS element (1;2). However, the mouse GnRHR promoter is activin regulated while the rat promoter is not. To investigate this divergence in activin regulation between the two promoters the 1 bp difference in the GRAS element was exchanged between the two promoters. The rat promoter with the mouse GRAS homolog did not gain activin regulation, while the mouse promoter with the rat GRAS element did lose activin regulation in accordance with the importance of GRAS. This was the first evidence that while necessary GRAS alone is not sufficient for activin regulation of the mouse GnRHR promoter (3). Using a series of chimeric exchanges and block replacements in the mouse promoter, I identified a new element I termed the Downstream Activin Response Element or DARE (4). I suggest that GRAS and DARE together comprise a unique activin responsive unit (ARU) in the proximal promoter of the murine GnRHR gene.

Although activin responsiveness of the murine GnRHR gene promoter has been extensively studied using the gonadotrope derived αT3-1 cell line, whether this response is evident in a more physiological context was unknown. Thus, I sought to determine if the activin responsive phenotype of the GnRHR gene promoter defined *in vitro* is recapitulated in transgenic mice. Transgenic mice harboring the wild type mouse GnRHR gene promoter fused to the cDNA for luciferase (-1900wt) (7) were infected with an adenoviral construct that expresses follistatin (Ad-follistatin). I find that adenoviral mediated over-expression of follistatin leads to an approximately 50% decrease in pituitary luciferase expression in the –1900wt mice. These data are the first to demonstrate activin responsiveness of a GnRHR promoter construct *in vivo*. Finally, I repeated this experimental paradigm in a separate line of transgenic mice harboring the 1900 bp promoter fragment containing a loss of function mutation in GRAS (-1900μGRAS). Consistent with the critical role of GRAS in mediating activin responsiveness *in vitro*, I find that pituitary expression of luciferase in the –1900μGRAS line is not affected by follistatin over-expression.

Brian D. Cherrington

Biomedical Sciences Program

Colorado State University

Fort Collins, Colorado 80523

Fall 2005

ACKNOWLEDGEMENTS

I would like to begin by thanking my committee members: Terry Nett, Bob Handa, Dick Bowen, and Paul Laybourn who all at one time or another have helped with some aspect of my project. Next, I would like to thank Colin Clay my advisor and friend. Without Colin's guidance and patience none of this would have been possible. Colin has been there for me through the best and worst of times. I would also like to thank everyone at ARBL past and present with whom I have interacted. In particular, I must mention a few friends who have always brightened my day: Paul Gordy, Ryan Ashly, and Marco Coutino da Silva. Thanks to everyone who has offered me technical assistance in completing my work such as all members of the Nett/Niswender and RIA lab. Thanks to Buffy Ellsworth who taught me so much during my first year in the lab and started my project in the first place. I also want to thank all the members of the Clay lab past and present: Jeremy Cantlon, Clay Lents, Kathy Davis, and Todd Farmerie. I would specifically like to thank Todd Farmerie for helping me with all my cloning schemes and his critical input on my project. Without Todd, I would not know as much about science, genealogy, or medieval history as I do today. Lastly, I would like to thank my family. My parents Debbie and Alan have always supported me mentally, physically and financially in every aspect of my life. I also want to thank my brother Kevin and my sister Andrea who have supported me in everything I have done. Most of all I would like

to thank Amy. Her encouragement and laughter have made the past four years a truly enjoyable experience.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

Since the initial isolation of GnRH in 1971 (8) a clear picture of the key role of this molecule in controlling reproductive function has emerged. The pulsatile discharge of GnRH from hypothalamic neurons not only stimulates but is obligatory for synthesis and secretion of LH and, to a lesser extent, FSH from gonadotrope cells of the anterior pituitary gland (9-12). In addition, expression of genes encoding both the common α and unique β subunits of LH are absolutely dependent on GnRH input (13).

Relative changes in GnRH secretion from the hypothalamus are clearly an important determinant of gonadotropin secretion (11;14-17). Similarly, changes in the number of pituitary receptors for GnRH have also been implicated as an important mechanism underlying the regulation of gonadotropin secretion (18). Thus, changes in pituitary content and secretion of LH are not only dependent on changes in GnRH availability but also the number of GnRH receptors available for binding and, consequently, the responsiveness of the pituitary to a given dose of GnRH (11)

Since the first availability of cDNA's encoding the GnRH receptor (GnRHR) over 10 years ago, a number of groups have demonstrated coordinate changes in GnRHR numbers and pituitary concentrations of GnRHR mRNA (19-24). Receiving particular attention is an increase in GnRHR gene expression during the pre-ovulatory period that leads to heightened responsiveness of the pituitary gland to GnRH (14;19;23;25). Of the

multiple endocrine inputs that have been implicated in affecting changes in GnRHR numbers, perhaps the most dramatic are those associated with estradiol-17β, activin, and GnRH itself (26;27).

Given the critical role of GnRH in control of reproductive function, much effort has been devoted to characterizing the molecular events that underlie regulation of GnRH and GnRHR expression. In regard to the latter, our laboratory initiated a systematic analysis of the regulatory elements and transcription factors that mediate cell-specific expression and endocrine responsiveness of the GnRHR gene. Much progress has been made. Perhaps the first significant breakthrough was the definition of a "tripartite" enhancer in the proximal promoter of the murine GnRHR gene (28). The individual components of this enhancer include a binding site for the nuclear orphan receptor steroidogenic factor-1 (SF-1), a canonical AP-1 site and a novel regulatory element termed the GnRH receptor activating sequence or GRAS (2;29;30). This combination of regulatory elements contributes to the tissue/cell-specific properties of this promoter and also mediates multiple endocrine inputs. For example, GnRH stimulation of transcriptional activity of the GnRHR promoter is predominantly organized at the AP-1 site and appears to require cJun N-terminal kinase (JNK) post-translational activation of JunD and FosB and increased expression of cFos (30). Although originally defined as an element that contributed to "basal" activity of the GnRHR gene promoter, it is now well established that GRAS is a complex enhancer that mediates activin responsiveness through the binding of both Smad and non-Smad protein partners including AP-1 and a member of the forkhead or winged-helix family of transcription factors (2). In regard to SF-1 regulation, it is interesting to note that this is a characteristic common to multiple

genes expressed in a gonadotrope specific fashion. These include the common glycoprotein hormone α-subunit, the unique LHβ subunit and the GnRHR gene (29;31;32). Finally, although the major research efforts have focused on the tripartite enhancer (GRAS, AP-1, SF-1), it is now clear that there are many other regulatory elements that contribute to the functional activity of the GnRHR gene promoter. These additional elements include potential binding sites for PitX1, Oct-1, LHX3, NF-Y (5;33;34) and, based on the work presented in this dissertation, an element I have termed the downstream activin regulatory element or DARE.

It is important to point out that the progress that has been made in characterizing the functional architecture of the murine GnRHR gene promoter has been due to several key developments. Of these, perhaps the key event was the development of αT3-1 cells – an immortalized cell line of gonadotrope origin that expresses the endogenous GnRHR gene (35). These cells have served as the fundamental model for studying the organization and activity of the GnRHR gene promoter. Having said this I should underscore that this model is not without some shortcomings. For example, while estrogen is one of the most important physiological signals for increasing GnRHR gene expression in the pituitary, estrogen responsiveness of the GnRHR gene is not detectable in αT3-1 cells. Thus, our laboratory has sought to develop alternative models that allow for evaluation of GnRHR promoter activity in a more physiological context. This was the impetus for the construction of multiple transgenic mouse lines harboring fusion genes containing both wild-type and mutated forms of the GnRHR gene promoter. These transgenic mouse lines have proven invaluable in not only validating the *in vitro* analysis of promoter function but also revealing hormonal regulation that is simply not evident in

the αT3-1 cell model (e.g. estrogen responsiveness) (7;36). One of the central goals of my research was to determine if the activin responsive phenotype of the GnRHR gene promoter is recapitulated in transgenic mice.

As discussed above, at the start of my doctoral work the prevailing view was that GRAS alone was the enhancer element responsible for conferring activin responsiveness to the proximal promoter of the murine GnRHR gene. Based on asking a simple question regarding a single nucleotide difference in mouse GRAS and the GRAS homolog in the non-activin responsive rat GnRHR promoter I have found that the prevailing view was incorrect. In the following studies I describe the identification and characterization of DARE – an element that, in combination with GRAS, is necessary for activin responsiveness of the mouse GnRHR gene promoter. Thus, one of the key findings of my research is that GRAS and DARE cooperate to form an activin responsive unit (ARU) or "enhanceosome" in the proximal promoter of the murine GnRHR gene.

CHAPTER 2

LITERATURE REVIEW

I. *Hypothalamic-Pituitary-Gonadal Axis - Overview*

The hypothalamic pituitary gonadal axis is critical for maintaining reproductive function in mammalian species. Central to this axis is the anterior pituitary gland composed of five hormone secreting cell types: corticotropes, lactotropes, somatotropes, thryrotropes and gonadotropes. Of utmost importance to reproductive mechanisms and Gonadotropin Releasing Hormone Receptor (GnRHR) physiology is the gonadotrope cell. GnRH is secreted by GnRH neurons in the hypothalamus and released into the hypophyseal circulation through which it is quickly delivered to the pituitary gland (37;38). GnRH binds GnRH receptors on the plasma membrane of gonadotropes and initiates multiple intracellular signaling cascades that culminate in increased synthesis and secretion of the gonadotropic hormones, luteinizing hormone (LH), follicle stimulating hormone (FSH) and an increase in GnRHR numbers (11;39- 41). Although the pulsatile discharge of GnRH from hypothalamic neurons is obligatory for synthesis and secretion of LH, FSH secretion is highly regulated by activin – a member of the TGFβ family of growth and differentiation factors (42;43). Interestingly, activin also regulates GnRHR numbers in the anterior pituitary gland. In rodents, activin levels increase during proestrus and this rise, coordinate with increased GnRH secretion, may

be necessary for increasing the number of GnRHRs to sensitize the gonadotrope for the preovulatory LH surge (44).

The gonadotropins, LH and FSH, are secreted into the systemic circulation and act at the gonads to promote steriodogenesis and gametogenesis (13). LH is responsible for stimulating ovulation and corpus luteum formation in females and androgen secretion in males (45). In females, FSH stimulates the growth and maturation of ovarian follicles, while in males it promotes spermatogenesis (46). In response to gonadotropic stimulation, gonadal steroids are synthesized and secreted into the systemic circulation (39). The gonadal steroids exert a number of biological effects throughout the body, however, in the context of the hypothalamic-pituitary-gonadal (HPG) axis, these hormones participate in a negative feedback loop where an increase in steroids results in a decrease in GnRH secretion from the hypothalamus and a decrease in gonadotropin secretion from the pituitary.

A. *Anterior Pituitary Gland*

The anterior pituitary gland is a central component of the hypothalamic-pituitary-gonadal axis (Figure 1). Containing a heterogeneous population of endocrine cells, the anterior pituitary is responsible for the synthesis and secretion of multiple hormones that are essential regulators of many physiological processes including growth, metabolism, reproduction, fluid and electrolyte homeostasis and lactation. The corticotrope represents 10% of the cells in the anterior pituitary gland and expresses proopiomelanocortin (POMC), a polyprotein that is proteolytically processed into adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone, endorphins, and

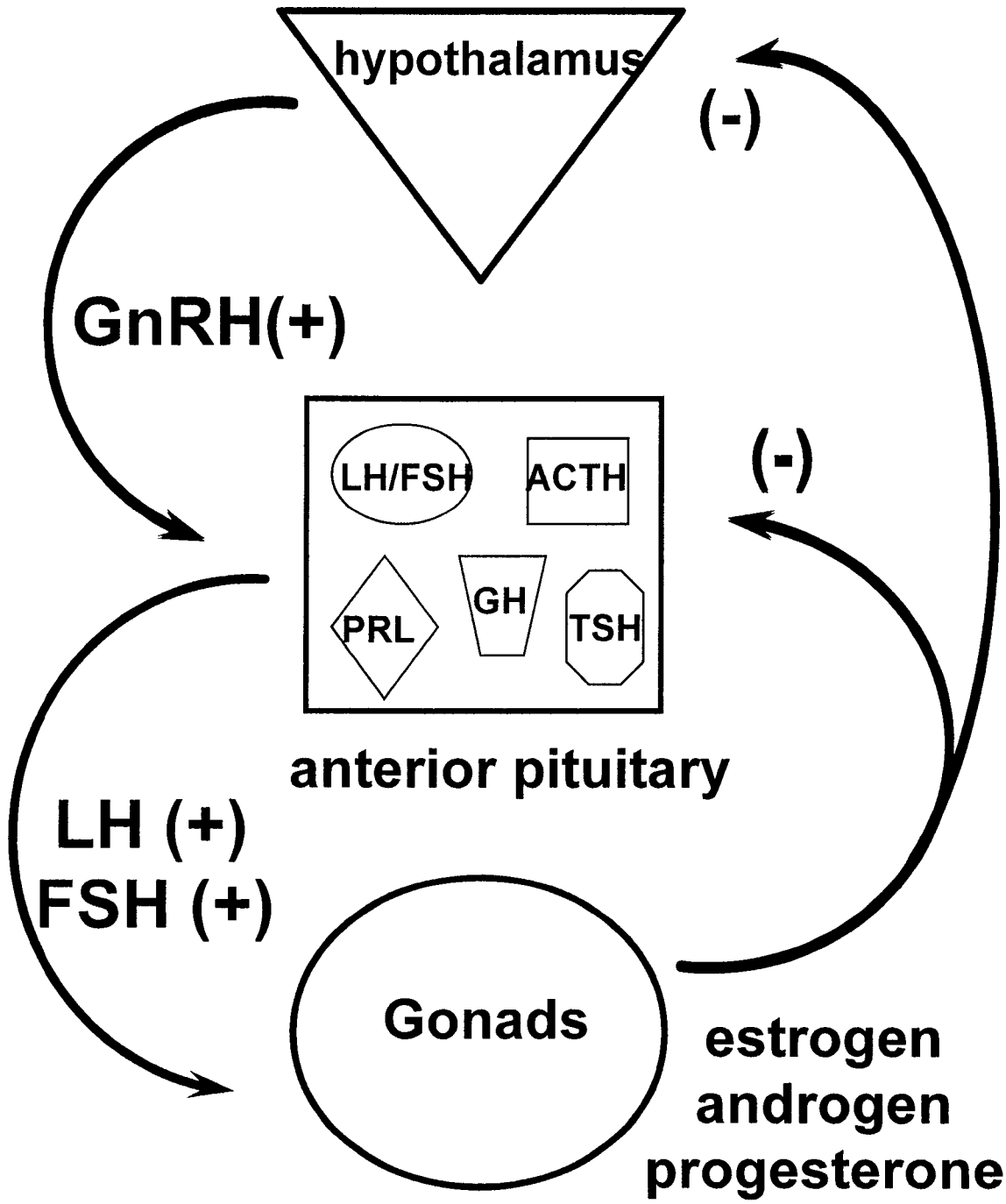

Figure 1. Hypothalamic-Pituitary-Gonadal Axis. The anterior pituitary gland represents a central component of the hypothalamic-pituitary-gonadal axis. GnRH secreted by the hypothalamus binds GnRH Receptors on gonadotropes in the anterior pituitary gland. In response to GnRH, the gonadotrope synthesizes and secretes LH and to some extent FSH. These hormones act on the gonads stimulating follicular development, spermatogenesis and steroid secretion-that can result in negative feedback at the level of the pituitary and hypothalamus.

enkephalins (47). Adrenocorticotropic hormone secretion into the systemic circulation stimulates the adrenal cortex to produce and secrete glucocorticoids in response to physical and psychological stress.

Lactotropes and somatotropes account for approximately 70% of the secretory cells found in the anterior pituitary and are responsible for the production of prolactin and growth hormone, respectively. Prolactin has been implicated in a number of physiological processes including metabolism, reproductive cycles in rodents and the development of mammary duct glands during pregnancy and milk production following parturition (47). In contrast to all other endocrine cell types in the anterior pituitary gland, hypothalamic regulation of lactotropes is predominantly inhibitory. In response to growth hormone releasing hormone somatotropes produce and secrete growth hormone, while somatostatin has the opposite affects on somatotrophs. Like prolactin, growth hormone interacts at multiple levels in metabolism where it is generally considered anabolic and promotes protein synthesis. Growth hormone is also the key stimulator of long-bone growth in childhood (47). Pituitary synthesis and secretion of GH is tightly regulated by hypothalamic GH releasing hormone (GHRH), somatostatin and the peripheral peptide hormone termed ghrelin (48). Thyrotropes are the least abundant secretory cells in the anterior pituitary comprising only 5-10% of the endocrine cell types. Thyroid-releasing hormone (TRH) from the hypothalamus stimulates thyrotropes to synthesize and secret thryroid-stimulating hormone (TSH) – a member of the glycoprotein hormone family. TSH exerts its primary biological effects at the thyroid gland and acts to increase the synthesis and secretion of thyroxine which is essential for normal metabolic processes as well as mental and physical development in children (47).

The last of the five hormone secreting cells of the anterior pituitary are gonadotropes. These cells comprise approximately 10% of the total endocrine cell population in the anterior pituitary gland. In response to GnRH released from the hypothalamus, gonadotropes synthesize and secrete the gonadotropic hormones LH and FSH. These hormones exert their primary biological effects at the ovary and testis and are critical for maintaining normal gonadal function including gametogenesis and steroidogenesis.

B. *Gonadotropes*

The unique phenotype of gonadotropes is defined by the expression of GnRHR on the plasma membrane and the synthesis and secretion of LH and FSH. From the standpoint of gene expression then this means that gonadotropes must express at least 4 unique gene products, the common α glycoprotein hormone subunit (αGSU), the unique β subunits of both LH and FSH and the GnRHR (Figure 2) (49). The gonadotrope is a target for many different endocrine inputs; however, none of these is more fundamental than GnRH. GnRH binding to its cognate membrane receptor leads to an increase in GnRHR numbers on the plasma membrane and increased expression of the different gonadotropin subunits. The GnRHR is a member of the rhodopsin like family of seven transmembrane domain G protein coupled receptors (50). Hormone binding the GnRHR activates $G\alpha_{q/11}$ which initiates multiple phospholipase activities leading to formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. Receptor activation also increases intracellular calcium concentrations through release of intracellular stores and opening of L-type calcium channels. These events underlie activation of protein kinase C (PKC)

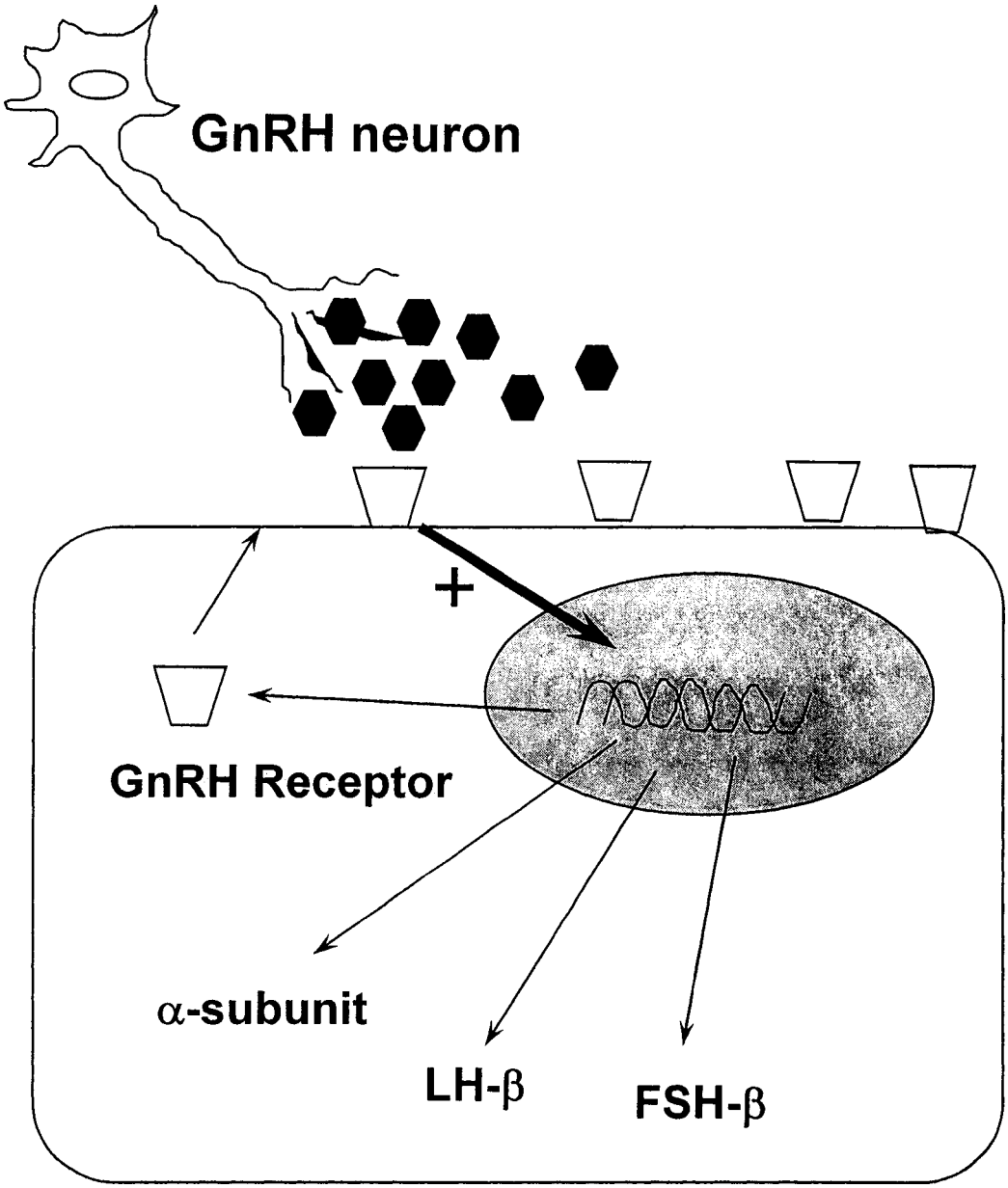

Figure 2. Model of a Gonadotrope. This diagram represents an idealized gonadotrope. Because it is the target of GnRH, it must express GnRH receptors. Binding of GnRH to GnRH receptors stimulates the expression of four genes: the glycoprotein α subunit, the LHβ and FSHβ subunits, and the GnRHR itself.

isoforms (51). GnRH activates MAPK intracellular signaling pathways including extracellular signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) in the gonadotrope derived αT3-1 cell line (52-54). Finally, GnRH induces the release of stored LH and FSH from secretory granules into the systemic circulation (9;12).

Since gonadotropes represent only a small portion of the endocrine cells found in the anterior pituitary gland, it is difficult to study gene regulation in gonadotropes using primary culture. To circumvent this problem, Windle et al. developed the gonadotrope derived αT3-1 cell line from tumors in the anterior pituitary gland of mice (35). The αT3-1 cell line represents early gonadotrope progenitors of approximately day 13 of embryonic development in the mouse pituitary. Similar to mature gonadotropes, αT3-1 cells express the α-subunit mRNA, α-subunit protein, GnRHR mRNA, GnRH receptors, activin, and activin receptors (35;55). However unlike mature gonadotropes, αT3-1 cells do not express the unique LHβ or FSHβ subunits necessary to form the mature gonadotropin hormones. Due to the lack of β subunit production there is concern that αT3-1 cells do not represent a suitable model for the fully differentiated gonadotrope (51). However, αT3-1 cells do recapitulate expression of the endogenous genes encoding the GnRHR and αGSU. In spite of their potential drawbacks, αT3-1 cells are widely used as a model for studying GnRH signal transduction and regulated expression of the genes encoding the GnRHR and αGSU (50;56;57). Due to limitations with αT3-1 cells, transgenic mice often provide a valuable tool to confirm *in vitro* promoter work. The use of transgenic mice to confirm *in vitro* promoter analysis will be discussed in chapter 5.

C. *Glycoprotein Hormones*

The glycoprotein hormones LH, FSH, TSH and chorionic gonadotropin (CG) are critical for reproduction, growth and metabolism, and the maintenance of pregnancy (39;58). The hormones are composed of two distinct subunits, the common αGSU and a unique β subunit. These subunits interact non-covalently to form the mature hormone. A unique characteristic of the glycoprotein hormones is the presence of carbohydrates moieties attached to the protein as the name implies (59). The common α subunit possesses two N-linked oligosaccharides while the β-subunits contain either one (LHβ and TSHβ) or two (FSHβ and CGβ) N-linked oligosaccharides. CGβ subunit contains an additional four O-linked carbohydrate moieties (59).

The common αGSU is encoded by a single copy gene that is expressed in pituitary gonadotropes and thyrotropes of all mammals and in the placental trophoblast of primates and horses (60). The gene contains four exons and three introns encoding a precursor protein of 116 amino acid residues (13). The mature α subunit contains 92 amino acids and two carbohydrate moieties as previously mentioned. The β-subunits of hCG and LH are members of a multigene family in which all of the genes contain three exons and two introns with the intron/exon boundaries conserved (61). Despite the high-homology of the primary amino acid sequence between hCGβ and LHβ, a functional difference between the two mature glycoprotein hormones exists. In particular, O-glycosylation of serine residues in the unique 26 amino acid carboxyl-terminal tail confers a far longer circulatory half-life of hCG as compared to LH (58). The FSHβ subunit is encoded by a single copy gene that also consists of three exons and two introns (62, 63). In addition, the intron/exon boundaries are conserved among FSHβ genes

across species. Overall, expression of the common α and unique β subunits of LH and FSH are increased by exposure of gonadotropes to GnRH. GnRH acting through the GnRHR activates signaling cascades which culminate at unique promoter regulatory elements located on the individual subunit genes to cause an increase in transcription.

II. *Transcriptional Regulation of Gonadotropin Subunit Genes*

Pituitary gonadotropes express the αGSU, the unique LH and FSH β subunits and the GnRHR. Regulation of transcription and secretion of LH, FSH, and the GnRHR is fundamental to understanding reproductive function in mammals. Transcriptional regulation of these different gene products in the pituitary gonadotrope involves unique and overlapping mechanisms. The molecular mechanisms by which the gonadotrope can produce these factors at any given time and at appropriate concentrations involve a tightly regulated system at the level of gene expression.

A. *The Glycoprotein Hormone α Subunit (αGSU)*

Expression of the α glycoprotein hormone subunit (αGSU) is critical for the ultimate biosynthesis of LH, FSH and CG. The αGSU is expressed in gonadotropes, thyrotropes, and trophoblasts and non-covalently interacts with the unique β subunits to form the mature glycoprotein hormones. The distinction must be made that tissue specific regulation of αGSU varies between the three different cell types. For the purpose of this dissertation, only expression in gonadotropes and trophoblasts will be discussed. Tissue specific expression of the α subunit in trophoblasts is mediated by three elements: a CCAAT box which binds α CCAAT binding factor (CBF), the junctional regulatory

element (JRE) which bind distal-less 3 (Dlx 3), and the trophoblast specific element (TSE) which binds activator protein 2 or AP-2 (64-67). In addition, the cyclic AMP response element (CRE) and α-activating element (αACT) contribute to αGSU expression in trophoblast cells as well as in gonadotrope cells (68;69).

In the gonadotrope, tandem cyclic AMP response elements (CRE)s are critical for activity of the human αGSU promoter (Figure 3) (57). These CREs can bind the CRE binding protein (CREB), CRE modulator, c-Jun, activating transcription factor (ATF)1 and ATF2 (70). In all other mammalian species studied, the αGSU promoter contains a single CRE homolog that differs from the human CRE by one base pair. This single CRE binds heterodimers of c-Jun /ATF2 with a higher affininty than CREB (71).

Basal expression of the mouse αGSU in the pituitary involves the pituitary glycoprotein hormone basal element or PGBE (Figure 3). PGBE binds the LIM-homeodomain transcription factor LH-2 (72). In addition to PGBE, basal regulation of the αGSU promoter in gonadotropes requires α-basal element 1 and 2 (αBE1 and 2) (Figure 3) (57). Furthermore, the gonadotope-specific element or GSE binds a member of the orphan family of nuclear receptors referred to as steroidogenic factor 1 or SF-1 (Figure 3)(31;73). SF-1 binding the human GSE contributes to pituitary specific expression of the αGSU gene. There are other elements that participate in αGSU expression in the gonadotrope but make only a minor contribution to basal activity. One such element is the αACT which binds the transcription factors GATA2 and GATA3 to increase expression of the αGSU (Figure 3)(69). Additionally, the loss of Pitx1 or basic helix-loop-helix protein expression results in attenuation of αGSU promoter activity in αT3-1 cells via a Pitx1 binding site and tandem E Boxes (Figure 3)(74;75). Thus, αGSU

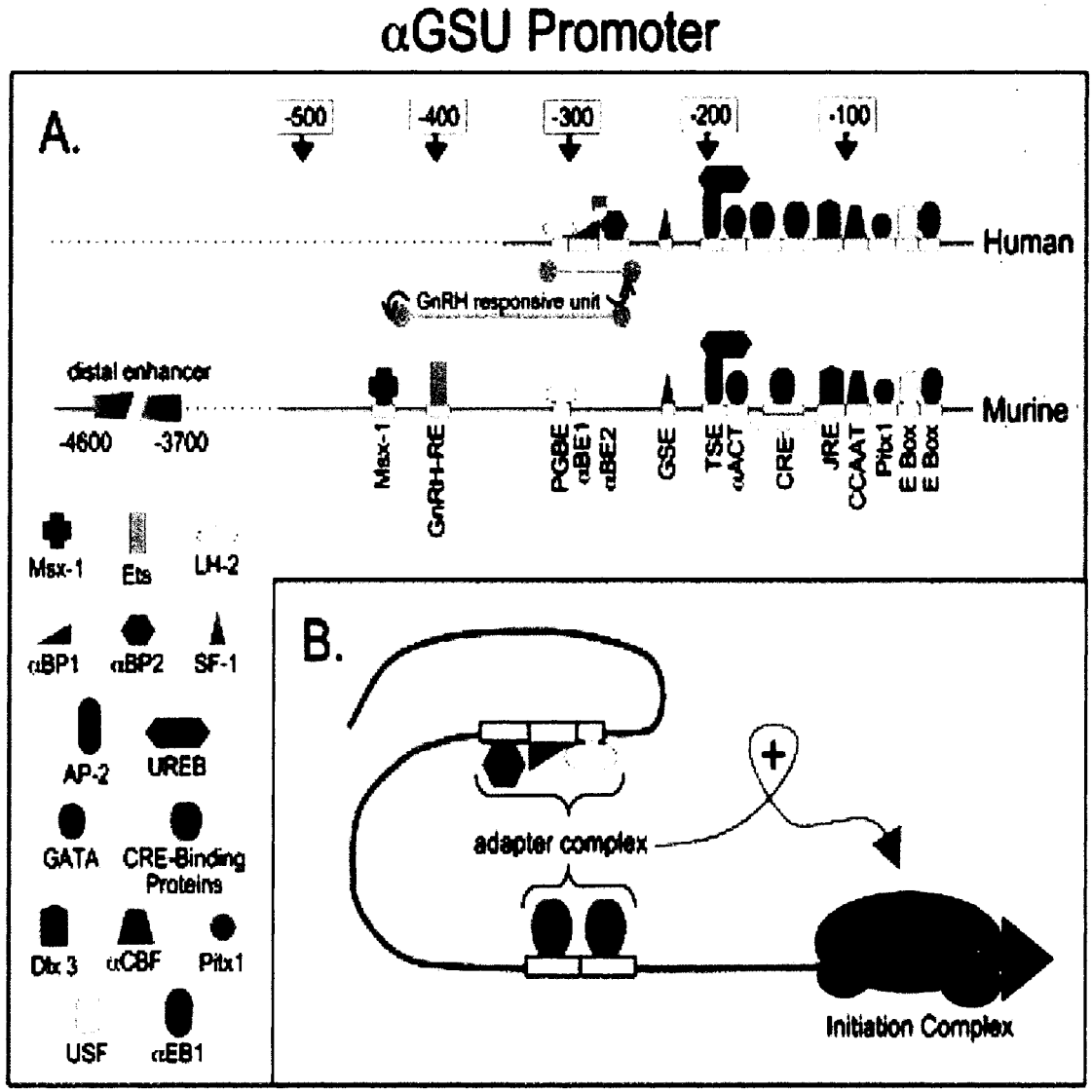

Figure 3. Diagram of the Glycoprotein Hormone α-Subunit Gene Promoter. This diagram represent a comparison of the human and mouse glycoprotein hormone α-subunits illustrating the defined promoter elements. Also illustrated are the transcription factors that bind the defined promoter elements (79).

expression is not controlled by a dominant element, but rather a combinatorial interplay between multiple elements and their binding proteins. In addition to basal regulation, a number of hormones are responsible for regulating expression of the αGSU.

GnRH responsiveness of the human and mouse αGSU promoter has been extensively studied, however, the mouse αGSU promoter displays a far more robust GnRH response compared to the human promoter. The decapeptide GnRH acting via the GnRHR stimulates αGSU expression through a mitogen activated protein kinase (MAPK) signaling cascade. Phosphorylation of ERK 1 and 2 leads to the activation of the MAPK-responsive transcription factor, Elk1. MAPK activity was mapped to a specific Ets binding site within the GnRH-RE in the mouse αGSU promoter (52). Thus, GnRH responsiveness of the mouse αGSU promoter appears to involve a consensus Ets binding site located in the GnRH response element (GnRH-RE) (Figure 3)(76;77). The true identity of the transcription factor binding this site *in vivo* is unknown, however an Ets family member is suspected due to the fact that a dominant-negative form blocks the GnRH response (52). A second GnRH responsive element in the mouse αGSU promoter is PGBE (Figure 3)(72;76). *In vivo*, the identity of the protein binding this element is also unclear but it likely binds a LIM-homeodomain transcription factor such as LH-2 or LHX3. In addition to GnRH-RE and PGBE, the GSE has recently been implicated in playing a minor role in GnRH responsiveness. Transgenic mice are important for confirming the physiologic relevance of all *in vitro* promoter studies. As such, 1500 bp of the human αGSU promoter fused to a chloramphenicol acetyl transferase (CAT) reporter was sufficient to target reporter activity to gonadotropes (60), and the transgene was responsive to GnRH (78).

In opposition to mechanisms that enhance transcription of the αGSU, there are mechanisms that repress transcription. Human αGSU transgenic mice which express the CAT reporter specifically in the pituitary show appropriately suppressed reporter activity after administration of testosterone. Suppression of human αGSU transcription proceeds through the androgen receptor (AR) in a ligand dependant manner, while estrogen receptors (ER) have no effect (79). AR likely suppresses αGSU expression at the level of the promoter while ER likely acts at the level of the hypothalamus. The human αGSU promoter contains two elements that mediate androgen dependent repression: the αBE and tandem CREs (Figure 3)(80). Although the promoter elements that mediate androgen repression of the αGSU are known, the absence of direct AR binding to the promoter suggests that ligand activated AR is part of a larger DNA-protein complex.

B. *The LHβ Subunit Gene*

Bioactive LH consists of the LHβ subunit non-covalently bound to the αGSU. In all species studied to date, the important regulatory elements for LHβ expression are located within 500 base pairs of the start site. The proximal 140 base pairs of the LHβ promoter are highly conserved across species and consist of tandem copies of transcription factor binding sites for an early growth response protein (Egr-1) and steroidogenic factor 1 (SF-1) (Figure 4) (32;81). Sandwiched between the tandem Egr and GSE elements is the binding site for PitX1 a *bicoid*-related homeodomain protein (Figure 4)(82). Upstream of the proximal 140 base pairs are additional regulatory elements that are not well conserved among species. In this distal region of the rat promoter, there is an element that binds the transcription factor protein specificity-1

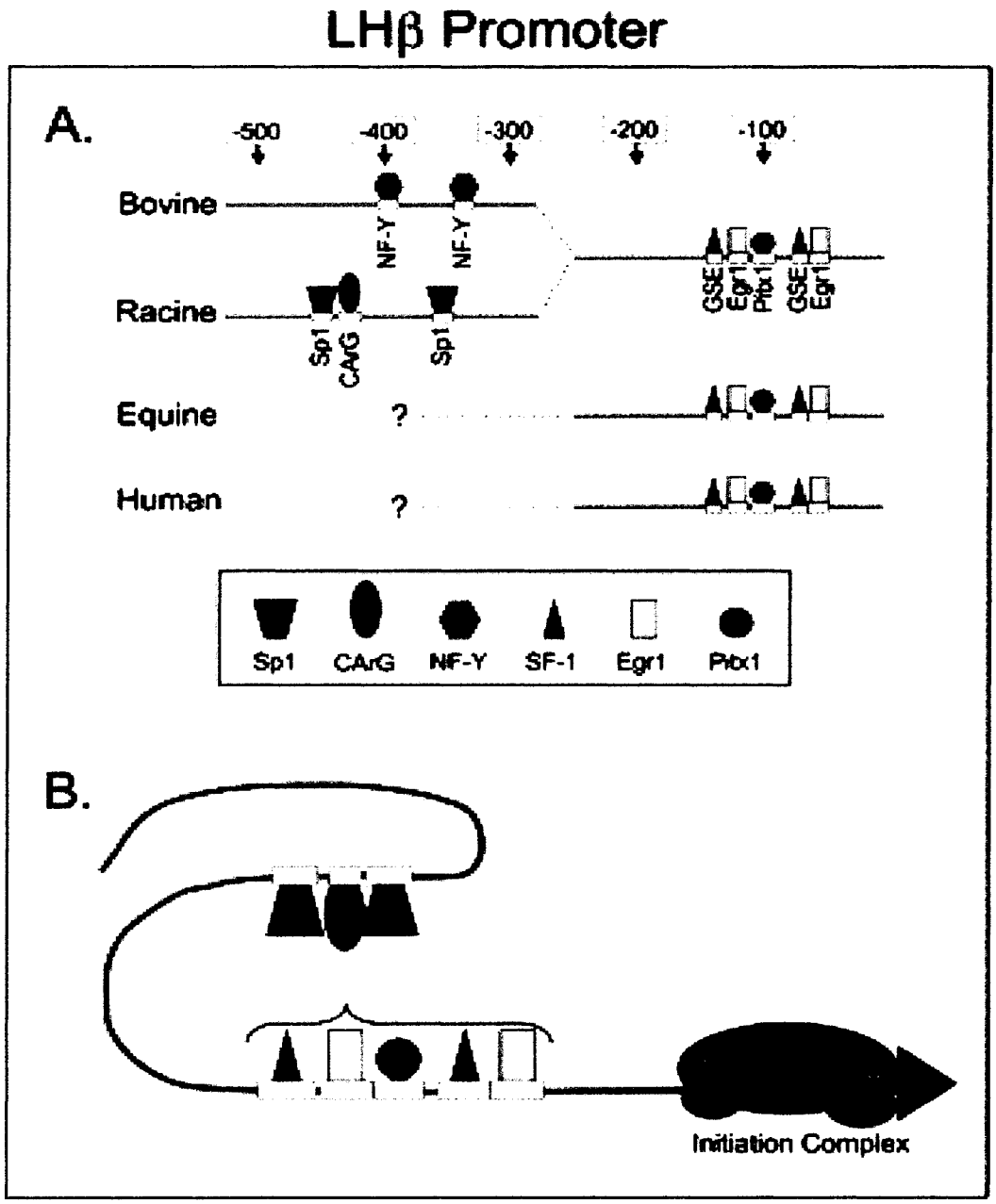

Figure 4. Diagram of the LHβ gene promoter. This diagram compares the bovine, racine, equine, and human LHβ gene promoters and shows the defined promoter elements. Also illustrated are the transcription factors that bind the promoter elements (79).

(Sp-1) and a CArG binding site (Figure 4)(83;84), whereas in the bovine promoter the same sequence contains a binding site for nuclear transcription factor Y (NF-Y) (Figure 4)(85). Transgenic mice harboring the bovine LHβ promoter fused to a CAT reporter containing either the NF-Y, SF-1, or Pitx1 site mutated had lower reporter activity when compared to the wild type transgene (32;82;85).

The LHβ promoter is highly sensitive to stimulation by GnRH, however the responsive regions vary among species. In the rat, two GnRH responsive regions were identified located -490 to -353 and -207 to –82 (86). The distal region responsive to GnRH in the rat LHβ promoter contains two Sp1 elements including an overlapping CArG box (Figure 4)(83;84), however the human, equine, and bovine promoters do not appear to contain this distal region of GnRH responsiveness. The proximal 140 base pairs of the bovine, equine, human, rat, and mouse LHβ promoters are highly conserved and mediate the GnRH signal. This 140 base pair sequence contains the SF-1, PitX1, and Egr-1 binding sequences previously described (Figure 4). Data from transgenic mice suggests the GSE and the PitX1 element or both are required for GnRH induced LHβ promoter activity (32;87). GnRH stimulation also results in an increase in Egr-1 mRNA and Egr-1 protein which appears tightly linked to an increase in LHβ synthesis (88). Overall, interaction between the proximal and distal regions of the LHβ promoter is necessary for full GnRH responsiveness.

Expression of the bovine LHβ promoter in transgenic mice is repressed by estrogen and testosterone (89). However, the promoter lacks binding sites for either ligand activated AR or ER (89). Therefore, the mechanisms for repression of the LHβ promoter could involve protein-protein interactions between steroid nuclear receptors and

transcription factors specific to the promoter. Cotransfection of AR, with over-expression of Egr-1, PitX1, and constitutively active SF-1 lacking the ligand binding domain, each individually rescued androgen mediated suppression of the bovine LHβ promoter (90). Furthermore, over-expression of full length SF-1 cannot rescue LHβ promoter activity, suggesting that the ligand binding domain most likely plays a role in the interaction that occurs between SF-1 and AR (90). Expression of the rat LHβ promoter is suppressed by androgen but not estrogen treatments while the divergent bovine LHβ promoter is suppressed by both (91). Inhibition in the rat promoter is thought to occur through an interaction between the steroid nuclear receptor and Sp1, possibly reducing cooperation between the proximal and distal GnRH response elements. Taken together, the bovine and rat studies suggest that AR suppresses the LHβ promoter via protein-protein interactions with specific DNA binding factors that link the proximal and distal regions of the promoter (90;91).

C. *The FSHβ Subunit Gene*

Despite its crucial role in mammalian reproduction, little is known about regulation of the FSHβ gene at the molecular level. The establishment of the gonadotrope derived LβT2 cell line has, however, allowed for more rapid progress in understanding of the molecular mechanisms underlying FSHβ transcription. Two elements contribute to basal and cell specific expression of the mouse FSHβ gene. The first element binds the orphan nuclear receptor steroidogenic factor 1 (SF-1), and the second site is for the heterotrimeric transcription factor NF-Y (92). Furthermore, direct interactions between SF-1 and NF-Y appear to be necessary for the functional

contribution of these proteins to basal transcriptional activity of the FSHβ gene (92). Basal activity of the human, racine, and porcine FSHβ promoters require binding sites for multiple homeodomain proteins. Specifically, the human and pig promoters contain binding sites for the LIM homeodomain transcription factor LHX3, while the rat promoter includes the binding site for the homeodomain protein Pitx-1 (6;93). An AP-1 site is implicated in basal and GnRH mediated FSHβ transcription in the mouse promoter. This AP-1 half site is juxtaposed to a CCAAT box that binds NF-Y (94). In the rat and sheep promoters, two AP-1 like enhancers are necessary for GnRH responsiveness in heterologous choriocarcinoma, HeLa, and COS-7 cells (95); however, based on analyses in transgenic mice the relative contribution of these AP-1 sites to GnRH responsiveness is questionable (96).

Although clearly responsive to GnRH, expression of FSHβ is more tightly coupled to the combination of activin and inhibin inputs to gonadotropes (96). These inputs reflect regulation at multiple levels including autocrine, paracrine as well as endocrine signaling. A member of the transforming growth factor (TGFβ) superfamily of growth and differentiation factors, activin up regulates FSHβ subunit mRNA, protein, and total FSH secretion (42;97). Consistent with this, mice deficient in the activin receptor have decreased FSHβ mRNA and circulating FSH levels (98;99). In contrast, inhibin opposes the stimulatory effects of activin on FSHβ gene expression by interfering with activin binding to the activin type II receptor.

As with other members of the TGFβ superfamily, activin signaling is initiated by both a type I and II receptor and subsequent phosphorylation and activation of multiple members of the Smad family of DNA binding proteins (100;101). In LβT2 cells, rat

FSHβ expression requires Smad3, but not Smad2, and is augmented by Smad4, the common Smad binding partner (102). Recently, Bailey et al identified three Smad binding elements (SBE) that are required for full activin responsiveness of the ovine FSHβ promoter (103). Interestingly, the most proximal site binds Smad 4 in association with the homeodomain proteins Pbx1 and Prep1 – a mechanism reminiscent of activin regulation of the murine GnRHR gene promoter (103)(see chapter 3). Finally, Spady et al showed that androgens stimulate expression of the ovine FSHβ gene in LβT2 cells, and mutation of either one of two androgen receptor binding sites within the promoter abolishes this stimulation (104). Androgen stimulation is dependent on an activin autocrine loop present in LβT2 cells as well as an activin response element in the ovine FSHβ gene. In transgenic mice harboring an ovine FSHβ transgene, expression and FSH hormone levels are diminished by follistatin, inhibin, and estradiol (to a lesser extent) similar to the endogenous FSHβ gene (96;105).

III. *Molecular Biology of the Murine GnRH Receptor Gene*

Changes in pituitary content and secretion of LH are not only dependent on changes in GnRH availability but also the number of GnRH receptors available for binding and, consequently, the responsiveness of the pituitary to a given dose of GnRH (11;17;19;106-108). Since the availability of cDNA's encoding the GnRHR, a number of groups have demonstrated coordinate changes in GnRHR numbers and pituitary concentrations of GnRHR mRNA (19-24). Thus, transcriptional regulation of the GnRHR gene appears to represent a key regulatory component of the sensitivity of the pituitary gland to GnRH input. As such, understanding the functional organization of the

GnRHR gene promoter is central to fully understanding the molecular events underlying hormonal regulation of GnRHR numbers in the anterior pituitary gland (18). Of the multiple hormones that have been implicated in affecting changes in pituitary levels of GnRH receptors perhaps the most dramatic effects are those mediated by estrogen, activin and GnRH itself (14;19;23;25).

A. *Functional Organization of the Murine GnRHR Gene Promoter*

Transcriptional activity of the mouse GnRHR gene promoter relies on multiple DNA regulatory elements arrayed across approximately 600 bp of proximal 5' flanking region (Figure 5). Collectively, these elements yield a combinatorial code sufficient to direct tissue-specific expression and mediate multiple endocrine inputs. Several years ago, Duval et al. proposed that transcriptional activity of the murine GnRHR promoter was predominantly mediated by 3 regulatory elements that comprise a "tripartite" enhancer (Figure 5)(28). The first of these, the gonadotrope specific element or GSE binds the nuclear orphan receptor SF-1 (Figure 5)(29). Thus, SF-1 responsiveness is characteristic of at least 3 genes expressed in a gonadotrope-specific fashion – the αGSU, the LHβ and GnRHR genes (29;109;110). Consistent with this, genetic "knock-out" of SF-1 leads to an absence of LH secretion and a hypogonadotropic-hypogonadal phenotype (111). Located upstream of the GSE is a canonical Activator Protein 1 (AP-1) site (Figure 5). This element binds multiple members of the Jun and Fos family of transcription factors and contributes not only to basal transcriptional activity of the GnRHR promoter but also mediates GnRH responsiveness of this gene (30). Finally, an element termed the GnRH Receptor Activating Sequence or GRAS was originally

Proximal Promoter of the Murine GnRHR Gene

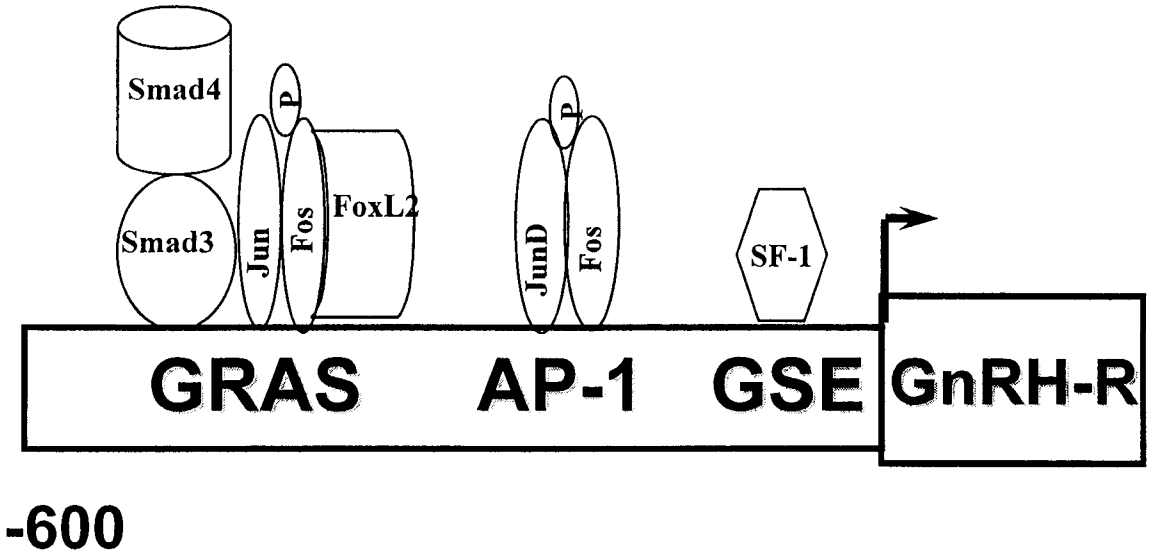

Figure 5. Model of the Proximal Promoter from the Murine GnRHR gene. GRAS, AP-1 and the GSE are all contained within 600 bp of proximal promoter from the murine GnRHR gene. The GSE binds SF-1, while AP-1 binds Jun and Fos heterodimers. GRAS is a tripartite element that binds Smads 3 and 4, Jun and Fos heterodimers, and the winged helix transcription factor FoxL2.

defined by scanning mutagenesis and was subsequently found to be a key element mediating activin input to the murine GnRHR gene promoter (1;28). GRAS is a composite regulatory element that consists of a Smad binding element (SBE) at the 5' end, immediately juxtaposed to a partial AP-1 site that binds Jun/Fos family members (Figure 5). Interestingly, mutations at the 3' end of GRAS do not block Smad 4 binding the SBE or Jun/Fos heterodimers binding at AP-1, but eliminate its functional activity (2). Thus, some other transcription factor(s) besides Smads and Jun/Fos must be playing a role at the GRAS element. Sequence analysis revealed that the 3' end of the GRAS element contains homology to the binding sites defined for the forkhead family of transcription factors. One member of the forkhead family of transcription factors, specifically FoxL2, is capable of interacting with the GRAS element (Figure 5)(112). FoxL2 activation is lost upon mutation of either the 5' or 3' end of the GRAS element indicating that it is necessary to interact in a multi-protein complex at the GRAS element. Overall, GRAS requires a diverse complex of transcription factors that probably act in concert to mediate the activin and GnRH responses of the mouse GnRHR promoter.

The studies described above were all based on promoter characterization in αT3-1 cells. Work in our laboratory has expanded *in vitro* promoter analysis to the use of transgenic mice. Specifically, 1900 bp of the mouse and 9100 bp of the sheep GnRHR gene promoters fused to the cDNA for luciferase are sufficient to confer tissue specific activity of the transgene in transgenic mice (36). These lines of transgenic mice have proven valuable for studying *in vivo* estrogen and GnRH regulation of the sheep and mouse GnRHR gene promoters respectively. Contributions of these transgenic mouse

models to estrogen and GnRH regulation of the GnRHR gene promoter will be discussed later in the literature review.

Over the past several years it has become clear that transcriptional regulation of the murine GnRHR gene is far more complex than the simple model of a "tripartite" enhancer (Figure 5). Recently, a NF-Y binding site and three homeodomain protein binding sites were identified that contribute to transcriptional activity of the murine GnRHR gene promoter (Figure 6) (4;5;33). Homeodomain proteins contain a highly conserved sequence of 60 amino acids encoding the DNA binding domain referred to as the homeodomain. The role of these proteins *in vivo* is to control the genetic determination of development and implementation of the genetic body plan. This, presumably, is why they are so highly conserved: they fulfill a function too vital to tolerate gross change in sequence or expression pattern. In the anterior pituitary gland, homeodomain proteins control pituitary development and the differentiation of the five hormone secreting cell types. More specifically, the homeodomain proteins Hesx-1/Rpx, LHX3, and Prop-1 are necessary for differentiation from progenitor cells to gonadotropes (113;114). Additionally, homeodomain proteins are necessary for transcription of the αGSU, LHβ, FSHβ, and GnRHR promoters (5;33;82;103;115;116).

Pitx-1, a *bicoid*-related homeodomain protein, activates the mouse GnRHR gene promoter through the sequences between -308 and –264 (Figure 6) (34). This region, termed the sequence underlying responsiveness to GnRH (SURGE-2) includes an AP-1 site, which is important for homologous GnRHR gene expression. Pitx-1 bound to this region with only low affinity. Kaiser et al suggest that activation of the mouse promoter by Pix1 occurs in part by DNA binding and in part by an action of Pitx-1 as a cofactor for

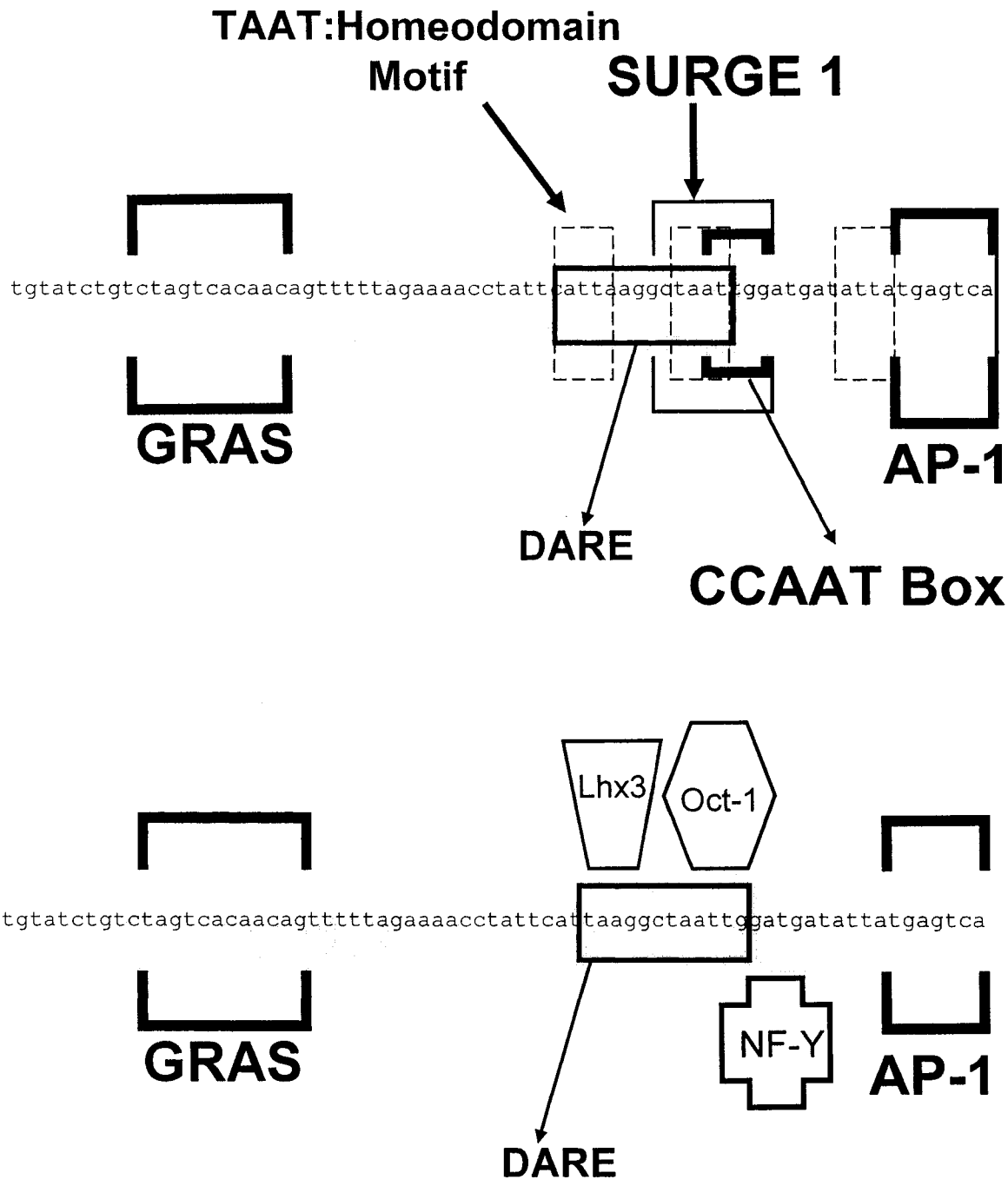

Figure 6. The 30 bp sequence between GRAS and AP-1 contains the binding sites for numerous transcription factors. The sequence between GRAS and AP-1contains three TAAT homeodomain motifs, the Downstream Activin Response Element (DARE), the Sequence Underlying Responsiveness to GnRH (SURGE), and a CCAAT box. The homeodomain proteins LHX3 and Oct-1 bind the TAAT repeats within DARE while the CCAAT box binds NF-Y.

AP-1, augmenting AP-1 activity through a novel protein-protein interaction between c-Jun and the homeodomain region of Pitx-1(34).

In the mouse GnRHR gene promoter, the thirty nucleotides located between the GRAS and AP-1 elements have been subject to intense investigation due to the presence of two TAAT repeats-the consensus binding site for homeodomain proteins (Figure 6). Recently, LHX3 has been shown to bind the first of the two TAAT repeats (in the negative orientation) and contribute to basal expression of the mouse GnRHR promoter (5). The second of the two TAAT repeats is designated the Sequence Underlying Responsiveness to GnRH or SURG-1 element (33). SURG-1 contains the second TAAT in the positive orientation in addition to a CCAAT box in the negative orientation. The POU homeodomain protein Oct-1 binds the TAAT sequence while the CCAAT box binds NF-Y (33). Interestingly, neither protein was shown to be activin regulated or required for activin responsiveness of the mouse GnRHR promoter, but both proteins contribute to basal and GnRH stimulated expression. In conclusion, the two TAAT motifs located between GRAS and AP-1 are important for basal and GnRH regulation of the mouse GnRHR promoter, however activin regulation of this region remains undefined.

B. *GnRH Responsiveness of the GnRHR Gene is Mediated at AP-1*

The GnRHR is a member of the rhodopsin like family of seven transmembrane domain G protein coupled receptors (50). Agonist binding to the GnRHR activates $G\alpha_{q/11}$ (117) which in turn activates phospholipase C activities leading to formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). These molecules act to amplify the

intracellular signal; IP3 causing the release of intracellular Ca2+ stores from the endoplasmic reticulum while DAG directly stimulates PKC. Active PKC in turn phosphorylates Ras and Raf to initiate multiple MAPK signaling cascades that ultimately activate the transcription factors Jun and Fos (118). Specifically, GnRH receptor occupancy results in activation of extracellular signal-regulated kinase (ERK) through a mechanism requiring calcium influx through L-type calcium channels in αT3-1 cells and primary rat pituitaries (119). GnRH induces activation of the c-Jun N-terminal (JNK) signaling cascade in a dose-, time-, and receptor-dependent manner in clonal αT3-1 cells and primary rat pituitaries (120). JNK activation by GnRH increases binding of JunD, FosB, and c-Fos at the AP-1 element in the mouse promoter (30). Activation of JunD and FosB occurs at the post-translational level, however c-Fos regulation occurs at the level of gene expression. Finally, GnRH responsiveness of the promoter is lost in αT3-1 cells expressing a dominant-negative JNK protein indicating an important role for JNK in GnRH regulation of the promoter.

Studies on GnRH regulation of the murine GnRHR promoter are complemented by *in vivo* experiments using transgenic mice harboring 1900 bp of the mouse GnRHR promoter fused to the coding sequence of luciferase. Transgenic mice containing the wild type GnRHR gene promoter (1900wt) specifically express the transgene in the pituitary, brain, and gonads recapitulating the sites of endogenous GnRHR gene expression (36). To study the *in vivo* role of AP-1, transgenic mice expressing the murine GnRHR promoter fused to the coding sequence of luciferase and containing a mutated AP-1 site (1900μAP-1) were generated. The 1900μAP-1 mice also displayed tissue specific expression of the transgene in the pituitary, brain, and gonads. 1900wt

transgenic mice respond by increasing luciferase expression when challenged with the GnRH agonist Dala-6 GnRH. This response, however, was lost in 1900μAP-1 transgenic mice confirming the role of AP-1 in mediating GnRH regulation *in vivo* (30).

The sequence of the GnRHR promoters is not well conserved between the human, rat, mouse, and ovine species. Although the endogenous genes obviously express in the whole animal and respond to GnRH, only the mouse, rat, and pig GnRHR promoters show significant activity in αT3-1 cells. Despite this, Leung et al have shown in heterologous cell lines of placental and ovarian origin that GnRH regulation of the human GnRHR promoter proceeds through a PKA cAMP dependent pathway that is not evident in the mouse (121). Using GGH3 cells, a somatotrope derived cell line stabily expressing the GnRHR, Conn et al found GnRH stimulated a cAMP signaling pathway that converges on an imperfect CRE in the mouse promoter (122). Work on the murine GnRHR promoter in the homologous αT3-1 gonadotrope derived cell line does not recapitulate activation by a cAMP dependent pathway. The rat GnRHR promoter is responsive to Pituitary Adenylate Cyclase Activating Peptide (PACAP). As the name implies, this peptide increases intracellular cAMP to heighten promoter expression via two regions termed PACAP Activating Response Element (PARE) I and PARE II(123). In the mouse GnRHR promoter, neither PARE I nor II mediate a cAMP response. Overall, there is divergence in GnRH and cAMP regulation of the human, ovine, racine and murine GnRHR; however the physiologic relevance of this divergence is unclear due to the use of heterologous cell lines and GnRHR promoter constructs that display little or no activity.

C. *The Molecular Mechanisms Underlying Estrogen Regulation of the GnRHR Gene Are Unknown*

Estradiol-17β exerts a profound effect on the number of GnRH receptors in the anterior pituitary gland (124-128). This effect of estrogen is generally recognized as one of the primary mechanisms underlying increased pituitary sensitivity to GnRH during the pre-ovulatory period and thus contributes to the high rates of LH secretion necessary for ovulation (14). Numerous studies have demonstrated coordinate changes in GnRHR mRNA and GnRHR numbers associated with estradiol treatment (18;19,21-24; 107). It is possible that the stimulatory effects of estrogen on GnRHR expression are mediated via a post-transcriptional mechanism such as mRNA stabilization; however, the ability of actinomycin D to block estrogen up-regulation of GnRHR numbers in primary cultures of pituitary cells does not support this theory (126;128). A canonical estrogen response element (ERE) is not present in the ovine GnRHR gene and, in fact, an ERE has not been identified in any GnRHR gene promoter reported. In line with this, ER binding to the ovine GnRHR promoter and estrogen regulation of this promoter using *in vitro* transient transfection paradigms is undetectable (36). As such, progress in identifying the molecular mechanisms underlying estrogen input to the GnRHR gene has been slow. However, transgenic mice represent a powerful model system in which estrogen regulation of GnRHR gene expression is reliably recapitulated.

As an alternative strategy to explore estrogen regulation of the GnRHR promoter, transgenic mice have been established harboring the ovine GnRHR gene promoter fused to the cDNA for luciferase. Interestingly, approximately 9,100 bp of 5' flanking region from the ovine GnRH receptor (oGnRHR) gene is devoid of transcriptional activity in

gonadotrope-derived cell lines and is not responsive to either estradiol or GnRH. In stark contrast, this same 9,100 bp promoter fragment directs tissue-specific expression of luciferase to the pituitary, brain, and gonads in multiple lines of transgenic mice recapitulating the endogenous sites of GnRHR gene expression. To test for hormonal regulation of the 9,100-bp promoter transgene, ovariectomized transgenic females were treated with a GnRH antiserum alone or in combination with estradiol and D-Ala-6 GnRH. Both hormonal treatments increased luciferase transgene expression in the pituitary mimicking the increase in GnRHR number seen in the whole animal but absent *in vitro* (36). Thus, the use of transgenic mice is important to confirm the physiological relevance of *in vitro* promoter studies.

D. *Activin Responsiveness of the Murine GnRHR Gene Promoter is Mediated at GRAS*

Activin, a member of the transforming growth factor (TGFβ) family of growth and differentiation factors, regulates the mouse GnRHR gene promoter through GRAS (112;129). As such, mutation of the GRAS element in the murine GnRHR promoter results in the total loss of activin regulation. Although GRAS clearly acts as an activin/TGFβ response element, it also mediates synergistic activation of the GnRHR promoter by activin and GnRH (130). Stimulation of αT3-1 cells with activin causes only a weak increase in mouse promoter-luciferase expression, however removal of activin by follistatin, a powerful activin binding protein, decreases basal expression by approximately 60% (4). The lack of responsiveness of αT3-1 cells to exogenous activin treatment is due to the fact that these cells, like *bona fide* gonadotropes, constitutively

produce and secrete activin (1;55). Biologically then, αT3-1 cells are constantly activin stimulated.

Activin binds its membrane receptor on gonadotropes and initiates an intracellular signaling cascade mediated by the Smad family of proteins (100; 101;131). Specifically, activin binds the extracellular activin type II receptor that in turn heterodimerizes with the type I receptor. Upon activation by the type II receptor, the type I receptor phosphorylates receptor mediated Smads (Smad 2 and Smad 3). In the case of αT3-1 cells and the GnRHR promoter, Smad 3 is phosphorylated by ligand bound activin receptor complexes and dimerizes with Smad 4, the common binding partner. This complex can then translocate to the nucleus where it binds a Smad binding element (SBE) located within GRAS to increase gene transcription (2).

Although GRAS clearly mediates activin input *in vitro*, the *in vivo* role of this element is less clear. Kumar et al found that in mice where the activin type II receptor was disrupted by homologous recombination no change in GnRHR expression was apparent (99). This data is in contrast to ours in which two lines of -1900μGRAS transgenic mice have significantly lower expression of pituitary luciferase than lines with the wild type transgene. Kumar's work offered no explanation of the relatively high embryonic mortality of the activin type II receptor knockouts or their phenotypes. Evidence suggests that the interaction between type II and type I activin receptors can be somewhat promiscuous (132;133). Thus the possibility exists that some of the components of activin/TGFβ signaling may be retained in the knock out mice. Therefore, we recognize the possibility that other TGFβ family members such as bone morphogenetic proteins (BMPs) might play a role in GnRHR gene regulation.

The proximal 600 bp of the mouse and rat GnRHR promoters are highly conserved including 100% conservation in the GSE and AP-1 elements. Despite this high degree of homology, the promoters differ in regard to activin regulation. In particular, the mouse GnRHR promoter is activin responsive while the rat promoter is not. This observation was intriguing due to the fact that the rat GRAS homolog contains only 1 bp difference from the mouse GRAS element, an established activin response element in the mouse GnRHR promoter. I hypothesized that exchanging the one nucleotide between the mouse and rat GRAS elements, effectively creating a rat GnRHR promoter with the mouse GRAS homolog, would be sufficient to confer activin regulation to the rat promoter. This hypothesis proved incorrect as the mouse GRAS homolog was not sufficient to confer activin regulation to the rat promoter. This was the first evidence that while necessary, GRAS alone does not account for activin regulation of the mouse GnRHR gene promoter (3). Furthermore, the divergence in activin responsiveness between the mouse and rat GnRHR genes suggests the presence of additional activin responsive regions in the mouse promoter. The focus of the work in this dissertation was to find and characterize the downstream activin responsive element (DARE) in the mouse promoter.

IV. *Statement of the Research Problem*

The pulsatile discharge of GnRH from hypothalamic neurons not only stimulates but is obligatory for synthesis and secretion of LH and, to a lesser extent, FSH from gonadotrope cells of the anterior pituitary gland (9-12). In addition, expression of genes encoding the common α subunit, unique β subunits, and GnRHR itself are absolutely

dependent on GnRH input (13). Given the central role of GnRH in reproduction much effort has been devoted toward understanding molecular and physiological regulation of GnRH and its pituitary receptor.

Although the rat and mouse GnRHR promoters are highly conserved, there is one striking difference. There is a 1 bp difference in the GRAS element between the rat and mouse promoters that we thought accounted for their difference in activin regulation. Specifically, the mouse promoter is activin responsive, while the rat GnRHR promoter is not. Since the GRAS element is known to mediate activin regulation of the mouse GnRHR promoter, we felt it was likely that the 1 bp transition accounted for the difference in activin regulation of the two promoters. However, exchanging the 1 bp between the rat and mouse promoter did not account for the difference in activin regulation. Thus, this was the first data to suggest that while mouse GRAS is necessary, it is not sufficient for activin responsiveness of the mouse promoter. Therefore, a central goal of my research was to identify the additional activin regulatory elements in the mouse GnRHR promoter. In the course of my research, I established that a downstream activin response element (DARE) is necessary for full activin regulation of the murine GnRHR gene promoter. Furthermore, a role for homeodomain proteins in mediating activin responsiveness of the murine GnRHR promoter through DARE is emerging. Details of these studies will be expounded upon in subsequent chapters of this dissertation.

In vivo activin regulation of gonadotropes and the GnRHR gene promoter is critical for reproductive function. As previously mentioned, it is vital to confirm *in vitro* promoter analysis with *in vivo* studies to confirm physiological relevance. Thus, murine -

1900wt transgenic mice and transgenic mice containing 1900 bp of the murine GnRHR promoter fused to the coding sequence of luciferase in which the GRAS element is mutated (1900μGRAS) provide a powerful model. Infection of both lines of transgenic mice with an adenovirus expressing the human 288 follistatin protein revealed the importance of the GRAS element in mediating activin regulation of the murine GnRHR promoter *in vivo*. Again, details of these studied will be discussed at length later in this dissertation (see chapter 5).

CHAPTER 3

ACTIVIN RESPONSIVENESS OF THE MURINE GONADOTROPIN-RELEASING HORMONE RECEPTOR GENE IS MEDIATED BY A COMPOSITE ENHANCER CONTAINING SPATIALLY DISTINCT REGULATORY ELEMENTS

INTRODUCTION

The interaction of gonadotropin-releasing hormone (GnRH) with specific, high-affinity receptors located on gonadotrope cells of the anterior pituitary gland is central to the regulation of reproductive function in mammals. The pulsatile discharge of GnRH from hypothalamic neurons not only stimulates but also is obligatory for synthesis and secretion of luteinizing hormone (LH) (9;134). Given the pivotal role of GnRH in reproduction much effort has been expended toward understanding the physiological consequences of regulation of GnRH and its cognate pituitary receptor. A member of the superfamily of hepathelical G-protein coupled receptors, the GnRH receptor (GnRHR) is coupled to activation of $G\alpha_{q/11}$ which initiates multiple intracellular responses including LH secretion and increased expression of the common α and unique LHβ subunit genes and the GnRHR gene itself (11;13;134). It is clear that relative changes in GnRH secretion from the hypothalamus are important determinants of LH secretion; however, changes in the number of pituitary receptors for GnRH are also implicated as an important mechanism underlying the regulation of gonadotropin secretion (21;22;135). Thus, both hypothalamic secretion of GnRH and pituitary concentration of GnRH receptors are targets for regulation. In regard to the latter, of the multiple inputs that have

been implicated in affecting changes in GnRHR numbers, perhaps the most dramatic are those associated with estradiol-17β, GnRH and activin (18;23;24;136).

A member of the transforming growth factor-β (TFGβ) family of signaling molecules, activin represents homo- or heterodimeric complexes of the different inhibin β-subunits and is implicated in a number of physiological processes ranging from embryonic development and tissue-patterning to the synthesis and secretion of follicle stimulating hormone (FSH) (137-139). As with other members of the TGFβ family, intracellular signaling by activin is initiated by binding to a Type II receptor at the plasma membrane and subsequent recruitment and phosphorylation of a Type I receptor (137;138). Functionally, this complex acts as a serine/threonine kinase and phosphorylates the activin/TGFβ restricted Smad proteins (Smad2 or 3) (131;137;138). These Smad proteins then typically associate with the common Smad partner (Smad4) and translocate from the cytoplasm to the nucleus. In the nucleus, Smads bind to specific DNA regulatory elements to alter the rate of transcription of target genes; however, as Smad proteins alone display relatively weak DNA binding transcriptional regulation by these proteins is often achieved via multi-protein complexes that contain both Smad and non-Smad protein partners (131;137;138).

Within the anterior pituitary gland the activin β-subunits are produced by gonadotrope cells as is the activin binding protein follistatin (137;140). The latter is considered to be a primary modulator of the biological effects of activin and thus prevents activin binding to its cognate receptor (44;141;142). In addition to gonadotrope expression, follistatin is also produced by pituitary folliculostellate cells (143;144). Thus, while initially described as an endocrine regulator of pituitary function, autocrine and

paracrine mechanisms are likely more important components of activin signaling in the pituitary gland (145;146). In this regard, it is clear that regulation of follicle stimulating hormone (FSH) production is a central biological role of activin in the pituitary (44). The effects of activin on FSH are evident as both increased secretion and enhanced expression of the FSHβ subunit gene (44;96;102). In addition to its effects on FSH, multiple studies have established that activin regulates GnRHR expression (1;2;55;129;147). At issue then are the mechanisms that account for activin regulation of FSHβ and GnRHR gene expression. To address the latter, we have examined transcriptional activity of the GnRHR gene promoter in the gonadotrope-derived αT3-1 cell line (35). Given that αT3-1 cells, like gonadotropes, express activin and activin receptors we used an indirect approach based on the ability of follistatin to bind and inactivate activin to localize activin responsiveness of the murine GnRHR gene to an element termed the GnRH receptor activating sequence or GRAS (1).

Consistent with its ability to mediate activin responsiveness, GRAS has been shown to bind members of the Smad family of transcription factors (2;129). However, the functional phenotype of GRAS depends not only on Smad binding but also AP-1 and FoxL2, a member of the winged-helix or forkhead family of transcription factors (2;129). Thus, as is the case with many activin/TGFβ response elements, GRAS is a composite enhancer whose functional activity is dependent on the binding of a Smad protein complex and multiple non-Smad protein partners (2;129). Furthermore, elimination of any one of these binding components eliminates the functional activity of GRAS (2). In light of this, we predicted that a single nucleotide difference between mouse GRAS and the rat GRAS homologue (148) would deprive the latter of activin responsiveness.

Consistent with this prediction we find that the rat GnRHR promoter is not responsive to activin. However, GRAS alone cannot account for the functional divergence in activin responsiveness of the proximal promoters of the mouse and rat GnRHR genes. This observation raised the possibility that, while necessary, GRAS may not be sufficient for activin responsiveness of the mouse GnRHR gene. Consistent with this possibility, we find that activin responsiveness of the mouse GnRHR gene requires not only GRAS but also a previously undefined element located approximately 15 bp downstream of GRAS that we term the downstream activin regulatory element (DARE). Based on the ability of DARE to interact with a recombinant homeodomain we suggest that activin regulation of the murine GnRHR gene is mediated by a complex "enhanceosome" (149) that includes spatially distinct Smad and homeodomain binding components – a scenario reminiscent of the mechanism recently described for activin regulation of the rat and ovine FSHβ subunit genes (102;103).

MATERIALS AND METHODS

Materials

αT3-1 cells were generously provided by Dr. Pamela Mellon (University of California San Diego). Dr. Wylie Vale (Salk Institute, La Jolla, CA) provided the adenoviral follistatin (AdCAFS288) and GFP (Ad-GFP) expression vectors (150) whereas purified LHX2 homeodomain and LHX2 antiserum (65) was kindly provided by Dr. Mark Roberson (Cornell University, Ithaca, NY). The NF-Y antibody was obtained from Rockland Inc. (Gilbertsville, PA). Oligonucleotides were obtained from Invitrogen (Carlsbad, CA) and Colorado State University Macromolecular Core Facility (Fort

Collins, CO). DNA sequencing was conducted by Macromolecular services at the University of California Davis. Restriction enzymes and DNA modifying enzymes were obtained from Fermentas (Hanover, MD) and New England Biolabs (Beverly, MA). Hyperfilm and G-25 Microspin columns were obtained from Amersham Biosciences (Piscataway, NJ). The amplification and purification of Ad-CAFS288 and Ad-GFP were performed as previously described (151).

Transfections and Adenovirus Infections

Culture of αT3-1 cells and transient transfections using SuperFect (Qiagen, Valencia, CA) were performed as previously described (1;2), except 0.8μg of the test luciferase plasmid and 0.2μg of RSV-β-galactosidase transfection efficiency control vector were used and after 3 h. incubation of cells with SuperFect/DNA mixture, media was replaced with growth media containing 1000 MOI (multiplicity of infection) of AdCAFS288 or the same MOI of an adenoviral construct expressing GFP. Cells were harvested 48 h post-infection and assayed for luciferase activity (1;2). Values were normalized for transfection efficiency by dividing the luciferase activity by β-galactosidase activity. Within a transfection, all treatments and vectors were tested in triplicate and all transfections were repeated at least 3 times using different plasmid preparations. Values are presented as the mean ± SEM.

Electrophoretic Mobility Shift Assay(EMSA)

Electrophoretic mobility shift assays (EMSA) were conducted as previously described (1;29;152;153). Briefly, αT3-1 nuclear extracts (2;29) were incubated with

Dignam D buffer (20 mM HEPES, 20% glycerol, 0.1 M KCl, 0.4 mM EDTA), poly(dI-dC) (2 μg), radiolabeled probe (100,000 cpm), and appropriate unlabeled competitor for 20 min at room temperature. For the NF-Y supershift, the established NF-Y binding site from the bovine LHβ-subunit gene (85) served as the radioactive probe and 2 μg of anti-NF-Y antibody or the same mass of a non-specific IgG was added after the 20 min incubation and then incubated for an additional 10 min. Complexes were resolved by electrophoresis in pre-run (100 V for 1 h) 5% polyacrylamide gels in 0.5X tris/glycine buffer (29) and visualized by autoradiography. In Figure 13, purified LHX2 homeodomain protein (65) was incubated at 4°C in Dignam buffer D (20 mM HEPES (pH 7.9), 20% glycerol (vol/vol), 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol) with 2 μg of poly (dI-dC) for 10 min. After incubation, radiolabeled probe (100,000 cpm) and appropriate amount of unlabeled competitor were added and incubated for 20 min. For supershift of LHX2 homeodomain protein, 1 μl of rabbit anti-LHX2 homeodomain antiserum or normal rabbit serum (NRS) was added and incubated for an additional 10 min. Reactions were electrophoresed in tris-glycine buffer for 2-2.5 h at 35 mA in 6% polyacrylamide gels. Gels were pre-run at 100 V for 1 h. Gels were transferred to blotting paper, dried, and exposed to Hyperfilm MP for approximately 16 h at –70°C. In all EMSA experiments, oligonucleotides were labeled using polynucleotide kinase and [γ-^{32}P]ATP. Double-stranded DNA probes were purified by centrifugation through a G-25 Microspin column.

Vector Construction

The plasmid pMGR-600Luc, with approximately 600 bp of the 5'-flanking region from the murine GnRHR gene fused to the cDNA encoding luciferase in the pGL3-Basic vector (Promega, Madison, WI)(152), and 3XGRAS-Luc (28;29) have been previously described. An analogous promoter vector for the rat was generated by digesting –1100 ratGnRHR (pLuc1.1GnRH-R BstEII, provided by R. Counis, Université Pierre et Marie Curie, Paris, France) with Xba I/Nco I and ligating into the Nhe I/Nco I sites of pGL3-Basic, to produce pRGR-600Luc.

The –600 vectors containing 1 bp exchange in the GRAS element were constructed by sequential rounds of polymerase chain reaction (PCR). Overlapping primers RMGs and RMGas, (in which the rat GRAS sequence is replaced by that of the mouse) were used in separate reactions with pGL3 flanking primers GL2 and RV3 and wild-type –1100 rat GnRHR promoter template to generate downstream (RMGs and GL2) and upstream (RGMas and RV3) fragments. Products were gel-isolated and used as template in a second PCR reaction using GL2 and RV3, and the product subcloned into pGEM-T Easy (Promega, Madison, WI). Presence of the mutation was confirmed by sequence analysis (Davis Sequencing, Davis, CA). A –600 promoter fragment of rat GnRHR promoter with murine GRAS was then excised by Xba I/Nco I digestion and cloned into the Nhe I/Nco I sites of pGL3-Basic (rat-GRAS-repair). A similar approach was performed using MRGs and MRGas and murine –600 GnRHR promoter to generate mouse GnRHR with the rat GRAS sequence, the final product being excised from pGEM-TEz with Kpn I/Nco I and subcloned into pGL3-Basic at Kpn I/Nco I (prGRAS/MGRLuc).

The –500 exchanges between the 5' end of the mouse and rat promoters were created by reciprocal exchanges of Kpn I/Mfe I (-600/-500) and Mfe I/Nco I (-500/-8) promoter fragments of pMGR-600Luc and rat-GRAS-repair vectors, ligated into pGL3-Basic at Kpn I/Nco I to generate pr(-500)MGR and pm(-500)RGR.

For the –250 exchanges, the fusion point selected represents a sequence conserved in both species where half-sites for the blunt-cutting restriction enzymes Ecl136 I and Sma I abut. Upstream fragments (-600/-250) were generated by PCR using templates pMGR-600Luc and rat-GRAS-repair, with primers RV3 and PIas, which incorporates at it's 5' end a full Ecl136 I site. The downstream fragments were created by PCR from pMGR-600Luc (oligonucleotides GL2 and mPIs) and pRGR-600Luc (GL2 and rPIs), incorporating a Sma I site at their upstream end. PCR products were cloned into pGEM-T Easy, and sequence confirmed by sequence analysis. Fragments excised with Kpn I and Ecl136 I (upstream) and Sma I/Nco I(downstream), and ligated together into pGL3-Basic, the blunt Sma I and Ecl136 I half-sites combining to regenerate the native sequence, generating pr(-250)MGR and pm(-250)RGR. The –150 exchanges used a similar approach, only PCR primers PIIas and RV3 (upstream) or PIIs and GL2 (downstream) were used to generate promoter fragments from pMGR-600Luc and rat-GRAS-repair that were cloned into pGEM-T Easy, excised, and joined in pGL3-Basic at compatible Bcl I and Bgl II sites derived from the PIIs and PIIas to generate pr(-150)MGR and pm(-150)RGR.

The 36 bp exchange clones were made by sequential rounds of PCR, as with the GRAS exchanges. Using pMGR-600Luc template, upstream (primers RV3 and SW1as) and downstream (GL2 and SW1s) fragments of the mouse promoter with 36 bp of rat

sequence substituted between GRAS and AP-1 elements were generated by PCR. These were gel isolated, and used as template in a second round of PCR, using RV3 and GL2. The same approach generated the analogous rat vector, using rat-GRAS-repair with RV3 and SW2as (upstream) and GL2 and SW2s (downstream). Products of the second round of PCR were subcloned into pGEM-T Easy, and transferred to pGL3 using Kpn I/Nco I sites, to generate pSW1MGRLuc and pSW2mGRAS/RGRLuc.

The block replacement construct pBR13 was previously described (29). Block replacement constructs pBR14, pBR15 and pBR16 were made by combining three pieces. An upstream fragment was generated by PCR using an oligonucleotide that incorporated a Hpa I site immediately downstream of the GRAS element (HPAas), using pMGR-600Luc as template. This product was cloned into pGEM T-Easy and excised with Sac I/Hpa I. A downstream fragment was attained by cutting pSW1MGRLuc (mouse promoter with 36 bp of the rat) with Ssp I, just upstream of the AP-1 element, and Nco I. The sequence between GRAS and AP-1 was generated by annealing sense and antisense oligonucleotides in which native sequence is replaced with the 8bp Not I restriction site (BR14s/BR14as, BR15s/BR15as, BR16s/BR16as) and the three promoter pieces were combined by either sequential or concurrent ligation, and inserted into Sac I/Nco I sites of pGL3-Basic.

The 2 bp mutants (14.1-5 and 15.1-4) were made by directly ligating an upstream PCR fragment generated with RV3 and the appropriate downstream primer (14.1n, 14.2n, 14.3n, 14.4n, 14.5n, 15.1n, 15.2n, 15.3n, 15.4n) to the Ssp I/Nco I fragment of pSW1MGRLuc, described above, cutting with Sac I/Nco I and ligating into pGL3-Basic. For the TAAT motif double mutant (14.4/15.4) and mouse-to-rat substitutions (-600–

Rat14.4, -600–Rat15.1/2) the analogous upstream PCR product (RV3 to 14.4/5,R/M14.4A or R/M15.1/2) added an Eco RV half-site. It was cloned into pGEM T-Easy, then excised with Sac I/EcoRV and ligated with the Ssp I/Nco I fragment of pSW1MGRLuc into pGL3-Basic.

Statistical Analysis

In every transfection, each treatment and vector was analyzed in triplicate and the experiments replicated three times using different plasmid preparations. Data are expressed as means $\pm$ the standard error of the mean. Student's T-test was used to compare the difference between cells infected with Ad-GFP and Ad-CAFS288 within a vector. In Figures 4, 5 and 8, data were analyzed by ANOVA and, when the f-test was significant ($P<0.05$), means were separated by Tukey's HSD or Duncan's Multiple Range Test.

RESULTS

Adenoviral delivery of follistatin is effective in attenuating transcriptional activity of the GnRHR gene promoter. As αT3-1 cells produce endogenous activin B these cells essentially exist in a constitutively activin-stimulated state (1). As such, we have utilized an indirect approach based on the addition of the activin binding protein follistatin to functionally map activin/TGFβ regulation of the GnRHR gene promoter - an approach that localized activin responsiveness of the GnRHR gene to GRAS (1). Unfortunately, this approach requires a continual supply of purified or recombinant

follistatin. As an alternative, we tested the efficacy of adenoviral delivery of human follistatin (AdCAFS288 – a generous gift from Dr. Wylie Vale) (150) in a transfection/infection paradigm. In our application, αT3-1 cells were transfected with either the mouse or rat –600 GnRHR promoters fused to luciferase, the mouse –600 promoter containing a loss of function mutation in GRAS (29) or 3 copies of GRAS fused to the rat prolactin (Prl) minimal promoter (28). Three hours following transfection, cells were infected with 1000 MOI of AdCAFS288 or the same MOI of an adenoviral construct expressing GFP. Consistent with earlier work using soluble recombinant follistatin (1), infection with AdCAFS288 led to an approximately 50% reduction in activity of the –600 wild-type mouse GnRHR promoter and a greater than 90% attenuation in activity of the 3XGRAS-LUC vector as compared to cells infected with Ad-GFP (Figure 7). Thus, adenoviral delivery of a follistatin cDNA is an effective paradigm for removal of activin input to αT3-1 cells. As expected, transcriptional activity of the –600 promoter containing mutated GRAS was unaffected by AdCAFS288. The proximal promoter of the rat GnRHR gene was not affected by follistatin expression.

A single nucleotide change in GRAS does not account for the functional divergence in activin responsiveness of the mouse and rat GnRHR gene promoters. Despite greater than 90% homology, the proximal promoters of the mouse and rat GnRHR genes are functionally divergent in regard to activin responsiveness - while the mouse promoter is activin responsive, the rat proximal promoter is not (Figure 7). A direct comparison of these promoters reveals perfect conservation of sequence in the SF-1 and AP-1 binding sites; however, the rat GRAS homologue differs from mouse by a single nucleotide transition (Figure 8). Given the requirement for GRAS in mediating

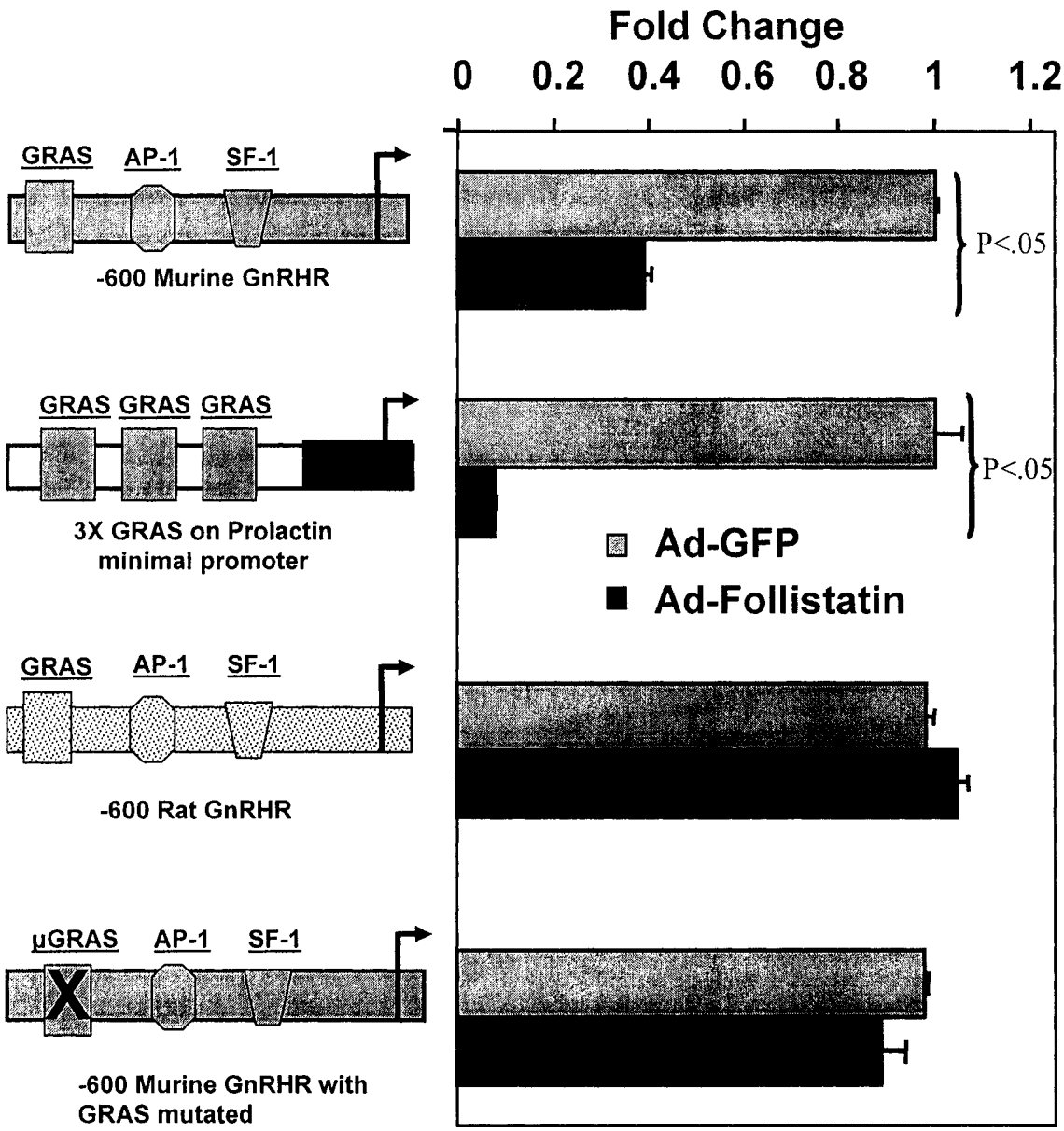

Figure 7. Adenoviral delivery of follistatin attenuates transcriptional activity of the proximal promoter of the mouse but not rat GnRHR genes. The indicated luciferase expression vectors were transiently transfected with pRSV-LacZ into αT3-1 cells. Three hours following transfection, cells were infected with either Ad-Follistatin or Ad-GFP. Cells were harvested 48 h after infection and cellular lysates were assayed for luciferase and β-galactosidase activity. Luciferase values were corrected for β-galactosidase activity, and values are expressed as fold change in adjusted luciferase activity in Ad-Follistatin vs. Ad-GFP infected cells. Values represent the mean ± sem of triplicate samples in 3 separate transfections. The relative positions of GRAS, AP-1 and SF-1 elements are indicated in the mouse and rat promoters.

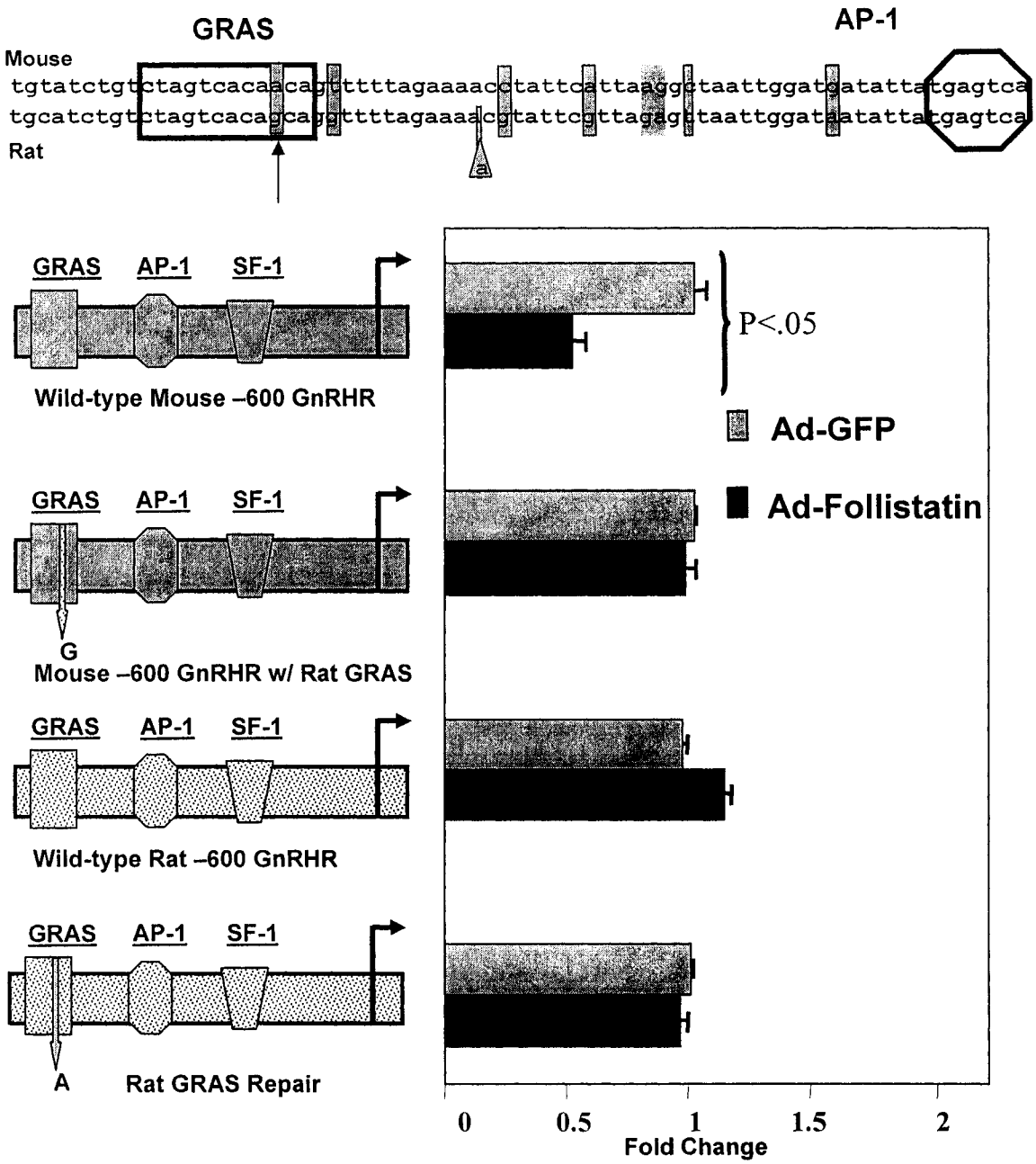

Figure 8. A single nucleotide change in GRAS does not account for the functional divergence in activin responsiveness of the mouse and rat GnRHR genes. αT3-1 cells were transfected with pRSV-LacZ and luciferase vectors containing approximately 600 bp of proximal promoter from either the mouse or rat GnRHR genes, the mouse promoter containing the rat GRAS homologue or the rat promoter containing mouse GRAS (rat-GRAS-repair). Three hours following transfection, cells were infected with either Ad-follistatin or Ad-GFP. Cells were harvested 48 h after infection and cellular lysates were assayed for luciferase and β-galactosidase activity. Luciferase values were corrected for β-galactosidase activity, and values are expressed as fold change in adjusted luciferase activity in Ad-follistatin vs. Ad-GFP infected cells. Values represent the mean ± sem of triplicate samples in 3 separate transfections.

activin regulation of the mouse GnRHR gene, we hypothesized that this single nucleotide change in the GRAS element would account for the divergence in activin responsiveness of the rat and mouse GnRHR promoters. To test this hypothesis, chimeric promoters were constructed in which the one bp difference in the GRAS element was reciprocally exchanged between the two promoters essentially creating a rat GnRHR promoter with an "intact" mouse GRAS (rat-GRAS-repair) and a mouse GnRHR promoter with the rat GRAS homologue. We reasoned that this 1 bp exchange would render the rat promoter activin responsive whereas the activin response of the mouse promoter would be lost. Consistent with this prediction, the transcriptional activity of the mouse promoter containing the rat GRAS homologue was unaffected by follistatin (Figure 8). Surprisingly, however, activin responsiveness was not conferred on the rat promoter containing intact mouse GRAS.

A 36 bp region located between GRAS and AP-1 is necessary for activin responsiveness of the mouse GnRHR gene promoter. The data in Figure 8 are subject to several interpretations. First, the rat promoter contains an element that actively represses the functional activity of GRAS. Alternatively, the rat promoter is lacking an additional regulatory element that is necessary for activin responsiveness – an element that would, presumably, be present in the mouse promoter. We reasoned that both possibilities would be testable by constructing a series of chimeric promoters in which progressive regions of proximal promoter were reciprocally exchanged between the mouse promoter and rat-GRAS-repair promoter. In short, we were screening for a promoter exchange that presented as both a loss and gain of function. The initial set of chimeric promoters represented exchanges at approximately -500, -250 and -150 bp

between the wild type mouse and rat promoters (Figure 9). There was no impact of exchanging the distal 100 bp regions on the follistatin response of either the mouse or rat-GRAS-repair promoters (Figure 9). In contrast, placement of approximately 250 or 350 bp of distal mouse sequence was sufficient to confer follistatin responsiveness to the proximal rat promoter. Thus, these exchanges defined a region between –500 and –250 in the mouse promoter that leads to a gain of activin regulation of the rat promoter. The converse result was observed when rat sequence was exchanged into the mouse promoter – either 250 or 350 bp of distal rat sequence eliminated follistatin response. As such, the critical regulatory information must reside between –500 and –250. Importantly, the contribution of at least a portion of this sequence was previously evaluated using a series of scanning mutations that placed the recognition site for NotI in 12 "block replacements" progressively from -500 to –365 (28). Only one of these (BR11) affected the follistatin/activin response of the mouse GnRHR gene promoter and, in fact, served to functionally identify GRAS (28). Thus, this prior work allowed us to direct further exchanges to the promoter regions residing downstream of GRAS between approximately –365 and –250. Also it was notable that this region contains several potential regulatory elements including a composite binding site for Oct-1 and NF-Y originally termed SURG-1 that contributes to GnRH responsiveness of the murine promoter (33). Thus, we first focused on this 36 bp region and constructed chimeric promoters in which the homologous sequence was reciprocally exchanged between the mouse GnRHR promoter and the rat-GRAS-repair promoter (Figure 10). This 36 bp reciprocal exchange revealed the desired phenotype, i.e., both a gain of function (follistatin responsiveness) of the rat-GRAS-repair promoter and a loss of function of the mouse promoter (Figure 10).

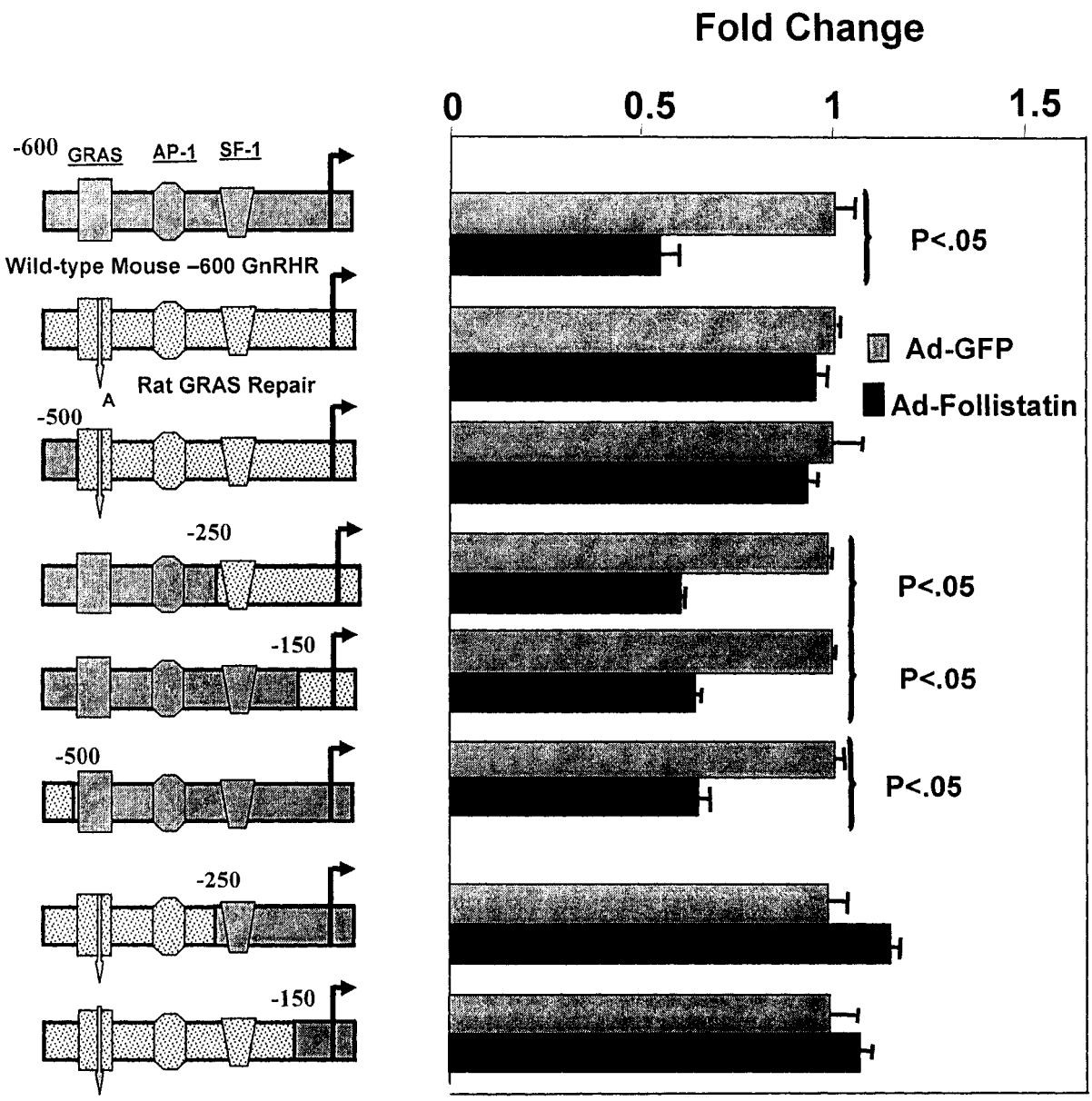

Figure 9. A 36 bp region located between GRAS and AP-1 is necessary for activin responsiveness of the mouse GnRHR gene promoter. αT3-1 cells were transfected with pRSV-LacZ, –600 wild-type mouse promoter, rat-GRAS-repair or the indicated chimeras of the mouse and rat GnRHR promoters. Three hours following transfection, cells were infected with either Ad-follistatin or Ad-GFP. Cells were harvested 48 h after infection and cellular lysates were assayed for luciferase and β-galactosidase activity. Luciferase values were corrected for β-galactosidase activity, and values are expressed as fold change in adjusted luciferase activity in Ad-follistatin vs. Ad-GFP infected cells. Values represent the mean ± sem of triplicate samples in 3 separate transfections.

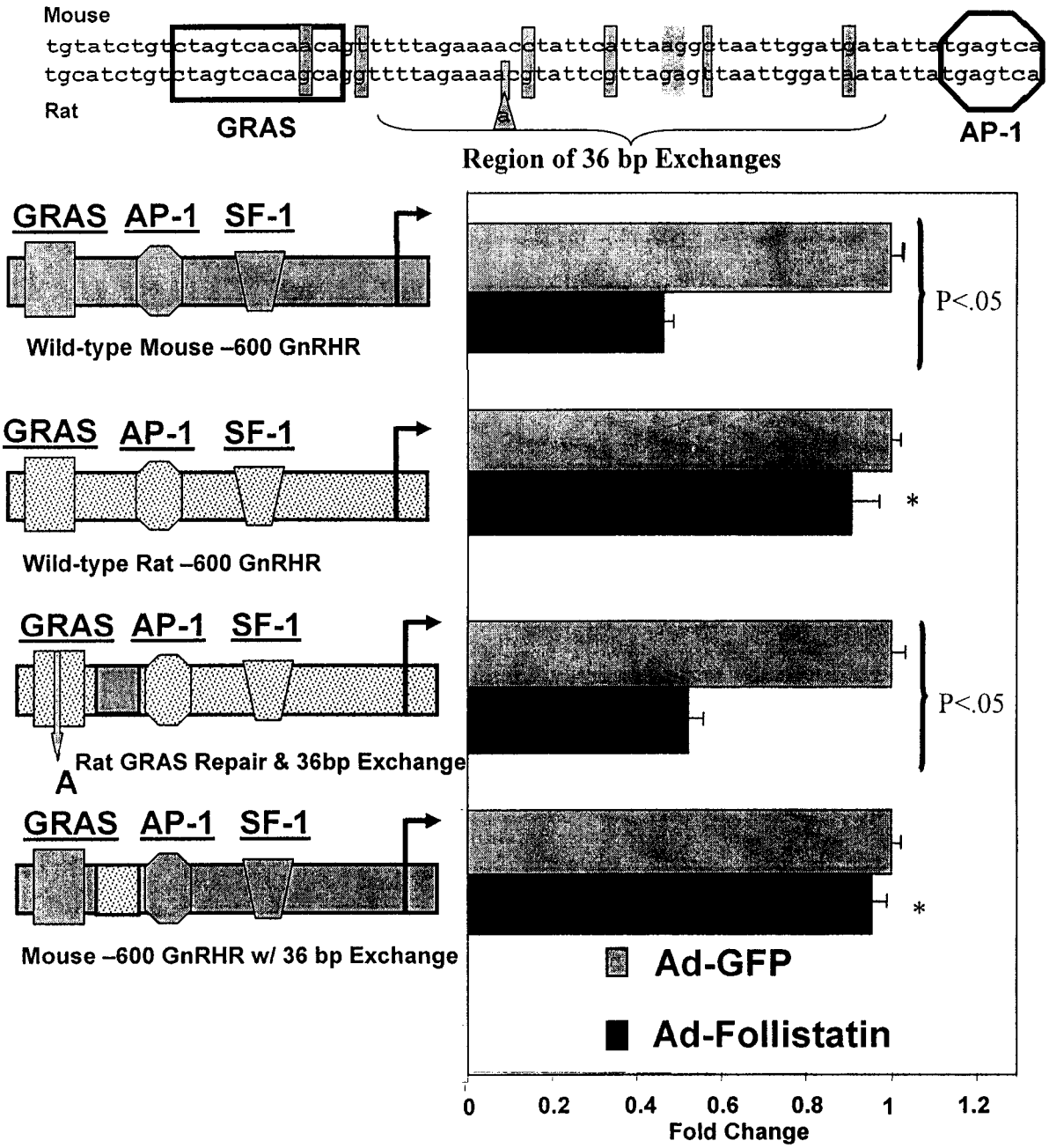

Figure 10. A 36 bp region between GRAS and AP-1 is necessary for activin responsiveness of the mouse GnRHR promoter and sufficient to confer activin responsiveness on the rat-GRAS-repair promoter. αT3-1 cells were transfected with pRSV-LacZ, the wild-type mouse and rat GnRHR promoters or the indicated chimeras of the mouse and rat promoters. Three hours following transfection, cells were infected with either Ad-follistatin or Ad-GFP. Cells were harvested 48 h after infection and cellular lysates were assayed for luciferase and β-galactosidase activity. Luciferase values were corrected for β-galactosidase activity, and values are expressed as fold change in adjusted luciferase activity in Ad-follistatin vs. Ad-GFP infected cells. Values represent the mean ± sem of triplicate samples in 3 separate transfections. * value is different ($p<0.05$) from fold-change of wild-type –600 mouse GnRHR promoter.

Thus, both GRAS and one or more regulatory elements arrayed within a 36 bp region located between GRAS and AP-1 would appear to be necessary for activin responsiveness of the mouse GnRHR gene.

The downstream activin regulatory element (DARE) localizes to an 18 bp sequence between –365 and –348 in the proximal promoter of the mouse GnRHR gene. The data in Figure 10 suggest the presence of an additional regulatory element located between –375 and –340 that cooperates with GRAS to confer activin responsiveness. To refine the functional boundaries of this accessory element(s) we next expanded the scanning mutagenesis approach that was used to define GRAS and placed the 8 bp recognition motif for Not I between –372 and –340 in the context of the mouse GnRHR promoter. In all, 4 separate mutations (BR13-BR16) were constructed (Figure 11). While BR13 and BR16 had no effect on activin responsiveness (follistatin repression), the follistatin response of the mouse promoter was eliminated by placement of Not I between –355 and –348 (BR15) and attenuated by BR14 (-365 to -358)(Figure 11). Thus, functional mapping localizes a novel downstream activin regulatory element (DARE) to a region between –365 and –348 in the mouse GnRHR gene promoter. Finally, it is interesting to note that, like mutations in GRAS, the BR14 and BR15 mutations decrease "basal" promoter activity to a level indistinguishable from that seen with follistatin treatment of the wild-type mouse GnRHR promoter (Figure 11).

NF-Y does not account for the functional properties of DARE. A canonical CCAAT motif located within the 36 bp exchange region has been shown to bind the CCAAT box binding factor NF-Y (33;154). Interestingly, the BR15 mutation effectively disrupts this motif and also disrupts activin responsiveness; however, BR16 also

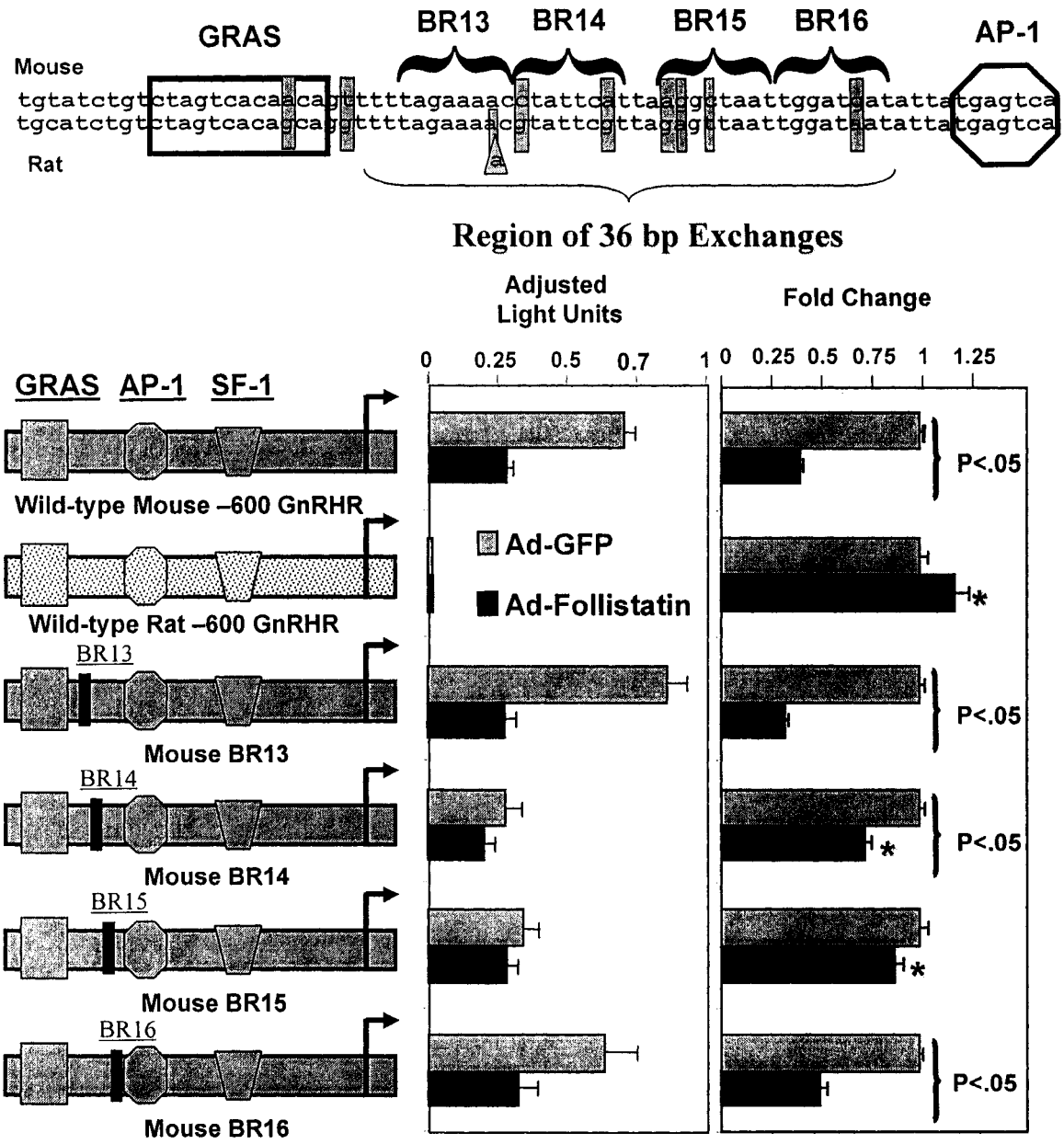

Figure 11. The downstream activin regulatory element (DARE) localizes to an 18 bp sequence between –365 and –348 in the proximal promoter of the mouse GnRHR gene. αT3-1 cells were transfected with pRSV-LacZ, the wild-type mouse or rat GnRHR promoters or mouse GnRHR promoters containing a series of scanning NotI mutations. Three hours following transfection, cells were infected with either Ad-follistatin or Ad-GFP. Cells were harvested 48 h after infection and cellular lysates were assayed for luciferase and β-galactosidase activity. Luciferase values were corrected for β-galactosidase activity, and values are expressed as both adjusted light units (left-hand panel) or fold change in adjusted luciferase activity in Ad-follistatin vs. Ad-GFP infected cells (right-hand panel). Values represent the mean ± sem of triplicate samples in 3 separate transfections. * value is different ($p<0.05$) from fold-change of wild-type –600 mouse GnRHR promoter.

eliminates the core CCAAT motif but has no effect on the follistatin response of the mouse promoter. Thus, it seems unlikely that this motif or NF-Y accounts for the functional properties of DARE. Nevertheless, we used EMSA to directly test the ability of DARE to displace NF-Y binding to an established NF-Y binding site in the LHβ subunit gene (85). Consistent with Keri et al. (85), a specific bound complex was evident with the LHβ probe and was shifted by anti-NF-Y antiserum (Figure 12). Homologous competition displaced binding to the radioactive probe in the supershifted complex. In contrast, while the BR14 oligonucleotide displayed a limited capacity to displace NF-Y binding to the radioactive probe, essentially no competition was evident with increasing concentrations of non-radioactive DARE, or DARE containing the BR15 and BR16 mutations. Thus, based on both the functional and EMSA data it would not appear that the CCAAT box or NF-Y contributes to the functional activity of DARE. Finally, in addition to the NF-Y containing complex, a faster migrating complex was evident with LHβ probe; however, as with NF-Y, the ability of the DARE competitors to displace binding at this complex was markedly reduced compared to the homologous competitor.

DARE is capable of binding the LHX2 homeodomain. In addition to the CCAAT motif, the region encompassed by DARE contains several TAAT motifs that are the core DNA binding sites of multiple homeodomain DNA binding proteins. Thus, we reasoned that DARE may represent a binding site for a homeobox binding protein. Consistent with this possibility we find that an *in vitro* translated homeodomain of the LIM-homeodomain protein LHX2 is capable of binding DARE in EMSA (Figure 13). A supershift resulting from the inclusion of rabbit anti-LHX2 homeodomain antiserum

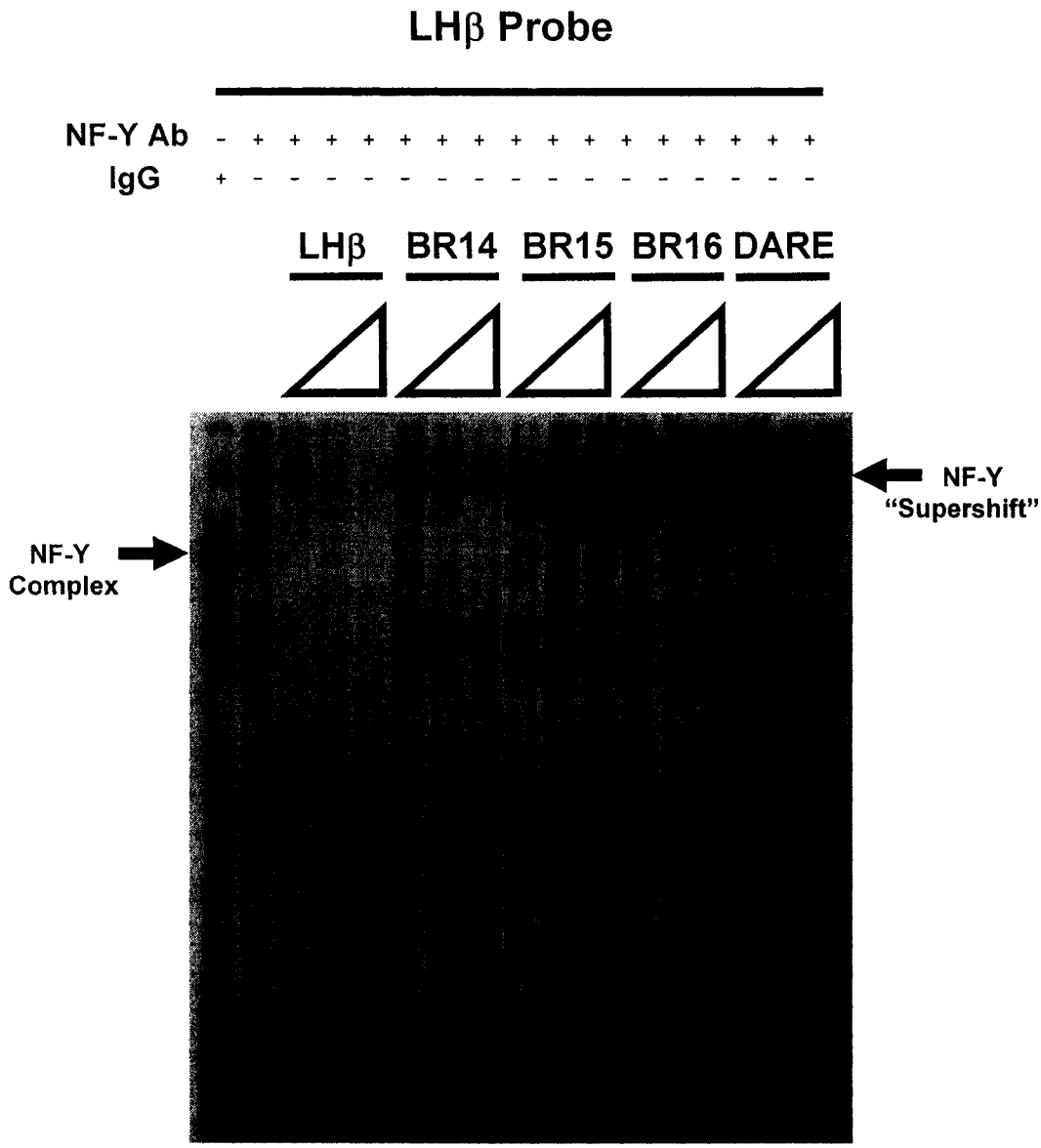

Figure 12. NF-Y does not interact with the CCAAT motif located in DARE. An established NF-Y binding site from the LHβ subunit gene was radiolabeled and incubated with αT3-1 nuclear protein alone or in the presence of either IgG or NF-Y antibody and subjected to electrophoresis in a non-denaturing polyacrylamide gel. Specificity of binding in the supershifted NF-Y containing complex was assessed by adding increasing concentrations of non-radioactive homologous DNA, DARE or DARE containing the indicated block replacement mutations.

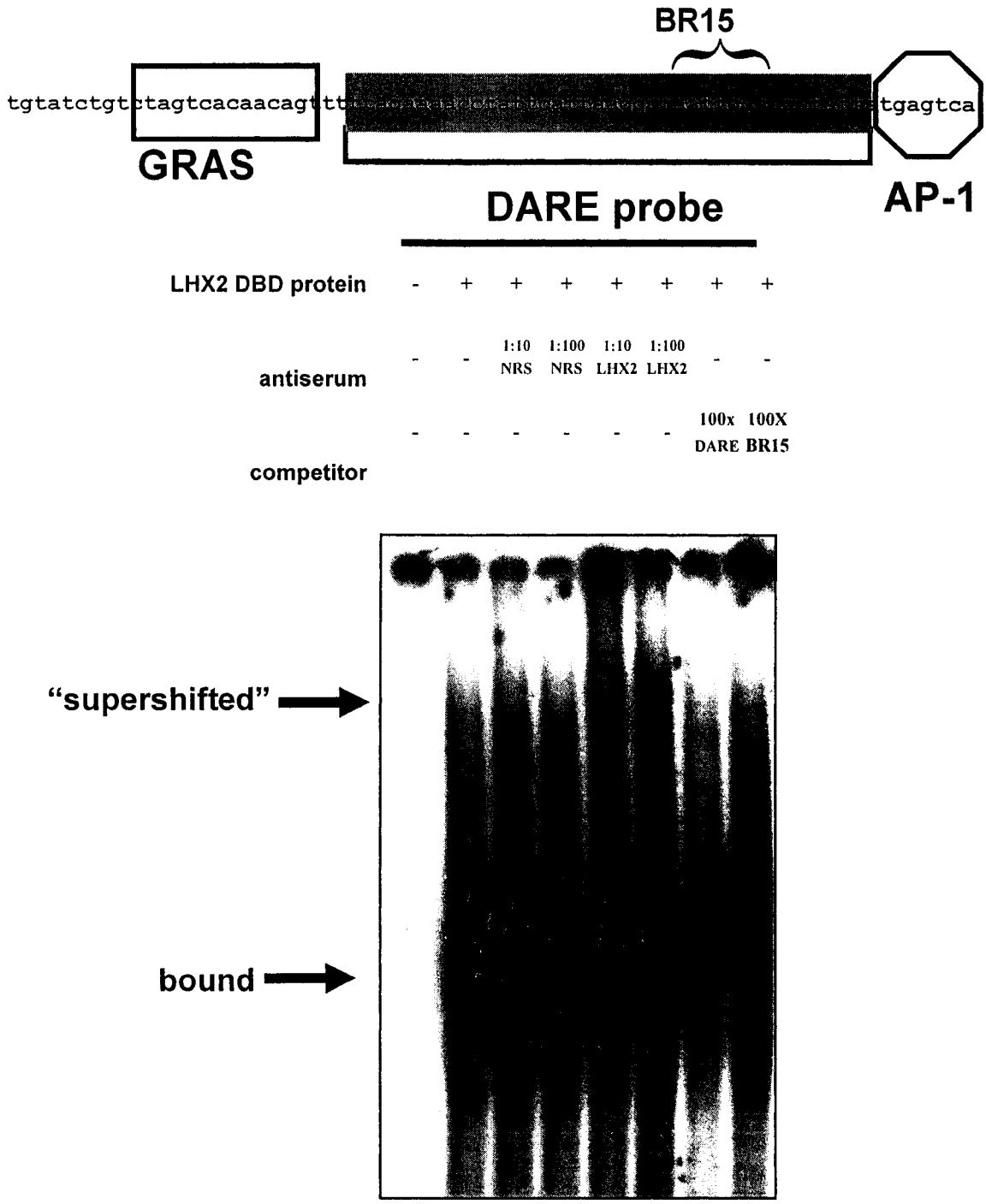

Figure 13. DARE is capable of binding *in vitro* translated LHX2 homeodomain. Radioactively labeled DNA encompassing DARE (shaded sequence) was incubated with *in vitro* translated homeodomain of LHX2 either alone or in the presence of a 1:10 or 1:100 dilution of either normal rabbit serum (NRS) or LHX2 homedomain antiserum. In the final 2 lanes, the inclusion of a 100X molar excess of either DARE (homologous competition) or DARE containing the BR15 mutation in the binding reaction was used to assess the specificity of the bound complex.

(155) but not an equal volume of normal rabbit serum (NRS) was used to confirm the identity of the bound complex. Also, consistent with the functional mapping, the LHX2 complex was displaced by homologous competition but not by BR15.

Disruption of the paired TAAT motifs in DARE eliminates activin responsiveness of the murine GnRHR gene promoter. The EMSA data suggests that DARE may represent a binding site for one or more homeodomain containing proteins. If correct, then we reasoned that the paired TAAT motifs disrupted by BR14 and BR15 would be key to the functional attributes of this element. To address this issue, a series of murine GnRHR promoters were constructed that contained 2 bp transversion mutations that spanned the entire BR14 and BR15 regions (designated μ14.1-5 and μ15.1-5, Figure 14). Although there was some attenuation in basal activity, the follistatin response of promoters containing the μ14.1, μ14.2, and μ14.3 mutations was not affected (Figure 14). In contrast, mutation of the TAAT motif in BR14 (μ14.4 and μ14.5) led to a significant reduction in both basal activity and the follistatin response compared to the wild-type mouse promoter. Interestingly, mutation of the 4 bases of sequence (μ15.1 and μ15.2) residing between the two TAAT motifs had little effect on either basal promoter activity or activin responsiveness. However, as was the case for μ14.4 and μ14.5, disruption of the core TAAT motif in the BR15 region (μ15.3 and μ15.4) reduced both basal activity and the follistatin response of the mouse GnRHR promoter. Based on these data, the TAAT motifs in the BR14 and BR15 regions both contribute to the functional activity of DARE. We next constructed a promoter that contains both the μ14.4 and μ15.4 mutations (μ14.4/15.4) thus effectively disrupting both TAAT sites in a single construct. Consistent with a combined role for both TAAT motifs, the μ14.4/15.4 promoter

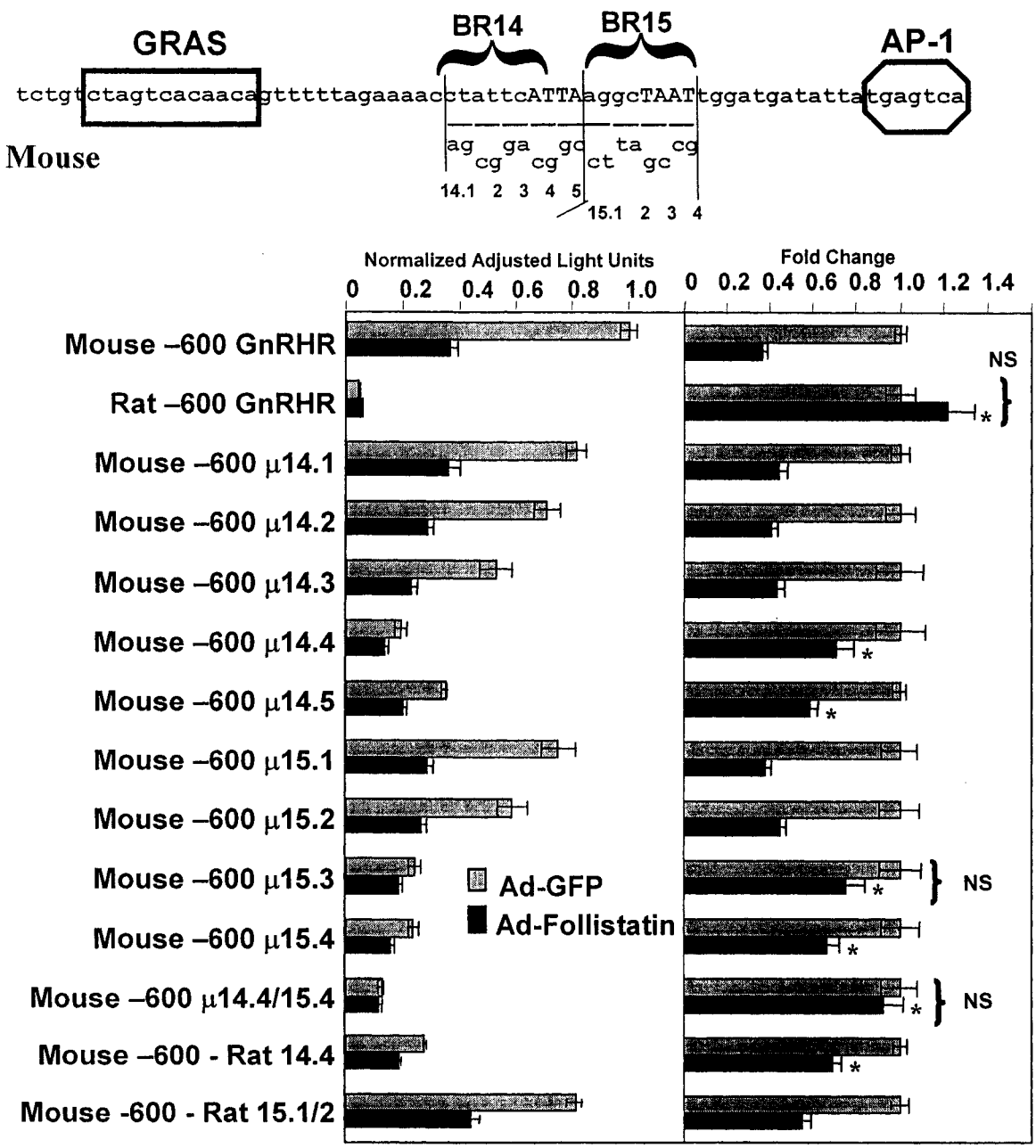

Figure 14. The TAAT motifs in DARE are critical for activin regulation of the mouse GnRHR promoter. αT3-1 cells were transfected with pRSV-LacZ, the wild-type mouse or rat GnRHR promoters or mouse GnRHR promoters containing a series of 2-bp mutations or rat-to-mouse substitutions. Three hours following transfection, cells were infected with either Ad-follistatin or Ad-GFP. Cells were harvested 48 h after infection and cellular lysates were assayed for luciferase and β-galactosidase activity. Luciferase values were corrected for β-galactosidase activity, and values are expressed as both adjusted light units (left-hand panel) or fold change in adjusted luciferase activity in Ad-follistatin vs. Ad-GFP infected cells (right-hand panel). Values represent the mean ± sem of triplicate samples in 3 separate transfections. * value is different ($p<0.05$) from fold-change of wild-type –600 mouse GnRHR promoter.

displayed the lowest basal activity and a complete loss of the follistatin response. Finally, as the sequence of the rat GnRHR promoter in the BR14 and BR15 regions diverges from the mouse at 5 nucleotides we next sought to determine if any of these differences may partially account for the lack of functional activity of the rat DARE homolog. Towards this end, we constructed a mouse promoter in which the A residue in BR14 was converted to G (Mouse –600 - Rat 14.4). Consistent with the dinucleotide transversions in μ14.4 and μ14.5, both basal activity and follistatin response of this construct was attenuated (Figure 14). In contrast, conversion of the 3 nucleotides in BR15 to the homologous rat sequence (mouse –600 - Rat 15.1/2) had little effect on either basal promoter activity or follistatin responsiveness - a result consistent with the μ15.1 and μ15.2 scanning mutants.

DISCUSSION

The TGFβ superfamily is important for tissue patterning and development in multiple organ systems including the reproductive axis (156). Specifically, sexual differentiation, primordial germ cell, gonadal, and pituitary, development are regulated by TGFβ family members (113;156). In the developing pituitary signaling gradients of bone morphogenetic proteins (BMPs) activate unique expression patterns of transcription factors that mediate a dorsal to ventral patterning of the hormone producing cell types (113;157). Of particular importance to reproductive function is the gonadotrope – a cell type that is uniquely characterized by expression of GnRH receptors and production of LH and FSH. The GnRHR mediates the primary stimulatory input to gonadotropes

whereas FSH is essential for later stages of ovarian follicular growth in females and regulation of Sertoli cell function in males (13;158). Activin is also produced by gonadotropes and is thought to affect changes in FSH and GnRHR expression via autocrine and paracrine mechanisms (1;159). In the case of FSHβ, removal of activin input through follistatin treatment results in a decrease in FSHβ mRNA and FSH (44). The production of mice deficient in the activin receptor Type II has provided *in vivo* confirmation for the key role of activin in regulation of FSH synthesis and secretion (99). As with the production of FSH, multiple reports have demonstrated activin regulation of GnRHR expression (1;2;55;129;147).

Consistent with their role in mediating intracellular signaling by TGFβ family members, activin regulation of both the FSHβ subunit and GnRHR genes is at least partially dependent on Smad proteins (2;103;129). In the case of the murine GnRHR gene, the effects of Smads are mediated by binding at the distal end of GRAS (2;129). However, as with many activin/TGFβ responsive elements GRAS is a composite regulatory element whose functional activity is dependent not only on Smad binding but also AP-1 and a forkhead DNA binding protein (2;129). Elimination of any one of these binding components leads to a complete loss of the functional activity of GRAS and activin responsiveness of the GnRHR gene promoter (2). Thus, functional mapping localized activin responsiveness to GRAS. As such, we were surprised to find that the single nucleotide divergence in the rat GRAS homologue could not account for the lack of activin responsiveness of the proximal promoter of the rat GnRHR gene. In fact, an activin responsive phenotype of the rat promoter only emerged with the inclusion of both "intact" GRAS and an additional 36 bp of downstream sequence from the mouse GnRHR

promoter. This comparative analysis and construction of mouse/rat chimeric promoters provided the key observations leading to the definition of a new regulatory element we have termed DARE. As is the case for GRAS, mutation of DARE leads to a loss of activin responsiveness of the mouse GnRHR gene promoter. Thus, neither GRAS nor DARE alone is sufficient for activin regulation. Rather, this functional phenotype requires both elements and, presumably, a unique configuration of multiple DNA binding proteins to form a complex activin/TFGβ responsive enhanceosome.

The presence of a core TAAT sequence is characteristic of DNA binding sites defined for a number of homeodomain proteins (160). The conservation of this signature motif in the promoter regions disrupted by the two loss of function mutations (BR14 and BR15) that defined the boundaries of DARE raised the possibility that DARE represents a binding site for a homeodomain protein. Consistent with this possibility, DARE was capable of interacting with the DNA binding domain of the LIM-homeodomain protein LHX2 in EMSA. Furthermore, 2 bp scanning mutagenesis established that the activin responsive properties of DARE is dependent on a contribution of both the distal and proximal TAAT motifs. As such, DARE may serve as a binding site for at least two homeodomain proteins. Alternatively, DARE might represent a binding site for homodimers or heterodimers of paired-like homeodomain proteins; however, the 4 bp separation of the TAAT motifs would not place this element in the more prototypical P2 or P3 binding sites characterized for the bicoid or Pax family of proteins (160). Finally, our mutagenesis studies suggest that the lack of functional activity of the rat DARE homolog is at least partially due to disruption of the more distal TAAT motif located in the BR14 region.

It is important to note that DARE partially overlaps with an element termed SURG-1 (5" CTAATTGGA 3') that was previously defined as contributing to GnRH responsiveness of the GnRHR promoter (161) and, more recently, shown to interact with NF-Y and Oct-1 (33). However, it seems unlikely that these proteins account for the contribution of DARE to activin responsiveness since the BR16 mutation effectively eliminates the binding sites defined for both NF-Y and Oct-1 (33) but has no effect on the follistatin response of the mouse promoter. Thus, although we certainly do not dispute a functional contribution of NF-Y/Oct-1 binding at SURG-1, it would appear that this event is independent of the contribution of DARE to activin responsiveness. In further support of this notion is that the NF-Y/Oct-1 binding site is conserved in the rat promoter; however, substitution of mouse DARE with the rat DARE homolog eliminated activin responsiveness of the mouse promoter.

Our data suggest that both GRAS and DARE participate in conferring activin responsiveness to the proximal promoter of the murine GnRHR gene. As such, this functional phenotype appears to require the formation of a Smad containing protein complex at GRAS and, potentially, a homeodomain protein complex at DARE. At issue, ultimately, is the mechanism(s) by which these spatially separate complexes cooperate to yield a functional activin/TGFβ responsive enhanceosome. It is certainly possible that components of the GRAS and DARE protein complexes directly interact. Smad proteins are clearly capable of interacting with a wide array of transcription factors including AP-1, forkhead DNA binding proteins and homeodomain proteins (2;162;163). For example, paired-like homeodomain proteins of the Mix family and forkhead transcription factors have been shown to recruit activated Smads to distinct promoter elements (163). Thus,

activin-mediated protein-protein interactions may be necessary to assemble the key binding components at GRAS and DARE and, ultimately, recruitment of the appropriate co-activator. If correct, such a mechanism would be strikingly similar to what was recently described for activin signaling to the FSHβ subunit gene in the gonadotrope derived LβT2 cell line. Specifically, Bailey et al. (103) reported that activin responsiveness of the ovine FSHβ gene promoter is partially dependent on a functional interaction between TALE homeodomain (Pbx1 and Prep1) and Smad proteins. Similarly, activin regulation of the rat FSHβ subunit gene requires both Smads and Pitx2, a member of the bicoid related homeodomain proteins (102).

Although activin is certainly capable of regulating expression of the GnRHR gene (1;2;55;147), Kumar et al. (99) recently reported that GnRHR expression was maintained in mice made deficient in the Type II activin receptor gene. As such, it is difficult to reach an unambiguous conclusion as to the precise physiological role of activin in affecting GnRHR gene expression. Several points are, however, important to consider. First, the pituitary phenotype in those embryos in which the knockout was lethal was not reported. Second, there is evidence that Type II receptors for TGFβ family members can be somewhat promiscuous in their interaction with Type I receptors (132;133). Thus, it is possible that components of activin signaling are retained in the activin receptor II null mice. Third, given that multiple TGFβ family members including bone morphogenetic protein 2 (BMP2), BMP4 and, more recently, BMP6 and BMP7, are involved in pituitary morphogenesis and development (114;157;164) it is possible that the key inputs to the GnRHR gene include not only activin but also other members of the TGFβ superfamily. There is evidence for intra-pituitary expression of BMP's and BMP receptors and, in fact,

expression of these proteins by gonadotropes themselves (132;165). At present, the complete array of TFGβ family members expressed by the αT3-1 cell line is unknown; however, follistatin is capable of binding both activin and BMP's (166). Thus, although we have shown that activin alone is capable of increasing transcriptional activity of the murine GnRHR promoter (1), it is possible that the effects of follistatin addition or over-expression are not confined to removing only activin input. As such, it would not seem judicious to dismiss a role for TGFβ family signaling in regulating transcriptional activity of the GnRHR gene. For example, the role of both TGFβ family signaling and homeodomain proteins in initiating cell-specific gene programs make the emerging properties of the activin responsive enhanceosome in the GnRHR gene an intriguing candidate for activation of GnRHR gene expression in the developing pituitary.

In summary, we have taken advantage of the divergence in activin responsiveness between the proximal promoters of the mouse and rat GnRHR genes to identify a new regulatory element in the mouse GnRHR promoter we have termed DARE. Both DARE and GRAS are necessary for activin/TGFβ responsiveness of the murine GnRHR gene promoter. We have not yet established the specific identity of the DARE binding components; however, it appears likely that this component(s) represents one or more members of the homeodomain protein family. Thus, GRAS and DARE together define a unique and complex activin/TGFβ responsive enhanceosome whose functional attributes are dependent on the binding of multiple classes of transcription factors at spatially distinct regulatory elements located in the proximal promoter of the murine GnRHR gene.

CHAPTER 4

ACTIVIN REGULATION OF THE MURINE GnRH RECEPTOR GENE PROMOTER REQUIRES A SPATIALLY DISTINCT ACTIVIN RESPONSIVE UNIT

INTRODUCTION

The binding of the hypothalamic peptide GnRH to specific, high-affinity receptors located on gonadotrope cells in the anterior pituitary gland is, perhaps, the central event in the regulation of reproductive function in mammals (37;38). Accordingly, much effort has been expended toward understanding the physiological consequences of regulation of GnRH and the GnRH receptor (GnRHR). In regard to the latter, much progress has been made in elucidating the molecular mechanisms underlying cell-specific and hormonal regulation of GnRHR gene expression. Central to progress in this arena is the relatively robust transcriptional activity of the murine GnRHR gene promoter in the gonadotrope derived αT3-1 cell line (152;167). This paradigm has allowed for the development of an increasingly refined map of the key regulatory elements that mediate transcriptional activity of the murine GnRHR gene. In this regard, over 5 years ago we defined a complex "tripartite" enhancer that mediates "basal" activity and endocrine responsiveness of the GnRHR gene promoter (28). The key components of this enhancer include a binding site for the nuclear orphan receptor

steroidogenic factor-1 (SF-1), a canonical AP-1 element and an element we termed the GnRH receptor activating sequence or GRAS (28;29;153). At least 2 of these elements also contribute to endocrine responsiveness of the GnRHR gene. Responsiveness to GnRH is dependent on recruitment of Jun/Fos family members to the AP-1 site (153) whereas activin responsiveness is dependent on GRAS (1). It has, however, become clear that the functional organization of the murine GnRHR gene promoter is far more complex as an increasingly broader array of regulatory elements are necessary for full transcriptional activity of this promoter. In particular, recent work has established the identity of multiple binding sites for homeodomain proteins including LHX3 and Oct-1 (5;33). Interestingly, at least 2 of these sites localized to paired homeodomain sites termed the downstream activin regulatory element or DARE. As the name implies, we have found that this element, like GRAS, is necessary for activin responsiveness of the GnRHR gene promoter (4). Thus, activin regulation of the murine GnRHR gene appears to require 2 spatially distinct regulatory motifs. An interest insight that has emerged from these studies is the view that activin input is a key component underlying overall transcriptional activity of the murine GnRHR gene promoter. Recent years have witnessed advances in our understanding of the auto- and paracrine roles of activin within the anterior pituitary gland. While clearly implicated in regulation of FSH synthesis and secretion (42;96;97), several studies have established that activin, a member of the TGFβ family of growth and differentiation factors, regulates the number of GnRH receptors on gonadotropes (55;136;168). Activin represents homo- or heterodimeric complexes of the different inhibin β-subunits (169). The activin binding protein, follistatin, is a primary modulator of the biological effects of activin by sequestering activin and preventing

activin binding to its cognate receptor (169). The activin β-subunits, as well as follistatin, are produced by gonadotropes in the anterior pituitary gland (170). Follistatin is also produced by folliculostellate cells (144). Thus, autocrine and paracrine mechanisms are an important component of activin signaling in the anterior pituitary. The cellular effects of activin are mediated by binding to and eliciting dimerization of Type I and Type II activin receptors to produce an active complex with ser/thr kinase activity (131). It is well established that activin stimulates transcription of the GnRHR gene (1;28;30;55;129) and that both activin and activin receptors are expressed in the αT3-1 cell line (55;169;170). This in itself presents an interesting problem in that αT3-1 cells exist in a constitutively activin stimulated state. Thus, the challenge is to discriminate between the contribution of any regulatory element to activin-dependent and activin-independent transcriptional activity of the GnRHR promoter. Our approach has been based on testing for reduced promoter activity after administration of follistatin (1) or adenoviral mediated over-expression of follistatin (4). Exactly this approach was used to define the contribution of GRAS and, more recently, DARE to the activin responsive phenotype of the murine GnRHR gene promoter.

Consistent with its contribution to activin responsiveness, GRAS interacts with Smad3 and Smad4; however, the functional activity of GRAS depends not only on Smad binding but also AP-1 and FoxL2, a member of the forkhead family of transcription factors (112). Thus, GRAS is a composite enhancer element capable of binding at least three different classes of transcription factors. The definition of DARE as a necessary component of what appears to be an increasingly complex activin responsive enhanceosome in the GnRHR gene promoter suggests that a functional interaction must

exist between GRAS and DARE. In this regard, it is interesting to note that GRAS and DARE are separated by 20 bp or 2 complete turns of the helix. Herein we ask if this spatial arrangement is important for the functional contribution of the GRAS-DARE enhanceosome to transcriptional activity of the murine GnRHR gene promoter. Additionally, based on scanning mutagenesis, 2 core homeodomain motifs (TAAT) separated by 4 bp appear to be key to the functional activity of DARE. Consistent with this, several homeodomain proteins including LHX2, LHX3 and Oct-1 have been implicated as binding components at DARE (4;5;33). As such, it is possible that multiple homeodomain proteins are interacting at DARE. Given the relative promiscuity of homeodomain binding sites it is difficult to unequivocally define the identity of the key transcription factor(s). Nevertheless, the presence of the paired TAAT motifs in DARE is striking. Thus, we also sought to determine if the juxtaposition of the 2 TAAT sites is important for the functional activity of DARE. Collectively, the goal of these studies is to refine our understanding of the functional organization of the GnRHR gene promoter that begins to define not just the identity of key regulatory elements but the spatial organization of these elements that leads to enhanceosomes whose properties are dependent on specific spatial combinations of regulatory elements.

Materials and Methods

Materials

αT3-1 cells were generously provided by Dr. Pamela Mellon (University of California San Diego). Dr. Wylie Vale (Salk Institute, La Jolla, CA) provided the adenoviral follistatin (AdCAFS288) and GFP (Ad-GFP) constructs (150). LHX3a and

LHX3a-KRAB expression vectors were a gift from Dr. Simon Rhodes (Indiana University, Indianapolis, IN) (6). Oligonucleotides were obtained from Invitrogen (Carlsbad, CA). DNA sequencing was conducted by Davis Sequencing, LLC (Davis, CA). Restriction enzymes and DNA modifying enzymes were obtained from Fermentas (Hanover, MD) and New England Biolabs (Beverly, MA). The amplification and purification of Ad-CAFS288 and Ad-GFP were performed as previously described (151). DMEM tissue culture media was purchased from Mediatech, Inc. (Herndon, VA) while serum for tissue culture was obtained from Gemini Bioproducts (Woodland, CA). Lastly, SuperFect was purchased from Qiagen Sciences (Valencia, CA).

Cell Culture and Transient Transfections

All cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. αT3-1 cells were maintained in high glucose DMEM containing 2mM glutamine 5% fetal bovine serum, 5% horse serum, 100 U/mL penicillin and 100 μg/mL streptomycin sulfate.

Transient transfections and over-expresion assays were performed according to a Qiagen SuperFect protocol. Briefly, αT3-1 cells were seeded one day prior to transfection at 80,000 cells per well. Eight hundred nanograms (.8μg) of promoter vectors and .2μg of RSV-β-galactosidase as a control for transfection efficiency were transfected per well. Cells were incubated with transfection mix for three hours at which time mix was replaced with αT3-1 media containing Ad-follistatin or Ad-GFP. In the case of over-expression experiments, .5μg of promoter reporter constructs, .5μg of expression vectors, and .2μg of RSV-β-galactosidase were all co-transfected. According

to Qiagen transient transfection protocol, cells were transfected for three hours at which time transfection media was removed and replaced with media containing 1000 MOI of either Ad-GFP or Ad-follistatin.

Forty eight (48) hours post infection, cells were harvested and assayed for luciferase activity (2;28). Luciferase activity was normalized for transfection efficiency by dividing the luciferase activity by β-galactosidase activity. All transfections were repeated at least 3 times using 2-3 different plasmid preparations. Values are presented as the mean ± SEM.

Vector Construction

The pMGR-600Luc, 3XGRAS-Luc, 2 bp mutants μ14.4 and μ15.4 and the double TAAT motif mutant μ4.4/μ15.4 were previously described (1;152;171). The plus and minus insertions between GRAS and DARE were created in different manners. The plasmids inserting 5, 10, 15, or 20 nucleotides between GRAS and DARE were constructed in a multistep process. The sequence between the NotI site of the BR13 mutant (29) and the GRAS element was replaced with oligonucleotides containing the additional sequence. Oligonucleotides were ligated to an upstream fragment (from a HpaI half site immediately downstream of GRAS to -600/SacI) previously described (4) and then ligated into the BR13 mutant cut with SacI and NcoI.

The constructs removing 5, 10, or 15 nucleotides between GRAS and DARE were created using PCR. The downstream fragments with deletion of nucleotides in the BR13 region were created by PCR using oligonucleotides containing deletions in the BR13

region and the GL2 primer. Resultant products were blunted with Klenow, cut NcoI, and ligated with the upstream SacI to Hpa fragment described above into pGL3.

Plus five (+5) and +10 TAAT spacer constructs were created by PCR. Upstream and downstream PCR fragments incorporated a HindIII site as part of the 5 or 10 base pair insert between the TAAT motifs. The upstream fragment was cut KpnI/HindIII and the downstream fragment cut HindIII/PstI. These two fragments were then ligated into pGL3 cut with KpnI/PstI.

Minimal promoter constructs containing one or three copies of the DARE element (1XDARE-Luc and 3XDARE-Luc) fused to the rat prolactin minimal promoter were constructed using oligonucleotides with sticky 5' Cla and 3' HindIII ends. Oligonucleotides were annealed and ligated into pBSK cut with ClaI/HindIII. The oligonucleotides were digested out of pBSK by KpnI/EcoRV and ligated into the rat prolactin minimal promoter cut with KpnI/EcoRV.

Statistical Analysis

In every transfection, each treatment and vector was analyzed in triplicate and the experiments replicated three times using different plasmid preparations. Data are expressed as means $\pm$ the standard error of the mean. Student's T-test was used to compare the difference between cells infected with Ad-GFP and Ad-follistatin (Ad-CAFS288) within a vector. Student-Newman-Keuls (SNK) Test was used to compare the activity of the mouse wild type promoter to other vectors.

Results

Separation of GRAS and DARE by half-turns of the helix attenuates transcriptional activity of the GnRHR. To investigate the spatial relationship between GRAS and DARE a series of promoters were constructed that reduced the separation by 5, 10 or 15 bp or increased the separation by 5, 10, 15 or 20 bp. Our choice of separating the two sites by multiples of 5 bp was to determine if there is a functional consequence of separation by half or full turns of the DNA helix. Each of the promoter constructs were co-transfected with RSV-LacZ into αT3-1 cells. At 3 h post-transfection, transfection reagent and media was replaced with media containing 1000 MOI of either Ad-GFP or Ad-follistatin (171). Media containing the adenovirus constructs remained on the cells for 48 hours after which cells were harvested in lysis buffer (4). Cellular lysates were analyzed for luciferase activity and adjusted for β-galactosidase activity as a control for transfection efficiency. Within an experiment, each vector was transfected in triplicate. All transfections were replicated a minimum of three times with at least two different plasmid preparations. Any spacer mutant that altered the spatial arrangement between GRAS and DARE by half-turns of the helix reduced transcriptional activity of the promoter in the presence of activin (Figure 15). Thus, the activity of the –5, -15, +5, and +15 promoter constructs was lower than the –600 wild-type promoter in Ad-GFP infected cells. In contrast, there was no effect of altering the spatial orientation of GRAS and DARE by full-turns of the helix (-10, +10 and +20 constructs). Thus, a critical spatial relationship exists between GRAS and DARE such that any half turn that presumably disrupts the sidedness of the elements reduced the effective functional contribution of the GRAS-DARE enhanceosome to transcriptional activity in the presence of activin

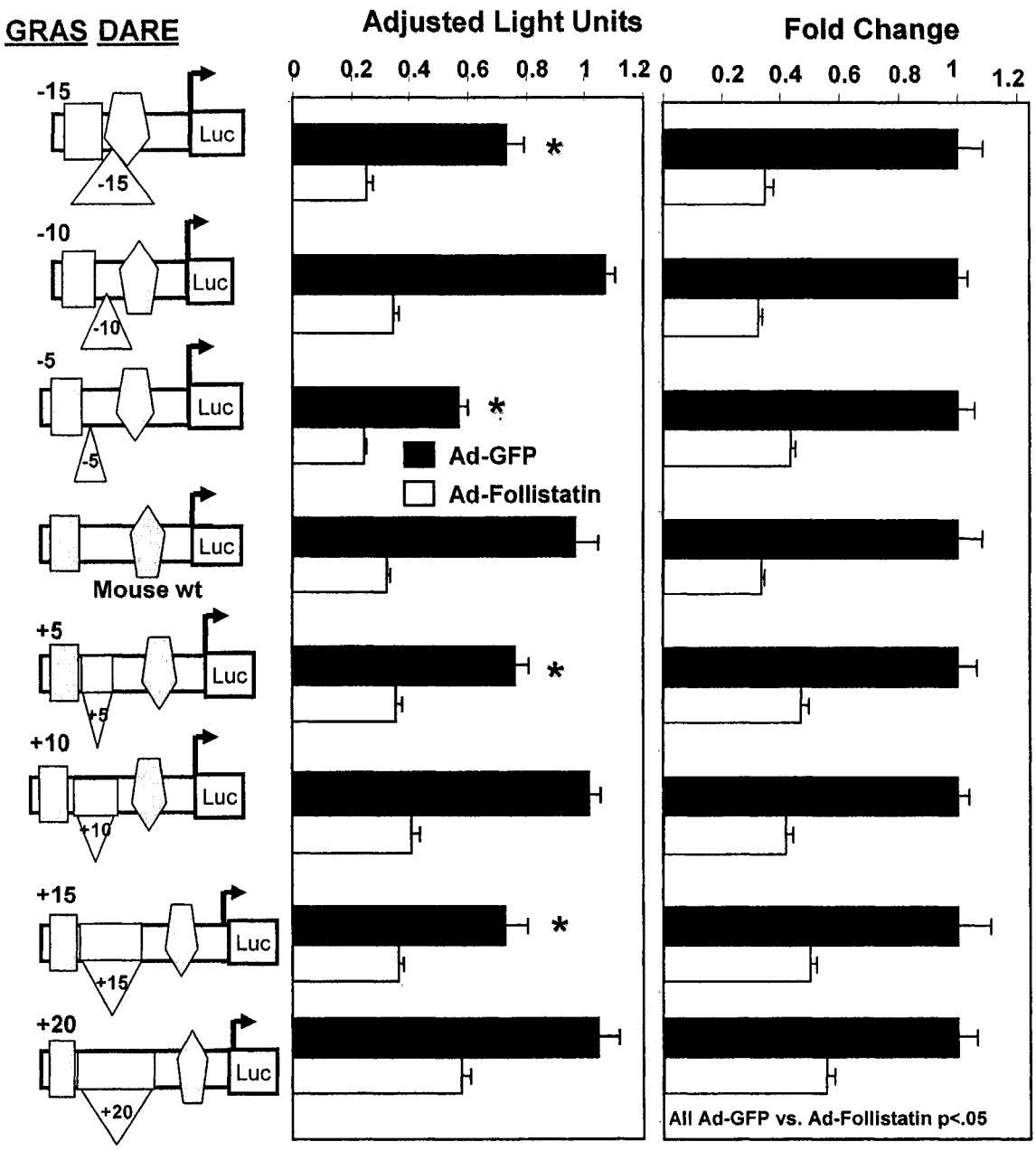

Figure 15. Activin regulation of the mouse GnRHR promoter is disrupted by the insertion of half turns in the DNA helix. Deletions of 5, 10, or 15 and insertions of 5, 10, 15, or 20 nucleotides were transiently transfected into αT3-1 cells. After 3 hrs, transfection mixture was replaced with media containing 1000 MOI of either Ad-GFP or Ad-follistatin. After an additional 48 hrs, cells were harvested and assayed for luciferase activity. Values represent mean +/- the standard error of the mean of triplicate samples. * represents values different ($p<.05$) from mouse wild type Ad-GFP infected construct.

Interestingly, however, while activin stimulated promoter activity is reduced by half-turn separations, a significant attenuation in promoter activity in the presence of follistatin suggests that all of the constructs appeared to retain functional responsiveness to activin.

Thus, altering the spatial relationship between GRAS and DARE reduces but does not eliminate the activin responsive properties of this complex enhanceosome. As would be predicted there was little impact of the spacer mutants on promoter activity in the absence of activin (Ad-follistatin infected cells).

Increasing the space between the two TAAT repeats in DARE alters both basal and activin regulation of the murine GnRHR promoter. The 4 bp separation of the TAAT motifs in DARE would not place this element in the prototypical P2 or P3 binding sites characterized for paired-like homeodomain proteins (160). Nevertheless, the juxtaposition of the 2 TAAT motifs is striking. Thus, we next sought to determine if this spatial relationship organization is relevant to the functional contribution of DARE by placing either 5 or 10 bp between the two TAAT repeats in DARE. Transcriptional activity of the spacer mutants was compared to the –600 wild-type murine GnRHR promoter and the same promoter region containing loss of function mutations in either one or both of the TAAT motifs (4) using the identical transfection/infection paradigm described for Figure 15. Insertion of either 5 or 10 bp between the two TAAT repeats in DARE led to an almost 50% reduction in transcriptional activity of the –600 promoter in the Ad-GFP infected cells (presence of activin) (Figure 16). Thus, the spatial orientation between the two TAAT repeats within DARE appears to be more critical for the

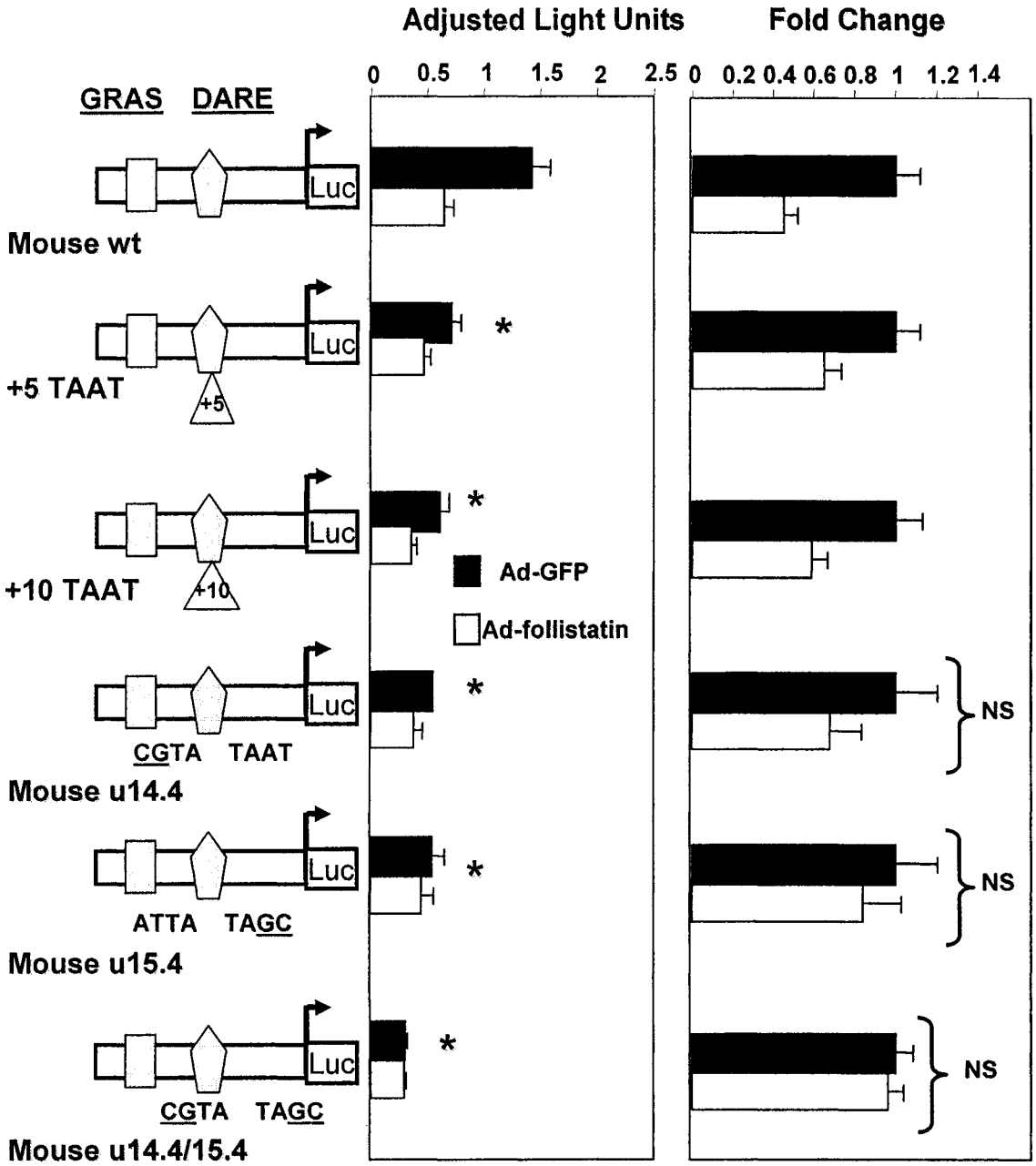

Figure 16. Separation of the TAAT repeats within DARE attenuates activin regulation of the promoter constructs. Constructs containing the insertions of 5 or 10 nucleotides within DARE and constructs containing mutations in the TAAT repeats were transiently transfected into αT3-1 cells. After 3 hrs, transfection mixture was replaced with media containing 1000 MOI of either Ad-GFP or Ad-follistatin. After an additional 48 hrs, cells were harvested and assayed for luciferase activity. Values represent mean ± the standard error of the mean of triplicate samples. * represents values different ($p<.05$) from mouse wild type Ad-GFP infected construct.

functional activity of this element than the spatial arrangement between the GRAS and DARE components of the combined enhanceosome. It is interesting that the decrease in promoter activity associated with the spacer mutations is similar to the impact of mutating either one of the two core TAAT motifs (μ14.4 and μ14.5) suggesting that the spatial separation yields a phenotype equivalent to the contribution of one homeodomain site within DARE. Consistent with this notion, similar to the effects of the μ14.4 and μ14.5 mutations, the follistatin response of the spacer mutants was attenuated but not lost. Only with mutation of both TAAT motifs (μ14.4/μ14.5) was follistatin regulation completely abrogated.

DARE confers LHX3 responsiveness on a heterologous promoter. At least 2 homeodomain proteins (LHX3 and Oct-1) have been implicated as binding components at DARE; however, whether either of these proteins may contribute to the activin responsive properties of DARE is not known. To first establish if DARE can serve as a functional response element for LHX3 we used a standard over-expression paradigm. Briefly, αT3-1 cells were co-transfected with expression vectors for LHX3 and the –600 murine GnRHR promoter. Three hours after transfection, cells were infected with either Ad-GFP or Ad-follistatin as described above. This analysis revealed a small but significant ($p<.05$) increase in promoter activity associated with LHX3 over-expression (Figure 17). Given these data, we next asked if DARE alone displays an LHX3 responsive phenotype when placed in the context of a minimal, heterologous promoter. We find that DARE alone or multimerized as 3 direct repeats confer LHX3 responsiveness on the minimal promoter from the rat Prl gene (Figure 18). In contrast,

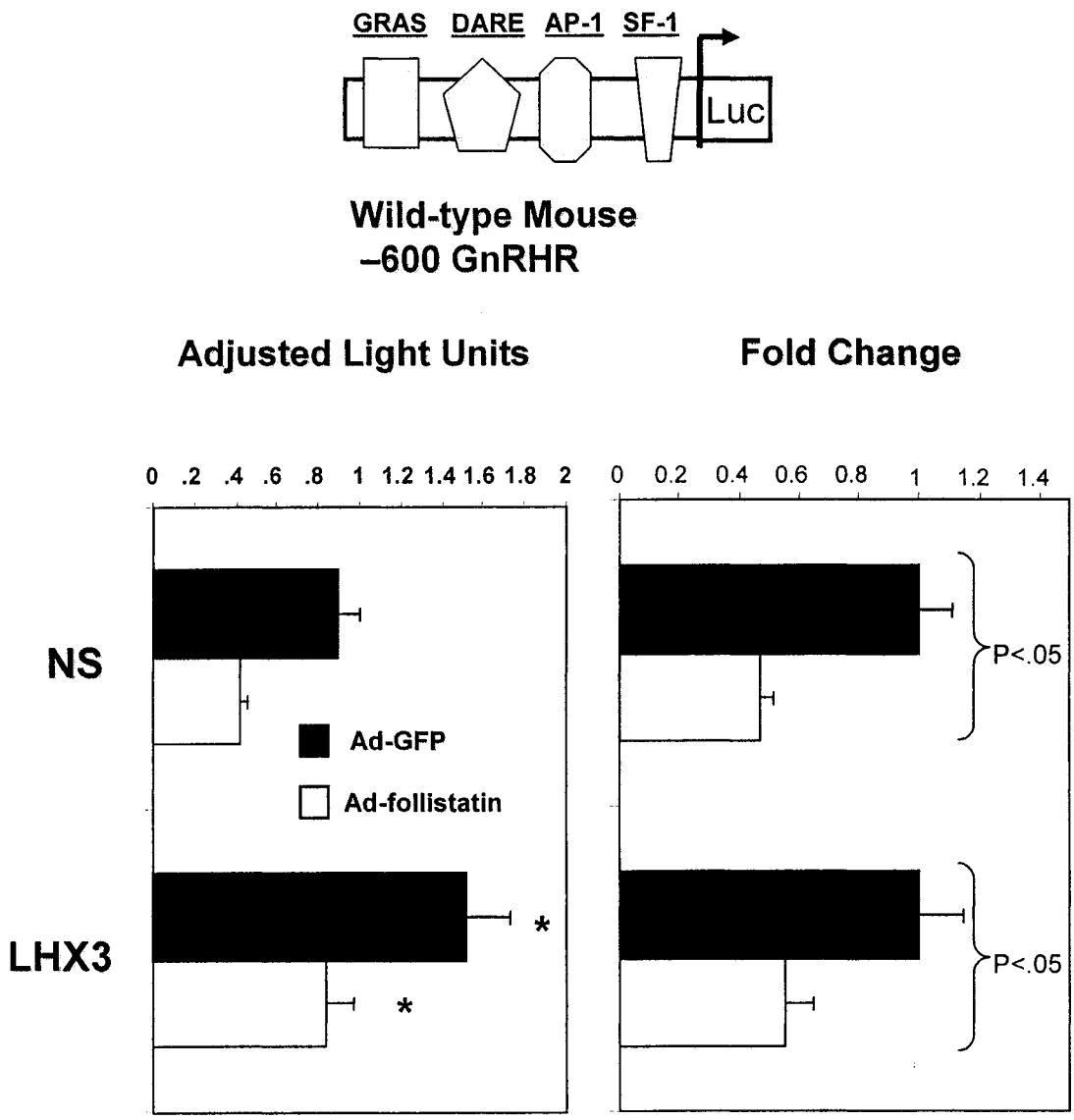

Figure 17. Over-expression of LHX3 stimulates the wild type mouse GnRHR promoter. The mouse wild type GnRHR promoter was co-transfected into αT3-1 cells with either a non-specific expression vector or expression vectors for LHX3. After 3 hrs, transfection mixture was replaced with media containing 1000 MOI of either Ad-GFP or Ad-follistatin. After an additional 48 hrs, cells were harvested and assayed for luciferase activity. Values represent mean ± the standard error of the mean of triplicate samples. * represents values different ($p<.05$) between non-specific expression vector and LHX3.

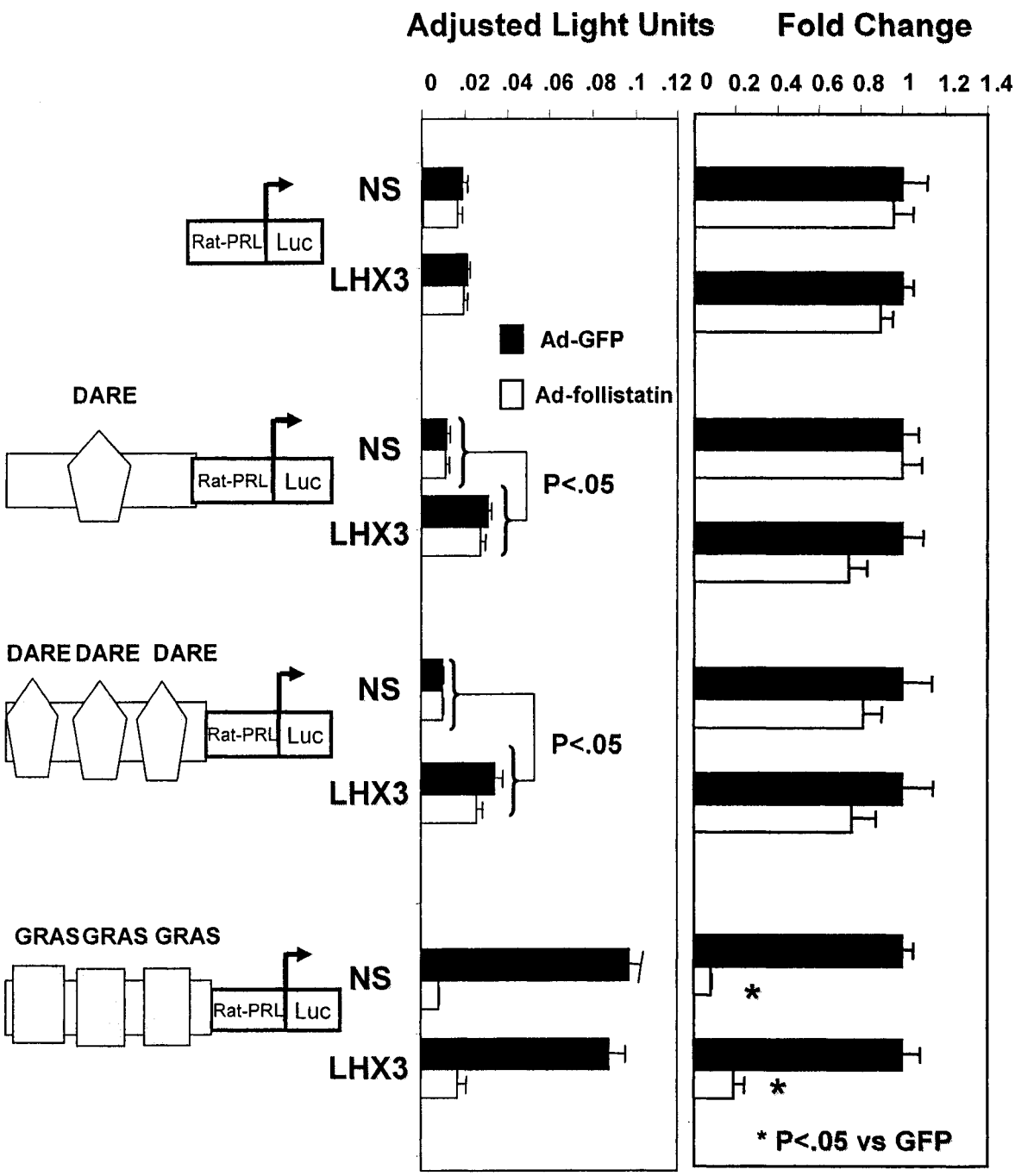

Figure 18. Over-expression of LHX3 can activate minimal promoter constructs through DARE. Minimal promoter constructs (rat prl empty, 1XDARE, 3XDARE, and 3XGRAS) were transiently co-transfected into αT3-1 cells with either a non-specific or LHX3 expression vector. After 3 hrs, transfection mixture was replaced with media containing 1000 MOI of either Ad-GFP or Ad-Follistatin. After an additional 48 hrs, cells were harvested and assayed for luciferase activity. Values represent mean ± the standard error of the mean of triplicate samples. * represents values different ($p<.05$) between Ad-GFP versus Ad-Follistatin..

the transcriptional activity of a construct containing 3 copies of GRAS fused to the Prl promoter (28) was not affected by LHX3 over-expression. Interestingly, in stark contrast to the 3XGRAS vector, neither the 1X DARE nor 3X DARE construct displayed responsiveness to follistatin. Thus, while DARE is sufficient for LHX3 responsiveness, an activin responsive phenotype appears to emerge only in combination with GRAS.

A dominant repressor of LHX3 attenuates transcriptional activity of the mouse GnRHR gene promoter. If LHX3 represents a key player in mediating the functional contribution of DARE then over-expression of dominant-negative forms of this protein should be revealed as a loss of transcriptional activity of the GnRHR gene promoter. To test this we evaluated the impact of an LHX3 fusion protein in which the coding sequence of LHX3 if fused to the transcriptional repressor domain or Kruppel domain of the human KOX1 gene (KRAB) (6). We find that the LHX3-KRAB fusion protein attenuates activity of the wild type mouse GnRHR gene promoter by approximately 65% (Figure 19). There was, however, no impact of LHX3-KRAB on the follistatin response of the –600 promoter. The ability of LHX3-KRAB to repress promoter activity was attenuated with mutation of either the distal (μ14.4) or proximal (μ15.4) TAAT sites and lost when both sites are mutated (μ14.4/μ15.4). Thus, the repressor activity of LHX3-KRAB on the murine GnRHR gene promoter is, at least partially, mediated at DARE. Importantly, however, the activin responsive phenotype of the GRAS-DARE enhanceosome does not appear to be significantly compromised in the presence of the LHX3-KRAB dominant-repressor.

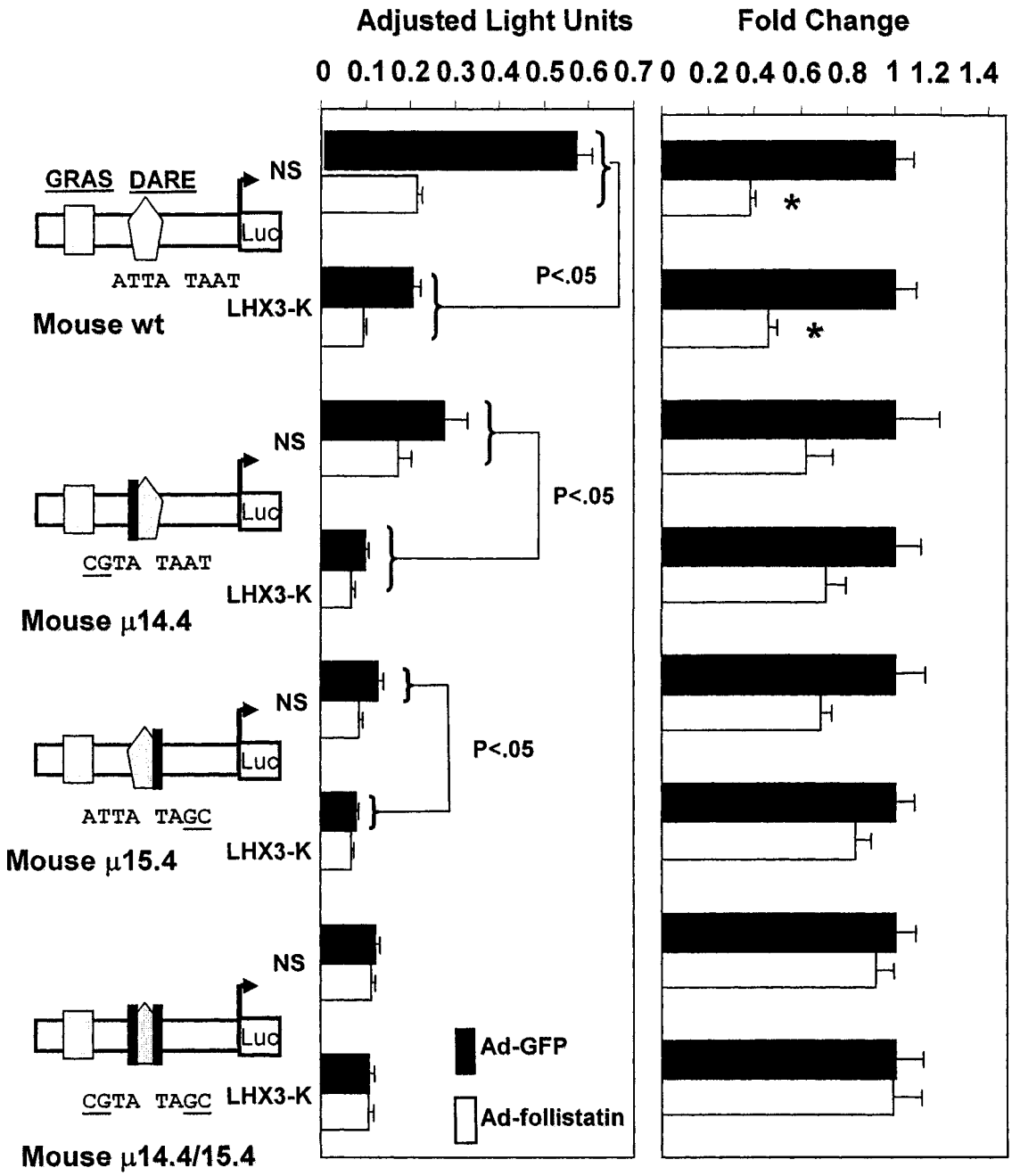

Figure 19. Over-expression of a dominant negative LHX3 fusion protein can decrease expression of constructs through DARE. The wild type mouse, μ14.4, μ15.4, and μ14.4/μ15.4 were transiently co-transfected into αT3-1 cells with either a non-specific or LHX3-KRAB expression vector. After 3 hrs, transfection mixture was replaced with media containing 1000 MOI of either Ad-GFP or Ad-follistatin. After an additional 48 hrs, cells were harvested and assayed for luciferase activity. Values represent mean ± the standard error of the mean of triplicate samples. * represents values different ($p<.05$) between Ad-GFP versus Ad-Follistatin.

Discussion

Approximately 10 years have passed since the initial description of a transcriptionally active promoter fragment from the murine GnRHR gene (152;167). During this period much progress has been made in constructing a functional map of the key regulatory elements that mediate both cell-specific regulation and endocrine responsiveness of this promoter. As in many class II promoters, it appears that much of the critical regulatory information is arrayed within a relatively confined region within 500 bp of the transcriptional start site (152). Such an arrangement is thought to yield a high "local" concentration of transcription factors and associated co-activators. As such, the transcriptional apparatus is positioned to respond rapidly to hormonal inputs that act to either increase or decrease the rate of gene expression. Our understanding of the GnRHR gene promoter suggests a hierarchy of cis-acting regulatory elements with both distinct and overlapping roles in affecting transcription. For example, AP-1 and GRAS make unique contributions in mediating either GnRH or activin responsiveness of the GnRHR gene; however, the combination of both regulatory elements yields a synergistic response to combined GnRH and activin treatment that is greater than the individual treatments (28;30;130). Thus, an emerging theme is that complex functional phenotypes displayed by eukaryotic promoters require specific combinations of individual regulatory elements. If correct, then not just the identification but the spatial organization of regulatory elements is likely to be an important component in fully developing a structural/functional map of the GnRHR gene promoter.

Certainly the combination of GRAS and DARE reflects the emergence of a functional property (i.e. activin responsiveness). In fact, there is no partial phenotype – when either

element is mutated the activin response is lost. Thus, the combination of GRAS and DARE defines a unique activin responsive unit (ARU). Presumably, this functional relationship reflects some level of protein-protein interaction either directly via interactions of transcription factors or presentation of a stereospecific face for binding of co-activator(s). If correct, then the specific architecture of the activin responsive unit may be important. The positioning of GRAS and DARE exactly 2 helical turns apart places the binding components on the same side of the helical backbone in the wild type promoter. I hypothesized that insertion or deletion of full turns in the DNA helix should not alter GRAS and DARE "sidedness" and allow for an activin response that is comparable to the mouse wild type promoter. The corollary of this hypothesis is that insertion or deletion of half turns in the DNA helix would eliminate or attenuate activin responsiveness of the GnRHR promoter. Consistent with this hypothesis, we find that separation of GRAS and DARE by half but not full helical turns attenuates the transcriptional activity of the GnRHR promoter in the presence of activin. These half helical turns would, presumably, place GRAS and DARE on opposite sides of the DNA helix resulting in either a loss of protein-protein contact between the key transcription factors or an inappropriate architecture for co-activator interaction with the ARU. Thus, helical orientation of GRAS and DARE is important for the full functional activity of the ARU in the murine GnRHR gene promoter. Not surprisingly, in the absence of activin (follistatin over-expression), spatial separation of GRAS and DARE had essentially no effect on promoter activity. Simply put, eliminating activin from the system negates the contribution of the GRAS-DARE ARU to promoter activity.

Another important spatial relationship appears to exist within the DARE component of the ARU. Specifically, spacing of the core TAAT motifs in DARE is critical for the functional contribution of this element to the ARU as a whole such that separation of the TAAT repeats by either five or ten bp leads to a significant decrease in activin stimulated transcriptional activity of the GnRHR promoter. It is interesting that this decrease in promoter function associated with the TAAT separations is comparable to the loss of activin regulated activity of constructs containing mutations in either one of the two TAAT repeats. Thus, spatial separation of the TAAT sites in DARE is functionally equivalent to mutating one of the motifs reflecting the contribution, presumably, of the single remaining homeodomain binding site. Interestingly, a single TAAT site within DARE appears to at least partially retain activin responsiveness of the ARU. That is, activin responsiveness is attenuated with spatial separation or mutation of the distal or proximal TAAT motifs (μ14.4 or μ15.4) but is lost only when both TAAT sites are eliminated (μ14.4/μ15.4). Finally, we should underscore that the effect of spatial separation of the distal and proximal TAAT sites in DARE would not appear to be due to disruption of a critical sequence since mutation of the 4 intervening nucleotides did not affect the functional activity of this element (171).

While the binding components at the distal member of the ARU (GRAS) have been extensively studied (112;129), it is clear that one of the key questions regarding DARE is the identity of the protein(s) that mediate the functional contribution of this element to activin responsiveness. Although the wide array of homeodomain proteins expressed in αT3-1 cells presents multiple candidates, we were particularly intrigued with a recent study in which LHX3 was identified as a potential binding protein at DARE (5).

The potential contribution of this protein to activin regulation of the mouse GnRHR gene promoter was not, however, specifically addressed – notwithstanding the fact that since αT3-1 cells constitutively secrete activin, mutational studies of the GnRHR promoter in this cell line are always in the context of activin stimulation.

Similar to McGillivray et al, we find that over-expression of LHX3 enhances transcriptional activity of the wild-type murine GnRHR promoter but not a promoter containing a loss of function mutation in DARE (5). Thus, the effect of LHX3 over-expression appears to be mediated at DARE. Consistent with this possibility, both a single copy of DARE (1X DARE) or multimerized elements (3X DARE) were capable of conferring LHX3 responsiveness on the rat prolactin minimal promoter. Given the composite nature of the ARU, it was not particularly surprising that activin responsiveness was not evident for either the 1X or 3X DARE construct. Thus, to explore the potential role of LHX3 to the functional attributes of the ARU, we utilized a dominant repressor of LHX3 in which the Kruppel domain of human KOX1 is fused to the first 230 amino acids of human LHX3a (LHX3a-KRAB) (6). Consistent with the ability of LHX3 to stimulate GnRHR promoter activity, over-expression of LHX3a-KRAB reduced transcriptional activity of the mouse wild type GnRHR gene promoter. The impact of the KRAB fusion protein was, however, attenuated with mutation of either the distal or proximal TAAT motifs and lost when both sites were mutated. Perhaps most important, however, was that activin responsiveness of the GnRHR promoter was not affected by the LHX3-KRAB fusion protein. Thus, although LHX3a appears to be capable of regulating transcriptional activity of the murine GnRHR promoter through DARE, these data do not support the notion that LHX3a is key to the contribution of DARE to the activin

responsive properties of the ARU. If correct, then we must be cautious in assigning any functional role for LHX3 at DARE.

It has become increasingly clear that complex functional phenotypes of promoters such as endocrine responsiveness are often dependent not on a single regulatory element but rather specific combinations of multiple elements that cooperate to form a hormone responsive unit. Consistent with this concept, activin responsiveness of the GnRHR gene promoter requires spatially distinct elements termed GRAS and DARE. Importantly, it is not simply the presence of these elements that yield an ARU but a specific helical orientation. This spatial arrangement presumably facilitates protein-protein contacts that are necessary for the functional attributes of the GnRHR ARU; however, the specifics of this mechanism await a clearer understanding of the key activin responsive binding components at DARE. In this regard, while LHX3 is capable of activating the GnRHR promoter through DARE, our data do not support the notion that this protein accounts for the functional role of this element as part of the ARU.

CHAPTER 5

ACTIVIN RESPONSIVENESS OF THE GnRH RECEPTOR GENE PROMOTER IS EVIDENT IN TRANSGENIC MICE

INTRODUCTION

Since its characterization in 1971, the key role of GnRH in controlling reproductive function has been well established. The pulsatile discharge of GnRH from hypothalamic neurons not only stimulates but is obligatory for the synthesis and secretion of luteinizing hormone (LH) from gonadotrope cells of the anterior pituitary gland (11;39;41). In addition, expression of the genes encoding the common glycoprotein α and unique β subunits of LH is dependent on GnRH input to gonadotropes (13). Given the central role of GnRH in reproduction, much effort has been devoted to defining the mechanisms underlying regulation of GnRH and the GnRH receptor (GnRHR). Relative changes in GnRH secretion are clearly an important determinant of gonadotropin secretion; however, changes in the number of GnRH receptors are also an important mechanism underlying regulation of gonadotropin secretion. Thus, changes in pituitary content and secretion of LH are not only dependent on changes in GnRH availability but also on the number of GnRH receptors available for binding and, consequently, the responsiveness of the pituitary to a given dose of GnRH (11;17;19;107). It is not surprising then that both GnRH and GnRHR expression are targets for endocrine

regulation. A number of groups have demonstrated coordinate changes in GnRHR numbers and pituitary concentrations of GnRHR mRNA (19-22;24). Of the multiple endocrine inputs that have been implicated in affecting changes in GnRHR expression, perhaps the most dramatic are those associated with estrogen, activin, and GnRH itself (26;27).

Functional activity of the murine GnRHR gene promoter in the gonadotrope derived αT3-1 cell lines has allowed much progress in characterizing the regulatory elements and transcription factors that mediate both cell-specific and hormonal regulation of this gene. For example, GnRH responsiveness is mediated at a canonical AP-1 site (30) and an element termed SURG-1 (154) whereas activin responsiveness requires a complex enhancer element termed the GnRHR activating sequence or GRAS (28). While the αT3-1 model has proven invaluable in characterization of the GnRHR promoter there is a concern as to the degree of conservation of the mechanisms defined *in vitro* to a more physiological context. Towards this end, we have constructed several transgenic mouse models to allow for *in vivo* evaluation of endocrine inputs to the GnRHR gene promoter. Using multiple hormone replacement paradigms we have been able to reliably recapitulate both GnRH and estrogen responsiveness of both the murine and ovine GnRHR genes (7;36). In addition, consistent with the *in vitro* studies, the introduction of a loss of function mutation in the AP-1 site eliminated GnRH regulation of the murine GnRHR gene promoter in transgenic mice (30). In the present studies, we sought to expand this paradigm to determine if activin responsiveness of the murine GnRHR gene promoter is recapitulated in transgenic mice.

Several studies have established that activin, a member of the transforming growth factor (TGFβ) family of growth and differentiation factors, regulates the number of GnRH receptors on gonadotropes (55;136;168). Activin represents homo- or heterodimeric complexes of the different inhibin β-subunits (169). The activin binding protein, follistatin, acts as a primary modulator of the biological effects of activin by sequestering activin and thus preventing activin binding to its cognate receptor (169). The activin β-subunits, as well as follistatin, are produced by gonadotropes in the anterior pituitary gland (170). Follistatin is also produced by pituitary folliculostellate cells (144). Thus, autocrine and paracrine mechanisms are considered to be an important component of activin signaling in the pituitary gland (44;159;172;173). The cellular effects of activin are mediated by binding to specific Type II receptors that combine with activin Type I receptors to produce an active complex with Ser/Thr kinase activity (131). Similar to gonadotropes, both activin and activin receptors are expressed in αT3-1 cells (55). Thus, our approach to studying activin regulation of the GnRHR gene promoter has been based on treating cells with follistatin and using the loss of promoter activity as an index of activin responsiveness. (1;130). More recently, we have utilized an adenoviral approach to deliver and express human follistatin 288 to bind and, thereby, inactivate activin (4). Exactly this approach was used to establish GRAS as necessary for activin responsiveness of the murine GnRHR gene promoter *in vitro*. Although the role of GRAS has been extensively studied, the contribution of this element to promoter activity *in vivo* is not known. Herein, we have adapted the adenoviral approach to establish that, as in αT3-1 cells, the murine GnRHR gene promoter is responsive to follistatin over-expression in transgenic mice. These are the first studies to confirm activin regulation of

the GnRHR gene promoter *in vivo*. Furthermore, we find that selective removal of GRAS appears to eliminate activin responsiveness of the murine GnRHR gene promoter. Thus, GRAS appears to be necessary for activin responsiveness of the murine GnRHR gene both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Dr. Wylie Vale (Salk Institute, La Jolla, CA) provided the adenoviral follistatin (AdCAFS288) and GFP (Ad-GFP) expression vectors (150). The amplification and purification of Ad-CAFS288 and Ad-GFP were performed as previously described (151).

Screening of transgenic mice

Generation and screening of the -1900wt transgenic mice line has been previously described (7). Briefly, to screen for transgenic mice genomic DNA was extracted from tail biopsies and analyzed for the presence of the transgene by slot blot hybridization (30) or PCR amplification using tail vein blood with a REDExtract-N-Amp Kit (Sigma, St. Louis, MO) employing primers specific to luciferase. Animals were maintained under a 14-h light, 10-h dark cycle and received food and water *ad libitum*. All experiments were performed under veterinary supervision with approval from the Colorado State University Animal Care and Use Committee and in accordance with the NIH Animal Care and Use Guidelines. All experiments were conducted using animals older than 6 wk of age.

Animal treatments

Experiment 1: Ad-Follistatin and Ad-GFP infection of non-transgenic mice.

Male and female non-transgenic mice were gonadectomized. One week post surgery, mice were infected interperitonially (ip) with either Ad-follistatin or Ad-GFP. Mice were split into three treatment groups per virus and injected with one of three increasing titers of virus: $3.9x10^{10}$, $8x10^{10}$, or $3.9x10^{11}$ pfu/ml in 200 μL of phosphate buffered saline (PBS). After three days of infection, trunk blood was collected and analyzed for FSH content by radioimmunoassay (RIA) (178).

Experiment 2: Ad-Follistatin and Ad-GFP infection of -1900 wt and -1900μGRAS transgenic mice.

Transgenic mice (-1900 wt and -1900μGRAS) were gonadectomized. After 7 days, half of the -1900 wt and half of the -1900μGRAS mice received a single ip injection of $3.9x10^{11}$ pfu/ml of Ad-GFP in 200 μL of PBS. The other half of the -1900 wt and -1900μGRAS transgenic mice received the same dose of Ad-follistatin. Infection lasted three days at which time pituitary; brain, liver, and trunk blood were collected. Serum was removed from whole blood and analyzed by RIA for LH and FSH concentration (178). Tissue samples were homogenized in lysis buffer and centrifuged at 14,000 rpm to pellet cellular debris. The resulting supernatants (20 μL) were added to 100 μL of Luciferin (Promega, Madison, WI) and analyzed for luciferase activity. Luciferase activity was adjusted for total protein content of the tissue sample determined by BCA protein assay (Pierce, Rockford, IL).

Statistical Analysis

Data are expressed as means ± the standard error of the mean. Student's T-test was used to compare, within tissue, the difference between mice infected with Ad-GFP versus Ad-follstatin.

Results

Adenoviral mediated over-expression of follistatin leads to a decrease in serum concentrations of FSH in FVB mice. We have established the utility of adenoviral-mediated delivery of follistatin to neutralize activin in αT3-1 cell cultures (4). To determine if this infection paradigm would effectively remove activin input to the pituitary we tested the effect of increasing viral load on serum concentrations of FSH in non-transgenic mice of the same genetic background in which the transgenic lines were constructed (FVB). Non-transgenic FVB mice were gonadectomized and allowed to recover from surgery for one week. Mice were treated with $3.9x10^{10}$, $8x10^{10}$, or $3.9x10^{11}$ pfu/mL of either Ad-follistatin or Ad-GFP. The adenovirus constructs were administered by IP injection in phosphate buffered saline (PBS). Three days following injection, trunk blood was collected and FSH concentrations were determined in serum by RIA. Regardless of viral dose, serum FSH levels tended to be lower in animals receiving Ad-follistatin as compared to the Ad-GFP control. At the $3.9x10^{11}$ pfu/ml dose, FSH levels were reduced by approximately 90 % ($p<.05$) in Ad-follistatin infected mice as compared to the Ad-GFP group (Figure 20). Thus, as in rats (150), *in vivo* infection with Ad-follistatin appears to effectively neutralize activin input to gonadotropes as revealed by reduced circulating levels of FSH. Subsequent studies in transgenic mice were conducted using the 3.9×10^{11} pfu/ml dose of Ad-follistatin and Ad-GFP. Furthermore,

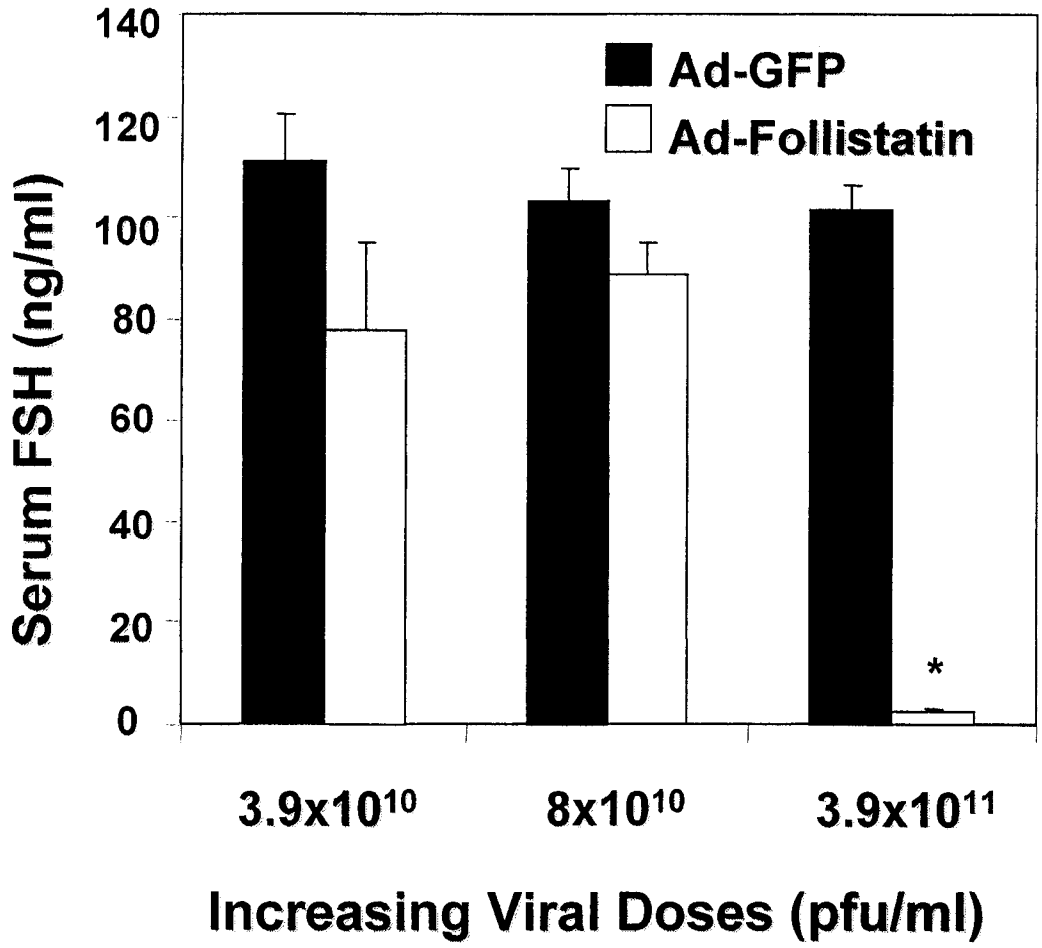

Figure 20. An Ad-follistatin dose of $3.9x10^{11}$ pfu/ml was sufficient to decrease circulating FSH levels in non-transgenic mice. Non-transgenic mice were gonadectomized and allowed to recover from surgery for one week. Mice were infected with either Ad-GFP or Ad-follistatin with one of three increasing concentrations of virus. After three days of infection mice were sacrificed and trunk blood collected. Serum was measured for FSH content. A dose of $3.9X10^{11}$ plaque-forming units/ml of Ad-follistatin was sufficient to reduce FSH compared to mice infected with the same dose of Ad-GFP. * represents a difference ($p<.01$) between Ad-GFP and Ad-follistatin infected mice.

we felt confident that we could use serum concentrations of FSH as a key biological control for activin neutralization in transgenic mice receiving the follistatin adenovirus.

1900 bp of proximal promoter from the murine GnRHR gene is responsive to follistatin over-expression in transgenic mice.

The ability of follistatin to attenuate transcriptional activity of the murine GnRHR gene promoter in αT3-1 cells is well established (1;171); however, whether an equivalent response is evident *in vivo* is not known. To address this question, we applied the adenoviral paradigm described above to transgenic mice harboring a transgene consisting of approximately 1900 bp of proximal promoter from the murine GnRHR gene fused to the cDNA encoding for luciferase (-1900wt-LUC) (Figure 21). The ability of this promoter fragment to direct tissue-specific expression of luciferase, as well as GnRH responsiveness, has been established (7). Transgenic mice (-1900wt-LUC) were gonadectomized and allowed to recover for one week prior to initiation of treatments. Mice were administered a single IP injection of $3.9x10^{11}$ pfu/ml of either Ad-follistatin or Ad-GFP. At three days post-infection, pituitary glands, brain and liver (negative control tissue) were harvested and tissue lysates analyzed for luciferase expression (7;30;36). Trunk blood samples were also collected and analyzed for serum concentrations of LH and FSH (see Figure 22 for experimental design). As above, follistatin over-expression reduced ($p<.05$) serum FSH levels. Serum concentrations of LH were not different ($p>.05$) between the Ad-follistatin and Ad-GFP treated groups. Pituitary luciferase expression was reduced by approximately 40% ($p<.05$) in animals receiving

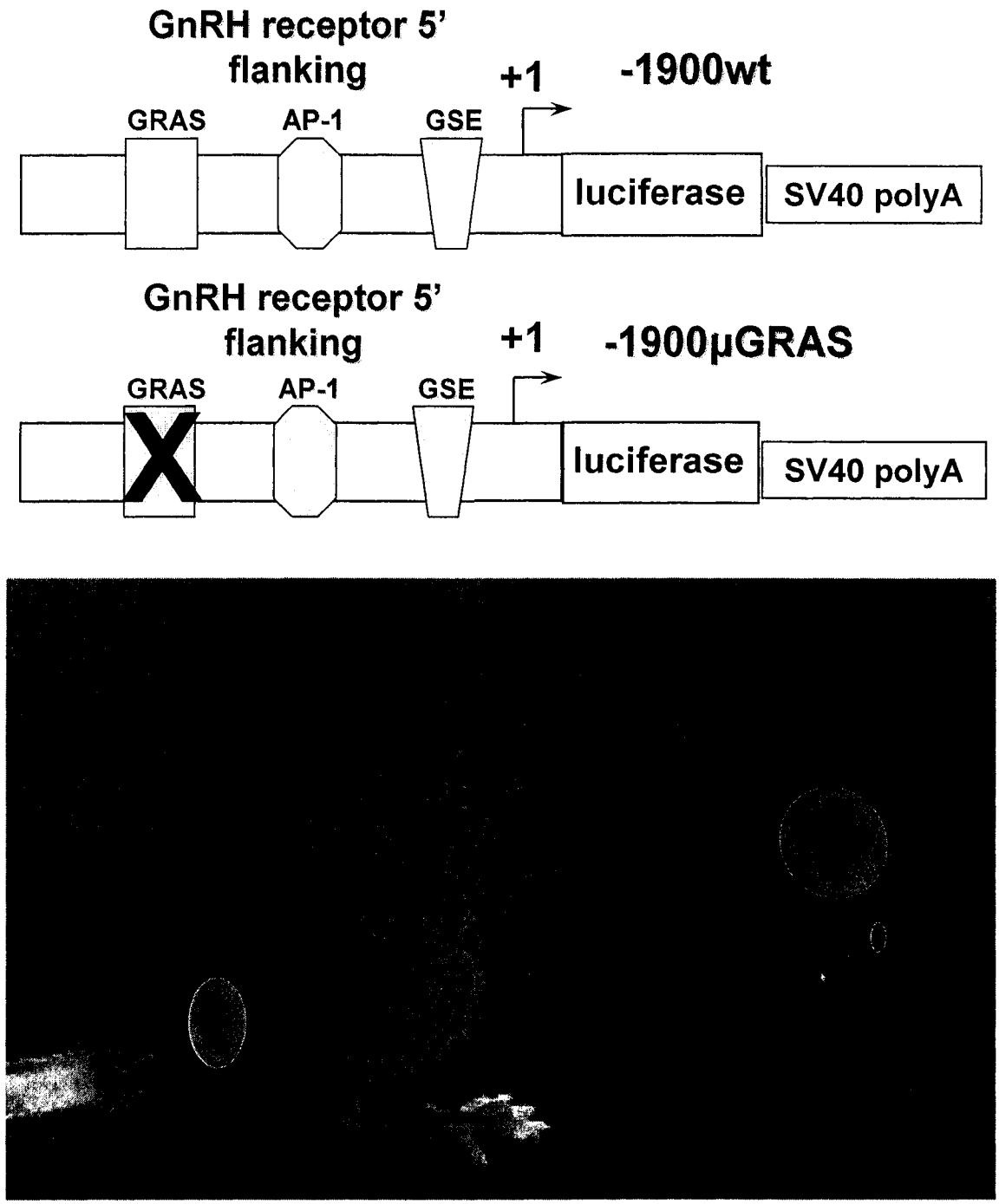

Figure 21. -1900wt and -1900μGRAS transgenic mice express the GnRHR promoter luciferase expressing transgene in the appropriate tissues. The -1900 wt transgene consists of 1900 bp of the 5' flanking region of the mouse GnRHR promoter fused to the cDNA encoding luciferase. The 1900μGRAS transgene contains the same 1900 bp of the mouse GnRHR promoter fused to luciferase in which the GRAS element is mutated. Both lines of transgenic mice express the transgene in the pituitary and gonads exactly recapitulating the sites of endogenous GnRHR expression.

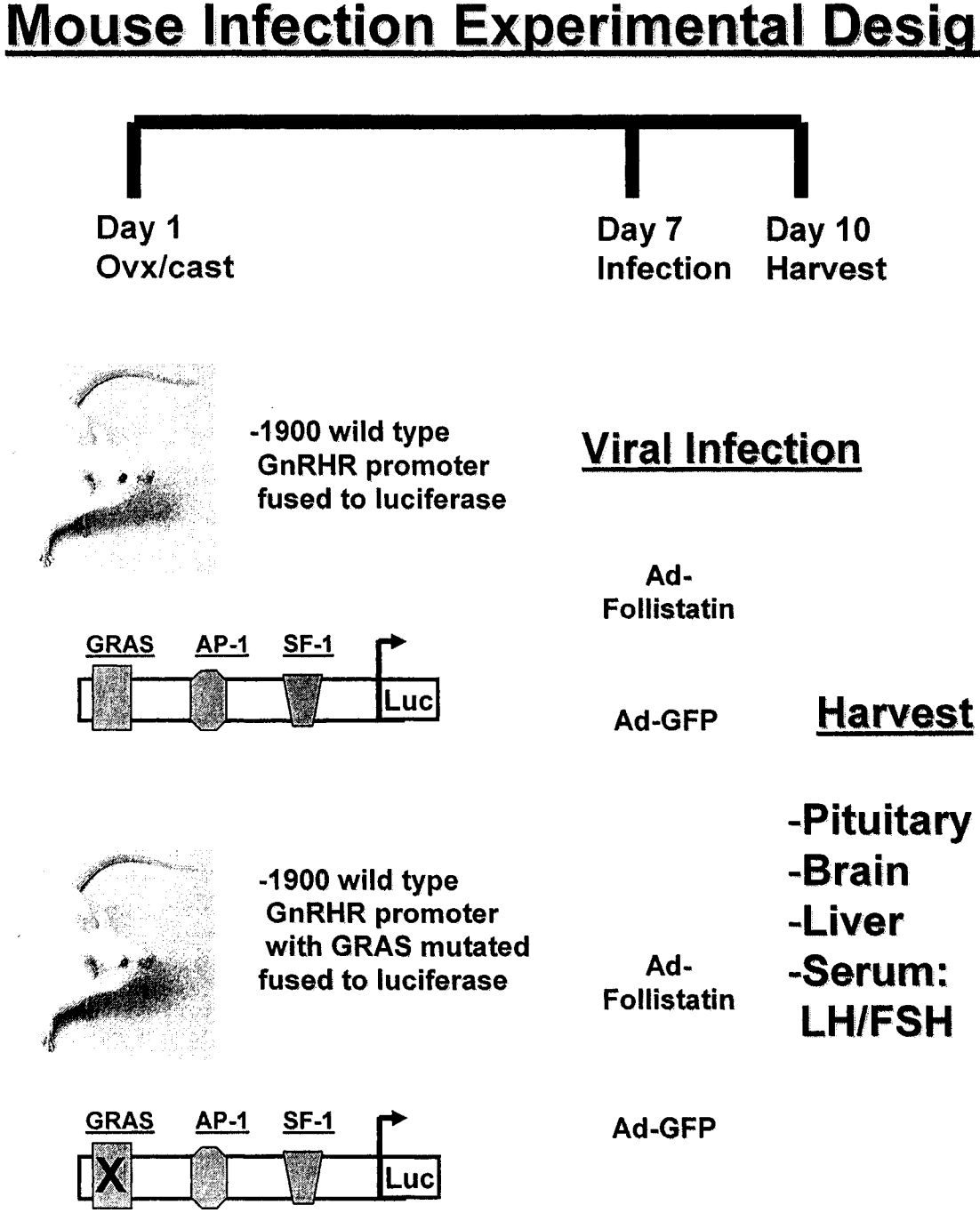

Figure 22. Experimental design of Ad-GFP and Ad-follistatin infection of the -1900wt and -1900μGRAS transgenic mice. Transgenic mice were gonadectomized and allowed to recover from surgery for one week. On day 7, mice were infected with either Ad-GFP or Ad-follistatin at the dose determined in the previous experiment. Mice were infected for a total of three days. On the third day post infection pituitary, brains, and liver were collected and analyzed for luciferase activity per mg of total protein. Trunk blood was also collected and analyzed for serum LH and FSH content.

Ad-follistatin compared to the Ad-GFP treatment group (Figure 23). This follistatin-mediated reduction in luciferase expression is almost exactly equivalent to that observed *in vitro* (171). There was no difference in brain expression of luciferase between the treatments; however, whether this is biologically relevant is problematic, as the adenoviral constructs may not cross the blood-brain barrier.

Selective removal of GRAS does not alter the tissue-specific properties of the murine GnRHR gene promoter. Mutation of GRAS leads to an approximately 50% loss of transcriptional activity of the murine GnRHR gene promoter. In addition, all activin responsiveness of this promoter is lost (1). At issue is whether a similar impact on promoter activity is evident *in vivo*. To address this, transgenic mice were constructed that harbored a transgene consisting of the same 1900 bp promoter fragment as in the –1900wt-LUC animals except for the presence of a loss of function mutation in GRAS (Figure21) (7). The -1900μGRAS transgenic mice were generated using standard micro-injection techniques and F1 progeny were screened for luciferase expression in pituitary, brain, heart, lung, liver, spleen, kidney, and gonads. Tissue expression of luciferase was considered significant when two times greater than the standard deviation of expression levels from the same tissues in non-transgenic mice (7;36). Of all the tissues screened, luciferase expression was above background only in the pituitary and gonads (Figure 24). Thus, the tissue-specific properties of the 1900 bp murine GnRHR gene promoter are retained despite the absence of GRAS. The reduced luciferase activity in tissues from the –1900μGRAS line as compared to the wild-type suggests that promoter function may be compromised in these animals; however, we cannot eliminate potential effects of integration site on transgene expression.

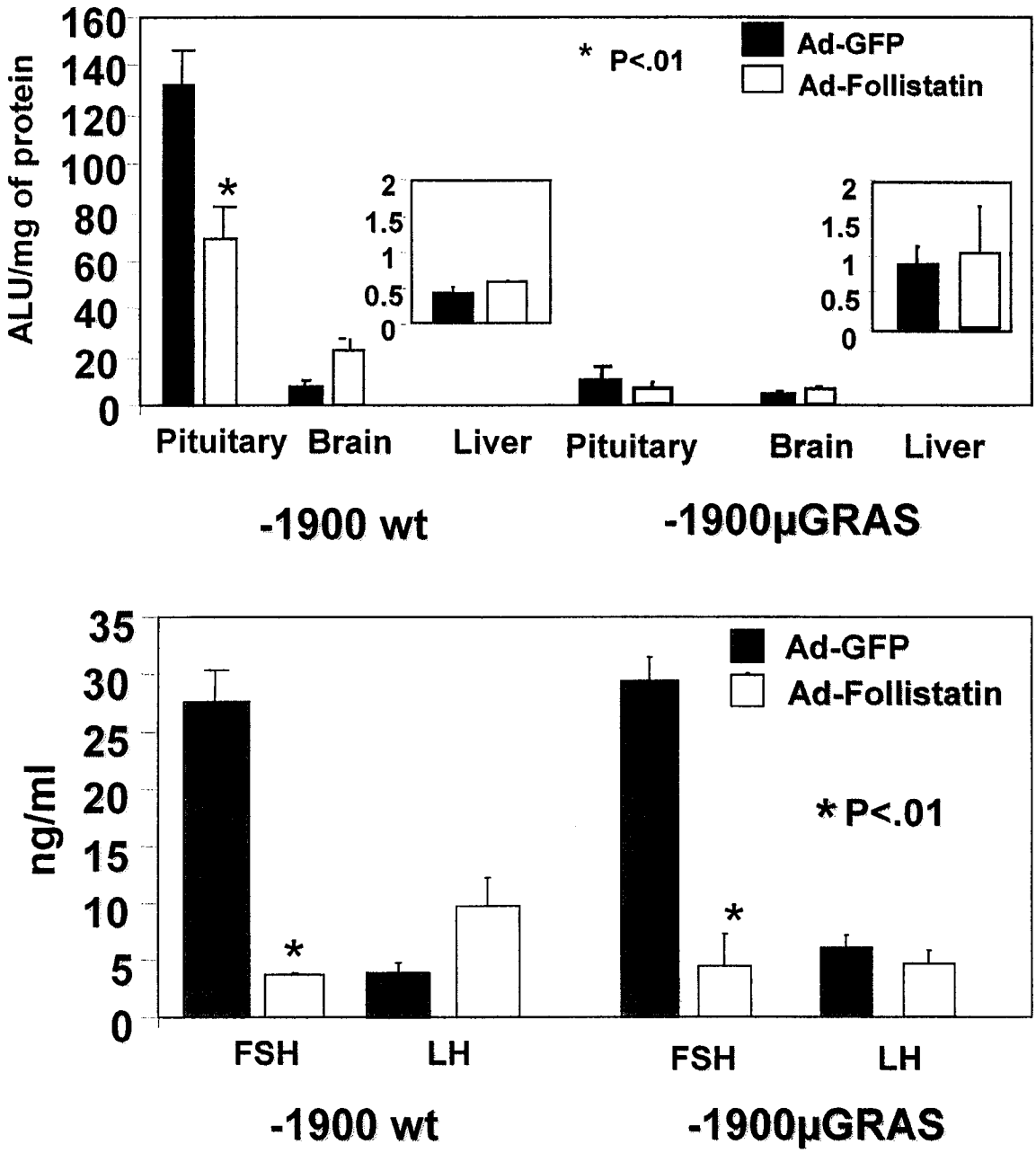

Figure 23. Ad-follistatin infection decreases GnRHR promoter luciferase transgene expression in the pituitary of -1900wt, but not -1900µGRAS, transgenic mice. The top panel represents luciferase activity of the transgene adjusted for total protein content from pituitary , brain, and liver samples infected with either Ad-GFP or Ad-follistatin from both the -1900 wt and -1900uGRAS lines of transgenic mice. The bottom panel represent the serum FSH and LH content of both lines of transgenic mice infected with either Ad-GFP or Ad-Follistatin. * represents a difference ($p<.01$) between Ad-GFP and Ad-follistatin infected mice.

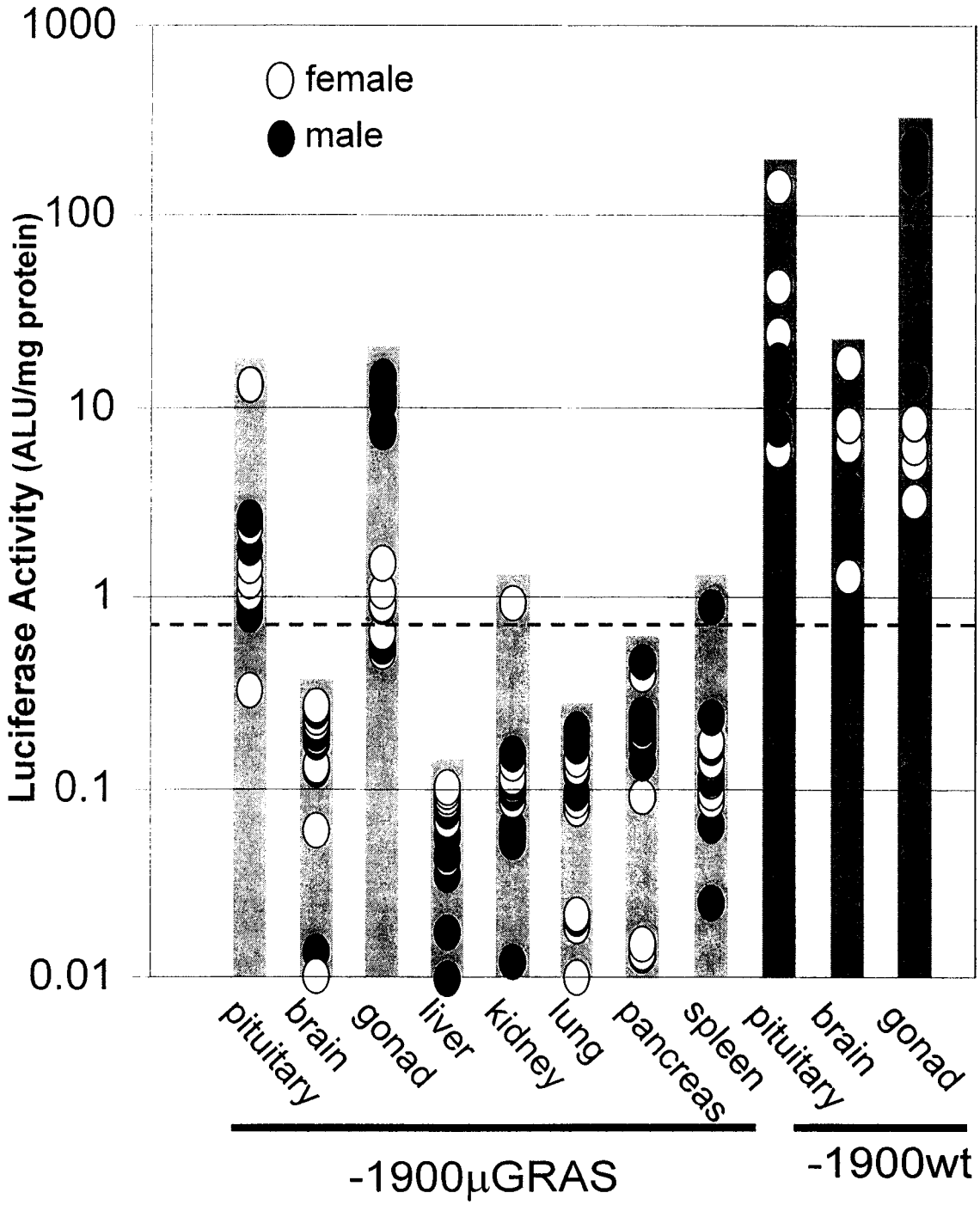

Figure 24. The -1900μGRAS transgenic mice express the transgene in the pituitary and gonads. Tissues from -1900μGRAS transgenic mice were homogenized in lysis buffer and aliquots measured for luciferase activity. Luciferase activity was adjusted for total protein content of the tissue sample. The dashed line represent two times the average standard deviation of similar tissue from non-transgenic mice. The three bars on the far right represent the equivalent tissues in the -1900wt line of transgenic mice.

Follistatin responsiveness of the –1900 bp murine GnRHR gene promoter containing a mutated GRAS is not evident in transgenic mice. To assess the contribution of GRAS to activin regulation of the mouse GnRHR promoter *in vivo*, transgenic mice harboring the –1900μGRAS promoter were subjected to the same experimental paradigm described for the –1900wt-LUC animals. Briefly, -1900μGRAS mice were gonadectomized. At one week following surgery, animals were administered a single IP injection of $3.9x10^{11}$ pfu/ml of Ad-GFP or Ad-follistatin. Three days following infection, pituitary glands, brain and liver were harvested and tissue lysates assayed for luciferase activity (7;30;36). Trunk blood samples were collected and analyzed for serum concentrations of LH and FSH (see Figure 22 for experimental design). In contrast to the –1900wt-LUC mice, there was no difference ($p>.05$) between pituitary luciferase expression in the -1900μGRAS transgenic mice infected with Ad-GFP compared to those animals receiving the follistatin adenovirus (Figure 23). Importantly, however, treatment of animals with Ad-follistatin led to an 85% decrease ($p<.05$) in serum concentrations of FSH while LH levels were unaffected. Thus, while follistatin over-expression effectively suppressed FSH levels, there was no effect on transgene expression in the pituitary.

Discussion

Activin and follistatin interact via auto- and paracrine pathways to modulate expression of activin responsive genes in gonadotrope cells of the anterior pituitary gland. Although the gene encoding FSHβ is a clear target of activin regulation (174), activin responsiveness appears to be fundamental to the transcriptional activity of the

GnRHR gene in the gonadotrope derived αT3-1 cell line (1;55). Consistent with this, follistatin mediated neutralization of secreted activin leads to an approximately 50% reduction in activity of the murine GnRHR promoter in αT3-1 cells. Similarly, the introduction of loss of function mutations in GRAS results in a 50% loss of GnRHR promoter activity and the elimination of activin responsiveness (1). Thus, the αT3-1 cell model has proven invaluable in characterizing the molecular mechanisms underlying activin regulation of the murine GnRHR gene. At issue, however, is the degree to which these mechanisms may be conserved when placed in a more physiological context. As such, the central issue we sought to address in these studies is whether activin responsiveness of the murine GnRHR gene promoter is detectable in transgenic mice.

Since αT3-1 cells constitutively secrete activin (1;55), our ability to investigate activin regulation of the GnRHR promoter in this cell line is based on adenoviral-mediated over-expression of the activin binding protein follistatin (4). Thus, our initial goal was to define an experimental paradigm that could effectively remove activin input to the mouse pituitary. Prior work had, in fact, established the utility of *in vivo* administration of Ad-follistatin in suppressing FSH secretion in rats (150). Consistent with this work, we find that IP administration of $3.9x10^{11}$ pfu/ml of Ad-follistatin to mice led to a significant reduction in serum concentrations of FSH whereas LH levels were unaffected. Based on these data, we suggest that IP administration of Ad-follistatin is an effective method for neutralizing activin input to gonadotropes and presented a unique and powerful avenue to evaluate activin responsiveness of the murine GnRHR gene promoter *in vivo*.

That activin stimulates transcriptional activity of the murine GnRHR gene in αT3-1 cells is indisputable (1;147); however, whether this response is equally evident in bona fide gonadotropes has never been addressed. Given clear evidence for discrepancies between *in vitro* and *in vivo* tests of promoter function (175;176) underscores the significance of directly addressing this issue. Importantly, we find that adenoviral mediated over-expression of follistatin results in an approximately 50% decrease in pituitary expression of luciferase – a reduction virtually identical to that determined in vitro (4). Thus, activin responsiveness of the GnRHR gene promoter is recapitulated in the pituitary glands of transgenic mice. Although no change in transgene expression was detectable in the brain, we are not able to conclude if this reflects tissue-specific properties of the activin response or poor partitioning of follistatin across the blood-brain barrier.

In vitro, GRAS is necessary for mediating activin regulation of the murine GnRHR gene promoter. Thus, while "basal" transcriptional activity is retained, mutation of GRAS eliminates the activin responsive phenotype of the GnRHR promoter (1). Similarly, we find that the –1900 murine GnRHR promoter containing a loss of function mutation in GRAS retains functional activity in transgenic mice and, like the wild-type promoter, directs expression of luciferase in the pituitary gland and gonads – all established sites of expression of the endogenous GnRHR gene. It is tempting to speculate that the lower tissue expression of luciferase in the μGRAS line compared to the -1900wt lines reflects compromised promoter activity; however, the potential effects of site of insertion preclude a firm conclusion in this regard. Certainly the most revealing experimental result with this line of mice was the absence of any change in pituitary

expression of luciferase associated with follistatin over-expression. As FSH levels were clearly reduced in these animals, the lack of effect of follistatin on expression of the transgene would not appear to be due to poor infection efficiency or blunted follistatin expression. Rather, the most straightforward interpretation of these results is that the selective removal of GRAS eliminates the activin responsive phenotype of the murine GnRHR gene promoter *in vivo*. If correct, then these data suggest that activin responsiveness; perhaps the central functional phenotype of the murine GnRHR gene promoter in αT3-1 cells is retained in transgenic mice. Furthermore, GRAS is likely a necessary component of the activin responsive enhanceosome both *in vitro* and *in vivo*.

Although activin is certainly capable of regulating expression of the GnRHR gene (1;2;55;147), Kumar et al. (99) reported that GnRHR expression was maintained in mice made deficient in the Type II activin receptor gene. As such, it is difficult to reach an unambiguous conclusion as to the precise physiological role of activin in affecting GnRHR gene expression. Several points are, however, important to consider. First, the pituitary phenotype in those embryos in which the knockout was lethal was not reported. Second, there is evidence that Type II receptors for TGFβ family members can be somewhat promiscuous in their interaction with Type I receptors (132;133). Thus, it is possible that components of activin signaling are retained in the activin receptor II null mice. Third, given that multiple TGFβ family members including bone morphogenetic protein 2 (BMP2), BMP4 and, more recently, BMP6 and BMP7, are involved in pituitary morphogenesis and development (114;157;164) it is possible that the key inputs to the GnRHR gene include not only activin but also other members of the TGFβ superfamily. There is evidence for intra-pituitary expression of BMP's and BMP receptors and, in fact,

expression of these proteins by gonadotropes themselves (132;165). At present, the complete array of TFGβ family members expressed by the αT3-1 cell line is unknown; however, follistatin is capable of binding both activin and BMP's (166). Thus, although we have shown that activin alone is capable of increasing transcriptional activity of the murine GnRHR promoter (1), it is possible that the effects of follistatin addition or over-expression are not confined to removing only activin input. As such, it would not seem judicious to dismiss a role for TGFβ family signaling in regulating transcriptional activity of the GnRHR gene. For example, the role of both TGFβ family signaling and homeodomain proteins in initiating cell-specific gene programs make the emerging properties of the activin responsive enhanceosome in the GnRHR gene an intriguing candidate for activation of GnRHR gene expression in the developing pituitary.

Activin responsiveness is fundamental to the functional properties of the murine GnRHR gene promoter in αT3-1 cells. Thus, considerable effort has been expended in elucidating the elements, proteins and mechanisms that mediate this response. Unfortunately, however, the degree of conservation of these mechanisms or, even more basic, activin responsiveness of the murine GnRHR promoter in anything other than transformed cell lines has never been established. Based on the present studies, we suggest that the activin responsive phenotype of the murine GnRHR gene promoter is evident *in vivo*. Also, since mutation of GRAS eliminates the activin response of the mouse promoter, it appears likely that key components of the mechanisms underlying activin signaling to the GnRHR that have been defined *in vitro* are conserved *in vivo*.

CHAPTER SIX

CONCLUSIONS

Almost a decade has passed since the initial identification of GRAS and subsequent characterization of this element as an activin responsive enhancer. I have shown that DARE is necessary, in addition to the GRAS element, for activin regulation of the mouse GnRHR gene promoter. Thus, I suggest that, although spatially separate, GRAS and DARE interact to form a complex activin responsive unit (ARU). This sort of arrangement is not without precedent and, in fact, is reminiscent of the multi-element glucocorticoid response unit (GRU) of the carbamoyl-phosphate synthase gene described by Schoneveld et al (177). I have also found that while GRAS and DARE are spatially distinct, the specific spatial relationship between these elements is important to the functional phenotype of the ARU. Separation of the elements by half but not full helical turns effectively attenuates the activin response of the proximal promoter from the murine GnRHR gene. It is interesting to note that the separation of GRAS and DARE by 20 bp in the mouse promoter conserves this important helical orientation. I have also studied the spatial relationship between the two homeobox motifs (TAAT) within DARE itself. Regardless of helical orientation, insertion of nucleotides between the two TAAT repeats decreases the activin response of the murine GnRHR gene promoter. In fact, altering the space between the TAAT repeats in DARE leads to a decrease in promoter activity that is equivalent to the effect of mutating either the distal or proximal motifs.

Thus, spatial separation of the TAAT sites yields a functional phenotype that would appear to reflect the contribution of a single homeobox rather than the contribution of the paired sites.

Although I have identified the elements of the ARU and their spatial relationship to one another, we are still not able to definitively name the protein or proteins responsible for mediating activin regulation of the mouse GnRHR promoter. Simultaneous with the publication of my work defining DARE as an activin regulatory element, two manuscripts were published that was directly relevant to my research. The first of these suggested that LHX3 could bind the distal TAAT motif in DARE (5). In the second, the authors suggested that Oct-1, in combination with NF-Y, interacted at the proximal end of DARE (33). Since the putative Oct-1/NF-Y binding site was retained in the non-functional rat DARE homolog, I focused my efforts on trying to determine if LHX3 might represent the key homeodomain protein that mediates the functional activity of DARE in the mouse GnRHR gene promoter. Utilizing over-expression and a dominant/negative form of LHX3, I was able to show that LHX3 could activate the mouse GnRHR promoter. In addition, LHX3 activation of the promoter was localized to the DARE element. However, since a dominant-negative repressor of LHX3 was unable to block the response of the mouse promoter to follistatin, it would not appear that this is the protein responsible for the activin responsive properties of DARE. As such, re-examining the potential role of Oct-1 at DARE is likely warranted. These experiments are far from completed and are not included in this dissertation. However, I have found that over-expression of Oct-1 does not enhance transcriptional activity of the mouse GnRHR promoter. I do not interpret this result as evidence for lack of Oct-1 regulation.

Rather, the levels of Oct-1 may be sufficiently high in αT3-1 cells such that the mechanism(s) requiring this protein are saturated. As no dominant/negative forms of Oct-1 have been defined, I have turned my attention to using inhibitory RNA (siRNA) to "knock-down" Oct-1 expression in the presence of activin. Although the results of the siRNA studies are mixed, my preliminary experiments suggest that Oct-1 siRNA attenuates transcriptional activity of the murine GnRHR gene promoter. If correct, then a role for Oct-1 in mediating activin responsiveness at DARE remains a distinct possibility. I am convinced that future studies need to continue to fully develop the Oct-1 siRNA strategy in the context of the follistatin infection paradigm I have developed.

In vivo confirmation of *in vitro* analyses of promoter function is, in my view, extremely important to reach any firm conclusions regarding physiological mechanisms and relevance. To this end, I have used the -1900wt and -1900μGRAS transgenic mice to show that adenoviral mediated delivery of follistatin is sufficient to reduce activin input to the mouse GnRHR promoter in the pituitary of transgenic mice. Additionally, I have shown that activin regulation of the mouse GnRHR promoter transgene in the pituitary proceeds through the GRAS element. *In vitro*, GnRH and activin have been shown to act synergistically to increase expression of the mouse GnRHR promoter. I believe it would be valuable to test this potential mechanism *in vivo*. As such, I have initiated a study in which GnRH and activin will be concomitantly neutralized in both the -1900wt and -1900μGRAS transgenic mice.

The mouse GnRHR gene promoter has been extensively studied. It has provided valuable insight into the mechanisms of hormonal regulation of GnRHR expression. However, the mouse GnRHR promoter is significantly different in sequence than other

mammalian GnRHR promoters like the bovine and human. Furthermore, the mouse promoter is not responsive to estrogen treatment either *in vitro* or *in vivo*. Estrogen dramatically increases the number of GnRHRs during the pre-ovulatory period. Yet, the mouse promoter in αT3-1 cells and in transgenic mice does not recapitulate this established endocrine response. Thus, expanding our studies to include more systematic analyses of GnRHR promoters from other species will likely be an important and valuable development. For example, in contrast to the murine GnRHR promoter, the ovine GnRHR gene promoter is highly responsive to estrogen in transgenic mice. In this instance, then, perhaps the most defensible position is to abandon the murine promoter and focus on the ovine gene.

Another compelling example of the value of the cross-species approach are studies I have recently initiated to examine activin responsiveness of GnRHR gene promoters cloned from three breeds of swine - meishan, index and control. Interestingly, although the sequence of the three promoters is highly conserved, they differ in regards to activin regulation. Specifically, the meishan breed not only has the most robust activity in the presence of activin, but also the lowest activity in the presence of follistatin infection. Thus, the meishan breed's GnRHR promoter has a more robust functional response to activin than the other two breeds. This intriguing observation may be correlated to an interesting phenotype displayed by the meishan breed. Specifically, the meishan breed displays an increased ovulation rate, i.e., high fecundity. Most exciting to me is that one of QTL's associated with increased ovulation maps to the GnRHR gene in the meishan genome. As such, increased fecundity in the meishan breed may be due to enhanced activin responsiveness of the GnRHR gene promoter in this breed of swine. If

correct, then one might predict higher levels of GnRHR gene expression in the meishan pituitary gland. In point of fact, pituitaries from meishan pigs have higher levels of GnRHR mRNA than either the index or control breeds. Thus, moving our thinking beyond the functional and intellectual limitations of the mouse model opens up exciting and intriguing avenues of research. It is my feeling that it will be exactly these sorts of approaches that will yield new and important information regarding the biology of GnRHR gene expression.

Reference List

1. **Duval DL, Ellsworth BS, Clay CM** 1999 Is gonadotrope expression of the gonadotropin releasing hormone receptor gene mediated by autocrine/paracrine stimulation of an Activin Response Element? Endocrinology 140:1949-1952

2. **Ellsworth BS, Burns AT, Escudero KW, Duval DL, Nelson SE, Clay CM** 2003 The gonadotropin releasing hormone (GnRH) receptor activating sequence (GRAS) is a composite regulatory element that interacts with multiple classes of transcription factors including SMADs, AP-1 and a forkhead DNA binding protein. Molecular and Cellular Endocrinology 206:93-111

3. **Cherrington BD, Farmerie TA, Clay C.M.,** Independent Mechanisms Underlie the Functional Divergence in Activin and PACAP Regulation of the Mouse and Rat GnRH Receptor (GnRHR) Genes. p P1 (Abstract)

4. **Cherrington BD, Farmerie TA, Lents CA, Cantlon JD, Roberson MS, Clay CM** 2005 Activin responsiveness of the murine gonadotropin releasing hormone receptor gene is mediated by a composite enhancer containing spatially distinct regulatory elements. Molecular Endocrinology

5. **McGillivray SM, Bailey JS, Ramezani R, Kirkwood BJ, Mellon PL** 2005 Mouse GnRH Receptor Gene Expression Is Mediated by the LHX3 Homeodomain Protein. Endocrinology

6. **West BE, Parker GE, Savage JJ, Kiratipranon P, Toomey KS, Beach LR, Colvin SC, Sloop KW, Rhodes SJ** 2004 Regulation of the follicle-stimulating hormone beta gene by the LHX3 LIM-homeodomain transcription factor. Endocrinology 145:4866-4879

7. **McCue JM, Quirk CC, Nelson SE, Bowen RA, Clay CM** 1997 Expression of a murine gonadotropin-releasing hormone receptor- luciferase fusion gene in transgenic mice is diminished by immunoneutralization of gonadotropin-releasing hormone. Endocrinology 138:3154-3160

8. **Matsuo H, Baba Y, Nair RM, Arimura A, Schally AV** 1971 Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. Biochemical and Biophysical Research Communications 43:1334-1339

9. **Brinkley HJ** 1981 Endocrine signalling and female reproduction. Biology of Reproduction 24:22-43

10. **Clarke IJ, Cummins JT, de Kretser DM** 1983 Pituitary gland function after disconnection from direct hypothalamic influences in the sheep. Neuroendocrinology 36:376-384

11. **Clayton RN, Catt KJ** 1981 Gonadotropin-releasing hormone receptors: characterization, physiological regulation and relationship to reproductive function. Endocrine Reviews 2:186-209

12. **Desjardins C** 1981 Endocrine signaling and male reproduction. Biology of Reproduction 24:1-21

13. **Gharib SD, Wierman ME, Shupnik MA, Chin WW** 1990 Molecular biology of the pituitary gonadotropins. Endocrine Reviews 11:177-190

14. **Crowder ME, Nett TM** 1984 Pituitary content of gonadotropins and receptors for gonadotropin-releasing hormone (GnRH) and hypothalamic content of GnRH during the periovulatory period of the ewe. Endocrinology 114:234-239

15. **Nett TM, Cermak D, Braden T, Manns J, Niswender G** 1988 Pituitary receptors for GnRH and estradiol, and pituitary content of gonadotropins in beef cows. II. Changes during the postpartum period. Domestic Animal Endocrinology 5:81-89

16. **Savoy-Moore RT, Schwartz NB, Duncan JA, Marshall JC** 1980 Pituitary gonadotropin-releasing hormone receptors during the rat estrous cycle. Science 209:942-944

17. **Wise ME, Nieman D, Stewart J, Nett TM** 1984 Effect of number of receptors for gonadotropin-releasing hormone on the release of luteinizing hormone. Biology of Reproduction 31:1007-1013

18. **Bauer-Dantoin AC, Weiss J, Jameson JL** 1995 Roles of estrogen, progesterone, and gonadotropin-releasing hormone (GnRH) in the control of pituitary GnRH receptor gene expression at the time of the preovulatory gonadotropin surges. Endocrinology 136:1014-1019

19. **Kaiser UB, Jakubowiak A, Steinberger A, Chin WW** 1993 Regulation of rat pituitary gonadotropin-releasing hormone receptor mRNA levels *in vivo* and *in vitro*. Endocrinology 133:931-934

20. **Turzillo AM, Campion CE, Clay CM, Nett TM** 1994 Regulation of gonadotropin-releasing hormone (GnRH) receptor messenger ribonucleic acid and GnRH receptors during the early preovulatory period in the ewe. Endocrinology 135:1353-1358

21. **Sealfon SC, Laws SC, Wu JC, Gillo B, Miller WL** 1990 Hormonal regulation of gonadotropin-releasing hormone receptors and messenger RNA activity in ovine pituitary culture. Molecular Endocrinology 4:1980-1987

22. **Bauer-Dantoin AC, Hollenberg AN, Jameson JL** 1993 Dynamic regulation of gonadotropin-releasing hormone receptor mRNA levels in the anterior pituitary gland during the rat estrous cycle. Endocrinology 133:1911-1914

23. **Brooks J, Taylor PL, Saunders PT, Eidne KA, Struthers WJ, McNeilly AS** 1993 Cloning and sequencing of the sheep pituitary gonadotropin-releasing hormone receptor and changes in expression of its mRNA during the estrous cycle. Molecular and Cellular Endocrinology 94:R23-R27

24. **Hamernik DL, Clay CM, Turzillo A, Van Kirk EA, Moss GE** 1995 Estradiol increases amounts of messenger ribonucleic acid for gonadotropin-releasing hormone receptors in sheep. Biology of Reproduction 53:179-185

25. **Koves K, Gottschall PE, Arimura A** 1989 Gonadotropin-releasing hormone binding sites in ovaries of normal cycling and persistent-estrus rats. Biology of Reproduction 41:505-511

26. **Katt JA, Duncan JA, Herbon L, Barkan A, Marshall JC** 1985 The frequency of gonadotropin-releasing hormone stimulation determines the number of pituitary gonadotropin-releasing hormone receptors. Endocrinology 116:2113-2115

27. **Frager MS, Pieper DR, Tonetta SA, Duncan JA, Marshall JC** 1981 Pituitary gonadotropin-releasing hormone receptors. Effects of castration, steroid replacement, and the role of gonadotropin- releasing hormone in modulating receptors in the rat. Journal of Clinical Investigation 67:615-623

28. **Duval DL, Nelson SE, Clay CM** 1997 The tripartite basal enhancer of the gonadotropin-releasing hormone (GnRH) receptor gene promoter regulates cell-specific expression through a novel GnRH receptor activating sequence. Molecular Endocrinology 11:1814-1821

29. **Duval DL, Nelson SE, Clay CM** 1997 A binding site for steroidogenic factor-1 is part of a complex enhancer that mediates expression of the murine gonadotropin-releasing hormone receptor gene. Biology of Reproduction 56:160-168

30. **Ellsworth BS, White BR, Burns AT, Cherrington BD, Otis AM, Clay CM** 2003 c-Jun N-terminal kinase activation of activator protein-1 underlies homologous regulation of the gonadotropin-releasing hormone receptor gene in alpha T3-1 cells. Endocrinology 144:839-849

31. **Barnhart KM, Mellon PL** 1994 The orphan nuclear receptor, steroidogenic factor-1, regulates the glycoprotein hormone alpha-subunit gene in pituitary gonadotropes. Molecular Endocrinology 8:878-885

32. **Keri RA, Nilson JH** 1996 A steroidogenic factor-1 binding site is required for activity of the luteinizing hormone beta subunit promoter in gonadotropes of transgenic mice. Journal of Biological Chemistry 271:10782-10785

33. **Kam KY, Jeong KH, Norwitz ER, Jorgensen EM, Kaiser UB** 2005 Oct-1 and Nuclear Factor Y Bind to the SURG-1 Element to Direct Basal and Gonadotropin-Releasing Hormone (GnRH)-Stimulated Mouse GnRH Receptor Gene Transcription. Molecular Endocrinology 19:148-162

34. **Jeong KH, Chin WW, Kaiser UB** 2004 Essential role of the homeodomain for pituitary homeobox 1 activation of mouse gonadotropin-releasing hormone receptor gene expression through interactions with c-Jun and DNA. Molecular and Cellular Biology 24:6127-6139

35. **Windle JJ, Weiner RI, Mellon PL** 1990 Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. Molecular Endocrinology 4:597-603

36. **Duval DL, Farris AR, Quirk CC, Nett TM, Hamernik DL, Clay CM** 2000 Responsiveness of the ovine gonadotropin-releasing hormone receptor gene to estradiol and gonadotropin-releasing hormone is not detectable in vitro but is revealed in transgenic mice. Endocrinology 141:1001-1010

37. **Clayton RN, Catt KJ** 1981 Regulation of pituitary gonadotropin-releasing hormone receptors by gonadal hormones. Endocrinology 108:887-895

38. **Ruf F, Fink MY, Sealfon SC** 2003 Structure of the GnRH receptor-stimulated signaling network: insights from genomics. Front Neuroendocrinol 2003 Jul ;24 (3):181 -99 24:181-199

39. **Pierce JG, Parsons TF** 1981 Glycoprotein hormones: structure and function. Annu Rev Biochem 50:465-495

40. **Dalkin AC, Haisenleder DJ, Ortolano GA, Ellis TR, Marshall JC** 1989 The frequency of gonadotropin-releasing-hormone stimulation differentially regulates gonadotropin subunit messenger ribonucleic acid expression . Endocrinology 125:917-924

41. **Braden TD, Conn PM** 1990 Altered rate of synthesis of gonadotropin-releasing hormone receptors: effects of homologous hormone appear independent of extracellular calcium. Endocrinology 126:2577-2582

42. **Gajewska A, Siawrys G, Bogacka I, Przala J, Lerrant Y, Counis R, Kochman K** 2002 In vivo modulation of follicle-stimulating hormone release and beta subunit gene expression by activin A and the GnRH agonist buserelin in female rats. Brain Res Bull 2002 Sep 15 ;58 (5):475 - 80 58:475-480

43. **Padmanabhan V, Battaglia D, Brown MB, Karsch FJ, Lee JS, Pan W, Phillips DJ, Van Cleeff J** 2002 Neuroendocrine control of follicle-stimulating hormone (FSH) secretion: II. Is follistatin-induced suppression of FSH secretion mediated via changes in activin availability and does it involve changes in gonadotropin-releasing hormone secretion? Biol Reprod 2002 May ;66 (5):1395 -402 66:1395-1402

44. **Besecke LM, Guendner MJ, Sluss PA, Polak AG, Woodruff TK, Jameson JL, Bauer-Dantoin AC, Weiss J** 1997 Pituitary follistatin regulates activin-mediated production of follicle-stimulating hormone during the rat estrous cycle. Endocrinology 138:2841-2848

45. **Arimura A, Schally AV, Saito T, Muller EE, Bowers CY** 1967 Induction of ovulation in rats by highly purified pig LH-releasing factor (LRF). Endocrinology 80:515-520

46. **Kalra SP, Prasad MR** 1967 Effect of FSH and testosterone propionate on spermatogenesis in immature rats treated with clomiphene. Endocrinology 81:965-975

47. **Reichlin S** 1989 Neuroendocrinology of the pituitary gland. Toxicol Pathol 17:250-255

48. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K** 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656-660

49. **Marian J, Conn PM** 1983 Subcellular localization of the receptor for gonadotropin-releasing hormone in pituitary and ovarian tissue. Endocrinology 112:104-112

50. **Tsutsumi M, Zhou W, Millar RP, Mellon PL, Roberts JL, Flanagan CA, Dong K, Gillo B, Sealfon SC** 1992 Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. Molecular Endocrinology 6 :1163-1169

51. **Kaiser UB, Conn PM, Chin WW** 1997 Studies of gonadotropin-releasing hormone (GnRH) action using GnRH receptor-expressing pituitary cell lines. Endocrine Reviews 18:46-70

52. **Roberson MS, Misra-Press A, Laurance ME, Stork PJS, Maurer RA** 1995 A role for mitogen-activated protein kinase in mediating activation of the glycoprotein hormone α-subunit promoter by gonadotropin-releasing hormone. Molecular and Cellular Biology 15:3531-3539

53. **Weck J, Fallest PC, Pitt LK, Shupnik MA** 1998 Differential gonadotropin-releasing hormone stimulation of rat luteinizing hormone subunit gene transcription by calcium influx and mitogen-activated protein kinase-signaling pathways. Molecular Endocrinology 12:451-457

54. **Levi NL, Hanoch T, Benard O, Rozenblat M, Harris D, Reiss N, Naor Z, Seger R** 1998 Stimulation of Jun N-terminal kinase (JNK) by gonadotropin-releasing hormone in pituitary alpha T3-1 cell line is mediated by protein kinase C, c-Src, and CDC42. Molecular Endocrinology 12:815-824

55. **Fernandez-Vazquez G, Kaiser UB, Albarracin CT, Chin WW** 1996 Transcriptional activation of the gonadotropin-releasing hormone receptor gene by activin A. Molecular Endocrinology 10:356-366

56. **Reinhart J, Mertz LM, Catt KJ** 1992 Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor. Journal of Biological Chemistry 267:21281-21284

57. **Heckert LL, Schultz K, Nilson JH** 1995 Different composite regulatory elements direct expression of the human alpha subunit gene to pituitary and placenta. Journal of Biological Chemistry 270:26497-26504

58. **Jameson JL, Hollenberg AN** 1993 Regulation of chorionic gonadotropin gene expression. Endocrine Reviews 14:203-221

59. **Baenziger J** 1990 The asparagine-linked oligosaccharides of the glycoprotein hormones. In: Chin WW, Boime I (eds). Glycoprotein Hormones.:11-18

60. **Bokar JA, Keri RA, Farmerie TA, Fenstermaker RA, Andersen B, Hamernik DL, Yun J, Wagner T, Nilson JH** 1989 Expression of the glycoprotein hormone alpha-subunit gene in the placenta requires a functional cyclic AMP response element, whereas a different cis-acting element mediates pituitary-specific expression. Molecular and Cellular Biology 9:5113-5122

61. **Policastro PF, Daniels-McQueen S, Carle G, Boime I** 1986 A map of the hCG beta-LH beta gene cluster. J Biol Chem 261:5907-5916

62. **Kim KE, Gordon DF, Maurer RA** 1988 Nucleotide sequence of the bovine gene for follicle-stimulating hormone beta-subunit. DNA 7:227-233

63. **Guzman K, miller cd, Phillips CL, Miller WL** 1991 The gene encoding ovine follicle-stimulating hormone beta: isolation, characterization and comparison to a related ovine genomic sequence. DNA and Cell Biology 10:593-601

64. **Kennedy GC, Andersen B, Nilson JH** 1990 The human alpha subunit glycoprotein hormone gene utilizes a unique CCAAT binding factor. Journal of Biological Chemistry 265:6279-6285

65. **Roberson MS, Meermann S, Morasso MI, Mulvaney-Musa JM, Zhang T** 2001 A role for the homeobox protein Distal-less 3 in the activation of the glycoprotein hormone alpha subunit gene in choriocarcinoma cells. Journal of Biological Chemistry 276 :10016-10024

66. **Jameson JL, Powers AC, Gallagher GD, Habener JF** 1989 Enhancer and promoter element interactions dictate cyclic adenosine monophosphate mediated and cell-specific expression of the glycoprotein hormone alpha-gene. Molecular Endocrinology 3:763-772

67. **Delegeane AM, Ferland LH, Mellon PL** 1987 Tissue-specific enhancer of the human glycoprotein hormone alpha-subunit gene: dependence on cyclic AMP-inducible elements. Molecular and Cellular Biology 7:3994-4002

68. **Nilson JH, Bokar JA, Andersen B, Bohinski R, Kennedy G, Keri RA, Farmerie TA, Fenstermaker RA** 1989 CRE-binding proteins interact cooperatively to enhance placental-specific expression of the glycoprotein hormone alpha-subunit gene. Ann N Y Acad Sci 564:77-85

69. **Steger DJ, Altschmied J, Büscher M, Mellon PL** 1991 Evolution of placenta-specific gene expression: Comparison of the equine and human gonadotropin α-subunit genes. Molecular Endocrinology 5:243-255

70. **Heckert LL, Schultz K, Nilson JH** 1996 The cAMP response elements of the alpha subunit gene bind similar proteins in trophoblasts and gonadotropes but have distinct functional sequence requirements. Journal of Biological Chemistry 271:31650-31656

71. **Drust DS, Troccoli NM, Jameson JL** 1991 Binding specificity of cyclic adenosine 3',5'-monophosphate-responsive element (CRE)-binding proteins and activating transcription factors to naturally occurring CRE sequence variants. Molecular Endocrinology 5:1541-1551

72. **Roberson MS, Schoderbek WE, Tremml G, Maurer RA** 1994 Activation of the glycoprotein hormone alpha-subunit promoter by a LIM- homeodomain transcription factor. Molecular and Cellular Biology 14:2985-2993

73. **Horn F, Windle JJ, Barnhart KM, Mellon PL** 1992 Tissue-specific gene expression in the pituitary: the glycoprotein hormone alpha-subunit gene is regulated by a gonadotrope-specific protein. Molecular and Cellular Biology 12:2143-2153

74. **Jackson SM, Gutierrez-Hartmann A, Hoeffler JP** 1995 Upstream stimulatory factor, a basic-helix-loop-helix-zipper protein, regulates the activity of the alpha-glycoprotein hormone subunit gene in pituitary cells. Molecular Endocrinology 9:278-291

75. **Jackson SM, Barnhart KM, Mellon PL, Gutierrez-Hartmann A, Hoeffler JP** 1993 Helix-loop-helix proteins are present and differentially expressed in different cell lines from the anterior pituitary. Molecular and Cellular Endocrinology 96:167-176

76. **Schoderbek WE, Roberson MS, Maurer RA** 1993 Two different DNA elements mediate gonadotropin releasing hormone effects on expression of the glycoprotein hormone α-subunit gene. J Biol Chem 268:3903-3910

77. **Sarapura VD, Wood WM, Gordon DF, Ridgway EC** 1992 A cell line that produces the glycoprotein hormone alpha-subunit contains specific nuclear factors similar to those present in thyrotropes. Thyroid 2:31-38

78. **Clay CM, Keri RA, Hamernik DL, Heckert LL, Nilson JH** 1994 Endocrine regulation of glycoprotein hormone alpha subunit gene expression in transgenic mice. In: Lustbader JW, Puett D, Ruddon RW (eds). Glycoprotein Hormones.Springer-Verlag, New York:19-35

79. **Jorgensen JS, Quirk CC, Nilson JH** 2004 Multiple and overlapping combinatorial codes orchestrate hormonal responsiveness and dictate cell-specific expression of the genes encoding luteinizing hormone. Endocrine Reviews 25:521-542

80. **Heckert LL, Wilson EM, Nilson JH** 1997 Transcriptional repression of the alpha-subunit gene by androgen receptor occurs independently of DNA binding but requires the DNA- binding and ligand-binding domains of the receptor. Molecular Endocrinology 11:1497-1506

81. **Tremblay JJ, Drouin J** 1999 Egr-1 is a downstream effector of GnRH and synergizes by direct interaction with Ptx1 and SF-1 to enhance luteinizing hormone beta gene transcription. Molecular and Cellular Biology 19:2567-2576

82. **Quirk CC, Lozada KL, Keri RA, Nilson JH** 2001 A single Pitx1 binding site is essential for activity of the LHbeta promoter in transgenic mice. Molecular Endocrinology 15:734-746

83. **Weck J, Anderson AC, Jenkins S, Fallest PC, Shupnik MA** 2000 Divergent and composite gonadotropin-releasing hormone-responsive elements in the rat luteinizing hormone subunit genes. Molecular Endocrinology 14:472-485

84. **Kaiser UB, Sabbagh E, Chen MT, Chin WW, Saunders BD** 1998 Sp1 binds to the rat luteinizing hormone beta (LHbeta) gene promoter and mediates gonadotropin-releasing hormone-stimulated expression of the LHbeta subunit gene. Journal of Biological Chemistry 273:12943-12951

85. **Keri RA, Bachmann DJ, Behrooz A, Herr BD, Ameduri RK, Quirk CC, Nilson JH** 2000 An NF-Y binding site is important for basal, but not gonadotropin-releasing hormone-stimulated, expression of the luteinizing hormone beta subunit gene. The Journal of Biological Chemistry 275:13082-13088

86. **Kaiser UB, Sabbagh E, Saunders BD, Chin WW** 1998 Identification of cis-acting deoxyribonucleic acid elements that mediate gonadotropin-releasing hormone stimulation of the rat luteinizing hormone beta-subunit gene. Endocrinology 139:2443-2451

87. **Halvorson LM, Kaiser UB, Chin WW** 1996 Stimulation of luteinizing hormone beta gene promoter activity by the orphan nuclear receptor, steroidogenic factor-1. Journal of Biological Chemistry 271:6645-6650

88. **Dorn C, Ou Q, Svaren J, Crawford PA, Sadovsky Y** 1999 Activation of luteinizing hormone beta gene by gonadotropin-releasing hormone requires the synergy of early growth response-1 and steroidogenic factor-1. Journal of Biological Chemistry 274:13870-13876

89. **Keri RA, Wolfe MW, Saunders TL, Anderson I, Kendall SK, Wagner T, Yeung J, Gorski J, Nett TM, Camper SA** 1994 The proximal promoter of the bovine luteinizing hormone beta-subunit gene confers gonadotrope-specific expression and regulation by gonadotropin-releasing hormone, testosterone, and 17 beta-estradiol in transgenic mice. Molecular Endocrinology 8:1807-1816

90. **Jorgensen JS, Nilson JH** 2001 AR suppresses transcription of the LHbeta subunit by interacting with steroidogenic factor-1. Molecular Endocrinology 15:1505-1516

91. **Curtin D, Jenkins S, Farmer S, Anderson AC, Haisenleder DJ, Rissman E, Wilson EM, Shupnik MA** 2001 Androgen suppression of GnRH-stimulated rat LHbeta gene transcription occurs through Sp1 sites in the distal GnRH-responsive promoter region. Molecular Endocrinology 15:1906-1917

92. **Jacobs SB, Coss D, McGillivray SM, Mellon PL** 2003 Nuclear factor Y and steroidogenic factor 1 physically and functionally interact to contribute to cell-specific expression of the mouse Follicle-stimulating hormone-beta gene. Molecular Endocrinology 17:1470-1483

93. **Zakaria MM, Jeong KH, Lacza C, Kaiser UB** 2002 Pituitary homeobox 1 activates the rat FSHbeta (rFSHbeta) gene through both direct and indirect interactions with the rFSHbeta gene promoter. Molecular Endocrinology 16:1840-1852

94. **Coss D, Jacobs SB, Bender CE, Mellon PL** 2004 A novel AP-1 site is critical for maximal induction of the follicle-stimulating hormone beta gene by gonadotropin-releasing hormone. Journal of Biological Chemistry 279:152-162

95. **Strahl BD, Huang HJ, Sebastian J, Ghosh BR, Miller WL** 1998 Transcriptional activation of the ovine follicle-stimulating hormone beta-subunit gene by gonadotropin-releasing hormone: involvement of two activating protein-1-binding sites and protein kinase C. Endocrinology 139:4455-4465

96. **Huang HJ, Sebastian J, Strahl BD, Wu JC, Miller WL** 2001 Transcriptional regulation of the ovine follicle-stimulating hormone-β gene by activin and gonadotropin-releasing hormone (GnRH): Involvement of tow prosimal activator protein-1 sites for GnRH stimulation. Endocrinology 142:2267-2274

97. **Weiss J, Crowley WF, Jr., Halvorson LM, Jameson JL** 1993 Perifusion of rat pituitary cells with gonadotropin-releasing hormone, activin, and inhibin reveals distinct effects on gonadotropin gene expression and secretion. Endocrinology 132:2307-2311

98. **Matzuk MM, Kumar TR, Bradley A** 1995 Different phenotypes for mice deficient in either activins or activin receptor type II [see comments]. Nature 374:356-360

99. **Kumar TR, Agno J, Janovick JA, Conn PM, Matzuk MM** 2003 Regulation of FSHbeta and GnRH receptor gene expression in activin receptor II knockout male mice. Molecular and Cellular Endocrinology 212:19-27

100. **Kretzschmar M, Massague J** 1998 SMADs: mediators and regulators of TGF-beta signaling. Curr Opin Genet Dev 8:103-111

101. **Baker JC, Harland RM** 1997 From receptor to nucleus: the Smad pathway. Curr Opin Genet Dev 7:467-473

102. **Suszko MI, Lo DJ, Suh H, Camper SA, Woodruff TK** 2003 Regulation of the rat follicle-stimulating hormone beta-subunit promoter by activin. Molecular Endocrinology 17:318-332

103. **Bailey JS, Rave-Harel N, McGillivray SM, Coss D, Mellon PL** 2004 Activin Regulation of the Follicle-Stimulating Hormone {beta}-Subunit Gene Involves Smads and the TALE Homeodomain Proteins Pbx1 and Prep1. Molecular Endocrinology 18:1158-1170

104. **Spady TJ, Shayya R, Thackray VG, Ehrensberger L, Bailey JS, Mellon PL** 2004 Androgen regulates follicle-stimulating hormone beta gene expression in an activin-dependent manner in immortalized gonadotropes. Molecular Endocrinology 18:925-940

105. **Huang HJ, Sebastian J, Strahl BD, Wu JC, Miller WL** 2001 The promoter for the ovine follicle-stimulating hormone-beta gene (FSHbeta) confers FSHbeta-like expression on luciferase in transgenic mice: regulatory studies in vivo and in vitro. Endocrinology 142:2260-2266

106. **Adams TE, Norman RL, Spies HG** 1981 Gonadotropin-releasing hormone receptor binding and pituitary responsiveness in estradiol-primed monkeys. Science 213:1388-1390

107. **Turzillo AM, Nolan TE, Nett TM** 1998 Regulation of gonadotropin-releasing hormone (GnRH) receptor gene expression in sheep: interaction of GnRH and estradiol. Endocrinology 139:4890-4894

108. **Smith MS** 1981 The effects of hight levels of progesterone secretion during lactation of the control of gonadotropin secretion in the rat. Endocrinology 109:1509-1517

109. **Parker KL, Schimmer BP** 1997 Steroidogenic factor 1: a key determinant of endocrine development and function. Endocrine Reviews 18:361-377

110. **Halvorson LM, Ito M, Jameson JL, Chin WW** 1998 Steroidogenic factor-1 and early growth response protein 1 act through two composite DNA binding sites to regulate luteinizing hormone beta- subunit gene expression. Journal of Biological Chemistry 273:14712-14720

111. **Zhao L, Bakke M, Krimkevich Y, Cushman LJ, Parlow AF, Camper SA, Parker KL** 2001 Steroidogenic factor 1 (SF1) is essential for pituitary gonadotrope function. Development 128:147-154

112. **Ellsworth BS, Burns AT, Duval DL, Clay CM,** Smad4 and Pituitary Forkhead Factor Interact to Mediate Transcriptional Activity at a Single Element in the GnRH Receptor (GnRHR) Gene. (Abstract)

113. **Dasen JS, Rosenfeld MG** 2001 Signaling and transcriptional mechanisms in pituitary development. Annu Rev Neurosci 24:327-355

114. **Dosen JS, Rosenfeld MG** 1999 Signaling mechanisms in pituitary morphogenesis and cell fate determination. Current Opinion in Structural Biology 11:669-677

115. **Sarapura VD, Strouth HL, Gordon DF, Wood WM, Ridgway EC** 1997 Msx1 is present in thyrotropic cells and binds to a consensus site on the glycoprotein hormone alpha-subunit promoter. Molecular Endocrinology 11:1782-1794

116. **Tremblay JJ, Lanctot C, Drouin J** 1998 The pan-pituitary activator of transcription, Ptx1 (pituitary homeobox 1), acts in synergy with SF-1 and Pit1 and is an upstream regulator of the Lim-homeodomain gene Lim3/Lhx3. Molecular Endocrinology 12:428-441

117. **Cornea A, Janovick JA, Stanislaus D, Conn PM** 1998 Redistribution of G(q/11)alpha in the pituitary gonadotrope in response to a gonadotropin-releasing hormone agonist. Endocrinology 139:397-402

118. **Naor Z, Benard O, Seger R** 2000 Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor. Trends in Endocrinology and Metabolism 11:91-99

119. **Mulvaney JM, Zhang T, Fewtrell C, Roberson MS** 1999 Calcium influx through L-type channels is required for selective activation of extracellular signal-regulated kinase by gonadotropin-releasing hormone. Journal of Biological Chemistry 274:29796-29804

120. **Mulvaney JM, Roberson MS** 2000 Divergent signaling pathways requiring discrete calcium signals mediate concurrent activation of two mitogen-activated protein kinases by gonadotropin-releasing hormone. Journal of Biological Chemistry 275:14182-14189

121. **Cheng KW, Leung PCK** 2001 Human gonadotropin-releasing hormone receptor gene transcription: up-regulation by 3',5'-cyclic adenosine monophosphate/protein kinase A pathway. Molecular and Cellular Endocrinology 181:15-26

122. **Maya-Nunez G, Conn PM** 1999 Transcriptional regulation of the gonadotropin-releasing hormone receptor gene is mediated in part by a putative repressor element and by the cyclic adenosine 3',5'-monophosphate response element. Endocrinology 140:3452-3458

123. **Pincas H, Laverriere JN, Counis R** 2001 Pituitary adenylate cyclase-activating polypeptide and cyclic adenosine 3',5'-monophosphate stimulate the promoter activity of the rat gonadotropin-releasing hormone receptor gene via a bipartite response element in gonadotrope-derived cells. Journal of Biological Chemistry 276:23562-23571

124. **Laws SC, Webster JC, Miller WL** 1990 Estradiol alters the effectiveness of gonadotropin-releasing hormone (GnRH) in ovine pituitary cultures: GnRH receptors versus responsiveness to GnRH. Endocrinology 127:381-386

125. **Nett TM, Flores JA, Carnevali F, Kile JP** 1990 Evidence for a direct negative effect of estradiol at the level of the pituitary gland in sheep. Biology of Reproduction 43:554-558

126. **Gregg DW, Nett TM** 1989 Direct effects of estradiol-17 beta on the number of gonadotropin-releasing hormone receptors in the ovine pituitary. Biology of Reproduction 40:288-293

127. **Simard J, Labrie F** 1985 Keoxifene shows pure antiestrogenic activity in pituitary gonadotrophs. Molecular and Cellular Endocrinology 39:141-144

128. **Gregg DW, Allen MC, Nett TM** 1990 Estradiol-induced increase in number of gonadotropin-releasing hormone receptors in cultured ovine pituitary cells. Biology of Reproduction 43:1032-1036

129. **Norwitz ER, Xu S, Xu J, Spiryda LB, Park JS, Jeong KH, McGee EA, Kaiser UB** 2002 Direct binding of AP-1 (Fos/Jun) proteins to a SMAD binding element facilitates both gonadotropin-releasing hormone (GnRH)- and activin-mediated transcriptional activation of the mouse GnRH receptor gene. Journal of Biological Chemistry 277:37469-37478

130. **Ellsworth BS, Nelson SE, Clay CM,** Synergistic activation of the gonadotropin-releasing hormone (GnRH) receptor by GnRH and activin is mediated at a single element. p 153 (Abstract)

131. **Padgett RW, Das P, Krishna S** 1998 TGF-beta signaling, Smads, and tumor suppressors. BioEssays 20:382-390

132. **Shimasaki S, Moore RK, Otsuka F, Erickson GF** 2004 The bone morphogenetic protein system in mammalian reproduction. Endocrine Reviews 25:72-101

133. **Candia AF, Watabe T, Hawley SH, Onichtchouk D, Zhang Y, Derynck R, Niehrs C, Cho KW** 1997 Cellular interpretation of multiple TGF-beta signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads. Development 124:4467-4480

134. **Clarke IJ, Cummins JT, Crowder ME, Nett TM** 1988 Pituitary receptors for gonadotropin-releasing hormone in relation to changes in pituitary and plasma gonadotropins in ovariectomized hypothalamo/pituitary-disconnected ewes. II. A marked rise in receptor number during the acute feedback effects of estradiol. Biology of Reproduction 39:349-354

135. **Turzillo AM, Nett TM** 1997 Effects of bovine follicular fluid and passive immunization against gonadotropin-releasing hormone (GnRH) on messenger ribonucleic acid for GnRH receptor and gonadotropin subunits in ovariectomized ewes. Biology of Reproduction 56:1537-1543

136. **Braden TD, Conn PM** 1992 Activin-A stimulates the synthesis of gonadotropin-releasing hormone receptors. Endocrinology 130:2101-2105

137. **Ethier JF, Findlay JK** 2001 Roles of activin and its signal transduction mechanisms in reproductive tissues. Reproduction 121:667-675

138. **Welt C, Sidis Y, Keutmann H, Schneyer A** 2002 Activins, inhibins, and follistatins: from endocrinology to signaling. A paradigm for the new millennium. Exp Biol Med (Maywood) 227:724-752

139. **Brown CW, Li L, Houston-Hawkins DE, Matzuk MM** 2003 Activins are critical modulators of growth and survival. Molecular Endocrinology 17:2404-2417

140. **Roberts V, Meunier H, Vaughan J, Rivier J, Rivier C, Vale W, Sawchenko P** 1989 Production and regulation of inhibin subunits in pituitary gonadotropes. Endocrinology 124:552-554

141. **Hashimoto O, Kawasaki N, Tsuchida K, Shimasaki S, Hayakawa T, Sugino H** 2000 Difference between follistatin isoforms in the inhibition of activin signalling: activin neutralizing activity of follistatin isoforms is dependent on their affinity for activin. Cell Signal 12:565-571

142. **Shimonaka M, Inouye S, Shimasaki S, Ling N** 1991 Follistatin binds to both activin and inhibin through the common beta-subunit. Endocrinology 128:3313-3315

143. **Danila DC, Zhang X, Zhou Y, Dickersin GR, Fletcher JA, Hedley-Whyte ET, Selig MK, Johnson SR, Klibanski A** 2000 A human pituitary tumor-derived folliculostellate cell line. The Journal of Clinical Endocrinology & Metabolism 85:1180-1187

144. **Kaiser UB, Lee BL, Carroll RS, Unabia G, Chin WW, Childs GV** 1992 Follistatin gene expression in the pituitary: Localization in gonadotropes and folliculostellate cells in diestrous rats . Endocrinology 130:3048-3056

145. **DePaolo LV, Bicsak TA, Erickson GF, Shimasaki S, Ling N** 1991 Follistatin and activin: A potential intrinsic regulatory system within diverse tissues. Proc Soc Exp Biol Med 198:500-512

146. **DePaolo LV** 1997 Inhibins, activins, and follistatins: the saga continues. Proc Soc Exp Biol Med 214:328-339

147. **Norwitz ER, Xu S, Jeong K, Bedecarrats GY, Winebrenner LD, Chin WW, Kaiser UB** 2002 Activin A augments GnRH-mediated transcriptional activation of the mouse GnRH receptor gene. Endocrinology 143:985-997

148. **Pincas H, Amoyel K, Counis R, Laverriere JN** 2001 Proximal cis-acting elements, including steroidogenic factor 1, mediate the efficiency of a distal enhancer in the promoter of the rat gonadotropin-releasing hormone receptor gene. Molecular Endocrinology 15:319-337

149. **Merika M, Thanos D** 2001 Enhanceosomes. Curr Opin Genet Dev 11:205-208

150. **Leal AM, Takabe K, Wang L, Donaldson CJ, MacConell LA, Bilezikjian LM, Verma IM, Vale W** 2002 Effect of adenovirus-mediated overexpression of follistatin and extracellular domain of activin receptor type II on gonadotropin secretion in vitro and in vivo. Endocrinology 143:964-969

151. **He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B** 1998 A simplified system for generating recombinant adenoviruses. Proceedings of the National Academy of Sciences (U S A) 95:2509-2514

152. **Clay CM, Nelson SE, DiGregorio GB, Campion CE, Wiedrmann AL, Nett RJ** 1995 Cell-specific expression of the mouse gonadotropin-releasing hormone (GnRH) receptor gene is conferred by elements residing within 500 bp of proximal 5'flanking region. Endocrine 3:615-622

153. **White BR, Duval DL, Mulvaney JM, Roberson MS, Clay CM** 1999 Homologous regulation of the gonadotropin-releasing hormone receptor gene is partially mediated by protein kinase C activation of an activator protein-1 element. Molecular Endocrinology 13:566-577

154. **Kam K, Jeong K, Norwitz ER, Kaiser UB,** Characterization and Roles of SURG-1 Binding Proteins in the Regulation of Mouse GnRH Receptor Gene Expression. p P3 (Abstract)

155. **Maurer R, Kim KE, Schoderbek WE, Roberson MS, Glenn DJ** 1999 Regulation of glycoprotein hormone alpha-subunit gene expression. Recent Progress in Hormone Research 54:455-484

156. **Chang H, Brown CW, Matzuk MM** 2002 Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocrine Reviews 23:787-823

157. **Scully KM, Rosenfeld MG** 2002 Pituitary development: regulatory codes in mammalian organogenesis. Science 295:2231-2235

158. **Kumar TR, Wang Y, Lu N, Matzuk MM** 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nature Genetics 15:201-204

159. **Besecke LM, Guendner MJ, Schneyer AL, Bauer-Dantoin AC, Jameson JL, Weiss J** 1996 Gonadotropin-releasing hormone regulates follicle-stimulating hormone-β gene expression through an activin/follistatin autocrine or paracrine loop. Endocrinology 137:3667-3673

160. **Tucker SC, Wisdom R** 1999 Site-specific heterodimerization by paired class homeodomain proteins mediates selective transcriptional responses. Journal of Biological Chemistry 274:32325-32332

161. **Norwitz ER, Cardona GR, Jeong KH, Chin WW** 1999 Identification and characterization of the gonadotropin-releasing hormone response elements in the mouse gonadotropin-releasing hormone receptor gene. Journal of Biological Chemistry 274:867-880

162. **Zhang Y, Feng X-H, Derynck R** 1998 Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-β-induced transcription. Nature 394:909-913

163. **Germain S, Howell M, Esslemont GM, Hill CS.** Homeodomain and winged-helix transcription factors recruit activated Smads to distinct promoter elements via a common Smad interaction

motif. Genes and Development 14, 435-451. 2000.

164. **Ericson J, Norlin S, Jessell TM, Edlund T** 1998 Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. Development 125:1005-1015

165. **Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF, Shimasaki S** 2000 Bone morphogenetic protein-15. Identification of target cells and biological functions. Journal of Biological Chemistry 275:39523-39528

166. **Iemura S, Yamamoto TS, Takagi C, Uchiyama H, Natsume T, Shimasaki S, Sugino H, Ueno N** 1998 Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. Proceedings of the National Academy of Sciences (U S A) 95:9337-9342

167. **Albarracin CT, Kaiser UB, Chin WW** 1994 Isolation and characterization of the 5'-flanking region of the mouse gonadotropin-releasing hormone receptor gene. Endocrinology 135:2300-2306

168. **Braden TD, Farnworth PG, Burger HG, Conn PM** 1990 Regulation of the synthetic rate of gonadotropin-releasing hormone receptors in rat pituitary cell cultures by inhibin. Endocrinology 127:2387-2392

169. **Ying SY** 1989 Inhibins, activins and follistatins. Journal of Steroid Biochemistry 33:705-713

170. **Roberts VJ, Peto CA, Vale W, Sawchenko PE** 1992 Inhibin/activin subunits are costored with FSH and LH in secretory granules of the rat anterior pituitary gland. Neuroendocrinology 56:214-224

171. **Cherrington BD, Farmerie TA, Lents CA, Cantlon JD, Roberson MS, Clay CM** 2005 Activin responsiveness of the murine gonadotropin-releasing hormone receptor gene is mediated by a composite enhancer containing spatially distinct regulatory elements. Molecular Endocrinology 19:898-912

172. **Chen C-LC** 1993 Inhibin and activin as paracrine/autocrine factors. Endocrinology 132:4-5

173. **Doi M, Igarashi M, Hasegawa Y, Eto Y, Shibai H, Miura T, Ibuki Y** 1992 *In vivo* action of activin-A on pituitary-gonadal system. Endocrinology 130:139-144

174. **Albanese C, Colin IM, Crowley WF, Ito M, Pestell RG, Weiss J, Jameson JL** 1996 The gonadotropin genes: evolution of distinct mechanisms for hormonal control. Recent Progress in Hormone Research 51:23-58

175. **Hamernik DL, Keri RA, Clay CM, Clay JN, Sherman GB, Sawyer HR, Jr., Nett TM, Nilson JH** 1992 Gonadotrope- and thyrotrope-specific expression of the human and bovine glycoprotein hormone α-subunit genes is regulated by distinct *cis*-acting elements. Molecular Endocrinology 6:1745-1755

176. **Swift GH, Kruse F, MacDonald RJ, Hammer RE** 1989 Differential requirements for cell-specific elastase I enhancer domains in transfected cells and transgenic mice. Genes and Development 3:687-696

177. **Schoneveld OJ, Gaemers IC, Hoogenkamp M, Lamers WH** 2005 The role of proximal-enhancer elements in the glucocorticoid regulation of carbamoylphosphate synthetase gene transcription from the upstream response unit. Biochimie 2005 Nov ;87 (11):1033 -40 Epub 2005 May 31 87:1033-1040

178. **Spies HG, Niswender GD** 1971 Levels of prolactin, LH, and FSH in the serum of intact and pelvic Neurectomized rats. Endocrinology 1971 Apr; 88(4): 937-43.