DISSERTATION

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

Submitted by

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

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ABSTRACT OF DISSERTATION

MOLECULAR MECHANISMS UNDERLYING HORMONAL REGULATION OF THE MURINE GONADOTROPIN-RELEASING HORMONE RECEPTOR GENE

Interaction of gonadotropin-releasing hormone (GnRH) with its receptor on the surface of gonadotropes represents a central point for regulation of reproductive function. Consequently, considerable effort has been devoted toward understanding the regulation of this hormone and its receptor. Toward this end, I have found that transcriptional activity of the murine GnRH receptor (GnRHR) gene is mediated by three elements: a binding site for steroidogenic factor-1 (SF-1), an AP-1 element and the GnRH receptor activating sequence (GRAS). Each of these elements contributes approximately equally to basal activity of the promoter (1). Activin, a member of the TGF-β family of growth and differentiation factors, stimulates expression of the murine GnRHR gene. I have established that 600 bp of 5' flanking sequence from this gene are sufficient to confer activin responsiveness in the gonadotrope-derived αT3-1 cell line. GRAS was both necessary and sufficient to confer activin responsiveness. At issue, then, was the identity of the DNA binding proteins necessary to mediate functional activity at GRAS. I have found that Smad4 interacts

at the 5' end of GRAS. Smad3 appears to interact at GRAS based on overexpression and yeast one-hybrid assays. Interestingly, GRAS also mediates a synergistic response to activin and GnRH. Consistent with GnRH regulation at GRAS, I have shown that Jun and Fos bind to the center of GRAS. Furthermore, a recently identified member of the forkhead family of transcription factors, FoxL2, can interact at the 3' end of GRAS. Thus, GRAS represents a composite regulatory element that is bound by a multi-factoral complex. The AP-1 element has been shown to mediate responsiveness to GnRH (2;3). I have found that GnRH responsiveness of the GnRHR gene is greatly attenuated by estradiol. Replacement of the AP-1 element with a cAMP response element (CRE) does not affect GnRH-responsiveness of the promoter, but does eliminate the effect of estradiol.

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that have enabled me to succeed in life. Most of all I would like to say how much I appreciate the support and encouragement that I have received from my husband, Greg Ellsworth, throughout my graduate career.

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CHAPTER ONE

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) was structurally characterized in 1971 (4), and has since been shown to play a central role in reproduction. The release of GnRH from the hypothalamus is necessary for synthesis and secretion of luteinizing hormone (LH) and, to a lesser extent, follicle-stimulating hormone (FSH) from the anterior pituitary gland (5-8). In addition, expression of genes encoding the subunits of LH is dependent on GnRH input (9).

Fluxuations in GnRH secretion from the hypothalamus (7;10-13) and changes in the number of pituitary receptors for GnRH are important for regulating gonadotropin secretion (14). Thus, changes in pituitary content and secretion of LH are not only dependent on changes in GnRH levels but also the number of GnRH receptors available for binding and, consequently, the responsiveness of the pituitary to a given dose of GnRH (7;13;15-18).

Since the isolation of cDNA's encoding the GnRHR (19), a number of groups have demonstrated coordinate changes in GnRHR numbers and levels of GnRHR mRNA (17;18;20-23). 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 (24-30). Thus,

multiple endocrine inputs are required for proper control of GnRHR gene expression.

In the following studies I have investigated the molecular mechanisms involved in activin, GnRH and estradiol regulation of the murine GnRHR gene. I have found that activin responsiveness is mediated at a novel element termed GnRH receptor activating sequence (GRAS) (1) and that this element is bound by members of a class of transcription factors referred to as Smads, AP-1 proteins and a member of the winged helix family of transcription factors. In addition to activin responsiveness, I demonstrate that GRAS mediates a synergistic response to activin and GnRH. Finally, these studies show that GnRH responsiveness is attentuated by treatment with estradiol and that this negative regulation by estradiol is mediated at the AP-1 element.

CHAPTER TWO

LITERATURE REVIEW

I. *Hypothalamic-Pituitary-Gonadal Axis*

Central to the hypothalamic-pituitary-gonadal axis is the anterior pituitary gland (Figure 1). This gland contains several hormone-secreting cell types: lactotropes, somatotropes, thyrotropes, corticotropes and gonadotropes. The cell type that will be the focus of this dissertation is the gonadotrope. GnRH secreted from the hypothalamus binds its receptor on the surface of gonadotropes causing a number of events to occur including an increase in the number of GnRHRs and synthesis and secretion of LH and to some extent FSH (Figure 2). FSH secretion is dependent upon the presence of activin. Activin levels increase transiently in early proestrus (31), which is when GnRH levels begin to rise (32). This coincident rise in activin and GnRH may be important for increasing the number of GnRHRs resulting in sensitization of the gonadotrope to GnRH thus contributing to the LH surge.

Gonadotropic hormones act on the gonads to stimulate spermatogenesis, folliculogenesis, ovulation and synthesis and secretion of steroids. LH stimulation of Leydig cells results in secretion of testosterone that is

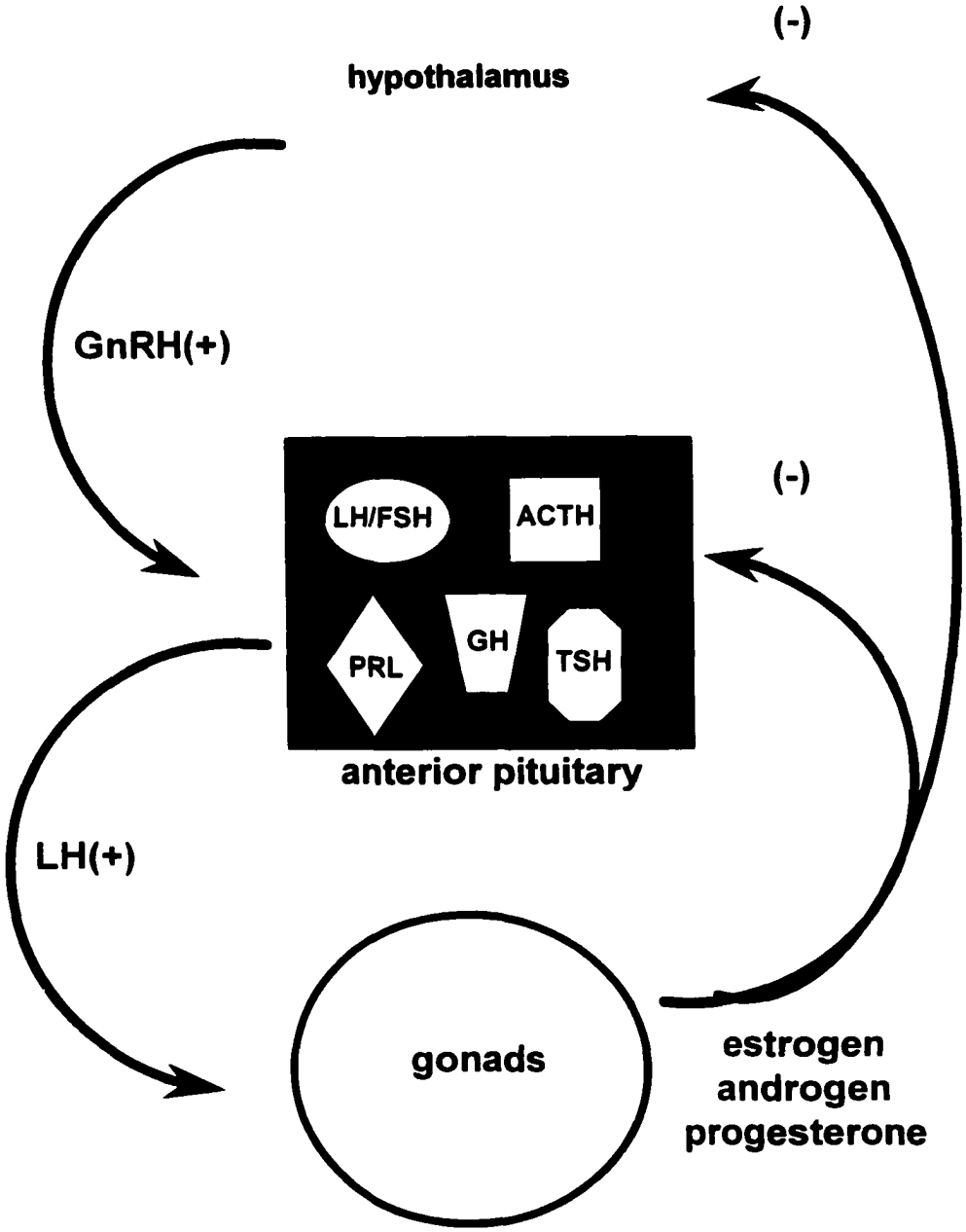

Figure 1. Hypothalamic-Pituitary-Gonadal Axis. Central to the hypothalamic-pituitary-gonadal axis is the anterior pituitary gland. This gland contains several cell type. The cells that are important for the work presented in this dissertation are gonadotrope cells. Gonadotropes are a target for input from GnRH secreted by the hypothalamus resulting in synthesis and secretion of LH and to some extent FSH. These gonadotropin 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 the hypothalamus.

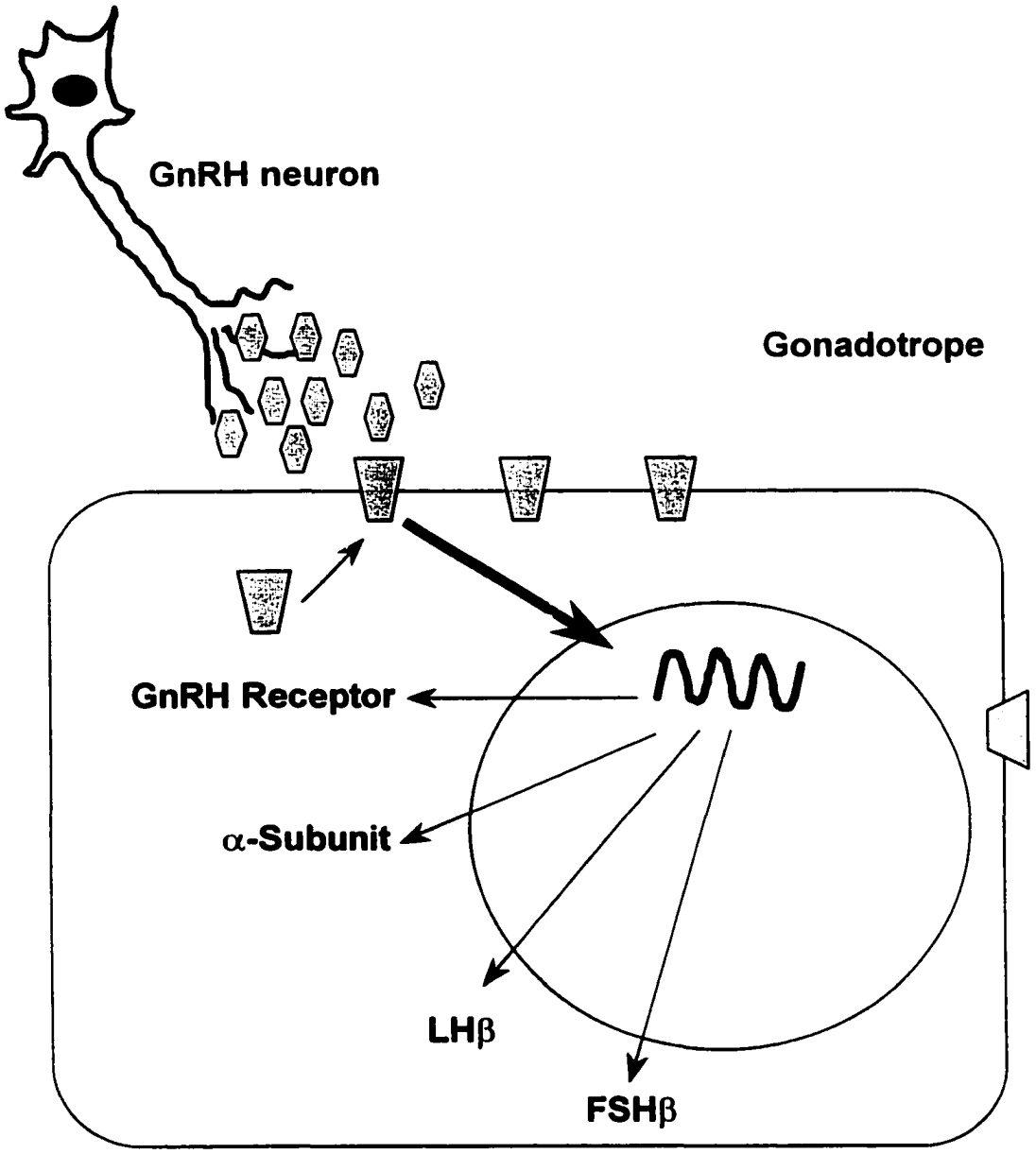

Figure 2. Model of a Gonadotrope. This diagram represents an idealized gonadotrope. Because it is a target for GnRH, it must express GnRH receptors. Upon binding to its receptor, GnRH stimulates the expression of four genes: the glycoprotein hormone α-subunit, LHβ, FSHβ and GnRHR itself.

essential for spermatogenesis (32). FSH acts on Sertoli cells that form a blood-testis barrier, acting as a filter that permits only certain substances to reach spermatocytes (32).

Folliculogenesis is defined as the development of a follicle from the primordial stage through a series of morphologically distinct forms: primary, preantral, antral and finally culminating in the Graafian or preovulatory follicle stage. Follicular development can be divided into three stages. Initiation occurs from birth to senescence and is independent of gonadotropic support. Progression is dependent on FSH stimulation. Maturation requries LH input (33-35). The LH surge results in initiation of luteinization, signals the ooctye to commence meiotic maturation and leads to rupture of the follicle wall (36;37).

Currently, the best characterized ovarian growth factors are insulin-like growth factor I and II (38) and members of the TGF-β superfamily of growth and differentiation factors (39). The TGF-β superfamily includes TGF-β, activin, and bone morphogenetic proteins (40). Activin stimulates FSH secretion in most species, while inhibin inhibits release of this hormone (32). GDF-9, a member of the BMP family is expressed specifically in the oocyte (41;42) and is required for early follicular growth (43;44).

Steroids have numerous effects throughout the body. A high intratesticular testosterone concentration is essential for spermatogenesis. Estradiol production is the hallmark of preovulatory follicular development (34). Steroids can participate in a negative feedback loop that affects the anterior pituitary and hypothalamus. One mechanism by which steroids act to down-regulate pituitary

function is to inhibit transcription of genes expressed in the pituitary including several genes expressed specifically in gonadotropes. The gonadotropic hormones are made up of a specific α glycoprotein hormone subunit and a unique β subunit.

Estradiol can augment GnRH-stimulated LH secretion, possibly due to stimulation of GnRHR gene expression (32). The effects of estradiol are not that simple, however. In primary cultures of rat pituitary cells, estradiol can both increase (chronic exposure) and decrease (short-term exposure) GnRHR numbers (45-47). While in the gonadotrope-derived αT3-1 cell line, the negative effect of estradiol on GnRHR numbers, but not the positive effect is recapitulated (47;48). This apparent contradiction may be explained if the up-regulation of GnRHR numbers seen in primary cultures occurs indirectly, involving steroid hormone effects on cells other than gonadotropes (47;48). Thus, one must consider the interaction of genes and their gene products not only in an individual cell, but in mixtures of cells.

II. Transcriptional Regulation of the Gonadotropin Subunit Genes

A. *Glycoprotein Hormone α Subunit Gene*

A great deal of effort has been expended to understand the molecular events underlying the ontogeny of pituitary gonadotropes and expression of their primary gene products: the glycoprotein hormone α subunit, the unique LHβ and FSHβ subunits, and the GnRHR (Figure 2). Basal expression of the glycoprotein hormone α-subunit gene appears to involve the pituitary glycoprotein hormone

basal element (PGBE). This element is bound by a LIM-homeodomain transcription factor referred to as LH-2. Overexpression of LH-2 can activate the α-subunit promoter in heterologous cells (49).

The α-subunit gene promoter contains a gonadotrope-specific element (GSE) that has been shown to bind the orphan nuclear receptor referred to as steroidogenic factor 1 (SF-1) (50). The GSE is required for tissue-specific expression of the α-subunit gene. In mice, disruption of the Ftz-F1 gene encoding SF-1 results in decreased but detectable levels of α-subunit (51). In addition to basal regulation, expression of the glycoprotein hormone α-subunit gene is influenced by a number of hormones.

GnRH stimulates the α-subunit gene via extracellular signal-regulated kinase (ERK); a member of the mitogen activated protein (MAP) kinase family (52). GnRH signaling to the α-subunit promoter involves Raf-1 kinase and Elk, signaling intermediates of the MAP kinase pathway (52). GnRH-responsiveness appears to involve a consensus Ets binding site (49). However, this site does not appear to bind an Ets protein and thus the GnRH stimulation of the glycoprotein hormone α-subunit promoter is most likely mediated by another transcription factor (53).

Androgen receptor (AR) can suppress transcription of the α glycoprotein hormone subunit gene in a ligand-dependent manner. This suppression appears to require two elements in the α-subunit promoter: the α basal element and tandem CREs. CREs have been shown to bind several members of the bZIP

family. Overexpression of cJun and activating transcription factor 2 (ATF2), but not CREB, reversed the inhibition caused by AR. And, in fact, AR interacts with cJun and ATF2 through protein-protein interactions (54).

B. *LHβ Subunit Gene*

Tandem copies of binding sites for early growth response protein-1 (Egr-1) and SF-1 are located in the LHβ promoter (55) and contribute to both basal activity and GnRH-responsiveness of the gene. Located between these elements is a single region that is capable of binding Pitx1. It appears that all of these sites interact cooperatively through a mechanism that does not require Pitx1 binding (55). The Pitx1 element is required not only for basal expression but also for GnRH regulation. A NF-Y binding site appears to be required for basal activity but not GnRH-regulation of the bovine LHβ-subunit gene (56).

GnRH stimulation of LHβ requires interactions between a complex distal response element containing two specificity protein-1 (Sp1) binding sites and a CArG box, and a proximal element with two bipartite binding sites for SF-1 and Egr-1 (57). The 3' Sp1 site is critical for basal expression. The 5' site partially overlaps a CArG box and mutation of the CArG element specifically eliminates the response to pulsatile GnRH but retains Sp1 binding. Thus a downstream element probably contributes to the full GnRH response (53).

Androgen treatment decreases GnRH stimulation of the rat LHβ gene by about 75% (57). The mechanism for this inhibition occurs primarily through direct interaction of androgen receptor with Sp1 with possible involvement of Egr-1 and

reduces cooperation between distal and proximal GnRH response elements (57). Androgen suppression can be rescued by overexpression of Egr-1, Pitx1 or a constitutively active SF-1 (SF-1ΔLBD) that has a truncated ligand binding domain (54). Furthermore, protein-protein interactions occur between AR and SF-1. Overexpression of a full-length SF-1 construct does not rescue LHβ activity, suggesting that the ligand-binding domain probably plays an important role in the interactions that occur between SF-1 and AR (54). Recently, a line of mice was created in which SF-1 was specifically disrupted in gonadotropes and thyrotropes (58). Thyrotropes do not express SF-1 and thus SF-1 expression was affected only in gonadotropes. Gonadotrope-specific disruption of the gene encoding for SF-1 results in a loss of immunoreactive LH (58).

C. *FSHβ Subunit Gene*

Inhibin regulates FSHβ mRNA stability and suppresses FSHβ gene transcription (59). SF-1 does not appear to directly regulate expression of FSHβ (60) however, in mice that do not express SF-1 in gonadotropes, immunoreactive FSH was virtually absent and serum FSH levels were dramatically reduced (58), suggesting that SF-1 is indirectly involved in FSHβ expression.

GnRH has been shown to stimulate the FSHβ gene via two AP-1 enhancer elements by a mechanism that involves PKC. These experiments were using HeLa cells that had been transfected with an expression vector for the GnRHR (61). The two AP-1 elements did not, however, appear to be important for GnRH stimulation of the FSHβ gene in a gonadotrope model (LβT2 cells), but

PKC was shown to be involved (62). GnRH induction of the FSHβ gene is inhibited by follistatin, suggesting its dependence on endogenous activin (63;64).

Activin appears to play a major role in transcriptional regulation of the FSHβ gene (63-65). Systemic administration of activin A to rats causes a significant rise in both FSHβ mRNA and serum FSH levels (66). These findings were confirmed using *in vitro* studies (67). Although the activin response element remains undefined (65), it appears to reside within 500 bp of proximal promoter from the ovine FSHβ gene (63;68).

III. Transcriptional Regulation of the GnRHR Gene

GnRH and its receptor play key roles in reproductive function of mammals. In light of this fact, considerable effort has been expended toward understanding the molecular mechanisms involved in regulation of GnRHR gene expression. Many of these studies have made use of the gonadotrope-derived αT3-1 cell line. These cells express GnRHR and therefore should contain all of the regulatory factors necessary for GnRHR gene expression (Figure 3). These cells also produce activin and its receptor and therefore exist in a constitutively activin-stimulated state.

The studies presented in this dissertation have focused on the molecular mechanisms involved in transcriptional regulation of the GnRHR gene. A number

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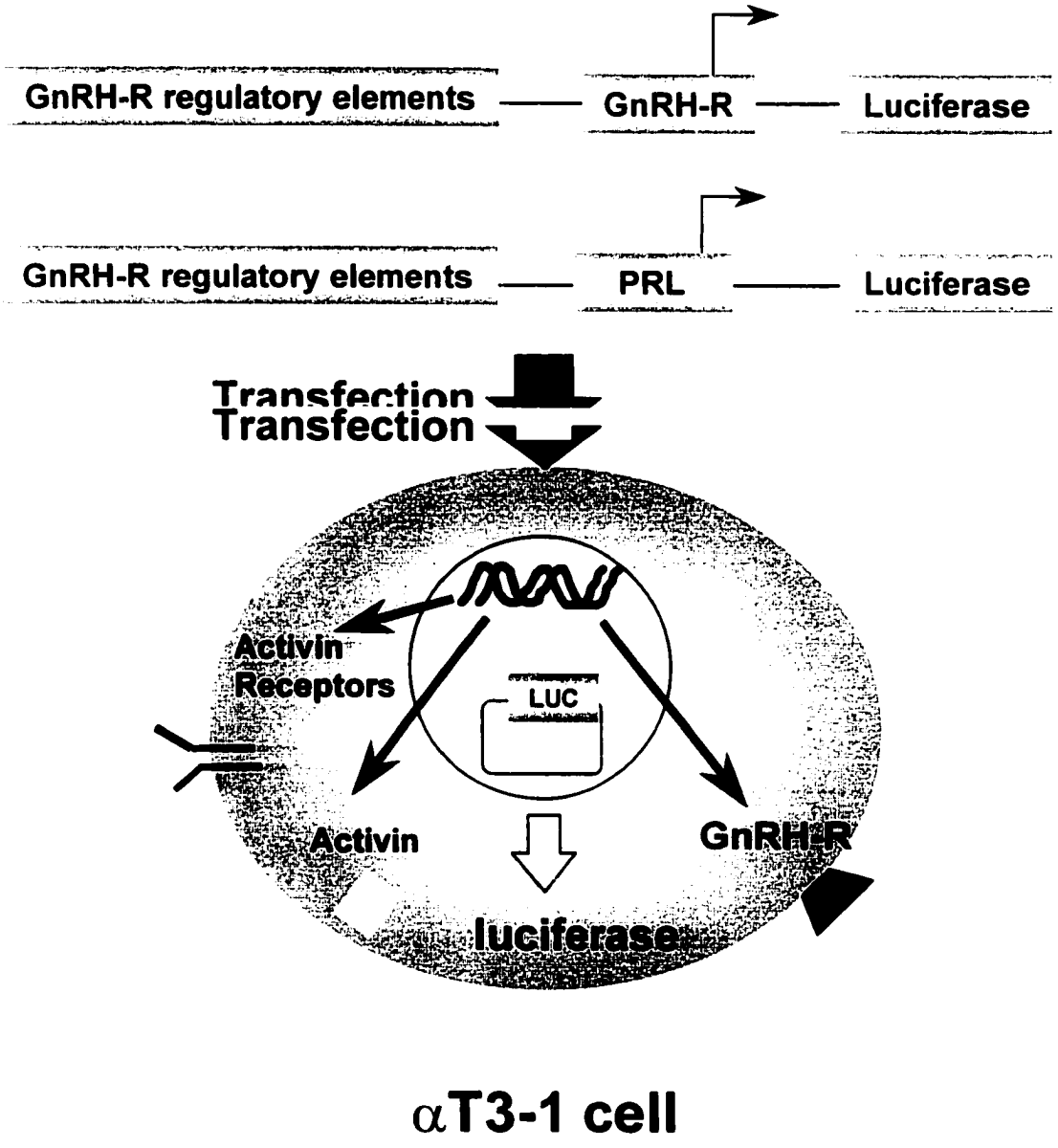


Figure 3. Model of an αT3-1 cell. αT3-1 cells express the GnRHR and therefore should contain all the transcriptional regulatory proteins necessary for expression of the GnRHR gene. Like gonadotropes, these cells produce activin and therefore exist in a constitutively activin-stimulated state.

of these studies investigated either the GnRHR promoter or individual cis-acting elements in transfection studies of αT3-1 cells (Figure 3). Basal activity of the murine GnRHR promoter in the gonadotrope-derived αT3-1 cell line (69) is mediated by a complex enhancer located within 500 bp of proximal promoter that includes a binding site for the nuclear orphan receptor steroidogenic factor-1 (SF-1), a consensus AP-1 element and a non-canonical element we have termed the GnRHR activating sequence or GRAS (1;70) (Figure 4). More recently, we have established that GnRH responsiveness of the GnRHR gene promoter is primarily conferred through protein kinase C (PKC) activation at the AP-1 element (Figure 4)(2). Thus, cell-specific and hormone-regulated expression partially converge at common elements in the proximal promoter of the GnRHR gene.

SF-1 was originally identified as a transcription factor that regulates steroid hydroxylase genes (71), but clearly it has other functions (44). Mice in which the SF-1 gene has been disrupted have no gonads, no adrenal glands, and abnormal pituitary and hypothalamic function (71;72). Selective disruption of the gene encoding SF-1 only in gonadotropes results in a marked reduction in GnRHR numbers (58).

A. Homologous Regulation of the GnRHR Gene

GnRH induced signal transduction partially occurs via coupling of the receptor with $G_{q\alpha}/G_{11\alpha}$ leading to stimulation of phospholipase activities, formation of inositol 1,4,5-trisphosphate and diacylglycerol, elevation of intracellular free calcium concentrations and activation of one or more isoforms of

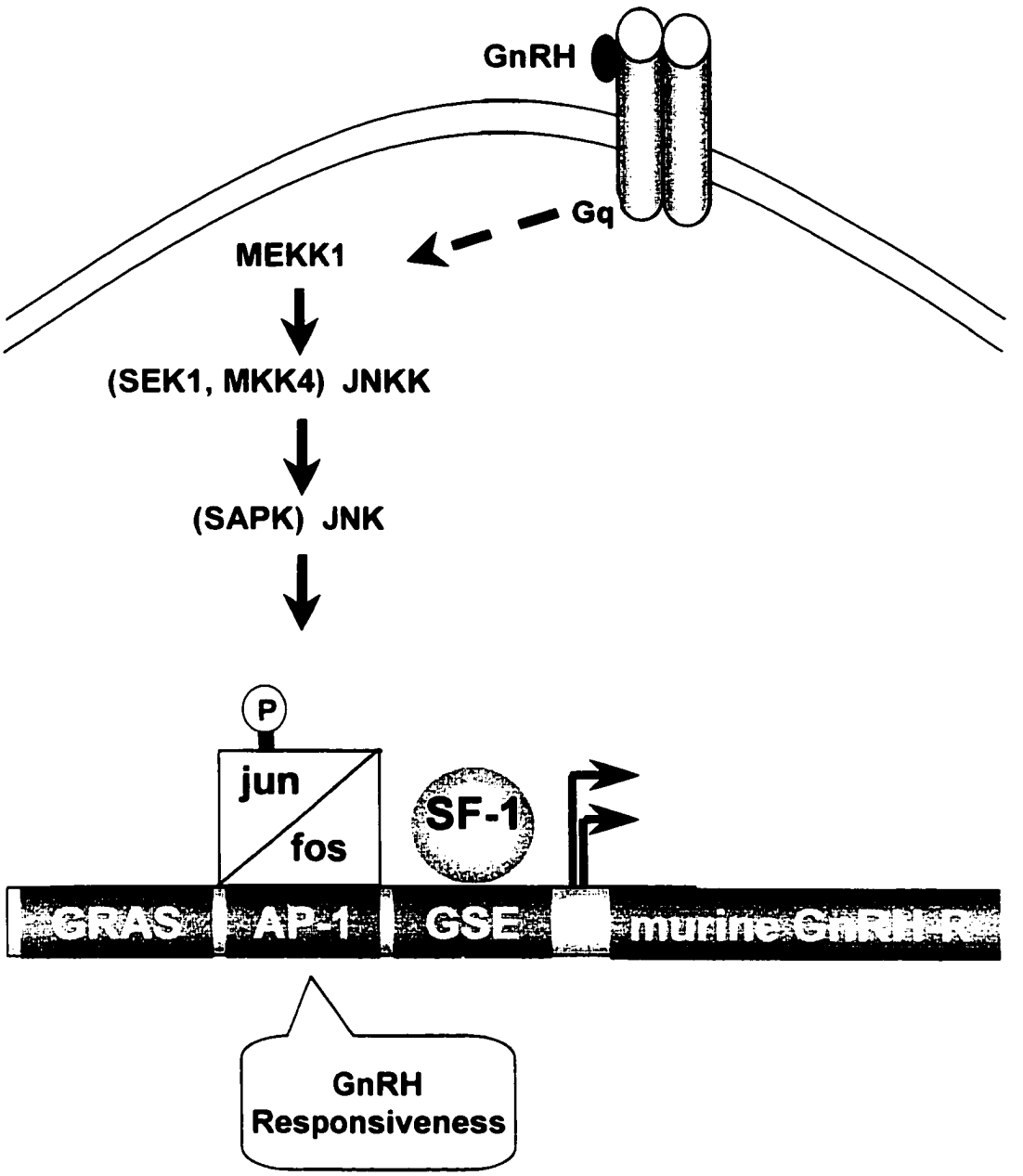


Figure 4. Model of the Proximal Promoter from the Murine GnRHR Gene. GRAS, AP-1 and the GSE are contained within 500 bp of proximal promoter from the murine GnRHR gene. The GSE has been shown to bind SF-1, while the AP-1 site binds Jun and Fos. AP-1 has been shown to mediate responsiveness to GnRH via the JNK pathway.

PKC (73;74). In αT3-1 cells, GnRH has been shown to stimulate multiple mitogen activated protein (MAP) kinase signaling cascades including extracellular signal regulated kinase (ERK) (52;75) , Jun N-terminal kinase (JNK) (76) and p38 MAP kinase (77).

Approximately 1900 bp of proximal 5' flanking region of the GnRHR gene is sufficient to confer GnRH responsiveness on a heterologous reporter gene in transgenic mice (78). The response of the GnRHR promoter to GnRH is ultimately mediated at the AP-1 element located at –336 and that GnRH-induced activation of this element is partially mediated by PKC (2;3). Mutation of the AP-1 element eliminates GnRH-responsiveness of the murine GnRHR promoter in transgenic mice (79). The specific components of the AP-1 binding complex include JunD, c-Fos, and FosB. The MAP kinase intermediate c-Jun N-terminal kinase (JNK) is required for GnRH induction of the GnRHR promoter (79).

B. Activin Stimulation of the Murine GnRHR Gene

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 (28-30). Activin represents homo- or heterodimeric complexes of the different inhibin β-subunits (80). The activin binding protein, follistatin, is thought to be a primary modulator of the biological effects of activin by sequestering activin and thus prevent activin from binding to its cognate receptor (80). The activin β-subunits, as well as follistatin, are produced by gonadotropes in the anterior pituitary gland (81). Follistatin is also produced by

pituitary folliculostellate cells (82). Thus, autocrine and paracrine mechanisms are considered to be an important component of activin signaling in the pituitary gland (31;64;83;84).

The cellular effects of activin are mediated by binding to specific Type I receptors that combine with activin receptor Type II to produce an active complex with serine/threonine kinase activity (85). Although it is known that activin stimulates transcription of the GnRHR gene (28) and that both activin and activin receptors are expressed in gonadotropes and αT3-1 cells (28;80;81), the mechanism(s) by which activin regulates gene expression in gonadotropes has remained undefined.

1. Smads Mediate Activin Signaling

TGFβ family signaling has been shown to occur through a family of proteins called Smads. The term Smad comes from the names of homologous proteins in *Drosophila* (MAD) and *C. Elegans* (Sma) (Sma + MAD = Smad). MAD (mothers against decapentaplegic) is a maternal enhancer protein that is important for signaling of TGF-β family members in *Drosophila* (85). Mutation of the gene encoding for Sma results in small body size in *C. Elegans* (85). To date, 8 Smad family members have been identified and separated into 3 groups (86-89). The first of these are referred to as receptor-regulated Smads and are required for signaling by bone morphogenetic proteins (Smad1, 5, 8) and TGFβ/activin (Smad2, 3) (86-89). Second, Smad4 appears to serve as a common partner in all of the Smad signaling cascades (90;91). Finally, Smad 6

and 7, which are inhibitory Smads, appear to block activation of, or signaling by, the receptor-regulated Smads (92-95). The developing paradigm for Smad signaling is that the activated Type I receptor phosphorylates a pathway specific Smad which complexes with Smad4 and is translocated to the nucleus (88;91;96). In the nucleus, these Smad complexes have been shown to stimulate transcription. Often, however, it appears that the functional effects of Smads on transcription require interactions with non-Smad transcription factors (97-99).

2. *Fork head Proteins can Cooperate with Smads*

Fork head / winged helix proteins are members of the helix-turn-helix protein superfamily (100). Fork head transcription factors are defined by a conserved DNA-binding domain found in the *Drosophila* homeotic gene fork head and rat hepatocyte nuclear factor-3 (HNF-3β) (101). The winged helix is comprised of three tightly packed α helical domains, of which the third establishes DNA base contacts within the major groove of its cognate recognition sequence (102). In *Xenopus* a transcription factor referred to as forkhead activin signal transducer-1 (FAST-1) has been shown to work with Smad2, a TGF-β signaling intermediate, to regulate activin-responsiveness of the *Mix.2* gene (101). Homologues of FAST-1 have been shown to work with Smads and mediate activin signaling in mice (103) and humans (104).

A member of the forkhead family of transcription factors has been implicated in pituitary organogenesis. Initial expression of a transcription factor referred to as FoxL2 coincides with the emergence of gonadotropes. Thus,

FoxL2 may be important for gonadotrope differentiation (105). Mutations in the gene encoding FoxL2 are responsible for a condition known as blepharophimosis/ptosis/epicanthus inversus syndrome (BPES) (106-109). BPES, which is characterized by distinctive eye abnormalities, occurs in two forms. In type I, the eyelid abnormality is associated with premature ovarian failure (either primary or secondary amenorrhea). Males are fertile. In BPES type II, only facial abnormalities are present (106;107). It is interesting to note that mice carrying mutations in the gene encoding the activin/inhibin βB subunit, exhibit eye lid abnormalities and females with impaired reproductive ability (110). One intriguing possibility is that FoxL2 mediates signaling for activin (107).

3. Other Smad Interacting Transcription Factors

Other types of proteins can also interact with Smads. Members of the Mix family of paired-like homeodomain transcription factors, Mixer (111) and Milk (112) have been shown to be important for recruiting Smad2 / Smad4 complexes to the activin responsive element of the *Xenopus goosecoid* promoter (113;114) by binding with the effector domain of Smad2 (113). Mixer and Milk contain short motifs in their carboxyl termini that are required for interaction with Smad2 (113). Interestingly, this motif is also found in the Smad-interacting winged-helix proteins *Xenopus* Fast-1, human Fast-1 and mouse Fast-2 (113). Thus, there are several proteins that contribute to Smad function. There are also proteins that antagonize Smad function.

The *ski* oncogene encodes for a leucine zipper protein that represses transcription by recruiting histone deacetylase (115;116). c-Ski can also act as a coactivator (115). Smad2/3 interacts with c-Ski through its C-terminal MH2 domain in a TGF-β-dependent manner (116). The c-Ski protein has been shown to bind a Smad binding element (GTCTAGAC), but mutation of the DNA binding domain does not eliminate the repression caused by c-Ski (115).

C. Estradiol Regulation of GnRHR Gene Expression

Estradiol-17β exerts a profound effect on the number of GnRH receptors in the pituitary gland (24;25;117-119). This phenomenon has received particular attention as it is thought to represent an important mechanism underlying increased pituitary sensitivity to GnRH during the pre-ovulatory period and thus contributes to the high rates of LH secretion necessary for ovulation (10). Since the availability of cDNA's encoding the GnRHR, a number of studies have demonstrated coordinate changes in GnRHR mRNA and GnRHR numbers associated with estradiol treatment (14;17;18;20-23;78;120). Although it is possible that estrogen exerts its stimulatory effects on GnRHR expression via a post-transcriptional mechanism such as mRNA stabilization, the ability of actinomycin D, an inhibitor of transcription, to block estrogen up-regulation of GnRHR numbers in primary cultures of pituitary cells does not support this contention (24;25).

Alternatively, it is possible that estrogen stimulates GnRHR gene expression. If correct, one might predict high affinity binding sites for estrogen

receptor within the GnRHR gene. Unfortunately, a canonical estrogen response element (ERE) has not been identified in any GnRHR gene reported to date nor has estrogen regulation of 1900 bp of proximal promoter from the murine GnRHR gene been detected in transient transfection paradigms (121). Estradiol responsiveness of the murine GnRHR gene has also been tested in transgenic mice. Mice containing 1900 bp of proximal murine GnRHR gene fused to luciferase were injected with pellets that released estradiol-17β. Luciferase expression in estradiol-treated mice was not different from that of control mice (121). Thus, despite the central role of estrogen in regulation of the GnRHR gene, the underlying mechanisms remain undefined.

IV. Unique Physical Characteristics of the GnRHR

The mammalian GnRHR, unlike most G-protein coupled receptors, lacks a C-terminal tail (122). Lack of the C-terminal tail results in resistance of the mammalian GnRHR to rapid desensitization (123). Normally the C-terminal tails of G-protein coupled receptors are phosphorylated in an agonist-dependent manner by G-protein receptor kinases (124). Once phosphorylated the tail is bound by β-arrestin that targets the desensitized receptor to clathrin-coated vesicles for internalization (125). The clathrin-coated vesicle containing the receptor is then "pinched off" from the plasma membrane by a dynamin collar (124). The mammalian GnRHR does not have a tail to be phosphorylated and this is the most likely mechanism for the lack of rapid desensitization. The

mammalian GnRHR is internalized normally, however, suggesting that the C-terminal tail is not involved in receptor internalization (123).

Both mammalian and non-mammalian receptors internalize via clathrin-coated vesicles, however mammalian GnRHR internalization is not dependent on dynamin (124). Interestingly, signaling of both mammalian and non-mammalian receptors does depend on dynamin (124).

V. Summary

Gonadotropes of the anterior pituitary gland express the glycoprotein hormone α-subunit, the LHβ and FSHβ subunits and the GnRHR. The pulsatile secretion of GnRH from the hypothalamus stimulates the synthesis and secretion of LH and to some extent, FSH (5-8). Thus, relative changes in GnRH secretion from the hypothalamus are clearly an important determinant of gonadotropin secretion (7;10-13). In addition, changes in the number of pituitary receptors for GnRH have also been implicated as an important factor underlying the regulation of gonadotropin secretion (14). Thus, changes in pituitary content and secretion of gonadotropins are not only dependent on changes in GnRH secretion but also the number of GnRHRs available for binding and, therefore, the responsiveness of the pituitary to a given dose of GnRH (7;13;15-18). Multiple endocrine inputs have been implicated in affecting changes in GnRHR numbers, primarily estradiol, activin and GnRH itself (24-26). A number of groups have demonstrated coordinate changes in GnRHR numbers and GnRHR mRNA

(14;17;20;21;23;78), suggesting that regulation of GnRHR numbers is, at least in part, under transcriptional control.

Much effort has been expended towards understanding the molecular mechanisms involved in transcriptional regulation of the GnRHR gene. It is now known that basal regulation of the GnRHR gene requires at least three elements: binding sites for SF-1 and AP-1 and an element termed GRAS. This gene is regulated by several hormonal inputs including estradiol-17β, activin and GnRH. In the following chapters I sought to identify some of the molecules and regulatory elements that mediate these cellular signals.

CHAPTER THREE

IS GONADOTROPE EXPRESSION OF THE GONADOTROPIN RELEASING HORMONE RECEPTOR GENE MEDIATED BY AUTOCRINE/PARACRINE STIMULATION OF AN ACTIVIN RESPONSE ELEMENT?

ABSTRACT

Expression of the GnRHR gene in gonadotropes is stimulated by activin. We sought to identify the cis-acting element(s) in the murine GnRHR gene promoter that confers activin responsiveness. Because αT3-1 cells, like gonadotropes, secrete activin, we examined the ability of the activin binding protein, follistatin, to block activin stimulation of the GnRHR gene. In transient transfection assays using approximately 600 bp of the murine GnRHR gene, treatment with increasing doses of follistatin resulted in a dose dependent decrease in promoter activity, suggesting that this region of the promoter is under positive regulation by activin. Mutation analysis demonstrated that GRAS is necessary for follistatin responsiveness of the murine GnRHR gene. Treatment of GRAS that has been isolated from the context of the wild type promoter with follistatin decreases enhancer activity significantly, while treatment with activin A results in a four-fold stimulation of GRAS enhancer activity, suggesting that

GRAS is sufficient to mediate activin responsiveness. Addition of excess activin A reverses follistatin inhibition, suggesting that the negative effects of follistatin are due to its ability to bind activin. In light of these data, we conclude that GRAS mediates activin responsiveness of the murine GnRHR gene.

INTRODUCTION

The hypothalamic-pituitary-gonadal axis controls reproductive functions. Central to this axis is the anterior pituitary gland. This gland contains several cell types including lactotropes, sommatotropes, corticotropes, thyrotropes and gonadotropes. Gonadotropes are defined by their expression of the gonadotropic subunits: luteinizing hormone (LH) β, follicle stimulating hormone (FSH) β and the common α subunit. As a target for GnRH, gonadotropes must also express GnRH receptors. GnRH secreted by the hypothalamus binds to specific receptors on the surface of gonadotropes, resulting in the synthesis and secretion of LH and to some extent FSH. These gonadotropin hormones act on the gonads to stimulate follicular development, spermatogenesis and the synthesis and secretion of steroids. Steroids can then participate in negative feedback at the level of the pituitary and hypothalamus. The magnitude of gonadotropin secretion is dependent upon the amount of GnRH released by the hypothalamus and the sensitivity of the gonadotropes as determined by the number of GnRH receptors. Consequently, regulation of the GnRHR gene represents a critical factor in reproductive function.

Upon binding to its receptor, GnRH stimulates the transcription of several genes including: the α-glycoprotein hormone subunit, LHβ, FSHβ, and the GnRH receptor, itself. The GnRHR gene is known to respond to a number of endocrine inputs including GnRH, estradiol and activin. Previously, we have found that approximately 500 bp of proximal 5' flanking region is required for cell specific expression of the murine GnRHR gene. Contained within this region is an element referred to as the gonadotrope-specific element that binds SF-1, a member of the orphan nuclear receptor family. Also contained within this region is a binding site for AP-1 and a non-canonical element we have termed the GnRHR activating sequence or GRAS. GnRH responsiveness of this promoter is mediated at AP-1 in transient transfection assays. The GnRHR gene is also regulated by GnRH in transgenic mice and mutation of AP-1 eliminates this regulation (79). Upon binding of GnRH to its receptor the cJun N-terminal kinase or JNK pathway is activated resulting in an increased recruitment of Jun and Fos binding at AP-1 (79).

A number of studies have indicated that transcription of the GnRHR gene is also regulated by activin. Activin is a member of the TGF-β superfamily of growth and differentiation factors. TGF-β family members have diverse functions during embryonic development and adult homeostasis (126). Activin represents homo- or heterodimeric complexes of the different inhibin β-subunits (80). The activin binding protein, follistatin, is thought to be a primary modulator of the biological effects of activin by sequestering activin and thus preventing it from binding to its cognate receptor (80). In the pituitary gland, the subunits for activin

as well as follistatin are produced by gonadotropes (81). Follistatin is also synthesized in folliculostellate cells (82). Thus, autocrine and paracrine mechanisms are an important component of activin signaling in the pituitary gland (31;64;83;84). Activin transduces its signal by binding to the type I receptor that then binds the type II receptor. Upon dimerization, the constitutively active type II receptors phosphorylate the type I receptor in a glycine-serine rich region, resulting in activation of the receptors serine-threonine kinase activity (87). These receptor subtypes have been identified in gonadotropes, including the gonadotrope-derived αT3-1 cell line. Although it is known that activin stimulation increases transcription of the GnRHR gene (28) and that both activin and activin receptors are expressed in gonadotropes and the gonadotrope-derived αT3-1 cell line (28;80;81), the mechanism(s) by which activin regulates gene expression in gonadotropes has not been elucidated. An important first step in characterizing these mechanisms is identification of elements that mediate activin responsiveness in gonadotrope-expressed genes. We have used the activin binding protein, follistatin in a number of the following studies to look at a decrease in the hyperstimulated activity of the GnRHR gene. Herein, we report the identification of an element in the GnRHR gene promoter that mediates activin responsiveness.

MATERIALS AND METHODS

Reagents: Recombinant Human activin A (*rh*activin A; 15365-36) and recombinant human follistatin (*rh*follistatin; B4384) were obtained through NHPP, NIDDK and Dr. A. F. Parlow.

Vector construction and transient transfections: The construction of luciferase expression vectors has been described (1;70). Cultures of αT3-1 cells were transfected using LipofectAMINE (Gibco BRL, Githersburg, MD) (70). Transfections included 1.4 μg of the test vector and 0.25 μg of pRSV-LacZ. Follistatin was administered immediately after the transfection mixture while Activin A was given 16 h after transfection. At the specified times following transfection, the cells were harvested and assayed for luciferase and β-galactosidase activity (70). Luciferase activity was normalized for transfection efficiency by dividing luciferase activity by β-galactosidase activity.

Statistical Analysis: The data (Figures 5, 10) were analyzed by one-way ANOVA with vector as the independent variable. If the F test was significant ($P<0.05$), means were separated using Dunnett's (Figure 5) or Tukey's (Figure 10) methods of multiple comparisons. In all other figures, treated and untreated vectors were compared using Student's T-test.

RESULTS

Follistatin treatment results in a dose dependent decrease in GnRHR promoter activity. A number of studies have suggested that activin regulates the number of GnRH receptors on gonadotropes (28-30). However, activin is

constitutively secreted by the gonadotrope-derived αT3-1 cells making activin studies in these cells difficult. To circumvent this problem, we used the activin binding protein, follistatin. Follistatin binds to activin and prevents it from interacting with its receptor. Thus, instead of looking at an increase in promoter activity with hormone treatment, we were looking at a reduction in promoter activity as follistatin sequestered activin from the cellular environment.

In this experiment, αT3-1 cells were transiently transfected with a luciferase reporter gene containing approximately 600 bp of wild-type murine GnRHR gene. Cells were then treated with increasing amounts of follistatin for 24 h then harvested and assayed for luciferase activity. Treatment of cells with 10 ng/mL of follistatin decreased promoter activity significantly (Figure 5). Activity of the promoter was decreased maximally by treatment with 50 ng/mL follistatin. These data suggest that this region of the promoter is sufficient to respond to follistatin treatment. In light of the fact that follistatin is an activin binding protein, we suggest that this region of the promoter contains an activin responsive region.

Mutation of GRAS blocks activin/follistatin responsiveness. Basal activity of the murine GnRHR gene is mediated by a complex enhancer containing three elements: binding sites for SF-1 and AP-1 and a non-canonical element termed GRAS. To determine if any of these regions was important for follistatin/activin responsiveness of the murine GnRHR gene, each of these elements was mutated by replacement with a restriction site for *Not*I (GRAS, SF-1) or *Eco*RI (AP-1). Mutation of any one of these elements decreases promoter activity by about half, while mutation of all three elements eliminates

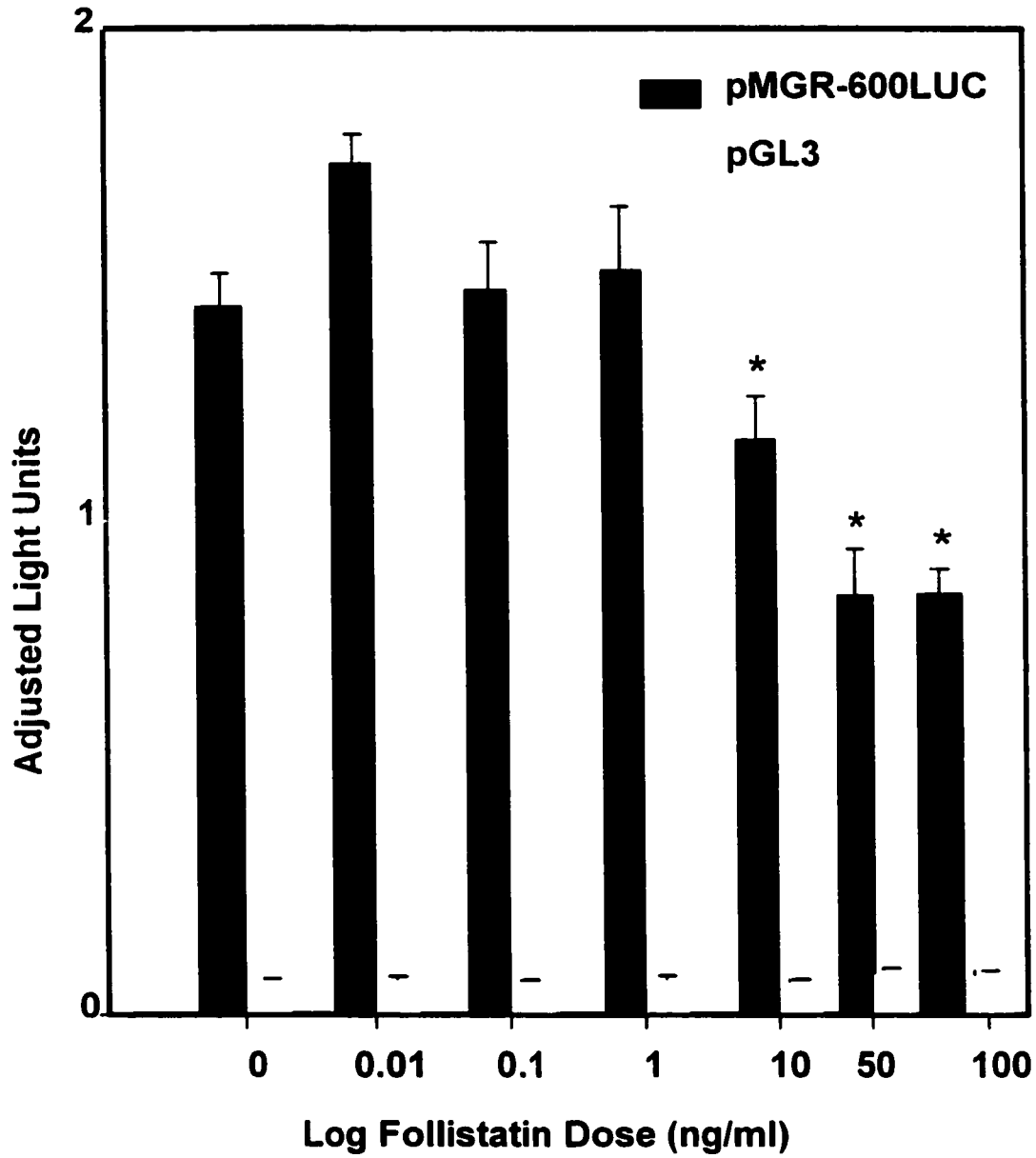


Figure 5. Follistatin treatment results in a dose dependent decrease in GnRHR promoter activity. Either the wild-type promoter (pMGR-600LUC) or the empty luciferase reporter construct (pGL3) were transiently transfected into αT3-1 cells and treated with increasing amounts of follistatin. After approximately 24 h, cells were harvested and assayed for luciferase activity. Values represent mean and SD of triplicate samples. * Represents values significantly different ($p<0.05$) from untreated pMGR-600LUC.

promoter activity (1). Cells were transiently transfected with the mutant vectors and treated with 100 ng/mL follistatin. After 24 h cells were harvested and assayed for luciferase activity. As expected, activity of the wild-type promoter was reduced by approximately 50% with follistatin treatment (Figure 6). Activity of vectors in which either the binding site for SF-1 or the binding site for AP-1 had been mutated was also decreased by approximately 50% indicating that neither of these elements is the activin/follistatin responsive element. In contrast, treatment of the vector in which GRAS had been mutated completely eliminated the ability of the promoter to respond to follistatin (Figure 6), suggesting that GRAS mediates responsiveness of the murine GnRHR promoter to activin/follistatin.

The activin/follistatin responsiveness region maps to the functional limits of GRAS. Previous studies using two-bp transversion mutations that scan GRAS have determined that the functional boundaries of GRAS are defined by mutations 3-8 (1) (shown in Figure 7). To determine if the activin/follistatin responsive region identified by the 8 bp *Not*I mutation correlates with the functional boundaries of GRAS, we tested these same two-bp transversion mutations for their ability to respond to activin/follistatin. Each mutation was transiently transfected into αT3-1 cells and treated with 100 ng/mL follistatin. As seen before, follistatin treatment of the wild-type promoter resulted in a 50% decrease in promoter activity while replacement of GRAS with a *Not*I recognition site eliminates responsiveness of the promoter to activin/follistatin. Looking at the two-bp transversion mutations we found that activin/follistatin responsiveness

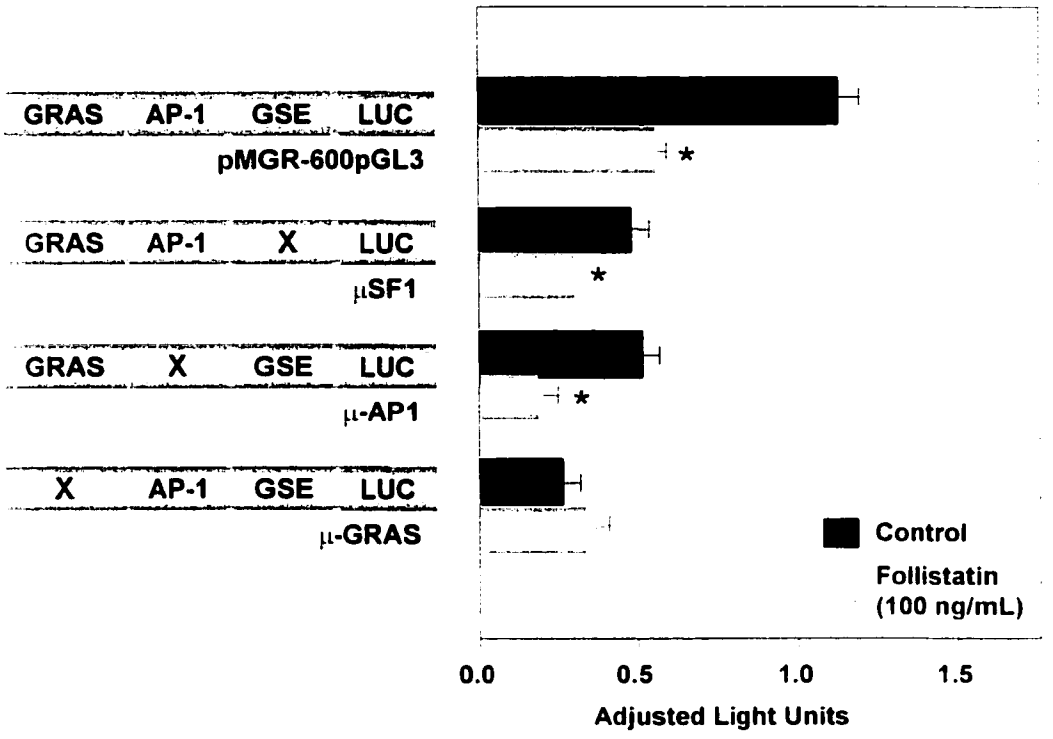


Figure 6. Mutation of GRAS blocks activin/follistatin responsiveness. Vectors were transiently transfected into αT3-1 cells and treated with follistatin. After approximately 24 h, cells were harvested and assayed for luciferase activity. Values represent the mean ± SEM for triplicate samples in transfections of 2-3 different plasmid preparations. *Represents values different ($p<0.01$) from untreated vector.

was retained in mutations 1, 2 and 9, but was lost at precisely the mutations that map the functional limits of GRAS (μGRAS 3-8) (Figure 7). Thus, the functional limits of GRAS co-localize to an activin/follistatin responsive element.

Follistatin treatment blocks the enhancer activity of GRAS. We have now established that GRAS is necessary to mediate activin/follistatin responsiveness of the murine GnRHR gene. To determine whether the presence of GRAS is sufficient to respond to activin/follistatin a luciferase reporter vector was used that contained 3 copies of GRAS fused to the minimal promoter for the rat prolactin gene (-33 to +13) (3XGRAS-PRLpGL3). As a negative control, a similar vector was used containing three direct repeats of the element mutated as in μGRAS-5 (3XμGRAS-5-PRLpGL3). These vectors were transiently transfected into αT3-1 cells and treated with 100 ng/mL follistatin. Basal activity of 3XGRAS-PRLpGL3 was nearly 100-fold greater than that of the mutant vector (Figure 8). Furthermore, the activity of 3XGRAS-PRLpGL3 was nearly blocked by follistatin treatment, but activity of the mutant vector was unaffected. Based on these data we conclude that GRAS is sufficient to confer activin/follistatin responsiveness to the murine GnRHR gene.

GRAS enhancer activity is stimulated by activin A. In the previous experiments, we have used the activin binding protein, follistatin to look indirectly at activin responsiveness of the murine GnRHR gene. We next sought to show that GRAS was stimulated by activin directly. Either 3XGRAS-PRLpGL3 or 3XμGRAS-5-PRLpGL3 were transfected into αT3-1 cells. After 16 h, cells were treated with 20 ng/mL activin. Due to the high levels of activin produced by αT3-1

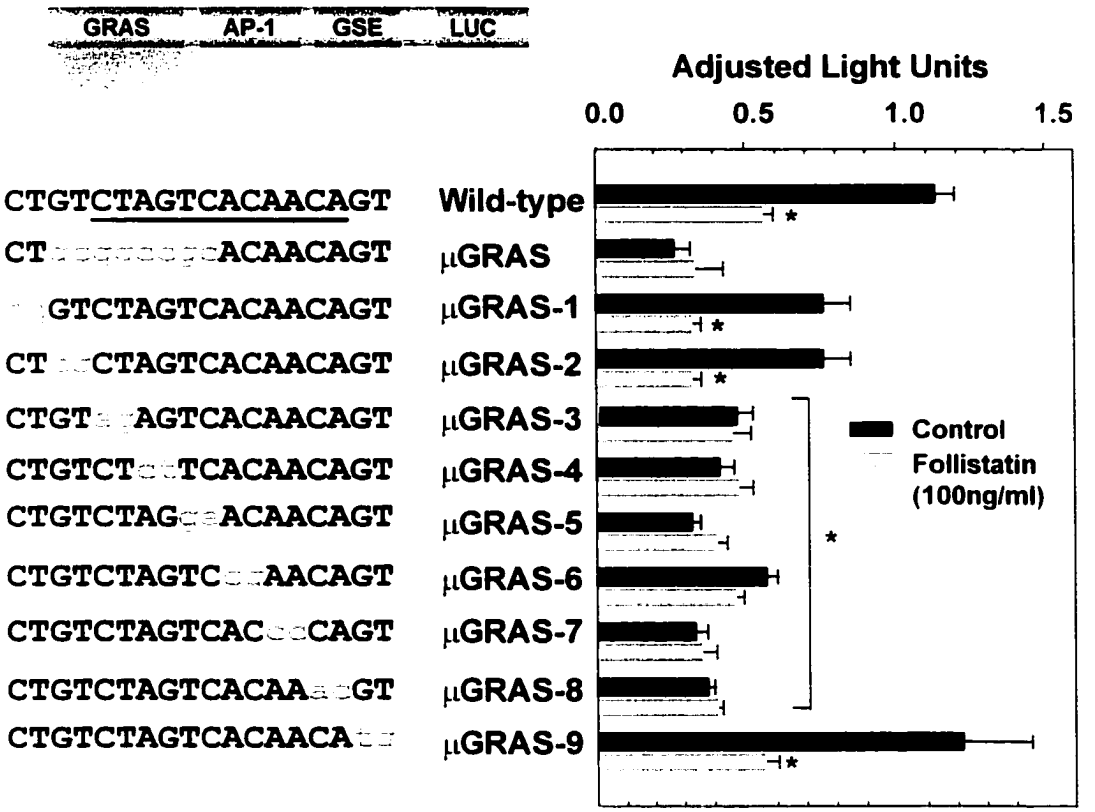


Figure 7. The activin/follistatin responsive region co-localizes with the functional limits of GRAS. Vectors were transiently transfected into αT3-1 cells and treated with follistatin. After approximately 24 h, cells were harvested and assayed for luciferase activity. Values represent the mean ± SEM for triplicate samples in transfections of 2-3 different plasmid preparations. *Represents values different ($p<0.01$) from untreated vector.

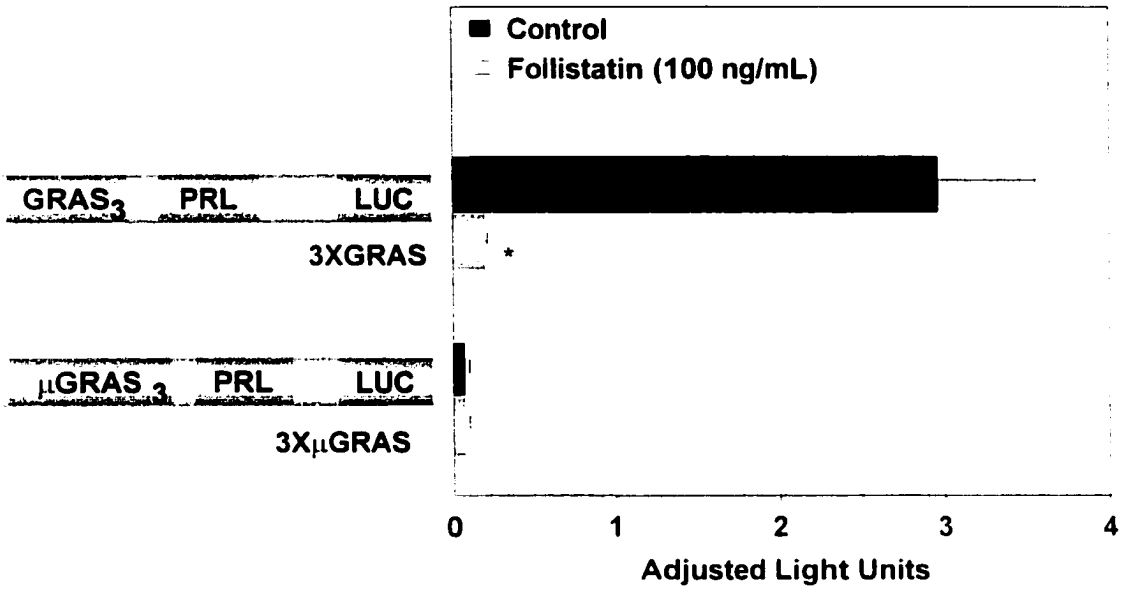


Figure 8. Follistatin treatment blocks enhancer activity of GRAS. Vectors were transiently transfected into αT3-1 cells and treated with 100 ng/mL follistatin. After approximately 24 h, cells were harvested and assayed for luciferase activity. Values represent mean ± SEM for triplicate values in transfectionsof 2-3 different plasmid preparations. *Represents values different ($p<0.01$) from untreated vector.

cells, treatments proceeded for 4 h, then cells were washed to remove endogenous activin and treatments were replaced and allowed to proceed for another 4 h. Cells were then harvested and assayed for luciferase and β-galactosidase activity. Activin treatment stimulated luciferase activity approximately 4-fold in cells transfected with 3XGRAS-PRLpGL3 but had no effect on luciferase activity in cells containing the mutant vector (Figure 9). Thus, GRAS represents an activin responsive element.

Activin treatment can reverse follistatin inhibition of GRAS. Presumably, follistatin is working by binding to activin and sequestering it from the cellular environment. If this is true, it should be possible to reverse follistatin inhibition by adding activin back into the system. To test this scenario, αT3-1 cells were transiently transfected with 3XGRAS-PRLpGL3. As before, treatment with 100 ng/mL of follistatin (3 nM) reduced luciferase expression significantly in cells containing 3XGRAS-PRLpGL3. Addition of increasing amounts of activin not only returned luciferase expression to its original level, but increased it, suggesting that the effects of follistatin are in fact due to its ability to bind and inactivate activin (Figure 10).

DISCUSSION

Previously, we proposed a model in which cell specific basal activity of the GnRHR gene promoter is primarily regulated by a tripartite enhancer composed of a binding site for SF-1, an AP-1 element, and the GRAS element. More recently, we have established that GnRH responsiveness of the GnRHR

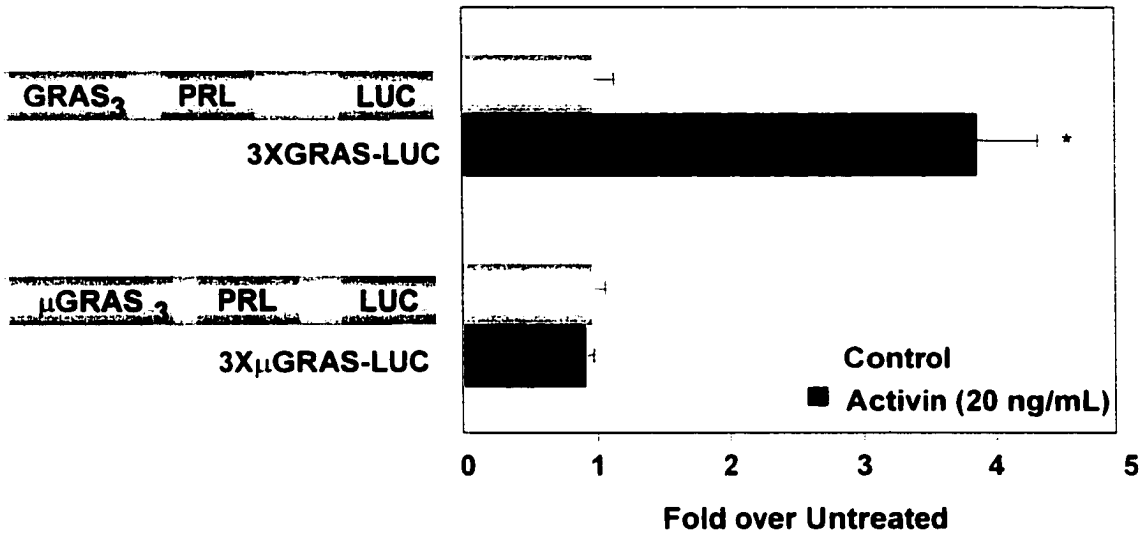


Figure 9. GRAS enhancer activity is stimulated by activin A. Either 3XGRAS or 3XμGRAS were transiently transfected into αT3-1 cells. After 16h, cells were treated with 20 ng/mL activin A for 4 h. Cells were then washed and given fresh media and treatments. After another 4 h cells were harvested and assayed for luciferase activity. Values represent mean ± SD of triplicate samples. *Represents values different ($p<0.01$) from untreated vector.

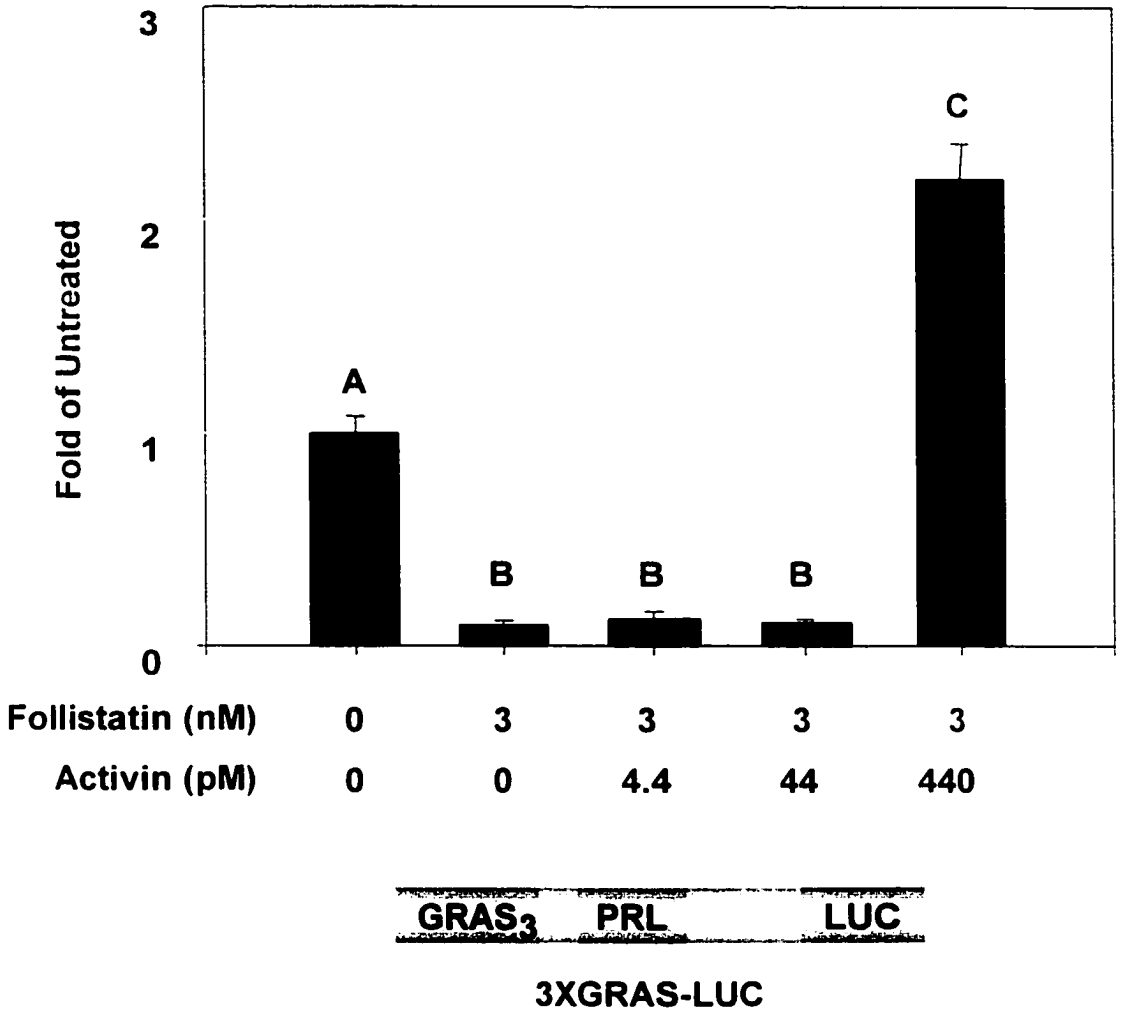


Figure 10. Activin treatment can overcome follistatin inhibition of GRAS. 3XGRAS was transiently transfected into αT3-1 cells and treated with 3 nM follistatin or increasing amounts of Activin A. After approximately 24 h, cells were harvested and assayed for luciferase activity. Values represent mean ± SD of fold change from untreated vector from triplicate samples. ^{A,B,C}Bars bearing different letters differ ($p<0.01$).

gene promoter is primarily conferred through protein kinase C activation of AP-1. Thus, cell specific and hormone-regulated expression partially converge at common elements within the proximal promoter of the GnRHR gene. In the current study, we have utilized the ability of follistatin to bind and inactivate activin to establish that GRAS confers activin responsiveness to the murine GnRHR gene. Because the activin α-subunits, as well as follistatin, are produced by gonadotropes in the anterior pituitary gland, all of the elements are in place for autocrine/paracrine regulation of GnRHR gene expression. The activin response element, GRAS, and αT3-1 cells represent an excellent means to study these potential autocrine / paracrine mechanisms.

As both activin and activin receptors are expressed in a broad range of tissues and are necessary for normal fetal growth and development (127), activin stimulation of GnRH receptor gene expression may play an important role during the development of reproductive function. Furthermore, while activin and inhibin subunit mRNA levels show little variation during the rat estrous cycle, follistatin levels increase during the ovulatory gonadotropin surge (128). This increase has been attributed to the stimulation of follistatin expression by GnRH (64;129). Thus, a regulatory feedback loop exists in which activin stimulation of the GnRH receptor can be controlled by follistatin levels in the pituitary gland, which in turn can be regulated by GnRH stimulation. Consequently, activin regulation of the GnRH receptor gene may serve an important role not only in basal expression of the GnRH receptor in gonadotropes, but also in the subsequent sensitivity of gonadotropes to hormonal stimulation.

Characterization of GRAS as an activin responsive element represents the first activin response element described in gonadotropes. Another issue, however, is the identity of the protein(s) that mediate activin signaling at GRAS. Perhaps the most likely candidates for such a role are members of a family of proteins termed Smads. The Smad proteins have been identified as the downstream effectors of TGF-β family signaling (85). To date, eight Smad family members have been identified and separated into three groups (86-89;130). The first of these are referred to as receptor regulated Smads as they appear to subserve signaling by bone morphogenetic proteins (Smad1, 5, 8) and TGF-β/activin (Smad2, 3) (86-89;130). Second, Smad4 appears to serve as a common partner in all of the Smad signaling cascades (91;131). Finally, Smad6 and 7 (inhibitory Smads) appear to block activation of, or signaling by, the pathway specific Smads (92-95). The developing paradigm for Smad signaling suggests that the activated Type I receptor phosphorylates a pathway specific Smad which complexes with Smad4 and is translocated to the nucleus (88;91;96). In the nucleus, these Smad complexes have been shown to stimulate transcription. Often, however, it appears that the functional effects of Smads on transcription require interactions with non-Smad transcription factors (97;98;132;133). Intriguingly, GRAS contains a near consensus Smad binding element (134). In addition to this Smad binding site, GRAS contains an additional 6 bp that are essential for functional activity. Therefore, in addition to Smads, GRAS likely binds an additional transcription factor that is critical for its activity. Thus, we suggest that GRAS represents a composite regulatory element whose

functional activity is dependent on the binding of a Smad protein complex and a non-Smad partner.

CHAPTER FOUR

THE GONADOTROPIN RELEASING HORMONE 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.

ABSTRACT

Activin responsiveness of the murine GnRH receptor gene promoter is mediated at a regulatory element we have termed the GnRH receptor activating sequence or GRAS. In the present studies we sought to define the complex of transcription factors that interact at this element. Consistent with activin regulation at GRAS, gel shift analyses and yeast one-hybrid assays reveal Smad4 interaction at the 5' end of GRAS. While overexpression of Smad3 activates a GRAS reporter, Smad3 binding at GRAS was not detectable. A functional interaction of Smad3 at GRAS was, however, detectable in yeast expressing Smad4. Thus, Smad3 interaction at GRAS appears to be dependent on the presence of Smad4. Mutations located at the 3' end of GRAS do not affect Smad binding but eliminate functional activity. Thus, Smad binding alone cannot account for the functional attributes of GRAS. Consistent with this notion, we find that AP-1 binding is immediately juxtaposed to and, in fact, partially overlaps the Smad binding site.

Finally, a recently identified member of the forkhead family of transcription factors, FoxL2, is also capable of interacting at GRAS. Furthermore, FoxL2 activation at GRAS is lost with mutation of either the 5' Smad binding site or a forkhead binding site homology located at the 3' end of the element. We suggest that GRAS is a composite regulatory element whose functional activity is dependent on the organization of a multi-protein complex consisting of Smads, AP-1 and a member of the forkhead family of DNA binding proteins.

INTRODUCTION

Central to the maintenance of reproductive function in mammals is the pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. Upon binding to receptors located on gonadotrope, GnRH not only stimulates, but is obligatory for synthesis and secretion of luteinizing hormone (LH) from the anterior pituitary gland (5;6;9;135;136). As such, the rate of LH secretion is dependent on both the amount of GnRH released by the hypothalamus and the concentration of GnRH receptors (GnRHR). For example, the high secretory rate of LH that is necessary for ovulation reflects both enhanced GnRH secretion and increased pituitary sensitivity to GnRH. In regard to the latter, multiple endocrine inputs, including estradiol-17β, progesterone, testosterone, inhibin, activin and GnRH itself, have been shown to mediate changes in GnRHR numbers (16;24;28-30;137-140). Furthermore, changes in GnRHR numbers are often coordinate with changes in GnRHR mRNA levels

suggesting that endocrine regulation of pituitary GnRHR numbers is mediated at the level of gene expression (14;17;20-23;78).

To examine the molecular mechanisms involved in transcriptional regulation of the GnRHR gene we have cloned the gene encoding the murine GnRHR (141) and have found that expression of the murine GnRHR gene in the gonadotrope-derived αT3-1 cell line (69) is dependent on a complex enhancer consisting of a binding site for steroidogenic factor 1 (SF-1), a canonical AP-1 element and a novel element we termed GnRHR activating sequence (GRAS) (1;142). This complex enhancer not only contributes to basal activity of the GnRHR promoter but also appears to mediate multiple endocrine inputs. For example, homologous regulation of the GnRHR gene by GnRH is mediated, at least in part, at AP-1 (2;3). Similarly, activin responsiveness of the GnRHR promoter is mediated at GRAS (142).

A member of the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors, activin is a critical regulator of cell proliferation and differentiation, developmental patterning, and tumor suppression (127;143;144). Upon binding to its receptor, activin initiates intracellular signaling cascades mediated either wholly or in part by members of the Smad family of proteins (87). The Smad proteins themselves have been categorized into several classes. The first of these represent the receptor-regulated Smads. This class is divided into the activin and TGF-β responsive Smads (Smad2 and Smad3) and the BMP responsive Smads (Smad1, Smad5 and Smad8). Smad4 is a common partner for the hormone responsive Smads and is important for

a recently identified member of the forkhead family of DNA binding proteins (105;107-109). Furthermore, the functional organization of this multi-protein complex appears to be highly interdependent in that mutations that eliminate the binding of any one of these components correspondingly eliminate the functional attributes of GRAS.

MATERIALS AND METHODS

Reagents: Recombinant human activin was obtained through NHPP, NIDDK and Dr. A. F. Parlow. Antibodies for Smad1 (catalog no. sc-7153X), Smad2/3 (catalog no. sc-6033X), Smad3 (catalog no. sc-6202X), Smad4 (catalog no. sc-1909X), Smad5 (catalog no. sc-7443), Smad7 (catalog no. sc-7004X), Smad8 (catalog no. sc-7442), Jun family members (catalog no. sc-44X), Fos family members (catalog no. sc-253X) and an anti-mouse antibody (catalog no. sc-2005) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody directed against the hemagglutinin (HA) epitope was obtained from Covance (Richmond, CA) (catalog no. MMS-101R). The cDNAs for Smad2 and Smad4 were generously supplied by Dr. Bert Vogelstein. The cDNA for Smad3 was kindly provided by Dr. R. Derynck.

Vector construction: The plasmid pMGR-600LUC consists of 600 bp of 5'-flanking region from the murine GnRHR gene fused to the cDNA encoding luciferase in the pGL3 basic vector (Promega, Madison, WI) (141). 3XGRAS-LUC (1;70), 4XμGRAS3-LUC and 4XμGRAS7-LUC were constructed as follows: multiple copies of the GRAS element (wild-type or with 2 bp mutations) were

concatamerized by phosphorylating one strand of a synthesized oligonucleotide containing the element using polynucleotide kinase, annealing the two synthesized strands, and ligating the mixture. Concatamers were subsequently ligated into pBlue-script SK (pBSK) digested with *Sma*I. The construction of multiple directionally inserted elements was verified by sequencing. The multimers were subsequently digested out of the pBSK vector and ligated into pGL3 containing the minimal promoter from the rat prolactin gene. The identity of all plasmids was verified by restriction enzyme digestion and sequencing. 3XGRAS-LacZ, 4XμGRAS3-LacZ and 4XμGRAS7-LacZ were prepared in a similar manner except after digestion from pBSK, they were ligated into pLacZi (Clontech, San Jose, CA).

Smad3 and 4 cDNAs were cloned into pGEM by PCR using primers that incorporated restriction sites for *Eco*R I and *Xho* I. Smads were then directionally cloned into pBD-GAL4 (Stratagene, La Jolla, CA) and pGAD-GL (Clontech, San Jose, CA). To make yeast expression vectors for Smad3 and Smad4 (pSmad3, pSmad4), the Gal4 DNA binding domain was excised from pBD-Smad3 and pBD-Smad4. pGEX-Smad3 and pGEX-Smad4: cDNAs for Smad3 and 4 were directionally cloned into the GST expression vector, pGEX-6P-1 (Amersham Pharmacia Biotech, Piscataway, NJ). GRASΔAP1 was made by dual rounds of PCR (primers: GRASΔAP1 Sense 5'-CTGTCTGACTCAAACAGTTTTTAGAAAACC, GRASΔAP1 Antisense 5'-CTGTTTGAGTCAGACAGATACAAAATGAAATA). The sequence of the AP-1 element is underlined.

pFoxL2-AD: the DNA binding domain for FoxL2 was cloned from αT3-1 mRNA by RT-PCR and ligated into pGEM-T easy (Promega, Madison, WI). pGEM-FoxL2 was digested with *Eco*RI and cloned into the *Eco*RI site of pGAD-GL (Clontech, San Jose, CA). HA-FoxL2: a full-length clone of FoxL2 was isolated from a genomic library based on its ability to hybridize to a radiolabeled probe consisting of the DNA binding domain of FoxL2. PCR was used to incorporate a *Bam*HI site into the 5' end of the fragment. The PCR product and a double-stranded oligonucleotide containing the coding sequence for the HA epitope (Sense: 5'-CTAGCATGTACCCCTACGACGTGCCCGACTACGCCG, Antisense: 5'- GATCCGGCGTAGTCGGGCACGTCGTAGGGGTACATG, HA sequence is underlined) were simultaneously ligated into pBK-CMV (Stratagene, La Jolla, CA). FoxL2-VP16 and Gal4- FoxL2: Primers used to generate the N-terminal PCR product were *Eco*RI Sense 5'-*GCGAATTC*ATGATGGCCAGCTAC-3' and *Sac*II Antisense 5'-GC*AAGCTT*GACCACCGCGGCTGCA-3'. This PCR product was gel purified and digested with *Eco*RI and *Hind*III and subcloned into pBlue-script KS II (pBS II) (Stratagene, La Jolla, CA). Primers used to generate the C-terminal PCR product were *Sac*II Sense 5'-*GCGAATTC*GGTGCAGCCGCGGTG and *Xho*I Antisense 5'-*ATCTCGAG*TCAGAGATCCAGACGCGAG -3'. This PCR product was digested with *Eco*RI and *Xho* I and also subcloned into pBS II. The N-terminal fragment was excised from pBS II with *Eco*RI and *Sac*II. The C-terminal fragment was excised from pBS II with *Sac*II and *Xho* I. The two fragments were ligated simultaneously into the *Eco*RI and *Sal*I sites of the mammalian two-hybrid

vectors pM and pVP16 (Clontech, San Jose, CA). FoxL2(N-225)-VP16: The N-terminal PCR subclone described above was digested with *Eco*RI and *Pst*I (nucleotide position 674) and the resulting DNA fragment was ligated into pVP16. FoxL2(N-138)-VP16: A PCR product was generated using the *Eco*RI sense primer from above and the antisense primer 5'-TAGTTGCCCTTCTCGAACATGTCC –3', with the 5' position corresponding to nucleotide 415. The PCR product was gel purified, digested with *Eco*RI, and subcloned into the *Eco*RI and *Hinc*II sites of pBS II. The DNA was excised with *Eco*RI and *Xho*I and ligated into the *Eco*RI and *Sal*I sites of pVP16. FoxL2(140-C)-VP16: A PCR product was generated using the *Eco*RI sense primer, 5'-*GCGAATTC*CGGCGCCGCCGCCGCAT-3' corresponding to nucleotide 416, and the antisense *Sac*II primer described above. The PCR product was digested with *Eco*RI and *Sac*II and the resulting DNA fragment was used to replace the N-terminal *Eco*RI-*Sac*II fragment in the full-length FoxL2-VP16 construct described above. Gal4-Smad3(MH2) and Smad3(MH2)-VP16: The human (Genbank accession # NM 005902) Smad3 MH2 domain DNA fragment was generated with the following PCR primers: Sense *Eco*RI primer 5'-*ATGAATTC*GCCTTCTGGTGCTCCATCTCCT-3' corresponding to nucleotide 754 (amino acid 230), Antisense C-terminal *Xba*I primer 5'-*GCTCTAGA*CTAAGACACACTGGAACAGCGGAT-3'. The PCR product was digested with *Eco*RI and *Xba*I and inserted into the pM and pVP16 vectors.

Cell Culture and Transient Transfections: All cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cultures of αT3-1 cells were

maintained in high glucose DMEM containing 2 mM glutamine, 5% fetal bovine serum, 5% horse serum, 100 U/mL penicillin and 100 μg/mL streptomycin sulfate (Mediatech, Herndon, VA). CHO cultures were maintained in high glucose DMEM containing 2 mM glutamine, 0.1 mM non-essential amino acids, 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Mediatech, Herndon, VA).

Transfections were performed using either SuperFect reagent (Qiagen, Valencia, CA) (Figures 16, 23, 24) or calcium phosphate-DNA co-precipitation (Figures 20, 21). SuperFect transfections included 1 μg of the test vector and 0.25 μg of pRSV-LacZ. Approximately 24 h after transfection, cells were harvested and assayed for luciferase activity (70). Luciferase activity was normalized for transfection efficiency by dividing the luciferase activity by β-galactosidase activity. In overexpression assays, 1 μg of the reporter vector and 0.25 μg of the overexpression vector was transfected (Figure 16).

A calcium-phosphate protocol was utilized for experiments examining GnRH regulation as described previously (2). Briefly, 5 x 10^5 cells were plated per well in 6-well plates. After approximately 18 h, cells were transfected by adding a mixture containing 1 μg reporter plasmid, 0.25 μg pRSV-LacZ, 124 mM $CaCl_2$, and 1X HBS (280 mM NaCl, 50 mM HEPES, 2.8 mM Na_2HPO_4). After 30 min, media containing appropriate treatments was added to cells and transfection mixture. Cells were incubated at 37°C for 6 h. Cells were then harvested and assayed for luciferase activity (2). Luciferase activity was normalized for

transfection efficiency by dividing the luciferase activity by β-galactosidase activity.

In mammalian one- and two-hybrid assays (Figures 23, 24), cells were transfected with 0.2 μg of each vector including pRSV-LacZ. Approximately 24 h after transfection, cells were harvested and assayed for luciferase activity (70). Luciferase activity was normalized for transfection efficiency by dividing the luciferase activity by β-galactosidase activity.

Preparation of nuclei and nuclear extracts: Nuclei were isolated as previously described (2;150). For preparation of nuclear extracts, cells were harvested in phosphate buffered saline (10 g/L NaCl, 0.224 g/L KCl, 1.42 g/L Na_2HPO_4, 0.272 g/L KH_2PO_4, pH 7.4) with 1 mM EDTA and pelleted by centrifugation in a clinical centrifuge approximately 1200 x g for 5 min. Cells were then resuspended in ice cold lysis buffer (70 mM β-glycerol phosphate, 2 mM $MgCl_2$, 0.5 mM EGTA, 15 mM HEPES, 100 mg/mL, 0.15 mM spermine, 0.5 mM spermidine, 2 mM sodium meta vanadate, 2 mM sodium fluoride, 2.5 μg/mL leupeptin, 2.5 μg/mL pepstatin, 1 mM PMSF, 5 mM benzamidine, 1 mM DTT, pH 7.8) and incubated on ice for 5 min. Cells were lysed with 10 strokes of a glass Dounce B homogenizer. The lysates were layered over a cushion consisting of 300 mg/mL sucrose in lysis buffer and centrifuged at 6650 x g in a JS7.5 Beckman rotor at 4°C. Pellets were resuspended in lysis buffer, further homogenized by 10 strokes of the Dounce homogenizer and layered over a sucrose cushion as above. The pellet was resuspended in lysis buffer plus 10% glycerol and 450 mM KCl. The solution was mixed by vortexing and then

incubated at 4°C with gentle mixing on a circular mixer for 30 min. The solution was then centrifuged at 338,000 x g at 4°C for 45 min in a 70.1 Ti Beckman rotor. Supernatants were recovered and protein concentrations were determined using bicinchoninic acid (BCA Assay, Pierce Chemical Co.).

Preparation of recombinant proteins: Superbroth (9.6 g/L Tryptone, 19.2g/L Yeast extract, 0.4% glycerol, 45 mM K_2HPO_4, 22 mM KH_2PO_4, 100 μg/mL ampicillin) was inoculated with E. coli (DH5α) previously transformed with either pGEX-Smad4 or pGEX-Smad3. Bacterial cultures were incubated at 37°C with shaking until an OD_{600} of 0.5-0.7. At this point 100 μM IPTG was added to induce protein expression and cultures were incubated at 30°C overnight with shaking. Bacteria were pelleted by centrifugation in a JA17 Beckman rotor at 5000 x g for 10 min at 4°C and resuspended in resuspension buffer (100 mM Tris pH 8.0, 0.85 mM phenylmethylsulfonyl fluoride). Lysozyme was added to 0.2 mg/mL and samples were sonicated (duty cycle 70%, output 6) for approximately 30s on ice. Triton-X was then added to 1% (v/v) and samples were sonicated as above. Samples were centrifuged at 25,000 x g for 10 min. at 4°C. Supernatant was incubated with glutathione resin in a rotating shaker at 4°C for 30 min. Glutathione residue was recovered by centrifugation in clinical centrifuge at approximately 1500 x g for 2 min. The recovered resin was washed twice with 5 volumes of wash buffer (50 mM Tris pH 8.0, 140 mM NaCl, 0.3 mM DTT). Smad4-GST protein was eluted by resuspending the resin in 5 mM glutathione in wash buffer. The mixture was agitated gently for 2 min, centrifuged for 10 s at

500 x g and the supernatant was collected. This elution step was repeated 2 more times and each elution was analyzed by SDS-PAGE. In the case of Smad3, the GST protein was cleaved from Smad3 using PreScission Protease (Amersham Pharmacia Biotech, Piscataway, NJ). As the GST protein was still bound to the glutathione resin, Smad3 was isolated by centrifuging the slurry at 500 x g for 10 s and the Smad3-containing supernatant was saved.

Electrophoretic Mobility Shift Assays (EMSA): EMSAs using nuclear extracts (2-12 μg protein) from αT3-1 cells (Figures 13, 19, 21, 22) were incubated with Dignam buffer D (20 mM HEPES, 20% glycerol, 0.1 M KCl, 0.4 mM EDTA), poly(dI-dC) (2 μg) (Amersham Pharmacia Biotech, Piscataway, NJ), radiolabeled probe (10 fmol, approximate specific activity of 2 x 10^4 cpm/fmol) and unlabeled competitor (where indicated) for 20 min at room temperature (70). EMSAs using nuclei (2,000,000 nuclei/reaction) from αT3-1 cells (Figure 12) were performed under precisely the same binding conditions as above.

For EMSAs performed with recombinant proteins (Figures 14, 17), proteins were incubated with binding buffer (25mM Tris-HCl pH 7.9, 80 mM NaCl, 35 mM KCl, 5 mM $MgCl_2$, 20% glycerol, 0.6 mg/mL BSA, 1 mM DTT), 2 μg poly(dI-dC), radiolabeled probe (10 fmol, approximate specific activity of 2 x 10^4 cpm/fmol) and, if included, unlabeled competitor (5 pmol) at room temperature for 20 min.

Where included, antibodies (2 μg) were added to the binding reactions after the 20 min incubation and then incubated for an additional 10 min. The one

exception is the pan-Jun antibody that was incubated with the extract for 10 min prior to the addition of radiolabeled probe.

In all of the mobility shift assays, free probe was separated from bound probe by electrophoresis for 1-2 h at 35 mA in 5% polyacrylamide gels that were pre-run at 100 V for 2 h in 50 mM Tris, 380 mM glycine, and 2 mM EDTA, pH 8. Gels were dried onto blotting paper and exposed to autoradiography film for approximately 16 h at -70°C with Dupont Cronex intensifying screens (Dupont, Boston, MA).

Radiolabeled probes were prepared by incubating double stranded oligonucleotides with [γ-^{32}P]ATP (4500 Ci/mmol; ICN, Irvine, CA) and T4 polynucleotide kinase at 37°C for 30 min. Radiolabeled DNA was separated from free nucleotides on a microspin G-25 column (Amersham Phamacia Biotech, Piscataway, NJ).

Yeast hybrid assays: YPAD broth (20g/L Difco peptone, 10 g/L yeast extract, pH 5.8, 2% (v/v) glucose) was inoculated with yeast (YM4271) and grown to an OD_{600} of 0.8-1.0. Yeast were rendered competent and transformed according to the Zymo Research protocol (Zymogen, Orange, CA). After transformation, yeast were plated onto SD Agar selection plates that were prepared with all essential amino acids except those supplied by transformation of the plasmid as follows: uracil (p3XGRAS-LacZ, p4XμGRAS3-LacZ, p3XμGRAS5-LacZ, p4XμGRAS7-LacZ), leucine (pSmad4-AD, pSmad3-AD, pFoxL2-AD) and tryptophan (pSmad4, pSmad3). Yeast were allowed to grow on selection plates for 3-5 days at 30°C. To assay for β-galactosidase, yeast were

transferred to filter paper (Whatman #1). Yeast adhering to the filter were lysed by freezing in liquid nitrogen. After lysis, filters were soaked in Z-buffer (16.1 g/L of Na_2HPO_4-$7H_2O$, 5.5 g/L of NaH_2PO_4-H_2O, 0.75 g/L of KCl, 0.246 g/L of $MgSO_4$, 0.27% β-mercaptoethanol, 33.4 mg/L X-gal) at room temperature. The time required for color development was used as the index of β-galactosidase expression. $^{+++}$ Indicates Lac-Z expression that was evident (blue-color) within 2 hours. $^{++}$ Indicates Lac-Z expression that was evident (blue-color) between 2 – 12 hours. $^{-}$ Indicates the absence of color development in advance of the negative control (absence of pGAD fusion construct). All yeast one-hybrid assays were replicated at least 3 times.

Western analysis: αT3-1 cells were transfected with HA-FoxL2 using SuperFect reagent. Approximately 24 h after transfection, cells were washed with ice-cold phosphate buffered saline (10 g/L NaCl, 0.224 g/L KCl, 1.42 g/L Na_2HPO_4, 0.272 g/L KH_2PO_4, pH 7.4) and lysed in RIPA buffer (20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP-40, 0.1% SDS, 0.5% deoxycholate, 2 mM EDTA, 5 mM sodium vanadate, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride) on ice. The cell lysates were collected and debris cleared by centrifugation. Proteins were resolved using denaturing PAGE followed by transfer to nitrocellulose membrane by electroblotting. Membranes were exposed to an antibody directed against the HA epitope at a concentration of 2 μg/mL in 5% non-fat milk in TBS-T (140 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 7.4). After washing 3 times in TBS-T, membranes were exposed to an

anti-mouse secondary in 5% non-fat milk in TBS-T at a concentration of 0.08 μg/mL for 2 h at room temperature.

Statistical analysis: Data were analyzed by SAS (151). The transfection data were analyzed by one-way ANOVA with vector as the independent variable. To compare each treatment to control, Dunnett's t test was used (Figure 16). For pairwise comparisons of data, Tukey's studentized range test was used (Figures 20, 22, 23). Due to heterogeneity of variance, the adjusted luciferase data in Figure 20A and Figure 22 were $\log_{10}$ transformed prior to analysis of variance.

RESULTS

Smad binding localizes to the 5' end of GRAS. We have established GRAS as an activin response element in the GnRHR gene (1;142). Consistent with this observation, the 5' end of GRAS is homologous to the binding motif characterized for members of the Smad family of transcription factors (152) (Figure 11). To determine if Smad proteins are capable of binding GRAS we conducted electrophoretic mobility shifts assays (EMSA) in which nuclei isolated from αT3-1 cells were incubated with a probe representing a consensus Smad binding site (152). As activin is constitutively secreted by αT3-1 cells (28), these nuclei are effectively activin-treated. Sequence specific binding was assessed by competition with excess unlabeled homologous and heterologous DNA. Two sequence specific binding complexes were identified (Figure 12A). Unlabeled DNA probes of 30 and 20 bp containing GRAS competed for binding to the Smad probe while a competitor containing an 8 bp *Not*I mutation of GRAS (μGRAS) (1)

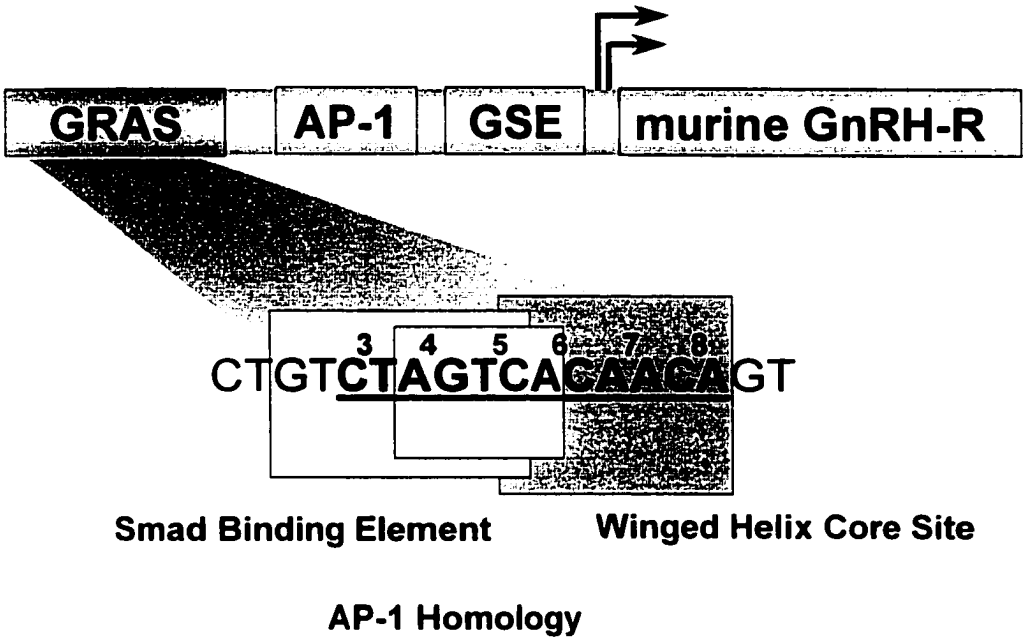


Figure 11. Homology of GRAS to a Smad Binding Element, an AP-1 site and a Winged Helix Core Site. Distal GRAS contains a near-consensus Smad3/4 binding site, highlighted here in a light gray box. The center of GRAS is homologous to an AP-1 half site (white box). The proximal region of GRAS is homologous to a winged helix core site (dark gray box). The numbers overlying the nucleotide sequence correspond to the 2 bp transversion mutations (μGRAS-3 – μGRAS-8) that were used to define the functional boundaries of GRAS (70).

did not compete for binding. Where indicated, antibodies directed against Smads 3 and 4 or an equal mass of IgG were added after the binding reaction and incubated for 15 min. The Smad3 antibody led to a supershift of the upper complex while the Smad4 antibody supershifted the lower complex. Antibodies against Smads 1 and 2 had no effect on binding (data not shown). Thus, Smad3 and Smad4 are present in nuclei from untreated (i.e. activin stimulated) αT3-1 cells and are capable of binding to GRAS. Furthermore, the ability of GRAS to effectively displace binding of Smad3 and Smad4 was compromised if 2 bp transversion mutations were localized to the 5' end of the element suggesting that Smad3 and Smad4 binding maybe confined to the distal end of GRAS (Figure 12B).

To directly assess the potential for Smad binding we next conducted an EMSA in which untreated αT3-1 nuclear extract was combined with radiolabeled GRAS. A super-shifted complex was evident when an antibody specific for Smad4 was added. No change was observed when antibodies that recognize Smads1, 2, 3, 5, 7 or 8 were added (Figure 13). Thus, Smad4 binding is detectable when GRAS is used as the radioactive probe in EMSA.

Based on the competition data presented in Figure 12B, Smad binding appears to localize to the 5' end of GRAS. To confirm this observation, recombinant Smad4 was produced as a fusion protein with glutathione s-transferase (rhSmad4) and examined for binding to GRAS in an EMSA. Consistent with the EMSA data using αT3-1 nuclear preparations, rhSmad4 displayed sequence specific binding to GRAS (Figure 14A). A supershift

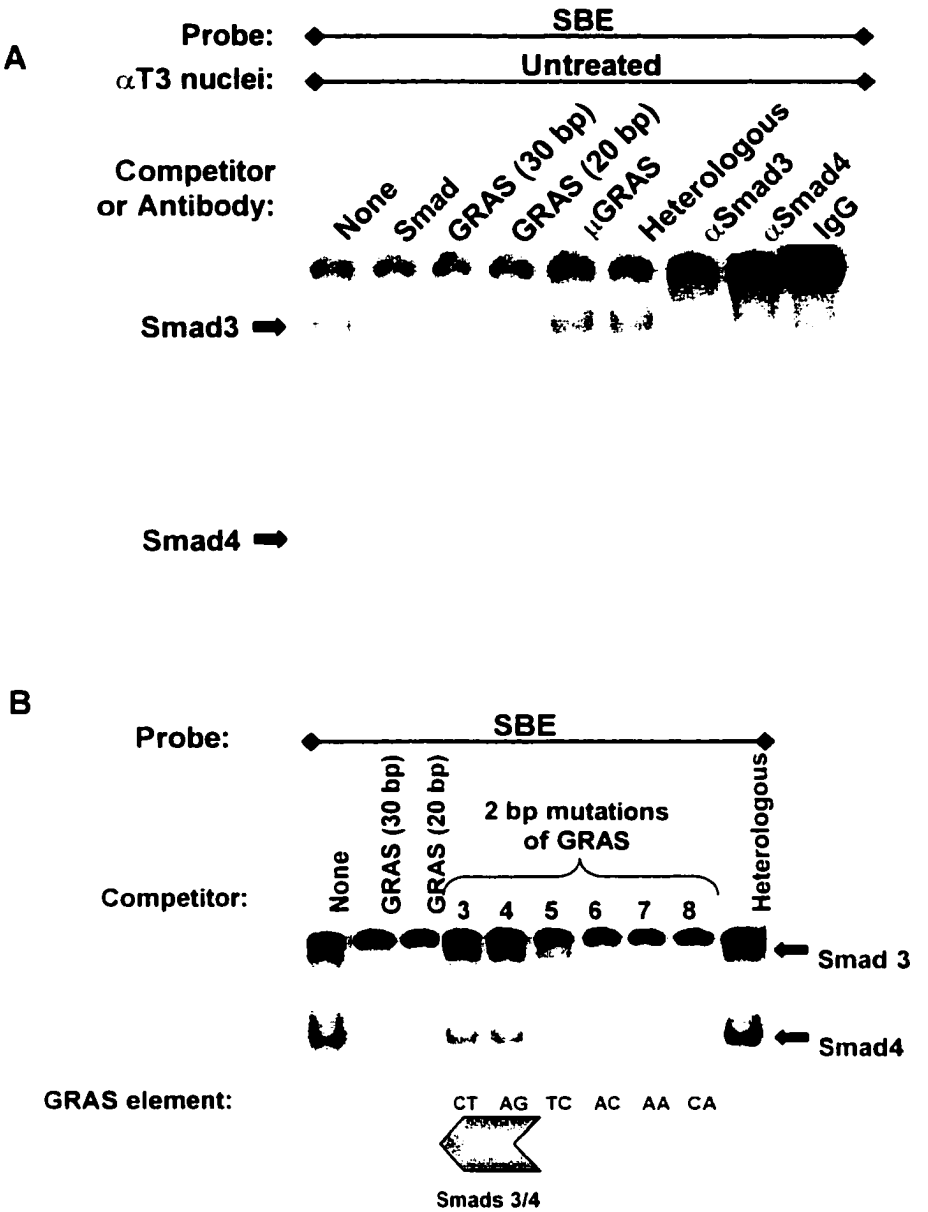


Figure 12. GRAS competes for Smad binding. Nuclei isolated from αT3-1 cells were incubated with a radioactive probe representing a consensus Smad binding site. A) Sequence-specific binding was assessed by competition with 500-fold molar excess of unlabeled homologous (SBE) and heterologous DNA. Unlabeled oligonucleotides of 30 and 20 bp containing GRAS or mutated GRAS (μGRAS) were also added as competitors. Where indicated, specific antibodies directed against Smad3 (αSmad3), Smad4 (αSmad4) or an equal mass of IgG were added after the binding reactions and incubated for a further 15 min. B) Unlabeled oligonucleotides containing wild-type GRAS, a series of 2 bp transversion mutations that defined the functional limits of GRAS or an unrelated sequence (heterologous) were added as competitors.

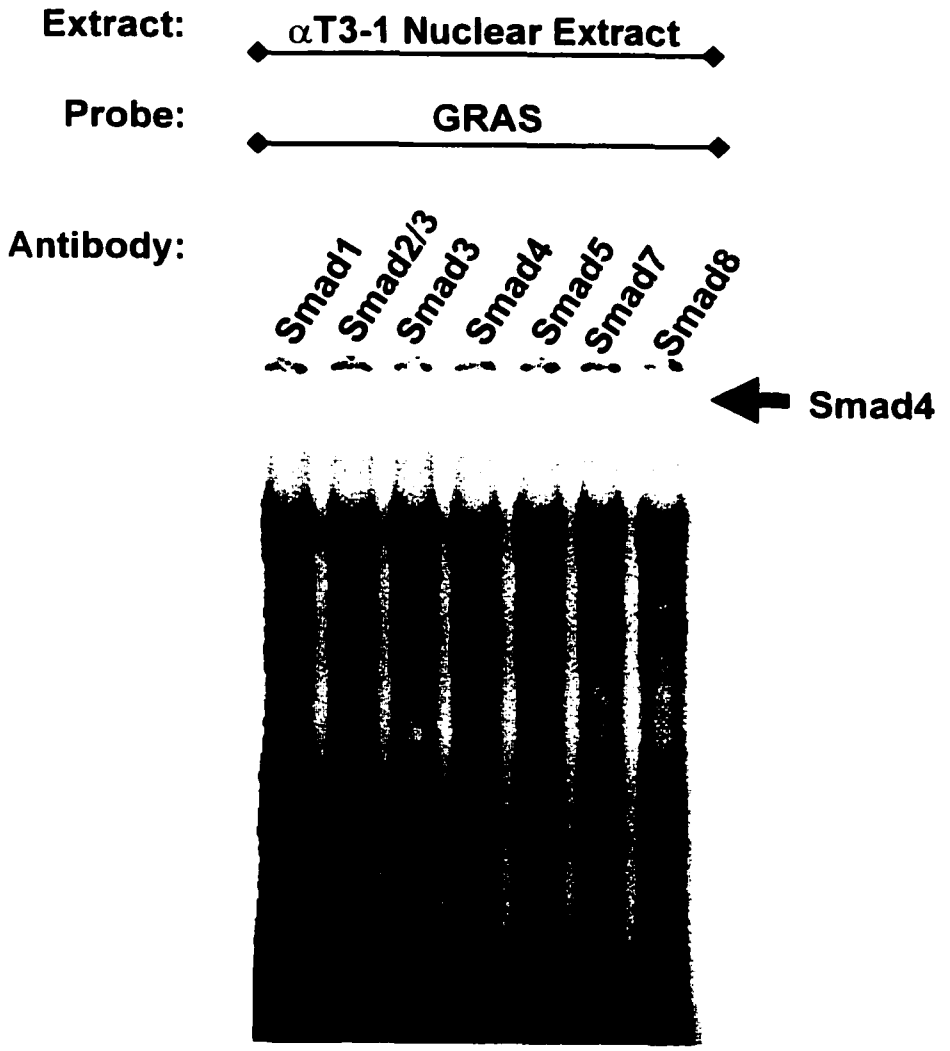


Figure 13. Smad4 directly binds GRAS. Nuclear extracts from αT3-1 cells were incubated with radiolabeled GRAS. Where indicated, antibodies directed against Smads1, 2, 3, 4, 5, 7, and 8 were added after the binding reactions and incubated for a further 15 min.

associated with the inclusion of an antibody directed against Smad4 confirmed the identity of Smad4 in the specific bound complex (Figure 14A). To localize binding of the recombinant protein, radiolabeled oligonucleotides containing either GRAS or a series of 2 bp mutations that scan the functional boundaries of the element (μGRAS-3 through μGRAS-8) (1) were incubated with rhSmad4. As in the competition analysis, transversion mutations located at the 5' end of GRAS (μGRAS-3 through μGRAS-5) diminished the ability of the oligonucleotide to bind rhSmad4 (Figure 14B). It would appear, however, that the effects of μGRAS-5 and μGRAS-6 on the binding of the recombinant protein were more pronounced than what was observed in the competition assay using the consensus Smad binding site as the radioactive probe (Figure 12B). A likely explanation for this difference is the presence of other GRAS binding components in the nuclei that stabilize the binding of Smad4. These components would not of course be present in the binding assays conducted with recombinant protein.

Functional activation by Smad4 is not affected by mutations at the 3' end of GRAS. Mutations at the proximal end of GRAS appear to have little effect on Smad binding yet abrogate functional activity of the element as effectively as mutations localized to the 5' end. To determine if a mutation that does not affect Smad binding (μGRAS-7) might affect the ability of Smad to functionally activate GRAS we utilized a yeast one-hybrid analysis. Three copies of GRAS were placed in a vector upstream of a minimal promoter that was fused to the gene for β-galactosidase (3XGRAS-LacZ). This DNA was stably integrated into the yeast strain YM4271. A vector containing human Smad4 fused to the GAL4 activation

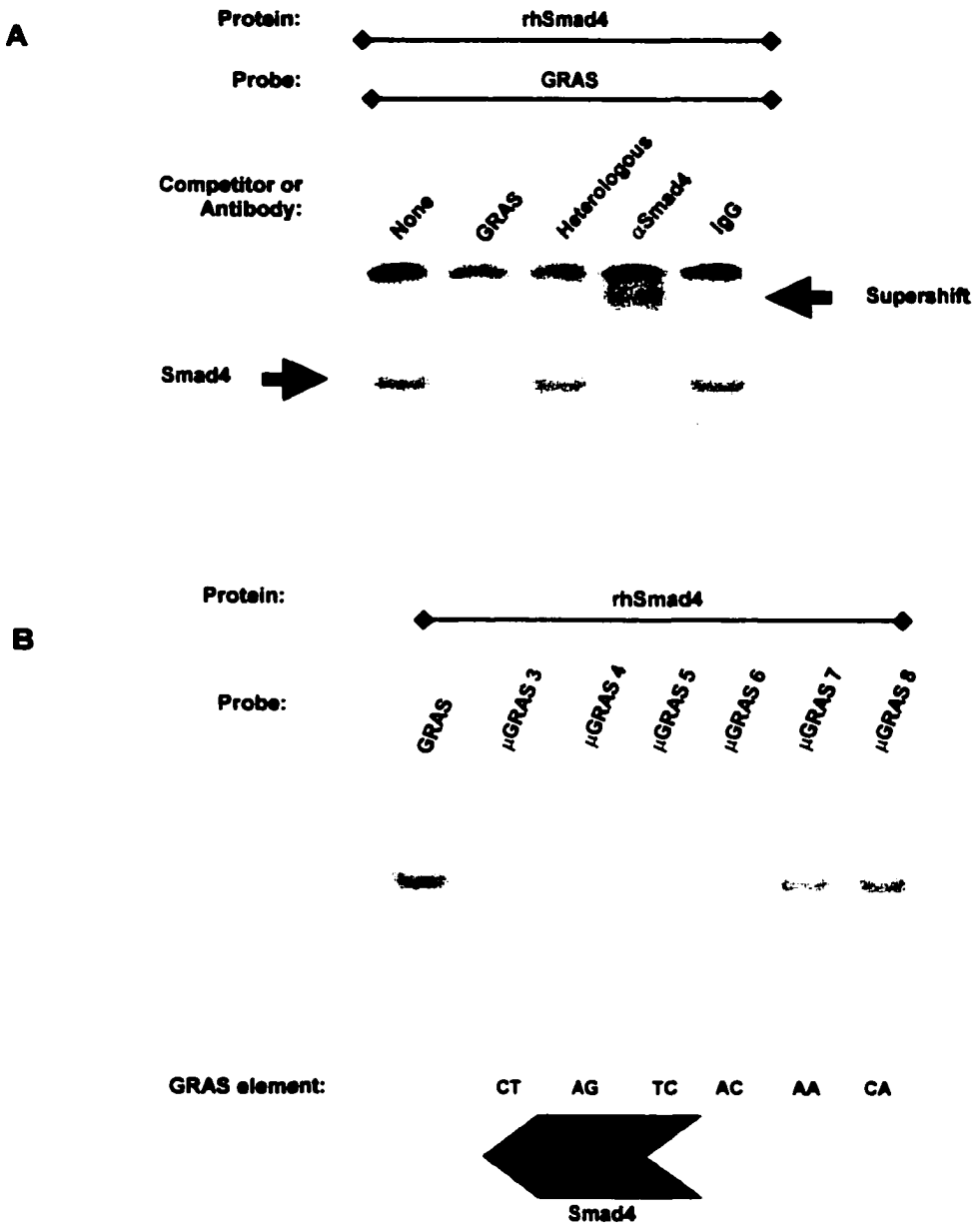


Figure 14. Smad4 binds the 5' end of GRAS. A) Radiolabeled GRAS was combined with rhSmad4. Unlabeled homologous or heterologous oligonucleotides were added as competitors at 500-fold molar excess. Where indicated, an anti-Smad4 antibody (αSmad4) or non-immune mouse IgG were added after the binding reactions. B) Bacterially expressed rhSmad4 was incubated with radiolabeled oligonucleotides containing GRAS or the series of 2 bp mutations that scan the functional limits of GRAS.

domain (pSmad4-AD) was transformed into these recombinant yeast strains and after approximately 3-5 days yeast were analyzed for β-galactosidase activity. Consistent with the binding data, the Smad4-AD fusion protein robustly activated LacZ expression from the reporter construct containing wild-type GRAS (Fig. 15). As expected, Smad4-AD was unable to activate the LacZ reporter harboring a distal mutation (μGRAS-3) that resulted in a loss of DNA binding. In contrast, the ability of Smad4-AD to transactivate at μGRAS-7 was not compromised. Thus, while mutations in proximal GRAS eliminate functional activity of the element (1;142) this effect is not reflected at the level of Smad binding or the ability of Smad4 to activate GRAS in a one-hybrid assay.

Overexpression of Smad3 stimulates functional activity of GRAS. Either Smad2 or Smad3 prototypically mediates activin signaling in cooperation with Smad4 (87). Consistent with this notion, GRAS is capable of displacing binding of Smad3 to a defined Smad binding motif (Figure 12A). However, we were unable to detect either Smad2 or Smad3 binding when GRAS itself was utilized as the radioactive probe in EMSA (Figure 13). To further explore a potential role for Smad3 at GRAS we tested the functional consequence of overexpression of both Smad3 and Smad2 in αT3-1 cells. Expression vectors for Smad3 or Smad2 either alone or in combination with an expression vector for Smad4 were co-transfected into αT3-1 cells with a luciferase reporter construct containing three copies of GRAS fused to the minimal promoter from the rat prolactin gene (3XGRAS-LUC) or 3 copies of GRAS containing a 2 bp loss of function mutation (3XμGRAS5-LUC) (1;142). Also, because αT3-1 cells constitutively produce

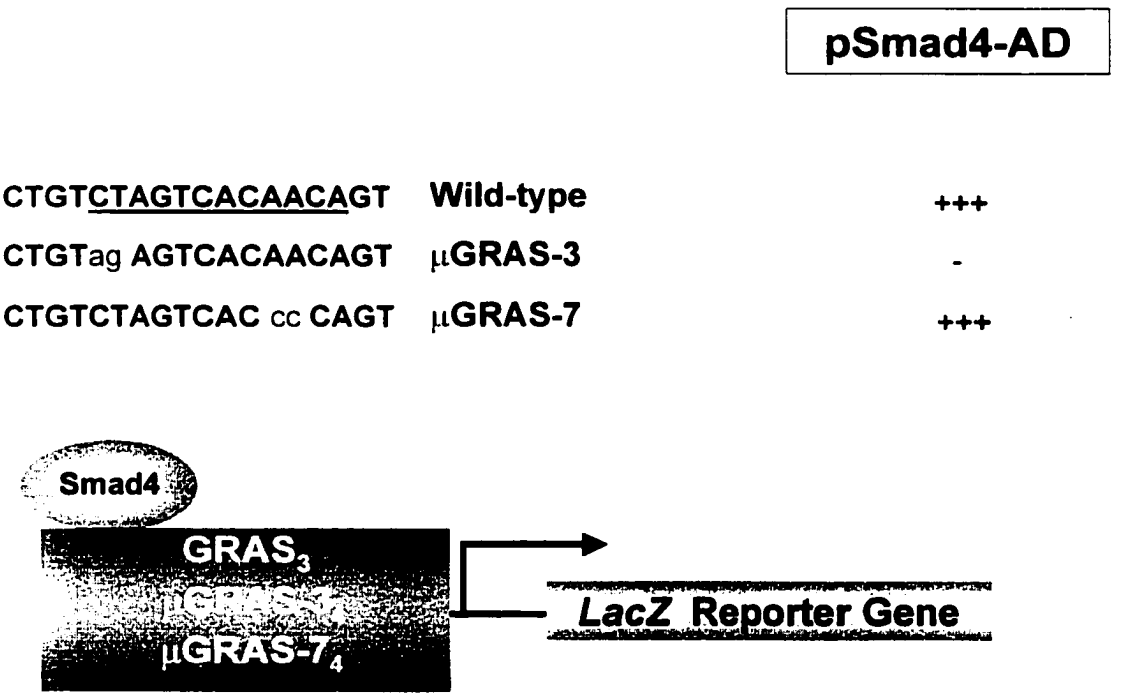


Figure 15. Smad4 activates at GRAS in a yeast one-hybrid assay. Multiple copies of GRAS, μGRAS-3 or μGRAS-7 were placed upstream of an expression cassette for β-galactosidase (LacZ) and stably integrated into the yeast strain YM4271. Full-length human Smad4 was fused to the GAL4 activation domain in a yeast expression vector (pSmad4-AD) and transformed into the recombinant yeast strains containing the indicated LacZ reporter constructs. After approximately 3-5 days of growth, yeast were assayed for LacZ expression as described in Materials and Methods. $^{+++}$ Indicates Lac-Z expression that was evident (blue-color) within 2 hours. $^{-}$ Indicates the absence of color development in advance of the negative control (reporter construct alone).

activin, we were concerned that the cells might exist in a maximally activin-activated state thus masking any further contribution of overexpressed protein. To circumvent this potential problem, cells were washed and cultured in fresh media approximately 18 hours after transfection. Five hours later, cells were harvested and assayed for luciferase activity. Overexpression of Smad3 increased ($p<0.05$) transcriptional activity of 3XGRAS-LUC (Figure 16). In contrast, promoter activity was unaffected by overexpression of Smad2 either alone or in combination with Smad4. Finally, the effects of Smad3 overexpression were specific to GRAS as the 2 bp loss of function mutation (μGRAS-5) rendered the luciferase reporter non-responsive.

Smad3 does not bind at GRAS as a recombinant protein. Although Smad3 could not be detected by EMSA using GRAS as a radioactive probe, the overexpression data are consistent with a functional role for Smad3 at GRAS. We next asked if binding at GRAS could be detected using purified, recombinant Smad3. Bacterially expressed rhSmad3 was incubated with radiolabeled GRAS and subjected to EMSA. In contrast to Smad4, no binding was detected at GRAS using the recombinant Smad3 protein (Figure 17). The absence of binding would not appear to be due to compromised integrity of the protein as the recombinant Smad3 was capable of binding to the consensus Smad binding element. The inclusion of Smad4 in the binding reaction did not recruit or facilitate Smad3 binding to GRAS (data not shown).

Smad3-Smad4 interaction at GRAS is detectable by one-hybrid analysis. Although overexpression of Smad3 leads to functional activation of GRAS we

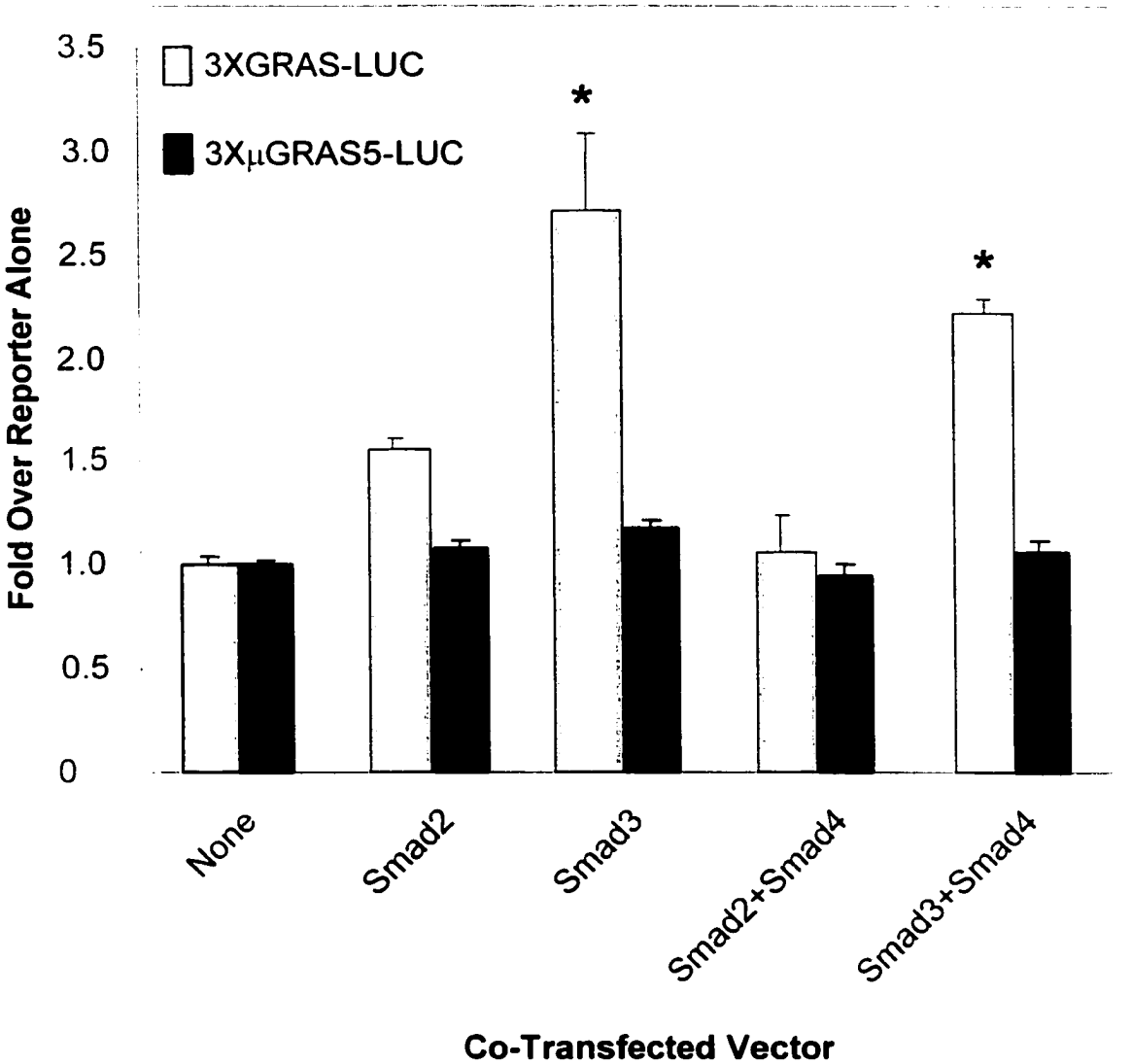


Figure 16. Overexpression of Smad3 stimulates functional activity of GRAS. Expression vectors for Smad2 and Smad3 either alone or in combination with an expression vector for Smad4 were co-transfected into αT3-1 cells with a reporter construct containing three copies of GRAS linked to a minimal promoter fused to luciferase (3XGRAS-LUC) or a similar construct containing a 2 bp transversion mutation in GRAS (3XμGRAS5-LUC). Cells were washed 18 h after transfection, and incubated for an additional five hours before harvesting. *$p<0.01$ compared to cells transfected with reporter alone.

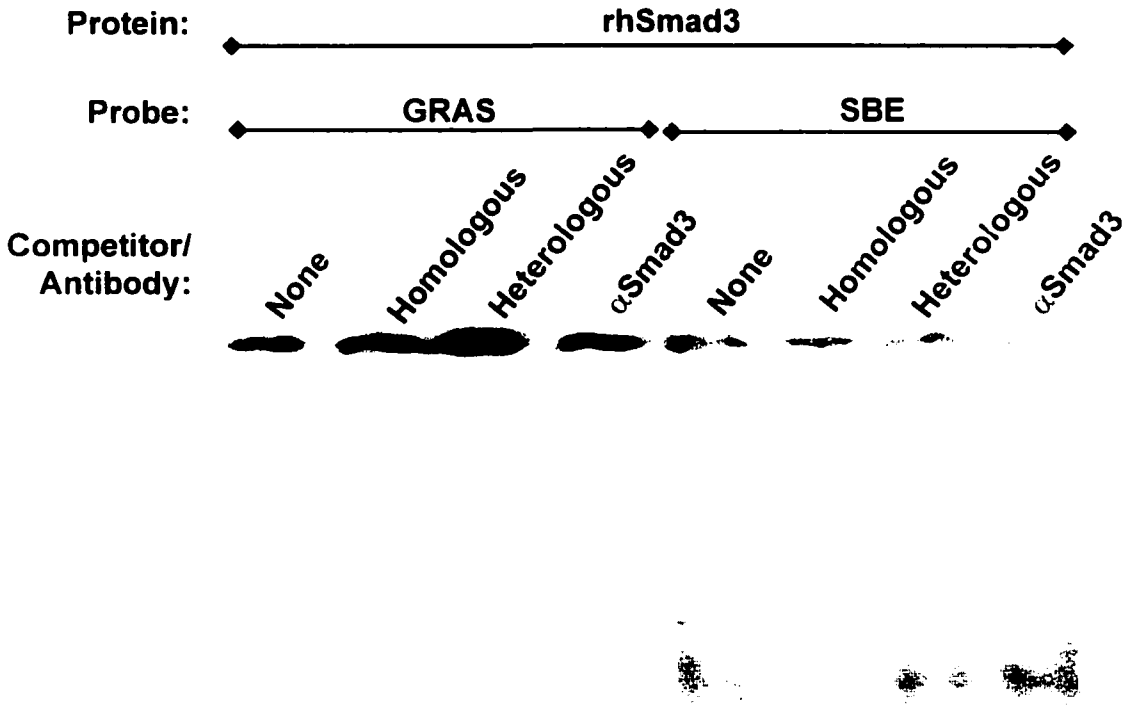


Figure 17. Smad3 does not bind at GRAS as a recombinant protein. Radiolabeled oligonucleotides containing either GRAS or a consensus Smad binding element (SBE) were incubated with bacterially expressed rhSmad3. Specificity of binding was assessed by the addition of non-radioactive competitor as indicated. Where indicated, an anti-Smad3 antibody (αSmad3) was added after the binding reactions.

were not able to directly demonstrate Smad3 binding. Thus, Smad3 activation at GRAS may be exerted independently of direct DNA binding but rather indirectly via protein-protein interaction with Smad4. To test this possibility we examined the ability of Smad3 fused to the transcriptional activation domain (AD) of Gal4 (pSmad3-AD) to activate 3XGRAS-LacZ in yeast one-hybrid analysis. Consistent with the binding data, Smad3-AD alone did not induce LacZ expression (Figure 18). Importantly, however, if Smad3-AD was introduced into yeast expressing Smad4 then LacZ expression was elicited. In this analysis, the co-expressed Smad4 is not fused to the Gal4-AD and, in contrast to the Smad4-AD fusion protein, does not activate LacZ expression from the 3XGRAS reporter construct. Thus, transcriptional activation of GRAS by Smad3 is dependent on the presence of Smad4.

AP-1 binding activity is localized to the center of GRAS. A functional interaction of Smad4 and Smad3 is organized at the distal end of GRAS. While consistent with the role of this element in mediating activin responsiveness of the GnRHR gene it would not appear that this event alone is sufficient to account for the functional activity of GRAS. Simply put, why do mutations that do not affect Smad binding eliminate enhancer activity of GRAS (e.g. μGRAS6-8) (142)? Perhaps the simplest explanation is that the functional activity of GRAS requires not only Smad binding but also the interaction of non-Smad protein partners. In fact, Norwitz et al. (167), recently used EMSA to detect AP-1 binding activity at GRAS. Thus, a Jun/Fos heterodimer may represent at least one component of the putative non-Smad protein partners. To test for Jun/Fos binding at GRAS,

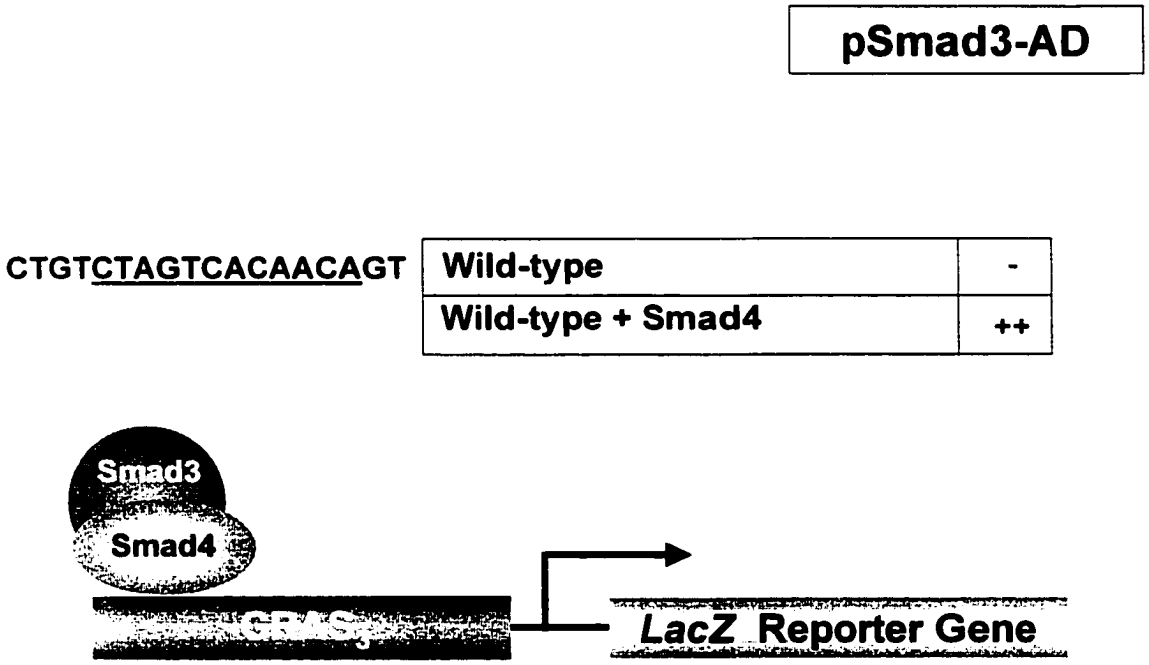


Figure 18. Smad3-Smad4 interaction at GRAS is detectable by one-hybrid analysis. Multiple copies of GRAS were placed upstream of an expression cassette for β-galactosidase (LacZ) and stably integrated into the yeast strain YM4271. Full-length hSmad3 was fused to the GAL4 activation domain in the yeast expression vector (pSmad3-AD). This construct was transformed into recombinant yeast strains transformed with the 3XGRAS-LacZ reporter construct either alone or in combination with an expression vector for Smad4. After approximately 3-5 days of growth, yeast were assayed for LacZ expression as described in Materials and Methods. $^{++}$ Indicates Lac-Z expression that was evident (blue-color) between 2 – 12 hours. $^{-}$ Indicates the absence of color development in advance of the negative control (absence of pSmad3-AD).

nuclear extract from αT3-1 cells that had been serum starved for 24 h and treated with 100 nM GnRH for 1 h was combined in a gel shift assay with a radiolabeled oligonucleotide containing GRAS (Figure 19A). Sequence specific binding was confirmed by addition of 500-fold molar excess of unlabeled homologous and heterologous DNA to the binding reaction. Most notable is that an oligonucleotide containing a canonical AP-1 site effectively displaced binding to the GRAS probe. Thus, binding activity at GRAS can be displaced by competition with AP-1. Consistent with this observation, the inclusion of an antibody directed against the DNA binding domain of Jun family members resulted in an abrogation of the slowest migrating complex whereas an antibody directed against a non-DNA binding domain epitope of Fos family members resulted in a supershift of the same complex (Figure 19A). The addition of non-immune mouse IgG had no effect on GRAS binding activity. To precisely map the AP-1 binding site, we next tested the ability of a series of oligonucleotides containing the 2 bp transversion mutations that delimit the functional boundaries of GRAS to displace binding of AP-1. GnRH-treated αT3-1 nuclear extract was combined with a radiolabeled oligonucleotide containing GRAS and the pan-Fos antibody as above. Since the Fos antibody results in a clear supershift rather than an abrogation we felt that this approach would facilitate interpretation. While the ability of μGRAS-3 and μGRAS-4 to displace Fos binding was clearly compromised the most profound effects were apparent with mutations placed at the center of the element (Figure 19B). Specifically, neither μGRAS-5 nor μGRAS-6 were capable of competing for Fos binding to the wild-type element.

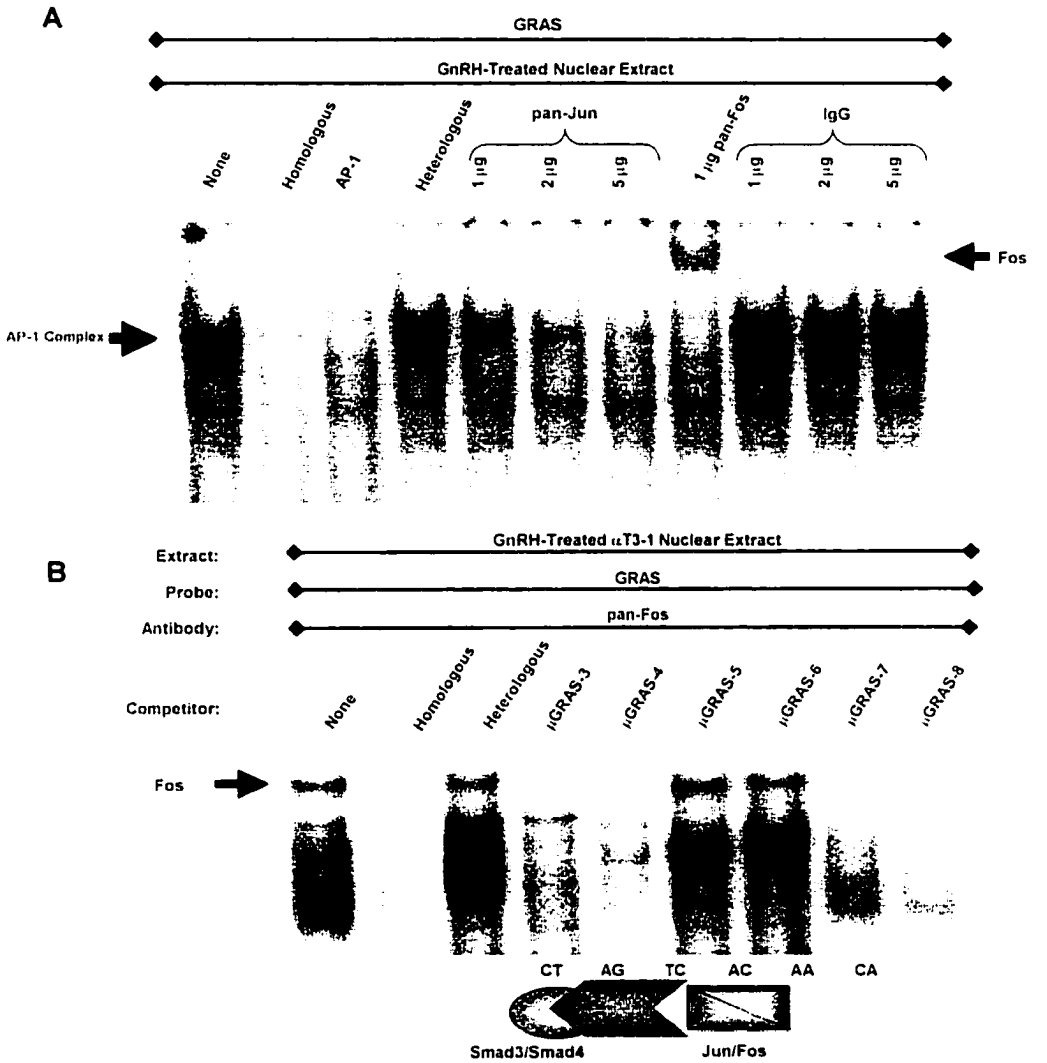


Figure 19. AP-1 binding is localized to the center of GRAS. A) Nuclear extract from αT3-1 cells that had been serum starved for 24 h and treated with 100 nM GnRH for 1 h was incubated with a radiolabeled oligonucleotide containing GRAS and subjected to EMSA. Where indicated, unlabeled oligonucleotides representing either GRAS (homologous), a canonical AP-1 binding site, or an unrelated sequence (heterologous) were added to the binding reactions in 500-fold molar excess. To identify the binding components, an antibody directed against the DNA binding domain of Jun family members (pan-Jun), an anti-Fos antibody (pan-Fos) or non-immune mouse IgGs were added to the binding reactions. B) Nuclear extract prepared from αT3-1 cells that had been serum starved for 24 h and treated with 100 nM GnRH for 1 h was incubated with a radiolabeled oligonucleotide containing GRAS and the pan-Fos antibody. The ability of unlabeled µGRAS-3, 4, 5, 6, 7 and 8 (500-fold molar excess) to displace the radioactive probe in the supershifted Fos complex was used to localize AP-1 binding at GRAS.

Thus, nucleotides comprising the center of the GRAS element and overlapping the Smad binding motif are essential for AP-1 binding.

GRAS is both necessary and sufficient for synergistic activation of the GnRHR promoter by activin and GnRH. Responsiveness of the GnRHR promoter to both activin and GnRH has been established (2;3;28;142;153;154); however, the elements that underlie activin and GnRH regulation are distinct. Specifically, GRAS has been shown to mediate the effects of activin whereas a more proximal AP-1 site is critical for GnRH responsiveness. More recently, however, several groups have suggested that co-administration of activin and GnRH leads to a synergistic activation of the GnRHR promoter. Consistent with this notion, we find that in a 6 h transfection protocol (2) the wild-type –600 promoter from the murine GnRHR gene (pMGR-600LUC) exhibits the expected response to GnRH and this GnRH response is significantly enhanced in the presence of activin (Figure 20A). The ability of activin to enhance the GnRH response is, however, lost if GRAS is replaced with a *Not*I recognition site (μGRAS). Thus, GRAS is necessary for activin augmentation of the GnRH response of the GnRHR promoter. To determine if GRAS is sufficient to mediate activin augmentation of GnRH responsiveness we next tested the effects of activin and GnRH treatment on the activity of the reporter construct containing 3 copies of GRAS linked to the minimal promoter from the rat prolactin gene (p3XGRAS-LUC). While neither GnRH nor activin alone affected transcriptional activity of p3XGRAS-LUC the combined treatment (GnRH + activin) resulted in a nearly 40-fold stimulation of luciferase expression (Figure 20B). This effect was specific to

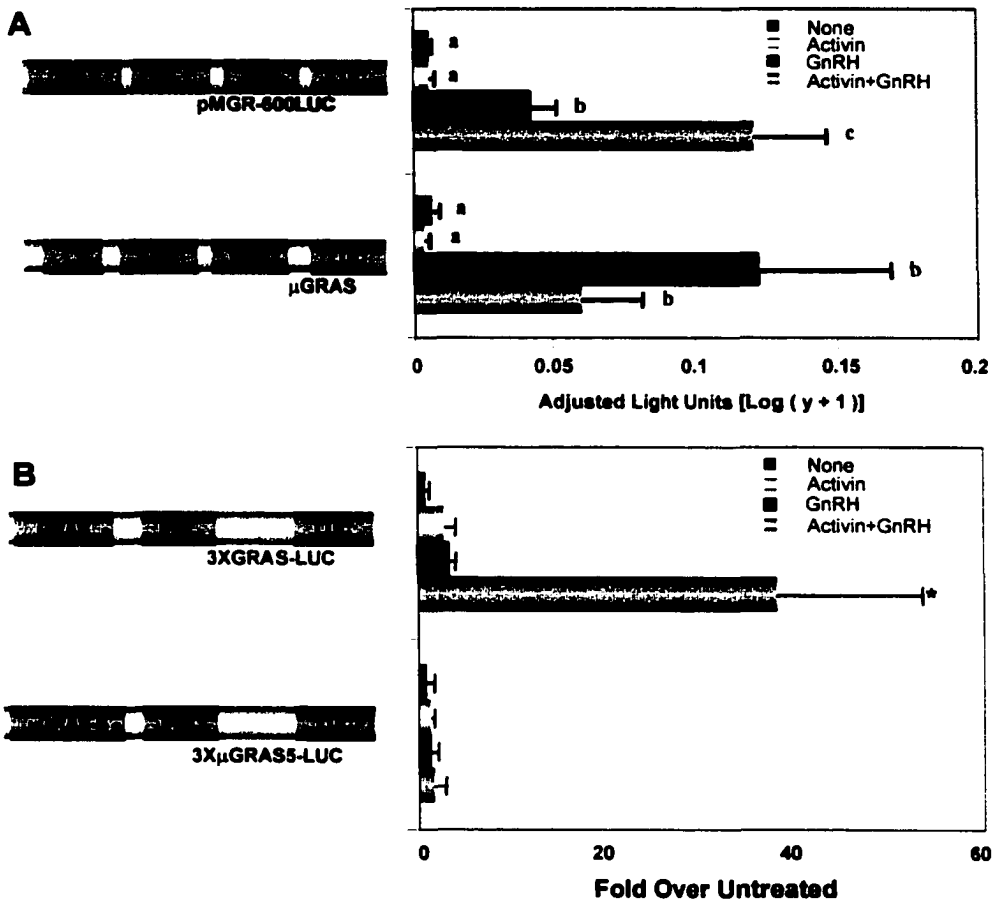


Figure 20. GRAS is both necessary and sufficient for synergistic activation of the GnRHR promoter by activin and GnRH. A) Constructs containing either 600 bp of proximal promoter fused to the cDNA for luciferase (pMGR-600LUC) or the same promoter region in which GRAS has been replace with a *NotI* recognition site (μGRAS) were co-transfected with pRSV-LacZ into αT3-1 cells. Thirty minutes after transfection, cells were treated with 20 ng/mL activin in the presence or absence of 100 nM GnRH. Six hours following treatment, cells were harvested and cellular lysates assayed for luciferase and LacZ expression. Luciferase data were divided by the β-galactosidase values to adjust for transfection efficiency. [a,b,c] Within vector, bars bearing different letters differ ($p<0.05$). B) Constructs containing 3 copies of wild-type GRAS or μGRAS-5 fused upstream of the prolactin minimal promoter linked to the cDNA encoding luciferase (3XGRAS-LUC, 3XμGRAS5-LUC) were co-transfected with pRSV-LacZ into αT3-1 cells. Thirty mintues after transfection, cells received 20 ng/mL activin in either the presence or absence of 100 nM GnRH. Six hours after the addition of treatments, cells were harvested and assayed for luciferase and LacZ expression. Luciferase values were divided by the β-galactosidase values to adjust for transfection efficiency. *$p<0.05$ as compared to untreated vector.

the wild-type GRAS element as none of the treatments affected luciferase expression in cells transfected with a similar reporter construct consisting of three copies of a GRAS containing a loss of function mutation in the AP-1 binding motif (3XμGRAS5-LUC). Thus, GRAS is both necessary and sufficient to mediate activin enhancement of GnRH responsiveness. The absence of an activin response in this experiment is due to the short transfection protocol that is necessary to observe GnRH regulation (2;3). Using a more standard 24 hour transfection protocol, activin responsiveness of GRAS is clearly demonstrable (142).

GnRH enhances AP-1 binding at GRAS but AP-1 alone is not sufficient for synergistic activation of the GnRHR gene by activin and GnRH. Both GnRH and activin regulation is organized at GRAS. The latter is, presumably, at least partially mediated by Smad3 and Smad4. Given that AP-1 is an established target for GnRH activation (77;155-158) we next sought to determine if GnRH stimulation of αT3-1 cells might be revealed at GRAS as an increase in AP-1 binding activity. To address this issue, an EMSA was performed in which radiolabeled GRAS was combined with nuclear extracts from αT3-1 cells that were serum-starved for 24 h and then either left untreated or treated with 100 nM GnRH. As above, the pan-Fos antibody was included in the binding reactions to more easily visualize the AP-1 complex. Consistent with the hypothesis that GnRH treatment would stimulate AP-1 binding at GRAS, the supershifted Fos complex was significantly enhanced in extracts from GnRH treated cells (Figure 21A).

While it is likely that Smad3 and Smad4 contribute to activin regulation at GRAS, TGF-β family members are capable of signaling via Smad independent activation of AP-1 (159-161). As such, we were interested in testing whether synergistic activation of the GnRHR promoter by activin and GnRH might be mediated by AP-1 alone. To address this, GRAS was replaced with a canonical AP-1 site in the context of 600 bp of proximal GnRHR promoter (GRASΔAP-1) thus effectively removing any Smad binding component while retaining a binding site for AP-1. As above, activin enhanced the GnRH response of the wild-type –600 promoter; however, this effect of activin was lost with the GRASΔAP-1 construct (Figure 21B). In fact, activin treatment led to a significant reduction in the GnRH response of the GRASΔAP-1 promoter. Thus, AP-1 alone is not sufficient to organize the ability of activin to enhance the GnRH response of the GnRHR promoter.

Interaction of a member of the forkhead family of DNA binding proteins is detectable at the proximal end of GRAS. The data in the preceding sections suggest that GRAS is a composite regulatory element that is capable of interacting with both Smad and AP-1 binding components. However, loss of function mutations located at the extreme 3' end of GRAS (142) appear to have little effect on either Smad or AP-1 binding. At issue then is whether an additional binding component localized to proximal GRAS is necessary for the functional activity of this element. In this regard, it is notable that the 3' end of GRAS is homologous to binding sites defined for the forkhead family of transcription factors (162). Furthermore, a functional interaction of forkhead

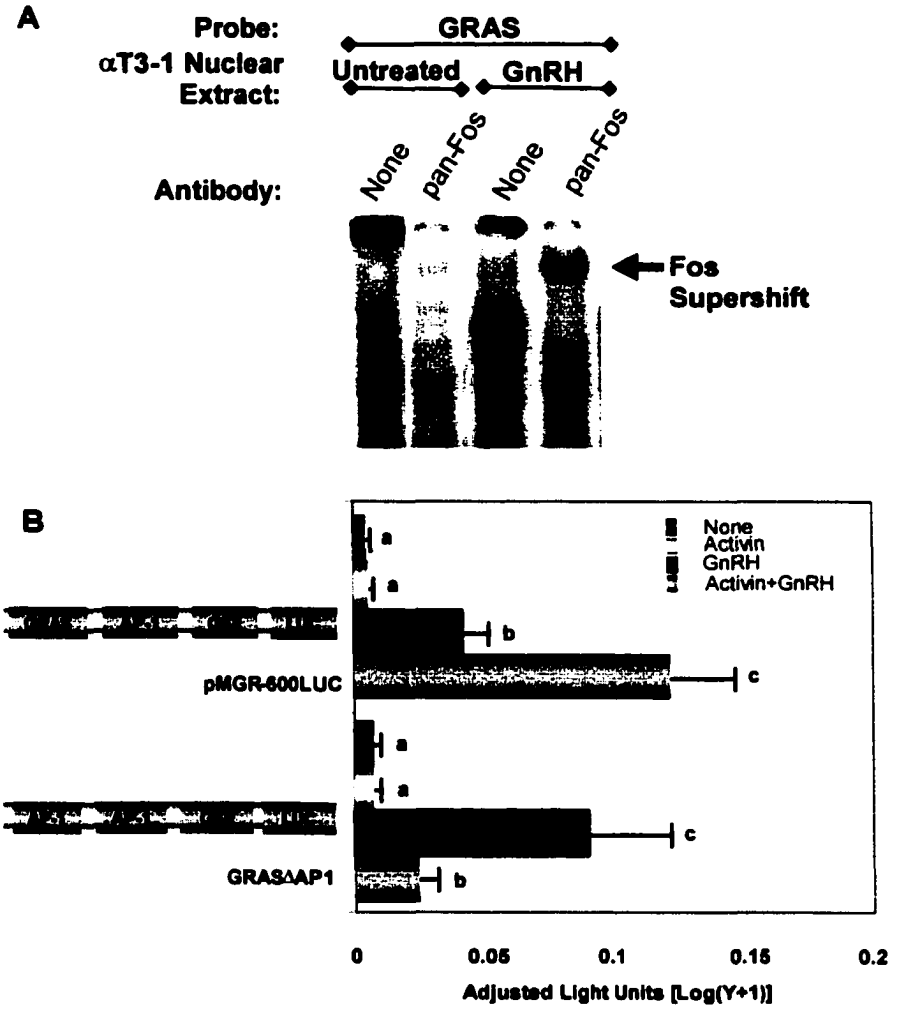


Figure 21. GnRH enhances AP-1 binding at GRAS but AP-1 alone is not sufficient for synergistic activation of the GnRHR gene by activin and GnRH. A) Nuclear extract from αT3-1 cells that were serum starved for 24 h and then either untreated or treated with 100 nM GnRH for 1 h were combined with radiolabeled oligonucleotides containing GRAS. An antibody that recognizes all members of the Fos family (pan-Fos) was added as indicated. B) Luciferase reporter constructs containing either the 600 bp wild-type promoter (pMGR-600LUC) or the same promoter in which GRAS was replaced with a canonical AP-1 binding site (GRASΔAP1), were co-transfected with pRSV-LacZ into αT3-1 cells. Thirty minutes following transfection, cells were treated with 20 ng/mL activin in the presence or absence of 100 nM GnRH. Six hours after treatments, cells were harvested and cellular lysates assayed for luciferase and β-galactosidase activity. Luciferase values were divided by β-galactosidase values to adjust for differences in transfection efficiency. ^{a,b,c} Within vector, bars bearing different letters differ ($p<0.05$).

proteins with Smads is critical in mediating TGF-β and activin signaling to multiple genes (101;104;163). Thus, we were intrigued with the possibility that proximal GRAS might represent a binding site for a member of the forkhead family of transcription factors. Of particular interest to us was the potential role for a relatively new member of the forkhead family referred to as FoxL2. Expression of FoxL2, originally termed pituitary forkhead factor or PFrk, immediately precedes the emergence of gonadotropes in the developing anterior pituitary gland (164). Also, FoxL2 expression in the developing pituitary is confined to the ventral cell types that are the progenitors of both gonadotropes and thyrotropes (105). As such, it seemed reasonable to predict that FoxL2 might contribute to the onset of expression of the central phenotypic markers of differentiated gonadotropes including the GnRHR gene. Thus, FoxL2 seemed an appealing candidate to examine for potential activity at proximal GRAS. Towards this end, we first used RT-PCR to isolate a partial coding sequence for FoxL2 from αT3-1 mRNA. As FoxL2 appears to exist as an intronless gene, the partial cDNA was then used to isolate the entire coding region from a mouse genomic library. Subsequently, FoxL2 expression in αT3-1 cells was confirmed by Northern analysis (data not shown).

To address binding of FoxL2 to GRAS, EMSA was performed using radiolabeled GRAS as the probe and nuclear extracts prepared from αT3-1 cells that were either untransfected or transfected with an expression vector for HA-tagged FoxL2. Expression of the fusion protein was confirmed by Western blotting (Figure 22A). The inclusion of an anti-HA antibody in the binding

reaction using nuclear extracts prepared from transfected cells retarded the migration of radiolabeled GRAS (Figure 22B). The electrophoretic mobility of radiolabeled GRAS was not affected by inclusion of anti-HA antibody in reactions using extracts from non-transfected cells. Thus, binding of FoxL2 at GRAS is detectable by EMSA.

We next utilized the yeast one-hybrid approach as an independent method to assess FoxL2 interaction at GRAS. In this assay, we fused coding sequence for the FoxL2 DNA binding domain to the activation domain of GAL4 (pFoxL2-AD). Transformation of this vector into yeast containing three copies of GRAS fused to a LacZ reporter gene did not elicit expression of LacZ (Figure 22C). To determine if the presence of Smads is necessary for FoxL2 interaction at GRAS, yeast were transformed with an expression vector for Smad4 alone or Smad4 plus an expression vector for Smad3. Transformation of FoxL2-AD into yeast expressing Smad4 resulted in a modest induction of LacZ expression that was greatly enhanced upon addition of Smad3. Finally, the ability of FoxL2-AD to activate LacZ expression was lost if a 2 bp mutation was introduced at the center of the winged-helix homology (μGRAS-7). Thus, in contrast to Smad3 and Smad4, FoxL2 interaction at GRAS is compromised by mutations localized to the 3' end of the element. Furthermore, it would appear that FoxL2 interaction at GRAS is dependent on the presence of Smads and, more specifically, Smad3.

FoxL2 interacts with the C-terminus of Smad3 in a mammalian two-hybrid assay. The yeast one-hybrid data suggest that the ability of FoxL2 to activate a multimerized GRAS reporter is dependent on the presence of Smad3. To further

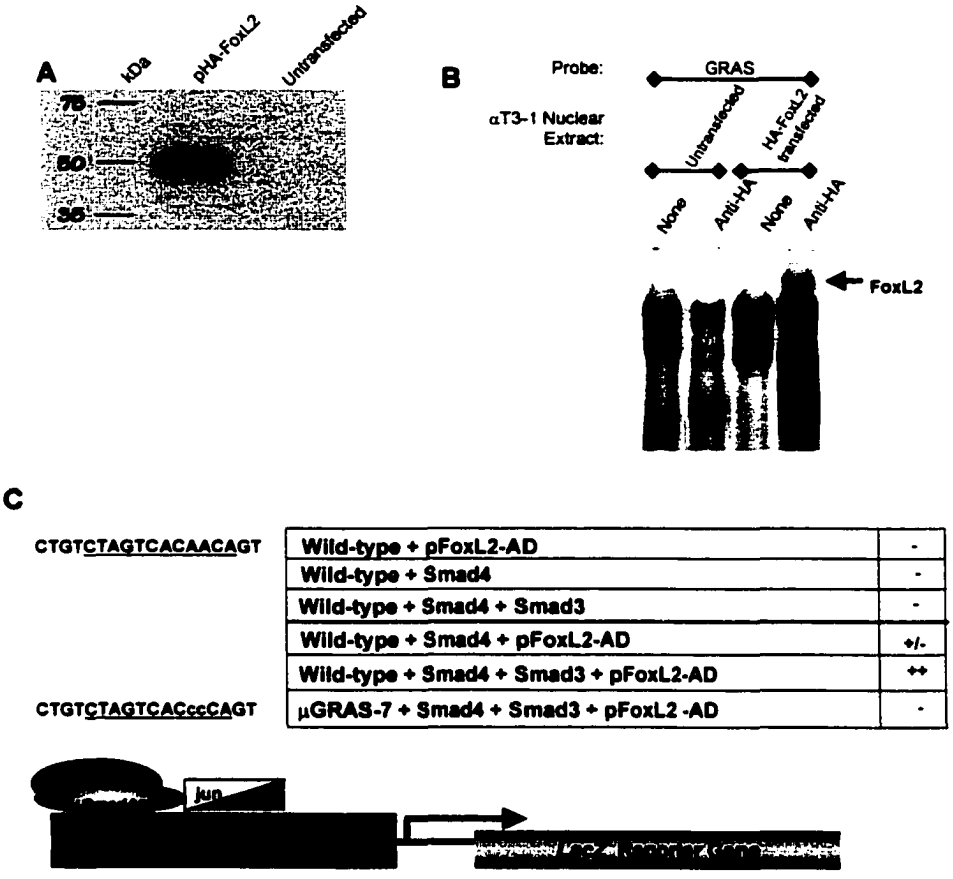


Figure 22. Interaction of a member of the forkhead family of DNA binding proteins is detectable at the proximal end of GRAS A) Cellular lysates from either untransfected αT3-1 cells or αT3-1 cells that were transfected with an expression vector for HA-tagged FoxL2 were analyzed by Western blotting using an anti-HA antibody. B) Nuclear extracts prepared from αT3-1 cells that were either untransfected or transfected with an expression vector for HA-FoxL2 were combined with a radiolabeled oligonucleotide containing GRAS. Where indicated, an anti-HA antibody was included in the binding reaction. C) Multiple copies of GRAS or μGRAS-7 were placed upstream of an expression cassette for β-galactosidase (LacZ) and stably integrated into the yeast strain YM4271. The cDNA encoding the DNA binding domain for FoxL2 was fused to the GAL4 activation domain in a yeast expression vector (pFoxL2-AD). This construct was transformed into the recombinant yeast strains transformed with the indicated LacZ reporter constructs. Where indicated, pFoxL2-AD was co-transformed with expression vectors for Smad4 and Smad3. After approximately 3-5 days of growth, yeast were assayed for LacZ expression as described in Materials and Methods. $^{-}$ Indicates that color development was never evident in advance of the negative control (absence of pFoxL2-AD). $^{+/-}$ Indicates Lac-Z expression that was evident (blue-color) within 12 hours and in advance of the negative controls in 6/12 assays. $^{++}$ Indicates Lac-Z expression that was evident within 2 – 12 hours.

explore this issue we examined the ability of FoxL2 to interact with Smad3 using a mammalian two-hybrid approach. In this assay, we fused various regions of FoxL2 to the transcriptional activation domain of VP16 and co-expressed these fusion proteins with the C-terminal (MH2) domain of Smad3 fused to the DNA binding domain of Gal4. The MH2 domain was utilized to eliminate the N-terminal DNA binding domain (MH1) that has been shown to inhibit a number of protein-protein interactions (165;166). Induction of luciferase expression from a Gal4-LUC reporter after transfection into CHO cells was used as the index of Smad3-FoxL2 interaction. Co-expression of Gal4-Smad3(MH2) and full-length FoxL2-VP16 resulted in an approximately 3-fold increase in luciferase expression over Gal4-Smad3(MH2) alone (Figure 23). Deletion of the C-terminal region of FoxL2 (N-225) led to an even greater induction of luciferase expression in the presence of the Gal4-Smad3(MH2) fusion protein suggesting that a C-terminal domain of FoxL2 may inhibit interaction with Smad3. Finally, deletion of the FoxL2 DNA binding domain (140-C) completely eliminated FoxL2-Smad3 interaction as assessed by induction of the Gal4-LUC reporter suggesting that this region is critical in mediating the association of Smad3 and FoxL2.

FoxL2 activates GRAS in a mammalian one-hybrid assay. Based on the EMSA and yeast one-hybrid and mammalian two-hybrid data it seemed reasonable to predict that overexpression of FoxL2 would stimulate transcriptional activity of the multimerized GRAS-luciferase reporter in αT3-1 cells. However, as forkhead proteins alone are often not potent transcriptional activators (126;130) we addressed this issue by co-transfecting αT3-1 cells with

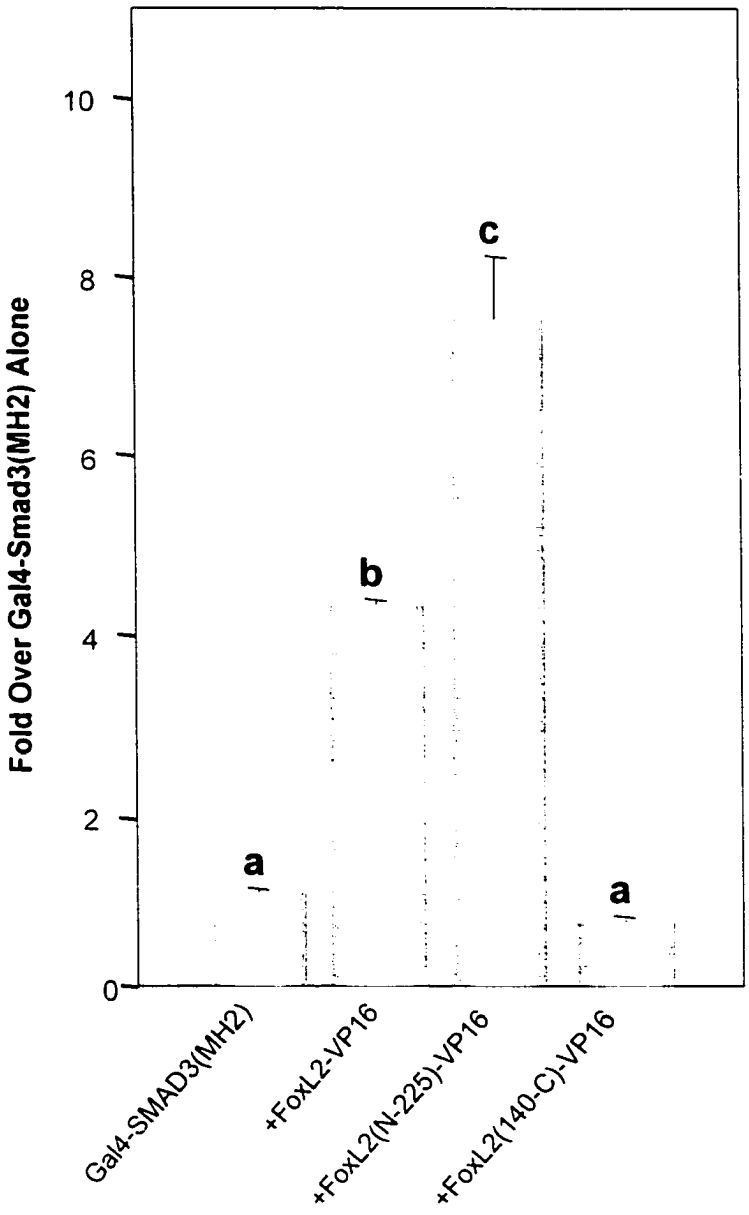


Figure 23. FoxL2 interacts with the C-terminus of Smad3 in a mammalian two-hybrid assay. An expression vector for the C-terminus of Smad3 fused to the Gal4 DNA binding domain (Gal4-Smad3MH2) was co-transfected into CHO cells with a Gal4-LUC reporter and expression vectors for the indicated fragments of FoxL2 fused to the activation domain of VP16. pRSV-LacZ was included in each transfection to account for differences in transfection efficiencies. Cells were harvested 24 h after transfection and assayed for luciferase and β-galactosidase activity. Luciferase values were divided by the corresponding β-galactosidase value. Data are expressed as the fold change in adjusted luciferase activity as compared to Gal4-Smad3(MH2) alone. ^{a,b,c} Bars bearing different letters differ ($p<0.05$).

the expression vector for the FoxL2-VP16 fusion protein and the 3XGRAS-LUC reporter vector. Overexpression of FoxL2-VP16 led to an approximately 25-fold activation of 3XGRAS-LUC (Figure 24). Furthermore, the stimulatory effect of the FoxL2 fusion protein was lost if mutations were introduced at either the 5' or 3' end of GRAS. Thus, both the Smad binding site and the winged-helix homology appear to be necessary for FoxL2 activation at GRAS in αT3-1 cells.

DISCUSSION

Approximately 5 years ago we used scanning mutagenesis to characterize a novel regulatory element located in the proximal promoter of the GnRHR gene that we termed the GnRH receptor activating sequence (GRAS) (1). Originally characterized as 1 of 3 regulatory elements that contribute to cell-specific activity of the GnRHR gene promoter it has since become evident that the functional role(s) of GRAS is more complex. For example, responsiveness of the GnRHR gene promoter to members of the TGF-β superfamily, in particular activin, is mediated at GRAS (142). Recently, GRAS has been shown to be responsive not only to activin but also GnRH (154;167). Thus, GRAS is emerging as a complex element that mediates responsiveness to multiple endocrine inputs. Here we have sought to identify the complement of proteins that interact at GRAS and find that the functional activity of this element depends on a complex array of transcription factors including Smads, AP-1 and a forkhead DNA binding protein. Furthermore, the functional attributes of GRAS are lost when the binding of any single component is eliminated.

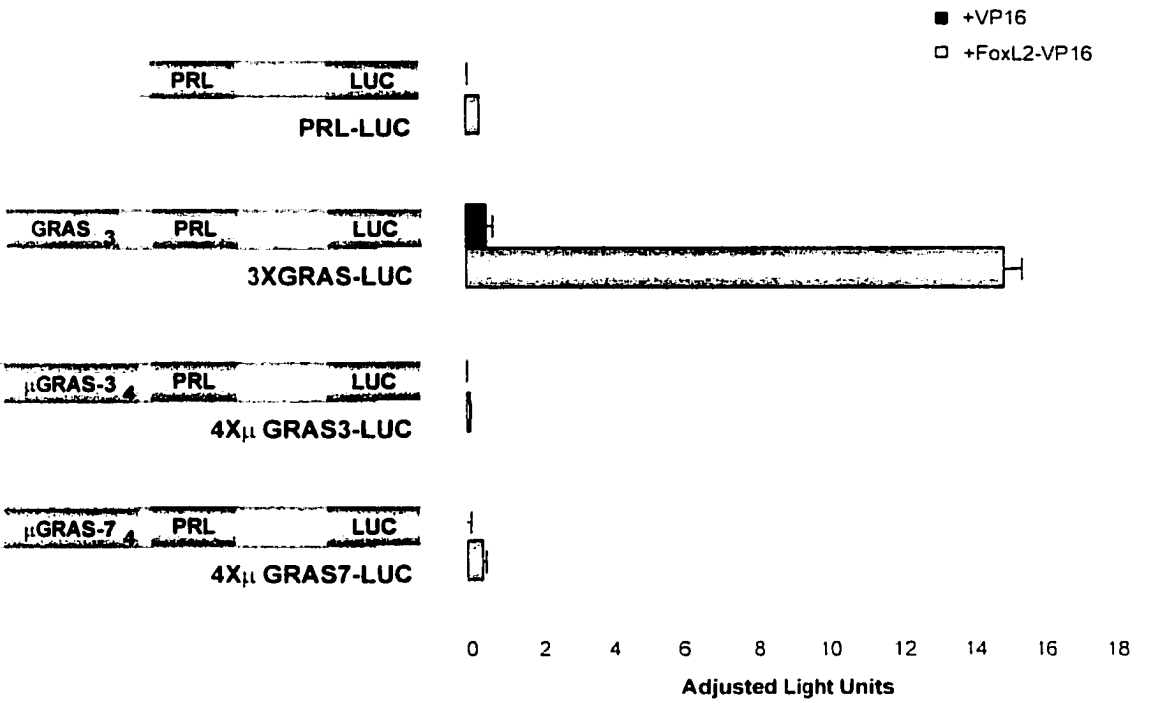


Figure 24. FoxL2 activates GRAS in a mammalian one-hybrid assay. Expression vectors for full-length FoxL2 fused to the activation domain from VP16 or the VP16 activation domain alone were co-transfected into αT3-1 cells with the indicated reporter constructs. pRSV-LacZ was included in each transfection. Cells were harvested 24 hours after transfection and assayed for luciferase and β-galactosidase activity. Luciferase values were divided by the corresponding β-galactosidase values to adjust for differences in transfection efficiency.

As an activin response element, members of the Smad family of TGF-β signaling proteins were clear candidates for contributing to the functional activity of GRAS. Of particular interest were the pathway specific Smads 2 and 3 which mediate TGF-β/activin signaling and Smad4 which serves as a common partner with the pathway specific Smads (130;132). Consistent with this we find that GRAS effectively displaces binding of Smad3 and Smad4 to a consensus Smad binding site (152). Furthermore, the ability of GRAS to displace Smad binding was minimally compromised by mutations at the 3' end of the element suggesting that Smad binding is largely mediated at the Smad binding site homology located at the 5' end of GRAS. With Smad4, we were able to confirm this observation using both recombinant protein and a yeast one-hybrid assay. However, defining the nature of the interaction of Smad3 with GRAS proved more difficult.

Both the EMSA competition assay and overexpression analysis are consistent with a functional role for Smad3 at GRAS. Nevertheless, Smad3 binding could not be directly detected at GRAS in EMSA using either nuclear extracts or recombinant protein. In regard to the latter, it is important to underscore that binding of recombinant Smad3 was detectable using the consensus Smad binding element (152). Thus, the absence of binding of the recombinant protein would appear to reflect an inherent inability of Smad3 to bind directly to GRAS. In point of fact, detecting Smad binding in EMSA is often problematic as Smad proteins are known to interact weakly with DNA, particularly at composite regulatory elements (126;134). To approach the issue of Smad3

interaction at GRAS from a functional standpoint we utilized the yeast one-hybrid assay. Consistent with the EMSA data, Smad3 alone was not able to activate the multimerized GRAS reporter in yeast. Only in the presence of Smad4 was functional activation of GRAS by Smad3 revealed - a result consistent with an inability of either native or overexpressed Smad3 to bind DNA independently of Smad4 (168).

We and others (154;167) have reported that GRAS not only mediates activin responsiveness but also enhances the GnRH response of the GnRHR gene promoter. Here we find that GRAS is both necessary and sufficient for activin augmentation of GnRH responsiveness. Importantly, however, the use of 2 bp scanning mutations suggest that Smad binding alone cannot account for the functional impact of GnRH and activin at GRAS. In this regard, we find that the distal end of GRAS represents immediately juxtaposed and partially overlapping binding sites for both Smads and AP-1 – an established target for GnRH activation (2;3;61). Consistent with this we find that GnRH enhances the binding of AP-1 at GRAS suggesting a potential mechanism that requires a functional interaction between a Smad3/Smad4 complex that conveys the activin signal and an AP-1 complex that conveys the GnRH signal. It is important to underscore, however, that from a functional standpoint these events are inseparable. That is, neither Smad binding alone nor AP-1 alone is sufficient to convey either an activin or GnRH signal to GRAS. In this regard, it is interesting that converting GRAS to a canonical AP-1 site not only eliminated synergistic activation of the GnRHR promoter but also led to a significant decrease in luciferase expression

as compared to GnRH treatment alone. This was an unexpected result for which we have no definite explanation. It may be that the attenuated GnRH response in the presence of activin reflects squelching whereby AP-1 is sequestered in non-productive interactions with Smads. A canonical AP-1 site located approximately 50 bp downstream of GRAS contributes to both "basal" activity and GnRH responsiveness of the GnRHR promoter (1;2). As AP-1 has been shown to interact with Smads (169-171) it is plausible that, upon the addition of activin, Smads are phosphorylated and translocate to the nucleus where protein-protein interactions engage AP-1 components in a non-productive complex. The effects of sequestering AP-1 might be exaggerated if the second, engineered AP-1 binding site was itself non-productive, i.e., capable of binding AP-1 but not contributing to promoter activity. Consistent with this possibility, the activity of the GnRHR promoter in which GRAS has been converted to a canonical AP-1 site (GRASΔAP-1) is identical to the same promoter containing a *Not*I recognition site in place of GRAS (data not shown). Thus, converting GRAS to AP-1 presents as a loss of function mutation in the GnRHR promoter.

As discussed above, Smad family members have been shown to interact directly with AP-1 proteins. For example, both Smad3 and Smad4 bind all three Jun family members: JunB, c-Jun and JunD (169). The Smad/AP-1 complex can then bind DNA and activate transcription (171;172). It is interesting to note that c-Jun has been shown to associate with the N-terminal and linker region of Smad3 but not with the C-terminus (171;172). However, as with other forkhead DNA binding proteins (113;163), we find an interaction of FoxL2 with the C-terminal

region of Smad3. Thus, different domains of Smad3 may mediate interactions with AP-1 and FoxL2 at GRAS.

We have demonstrated that both Smads and AP-1 proteins bind at GRAS; the question remains, however, is whether these proteins simultaneously occupy GRAS. In previous work aimed at determining the crystal structure of Smad3 binding at a Smad binding element in the human collagenase I promoter, Shi et al. (134) addressed the likelihood of Smad3 binding if a Jun/Fos heterodimer were docked to an AP-1 site that overlaps with the last base of a Smad binding site. While their analysis indicated that the N-terminal eight residues of Fos would sterically clash with the MH1 domain of Smad3, they suggested that Smads and AP-1 could coexist on this site if the N-terminal residues of Fos adopt a different conformation (134;165). In fact, TGF-β regulation at the collagenase I Smad binding element was subsequently shown to be mediated by the binding of both Smads and AP-1 as evident in EMSA and DNAse footprinting analysis (165;171). GRAS is reminiscent of this scenario in that the Smad binding motif overlaps a binding site for AP-1.

While it is tempting to focus on Smad and AP-1 interactions at GRAS it is also clear that these components are only part of the complex that is necessary for the functional activity of this element. That is, mutations localized to the proximal end of GRAS (μGRAS-7 and μGRAS-8) do not appear to influence the interactions of either Smads or AP-1 at GRAS yet are as effective in abrogating the functional activity of this element as mutations that eliminate Smad and/or AP-1 binding. For several reasons we have been attracted to the possibility that

proximal GRAS represents a binding site for a member of the forkhead family of DNA binding proteins. First, forkhead proteins were among the first proteins shown to functionally interact with Smads and contribute to TGF-β family signaling (98;101;104;146). Second, the sequence of proximal GRAS displays homology to a forkhead DNA binding site (162). Here we find that a likely candidate for mediating the functional activity localized to proximal GRAS is a fairly new member of the forkhead family of proteins termed FoxL2. First identified as a transcript expressed in the embryonic pituitary FoxL2 has been implicated in mediating the final differentiation of gonadotropes (105). As far as we know, GRAS represents the first potential target for FoxL2 activation in gonadotropes. Finally, in a manner similar to FAST1 and FAST2, FoxL2 interaction at GRAS appears to be dependent on the presence of Smads further underscoring the functional interdependence of the multi-protein complex organized at GRAS.

Although first defined as a DNA regulatory element in the GnRHR gene over 5 years ago, only recently has there been substantive progress in defining the multiple roles of GRAS and the multiple families of proteins that subserve these roles. Herein, we have demonstrated that GRAS is capable of interacting with at least 3 classes of transcription factors including Smads, AP-1 and a forkhead DNA binding protein, FoxL2. This is the first demonstration of these 3 classes of DNA binding proteins interacting at a single regulatory element. Thus, GRAS represents a composite enhancer whose functional activity is dependent on a multi-protein complex. We propose that this complex presents a unique

contour of transcription factors that underlies the functional contributions of GRAS to expression of the GnRHR gene.

CHAPTER FIVE

NEGATIVE REGULATION OF GONADOTROPIN RELEASING HORMONE RECEPTOR GENE EXPRESSION BY ESTRADIOL-17β

ABSTRACT

Short-term exposure to estradiol has been shown to decrease gonadotropin-releasing hormone receptor (GnRHR) numbers in primary pituitary cultures. We find that estradiol treatment attenuates GnRH stimulation of the GnRHR gene. Western analysis reveals no change in activation of the MAP kinases ERK or JNK by estradiol either alone or in the presence of GnRH. GnRH stimulation of the GnRHR promoter is known to be mediated, at least in part, via an AP-1 binding site. We sought to determine whether the negative effect of estradiol required AP-1. Mutation of AP-1 eliminates GnRH-responsiveness of the GnRHR gene, thus simple mutational analysis was not an acceptable approach for investigating the requirement for AP-1 in estradiol signaling to the GnRHR gene. To circumvent this difficulty we replaced the AP-1 site with a CRE (AP1ΔCRE). This construct had a similar level of basal activity to the wild-type promoter, was responsive to forskolin and able to bind CREB and ATF-2 in a binding assay. Like the wild-type promoter, AP1ΔCRE was responsive to GnRH and bound by Jun and Fos. We were intrigued to find that GnRH stimulation of

AP1ΔCRE is not attenuated by estradiol. In light of these data we suggest that negative regulation of the murine GnRHR by estradiol in αT3-1 cells is mediated specifically by AP-1.

INTRODUCTION

The past several years have witnessed a great deal of progress in our understanding of molecular events underlying the ontogeny of pituitary gonadotropes and expression of their primary gene products: the glycoprotein hormone α-subunit, the unique LHβ and FSHβ subunits, and the GnRHR. In regard to the latter, we have found that basal activity of the murine GnRHR promoter in the gonadotrope-derived αT3-1 cell line (69) is mediated by a cell-specific enhancer located within 500 bp of proximal promoter that includes a binding site for the nuclear orphan receptor, steroidogenic factor-1 (SF-1), a consensus AP-1 element and a non-canonical element we have termed GnRHR activating sequence (GRAS). In addition to its role in "basal" transcriptional activity, AP-1 also mediates GnRH responsiveness. Specifically, GnRH regulation of the GnRHR gene is dependent on protein kinase C (PKC) activation and subsequent recruitment of both a Jun and Fos component to the AP-1 element (2;3).

In primary cultures of rat pituitary cells, chronic estradiol treatment increases while short-term treatment decreases GnRHR numbers (45;46). In αT3-1 cells the negative, but not the positive effect of estradiol is recapitulated (48). This discrepancy may due to indirect effects of estradiol involving non-

gonadotropes. Since the availability of cDNA's encoding the GnRHR, a number of studies have demonstrated coordinate changes in GnRHR mRNA and GnRHR numbers associated with estradiol treatment (14;17;18;20-23). Thus, the effects of estradiol on GnRHR numbers are at least in part transcriptional. The goal of the current studies was to more closely examine the mechanisms involved in estradiol inhibition of GnRH regulation of the GnRHR gene. We found that although replacement of AP-1 with a CRE eliminates neither basal nor GnRH-stimulated expression of the GnRHR gene, this mutation completely eliminated the negative effect of estradiol on GnRHR expression.

MATERIALS AND METHODS

Reagents: Forskolin was purchased from Sigma Chemical Co. (St. Louis, MO). GnRH was obtained from Bachem (Philadelphia, PA). GF109203X (Bisindolylmaleimide I) was purchased from Calbiochem (La Jolla, CA). Antibodies for used for EMSA: c-Jun/AP-1 (catalog no. sc-44X, broadly reactive with c-Jun, Jun B and Jun D p39), c-Fos (catalog no. sc-253X, broadly reactive with c-Fos, Fos B, Fra-1 and Fra-2), CREB (catalog no. sc-186X), and ATF-2 (catalog no. sc-187X) and for immunoblot analysis: c-Fos (catalog no. sc-253, broadly reactive with c-Fos, Fos B, Fra-1 and Fra-2), and anti-rabbit (catalog no. sc-2004) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Vector construction: The construction of luciferase expression vectors has been described (1;70). Element switches were made using dual rounds of PCR (primers: AP1ΔCRE Sense 5'-TATTATGACGTCACTTTC, AP1ΔCRE Antisense

5'-GAAAGTGACGTCATAATA, SF1ΔAP1 Sense 5'-CACTTGACTCACAGGAGGGCTTTGG, SF1ΔAP1 Antisense 5'-CCCTCCTGTGAGTCAAGTGTAACCGTAGC, GRASΔAP1 Sense 5'-CTGTCTGACTCAAACAGTTTTTAGAAAACC, GRASΔAP1 Antisense 5'-CTGTTTGAGTCAGACAGATACAAAATGAAATA).

Cell Culture and Transient Transfections: All cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cultures of αT3-1 cells were maintained in high-glucose DMEM containing 2 mM glutamine, 5% FBS, 5% horse serum, 100 U/mL penicillin and 100 μg/mL streptomycin sulfate (Mediatech, Herndon, VA). The plasmids were transfected using LipofectAMINE (GIBCO/BRL Life Technologies, Gaithersburg, MD) {Duval, Nelson, et al. 1997 ID: 25}. Transfections included 1.4 μg of the test vector and 0.25 μg of pRSV-LacZ. Approximately 24 h after transfection, cells were harvested and assayed for luciferase activity (70). Transient transfections involving forskolin treatment were performed as above except that 18 h after transfection, cells were treated with 10μM forskolin. After another 6 h cells were harvested and assayed for luciferase activity. Transient transfections involving GnRH treatment were carried out using a calcium phosphate/DNA co-precipitation method (2;173). Briefly, the day prior to transfection 2 x 10^6 cells were plated in 100 mm tissue culture dishes. Complete media was removed and calcium phosphate/DNA precipitates in a total volume of 1 mL were aded to the plates. At 30 min, post-transfection, media was added and cells were treated for 6 h with either GnRH or the treatment as indicated. When GF109203X was used it was added 15 min prior to

GnRH treatments. After 6 h of treatment, cells were washed twice with ice-cold phosphate-buffered saline (PBS) (10 g/L NaCl, 0.224 g/L KCl, 1.42 g/L Na_2HPO_4, 0.272 g/L KH_2PO_4, pH 7.4) and lysed in 400 μL of 25 mM glycyl-glycine (pH 7.8), 15 mM $MgSO_4$, 1% Triton-X100 and 1 mM dithiothreitol (DTT) (174). Lysates were cleared by centrifugation at 16,000 x g for 2 min. Lysates (40 and 100 μL for luciferase and β-galactosidase, respectively) were assayed according to manufacturer's instructions for luciferase (Promega, Madison, WI) and β-galactosidase (Tropix, Bedford, MA) activity using a Turner 20D luminometer (Turner Designs, Sunnyvale, CA). Luciferase values were normalized for transfection efficiency by dividing the luciferase activity by β-galactosidase activity (1;2).

Preparation of nuclear extracts: For preparation of nuclear extracts, cells were harvested in ice-cold PBS with 1 mM EDTA and pelleted by centrifugation in a clinical centrifuge approximately 1200 x g for 5 min. Cells were then resuspended in ice-cold lysis buffer (70 mM β-glycerol phosphate, 2 mM $MgCl_2$, 0.5 mM EGTA, 15 mM HEPES, 100 mg/mL, 0.15 mM spermine, 0.5 mM spermidine, 2 mM sodium meta vanadate, 2 mM sodium fluoride, 2.5 μg/mL leupeptin, 2.5 μg/mL pepstatin, 1 mM PMSF, 5 mM benzamidine, 1 mM DTT, pH 7.8) and incubated on ice for 5 min. Cells were lysed with 10 strokes of a glass Dounce B homogenizer. The lysates were layered over a cushion consisting of 300 mg/mL sucrose in lysis buffer and centrifuged at 6650 x g in a JS7.5 Beckman rotor at 4°C. Pellets were resuspended in lysis buffer, further homogenized by 10 strokes of the Dounce homogenizer and layered over a

sucrose cushion as above. The pellet was resuspended in lysis buffer plus 10% glycerol and 450 mM KCl. The solution was mixed by vortexing and then incubated at 4°C with gentle mixing on a circular mixer for 30 min. The solution was then centrifuged at 338,000 x g at 4°C for 45 min in a 70.1 Ti Beckman rotor. Supernatants were recovered and protein concentrations were determined using bicinchoninic acid (BCA Assay, Pierce, Rockford, IL)

Electrophoretic Mobility Shift Assays (EMSA): EMSAs were conducted as previously described (175). Nuclear extracts (2-12 μg) from αT3-1 cells were combined with Dignam buffer D (20 mM HEPES, 20% glycerol, 0.1 M KCl, 0.4 mM EDTA) and poly(dI-dC) (2 μg) (Amersham Pharmacia Biotech, Piscataway, NJ). Reactions were placed on ice for 10 min then combined with a random prime labeled probe (10 fmol, approximate specific activity of 2 x 10^4 cpm/fmol) (AP1ΔCRE Sense 5'-TATTATGACGTCACTTTC, AP1ΔCRE Antisense 5'-GAAAGTGACGTCATAATA, AP1 Sense 5'-TATTATGAGTCACTTTC, AP1 Antisense 5'-GAAAGTGACTCATAATA) and competitor, where indicated (5 pmol). Reactions were then incubated at room temperature for 20 min. Antibodies (Fos, CREB, ATF-2) (2 μg) were added at this time and reactions were incubated at room temperature for another 15 min. For antibodies against the Jun family members, the nuclear extract mixture was incubated with 2μg of antibody for 15 min at room temperature before the addition of radiolabeled probe (10 fmol, approximate specific activity of 2 x 10^4 cpm/fmol). Free probe was separated from bound probe by electrophoresis for 1-2 h at 40 mA in 5% polyacrylamide gels that were pre-run at 100 V for 30 min in 25 mM Tris, 190 mM

glycine, and 1 mM EDTA (pH 8). Gels were transferred to blotting paper, dried, and exposed to Hyperfilm MP (Amersham, Arlington Heights, IL) for approximately 6 h at -70°C with Dupont Cronex intensifying screens (Dupont, Boston, MA). Radiolabeled probes were prepared by labeling double-stranded oligonucleotides with [γ-^{32}P]ATP (4500 Ci/mmol; ICN, Irvine, CA) and T4 polynucleotide. Double-stranded DNA probes were separated from free nucleotides by centrifugation on a G-25 Microspin column (Amersham Pharmacia Biotech, Piscataway, NJ).

Pull-down assays: Pull-down assays were performed as previously described (176). Briefly, biotinylated oligonucleotides (biot-AP1ΔCRE Sense, AP1ΔCRE Antisense as shown above) were combined in binding buffer (25 mM Tris-Cl pH 7.5, 80 mM NaCl, 35 mM KCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, 10 μg/mL polydIdC, 0.3 mg/mL BSA, 2% NP40), heated to 100°C and allowed to cool slowly to room temperature. After annealing, double-stranded oligonucleotides were combined with metallic streptavidin beads (Promega CAS 9013-20-1) and incubated with shaking for 1 h at 4°C. Beads were washed 5 times in binding buffer and combined with 100 μg αT3-1 nuclear extract that was treated with 100 nM GnRH (0, 1, 6 h). Samples were incubated with shaking at 4°C for 2 h. Beads were washed five times with binding buffer and resuspended in 2X SDS sample buffer (50 mM Tris-Cl pH 6.8, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 1% (w/v) SDS). Samples were boiled for 10 min and immediately run on an SDS-PAGE. Proteins were transferred to nitrocellulose membrane by electroblotting. Membranes were blocked in TBS-T (140 mM NaCl,

10 mM Tris, 0.1% Tween 20, pH 7.4) plus 5% non-fat dry milk for 1 h then exposed to an antibody directed against all members of the Fos family (sc-253) (1:20,000 dilution) in 5% milk in TBS-T for 4 h at room temperature. Membranes were washed three times in TBS-T and incubated in TBS-T + 5% milk with anti-rabbit antibody (sc-2004) (1:5000 dilution) for 1 h at room temperature. Again, membranes were washed three times and proteins were visualized by SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL).

JNK and ERK activation assays: αT3–1 cells were grown to approximately 70% confluence and serum starved for 2 h prior to drug treatment and lysis. Cells were treated with 100 nM GnRH for 30 min. In addition, control vehicle (dimethyl sulfoxide [DMSO]) was applied to the cells receiving no drug treatment. Following treatment, cells were washed with ice-cold buffer containing 150 mM NaCl and 10 mM HEPES (pH 7.5) and lysed in radio-immunoprecipitation assay (RIPA) buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP40, 0.1% SDS, 0.5% deoxycholate, 2 mM EDTA, 5 mM sodium vanadate, 5 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice. The cell lysates were collected and debris cleared by centrifugation.

For ERK analyses, 10 μL of lysate was loaded onto a SDS-PAGE gel with a 10% acrylamide running gel section and a 5% acrylamide stack. Proteins were then transferred to nitrocellulose from Osmonics, and membranes were blocked in TBS-T (140 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 7.4) + 5% dry, non-fat milk for 30 min. Phosphorylated ERK analyses were incubated for 2 h at room temperature on an orbital shaker with a phospho-ERK antibody (1:1000 dilution)

obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Blots were then washed 6 times with TBS-T before incubating for 1 hour in TBS-T + 5% milk with anti-mouse IgG-HRP (1:2,000 dilution). Again, membranes were washed 6 times and proteins were visualized by SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). Blots were stripped at room temperature for 30 min on an orbital shaker with 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7) heated to 50°C. After washing the membranes twice, blots were reprobed with an antibody (1:10,000 dilution) which detects relative amounts of ERK protein independent of phosphorylation state (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-rabbit IgG-HRP (1:2,000 dilution) following the same general protocol.

JNK activation assays were performed in a similar manner, excepting the following differences: 20 μL of lysate was loaded onto gels, antibodies were obtained from Cell Signaling Technology (Beverly, MA) and primary antibodies were incubated with membranes in TBS-T + 5% BSA at 4°C overnight.

Statistical analysis: Data were analyzed by SAS (151). The transfection data were analyzed by one-way ANOVA with vector as the independent variable. Means were separated using Dunnett's t-test (Figures 25, 27, 30, 32). In Figure 28 treated and untreated vectors were compared using Student's t-test.

RESULTS

Estradiol treatment attenuates GnRH-responsiveness. A number of studies have demonstrated coordinate changes in GnRHR mRNA and GnRHR

numbers associated with estradiol treatment (20;23;25;117;120;138;177). Unfortunately, we have not been able to detect estradiol regulation of the murine GnRHR gene in either transient transfection paradigms or transgenic mice (121). However, hormones are not present as isolated entities in living organisms. They are present as complex mixtures that interact to modify one another's actions. To determine if estradiol had an effect on activity of the murine GnRHR promoter in the presence of GnRH, a vector containing 600 bp of proximal wild type GnRHR promoter (pMGR-600LUC) was transfected into αT3-1 cells and treated with GnRH and increasing amounts of estradiol-17β for 6 h. Treatment with 10 nM estradiol significantly reduced GnRH stimulation (Figure 25). Treatment with 100 nM severely attenuated promoter activity compared to GnRH alone.

GnRH stimulates the GnRHR gene via phosphorylation of JNK (79). We next sought to determine whether estradiol might have any effect on GnRH stimulated JNK phosphorylation. Western analysis was performed on αT3-1 cells that were treated with 100 nM estradiol, 100 nM GnRH or estradiol and GnRH together. Antibodies against either the phosphorylated (activated) form of JNK or the phosphorylated form of ERK were used. Estradiol treatment did not activate JNK or ERK, nor did estradiol have any effect on GnRH-induced activation of JNK and ERK (Figure 26). Thus, the effects of estradiol on GnRH signaling would appear to lie downstream of JNK.

AP-1 is not specifically required for basal activity of the promoter. GnRH stimulation of the GnRHR promoter is mediated, at least in part, via an AP-1 binding site. We next sought to determine whether the negative effect of estradiol

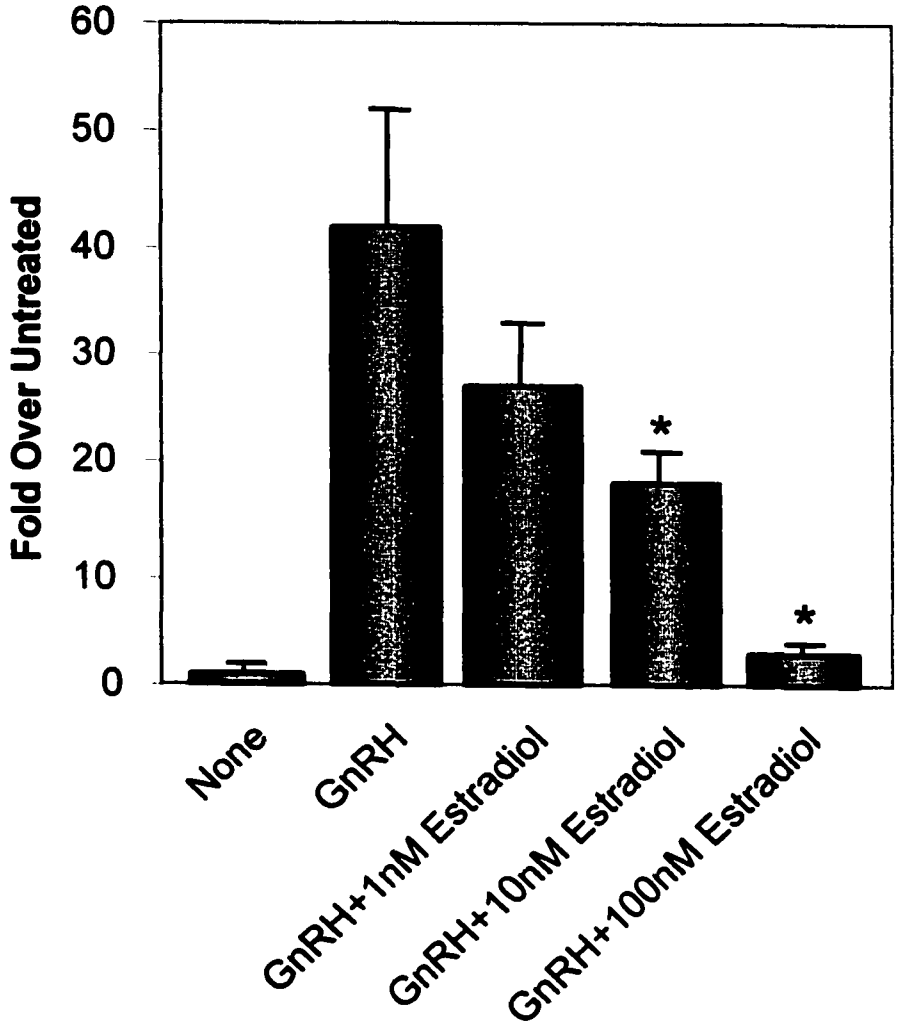


Figure 25. Estradiol treatment attenuates GnRH responsiveness. αT3-1 cells were transiently transfected with pMGR-600LUC and treated with 100 nM GnRH and 1-100 nM Estradiol-17β. Six hours after treatment cells were harvested and assayed for luciferase and β-galactosidase activity. *$p<0.05$ as compared to GnRH alone.

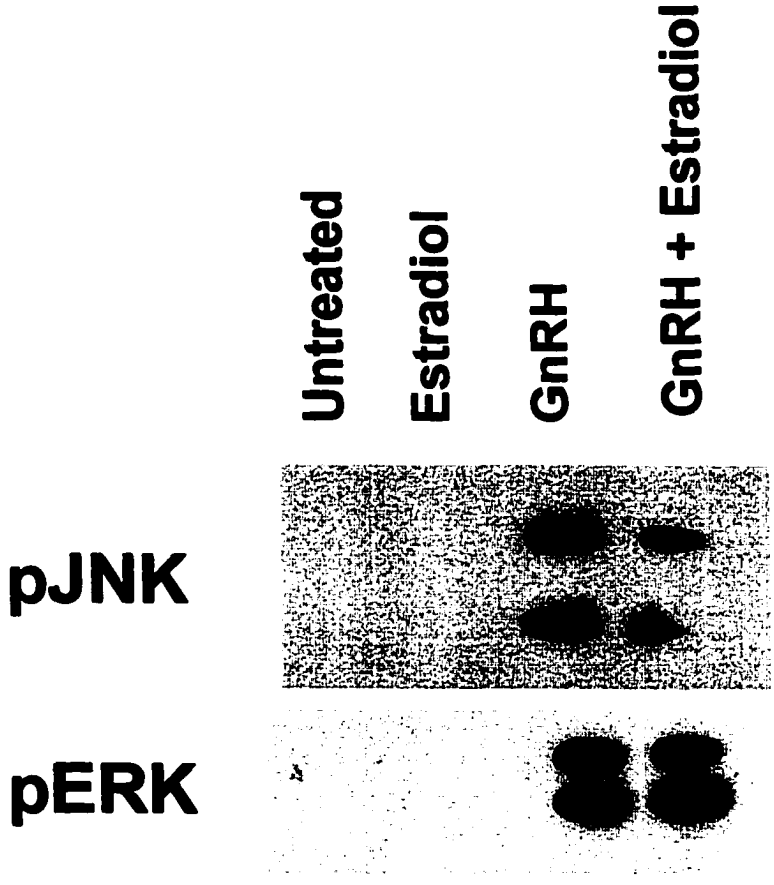


Figure 26. Estradiol treatment does not affect phosphorylation of JNK. αT3-1 cells were serum starved for 2 h and treated with 100 nM GnRH in the presence or absence of 100 nM estradiol for 1 h. Cells were then lysed in RIPA buffer and debris cleared by centrifugation. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were probed with either a phospho-specific ERK antibody (pERK) or a phospho-specific JNK (pJNK) antibody that recognizes the dual phosphorylated forms of ERK or JNK.

was mediated at the AP-1 binding site in the GnRHR promoter. Mutation of AP-1 eliminates GnRH-responsiveness of the GnRHR gene, thus simple mutational analysis was not an acceptable approach for looking at the requirement for AP-1 in estradiol signaling to the GnRHR gene. We sought to circumvent this difficulty by replacing the AP-1 site with a CRE (AP1ΔCRE). This approach was performed in hopes that we could change the identity of the element without losing GnRH-responsiveness. When compared to the wild type promoter, replacement of the AP-1 element with a CRE did not affect basal activity of the promoter (Figure 27). To determine if the requirements for the other elements in the GnRHR cell specific enhancer were as flexible as those for AP-1, we substituted GRAS and SF-1 with an AP-1 site. These element switches were not different from null mutations of the elements (Figure 27). Thus, while GRAS and SF-1 cannot be replaced with an AP-1 site and retain promoter function, AP-1 can be replaced with a CRE, suggesting that a certain degree of plasticity exists in the functional requirement for AP-1 in the GnRHR gene.

AP1ΔCRE has the characteristics of a CRE. Considering replacement of the AP-1 binding site with a CRE did not alter basal activity of the promoter, we next sought to confirm that our CRE was functioning as a CRE. We treated the AP1ΔCRE mutation with forskolin, which activates cAMP and therefore should stimulate the CRE. Promoter activity of the AP1ΔCRE mutation was increased approximately four-fold by forskolin treatment, while the wild-type promoter was unresponsive (Figure 28). To determine if this forskolin responsiveness could be due to binding of CRE binding protein (CREB), a

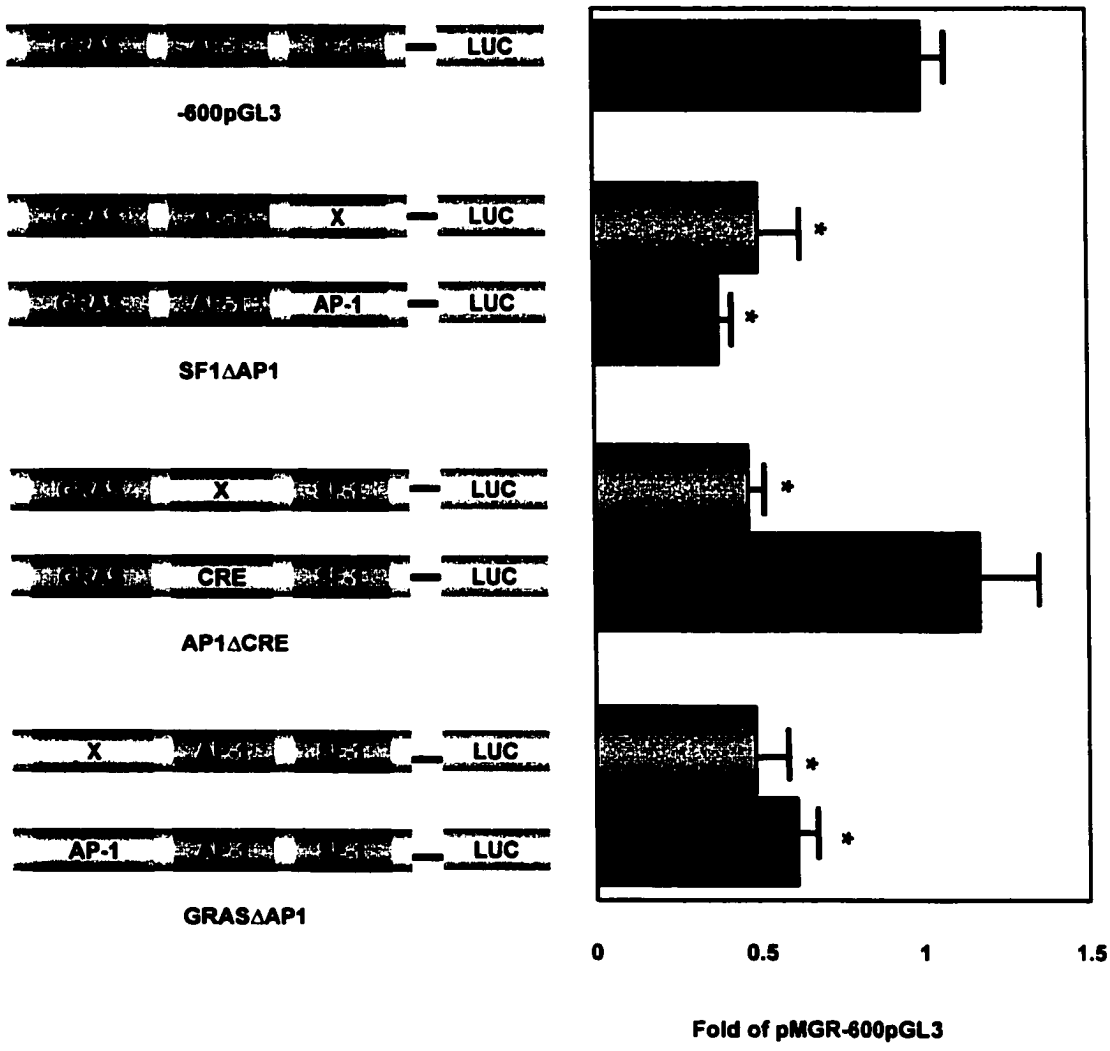


Figure 27. AP-1 is not specifically required for basal activity of the promoter. Three vectors were constructed in which GRAS or SF-1 were replaced with an AP-1 element (GRASΔAP1 and SF1ΔAP1, respectively) or the AP-1 element with a CRE (AP1ΔCRE) each in the context of 600 bp of 5' flanking sequence. These constructs were compared to vectors in which GRAS, SF-1 or AP-1 were replaced with a null mutation. All constructs were transiently transfected into αT3-1 cells and assayed for luciferase activity. Values represent mean ± SEM for triplicate samples. * $p<0.01$.

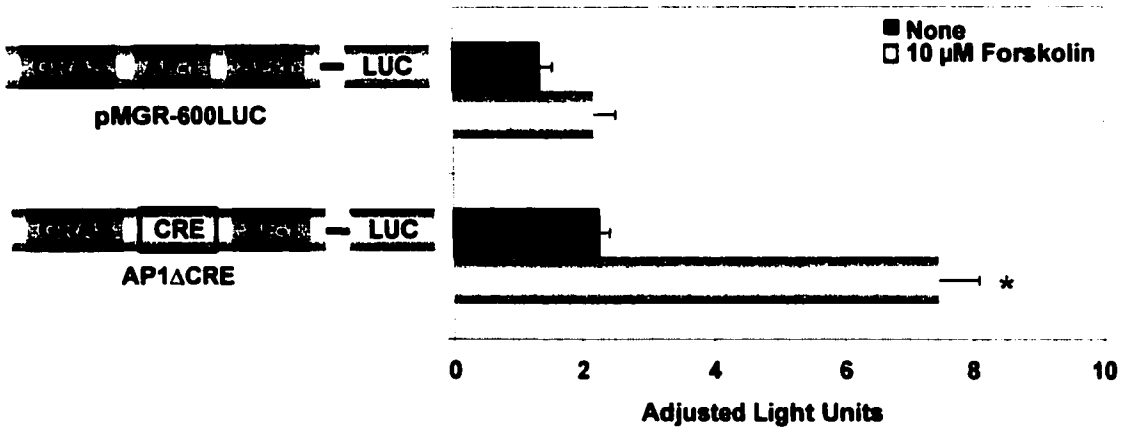


Figure 28. AP1ΔCRE responds to forskolin. Vectors containing the AP1ΔCRE mutation or the wild type promoter were transiently transfected into αT3-1 cells, treated with 10 μM forskolin for six hours, and assayed for luciferase activity. Values represent mean ± SEM for triplicate samples. * Represents values different ($p<0.01$) from untreated for each vector.

binding assay was performed. CREB and ATF-2 were able to bind AP1ΔCRE but not AP-1 (Figure 29). This shows that the CRE is demonstrating the characteristics of a classic CRE.

The AP-1ΔCRE mutation retains GnRH responsiveness. When we found that the AP1ΔCRE mutation did not diminish basal activity of the promoter, we realized that this represented a unique opportunity to determine the specific requirements for function of the AP-1 element. While a CRE and an AP-1 element both bind b-zip transcription factors, they generally respond to different stimuli. We wanted to know if the specificity of the AP-1 element is important for GnRH responsiveness even though it is not necessary for basal activity. To answer this question, the AP-1ΔCRE vector was transiently transfected into αT3-1 cells. The mutated promoter was stimulated by treatment with GnRH to a level not different from the wild-type promoter (Figure 30). Likewise, the stimulation of AP-1ΔCRE by GnRH is obliterated by treatment with the PKC inhibitor Bisindolylmaleimide I (GF 109203X). Thus, activation of AP1ΔCRE, like the wild-type promoter, appears to proceed through a PKC-dependent mechanism.

AP1ΔCRE binds Jun and Fos. When AP-1 was replaced with a CRE, the switch did not affect basal activity or GnRH responsiveness of the promoter. This prompted us to ask if this CRE could bind Jun and Fos components. Interestingly, both Jun and Fos were shown to bind AP1ΔCRE in a binding assay (Figure 31A). These are the components that are known to bind the wild type AP-1 (174). Binding of Jun at a CRE is well established, however there is less data to support Fos-binding at a CRE (176). To confirm that Fos was binding to our

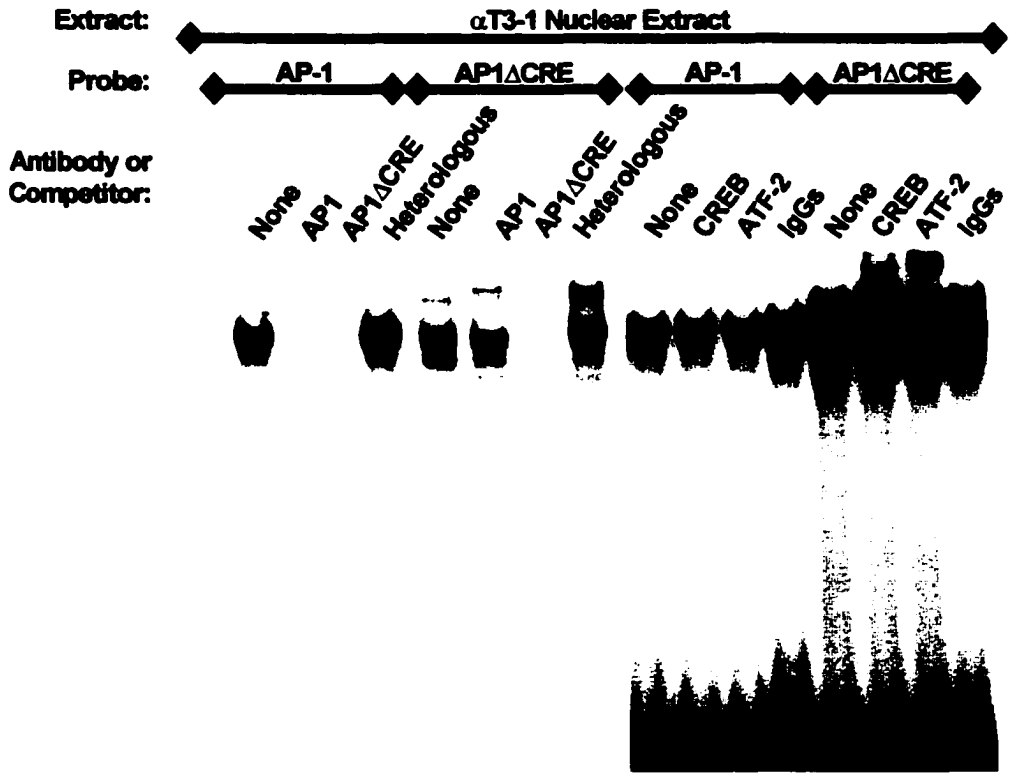


Figure 29. AP1ΔCRE binds CREB and ATF-2. Nuclear extracts from αT3-1 cells were incubated with a radiolabeled probe consisting of the consensus AP-1 element from the murine GnRHR gene promoter or the AP1ΔCRE mutation. A 500-fold molar excess of unlabeled oligonucleotides containing AP1, AP1ΔCRE or heterologous DNA, and antibodies to CREB and ATF-2 were added where indicated.

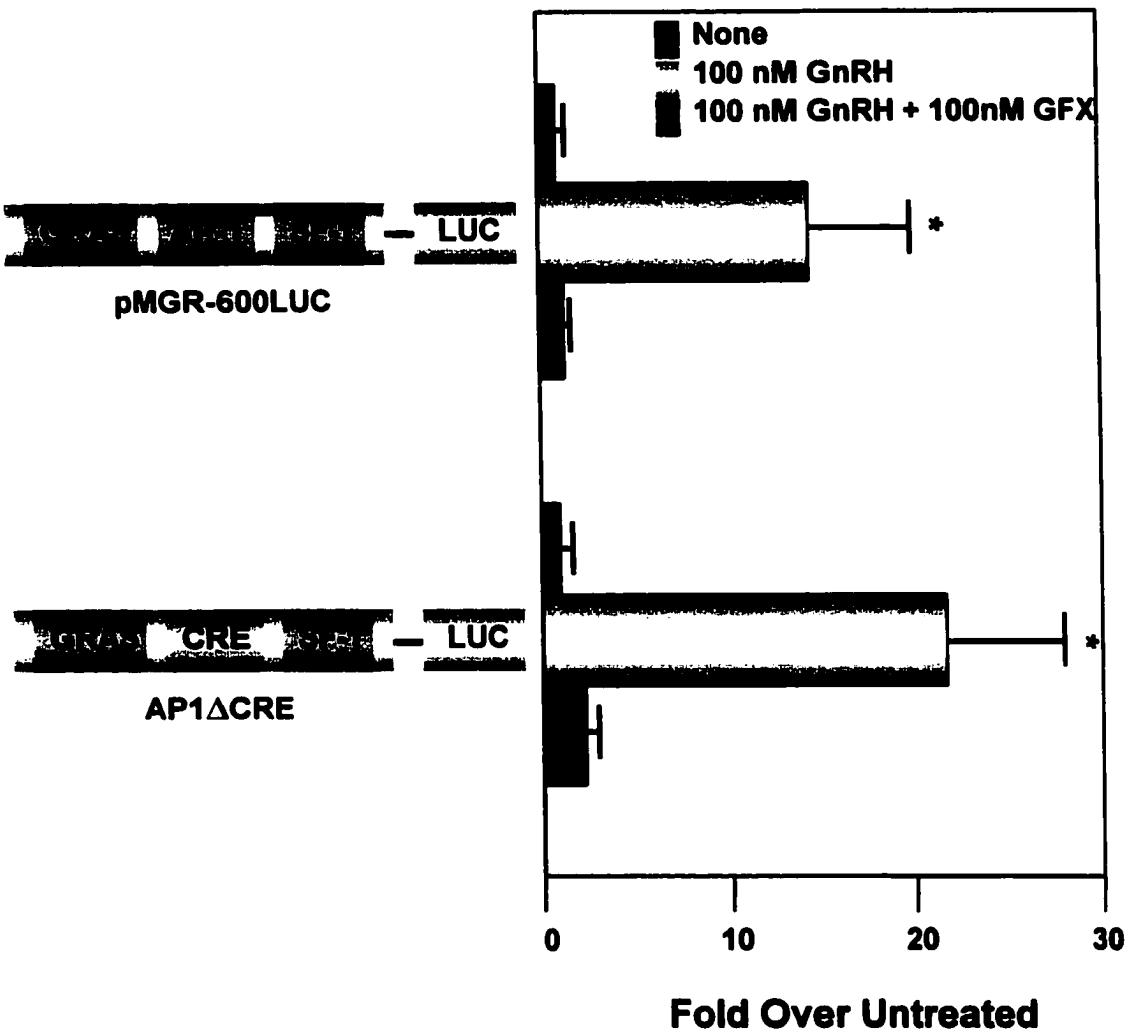
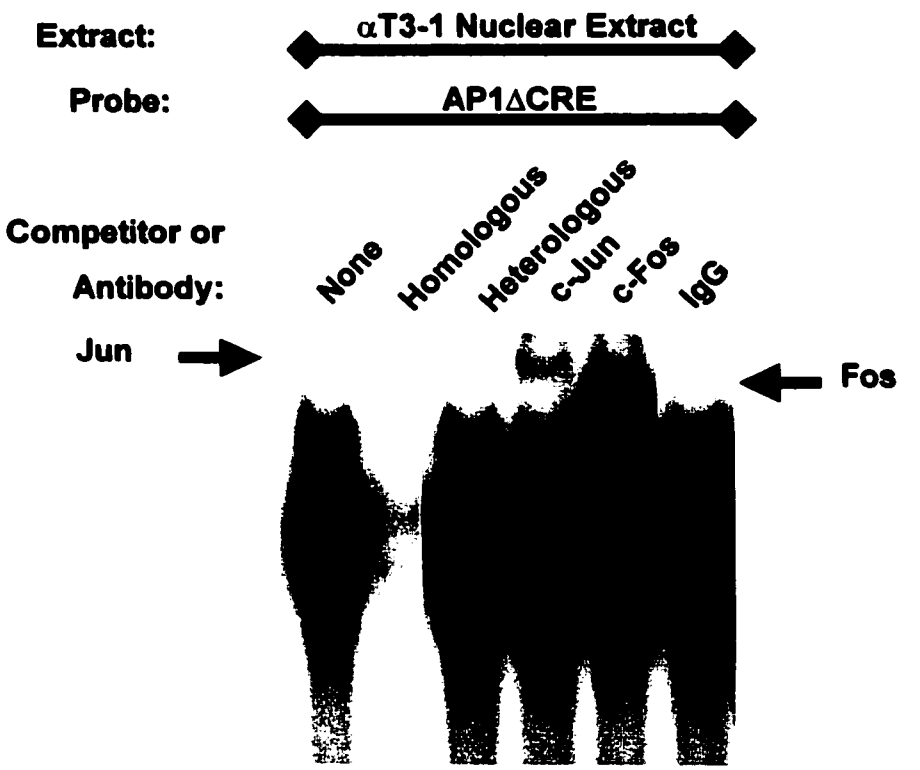


Figure 30. AP1ΔCRE retains GnRH responsiveness. Vectors containing the AP-1ΔCRE mutation or the wild type promoter were transiently transfected into αT3-1 cells, treated with 100 nM GnRH for 6 hrs, and assayed for luciferase activity. Where indicated, samples were pretreated with the specific PKC inhibitor Bisindolylmaleimide I (GFX) (100 nM) for 15 min before GnRH was added. Values represent mean ± SEM for triplicate samples. * Represents values different ($p<0.01$) from untreated for each vector.

CRE we performed a pull-down assay. A biotinylated oligonucleotide containing the AP1ΔCRE fragment was combined with nuclear extract from GnRH-treated cells. DNA/extract mixtures were combined with streptavidin linked to agarose beads and washed several times. Reactions were then analyzed by an immunoblot assay. Detection was performed with an antibody that recognizes all members of the Fos family. Fos binding to AP1ΔCRE was detected in extracts from cells treated with 100 nM GnRH for 1 h, but not in untreated extracts or extracts from cells treated with GnRH for 6 h (Figure 31B). This assay confirmed our findings that Fos binds to AP1ΔCRE upon GnRH stimulation. Thus, replacement of the AP-1 site with a CRE does not alter the ability of Jun and Fos to bind.

AP1ΔCRE is not negatively regulated by estradiol. Now that we had characterized the AP1ΔCRE mutation, we sought to determine the effect of estradiol on GnRH regulation of this construct. To determine whether the inhibition caused by estradiol was mediated at AP-1, we compared estradiol treatment of the wild-type promoter to AP1ΔCRE in transient transfections. Vectors were transfected into αT3-1 cells and treated with GnRH and estradiol. Intriguingly, estradiol treatment did not block GnRH responsiveness of AP1ΔCRE (Figure 32). Based on this finding, we suggest that estradiol inhibition of the GnRH response specifically requires an intact AP-1 binding site.

A



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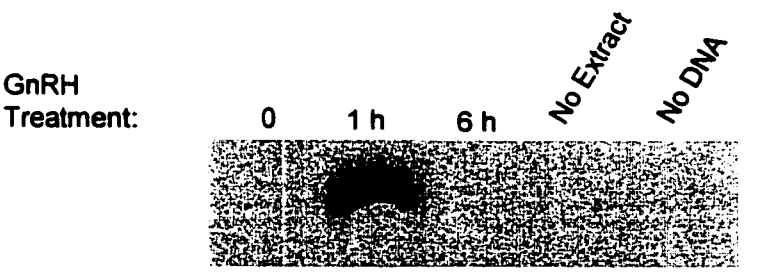

Figure 31. AP1ΔCRE Binds Jun and Fos. A) Nuclear extracts from αT3-1 cells were incubated with radiolabeled oligonucleotides containing the AP1ΔCRE mutation. A 500-fold molar excess of unlabeled homologous or heterologous oligonucleotides, or pan-Jun or pan-Fos antibodies were added where indicated. B) GnRH-treated (0, 1, 6 h) αT3-1 nuclear extracts were combined with a biotinylated oligonucleotide containing the AP1ΔCRE mutation. Western analysis using a general Fos antibody detected induction of Fos binding at 1 h of GnRH treatment.

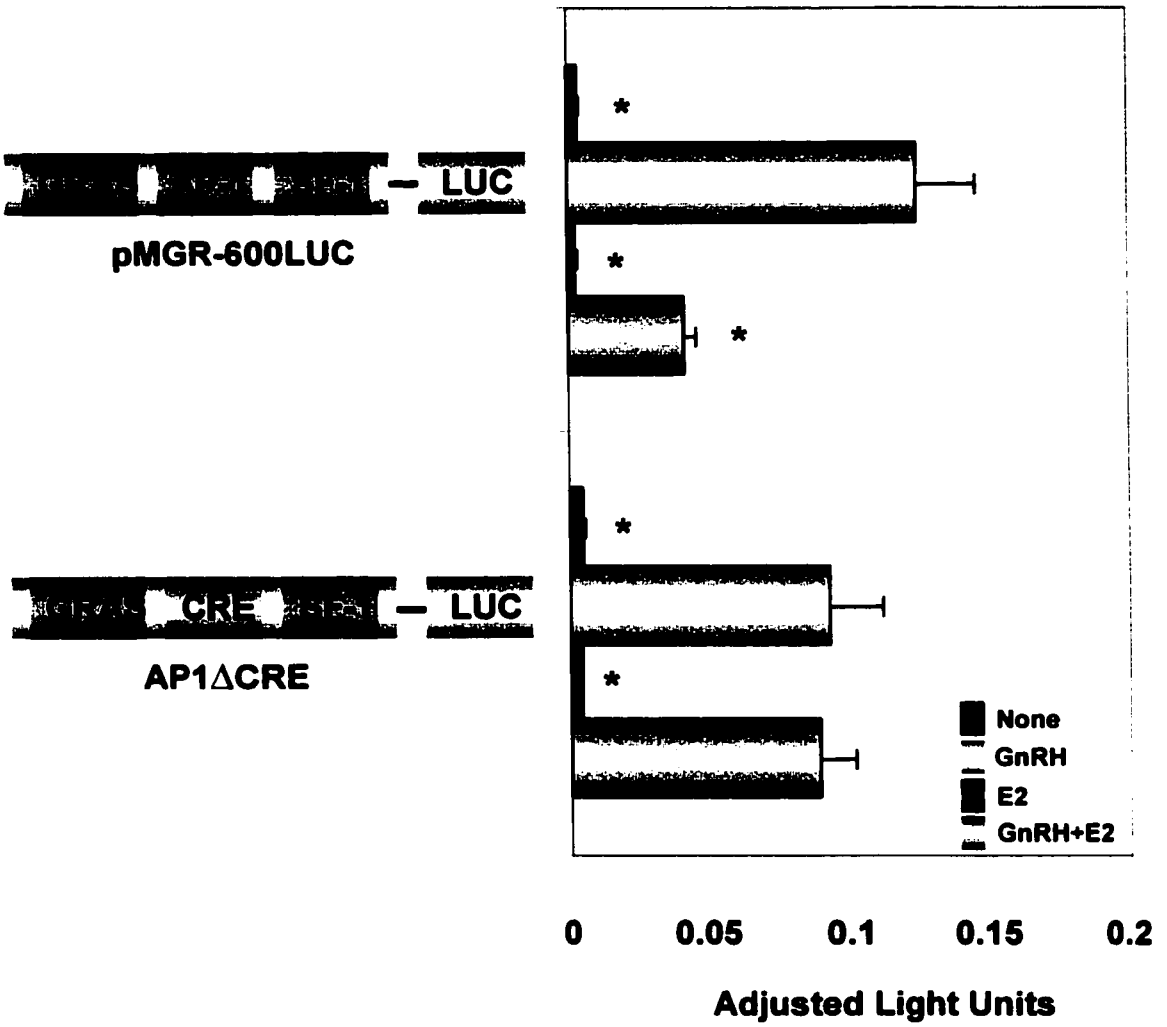

Figure 32. AP1ΔCRE is not negatively regulated by estradiol. αT3-1 cells were transiently transfected with either pMGR-600LUC or AP1ΔCRE. Cells were treated with 100 nM GnRH in the presence or the absence of 100 nM Estradiol-17β. Six hours after transfection, cells were harvested and assayed for luciferase activity.

DISCUSSION

We have found that estradiol blocks GnRH stimulation of GnRHR gene expression. This is consistent with previous findings that estradiol reduces GnRHR numbers in αT3-1 cells (47;48). In primary cultures of rat pituitary cells, chronic exposure to estradiol can increase GnRHR numbers while short-term exposure decreases GnRHR numbers (45-47). Thus, gonadotropes and αT3-1 cells both exhibit negative responses to estradiol, but gonadotropes also exhibit a positive response to estradiol that is not seen in αT3-1 cells. This discrepancy may be explained if the up-regulation of GnRHR numbers seen in primary cultures occurs indirectly, involving steroid hormone effects on cells other than gonadotropes (47).

Replacement of the AP-1 site with a CRE does not affect basal activity of the murine GnRHR promoter. The AP1ΔCRE construct gained the ability to respond to forskolin and to bind CREB and ATF-2. It is interesting to note that the murine GnRHR gene already contains one CRE and this CRE is not sufficient to confer forskolin responsiveness. In addition, the promoter retained GnRH responsiveness and, interestingly, retained the ability to bind Jun and Fos. This is in contrast with the human glycoprotein hormone α-subunit promoter in which the CRE could not be replaced by an AP-1 element without a loss of promoter activity (178). That functional activity of the AP-1 element can be replaced by a CRE suggests a certain level of plasticity in the murine GnRHR composite enhancer. It is also interesting to note that the gain of cAMP responsiveness is not associated with a loss of GnRH responsiveness and that the GnRH response

remains dependent on PKC. The fact that we could replace AP-1 with a CRE and retain hormonal responsiveness and basal expression, while others lost expression when testing the reverse (179) suggests that the functions of a CRE may be much broader than that of an AP-1 binding site. The requirements for GRAS and SF-1, however, appear to be much more rigid. We found that replacing either GRAS or SF-1 with a consensus AP-1 element reduced promoter activity to a level similar to null mutation of the element. GRAS contains homologies to an AP-1 half site, so the change in sequence was not dramatic, however, it was enough of a change to eliminate function of this promoter region.

Interestingly, although GnRH responsiveness was retained when the AP-1 binding site was replaced with a CRE, the negative effect exerted by estradiol was lost, suggesting that the AP-1 binding site is not specifically required for GnRH-responsiveness, but is specifically required to mediate the negative effect of estradiol. These data would also suggest that the negative effect of estradiol is mediated, at least in part, at the AP-1 binding site. Estradiol did not affect GnRH-induced activation of JNK. Thus, the target for estradiol inhibition must lie between JNK and AP-1. There is evidence to suggest that estradiol treatment of αT3-1 cells shifts the dose-response curve for GnRH-stimulated inositol phosphate (IP) accumulation rightward, increasing the EC50 for this GnRH effect by approximately 20-fold (48). Upon treatment with GnRH, phospholipase C breaks phosphatidyl inositol down into diacylglycerol (DAG) and inositol tri-phosphate (IP_3) (74;153). DAG activates PKC, which is important for ERK phosporylation while IP_3 results in release of intracellular Ca^{2+} stores resulting in

activation of the JNK pathway (74;153;155). Thus, McArdle's data showing that estradiol treatment inhibits GnRH stimulation of IP accumulation seems to contradict the fact that JNK activation is not affected by estradiol.

In summary, these data add to the emerging picture of genetic elements and biochemical pathways that underlie the intricate regulation of gene expression. The studies presented in this manuscript give us a glimpse of the different regulatory pathways and how they integrate, adding key elements to the increasingly complex but increasingly complete picture of the interactions that exist between GnRH and estradiol signaling pathways. We have shown that the negative effect of estradiol on GnRHR expression could not be recapitulated after replacement of the AP-1 element with a CRE. Thus, although GnRH stimulation of the murine GnRHR gene can be mediated via a CRE, negative regulation of this gene by estradiol specifically requires the presence of the AP-1 element.

CHAPTER SIX

CONCLUSIONS

GRAS was originally defined six years ago. Over the past several years, I have shown that GRAS represents an activin responsive element. Because the activin α-subunits, as well as the activin binding protein follistatin, are produced by gonadotropes, all of the elements are in place for autocrine/paracrine regulation of the GnRHR gene. GRAS represents the first activin response element characterized in gonadotropes.

The next issue I considered was the identity of the protein(s) that mediate activin signaling at GRAS. As signaling intermediates for activin, Smads were an obvious candidate. Consistent with this, I have found that Smad4 interacts at the 5' end of the element. Smad3 could not be detected binding in a gel shift when GRAS was used as radiolabeled probe. However, GRAS effectively displaces binding of Smad3 to a consensus Smad binding element. Furthermore, overexpression of Smad3 stimulates enhancer activity of GRAS suggesting that Smad3 may be involved in activation at GRAS. Although I have presented evidence to support a role for Smad3 at GRAS, I have not ruled out involvement of Smad2. It would be interesting to investigate whether Smad2 contributes to GRAS function. Perhaps the simplest approach to this question would be to determine whether Smad2 interacts at GRAS in a yeast one-hybrid assay.

Although unresponsive to GnRH alone, GRAS mediates a synergistic response to activin and GnRH together. Consistent with GnRH regulation at GRAS, I have found that Jun and Fos bind to the AP-1 half site located in the center of GRAS. The next issue is the signaling intermediates that are required for propagation of the signal from receptor to transcription factor. GnRH signaling to Jun/Fos components has been shown to occur via MAP kinases (52;75-77;155;156). Future experiments should investigate whether GnRH communicates to GRAS via MAP kinases and if they are, the identity of the specific signaling intermediates involved should be determined.

FoxL2, a member of the forkhead family of transcription factors binds to the 3' end of GRAS. Little is known about signaling to FoxL2. Research is needed to explore the molecular mechanisms involved in FoxL2 signaling. There is evidence to suggest that members of the forkhead family are phosphorylated upon hormone treatment which results in a change in their cellular distribution (180). Furthermore, the interaction of FAST-2 with Smad2 is enhanced by ligand treatment (146). Future studies should be directed toward understanding the effect of ligand treatment on Smad-FoxL2 interactions and investigating protein-protein interactions between FoxL2 and the individual Jun/Fos components binding at GRAS.

Finally, GRAS responds to activin and mediates a synergistic response to activin and GnRH. These different responses are likely a product of different transcription factors binding at GRAS. Based on the data I have presented in this

manuscript it seems likely that synergistic activation at GRAS requires AP-1 binding, while activin regulation does not. Further investigation of the identity of transcription factors binding at GRAS during each of these responses would deepen our understanding of how GRAS mediates these two different hormonal inputs.

These findings should be confirmed in transgenic mice. Mice are currently being generated that contain a luciferase reporter construct down stream of the murine GnRHR gene promoter that has a *Not*I site in place of GRAS (μGRAS). These mice should be studied to determine the effect of mutating GRAS *in vivo*. Using mice containing the wild-type promoter fused to luciferase (-1900wt), one could study follistatin/activin responsiveness of this promoter region. These mice could be injected with follistatin and their tissues assayed for luciferase expression. Besecke *et al.*, injected follistatin into rats to study the effects of activin on FSH production (31). The effects of follistatin could be compared in –1900wt and in μGRAS mice.

I have demonstrated that GRAS is capable of interacting with at least three classes of transcription factors. These families of transcription factors have not previously been shown to bind to a single element. Thus, GRAS represents a complex enhancer whose functional activity is dependent on a unique contour of transcription factors. It seems likely that this unique conformation of proteins are essential for recruitment of the appropriate cofactor(s) and thus stimulation of the enhancer activity of GRAS. Future studies should investigate the identity of these cofactors and how they interact at GRAS.

In addition to activin, the GnRHR gene is also stimulated by GnRH. GnRH responsiveness of the murine GnRHR gene is mediated at the AP-1 element (2;3). I have shown that this response is attenuated by treatment with estradiol. Replacing the AP-1 element with a CRE does not affect GnRH responsiveness but blocks the negative effect of estradiol, suggesting that the effect of estradiol has a strict sequence requirement for AP-1 while the GnRH response does not.

McArdle *et al.* (48), has suggested that estradiol treatment blocks GnRH-induced accumulation of inositol phosphates. However, one might expect this to block ERK or JNK, but this is in contrast to my results. Further work needs to be done to elucidate the point at which estradiol signaling cross-talks with GnRH signaling. My data suggest that AP-1 is not required for GnRH stimulation but is required for estradiol inhibition of the GnRHR gene. Thus, cross-talk between GnRH and estradiol may occur at the level of the DNA binding components. I found that the CRE bound not only Jun and Fos, but also CREB and ATF-2. It may be useful to determine whether GnRH treatment stimulates CREB and ATF-2. If GnRH signaling to a CRE is mediated via fundamentally different mechanisms than GnRH signaling to AP-1 then understanding these differences may highlight the point at which estradiol has its effect. Finally, these experiments should also be confirmed in transgenic mice: –1900wt mice would be treated with either GnRH alone or GnRH in combination with estradiol. Presumably, estradiol treatment would attenuate the GnRH response.

The binding of GnRH to receptors on the surface of gonadotropes is obligatory for synthesis and secretion of LH and FSH, and thus normal

reproductive function. The magnitude of gonadotropin secretion is dependent upon both the amount of GnRH secreted from the hypothalamus and the number of GnRH receptors, i.e. the sensitivity of the pituitary to a given dose of GnRH. Consequently, regulation of the GnRHR gene represents an important point for regulation of reproductive function. The studies presented in this dissertation further the understanding of how expression of the GnRHR gene is regulated.

CHAPTER SEVEN

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