

ABSTRACT

JIA, JINGJING. Analysis of Gonadotrope-Specific Expression of Ovine Follicle Stimulating Hormone Beta Subunit Using Adenoviral Expression Constructs in Purified Primary Murine Gonadotropes. (Under the direction of Dr. William L. Miller).

The beta subunit of follicle stimulating hormone (FSHB) is expressed specifically in pituitary gonadotropes in all vertebrates. Previous transgenic work has shown that -1866 bp of the ovine FSHB promoter (but not -750 bp) can direct gonadotrope-specific expression of luciferase (Luc) in the mouse. The studies presented here use purified primary gonadotropes (and non-gonadotropes) as a model for identifying sequences between -1866 bp and -750 bp of the ovine FSHB promoter that are necessary for gonadotrope targeted FSHB expression. Three ovine FSHBLuc constructs were prepared to analyze cell specific expression in purified primary adult mouse gonadotropes and pituitary non-gonadotropes since these cells normally express or repress expression of ovine FSHBLuc transgenes *in vivo*. Adenovirus was used to introduce the following constructs into the primary cells: -2871oFSHBLuc-V, -750oFSHBLuc-V or -232oFSHBLuc-V. All three were expressed in both gonadotropes and non-gonadotropes at essentially the same high levels, but induction by activin A (50 ng/ml) or inhibition by gonadotropin releasing hormone (GnRH:100nM) was observed only in gonadotropes. Therefore, hormone-specific regulation was observed, but gonadotrope-specific expression was not. Surprisingly, GnRH did not inhibit gonadotrope expression of -232oFSHBLuc-V, but it did inhibit expression of both -2871oFSHBLuc-V and -750oFSHBLuc-V. These data provide the first evidence for promoter-directed GnRH inhibition. In summary, the results suggest that sequences between -1866 bp and -750 bp of the ovine FSHB promoter are involved with chromatin remodeling that occurs during embryogenesis and can only be identified currently using transgenic techniques. In addition,

a negative GnRH response element was detected between -750 bp and -232 bp of the ovine FSHB promoter. GnRH has been studied extensively for its ability to secrete gonadotropins, but rarely for its ability to inhibit FSH production. GnRH is often used medically to shrink reproductive tumors (breast or prostate cancer) by inhibiting FSH which ultimately prevents the ovaries and testes from producing their steroid hormones (estrogens/testosterone) that stimulate tumor growth. For many reasons, the nature of FSHB inhibition by GnRH is important to understand.

Analysis of Gonadotrope-Specific Expression of Ovine Follicle Stimulating Hormone Beta Subunit Using Adenoviral Expression Constructs in Purified Primary Murine Gonadotropes

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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Biochemistry

Raleigh, North Carolina

2011

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DEDICATION

I dedicate this thesis to my mother and father for their support, trust and endless love. It is also dedicated to my grandmother for raising and enlightening me, and to my uncle for his inspiration. Also I want to thank my boyfriend for his support which is always present.

BIOGRAPHY

Jingjing Jia received her Bachelor of Science degree in July, 2005 in Biological Science from Fudan University. In August 2005, she joined the Biochemistry Department of North Carolina State University, and her Ph.D. thesis was under the supervision of Dr. William L. Miller. It was during the graduate school time that her interests in biology were fully realized and developed. Now in the fall of 2010, Jingjing is completing her graduate studies with a Ph.D. in Biochemistry and continuing her education at the postdoctoral research level.

ACKNOWLEDGMENTS

First, I would like to thank Dr. William L. Miller, my Ph.D. advisor. He has been so patient and knowledgeable and has guided me into cutting-edge research in molecular biochemistry. He supported me always even through the most difficult times. He has been a great guide and also a good friend.

Second, I would like to thank Dr. Jesse A. Gore, a former Ph.D. student in Dr. Miller's laboratory. Jesse helped me through the early years in Dr. Miller's lab, in terms of bench work techniques and writing skills in English. He was the most friendly and helpful team member I could have ever wished to work with.

I would like to thank Dr. Dennis T. Brown. Without his advice and financial support, I could not have finished my work. I will always remember with gratitude and respect his dedication and devotion to me and to the entire department of Molecular and Structural Biochemistry.

I also thank former laboratory members including Joyce Wu and Sang-oh Han. They offered me incredibly useful help during my early times in the laboratory.

My Ph. D. work could not have been completed by myself. It is only with the support and help from so many people that I was able to accomplish my work. I enjoyed my life at NCSU and appreciate all who took care of me and encouraged me. I had a great experience.

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CHAPTER 1

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CHAPTER 1

Literature Review

I. Introduction

I-A. FSH Structure

Follicle Stimulating Hormone (FSH) is a glycoprotein heterodimer composed of two subunits (α/β) similar to luteinizing hormone (LH), chorionic gonadotropin, and thyroid stimulating hormone (TSH). The FSH beta subunit (FSH β), like the other beta subunits, gives specificity to its hormone and also regulates overall hormone expression through induction or repression of its transcription [1].

I-B. FSH Function

FSH and LH are produced only in gonadotropes of the anterior pituitary in vertebrates. FSH acts on sertoli cells (testes) or granulosa cells (ovary) to regulate sperm and egg production, respectively [1]. Female mice null for FSH β do not develop eggs, much like women lacking FSH β receptors [2]. Male mice without FSH β are fertile, but have reduced sperm numbers and sperm motility [2].

I-C. FSH Hormone Regulation

FSH was discovered > 40 years ago and its role in reproduction is well known. Regulation of its production/secretion is depicted below (Figure 1). FSH production is primarily controlled by FSH β transcription which is reflected in constitutive secretion of FSH from gonadotropes. At least 6 hormones are involved either directly or indirectly in FSH β regulation: hypothalamic gonadotropin releasing hormone (GnRH); gonadal inhibin and steroids; pituitary-made activins, follistatin and, perhaps, bone morphogenetic proteins (BMPs). The critical autocrine/paracrine pituitary-made hormones are the most difficult to identify, quantify and study. Each hormone regulator in Figure 1 is discussed below.

I-D. FSH β expression in gonadotropes

There are two promoter sequences between -167/-50 bp required for gonadotrope expression of ovine FSH β in mice [3,4]. Between -1866/-750 bp there are more sequences required for gonadotrope expression (5; unpublished work in this laboratory by F. Shaffiee Kermani). Transformed gonadotropes (L β T2 cells) cannot be used to identify these sequences and transgenic studies are too expensive. A new in vitro model would be helpful for identifying these sequences.

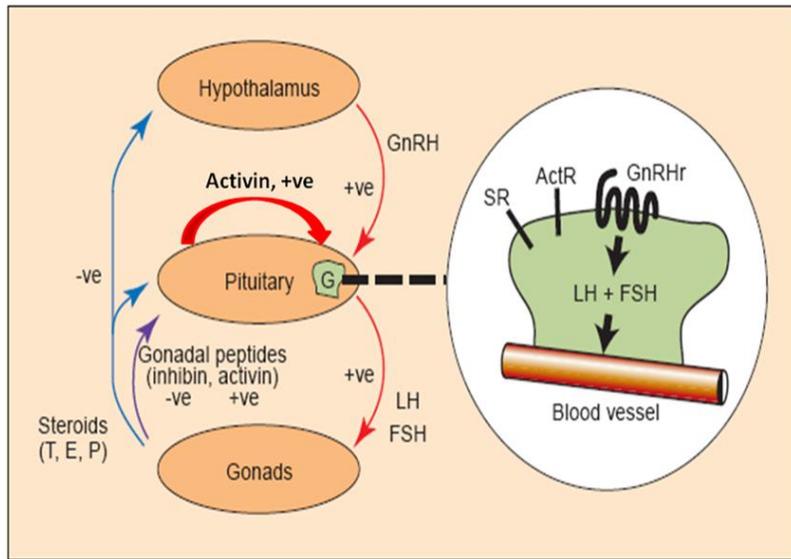


Figure 1: Diagram of positive and negative regulation of FSH and LH within the hypothalamic-pituitary-gonadal (HPG) axis. **SR** = Steroid receptors; **ActR** = Activin receptor; **GnRHr** = GnRH receptor; **T** = testosterone; **E** = estrogens; **P** = progesterone; **BMPs** = bone morphogenetic proteins; **folliclestimin** = binds and inactivates activin; +ve = induction; -ve = repression.

I-E. Models for studying FSH β regulation

L β T2 cells (transformed embryonic mouse gonadotropes) provide the best cell line for studying FSH β expression. These cells produce FSH β and respond to GnRH, activin, BMPs and inhibin. Like any transformed cell line, however, L β T2 cells exhibit some differences in their signaling pathways from primary gonadotropes [6] so they have been helpful, but are unpredictably unreliable for analyzing FSH β regulation. Originally, L β T2 cells held great

promise for identifying all promoter sequences required for gonadotrope expression of FSH β in vivo (40), but they do not seem to recognize sites between -1871/-750 bp as being important so they cannot be used to identify them.

Transgenic expression of ovine FSH β (promoter)-Luciferase constructs in mice has been very useful [4,5]. Such studies are the “gold standard” in our laboratory for showing the physiological relevance of a particular DNA element in the ovine FSH β promoter. Transgenic work is very time-consuming, however, and costly. Furthermore, transgenic studies cannot help dissect the autocrine/paracrine interactions in the pituitary regulating FSH β expression. Therefore, transgenic studies have limitations also.

Purified primary gonadotropes are now available in limited numbers for studying FSH β regulation [7]. Gonadotropes comprise only 4-6% of pituitary cells (~ 20,000-30,000/mouse), but they produce on a per cell basis about 1000x the FSH β made in L β T2 cells [3,5]. Furthermore, their signaling pathways are those used by gonadotropes in vivo.

I-F. *The purpose of this thesis*

The purpose of this thesis was to determine the usefulness of primary gonadotropes infected with adenoviral FSH β -promoter/reporter constructs for studying hormone regulation of FSH β transcription and identifying all promoter elements needed for gonadotrope expression of ovine FSH β .

II. Regulation of ovine FSH β transcription by GnRH

II-A. *GnRH INDUCTION of FSH β transcription (General)*

Mice without GnRH (GnRH null mice), have serum levels for FSH and LH that are ~13% and < 1%, respectively, compared to normal mice [8]. Many studies have tried to understand how GnRH regulates serum levels of FSH and LH. To complicate matters, GnRH is a secretagogue required for LH secretion while FSH is secreted primarily by a constitutive pathway [9]. Even more complication comes with the knowledge that LH and FSH are sometimes regulated differentially during the female reproductive cycle. Finally, GnRH

induces many genes that may affect general gonadotrope function and protein synthesis. Here we present data concerning GnRH induction of FSH β transcription.

GnRH is a decapeptide made in the hypothalamus and released by a biological “pulse generator” in 5 minute intervals once every hour, or more rapidly during ovulation in females. Short term treatment with GnRH (designed to mimic one pulse) increased FSH β mRNA by 3-fold in castrate, testosterone-treated, GnRH-deficient male rats [10]. Two-fold induction of FSH β mRNA was obtained in another experimental model: primary rat pituitary cultures perfused with hourly 5 minute pulses of GnRH for 10 h [11, 12]. The generally accepted conclusion from in vitro work is that GnRH can induce FSH β transcription by 2- to 3-fold or maybe more based on the GnRH-null model.

II-A1. Signaling associated with GnRH induction of FSH β transcription

GnRH binds a G protein-coupled receptor that activates G_q/G₁₁ heterotrimeric G proteins [13]. Such activation causes phospholipase C- β (PLC β) to generate secondary messengers inositol 1,4,5-trisphosphate (IP₃), diacylglycerol (DAG) and calcium which collectively activate PKC [14,15,16,17] and downstream MAP Kinases including ERK, JNK and p38 [15,17]. Genomic and biological studies with L β T2 cells confirm this and show that PKC, ERK, JNK, p38, and c-Src are associated with GnRH induction of FSH β [14]. The proposed signaling network is shown in Figure 2 below.

Figure 2 indicates that ERK1/2 contributes more to FSH β expression than JNK or p38 MAPK. Data supporting this concept comes from transfection studies in L β T2 cells using FSH β -reporter constructs from sheep, mouse, rat and human showing that inhibition of the upstream kinase for ERK1/2 caused the greatest reduction in GnRH-stimulated FSH β promoter activity [18]. Data showing that GnRH activates ERK1/2 in vivo came from a study in which GnRH-deficient castrate, testosterone-treated male rats were treated with GnRH and ERK1/2 was activated [19]. Surprisingly, however, mice completely lacking ERK1/2 in gonadotropes still have normal serum FSH levels even though LH levels were inhibited [20]. This discrepancy may reflect compensation by other kinases or incorrect signaling in L β T2 cells.

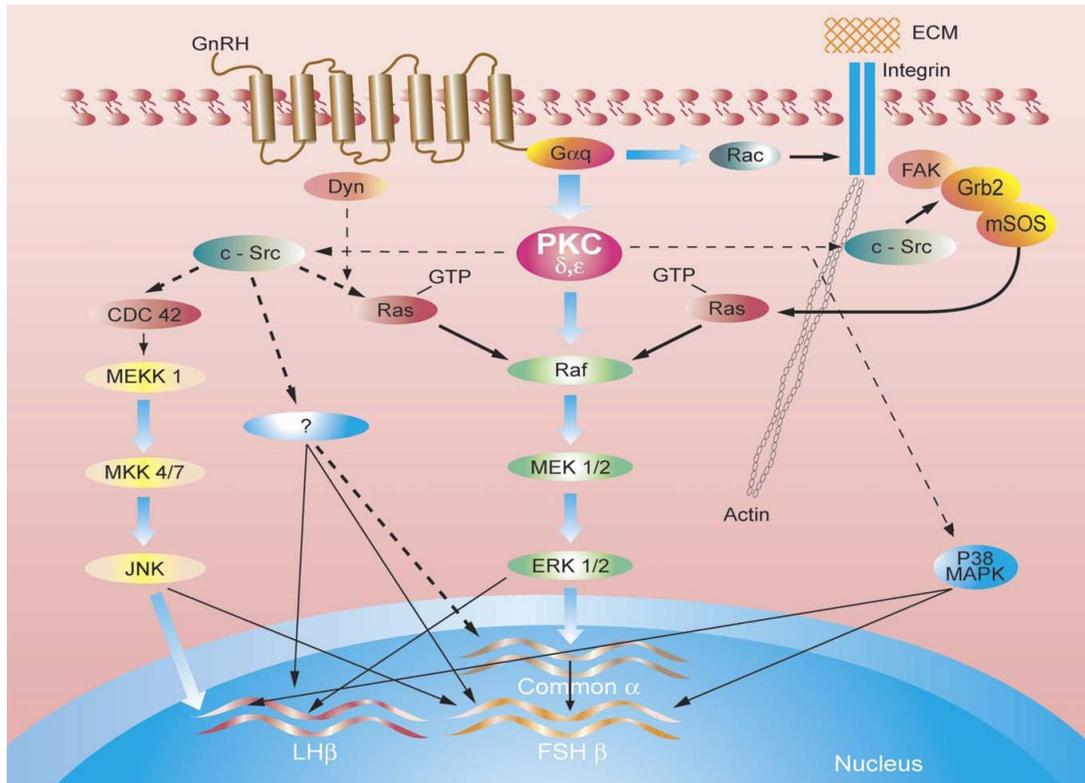


Figure 2: Schematic of GnRH-stimulated transcription regulation of FSH β , LH β and the common glycoprotein alpha subunit (GSU α) in L β T2 transformed embryonic gonadotropes [15].

II-A2. FSH β promoter sequences associated with GnRH induction

A second approach to identifying important signaling pathways is to identify elements on the FSH β promoter that are critical for GnRH induction of FSH β . Using the ovine FSH β promoter, two sites at -120 bp and -83 bp were found to bind activator protein-1 (AP-1) and both are necessary for GnRH induction in HeLa, JAR and COS cells [21,22]. AP-1 enhancer sites bind AP-1 family members, including c-fos and c-jun, which are kinase substrates of ERK, JNK and p38. Given the information in Figure 2 (above), it is reasonable to deduce that AP-1 sites are likely to drive GnRH stimulation. Studies with both human and murine FSH β promoters in L β T2 cells also identified binding sites for AP-1 family members in the same

regions found in the ovine FSH β promoter that can mediate GnRH induction of FSH β promoter/reporter constructs. Therefore, promoter analysis and signaling studies in L β T2 cells all support a similar AP-1 mechanism driving GnRH-mediated induction of FSH β transcription. Essentially all GnRH/FSH literature universally affirms this viewpoint, but it is all based on studies in L β T2 cells.

II-A3. Physiologic importance of GnRH induction of FSH β transcription

Strangely, transgenic mouse studies with ovine FSH β -promoter/luciferase constructs could not confirm the in vitro studies. In the transgenic studies, pituitary expression of wild type ovine FSH β promoter/luciferase was compared to expression of the AP-1 mutated construct. Both wild type and AP-1 mutated constructs were expressed equally well in vivo. Although variation between founders was large, a significant difference should have been observed if GnRH really played a major direct inductive role in FSH β transcription in vivo. The absence of an effect could mean that a significant portion of GnRH induction is mediated indirectly through pleiotropic effects on the gonadotrope and not specifically on FSH β transcription. It could also mean that the incorrect “AP-1 site” was targeted for mutation and that other sites exist although no others have been identified on the ovine FSH β promoter [5]. If the site is correct, it is strange that destruction of a site so tightly linked to GnRH induction of FSH β transcription in transformed cells (HeLa, JAR, COS, L β T2) would not decrease FSH β -driven transcription when placed into a physiological setting, if, in fact, GnRH directly and specifically induces FSH β transcription in vivo. It is noteworthy that a similar transgenic approach to analyzing an actin responsive site (-167 bp) on the same ovine FSH β promoter/Luciferase construct showed a > 99% decrease in FSH β expression in vivo when the mutated construct was tested [3].

II-B. GnRH INHIBITION of ovine FSH β transcription

Reproductive cancers of the breast and prostate are sometimes treated with supraphysiologic levels of GnRH. This pharmacologically castrates the patient which rapidly reduces estrogens and androgens that stimulate reproductive cancers. Pharmacological castration occurs because chronic GnRH down regulates gonadotropes which then reduce LH and FSH

production which, in turn, stops gonadal function. The scientific community generally assumes that GnRH simply down-regulates GnRH receptors and/or their associated signaling pathways in gonadotropes in vivo, but evidence from our laboratory suggests down regulation can be an active process also.

Our first evidence for GnRH-mediated inhibition of FSH β transcription came from in vitro studies with cultures of pituitaries from transgenic mice expressing FSH β promoter/Luciferase with mutated AP-1 sites. Addition of GnRH to these cultures rapidly (within 4 h) reduced luciferase expression > 50% [3,26]. The results indicated that GnRH actively inhibited FSH β transcription since no GnRH was present in pituitary cultures to “down regulate.” These experiments were followed by studies with pituitaries from transgenic mice that contained the wild type FSH β promoter/luciferase construct and inhibition was also observed, but at higher levels of GnRH associated with the supraphysiologic levels used for treating reproductive cancers.

II-C. Summary for GnRH regulation of FSH β transcription

Significant evidence indicates that GnRH can induce transcription of FSH β by 2- to 3-fold or more. GnRH action is complicated since it activates several interrelated signaling pathways and more than 70 genes in gonadotropes that may contribute directly or indirectly to FSH β expression. Destruction of two AP-1 sites in an ovine FSH β promoter/luciferase transgene had no observable effect on its pituitary expression, however. This suggests that GnRH does not induce FSH β transcription directly through a promoter sequence, but rather through indirect pleiotropic mechanisms. **Evidence from our laboratory shows that GnRH can actively inhibit FSH β transcription. Data in this thesis suggest that a specific ovine FSH β promoter sequence is responsible for this active negative regulation.**

III. Activin induction of ovine FSH β transcription

III-A. Activins, BMPs, Inhibins and Follistatins (General)

Inhibin, activin and bone morphogenetic proteins (BMPs) are members of the transforming growth factor beta (TGF β) superfamily. Activin is the most potent inducer of FSH β , but BMPs also induce FSH β in L β T2 cells at higher concentrations [24]. Inhibin is a TGF β member, but it inhibits FSH β transcription by binding a betaglycan that interferes with activin and BMP receptors. Follistatin is not a TGF β family member, but tightly binds activins and BMPs preventing them from activating their receptors. Therefore, activin and BMPs induce FSH β transcription while inhibin and follistatin inhibit it.

III-A1. TGF β hormone structures

There are 43 TGF β family members. One is a α -chain and all the others are β -chains. Activin is a β/β dimer, but there are two activin subunits (A & B). Therefore, there are three activins: AA, BB and AB. BMPs are β/β dimers and are thought to be homodimers, but may cross link to many other TGF β family members. Inhibin is an α/β dimer where the β -subunit comes from the activin subfamily [25]. Follistatin, as noted above, is not a TGF β family member. It is a single-chain glycoprotein with MW ranging from 31kDa or 49kDa due to alternate mRNA splicing and variable glycosylation [26].

III-A2. TGF β hormone functions

Activin B is considered the primary inducer of FSH β in vivo based on monoclonal antibody neutralization studies in rats and rat pituitary cultures at the Salk Institute. There is no question that Activin A and B are the most potent inducers of FSH β in L β T2 cells in pituitary culture and in vivo. It is interesting, however, that mice with a knockout for activin B produce more FSH β than wild type mice. Our laboratory has found that BMPs 6 and 7 also increase FSH β expression in mouse pituitary cultures and L β T2 cells, but at much higher concentrations (100x). Activin A is less expensive than activin B and is actually more potent than activin B in tissue culture. Since it binds the same receptor as activin B, activin A has been used exclusively for studies on FSH β transcription in this thesis.

III-A3. Tissue source of activins, BMPs, inhibin and follistatin

Activins, BMPs, inhibins and follistatin are all made in gonadotropes and, perhaps, other pituitary cell types as well. They are autocrine/paracrine factors. Inhibin is also an endocrine hormone made in the gonads and travels through the blood stream to inhibit FSH β transcription in the pituitary. Gonadal castration removes this source of FSH β inhibition and FSH levels increase.

III-B. Activin signaling pathways (General)

Activin A activates two basic signaling pathways by binding its type II and type I receptors. The classic pathway is smad-dependent and the other works through TAK1 (smad-independent) (see Figure 3 below). Both pathways can act together or independently. A schematic is shown below depicting smad-dependent and smad-independent pathways (TAK1).

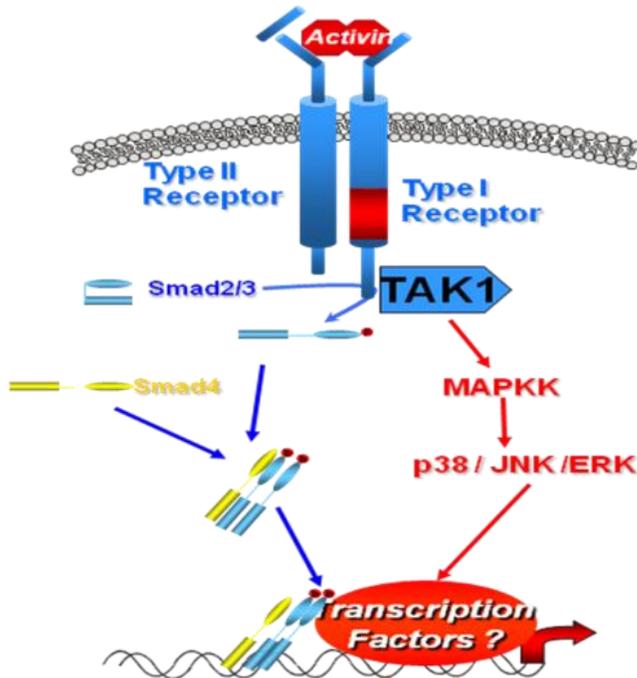


Figure 3: Activin pathways that may induce FSH β transcription. The classic activin pathway involves recruitment and phosphorylation of Smad2/3 by the autophosphorylated type I receptor. Then Smad2/3 complexes with Smad4 (co-smad) and moves to the nucleus where it binds to the FSH β promoter along with a partner transcription factor. An second pathway involves activation of “TGF β activated kinase 1” (TAK1) which activates downstream kinases and transcription factors that act in conjunction with or separately from smads.

III-B1. Smads

There are 8 homologous smads in mammals [55]. These fall into 3 categories. Smads 1, 2, 3, 5 and 8 are receptor-activated Smads (R-Smads), among which Smads 1, 5 and 8 are phosphorylated by the BMP type I receptor, while Smads 2 and 3 are activated by the activin type I receptor. Smads 6 and 7 are inhibitory Smads (I-Smads) and act as pseudo-substrates for type I receptors. R-Smads and Smad4 are widely expressed in almost all cell types, while I-Smads are transcriptionally regulated by TGF β pathway [56]. Smad complexes do not have a high binding affinity for their consensus DNA binding sequences [27], so they usually require specific partner proteins to stabilize DNA binding. This combination also confers cell-specific effects.

III-B2. Smad partner proteins

The short 4 bp smad binding site (AGAC) occurs frequently in the genome, but needs to be paired with an adjacent sequence that binds a partner protein to stabilize the complex [27]. This complex provides specificity and allows activin to induce and repress over 100 genes in gonadotropes [28]. Common partner proteins are FAST-1, HDACs and RUNX family members [29].

III-B3. Smad-Independent pathways

It has been reported TGF- β can activate MAPK pathways (Figures 2 & 3), including ERK, JNK, and p38 (MAPKK) [30]. It has been shown in L β T2 cells and sheep pituitary cultures, that both p38 and ERK1/2 can be rapidly activated by activin [31]. Smad independent JUN stimulation by TGF- β has been reported and both Smad and MAPK pathways participate in TGF- β induced epithelial-to-mesenchymal transdifferentiation [30]. The MAPK kinase kinase (MAPKKK) linking the TGF- β receptor and many MAPKKs was discovered to be “TGF- β Activating Kinase-1” (TAK1) and there is evidence that TAK1 binding proteins (TAB1, TAB2, TAB3) all play a role in TGF β initiated MAPK pathway activation.

Studies in our laboratory used a TAK1 inhibitor and smad dominant negative constructs to show that TAK1 plays a major role in ovine FSH β Transcription. Furthermore, time-course data suggested that a transcription factor was likely to be activated to partner with smads3/4 to produce the increase in FSH β transcription.

III-C. Activin induction of ovine FSH β transcription - L β T2 and transgenic studies

Data from our laboratory using L β T2 cells indicate that ovine FSH β promoter sequences from -169/-159 bp are required for activin induction of FSH β . There is a putative smad binding element from -162/159 bp and an adjacent putative RUNX1 binding site from -172/-163 bp. RUNX1 is known to interact with smads.

Transgenic mice were produced carrying the ovine FSH β promoter/luciferase construct with 4 mutated nucleotide pairs from -168/-165 bp. Expression of this construct was decreased by >99.9% in 8 of 10 founder lines. The two remaining lines showed a decrease of 99%.

III-D. Summary for activin regulation of FSH β transcription

Activin is the most potent inducer of FSH β transcription currently known. Data from our laboratory suggest that smad3/4 and a TAK1-mediated pathway are needed for inducing transcription of ovine FSH β transcription. Data from L β T2 cells and transgenic mice pinpoint a site from -172/-163 that is needed for activin induction and > 99% of FSH β expression in vivo. This site contains a putative smad binding element with adjacent putative binding site for RUNX1. A recent report using the porcine FSH β promoter suggests that FoxL2 is likely to be the natural smad binding partner at this site. **We conclude that this putative smad/smad partner binding region (-172/-159 bp) is essential for FSH β transcription. It is one of several sites required for significant expression of FSH β in vivo.**

IV. Steroid hormone regulation of ovine FSH β transcription

IV-A. Estrogens, progestins, testosterone (General)

Many studies have been devoted to the nature and importance of FSH β regulation by gonadal steroids in rodents, sheep and humans. The first indications that gonadal steroids altered

FSH β expression were observed in gonadectomized animals. After gonadectomy FSH β mRNA was elevated and treating castrated rats with estradiol or testosterone reduced FSH β expression, suggesting a physiological inhibitory role for steroid hormones [32-34]. By contrast, ovarian estrogens can increase secretion of LH to trigger the LH surge that causes ovulation. This suggests a stimulatory role for, at least, estrogens. There are significant species differences that occur at the hypothalamus and the pituitary. This section will focus only on steroid hormone effects that alter ovine FSH β transcription directly at the pituitary level.

IV-A1. Inhibition of FSH β transcription by estradiol and progesterone

Reports of steroid hormone regulation of ovine FSH β expression come exclusively from our laboratory. Both estradiol and progesterone inhibit FSH β transcription in primary ovine pituitary cultures. Inhibition begins within 2 h and reaches 68% and 58% after 24 h with estradiol or progesterone, respectively [35,36]. Inhibition is completely reversible and transcription run-on experiments showed that inhibition was directly at the level of transcriptional [36]. The ED50s for both hormones are within their physiological ranges suggesting that this steroid regulation is physiologically relevant [35,36].

IV-A2. Inhibition by progesterone

The progesterone receptor has 6 authentic binding sites in the ovine FSH β promoter and two surround the activin responsive element between -172/-158 bp [37]. One spans sequences -153/-139 bp and the other includes sequences from -212/-197 bp. Since inhibition by steroid hormones generally comes from interference with inducer transcription factors, it is possible that these binding sites cause the progesterone receptor to interfere with activin-mediated smad/smad partner binding to inhibit FSH β transcription.

IV-A3. Inhibition by estradiol

Estrogen receptors have no binding sites on the FSH β promoter (-1 to -4741 bp). Steroid hormones can still inhibit transcription when no binding sites exist because their receptors can interfere with inducers and the estrogen receptor can bind smad3 [38]. It was surprisingly

to us that when 4.7 kb of the ovine FSH β promoter/luciferase gene was expressed in mice as a transgene, inhibition by estradiol disappeared. That is, estradiol treatment of pituitary cultures from the transgenic mice caused no inhibition of luciferase expression. It is possible that coactivators or corepressors are different in sheep versus mouse gonadotropes which might explain this species difference. Alternatively, there may be upstream FSH β promoter sequences responsible for estrogen inhibition of ovine FSH β expression.

IV-A4. Summary for steroid hormone regulation of FSH β

Steroid hormones can regulate FSH β transcription either indirectly by altering GnRH expression/secretion or directly by regulating FSH β transcription. There are significant species differences in the roles gonadal steroids play in FSH β regulation, but it is clear that physiological levels of estrogens and progesterone can directly inhibit FSH β in ovine gonadotropes. Inhibition presumably involves interference with smad/smad partner binding to the FSH β promoter or by sterically blocking smad/smad partner interactions with coactivators.

V. Gonadotrope specific expression of the ovine FSH β gene

V-A. Gonadotrope specific expression of FSH β (General)

FSH β is expressed only in gonadotropes of the anterior pituitary in vivo [39]. This specificity depends on just 4741 bp of ovine FSH β promoter which can target luciferase expression >1000x to gonadotropes in transgenic mice than any other cell type tested (heart, liver, kidney, pancreas, lung, testis, ovary, brain) (10). We currently know that a Pitx1 site at -60 bp of the ovine FSH β promoter is required for basal expression. Without it, expression of ovine FSH β -Luciferase is decreased by 99% in transgenic mice. An even more important site resides at -172 bp which involves activin-activated Smad/partner binding transcription factors. Without this site, > 99% of ovine FSH β -luciferase expression is lost. Others have studied other proximal promoter sequences that seem important (see SF-1, NP-Y, LHX3 below), but none of these have been tested for physiological significance in vivo. Somewhere

between -1800 bp and -750 bp there are elements that are also needed for ovine FSH β -Luciferase expression in transgenic mice.

Surprisingly, a number of transformed cell lines all express ovine FSH β -luciferase equally well whether they contain sequences between -1800/-750 bp or not. In vitro studies using L β T2, α T3-1, AtT20, GT1-7 CV1 and NIH3T3 cells have not been able to identify the important regulatory sites that occur between -750 bp and -1800 bp. Expression is only 3-fold different between L β T2 cells (transformed gonadotropes) and NIH3T3 cells (human fibroblasts) [40]. It is clear that the specificity seen in vivo is not observed in transformed cell lines. **As noted above, a major goal of this thesis work was to determine if adult primary gonadotropes have the ability to differentially express oFSH β compared to other primary cells or if cell targeting occurs before cells are fully mature, perhaps, during embryonic development.**

V-B. Transcription factors associated with differentiation of pituitary cells during embryogenesis

Figure 4 below shows the expression pattern of transcription factors known to be important for the development of most anterior pituitary cells. The anterior pituitary originally develops from stomodeum and its epithelial derivative, Rathke's pouch. The first identification of Rathke's pouch in mice is at e8.5 when pituitary differentiation begins [41]. The mature anterior pituitary contains multiple cell lineages including corticotropes, thyrotropes, somatotropes, lactotropes and gonadotropes. These cells are very different and are regulated by different transcription factors/activators expressed at certain stages between embryonic days 10.5-15 (e10.5-e15). These transcriptional factors not only play roles in the development of cell-lineages, but also contribute to cell-specific gene transcription by synergy with other transcription factors including cell-lineage restricted factors and signaling pathway members [41-43]. Several genes associated with certain cell types have been investigated with regard to their cell specific expression and they have been useful as models for showing how transcriptional factors can synergistically binding certain locations on gene promoters to promote cell-specific expression (see Figure 4 below).

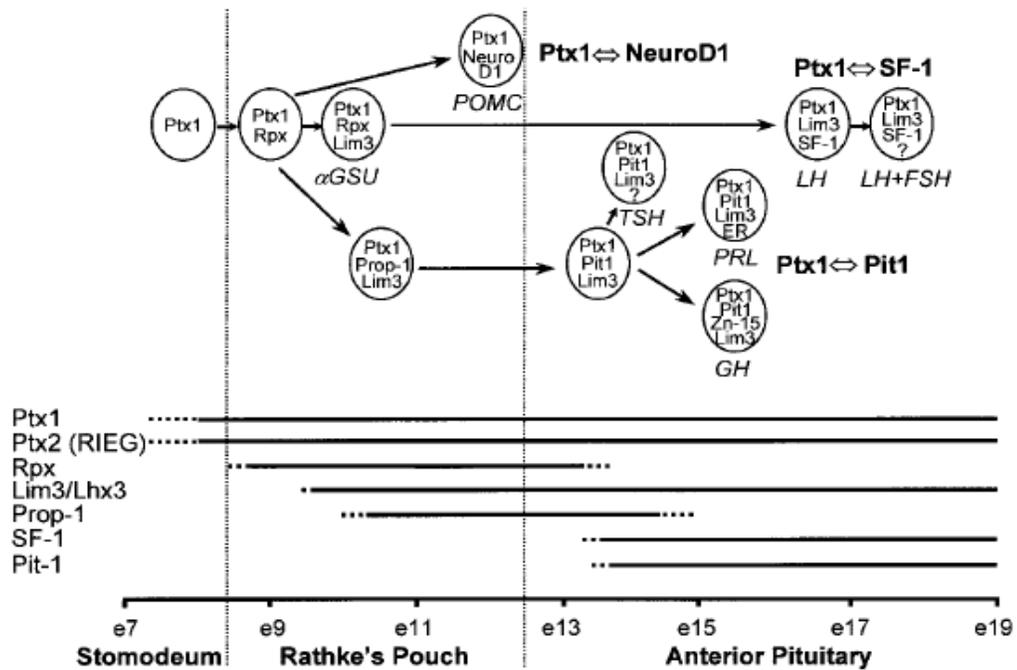


Figure 4: Regulatory factors during pituitary development. **Top**, representation of putative cellular intermediates during pituitary differentiation indicating transcription factors expressed at each stage. For terminally differentiated cells, factors shown previously or in the present work to activate transcription synergistically with Ptx1 are shown in bold. **Bottom**, Timing of onset and extinction for pituitary transcription factors [41].

For convenience of reference, the following figure shows the positions of many of the above transcription factor binding sites in ovine, porcine, human and murine FSH β promoters.

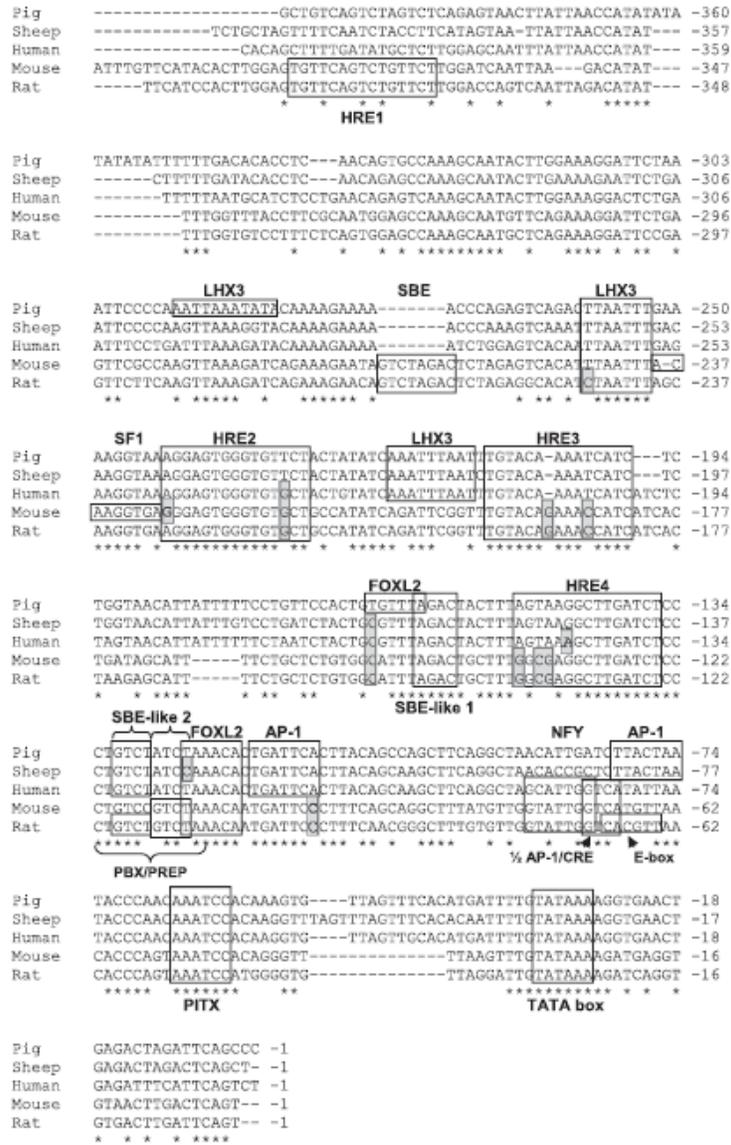


Figure 5: Alignment of proximal *Fshb/FSHB* promoters in pig, sheep, human, mouse, and rat. The proximal 400 bp from the 5' flanking regions of the *Fshb/FSHB* genes in the indicated species were aligned using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Defined cis-elements (as described in the text) are labeled and boxed (with the exception of the PBX/PREP site in sheep, which is marked with a bracket to avoid cluttering the figure). Some nucleotides are boxed in gray to reflect differences from the experimentally defined cis-elements in other species. Nucleotides are numbered at the right relative to the start of transcription. Nucleotides conserved across species are marked with an asterisk (*) and gaps (-) have been introduced to facilitate the alignment.

V-B1. *Pitx1/2 (pituitary homeobox 1/2) is required for expression of ovine FSH β*

Two laboratories have identified a highly conserved Pitx1 site in the proximal promoter of FSH β promoters (-53 bp for the rat; -60 bp for the sheep). It is important for expression in L β T2 cells. The first laboratory worked with rat FSH β constructs and determined this Pitx1/2 site to be important for basal expression as well as GnRH-regulated expression [44]. The second laboratory identified the -60 bp site as being necessary for basal expression and activin-induced expression [45]. Our laboratory followed up on these studies to show that the Pitx1 site at -60 bp in the ovine FSH β promoter is required for 99% of FSH β expression in gonadotropes in transgenic mice (Sang-oh Han and Jingjing Jia in our laboratory, manuscript ready for submission).

Pitx1 and Pitx2 are members of the *bicoid* subfamily of homeodomain (HD) proteins. They are pan-pituitary regulators of transcription and emerge as early as the stomodeum ~e7.5. Pitx1 is the predominate Pitx species in gonadotropes; Pitx2 is minor, but is expressed like Pitx1. Other research groups have used in vitro cell culture studies to show that Pitx1/2 can activate most, if not all pituitary hormone gene promoters including GSU α , LH β , FSH β , TSH β , GH, PRL and POMC. As expected, Pitx binding sites can be found on the promoters of all these genes. A striking characteristic of Pitx proteins is that they partner with cell/pathway restricted factors to direct cell specific gene expression. An interaction between Pitx1 and SF1 (Steroidogenic Factor 1) on the LH β promoter enhances LH β transcription. SF1 is uniquely expressed in gonadotropes although this synergy was not observed on the promoters of either GSU α or FSH β . [41,42,46].

In summary, Pitx1/2 is necessary for 99% of FSH β expression, but there is no evidence to indicate it has anything to do with gonadotrope specific expression since it also appears in almost all pituitary cell types. It is present in fully mature, adult gonadotropes.

V-B2. *Steroidogenic Factor 1 (SF1)*

SF-1 is an orphan nuclear receptor specifically expressed in murine gonadotropes from e13.5. It is expressed in non-pituitary organs also, including adrenals and gonads [47]. SF-1 binding sites have been identified on many gonadotrope gene promoters, such as GSU α , LH β , FSH β

and GnRH receptor. SF-1 null mice are infertile with decreased levels of LH β , FSH β , GSU α and GnRH indicating its contribution to gonadotrope function. However, SF-1 itself is not enough to confer gonadotrope specific expression to a gene since it appears outside the pituitary.

SF-1 synergy with Pitx1 is important for LH β expression in L β T2 cells, but not for FSH β expression [84]. The SF-1 transcription factor might interact with other factors bound to the murine FSH β promoter, however. SF-1 sites are located at -341 and -239 and mutations of both sites plus a NF-Y (nuclear factor Y) binding site at -76 caused a 50% decrease in FSH β promoter activity in L β T2 cells [48]. NF-Y is an important basal regulatory transcription factor for many genes and it can interact with several key components of the transcription machinery such as TATA binding protein (TBP) and CREB binding protein (CBP). In vitro data suggest that NF-Y and SF-1 cooperate to create gonadotrope specific expression of FSH β , but the binding sites for NF-Y and SF-1 are not conserved across species, so this hypothesis is still waiting for *in vivo* data support.

In summary, SF-1 may cooperate with other transcription factors such as Pitx1 or NF-Y to help create gonadotrope specific expression. This seems true for LH β expression, but has not yet been proven for FSH β transcription. SF-1 is present in fully mature adult gonadotropes.

V-B3. LIM-Homeodomain transcription factor 3 (LHX3)

LHX3 is expressed in Rathke's pouch, the progenitor of the anterior pituitary, as early as e9.5 and its expression is sustained throughout pituitary development [39]. It is essential for the differentiation and proliferation of several pituitary cell lineages except the corticotropes [57]. LHX3 null mice showed arrested pituitary development at the stage of Rathke's pouch [39]. LHX3 can bind to the pituitary glycoprotein basal element (PGBE) on the promoter of GSU α , which is required for specific thyrotrope expression of GSU α [49]. It can stimulate human FSH β expression in non-gonadotrope cell lines, such as HEK293 cells, but there is no solid data showing LHX3 has a role in vivo. LHX3 can also induce both human and porcine FSH β genes in L β T2 cells, but not the LH β gene which is also gonadotrope specific [50].

There are six LHX3 binding sites on the porcine FSH β promoter. Three are proximal, two are between -750 bp and -1800 bp and one is distal at -5039 bp. Mutation of all three proximal sites decreases expression of porcine FSH β by 90% in L β T2 cells. Deletions of the distal site at -838 bp also decrease expression significantly, but the more distal sites seem to have little inductive effects [51].

In summary, LHX3 transcription factors can bind porcine, ovine, human and murine FSH β promoters. Two of the proximal sites are conserved in pigs, sheep and humans (Fig 5). One distal site between -750 and -1800 bp of the porcine FSH β promoter (-838 bp) seems important for inducing porcine FSH β . LHX3 is present in fully mature adult gonadotropes.

V-B4. Pit-1 homeodomain factor (Prop-1)

Prop-1 is expressed in the embryonic murine pituitary as early as e10, but is turned off after e15, which is before the expression of all the pituitary hormone genes [39]. It is reported to be present in adult pituitary cells in the rat, however [52]. Prop-1 can interact with Rpx/Hesx1, which is also expressed in the early pituitary (see Fig 4), to activate Pit-1 which results in the terminal differentiation of the Pit-1 lineages including thyrotropes, somatotropes and lactotropes [53]. In Ames df mice with a mutated Prop-1 gene, hypogonadism was observed indicating that Prop-1 is important for gonadotrope function [54]. Prop-1 was discovered to bind on the porcine FSH β promoter between -750 and -850 [52], where there also appears a binding site for LHX3. No evidence shows that Prop-1 and LHX3 synergize together to direct porcine FSH β expression. Scanning of the ovine FSH β promoter in the -750/-850 region shows no Prop 1 site, so it is likely that the ovine, porcine and human FSH β genes are regulated differently. The human FSH β gene is expressed specifically in transgenic mouse gonadotropes due to a sequence in the 3' portion of the gene which has no obvious homology to either the porcine or ovine promoter elements.

In summary, three of four protein factors mentioned above belong to the homeobox domain superfamily. It is obvious that these factors are critical for pituitary development and differentiation. They prefer to function in a fashion of synergy with a partner protein factor, however, and bind to certain locations on the gene promoter. More background knowledge is

needed to understand how these molecules actually create cell-specific expression of the many pituitary hormones, including FSH β .

VI. Adenovirus mediated expression of genes in primary cells

VI-A. Why use viral expression vectors?

Germline transgenesis can generate useful and physiologically relevant animal models, but it is costly and time-consuming. A less costly, more rapid way to add genes to adult animals involves genetic engineering vectors such as viruses. Viral delivery is evolving rapidly because of its high efficiency compared with non-viral methods. Viruses can achieve a high rate of infection *in vivo* and sustain it over many months. Viral vectors have been developed from adenoviruses, retroviruses, adeno-associated viruses (AAV), herpes simplex viruses (HSV) and alphavirus, each of them has their advantages and disadvantages.

VI-B. Adenoviruses versus lentiviruses

Adenoviruses and lentiviruses are the two major viral types commercially available to express exogenous genes in transformed cell lines and primary cells. Lentiviruses are used to generate stabilized cell lines which can constitutively express the delivered foreign gene, since lentiviruses can integrate into the host cell genome. However, lentiviruses can only infect cells undergoing mitosis, thus they are not appropriate for infecting most primary cells *in vitro* since these cells are usually post-mitotic. Adenoviruses have many advantages over other viral types, since they are not cell-cycle dependent and the gene transfer efficiency is high [55].

VI-C. Adenoviruses

The current research on gene transfer mainly utilizes the human type-5 adenovirus. The virion particles have 12 fiber proteins from each apical penton base. These facilitate the entry of virion particles into endosomal vesicles. The adenoviral genome is a linear double stranded DNA of 30-34kb in length with inverted terminal repeats (ITR) at both ends. The genome can encode 5 early genes (E1A, E1B, E2, E3 and E4) which are expressed before

viral DNA replication. E1 is the element that is responsible for viral genome replication [56]. To generate a safe tool for delivering DNA, E1 is always deleted. Thus, the viral particles can only propagate in a complementing cell line that contains E1 in its genome, such as the HEK293 cell line. In the system I used for this study (from Clontec), both E1 and E3 elements are deleted which blocks viral replication and also allows the virus to carry up to 8kb of foreign DNA.

VI-D. Examples of Adenoviral use in somatic cells

Adenoviruses have been used for selectively destroying animal tumors. Specific tumor gene inhibitors or toxic RNAs can be delivered exclusively into tumors under a tumor specific promoter by adenoviruses. The major problem is that viruses can initiate a strong immune response which can lead to huge losses of virion particles from the infected cells resulting in rapid degradation of the toxic effect. Manipulation of the viral genome is still needed to overcome this problem of virus rejection [55].

Adenoviruses can also be employed for somatic gene transfer and expression within the physiological context of the organism. By injecting an adenovirus that can express a eukaryotic gene into an animal model, the gene can be expressed in vivo. This method can be used to broadly study signal transduction and gene function instead of using transgenic animals.

A recent report showed that adenoviral vectors can be used to probe promoter activity in primary immune cells. Viral transfer of a NF-AT (nuclear factor of activated T cells) promoter into primary T cells showed induction in this promoter, and even further they studied an inducible IL-12/23 p40 promoter in primary dendritic cells and macrophages by adenoviral delivery [57]. This study opened a window for using adenoviruses to study gene specific expression and regulation. Since transformed cell lines are not always representative of primary cells in terms of over-expressed or inhibited genes, it is really helpful to be able to manipulate primary cells. Adenoviruses provide just such a tool.

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CHAPTER 2

Analysis of Gonadotrope-Specific Expression of Ovine Follicle Stimulating Hormone Beta Subunit Using Transgenics and Adenoviral Expression Constructs in Purified Primary Murine Gonadotropes

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I. Abstract

The beta subunit of follicle stimulating hormone (FSHB) is expressed specifically in pituitary gonadotropes in all vertebrates. Previous transgenic work showed that -4741 bp of the ovine FSHB promoter (but not -750 bp) can direct gonadotrope-specific expression of luciferase (Luc) in the mouse. The studies presented here extend that work, first by testing two ovine FSHBLuc transgenes with shorter promoters that lack upstream ovine sequences (5' of -2361 bp or -1866 bp). The omitted upstream sequences contain regions of high homology conserved in ovine, porcine and human FSHB promoters which made them potential candidates for controlling gonadotrope-specific expression. Their deletion, however, did not alter expression. Second, three ovine FSHBLuc constructs were made to analyze cell specific expression in purified primary adult mouse gonadotropes and pituitary non-gonadotropes since these cells normally express or repress expression of ovine FSHBLuc transgenes in vivo. Adenovirus was used to introduce the following constructs into the primary cells: -2871oFSHBLuc-V, -750oFSHBLuc-V or -232oFSHBLuc-V. All three were expressed in both gonadotropes and non-gonadotropes at fairly equivalent and high levels, but induction by activin A (50 ng/ml) or inhibition by GnRH (100 nM) was observed only in gonadotropes. Therefore, hormone-specific regulation was observed, but gonadotrope-specific expression was not. Surprisingly, GnRH did not inhibit gonadotrope expression of -232oFSHBLuc-V, but it did inhibit expression of both -2871oFSHBLuc-V and -750oFSHBLuc-V. This provides the first evidence for promoter-directed GnRH inhibition. In summary, the results indicate there are sequences between -1866 bp and -750 bp of the ovine FSHB promoter that are necessary for gonadotrope-specific expression of ovine FSHB. These sequences are likely to function during embryonic development since neither adult gonadotropes nor non-gonadotropes could use the promoter information to create gonadotrope-specific expression. In addition, a negative GnRH response element was detected between -750 bp and -232 bp of the ovine FSHB promoter.

II. Introduction

It is well known that follicle stimulating hormone (FSH) is important for reproduction in all vertebrates. It is expressed and released only from pituitary gonadotropes and is secreted primarily through a constitutive pathway [1] so serum FSH usually mirrors gene expression. FSH is an α/β heterodimer and transcription of its beta subunit (FSHB) is rate limiting for overall FSH expression.

Transcription of FSHB is controlled primarily by the presence or absence of two hormones: activin and GnRH. Each hormone helps induce FSHB transcription in physiologically important ways which also contributes to its gonadotrope-specific expression through hormone responsive elements on the FSHB promoter. Furthermore, there is at least one constitutively active transcription factor (Pitx1) that is essential for FSHB expression. Transfection studies using mutated oFSHBLuc constructs in L β T2 cells (transformed embryonic mouse gonadotropes) have already helped define one activin responsive element in the ovine FSHB promoter at -172/-158 bp. Follow-up studies using the same mutated oFSHBLuc constructs in transgenic mice showed that without this site, only 0.1-1% of ovine FSHB expression is observed in gonadotropes in vivo [2]. One Pitx1 site was also located using mutated oFSHBLuc transfected into L β T2 cells (-60/-53 bp) [3-5], and follow-up transgenic studies showed that only 1% of expression is observed in mouse pituitaries when this site is selectively destroyed by mutation (Sang-oh Han et al., our laboratory, manuscript in review). To date, a specific site has not yet been identified for either positive or negative regulation by GnRH on the ovine FSHB promoter [6-8] and some of its effects on FSHB expression may be indirect [9].

Strong evidence exists for other site(s) that are critical for gonadotrope-specific expression of ovine FSHB. These data come from studies which used -4741 bp of ovine FSHB promoter to express luciferase (-4147oFSHBLuc) in transgenic mice [10]. This transgene expressed high levels of luciferase activity (1-10 million RLU/mg protein), but no expression was observed when -750oFSHBLuc was used as a transgene [10]. Therefore, there must be sequences between -4741 bp and -750 bp that are absolutely necessary for FSHB expression in vivo.

In vitro studies with L β T2 cells seemed promising for defining all sequences needed for regulating and expressing FSHB [11], but all cell lines tested to date also express -4741oFSHBLuc fairly well. The only hormones that alter expression in L β T2 cells versus other transformed cells are activin (5- to 8-fold induction) or GnRH (\pm 2-fold difference depending on concentration). Since the difference between expression of the -4147oFSHBLuc transgene in gonadotropes and many other tissues is \sim 1000-fold, the 2- to 3-fold changes observed between expression of -4741oFSHLuc in L β T2 cells and other transformed cells seems very small. Therefore, transformed gonadotropes have not been useful for defining the regulatory site(s) between -4741 bp and -750 bp on the ovine FSHB promoter that creates high level gonadotrope targeting of FSHB expression in vivo.

Recent transgenic studies in the rat implicate Prop-1 sites between -850 bp and -750 bp of the porcine FSHB promoter as being critical for expression of porcine FSHB [12, 13], but the ovine FSHB promoter has no similar Prop-1 sites. The gene encoding human FSHB needs only -350 bp of 5' promoter sequences, but absolutely requires 3' sequences between +2138 bp and +3142 bp for transgenic expression in the mouse pituitary [14]. Again, sequences within this region show no obvious homology to ovine promoter sequences. Therefore, the transgenic studies completed to date with the ovine FSHB gene have not defined specific sequences between -750 bp and -4741 bp needed for gonadotrope-specific expression.

The studies reported here used a two pronged approach for defining sites on the ovine FSHB promoter needed for gonadotrope-specific expression. One was computer-driven and discovered conserved sequence patterns in the ovine, porcine and human upstream 5' promoters. These sequences were deleted from the wild type oFSHBLuc construct and the remaining construct was used as a transgene to determine if the deleted sequences were important for gonadotrope-specific expression. The second approach used viral transmission of oFSHBLuc constructs into primary gonadotropes reasoning that primary cells had the best chance of producing regulatory factors that direct gonadotrope-specific expression. Although L β T2 cells are gonadotropes, they are transformed and might not produce one or more of these critical factors. To prove that the viral constructs (oFSHBLuc-V) were regulated normally in gonadotropes (or non-gonadotropes), infected primary cells were treated with

follistatin (to block any autocrine activin action), activin or activin plus inhibitory levels of GnRH.

The transgenic data focus attention on a 1.1 kb region in the ovine FSHB promoter that is needed for gonadotrope-specific expression of ovine FSHB *in vivo*. The adenoviral oFSHBLuc –V constructs expressed in primary gonadotropes provide an effective and useful way to analyze gene expression that is regulated by signaling pathways used by gonadotropes on a day-to-day basis *in vivo*.

III. Materials and Methods

III-A. Reagents

Recombinant human activin A and follistatin (288) were obtained from R&D Systems (Minneapolis, MN). Gonadotropin releasing hormone (GnRH), oligonucleotide primers for preparing viral constructs, collagenase Type I and Pancreatin were all purchased from Sigma-Aldrich (St Louis, MO). Fugene 6 was purchased from Roche Applied Science (Indianapolis, IN) and restriction enzymes plus luciferase assay kits were from Promega (San Luis Obispo, CA). Integrated DNA Technologies was the source of oligos for PCR. Dulbecco's modified eagle medium and fetal bovine serum were bought from Invitrogen (Carlsbad, CA). Common reagents such as buffers, growth media and agar were purchased from Fisher Scientific, (Inc. Pittsburgh, PA).

III-B. Generation of promoter-reporter plasmids, transgenes and transgenic mice

The wild type ovine FSHB promoter-reporter plasmid (-4741oFSHBLuc) was described previously [10]. Briefly, it contained 4741 bp of the ovine FSHB promoter plus intron 1 driving expression of a luciferase gene in the GL3 basic vector. This plasmid was used to generate the LO- and LS-oFSHBLuc plasmid constructs that contain either -2361 bp or -1866 bp, respectively. Using site directed mutagenesis, a KpnI restriction site was produced at -2361 or -1866 and plasmids were digested and re-ligated to produce LO- or LS-oFSH β Luc, respectively.

To generate transgenic mice, the constructs were digested with KpnI and BamHI to release them from the plasmid backbone. The digests were sent to the University of North Carolina for purification and injection into the pronuclei of fertilized B6SJL mouse eggs. Transgenic mice were genotyped as described previously [10]. All transgenic mice were maintained and studied with the approval and oversight of the Institutional Animal Use Committee at the University of North Carolina, Chapel Hill, NC, or North Carolina State University. All transgenic mice were bred and cared for at the Biological Resource Facility of North Carolina State University.

III-C. Pituitary cell cultures

Mice carrying LO- or LS-oFSHBLuc transgenes were sacrificed at 7-40 weeks of age using CO₂, their pituitaries were collected within 5 min and then dispersed into single cell suspensions as described previously [10]. Briefly, pituitaries were sliced into small pieces and digested with collagenase type I (Sigma-Aldrich, Woodlands, TX) and Pancreatin (Life Technologies, Inc). The yield was approximately 5x10⁵ cells/pituitary. Cells were then plated in 96-well Primaria tissue culture plates (Becton Dickinson & Co., Franklin Lakes, NJ) at a density of 30,000 cells/well in 50 µl of medium 199 containing 10% charcoal-treated sheep serum plus 100 U/ml penicillin G and 100 µg/ml streptomycin (Sigma-Aldrich, Woodlands, TX) and allowed to attach for 2 days at 37° C under an atmosphere of 95% air: 5% CO₂ in a humidified chamber before treatment. Cells were treated with hormones at the indicated doses and times as described in the figure legends. Treatments were terminated by lysis in 50 µl of 1x passive lysis buffer (Promega Co., Madison, WI), and 15 µl of each cell lysate was assayed for luciferase activity. All of the experiments were performed at least three times and each treatment was assayed in triplicate or quadruplicate.

III-D. Luciferase assay

For in vivo experiments, mouse pituitaries or other tissues (0.3-1-mg) were snap-frozen in liquid nitrogen before homogenization in 200 µl of cell lysis reagent (Promega Co., Madison, WI). Cell debris was removed by centrifugation at 10,000 x g for 20 sec, and then 10 µl of all cell lysates were immediately assayed for luciferase activity using the luciferase assay system (Promega Co., Madison, WI). Activity was measured for 20 sec using an automated 1420 Victor-Light micro plate luminometer (PerkinElmer, Waltham, MA). Finally, protein was assayed using a Qubit fluorometer (Invitrogen, Carlsbad, CA). For primary pituitary cultures, cells were lysed using 50 µl passive Lysis solution (promega Co., Madison, WI) and 15 µl were analyzed in duplicate using the luciferase assay system.

III-E. Truncated promoter-luciferase construct (plasmids)

Three plasmids containing different lengths of the ovine FSH β promoter controlling luciferase expression were produced as reported previously (-215oFSHLuc, -748oFSHBLuc, and -2932oFSHBLuc) [15].

III-F. Cell culture and transient transfection

Transformed L β T2 cells were obtained as a kind gift from the laboratory of Dr. Pamela Mellon [16]. Cell culture and transfections with Fugene 6 (Roche Molecular Biochemicals, Basel Switzerland) were described previously [2].

III-G. Gonadotrope purification from H2Kk transgenic mice

Gonadotropes were purified from hemizygous H2Kk transgenic mice that were 7-50 weeks old [17]. The method for dispersing pituitary cells was the same as that outlined above (Pituitary cell culture). After dispersion, gonadotropes were separated from non-gonadotropes with two cycles of purification as previously reported [17]. Non-gonadotropes were used here to show expression of oFSHBLuc-V constructs in inappropriate cell types so non-gonadotropes were also purified through two cycles to deplete them of as many gonadotropes as possible. All cells were cultured as noted above.

III-H. Adenoviral constructs and adenovirus amplification

Three adenoviral constructs were obtained using Adeno-XTM Expression Systems from Clontech (Mountain View, CA). These adenoviral constructs contained -232oFSHLuc, -750oFSHLuc and -2871oFSHBLuc in a viral context. Two of the constructs (-750oFSHLuc-V and -2871oFSHBLuc-V) were generated using the Adeno-XTM Expression System II. These truncated promoters plus intron 1 connected to the luciferase gene were amplified using Advantage-HF 2 PCR Kit (Clontech, Mountain View, CA) with the following primers: 5'-acggtaccggacatatgaatgcatcagctagcaaaca -3'(-2871 forward),

5'-gaattccgggcatatggaattacacggcatctttc-3'(-2871 & -750 reverse),

5'-acggtaccggacatatgcatggagctcttagtctact-3' (-750 forward).

Then the amplified sequences were inserted into the promoterless pDNR-1r donor vector (System II) using the In-Fusion Advantage PCR Cloning Kit (Clontech, Mountain View, CA), and target sequences on the donor vector were put into the adenoviral backbone by Cre recombinase (System II). The insertion was confirmed by PCR analysis using the Adeno-X™ PCR screening primer set (Clontech, Mountain View, CA).

The third adenoviral construct (-232oFSHBLuc-V) was produced using the Adeno-X™ Expression System I since Clonotech system II was discontinued. The differences between System I and II were: System I was not designed for promoterless use so the CMV promoter had to be removed on the shuttle plasmid. Moreover, System I cannot use the efficient recombinase technique. As with system II, the -232oFSHBLuc construct was amplified using the Advantage-HF 2 PCR Kit (Clontech) with the following primers:

5'-ttgattattgactagtagcaaggtaaaggagtggtgg-3' (-232 forward) and

5'-ccgttaaacgctagctcttatcatgtctgctcgaa-3' (-232 reverse).

The pShuttle2 plasmid was cut with SpeI and NheI (Promega) to remove the CMV promoter. The amplified sequence was infused into pShuttle2 using the Fusion Advantage PCR Cloning Kit (Clontech). Finally, the sequence was cut out and ligated into the adenoviral backbone according to the protocol. This insertion of the foreign DNA was confirmed by PCR using the Adeno-X™ PCR screening primer set (Clontech, Mountain View, CA).

When all the three adenoviral constructs were made, they were transfected into an early batch of HEK293 cells fresh from ATCC which were cultured the same as LβT2 cells (see above). The viruses were amplified and harvested according to the Clonotech protocol. After 3-4 generations of amplification, the virus titers reached 10⁹ ifu/ml.

III-I. Virus titering

Virus titers were determined using the Adeno-X™ Rapid Titer Kit (Clontech, Mountain View, CA). All three viral constructs were adjusted to the same titer of 10⁸ ifu/ml.

III-J. Infecting primary cell cultures

Primary cell culture was performed as described above. The infection was with the MOI of ~100 ifu/cell in a volume of 10ul.

III-K. Cellular mRNA extraction and Real Time-rtPCR

Cellular mRNA was extracted using TRI REAGENT (MRC.Inc., Cincinnati, OH). Then mRNA was converted into cDNA using the iScript™ cDNA Synthesis Kit (BIO-RAD, Hercules, CA). For real time PCR, the setting for each well in the 96-well plate was: 1ul cDNA, 15ul Universal PCR Master Mix (TaqMan, Carlsbad, CA), 13.7ul dH₂O, 0.1ul of each primer and probe (IDT, Newark, NJ). The primers and probes for 18S, mouse prolactin cDNA, and mouse FSHB cDNA were as follows:

the primers and probe for 18S were

5'-gaaactgcgaatggctcattaa-3' (forward),

5'-gaatcaccacagttatccaagtagga-3' (reverse),

and 5'-/56FAM/atggttcctttggctcgcctcc/3BHQ_1/-3' (probe);

for mouse prolactin they were

5'-tctcaaggtcctgaggtgccaaat-3' (forward),

5'-caattgcaccaagcatgcactga-3' (reverse) and

5'-/56-FAM/acaactgctaaaccacattcagcca/3BHQ_1/-3' (probe);

for mouse FSHB they were

5'-agagaaggaagagtgccgtttctg -3' (forward),

5'- acatactttctgggtattgggccg-3' (reverse),

and 5'-/56FAM/atcaataccacttggtgtcgggcta/3BHQ_1/-3' (probe);

for the H2Kk they were

5'-agacaaggcagctgtctacggaaa-3' (forward),

5'-gcagattgctctccagcaacagaa-3' (reverse) and

5'-/56 FAM/agcatccacagttaccaagtgcc/3BHQ_1/-3' (probe).

The PCR cycle setting was 50C for 2 minutes, 95C for 10 minutes, then 95C for 15 seconds for 40 cycles followed by 60 C for 1 minute.

III-L. Statistical analysis

All experiments were performed either in triplicate or quadruplicate and repeated 3 times or more. The data in Table 1 represent means \pm sems. Where there are multiple comparisons, data were analyzed using one-way ANOVA with Tukey's multiple comparison test according to the Prism version 4 (GraphPad Software, Inc., San Diego CA.)

IV. Results

IV-A. Specific pituitary expression of -2339oFSHBLuc and -1866oFSHBLuc in transgenic mice

Previous research showed that -4741 bp of ovine FSH β promoter directs robust expression of the luciferase reporter gene specifically to the pituitary, but -750 bp of the same promoter cannot generate any expression. Genomic sequence comparisons of human, pig and sheep FSHB promoters found unique promoter regions that show $\geq 75\%$ homology in all three species in 1.1 kb of the distal promoter (Figure 1). This region contains three subdomains of ~100-200 bp each and resides several kb upstream of the FSHB start site. High homology was also observed between the ovine and porcine promoters between -2483 bp and 2070 bp. Therefore, computer analysis showed that the porcine, ovine and human FSHB promoters contained high homology regions distal to -2339 bp and -1866 bp (Figure 1).

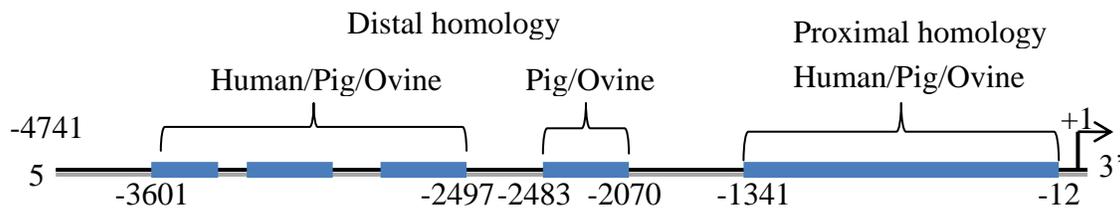


Figure 1: Regions of the ovine FSHB promoter with $\geq 75\%$ sequence homology to porcine and/or human 5' promoter regions. 4.7 kb of ovine FSHB promoter was aligned with 10 kb of human (Ensembl transcript ID: ENST0000025122) and 5.7 kb of porcine (NCBI Acc# D00621.1) FSHB promoters using BLAST 2. Regions between -3.6 and -2.5 kb of the ovine FSHB promoter are shown that correspond to similar sequences in the human (-5 to -6.1 kb) and porcine (-4.6 to -5.7 kb) FSHB promoters. A fourth region of high homology between porcine and ovine FSHB promoters was found between -2.48 kb and -2.07 kb.

To determine if the highlighted distal regions in Figure 1 are critical for FSHB expression, two transgenic mouse lines were produced that contained -2339 bp of 5' promoter (LO) and -1866 bp of 5' promoter (LS). The LO promoter lacks the 3 upstream high homology sections and LS lacks all 4 distal high homology sections. Three founder lines were produced from each oFSHBLuc construct. All were verified to contain the correct construct using PCR and

all founders were fertile with a normal transmission frequency. Pituitaries and other tissues from the hemizygous offspring of all founder lines were analyzed for luciferase activity (Table 1). The results show that both the LO and LS transgenic lines expressed luciferase primarily in the pituitary at a level near that produced by the -4741oFSHBLuc transgene [10]. In most cases expression in the pituitary was >100x that in any other tissues except occasionally in brain tissue. Even with -4741oFSHLuc, there was occasional high expression in the frontal lobe of the brain.

Table 1: Expression of LS and LO oFSHBLuc transgenes in mouse tissues. Tissues were harvested from mice at least 7 weeks old and lysates were assayed for luciferase activity and protein. Values are luciferase activity expressed as relative light units (RLU) x 10⁻⁴/mg/protein. Values for the LS lines represent the mean ± SEM for three animals. LO data are from one mouse each.

Founder	Sex	Pituitary	Brain	Lung	Liver	Heart	Spleen	Gonad
LS 41716	F	1407±729	7±2	1±0.4	0	0	2±1	4±1
LS 41721	M	129±29	89±23	0	0	0	0	3±1
LS 41756	M	31±7	1±0.4	0	2±1	0	0	0
LO 41760	F	69	0	0	0	0	0	1
LO 41765	F	263	1	0	0	0	0	0
LO 41774	F	206	73	0	0	0	0	0

Finally, to show that LO and LS transgenes were regulated by activin and GnRH the same way as -4741oFSHBLuc, dispersed cultures were produced from the pituitaries of offspring from founder 41716 (see Table 1). The results from these cultures are shown in Figure 2. Since mouse pituitary cultures produce activin from paracrine and/or autocrine sources, cultures were treated with follistatin to incapacitate culture-made activin so gonadotropes produce basal levels of luciferase. Activin induced luciferase 7.5-fold which was observed for the -4741oFSHBLuc transgene [10] and, finally, 100 nM GnRH inhibited luciferase as expected (Figure 2). These results are comparable to the regulation observed for the -4741oFSHBLuc transgene previously reported [9, 10].

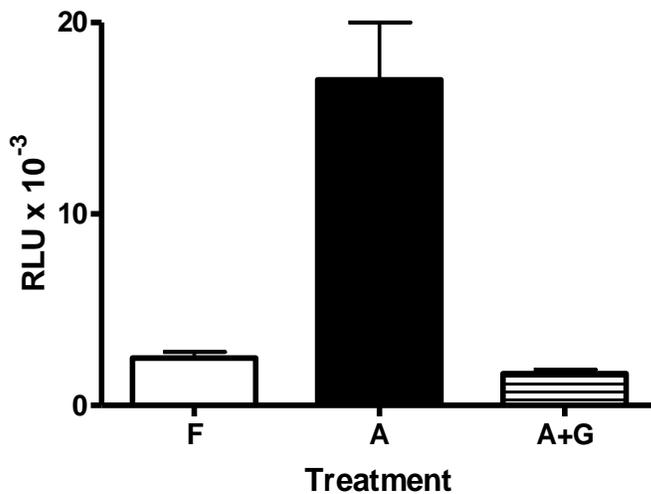


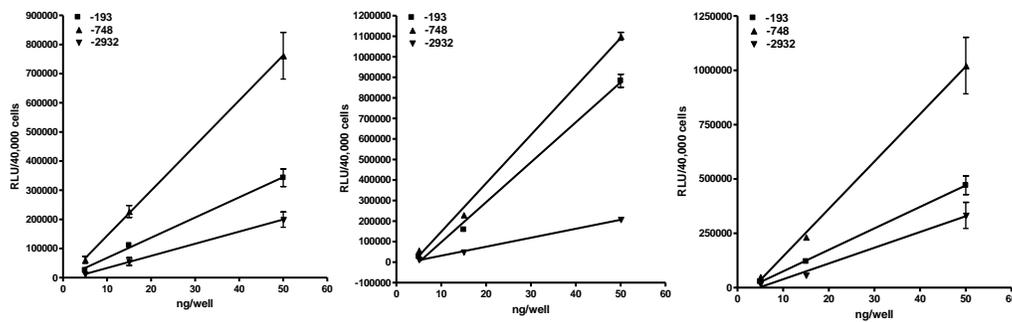
Figure 2: Hormonal regulation of LS-oFSHBLuc in dispersed pituitary cell culture. Pituitary cells from transgenic mouse expressing LS-oFSH β Luc were dispersed and plated at a density of 30,000/well. After 2 days, cells were treated with 250 ng/ml of follistatin (F), 50 ng/ml of activin (A) or 50 ng/ml of activin + 100 nM GnRH (A+G) for 20 h. Cell lysates were assayed for luciferase activity. Values are luciferase activity expressed as relative light units (RLU) and represent the mean \pm sem for three independent experiments each performed in triplicate.

IV-B. Comparison of plasmid and adenoviral oFSHBLuc constructs in L β T2 cells

Distinct from plasmids, adenoviruses are organisms that can infect living cells, then replicate their genome and assemble new viral particles. The viruses used in this study cannot replicate in primary mammalian cells, but there is the possibility that results from plasmids and viral constructs might yield different results. One criterion for judging whether adenoviruses are useful tools for introducing foreign genes into a cell is to see whether the viral constructs behave the same as plasmids. Since plasmids cannot be transfected into primary cells, the comparison was made in transformed L β T2 cells. Three plasmid constructs were made and tested: -193oFSHBLuc, -748oFSHBLuc and -2932oFSHBLuc. Three similar viral constructs were produced and tested: -232oFSHBLuc-V, -750oFSHBLuc-V and -2871oFSHBLuc-V where the “V” designates a viral construct.

Plasmid and viral constructs were first tested using dose-response experiments at three concentrations of plasmid (5ng, 15ng and 50ng) and virus (1ul, 3ul and 10ul of each virus with a titer of 10^8 ifu/ml). Each construct was used to express luciferase in 40,000 L β T2 cells and then the cells were treated with 50ng/ml activin A for 24 hours. All plasmid and viral constructs produced linear dose-responses with luciferase expression being similar for both plasmids and viruses, except for the longest oFSHBLuc viral construct which was expressed at a significantly lower level than its equivalent plasmid construct (compare Figures 3A and 4A).

A.



B.

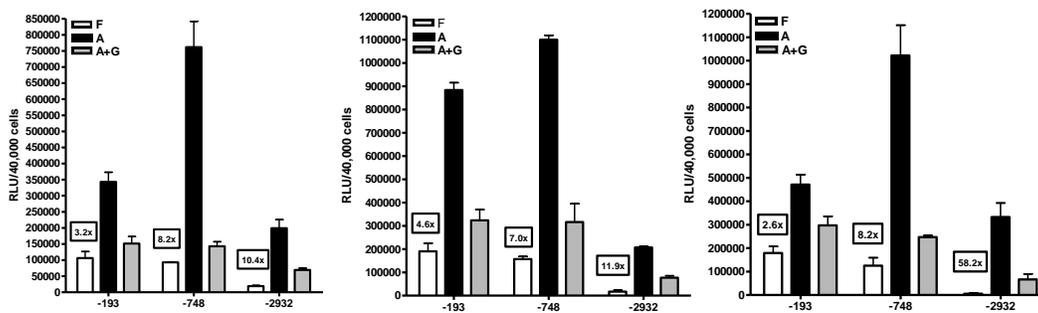
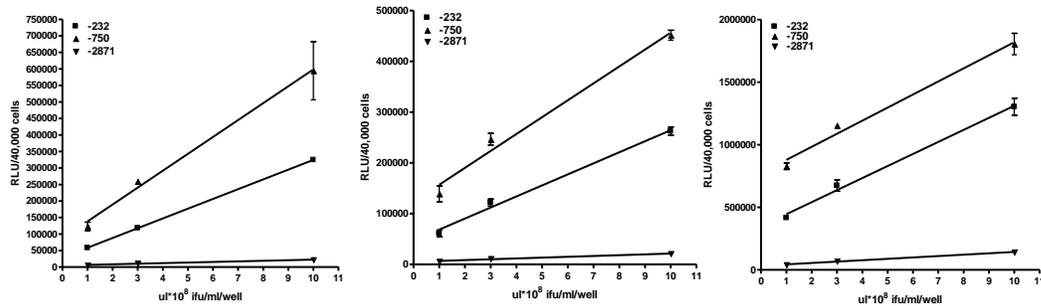


Figure 3A: Dose-response of plasmid transfection in L β T2 cells. L β T2 cells (40,000 cells/well) were plated and transiently transfected with -193oFSHBLuc, -748oFSHBLuc or -2932oFSHBLuc. Different amounts of DNA (5, 15 or 50ng/well) were tested. After a 12 hour transfection, cells were treated with activin A (50ng/ml) for 24 h, and then assayed for luciferase activity.

Figure 3B: Hormonal regulation of L β T2 cells transfected with plasmids. L β T2 cells transfected with the 3 plasmid constructs at 50 ng/well (see Fig 3A) were treated with follistatin (250ng/ml), activin A (50ng/ml) or activin A (50ng/ml) + GnRH (100nM) for 24 hours after a 12 hour transfection, and then assayed for luciferase activity. The framed text indicates activin A induction for each plasmid construct.

The effects of activin A and GnRH were then compared using the highest levels of plasmid or virus used for the dose-response experiments. Again 40,000 cells were treated with plasmid or virus and then cells were treated with follistatin, (250 ng/ml), activin (50 ng/ml) or activin plus GnRH (100 nM) for 24 hours. In all cases the plasmids and viruses behaved similarly (compare 3B and 4B).

A.



B.

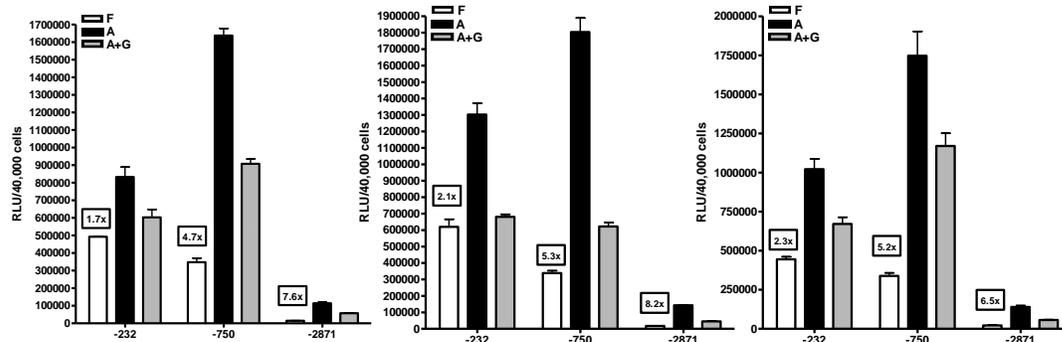


Figure 4A: Dose-response for viral infection in LβT2 cells. LβT2 cells (40,000 cells/well) were plated and infected with -232oFSHBLuc-V, -750oFSHBLuc-V or -2871oFSHBLuc-V. Different amounts of viral constructs (1, 3 and 10ul/well of 10^8 ifu/ml) were tested. After a 12 hour infection, cells were treated with activin A (50ng/ml) for 24 hours, and then assayed for luciferase activity.

Figure 4B: Hormonal regulation of the LβT2 cells infected with viral constructs. LβT2 cells infected with 10 ul of 232oFSHBLuc-V, -750oFSHBLuc-V or -2871oFSHBLuc-V (see Fig 4A) were treated with follistatin (250ng/ml), activin A (50ng/ml) or activin A (50ng/ml) + GnRH (100nM) respectively for 24 hours after a 12 hour infection, and then assayed for luciferase activity. The framed text indicates the activin A induction for each viral construct.

IV-C. Testing the purity of gonadotropes obtained from H2Kk transgenic mice

Gonadotrope and non-gonadotrope populations were assayed by real-time rtPCR for murine prolactin and FSHB mRNAs plus 18s RNA (internal standard for both PCR assays). Lactotropes comprise 30-40% of mouse pituitary cells and FSHB comes solely from gonadotropes which comprise ~5% mouse pituitary cells. Based on the percent decrease in prolactin mRNA between flow through cells and the gonadotrope fraction, the purity of gonadotropes was calculated to be 96%, 89% and 91% for preparations I, II and III, respectively. These preparations were used to generate the combined data in Figures 6A and 7A. The data in Figures 6B and 7B were from preparation II.

The levels of FSHB mRNA in the gonadotrope and non-gonadotrope fractions are shown in Figure 5 for preparations I, II and III featured in Figures 6 and 7 as noted above. The level of FSHB mRNA was 30- to 60-fold higher in the gonadotrope fractions compared to the non-gonadotropes. (Figure 5).

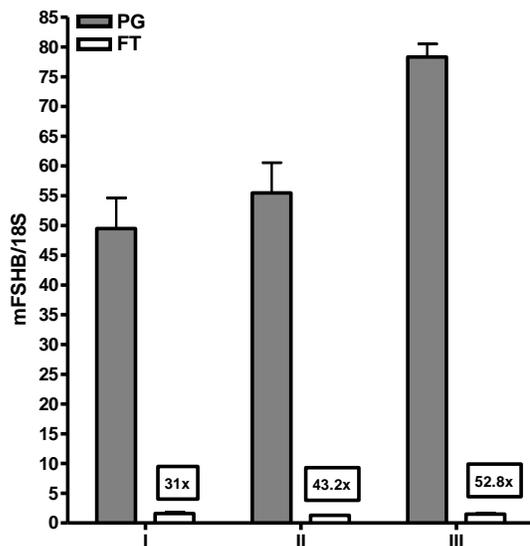


Figure 5: Gonadotrope purification from H2Kk mice verified by RT-rtPCR. Both primary gonadotropes and flow through cells (non-gonadotropes) went through two cycles of purification from H2Kk transgenic mice and were plated out as 10,000 cells/well, and then treated with activin A (50ng/ml) for 48 hours. Then the cellular mRNA was extracted and analyzed by Real-time rt PCR for prolactin/18S and FSHB/18S. Figure 5 only shows the results for FSHB/18S in both the purified gonadotropes and flow through cells in all three experiments. The framed text indicates the difference of mFSHB/18S between the gonadotropes and flow through cells.

IV-D. Expression and regulation of viral oFSHBLuc-V constructs in purified primary gonadotropes

The data in Figure 6A are the normalized composite results from three separate experiments showing expression of the three viral constructs in purified gonadotropes and comparing the results of follistatin, activin and activin plus GnRH treatments. The data in Figure 6B are non-normalized data from a single experiment. The data in Figure 6B show clearly that -232oFSHBLuc-V and -750oFSHBLuc-V are expressed better than the longer -2871oFSHBLuc-V construct. Therefore, the same results are observed in primary cells as in L β T2 cells meaning that expression in primary cells does not resemble that observed in vivo. The shorter two constructs should not have been expressed.

It should be noted that fold-induction by activin decreases as the promoter sequences become shorter which was previously documented in L β T2 cells for plasmid constructs [15]. As expected GnRH inhibited the -750oFSHBLuc-V and -2871oFSHBLuc-V constructs, but surprisingly it could not inhibit the -232oFSHBLuc-V construct. This phenomenon was not observed in L β T2 cells and suggests a difference between analysis in primary and L β T2 transformed gonadotropes.

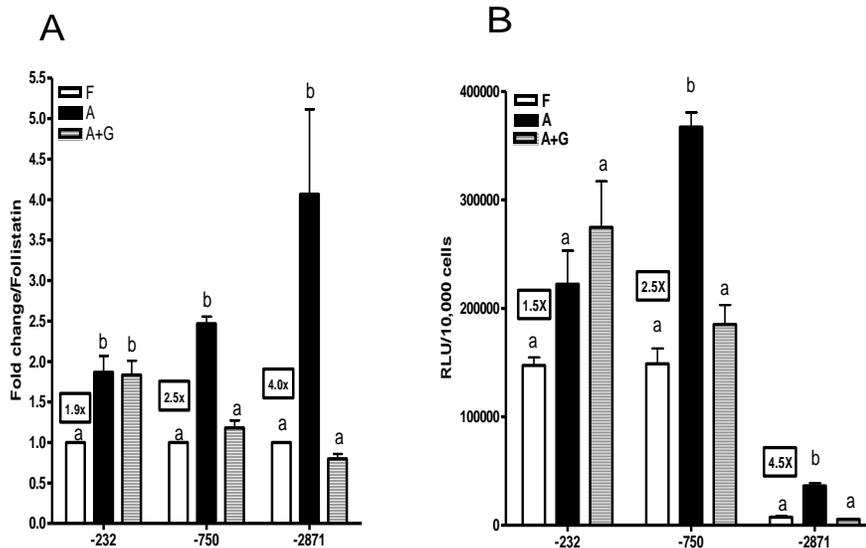


Figure 6: Adenoviral infection of purified gonadotropes. Primary gonadotropes (10,000 cells/well) were plated and infected with -232oFSHBLuc-V, -750oFSHBLuc-V or -2871oFSHBLuc-V. After a 24 hour infection, cells were treated with follistatin (250ng/ml), activin A (50ng/ml) or activin A (50ng/ml) + GnRH (100nM) respectively for 48 hours. Experiments were performed in quadruplicate and repeated 3 times. **Figure 6A** shows combined results for three individual experiments. Follistatin results were normalized to 1. The framed text indicates the activin A induction for each viral construct. Bars with different letters are significantly different ($P < 0.05$). Bars with the same letters are not different from each other ($P > 0.05$). **Figure 6B** shows original data from one of the three independent experimental replicates. The framed text indicates the activin A induction for each viral construct. Bars with different letters are significantly different ($P < 0.05$). Bars with same letters are not different from each other ($P > 0.05$).

IV-E. Expression and regulation of viral oFSHBLuc-V constructs in non-gonadotropes

Figure 7A shows normalized composite data for results from three separate experiments showing expression of the three viral constructs in non-gonadotropes. Figure 7B is a representative single experiment showing original data. In both figures (A&B) it is clear that there is considerable expression of all ovine FSHBLuc-V constructs in non-gonadotropes. There should not have been any expression in these cells if proper cell-specific targeting was occurring. A statistical comparison of the data in Figures 6 and 7 indicates that basal

expression of oFSHBLuc-V constructs is not significantly different in gonadotropes compared to non-gonadotropes. Finally, it should be noted that neither activin nor GnRH (100 nM) had any significant effect on expression in non-gonadotropes.

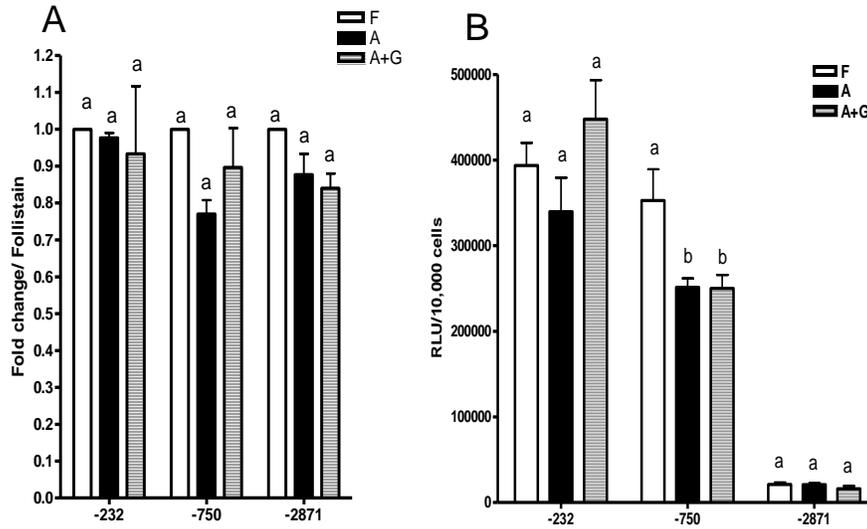


Figure 7: Adenoviral infection of non-gonadotropes (flow through cells). Non-gonadotropes (10,000 cells/well) were plated and infected with -232oFSHBLuc-V, -750oFSHBLuc-V or -2871oFSHBLuc-V. After a 24 hour infection, cells were treated with follistatin (250ng/ml), activin A (50ng/ml) or activin A (50ng/ml) + GnRH (100nM) for 48 hours. Experiments were performed in quadruplicate and repeated 3 times. **Figure 7A** shows combined results for three individual experiments. Follistatin results were normalized to 1. Different letters indicate significantly different means ($P < 0.05$). Bars with same letters are not different from each other ($P > 0.05$). **Figure 7B** represents data from one representative replicate. Different letters indicate significantly different means ($P < 0.05$). Bars with same letters are not significantly different from each other ($P > 0.05$).

V. Discussion

V-A. Cell Specific Expression – the transgenic approach

Prior to this study, 12 founder mice were produced that contained 4741 bp of ovine FSHB promoter linked to the luciferase gene and all but two expressed high levels of luciferase (1-10 million RLU/mg protein) in the pituitary. This was at least 100x the activity in all other tissues tested, except occasionally the frontal lobe of the mouse brain [8, 10]. Considering that only 1/20th (~5 %) of pituitary cells are gonadotropes and they are the only pituitary cells producing luciferase [17], the specificity of expression for -4741oFSHBLuc in gonadotropes is $20 \times 100 = 2,000:1$.

Three transgenic founders containing -750 bp of the oFSHB promoter were also produced and none expressed luciferase in any tissue. These data indicated that sequences important for FSHB expression exist between -750 bp and -4741 bp of the ovine FSHB promoter. One of the -4741oFSHBLuc transgenes that was not expressed in vivo had a deletion from -2755 bp to -3275 bp suggesting that important elements within this sequence might be especially important for gonadotrope expression. Figure 1 of this paper shows that the ovine FSHB promoter contains three sequences between -2755 bp and -3275 bp that have high homology to similar sequences on the human and porcine FSHB promoters. This region (1.1 kb) with internal 100-200 bp sequences resembles a classical Locus Control region (LCRs).

Locus Control Regions are usually found in stretches of DNA that are 1.1 kb long (18-22), reside 8 kb to 60 kb from the genes they control [19-22], direct cell-specific expression [23, 24] and are highly conserved across species [25]. Even though the FSHB gene is a single copy gene, it seemed possible that it might be controlled in part by a classic LCR, especially since gonadotrope expression of the human FSHB gene requires relatively non-descript sequences in a 1 kb region beyond its coding sequence (+2138 bp and +3142 bp) [13]. The 5' sequences of the human promoter that are homologous to the ovine sequences are so far upstream on the 5' end (-5 to -6.1 kb) they have never been included or tested for importance in any human FSHBLuc transgene. The concept that these conserved sequences might help

control FSHB expression in many vertebrates needed to be tested. Therefore, we produced transgenics that carried ovine FSHBLuc constructs missing the conserved homologous regions: -2361oFSHBLuc and -1866oFSHBLuc.

The transgenic data presented in this report show that the hypothetical LCRs are not needed for specific FSHB expression in gonadotropes. The data do indicate, however, that sequences between -1866 bp and -750 bp are responsible for gonadotrope-specific expression of ovine FSHB. Since the porcine promoter sequence is different from the ovine sequence in this region and the human FSHB gene requires 3' sequences for expression, it may be that unique solutions exist for the expression of many vertebrate FSHB genes. This latter scenario seems highly unlikely, however, for a gene so important for preservation of the species.

VB. Cell Specific Expression – the primary gonadotrope approach

Since the homology approach was unable to locate sequences needed for gonadotrope-specific expression of ovine FSHB, and since L β T2 cells have not been useful for identifying sequences that control gonadotrope-specific expression, we turned to the use of primary gonadotropes believing they might contain the information (transcription factors) needed to create gonadotrope specific expression. The following 3 adenoviral constructs were made for expression in primary gonadotropes (and non-gonadotropes) to study gonadotrope specific expression of ovine FSHB: -232oFSHBLuc-V, -750oFSHBLuc-V, and -2871oFSHBLuc-V.

Just as in L β T2 cells, however, each of the above constructs was expressed in purified primary gonadotropes. Only -2871oFSHBLuc-V should have been expressed in gonadotropes and none should have been expressed in non-gonadotropes if adult cells had the ability to create gonadotrope-specific expression. All three constructs were expressed in primary non-gonadotrope pituitary cells at essentially the same basal levels.

Therefore, the use of primary cells (gonadotropes and non-gonadotropes) is not useful for defining the type of control used by sequences between -1866 bp and -750 bp. This type of control must not come from the presence or absence of certain constitutively active transcription factors that provide constant stimulation of transcription. This type of stimulation is exemplified by the Pitx1 transcription factor working at -60 bp in the ovine FSHB promoter. The type of expression controlled by sequences between -1866 bp and -750 bp must be different.

Adenoviral DNA is foreign DNA which did not experience primary cell differentiation and development during embryogenesis. Current research shows that chromatin structure changes during embryogenesis caused by temporal changes in transcriptional factors that can close down chromatin permanently or keep it available permanently for expression. Such processes occur throughout embryogenesis. Hoxb1, for example, can recruit Pbx and Meis to activate many promoters during zebra fish embryogenesis. Each promoter is bound by a complex of Hoxb1, Pbx and Meis, and Meis plays an essential role in acetylating and remodeling the chromatin so that transcription is turned on [26]. Likewise, Brg1, a chromatin remodeling protein needs Cdx2 to repress Oct4 in mouse blastocysts [27]. Cdx2 is also a homeobox family member and has been shown to interact with EP300, CBP and other histone acetyltransferases. These are two examples of chromatin alteration during embryogenesis under the control of a homeobox proteins. Homeobox genes are also important in pituitary embryogenesis, which begins with Rathkes' pouch that first appears at e8.5 in the mouse. A cascade of tightly controlled homeobox transcription factors participate in the permanent differentiation of gonadotropes and specific expression of the FSH β gene that includes Ptx-1, Rpx/Hesx-1, SF-1, LHX3 and prop-1. These transcription factors are not expressed at the same time during embryogenesis, and some of them are even turned off prior to birth, such as prop-1 and Rpx/Hesx-1 (1 report indicates prop-1 is present in the postnatal rat pituitary).

Since neither the -215oFSHLuc nor the -750oFSHLuc can be expressed in vivo but the -1866oFSHLuc can, it is reasonable to speculate that there is a site between -750bp and -1866bp that controls permanent inactivation of the ovine FSHB gene or its permanent expression like genomic imprinting involving epigenetic changes [28, 29]. If this is true, it is not difficult to understand why all the viral constructs were expressed in primary gonadotropes since they do not have the inhibitory mechanism between -750bp and -1866bp which is controlled during embryogenesis.

Finally, aside from the inability of primary gonadotropes to help identify developmental changes that control gonadotrope-specific expression, it appears there is little difference in regulatory studies using adenoviral ovine FSHBLuc constructs in primary gonadotropes or plasmids in L β T2 cells, except that L β T2 cells are much easier to work with and plasmids are easier to prepare than viruses. Activin A induces the expression of both viral and plasmid constructs, and 100 nM GnRH inhibits activin-induced FSHB expression equally well in both systems with one exception: it does not inhibit expression of the shortest adenoviral construct (-232oFSHBLuc-V) in primary gonadotropes, while it does block expression of both adenoviral and plasmid constructs in L β T2 cells. This difference occurs because of cell type. It seems like a small difference, but the implication is large as noted below.

The data with primary gonadotropes suggest there are promoter sequences between -232 bp and -750 bp of the ovine FSHB promoter that mediate inhibition by GnRH in primary gonadotropes. By contrast, inhibition of all constructs in L β T2 cells may occur through signaling that affects whole cell characteristics. There is a huge difference between these two mechanisms and the data suggest primary gonadotropes are the cells needed to define the promoter mechanism that causes GnRH-mediated inhibition of FSHB in vivo.

VI. Future directions for my research.

Negative regulation of FSH by GnRH is not well understood, but GnRH has such an important effect on FSH that its negative action is extremely important to understand. Since the sequence between -232 bp and -750 bp on the ovine FSH β promoter was shown to be associated with GnRH negative regulation, detailed analysis of this sequence would be the first priority. Multiple deletion mutants of ovine FSHB promoter-luciferase constructs (viral) would be tested in purified primary gonadotropes to identify the sequences required for inhibition of FSH β expression by GnRH. GnRH activates many MAP kinases (ERK, JNK, p38 and others?) but it is not known what transcription factors they influence to directly inhibit FSHB transcription. Based on preliminary time-course data (not shown), 100 nM GnRH fully inhibits FSHB expression within 2 h after treatment, and inhibition lasts, at least, for 48 h. GnRH could do this by inducing an early response gene product that binds to the promoter or it could cause a constitutive transcription factor to be modified to bind to the FSHB promoter. No matter what occurs, it will be novel.

Gonadotrope-specific expression of the ovine FSHB promoter is controlled by sequences between -750 bp and -1866 bp. To fully understand FSHB expression, it is important to identify these sequences, but data from this thesis indicates that primary gonadotropes cannot be used to identify them. These sequences (sites) are likely to control changes in chromatin structure that occur during embryonic development. One way to identify these sequences is to construct a series of ovine FSHB-promoter-reporter constructs that lack sequences between -750 bp and -1866 bp and test their expression as transgenes in mice. This approach should identify the specific sequences, but such a process would be very expensive and time consuming.

A second approach would be to identify binding sites for Prop1 and Rpx-Hesx1 between -1866 bp and -750 bp of the ovine FSHB promoter since similar sites are associated with gonadotrope specific expression of porcine FSHB. These transcription factors are transiently expressed during embryonic development, but then disappear before parturition (Ref 41 in

the literature review). As suggested by Kato's laboratory (ref 52 in the literature review) Prop1 and Rpx-Hesx1 bind to sequences between -850 and -750 in the porcine FSHB gene and these sequences are needed to express porcine FSHB as a transgene in rats. These same sequences do not appear, however, in the ovine promoter between -750 bp and -850 bp, but may appear elsewhere. These sites would have to be found using EMSA analysis of DNA fragments incubated with nuclear extracts rich in Prop1 and/or Rpx-Hesx1. If specific binding sites are found in the ovine promoter these sites could be tested using a transgenic approach (see above) that includes only mutations of these binding sites.

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APPENDICES

APPENDIX A

Elucidation of Mechanisms of the Reciprocal Cross Talk between Gonadotropin-Releasing Hormone and Prostaglandin Receptors

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Published in Endocrinology, 2010, Vol 151:2700-2712.

Elucidation of Mechanisms of the Reciprocal Cross Talk between Gonadotropin-Releasing Hormone and Prostaglandin Receptors

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We recently described a novel GnRH receptor signaling pathway mediated by the prostaglandins (PGs) $F_{2\alpha}$ and PGI_2 , which acts through an autocrine/paracrine modality to limit autoregulation of the GnRH receptor and inhibit LH but not FSH release. Here we further explore the cross talk between GnRH and the PG receptors. GnRH stimulates arachidonic acid (AA) release from $L\beta T2$ gonadotrope cells via the Ca^{2+} -independent phospholipase A_2 (iPLA₂) and not via the more common Ca^{2+} -dependent cytosolic phospholipase $A_2\alpha$ (cPLA₂ α). AA release was followed by a marked induction of cyclooxygenase (COX)-1 and COX-2 by GnRH via the protein kinase C/c-Src/phosphatidylinositol 3-kinase/MAPK pathway. COX-2 transcription by GnRH is mediated by the two nuclear factor- κB sites and the CCAAT/enhancer-binding protein site within its promoter. Indeed, GnRH stimulates p65/RelA phosphorylation (22-fold) in $L\beta T2$ cells and the two nuclear factor- κB sites apparently act as a composite response element. Although GnRH stimulates cAMP formation in $L\beta T2$ cells, we found no role for cAMP acting via the cAMP response element site in the COX-2 promoter. $PGF_{2\alpha}$, PGI_2 , or PGE_2 had no effect on GnRH-stimulated ERK, c-Jun N-terminal kinase, and p38MAPK activation or on GnRH- and high K^+ -stimulated intracellular Ca^{2+} elevation in $L\beta T2$ and gonadotropes in primary culture. Although, $PGF_{2\alpha}$, PGI_2 , and PGE_2 reduced GnRH-stimulated cAMP formation, we could not correlate it to the inhibition of GnRH receptor expression, which is exerted only by $PGF_{2\alpha}$ and PGI_2 . Hence, the inhibition by $PGF_{2\alpha}$ and PGI_2 of the autoregulation of GnRH receptor expression is most likely mediated via inhibition of GnRH-stimulated phosphoinositide turnover and not by inhibition of Ca^{2+} elevation and MAPK activation. (*Endocrinology* 151: 2700–2712, 2010)

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Copyright © 2010 by The Endocrine Society
doi: 10.1210/en.2009-1335 Received November 17, 2009. Accepted March 23, 2010.
First Published Online April 14, 2010

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Abbreviations: AA, Arachidonic acid; AACOCF₃, arachidonyl trifluoromethyl ketone; BEL, bromoenol lactone; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; C/EBP, CCAAT/enhancer-binding protein; COX, cyclooxygenase; cPLA₂ α , cytosolic phospholipase $A_2\alpha$; EGF, epidermal growth factor; EP, E prostanoic acid; FCS, fetal calf serum; GPCR, G protein-coupled receptor; iPLA₂, calcium-independent phospholipase A_2 ; JNK, c-Jun N-terminal kinase; MEK, MAPK kinase; NF κ B, nuclear factor- κ B; PG, prostaglandin; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLA₂, phospholipase A_2 ; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; Q-PCR, quantitative PCR; sPLA₂, secretory PLA₂; TE-PC, thioetheramide phosphatidylcholine.

Eicosanoids are metabolites of arachidonic acid (AA), which are potent mediators of inflammation and signal transduction. AA is released from the sn-2 position of membrane phospholipids by phospholipase A₂ (PLA₂). This superfamily of enzymes consists of 14 groups (I–XIV) (1, 2). The low-molecular-mass (~12–19 kDa) secretory PLA₂ (sPLA₂) are all related evolutionarily; share a common catalytic histidine; require millimole Ca²⁺ for activation; have conserved five to seven disulfide bonds; are secreted proteins; and fall into the groups I, II, III, V, and IX–XIV. A high-molecular-mass (60–110 kDa) family of calcium-dependent (require μM Ca²⁺ for activation) cytosolic PLA₂ (cPLA₂), use a catalytic serine, have a high specificity to AA, are activated by Ser505 phosphorylation, and are designated as group IV (A; B; C; or α-, β-, γcPLA₂). The calcium-independent (iPLA₂) (group VI) contain eight ankyrin repeats, have a lipase consensus sequence with an active serine, play a role in phospholipid remodeling and homeostasis, and fall into the subgroups (A-1, A-2, and B) (1, 2).

Once liberated, AA is converted to prostanoids [prostaglandins (PGs) and thromboxanes] via cyclooxygenase (COX)-1, COX-2, and PG synthases. AA is also converted to lipoxygenase products via a family of lipoxygenase enzymes such as 5-lipoxygenase and to epoxygenase products via the cytochrome p450 epoxygenase (cyp2C11) (1, 2).

We have previously shown that GnRH stimulates AA release in cultured rat pituitary cells (3), but the nature of the enzymes involved were not known. We have also shown the formation of 5-lipoxygenase products by GnRH in cultured rat pituitary cells, which participate in GnRH-induced gonadotropin synthesis and release (4). Later, we found that 12-lipoxygenase expression is elevated by GnRH in LβT2 cells (5). More recently we demonstrated a novel GnRH signaling pathway mediated by PGF_{2α} and PGI₂, which limit the homologous regulation of GnRH receptor, whereas PGF_{2α} also exerts selective inhibition of LH release (6). Here we report that GnRH stimulates AA release via iPLA₂ and not via the more common cPLA_{2α}. GnRH stimulates COX-1 and COX-2 expression via the protein kinase C (PKC)/c-Src/phosphatidylinositol 3-kinase (PI3K)/MAPK pathway. COX-2 transcription is mediated apparently by the nuclear factor-κB (NFκB) and the CCAAT/enhancer-binding protein (C/EBP) sites. The documented inhibition by PGF_{2α} and PGI₂ of the autoregulation of GnRH receptor expression is most likely mediated via inhibition of GnRH-stimulated phosphoinositide turnover and not by inhibition of Ca²⁺ elevation, cAMP formation, and MAPK activation.

Materials and Methods

Materials

Culture medium was from Invitrogen Inc. (Paisley, UK). Penicillin-streptomycin and fetal calf serum (FCS) were from PAA Laboratories Ltd. (Middlesex, UK). GnRH was from Peninsula (St. Helens, UK). PGE₂, the stable PGI₂ analog iloprost, PGF_{2α}, arachidonyl trifluoromethyl ketone (AACOCF₃), thioetheramide-phosphatidylcholine (TE-PC), and bromoenol lactone (BEL) were from Cayman Chemical Co. (Alexis Corp., Nottingham, UK). PD980595, a peptide c-Jun N-terminal kinase (JNK) inhibitor (420116; Calbiochem, Nottingham, UK), SB203580, GF109203X, wortmannin, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*] pyrimidine (PP2), AG1478, and U73122 were from Calbiochem.

Goat polyclonal anti-cPLA₂ was from Santa Cruz (Autogen Bioclear, Calne, UK). Antiphospho-Ser505-cPLA₂ and antiphospho-Ser536-p65/RelA antibodies were from Cell Signaling (Beverly, MA). Secondary horseradish peroxidase-conjugated goat antimouse antibodies or goat antirabbit antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Mouse monoclonal antiactive (double phosphorylated) MAPKs (ERK, JNK, and p38) and rabbit antigerenal MAPKs antibodies were obtained from Sigma (Rehovot, Israel). The selective epidermal growth factor (EGF) receptor inhibitor, AG 1478, was kindly provided by Drs. A. Gazit and A. Levitzky (Hebrew University, Jerusalem, Israel).

The human COX-2 promoter -2307/-49 [pGL3.C2.2 (P6P5)] was obtained from R. Newton (University of Warwick, Coventry, UK). The COX2-LUC promoter, 5' deletions and NFκB mutants were obtained from Dr. K. Wu (The University of Texas, Dallas, TX). Using -891 to +9 COX2 human promoter construct, a C/EBP mutant 1-TTACGCAAT to TTACTagcT and a C/EBP mutant 2-TTACGCAAT to gcgatagcT were generated using oligonucleotide-directed mutagenesis. FuGENE6 was from Roche Applied Science (Indianapolis, IN). The dual-luciferase reporter assay system was obtained from Promega (Madison, WI).

Cell culture and transfection

LβT2 cells were kindly obtained from P. Mellon (University of California, San Diego, San Diego, CA). The cells were cultured in matrigel (Becton Dickinson, Oxford, UK)-coated plastic flasks in DMEM/10% FCS/glutamine medium (Sigma, Poole, UK), with 1% antibiotics (stock 500 IU/ml penicillin and 500 μg/ml streptomycin) at 37°C and 5% CO₂ (vol/vol) (7). Calcium measurements were also performed on anterior pituitary cells from postpubertal 8-wk-old female Sprague Dawley rats obtained from Taconic Farm (Germantown, MD). The rats were cared for and killed according to the rules and regulations of the institutional animal care and use committee of National Institute of Child Health and Human Development, National Institutes of Health. Pituitary cells were dispersed and cultured in medium 199 containing Earle's salts, sodium bicarbonate, 10% heat-inactivated horse serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Experiments were approved by the National Institute of Child Health and Human Development, National Institutes of Health Animal Care. Primary mouse gonadotropes were purified as reported (8). The transgenic mice were housed in the Biological Resource Facility of North Carolina State University and were cared for and killed according to the rules and regu-

lations of the Institutional Animal Care and Use Committee of North Carolina State University (Raleigh, NC). Quantitative Taqman RT-PCR showed the ratio of prolactin to 18s RNA was 18-fold higher in unpurified mouse pituitary cells than in the purified gonadotrope fraction after 2 d in culture. Thus, the gonadotropes were considered to be 95% or more pure.

Measurement of AA release

Cells were incubated in serum-free DMEM, 0.2% FCS containing [³H]AA (0.5 μ Ci/ml) for 12–16 h at 37 C. The labeled cells were washed three times with DMEM containing 0.2% fatty acid-free BSA. Cells were then stimulated with GnRH (100 nM). To identify the PLA₂ involved in AA release, the labeled cells were washed and pretreated with selective inhibitors for various PLA₂ enzymes before GnRH was added for another 15 min. The media were collected and centrifuged for 5 min at 12,000 \times g. The cells were solubilized with 1 ml of 0.5% NaOH. Supernatant and cells were counted in a liquid scintillation counter to verify total AA labeling.

PCR

Qualitative and quantitative RT-PCR for COX-1, COX-2, and PLA₂ family members were carried out as recently described (6). The sequence of the primers (PLA₂ family members, forward and reverse primers, respectively) was as follows: cPLA₂ α (IV A), 5'-AGCGGT AGC AGA TCC AGA TGA-3', 5'-CAA TCC TTC TCC ACA TCA GG-3'; cPLA₂ β (IV B), 5'-CAG CTT CTG TCT GAC ATT GAG T-3', 5'-CGC ACT GTG CAG TGA AGG G-3'; cPLA₂ γ (IV C), 5'-CTT GGC CTG TTG GAT GCT GT-3', 5'-AAT GGC TGC AAA GAT GGG ATA G-3'; sPLA₂ (II E), 5'-CTG GAA AAG TAC CTC TTC TCT-3', 5'-TCA GCA GGG TGG GGT GGG-3'; sPLA₂ (V), 5'-CTG GCT TGG TTC CTG GCT T-3', 5'-TAG ACC AGC TTC CGG TCA C-3'; iPLA₂ β (VI A), 5'-GTG AAG AAA CTC TCC ATA GTC GT-3', 5'-GCT GGA CAA GCT TCT GGA ACT C-3'; and iPLA₂ γ (VI B), 5'-CAT GCC GCT GGA TGA ATG TGA-3', 5'-CCT CTG TAT CTG TAG CAC TGC-3'. The sequence of the primers (COX-1 and COX-2 forward and reverse primers, respectively) was as follows: COX-1, 5'-TGG AAC GAC AGT ATC ACC TGC GGC-3', 5'-ATA AGG ATG AGG CGA GTG GTC TGG-3'; and COX-2, 5'-TGC CAC CTC TGC GAT GCT CTT CC-3', 5'-CAG ACT CCC TTG AAG TGG GTC AGG-3'.

For the quantitative PCR, the sequences of the COX-1 and COX-2 primers (forward, reverse, and probe, 6-carboxy fluorescein labeled) were: COX-1, 5'-AGA AGT ACT CAT GCG CCT GGT AC-3', 5'-GTT GTA GGT CGG AGG GC-3', 5'-CAC AGT GCG GTC CAA CCT TAT CCC C-3'; and COX-2, 5'-GCT TCG GGA GCA CAA CAG A-3', 5'-TGG TTT GGA ATA GTT GCT CAT CAC-3', 5'-TGT GCG ACA TAC TCA AGC AGG AGC ATC-3'. Quantitative RT-PCR for PG receptors were carried out by the SYBR Green method: RNA samples were quantified and reverse transcribed using Verso RT-PCR (Thermo Scientific, Swedesboro, NJ) according to the manufacturer's instructions. The reaction mix for the PCR consisted of 1 \times Absolute Fast QPCR master mix (Thermo Scientific), forward and reverse primers for mouse E prostanoic 2 or 4 (150 nM). The reaction mix was aliquoted into PCR tubes (19 μ l) and 1 μ l of cDNA (50 ng) was added. Triplicates (20 μ l samples) plus positive and negative controls were placed in a PCR plate and wells were sealed with optical caps. The PCRs were carried out using an ABI Prism 7000 (Applied Biosystems, Warrington, UK).

All primers were designed using the PRIMER express program (Applied Biosystems). The sequence of the mEP2 primers (forward and reverse primers, respectively) was as follows: 5'-ATC ACC TTC GCC ATT GCT C-3'; 5'-GGT GGC CTA AGT ATG CAA A-3'. The sequence of the mEP4 primers (forward and reverse primers, respectively) was as follows: 5'-TCATCT TAC TCA TCG CCA CCT-3'; 5'-TTC ACC ACG TTT GGC TGA TA-3'. Data were analyzed and processed using ABI Prism 7000 sequence detector (Applied Biosystems) according to the manufacturer's instructions. Results were expressed relative to a cDNA obtained from a single sample of β -actin.

Promoter activity assays

L β T2 cells were grown as above and transfected using FuGENE6 (Roche Applied Science) with the various plasmids. Promoter activity assays were carried out using the dual-luciferase reporter assay as recently described (6).

Activation of MAPK cascades

MAPKs (ERK, JNK, and p38) activity was determined as previously described (7).

cAMP formation

L β T2 cells were incubated with GnRH (100 nM) in the presence and absence of PGE₂, iloprost, or PGF₂ α for 30 min and cAMP production was determined by ELISA.

Ca²⁺ measurements

Cells (70,000) were plated on 25-mm coverslips, and on the next day, the culture medium was replaced by Krebs Ringer medium (in millimoles: 145 NaCl, 4.5 KCl, 10 HEPES, 10 glucose, 0.8 MgCl₂, 2 CaCl₂) containing 2.5 μ M fura-2AM (Invitrogen, Carlsbad, CA) for 1 h at room temperature. After washing the coverslips with the dye-free Krebs Ringer media, they were mounted on the stage of an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) attached to an Atofluor digital fluorescence microscopy system (Ato Instruments, Rockville, MD). Cells were examined under a \times 40 oil immersion objective during exposure to alternating 340 and 380 nm excitation beams, and the intensity of light emission at 520 nm (F₃₄₀ and F₃₈₀) was followed in several single cells simultaneously at the rate of one point per second.

Data analysis

Results from three experiments were expressed as mean \pm SEM. Where appropriate, data were subjected to statistical analysis using ANOVA and Dunnett's *post hoc* multiple comparison test for comparison to a control. Statistical significance accepted when $P < 0.05$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Results

GnRH stimulates AA release through iPLA₂

We first tested the effect of GnRH on AA release as a general measure for PLA₂ activity (3, 9) (Fig. 1A). GnRH stimulates AA release from the L β T2 gonadotrope cells, with maximal effect detected after 15 min of incubation (Fig. 1A). Similar effects were observed with the α T3-1

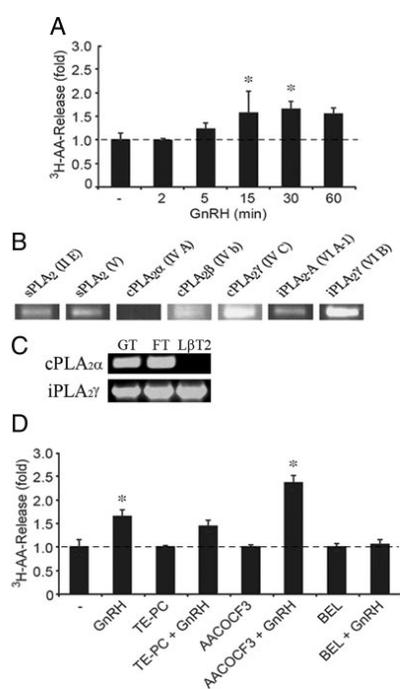


FIG. 1. A, Time response of AA release by GnRH. Subconfluent L β T2 cells were preincubated with [3 H]AA (0.5 mCi/ml) for 12–16 h at 37 C. The labeled cells were washed and then stimulated with GnRH (100 nM) for the time indicated. The cells were solubilized with 1 ml of 0.5% NaOH and [3 H]AA release was determined. In this and subsequent experiments, results from three experiments are shown as mean \pm SEM. *, $P < 0.05$ control vs. GnRH treatment. B and C, Qualitative RT-PCR of various PLA₂ enzymes in L β T2 cells (B) and mouse-purified gonadotropes (C). Total RNA isolated from subconfluent L β T2 cells or cultured mouse-purified gonadotropes was reverse transcribed, and aliquots of single-stranded cDNA were subjected to PCR (in duplicate, with negative controls) using appropriate oligonucleotide primers. PCR products were analyzed by UV fluorescence of ethidium bromide-stained DNA after agarose gel electrophoresis alongside DNA size markers (100-bp ladder; Promega, Southampton, UK). GT, Mouse-purified gonadotropes; FT, other pituitary cells. D, Effect of PLA₂ inhibitors on GnRH-stimulated AA release. Subconfluent L β T2 cells were preincubated with [3 H]AA (0.5 mCi/ml) for 12–16 h at 37 C. The labeled cells were washed and pretreated with the selective inhibitors for cPLA₂ (AACOCF₃, 25 μ M), sPLA₂ (TE-PC, 25 μ M), and iPLA₂ (BEL, 20 μ M) for 15 min before GnRH (100 nM) was added for another 15 min. The cells were solubilized with 1 ml of 0.5% NaOH and [3 H]AA release was determined as above. *, $P < 0.05$ vs. GnRH treatment.

gonadotrope cell line (data not shown). We assumed that the likely candidate to mediate AA release is the G protein-coupled receptor (GPCR)-activated 85-kDa cPLA₂ α , a ubiquitous enzyme, known to be activated by various GPCRs via Ca²⁺ and Ser505 phosphorylation by MAPK (1, 10–12). We therefore treated L β T2 cells with a range of concentrations of GnRH for various time periods and

blotted the cell extract with antiphospho-Ser505-cPLA₂ antibody. Surprisingly, no bands were detected (not shown).

We then repeated the experiment and immunostained the cells with anti-cPLA₂ to detect possible translocation of cPLA₂ α from the cytosol to the Golgi apparatus/perinuclear region as observed in ceramide 1-phosphate-activated A549 cells (9). Again, no specific immunostaining could be detected (not shown). We therefore decided to reexamine the dogma that cPLA₂ α is ubiquitously expressed (1). As shown in Fig. 1B, RT-PCR analysis revealed that cPLA₂ α (group IV A) (1) is not expressed in the L β T2 cells. However, we could detect the expression of cPLA₂ β (IV B) and cPLA₂ γ (IV C) in the cells. The lack of reaction with antiphospho-Ser505-cPLA₂ and anti-cPLA₂ is now resolved because the commercially available antibodies to cPLA₂ react only with cPLA₂ α . In addition, we found that sPLA₂ (II E), sPLA₂ (V), iPLA₂ β (VI A), and iPLA₂ γ (VI B) are also expressed in the cells, whereas other members of the superfamily (1, 10) were not analyzed. However, mouse-purified gonadotropes do express cPLA₂ α and iPLA₂ γ (Fig. 1C). Still, although the gonadotropes are highly enriched, the cells are not purified to homogeneity. Thus, the presence of cPLA₂ α in this preparation may still reflect the presence of nongonadotropes in the sample in Fig. 1C.

We then resorted to the use of chemical inhibitors of the various PLA₂ enzymes to dissect out the nature of the enzyme involved in AA release. L β T2 cells were pretreated for 15 min with the selective inhibitors for cPLA₂, AACOCF₃, for sPLA₂, TE-PC, and for iPLA₂, BEL (1, 10) followed by stimulation with GnRH for 15 min (Fig. 1D). Stimulation of AA release by GnRH was abolished by BEL but not the other inhibitors, implicating iPLA₂ in the GnRH response in L β T2 cells. The results are interesting because very little is known about the involvement of iPLA₂ in GPCR actions compared with cPLA₂ α , which is the main player in GPCR induction of AA release (1, 10–12). Because COX-1 and COX-2 are downstream to PLA₂ in the eicosanoid synthesis pathway, we looked for activation of the COX enzymes by GnRH.

GnRH activates COX-1 and COX-2 expression

It is thought that COX-1 is constitutively expressed and COX-2 is hormone regulated (13), and we decided to check whether this is the case in GnRH-stimulated L β T2 cells. RT-PCR analysis revealed undetectable levels of COX-1 under basal conditions with an increase after 24 h of incubation in serum-free medium, which was further enhanced by GnRH (Fig. 2A). Unlike COX-1, basal COX-2 levels were not detected at 24 h, but GnRH produced a marked induction of COX-2 with a peak at 24 h

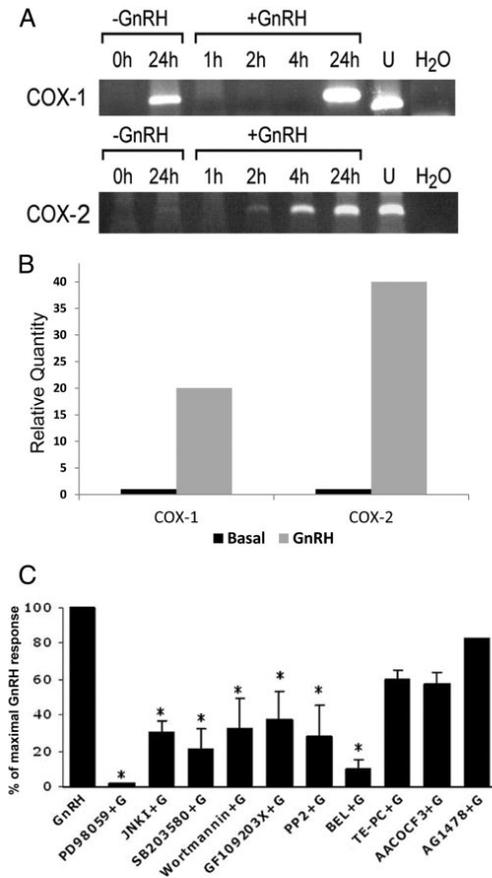


FIG. 2. GnRH induction of COX-1 activity. **A**, Effect of GnRH on COX-1 induction as revealed by RT-PCR. Subconfluent L β T2 cells were incubated with GnRH (10 nM) for the time indicated. COX-1 was then determined by qualitative RT-PCR. Negative (H₂O) and positive [uterus (U)] controls are also shown. **B** and **C**, Q-PCR for COX-1 induction by GnRH. Subconfluent L β T2 cells were stimulated with GnRH (100 nM for 8 h) (**B**) or pretreated for 20 min with the following selective inhibitors: for MEK (PD98059, 50 μ M), JNK (JNK inhibitory peptide, 2 μ M), p38 (SB203580, 10 μ M), PI3K (wortmannin, 25 nM), PKC (GF109203X, 3 μ M), c-Src (PP2, 5 μ M), iPLA₂ (BEL, 20 μ M), sPLA₂ (TE-PC, 25 μ M), cPLA₂ (AACOCF₃, 25 μ M), and EGF receptor kinase (AG1478, 5 μ M). Cells were then stimulated with GnRH (100 nM for 8 h) and COX-1 was determined by Q-PCR (**C**). The effect of GnRH was expressed at the 100% by calculating the fold change (induced/basal). *, $P < 0.05$ control vs. GnRH treatment; G, GnRH.

(6). Further quantitative PCR (Q-PCR) revealed marked activation of both COX-1 and COX-2 (6) by GnRH. COX-1 induction by GnRH at 8 h was robust (20-fold) (Fig. 2B). This stimulatory effect was reduced by the selective inhibitors for MAPK kinase (MEK), PD98059, for JNK, JNK inhibitor, for PKC, GF109203X, for

p38MAPK, SB203580, for PI3K, wortmannin, and for c-Src, PP2, with no significant effect on basal levels (not shown) but not with the EGF receptor kinase inhibitor, AG1478 (Fig. 2C). COX-2 induction by GnRH at 8 h was more robust (40-fold) and was reduced by the same inhibitors (6). The data implicate the known GnRH-activated PKC/c-Src/MAPK pathway (14–18) in GnRH induction of COX-1 and COX-2. The substantial inhibition (50–75%) by all the inhibitors implies that the various MAPK cascades do not converge on the same signaling molecules but rather act in parallel pathways to activate transcription factors, which act as a composite response

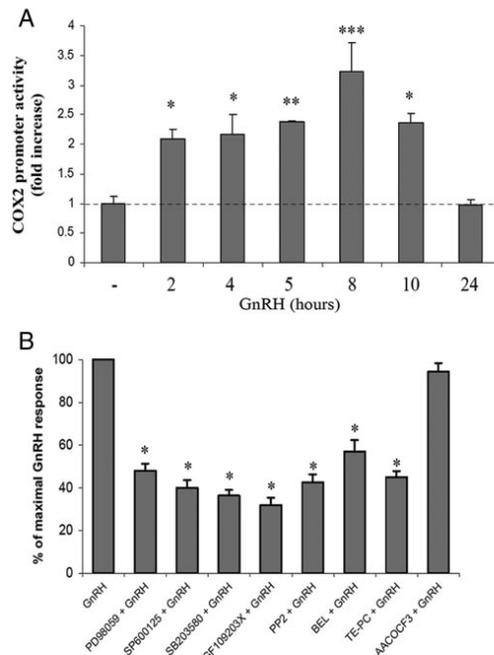


FIG. 3. Effect of GnRH on COX-2 promoter activity. **A**, Subconfluent L β T2 cells were transfected with 0.33 mg of -2307/+49 human COX-2 promoter [pGL3.C2.2 (P6P5)]. Renilla (33 ng) expression vector was also included as a measure of transfection efficiency and as an internal control. GnRH (100 nM) was added for the indicated time points, and cells were then harvested and assayed using a dual-luciferase reporter assay kit. Luciferase activity was normalized for renilla to correct for transfection efficiency. Results for promoter activity are expressed as fold increase relative to untreated controls. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ control vs. GnRH treatment. **B**, Subconfluent L β T2 cells were transfected as described above and were pretreated for 20 min with the following selective inhibitors: for MEK/ERK (PD98059, 25 μ M), JNK (SP600125, 10 μ M), p38 (SB203580, 10 μ M), PKC (GF109203X, 3 μ M), c-Src (PP2, 5 μ M), iPLA₂ (BEL, 20 μ M), sPLA₂ (TE-PC, 25 μ M), and cPLA₂ (AACOCF₃, 25 μ M). Cells were then stimulated with GnRH (100 nM for 8 h), and promoter activity was determined using dual-luciferase reporter assay as above. *, $P < 0.05$; ***, $P < 0.001$ control vs. GnRH treatment.

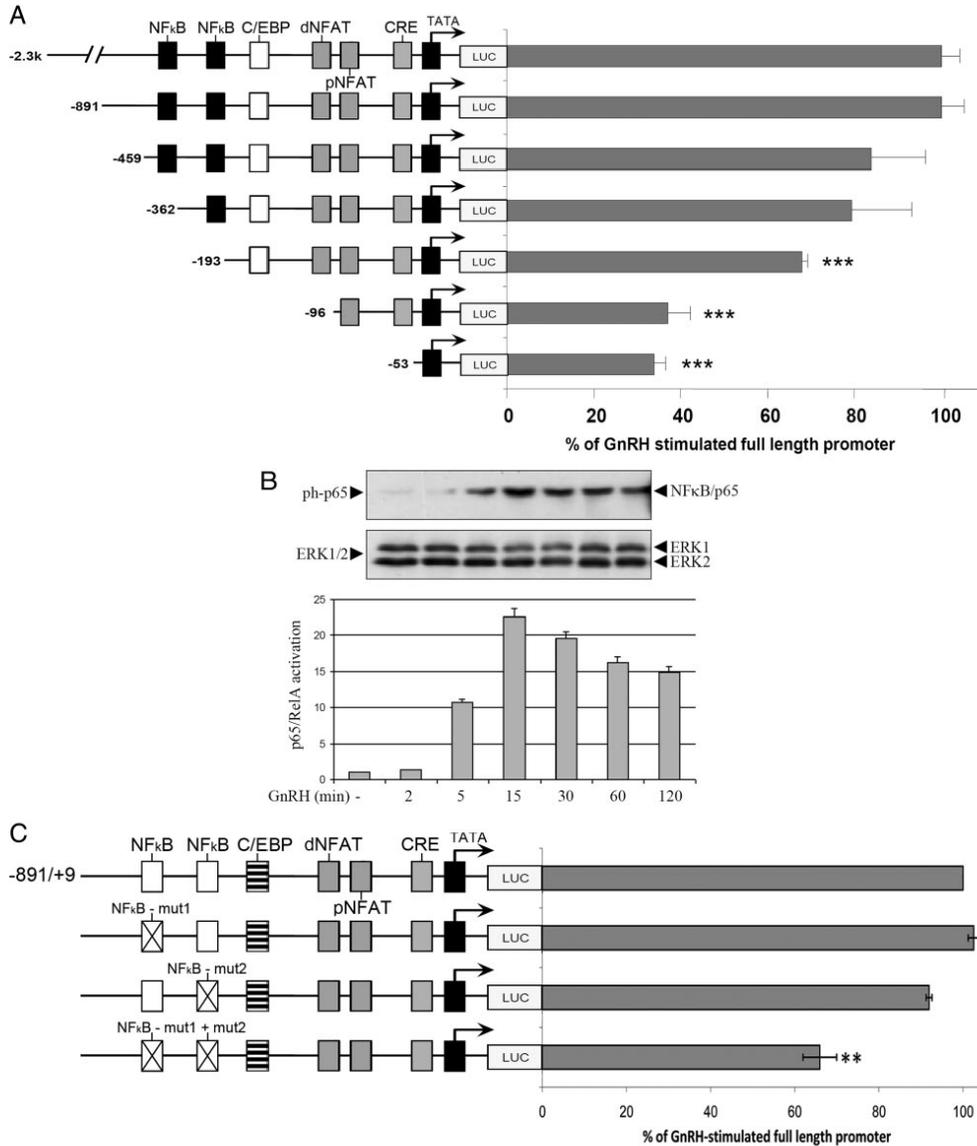


FIG. 4. Effect of GnRH on COX-2 promoter activity. **A**, Deletion analysis: subconfluent L8T2 cells were transfected with 0.33 mg of $-2307/+49$ human COX-2 promoter [pGL3.C2.2 (P6P5)] as described above or with the indicated 5'-deletions. Cells were then stimulated with GnRH (100 nM for 8 h), and promoter activity was determined using dual-luciferase reporter assay as above. **, $P < 0.01$; ***, $P < 0.001$. **B**, Effect of GnRH on NFκB activation. Subconfluent L8T2 cells were treated with GnRH (10 nM) for the time indicated, and NFκB activation was determined by Western blotting with antiphospho-p65/RelA antibody. Antigen ERK was used also for equal loading. Results are the mean \pm SEM from three experiments, each done in duplicates. **C**, Effect of NFκB mutation on GnRH-induced COX-2 promoter activity. Subconfluent L8T2 cells were transfected with 0.33 mg of $-891/+9$ human COX-2 promoter as described above or with the indicated NFκB mutants. Cells were then stimulated with GnRH (100 nM for 8 h), and promoter activity was determined using dual-luciferase reporter assay as above. **, $P < 0.01$. **D**, Effect of C/EBP mutation on GnRH-induced COX-2 promoter activity. Subconfluent L8T2 cells were transfected with 0.33 mg of $-891/+9$ human COX-2 promoter as described above or with the indicated C/EBP mutants as described in *Materials and Methods*. Cells were then stimulated with GnRH (100 nM for 8 h), and promoter activity was determined using the dual-luciferase reporter assay as above. **, $P < 0.01$; *, $P < 0.05$. NFAT, Nuclear factor of activated T cells; dNFAT, distal NFAT site; pNFAT, proximal NFAT site.

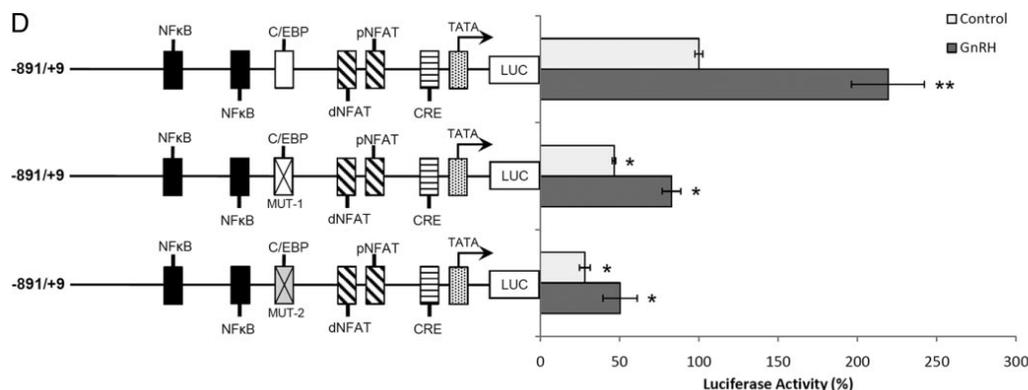


FIG. 4. Continued.

element during activation of COX-1 and COX-2 gene expression. The data are also in line with our observation that activation of MAPK by GnRH in gonadotropes is not mediated by transactivation of the EGF receptor (7, 19). We also examined the role of PLA₂, which has been implicated in COX-2 induction (20). The selective inhibitor for iPLA₂, BEL, but not for sPLA₂, TE-PC, and cPLA₂, AACOCF₃, reduced COX-1 induction by GnRH (Fig. 2C). Hence, iPLA₂ seems to be involved in both AA release and COX-1 induction. Also, the dogma that COX-1 is constitutively expressed and COX-2 is hormone regulated do not hold for GnRH-stimulated LβT2 cells because COX-1 is also regulated.

GnRH stimulates COX-2 promoter activity

We then examined the effect of GnRH on COX-2 promoter activity. LβT2 cells were transiently transfected with the COX-2 promoter and treated with GnRH for various time periods (Fig. 3A). Activation was rapid and was observed already at 2 h (2-fold, $P < 0.05$) with maximal activation observed after 8 h (3-fold, $P < 0.001$) with a decline to basal levels at 24 h. This stimulatory effect was reduced by the selective inhibitors for MEK, PD98059, for JNK, SP600125, for p38, SB203580, for PKC, GF109203X, and for c-Src, PP2, implicating the known GnRH-activated PKC/c-Src/MAPK pathway in the transcriptional control of the COX-2 promoter activity (Fig. 3B). Also, the specific inhibitors for iPLA₂, BEL, and for sPLA₂, TE-PC, but not for cPLA₂, AACOCF₃, reduced GnRH-induced COX-2 promoter activity (Fig. 3B). Addition of inhibitors had no effect on baseline levels.

5'-Deletion analysis of the COX-2 promoter

We then used 5'-deletion analysis to map the GnRH-responsive regions (Fig. 4A). LβT2 cells were transfected with luciferase reporter vectors containing successive 5'-

deletions from the COX-2 promoter and then stimulated with GnRH (Fig. 4A). GnRH responsiveness showed no decrease with the first deletion to -891 and a small but not significant decrease with the second deletion to -459. Even deletion of the first NFκB site (-362) had no significant reduction on promoter activity, which, however, was already reduced by 20% by the successive deletions. Further deletion of the second NFκB site from the COX-2 promoter (-193) reduced the GnRH response to 67% of the full activity ($P < 0.001$). Additional reduction of the promoter size to -96, which removed the C/EBP binding site, resulted in 37% of the full GnRH response ($P < 0.001$). Finally, the removal of the cAMP response element (-53) had no further inhibition on the GnRH response. Thus, the GnRH response maps to the -362/-193 region including the second NFκB site and the -193/-96 region including the C/EBP. The implication of NFκB as a mediator in COX-2 expression was interesting, and we therefore set first to check the activation by GnRH of NFκB in LβT2 cells.

GnRH activates NFκB in LβT2 cells

NFκB resides in the cytoplasm in an inactive form bound by its inhibitory proteins, members of the IκB family. A variety of cellular stimuli cause the dissociation of NFκB from inhibitory-κB, allowing NFκB to translocate to the nucleus and regulate gene expression. A number of protein kinases can phosphorylate p65/RelA and consequently potentiate the transcriptional activity of the NF-κB complexes. Because NFκB is implicated in COX-2 transcription (21), we looked for its activation by following the phosphorylation of the p65 subunit of NFκB. Interestingly, GnRH rapidly stimulated p65 phosphorylation (22-fold) the maximal effect being detected after 15 min, declining thereafter (Fig. 4B). To further verify the NFκB involvement in GnRH-induced COX-2 transcrip-

tion, we transfected the cells with wild-type or NF κ B site-directed mutant promoters and analyzed the GnRH effect. The mutation of the first NF κ B site had no effect on the GnRH response (Fig. 4C), in agreement with the 5'-deletion analysis of the COX-2 promoter (Fig. 4A). The mutation of the second NF κ B site alone resulted in 91% of GnRH-stimulated full-length promoter activity. Only double mutation of the two NF κ B sites resulted in 66% of GnRH-stimulated full-length promoter activation (Fig. 4C), in perfect agreement with the deletion analysis data (67%; Fig. 4A). However, unlike the 5'-deletion analysis, only the double mutation revealed that the two NF κ B sites, which apparently act as a composite response element, are both required and contribute some 34% to GnRH activation of COX-2 transcription.

Mutation of the C/EBP reveals its role in the GnRH response

The 5'-deletion analysis shown above implicated the C/EBP region in GnRH activation of the COX-2 promoter (Fig. 4A). To implicate the C/EBP site in GnRH-induced COX-2 transcription, we transfected the cells with wild-

type or C/EBP site-directed mutant promoters and analyzed the GnRH response. The first mutation of the C/EBP site (MUT-1) reduced by 50% ($P < 0.05$) the basal activity of the $-891/+9$ promoter (Fig. 4D). Furthermore, the GnRH response (2.2-fold) was also reduced. Maximal activity was reduced by 62%, whereas the fold increase was reduced to 1.75-fold ($P < 0.05$). The second mutation of the C/EBP site (MUT-2) reduced by 80% the basal activity

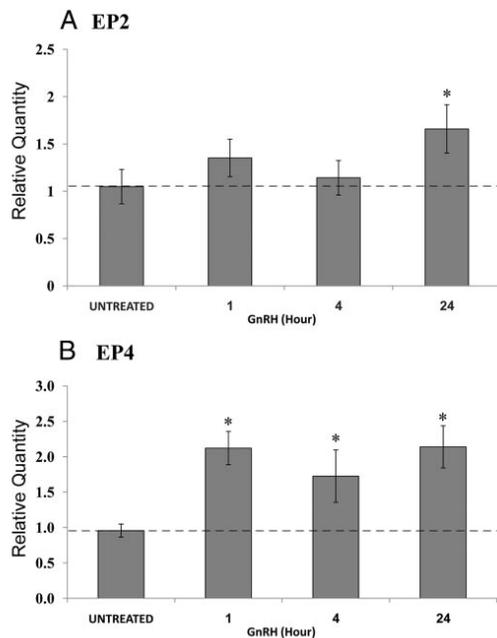


FIG. 5. Effect of GnRH on EP2 and EP4 prostanoid receptor expression in L β T2 cells. Subconfluent L β T2 cells were treated with GnRH (100 nM) for the time indicated. Expression of the PGE $_2$ receptors, EP2 and EP4, was analyzed by Q-PCR as described in *Materials and Methods*. Results are the mean \pm SEM from three experiments. Other prostanoid receptors were not affected by the GnRH treatment. *, $P < 0.05$ control vs. GnRH treatment.

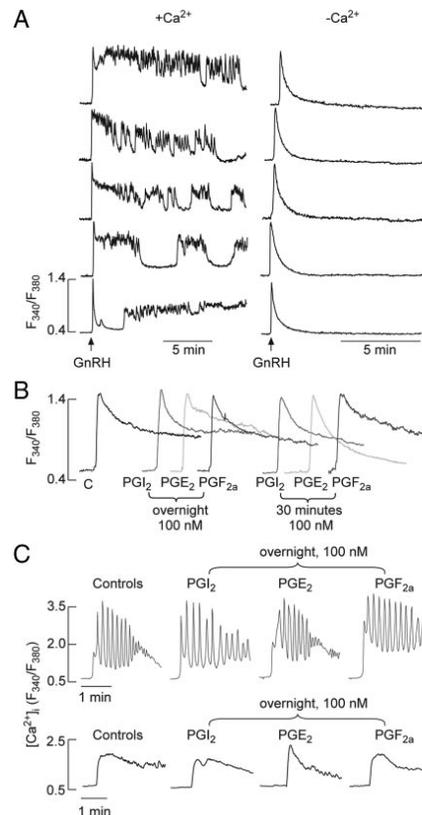


FIG. 6. Effect of PGs on GnRH-induced calcium signaling in L β T2 cells and rat pituitary gonadotropes. **A**, One hundred nanomoles GnRH induced a spike followed by fluctuations in $[Ca^{2+}]_i$ levels in cells bathed in 2 mM Ca^{2+}_e (*left traces*) and a spike followed by values that are equal or less than basal $[Ca^{2+}]_i$ in cells bathed in Ca^{2+}_e -deficient medium (*right traces*). Shown are five representative traces for each extracellular Ca^{2+}_e condition. **B**, Average traces ($n \geq 15$ in one of three similar experiments) of 10 nM GnRH-induced $[Ca^{2+}]_i$ responses in Ca^{2+}_e -containing controls and cells treated by PGs overnight (*left traces*) or for 30 min (*right traces*) before addition of agonist. **C** (*top panel*), Typical patterns of 1 nM GnRH-induced Ca^{2+}_e oscillations in rat pituitary gonadotropes 24 h after dispersion in controls and cells treated with PGs overnight. Traces shown are representative from at least 15 cells per treatment. **C** (*bottom panel*), The lack of effects of PGs on high (25 mM) K^+ -induced rise in $[Ca^{2+}]_i$. Average traces ($n \geq 15$ in one of three similar experiments) are shown.

of the $-891/+9$ promoter ($P < 0.05$), and the maximal activity was reduced by 77%, whereas the fold increase was reduced to 1.78-fold ($P < 0.05$) (Fig. 4D). Thus, the C/EBP site contributes some 65% of the basal activity and at least 20% of the GnRH-stimulated promoter activity in good agreement with the deletion analysis data.

Cross talk at the PG receptor level

We recently demonstrated the presence of the PGE₂ receptors E prostanoic (EP)-1, EP2, EP3, and EP4, the PGF_{2α} receptor F prostanoic, and the PGI₂ receptors I prostanoic in LβT2 cells (6). We also documented that PGF_{2α} and PGI₂ reduce the homologous induction of the GnRH receptor (6). It was therefore interesting to analyze

the effect of GnRH treatment on PG receptor expression. Q-PCR analysis showed consistent and significant effects only for the regulation of EP2 and EP4 (Fig. 5). A relatively rapid stimulatory effect was observed for GnRH-induced EP4 expression (2-fold at 1 h), which lasted for 24 h (Fig. 5B). The effect observed for EP2 was much slower and was detected after 24 h of incubation (Fig. 5A).

Cross talk at the calcium level

GnRH elevates intracellular Ca²⁺ concentrations ([Ca²⁺]_i), which leads to elevation of gonadotropin release (18). Therefore, we examined the cross talk between GnRH, PGE₂, PGF_{2α}, and PGI₂ in terms of Ca²⁺ signaling using LβT2 cells and gonadotropes from primary cultures.

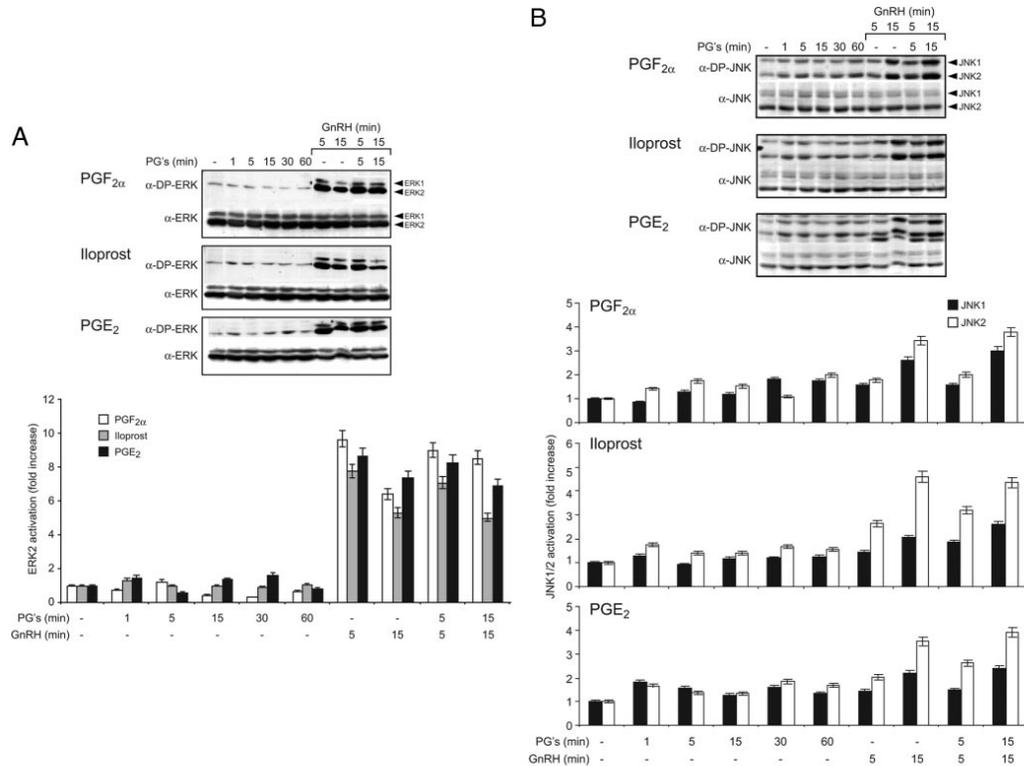


FIG. 7. Effect of PGs on basal and GnRH-stimulated ERK1/2, JNK1/2, and p38 activity. **A**, Subconfluent LβT2 cells were treated with PGF_{2α}, iloprost, or PGE₂ (100 nM) for the time indicated. In addition, some groups, which were pretreated with the PGs for 5 and 15 min, were also treated later by addition of GnRH (100 nM) for 5 and 15 min. ERK1/2 activation was determined by Western blotting with antidi-phospho-ERK antibodies. The membranes were reblotted with antigenal ERK antibody for equal loading. Results are the mean ± SEM from three experiments. **B**, Subconfluent LβT2 cells were treated with PGF_{2α}, iloprost, or PGE₂ (100 nM) for the time indicated. In addition, some groups, which were pretreated with the PGs for 5 and 15 min, were treated later by addition of GnRH (100 nM) for 5 and 15 min. JNK1/2 activation was determined by Western blotting with antidi-phospho-JNK antibodies. The membranes were reblotted with antigenal JNK antibody for equal loading. Results are the mean ± SEM from three experiments. **C**, Subconfluent LβT2 cells were treated with PGF_{2α}, iloprost, or PGE₂ (100 nM) for the time indicated. In addition, some groups, which were pretreated with the PGs for 5 and 15 min, were treated later by addition of GnRH (100 nM) for 5 and 15 min. p38 activation was determined by Western blotting with antidi-phospho-p38 antibodies. The membranes were reblotted with antigenal p38 antibody for equal loading. Results are the mean ± SEM from three experiments.

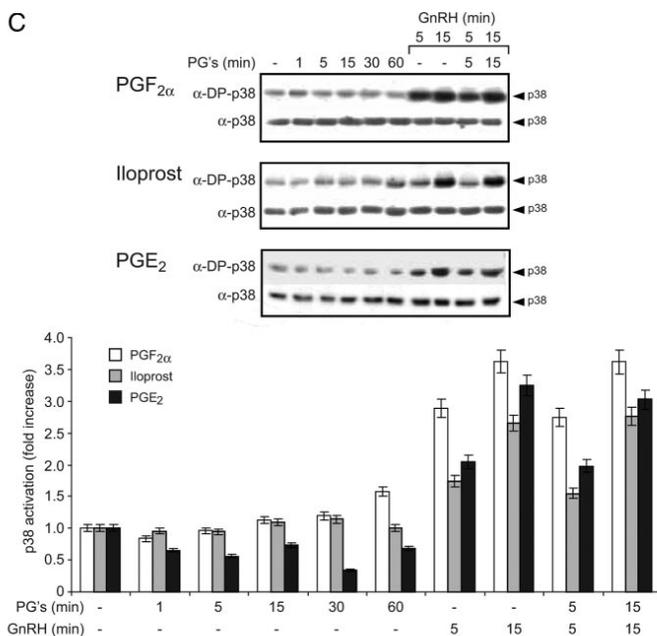


FIG. 7. Continued.

Figure 6A illustrates typical patterns of GnRH-induced Ca^{2+} signaling in $\text{L}\beta\text{T}2$ cells bathed in 2 mM Ca^{2+} -containing medium (Fig. 6A, left panel) and Ca^{2+} -deficient medium (Fig. 6A, right panel). In both experimental conditions, GnRH induced a rapid spike response. In Ca^{2+} -containing medium, the spike response was followed by fluctuations in $[\text{Ca}^{2+}]_i$ that resembled that observed in spontaneously active lactotrophs and somatotrophs in primary culture. In Ca^{2+} -deficient medium, the spike rise in $[\text{Ca}^{2+}]_i$ was followed by a decay to basal or below basal level within 2–3 min. Preincubation of cells with 100 nM $\text{PGF}_{2\alpha}$ or the stable PGI_2 analog iloprost or PGE_2 did not affect GnRH-induced elevation in $[\text{Ca}^{2+}]_i$. Figure 6B shows the average traces of 10 nM GnRH-induced Ca^{2+} responses in controls and cells treated overnight (Fig. 6B, left) or for 30 min (Fig. 6B, right) with the above PGs. In contrast to $\text{L}\beta\text{T}2$ cells, pituitary gonadotrophs in primary cultures respond to GnRH with oscillatory Ca^{2+} signals with the frequency determined by agonist concentration. Figure 6C (top panel) shows typical pattern of Ca^{2+} response in gonadotrophs from postpubertal female rats. The overnight treatments with the three PGs did not alter the pattern of Ca^{2+} signals triggered by 1 nM GnRH. The lack of effects of the PGs was also evident in cells treated with 10 nM GnRH (data not shown). To examine the possible effect of PGI_2 , PGE_2 , and $\text{PGF}_{2\alpha}$ on voltage-gated Ca^{2+} influx, we also examined the pattern of high (25 mM)

K^+ -induced Ca^{2+} signals in controls and treated cells. Figure 6C (bottom panel) shows no difference in the pattern of Ca^{2+} transients in controls and PG-pretreated cells.

Cross talk at the MAPK level

We and others have reported that GnRH stimulates MAPK (ERK, JNK, and p38) activation and that MAPKs play a role in GnRH-induced gonadotropin gene expression (18). Therefore, it was interesting to test the cross talk between GnRH, PGE_2 , $\text{PGF}_{2\alpha}$, and PGI_2 in terms of MAPK activation. $\text{PGF}_{2\alpha}$, iloprost, and PGE_2 had no detectable effect on ERK1/2 activity at 1–60 min of incubation (Fig. 7A). On the other hand, GnRH produced a robust activation of ERK1/2 at 5 and 15 min of incubation. Preincubation with $\text{PGF}_{2\alpha}$, iloprost, and PGE_2 had no inhibitory effect on GnRH-induced ERK1/2 activity (Fig. 7A).

$\text{PGF}_{2\alpha}$, iloprost, and PGE_2 had a small but not significant effect on JNK1/2 activity (Fig. 7B). On the other hand, GnRH enhanced the activation of JNK1/2, in particular at 15 min of incubation. Preincubation with $\text{PGF}_{2\alpha}$, iloprost, and PGE_2 for 5 or 15 min had no inhibitory effect on GnRH-induced JNK1/2 activity (Fig. 7B).

$\text{PGF}_{2\alpha}$, iloprost, and PGE_2 had no detectable effect on p38MAPK activity (Fig. 7C). On the other hand, GnRH markedly stimulated p38 activation at both 5 and 15 min of incubation. Preincubation with $\text{PGF}_{2\alpha}$, iloprost, and PGE_2 for 5 or 15 min had no inhibitory effect on GnRH-induced p38MAPK activity (Fig. 7C).

Cross talk at the cAMP level

We also studied the cross talk between GnRH, PGE_2 , $\text{PGF}_{2\alpha}$, and PGI_2 in terms of cAMP formation. Incubation of $\text{L}\beta\text{T}2$ cells with GnRH, PGE_2 , $\text{PGF}_{2\alpha}$, and iloprost at increasing doses for 30 min resulted in enhanced cAMP formation only in GnRH-treated cells (5-fold) (Fig. 8A). Combined treatment of the cells with GnRH and each of the PGs tested here resulted in inhibition of the stimulatory effect of GnRH on cAMP production (Fig. 8B).

Discussion

Most GPCR-activated AA release is catalyzed by the 85-kDa $\text{cPLA}_{2\alpha}$, which is known to be activated by Ca^{2+} and

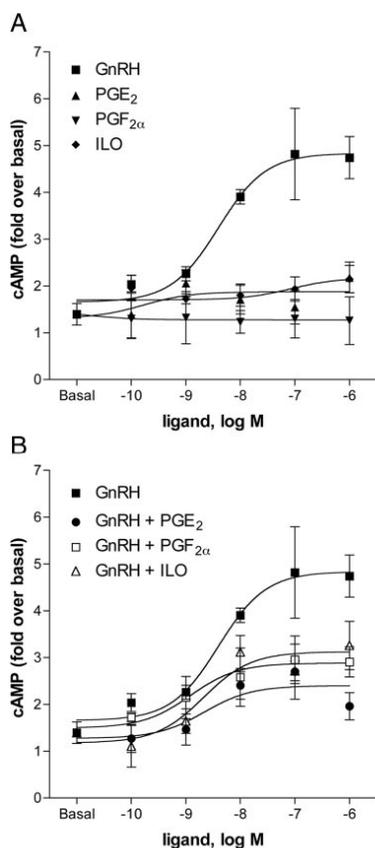


FIG. 8. Effect of PGs on GnRH-induced cAMP formation in L β T2 gonadotrophs. A, Subconfluent L β T2 cells were treated with increasing doses of PGF_{2 α} , iloprost (ILO), PGE₂, or GnRH for 30 min, and cAMP levels were determined. B, Subconfluent L β T2 cells were treated with increasing doses of GnRH with or without PGF_{2 α} , iloprost, or PGE₂ (100 nM each) for 30 min, and cAMP levels were determined. Results are the mean \pm SEM from three experiments.

Ser505 phosphorylation by MAPK (10). Because GnRH activates MAPK (14), we presumed this might be the mechanism of GnRH stimulation of AA production. However, we could not detect Ser505-cPLA_{2 α} phosphorylation in GnRH-treated L β T2 cells or translocation of cPLA_{2 α} from the cytosol to the Golgi as in other GPCRs (9). We further confirmed that there is no detectable expression of the cPLA_{2 α} (IV A) in L β T2 gonadotrope cells. Because cPLA_{2 α} is thought to be ubiquitously expressed, this presented a conundrum. We therefore resorted to the use of chemical inhibitors of the various PLA₂ enzymes (1, 10) to identify the nature of the enzyme involved in AA release. Only BEL, a selective and mechanism-based inhibitor for iPLA₂, significantly inhibited GnRH-stimulated AA

release, whereas inhibitors for cPLA₂ (AACOCF₃) and sPLA₂ (TE-PC) had no effect. BEL is a 1000-fold more selective inhibitor for iPLA₂ than for cPLA₂ (22) and 10-fold more selective for iPLA_{2 β} (VI A) than iPLA_{2 γ} (VI B) (23). Thus, the more likely PLA₂ to be involved in GnRH stimulation of AA production in L β T2 cells is iPLA_{2 β} (VI A) and not the ubiquitous cPLA_{2 α} . However, because we could detect cPLA_{2 α} in purified mouse gonadotropes, further studies are required to also confirm the above findings in purified gonadotrope cells.

It is thought that COX-1 is constitutively expressed and COX-2 is hormone regulated (13). Therefore, it was surprising to find out by Q-PCR a 20-fold increase in COX-1 gene expression in GnRH-treated cells. The use of pharmacological inhibitors revealed a role for the PKC/PI3K/c-Src/MAPK pathway but not for EGF receptor kinase in the GnRH induction of COX-1, in line with our claim that activation of MAPK by GnRH in gonadotropes is mediated by PKC and c-Src but not via transactivation of the EGF receptor (7, 14, 19). It should be noted that MAPKs, PI3K and c-Src, have previously been implicated in the induction of COX-2 (6, 24–26). Hence, both COX-1 and COX-2 seem to be similarly regulated. Because PLA₂ has been implicated in COX-2 induction (20), we tested its role in COX-1 induction by GnRH. As with the release of AA, we found a role only for iPLA₂ but not for sPLA₂ or cPLA₂. Hence, iPLA₂ mediates GnRH stimulation of AA release and is also involved in the induction of COX-1, possibly by a feed-forward mechanism via the newly formed PGs.

Several studies elucidated regulatory elements for the COX-2 but not the COX-1 promoter, and we therefore aimed to identify the relevant regulatory elements for GnRH activation of the COX-2 promoter. We first noted a relatively rapid activation of the COX-2 promoter by GnRH (2 h), which is in agreement with another report that documented that the induction of COX-2 by angiotensinII reflects an immediate-early gene response (27). As with COX-2 mRNA elevation, COX-2 transcription by GnRH is also mediated by the PKC/c-Src/MAPK pathway. Also because NF κ B is implicated in COX-2 transcription (21), we first checked whether GnRH activates NF κ B. Indeed, GnRH stimulated a robust increase in p65/Rel A phosphorylation, which indicates NF κ B activation. Although successive 5' deletion analysis of the COX-2 promoter has implicated the second NF κ B site in the GnRH response, it was the use of single and double mutants of the NF κ B sites that revealed that the presence of both sites is required for activation of the promoter by GnRH. Hence, the two NF κ B sites seem to act as a composite response element, contributing to some 44% of GnRH activation of COX-2 transcription. In addition to the contribution of

the double-NF κ B sites, the C/EBP site contributes another 25% to the GnRH response as evident by 5'-deletion analysis and mutation of the C/EBP site. On the other hand, the cAMP response element seems to have no contribution to the GnRH response. Indeed, NF κ B and C/EBP have been shown to induce COX-2 expression in a cooperative manner (28). Hence, the rise in cAMP levels by GnRH in L β T2 cells observed here does not contribute to further activation of the COX-2 promoter. Still, some 30% of the GnRH response is apparently mediated by elements within the 53 bp up to the replication start site.

The PGs produced by GnRH in L β T2 cells exert multiple effects on GnRH actions (6). PGF $_{2\alpha}$ and PGI $_2$ but not PGE $_2$ inhibited the homologous induction of the GnRH receptor. PGF $_{2\alpha}$ but not PGI $_2$ or PGE $_2$ reduced GnRH induction of LH β gene expression and inhibited GnRH-induced LH but not FSH secretion (6). We therefore searched for the mechanisms involved in the autocrine-negative feedback mechanism of the PGs as above. We ruled out the activation by GnRH of the various MAPKs (ERK, JNK, and p38) as the potential site for the negative cross talk between PGs and GnRH receptors because no inhibition by the PGs of GnRH-stimulated MAPK activation has been observed here. Similarly, we ruled out elevation of Ca $^{2+}$ levels by GnRH because the PGs had no inhibitory effect. Although cAMP production by GnRH was reduced by the PGs, we had to rule out this site also because PGF $_{2\alpha}$ and PGI $_2$ but not PGE $_2$ inhibited GnRH receptor expression, whereas all three PGs reduced GnRH-induced cAMP production. We are therefore left with inhibition of phosphoinositide turnover as the most likely mechanism responsible for the inhibitory cross talk (6). Indeed, PGF $_{2\alpha}$ and PGI $_2$ but not PGE $_2$ inhibited GnRH-stimulated phosphoinositide turnover (6). Furthermore, we could link the inhibition of phosphoinositide turnover to inhibition of GnRH receptor expression because the phospholipase C β inhibitor U73122 produced a dose-related parallel inhibition of GnRH-induced phosphoinositide turnover and GnRH receptor expression (6). Thus, inhibition of GnRH receptor expression by PGF $_{2\alpha}$ and PGI $_2$ appears to be mediated by inhibition of phosphoinositide turnover.

Still, our findings present an enigma because the dogma is that EP2 and EP4 activation by PGE $_2$ and IP activation by PGI $_2$ results in cAMP formation (29). In contrast, we report here that PGF $_{2\alpha}$, PGI $_2$, and PGE $_2$ inhibit GnRH stimulation of cAMP formation. It should be noted that L β T2 cells express the PGE $_2$ receptors, EP1, EP2, EP3, EP4; the PGI $_2$ receptor IP; and the PGF $_{2\alpha}$ receptor FP (6). It is therefore likely that the presence of multiple receptors in the same cell may result in a different signaling profile from that obtained by overexpressing a single prostanoid

receptor. Interestingly, although GnRH and pituitary adenylyl cyclase-activating polypeptide are both known to stimulate cAMP formation in L β T2 cells, cocubation of both resulted in GnRH inhibition of pituitary adenylyl cyclase-activating polypeptide-induced cAMP formation (30).

The PG-GnRH receptor cross talk examined here and previously (6) provides a novel inside-out molecular mechanism to regulate GnRH receptor number and to mediate the differential LH and FSH secretion during GnRH action.

Acknowledgments

We thank Fiorenza Prezeddecki and Sharon Battersby for excellent technical assistance. We thank P. Mellon for the cell lines and R. Newton, K. Wu, and M. A. Iniguez for the COX-2 plasmids.

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This work was supported by the Medical Research Council (United Kingdom) (to R.P.M.), the Israel Science Foundation (Grant 221/05), the German-Israeli Foundation for Research and Development (Grant I-751-168.2/2002), U.S.-Israel Binational Science Foundation (Grant 2007057), and the Adams Super-Center for Brain Studies at Tel-Aviv University (to Z.N.).

Disclosure Summary: M.N., B.S., R.F., A.J.P., H.N.J., K.M., J.J., M.T., S.S., N.S., and Z.N. have nothing to disclose. R.P.M. consults for Ardana PLC.

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Appendix B

Expression and Regulation of the β -Subunit of oFSH Relies Heavily on a Promoter Sequence Likely to Bind Smad-Associated Proteins

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Published in Endocrinology, 2007, Vol. 148: 4500-4508.

Expression and Regulation of the β -Subunit of Ovine Follicle-Stimulating Hormone Relies Heavily on a Promoter Sequence Likely to Bind Smad-Associated Proteins

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FSH is essential for normal gonadal function in mammals. Expression of its β -subunit (FSHB) controls overall production/secretion of FSH and is induced by activin. Studies with ovine *FSHB* promoter/reporter constructs in $L\beta T2$ gonadotropes show that induction by activin requires a putative Smad binding element in the ovine *FSHB* promoter (-162AGAC-159). Similar studies reported here show that another site, juxtaposed to the Smad binding element, was also required for 81% activin induction in $L\beta T2$ cells. This site was similar to several that bind proteins known to partner with Smads. When this site (-171ACTgegtTT-163) was mutated by changing the nucleotides shown in lowercase letters, the resulting ovine-derived construct (mut-*oFSHBLuc*) was expressed poorly as a transgene in primary mouse gonadotropes (<0.001 times compared with ovine wild-type transgenes).

This decrease in expression demonstrated the importance of this site for activin induction and, perhaps, basal expression, although studies with $L\beta T2$ cells did not suggest this latter possibility. Expression of mut-*oFSHBLuc* in male mouse gonadotropes *in vivo* was at least 644 times greater than expression in all but one nongonadotrope tissue tested, indicating that mut-*oFSHBLuc* retained significant gonadotrope-specific expression. An increase in FSHB expression occurs during estrus in mice and is faithfully reproduced with wild-type ovine *FSHBLuc* transgenes, but not with mut-*oFSHBLuc*, indicating that the mutated site is needed for this secondary FSH surge. These data suggest that activin gathers Smads and Smad-associated proteins at the -171/-159 promoter region to regulate expression of the ovine FSHB and overall FSH production. (*Endocrinology* 148: 4500–4508, 2007)

FSH IS ESSENTIAL for ovarian follicular development, and enhances the maturation and performance of sperm (1). Because of its importance, FSH is carefully regulated by more than six reproductive hormones that ensure its correct production and secretion. One of these hormones, activin, is recognized as an important inducer of FSH. Studies by others show that follistatin and inhibin (known inhibitors of activin action) decrease FSH production by 50% in primary rat pituitary cultures (2) and lower serum levels of FSH in ovariectomized rats by 50–60% within 4–5 h (3). More recent data show that mice lacking type II activin receptors produce significantly less FSH than normal (~34% normal males) (4). In an attempt to learn more about the molecular mechanism(s) used by activin at the gene level, studies have recently focused on transcriptional regulation of the FSH β -subunit (FSHB), which is rate limiting for overall FSH production.

Ovine *FSHB* promoter/reporter constructs (*oFSHBLuc*; 4.7 kb of the ovine *FSHB* promoter plus intron 1 attached to the luciferase gene) are expressed in $L\beta T2$ transformed gonadotropes in ways that permit identification of promoter elements associated with induction by activin. Because Smads

are recognized activin-activated transcription factors, putative Smad binding elements (SBEs) were sought and found in the proximal promoter of ovine *FSHB*. Destruction of the putative SBE at -162 bp (AGAC) is especially effective in blocking activin induction of reporter genes driven by the ovine *FSHB* promoter (5).

Similar studies in our laboratory, reported here, had discovered sequences adjacent to the -162-bp consensus Smad binding site that were also important for ovine FSHB expression in $L\beta T2$ cells in culture (6). This DNA sequence was recently identified by computer analysis to be a Runx1 binding site (7). This correlation is significant because Runx family members are known to bind the same sequence and interact with Smads through their Runt homology domains (8, 9). However, the correlation does not prove that any Runx family member binds this sequence. In fact, the DNA sequence of interest also resembles binding sites for forkhead proteins such as FAST-1 (FoxH1), a well-characterized DNA binding partner of Smads 2 and 3 that mediates many actions of TGF β 1 and activin (9, 10). Therefore, it is possible that forkhead transcription factors may, in fact, act through the putative Runx1 binding site to partner with Smads for the induction of FSHB in gonadotropes. The important point is that the sequence of interest in this report is associated with transcription factors known to partner with Smads.

To date, no laboratory using methods such as EMSA has been able to demonstrate that activin promotes the association of any protein to either the putative Runx1 or SBE

First Published Online June 21, 2007

Abbreviations: ECM, Estrous Cycle Monitor; FSHB, FSH β -subunit; RLU, relative light unit; SBE, Smad binding element.

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

binding site. Therefore, both binding sites remain “putative” and will be referred to this way herein because their abilities to bind these proteins have not been established. In addition, the importance of these sites has been established only in L β T2 cells. Thus, it is important to show the physiological relevance of this promoter region for FSHB expression and activin induction *in vivo*. The studies described here were designed to demonstrate the importance of this novel sequence *in vitro* and also in transgenic mice *in vivo*.

Materials and Methods

Hormones (activin A, follistatin-288, and D-Lys⁶-GnRH)

Recombinant human follistatin-288 was provided by the National Pituitary and Hormone Program of the National Institute of Diabetes and Digestive and Kidney Diseases. Recombinant human activin A was obtained from R&D Systems, Inc. (Minneapolis, MN) and was dissolved in PBS containing 0.1% serum albumin. [D-Lys⁶]-GnRH (referred to as GnRH in this text) was purchased from Sigma Chemical Co. (St. Louis, MO) and was dissolved in 0.01 M acetic acid before use.

Universal RIA for FSH (pan-FSH RIA)

Sheep and mouse FSH were measured using a pan-FSH RIA distributed by the National Institute of Diabetes and Digestive and Kidney Diseases using a double-antibody method as previously reported (11). Purified mouse FSH (AFP-5308D) was used as tracer and standard for the mouse assay, and 95% pure ovine FSH standard was used for the sheep assay. Intraassay variation for each RIA was less than 10%, and assay slopes were more than one.

Plasmid constructs

Our wt-oFSHBLuc, reported in 2001 (11), was used in this study to transiently transfect L β T2 cells and also to produce all the other constructs in this study. This wild-type construct contains 4.7-kb ovine FSHB promoter plus intron 1 driving luciferase expression. The constructs used in Fig. 2 were made from wt-oFSHBLuc by producing point mutations that created novel restriction sites: constructs 1, 4, 5, 6, 9, 10, 11, and 12 contained distinguishing Bgl2 sites; construct 2 had a novel Mlu1 site; construct 3 had an Xho1 site; and construct 8 had a new EcoRI site. All plasmids were sequenced and shown to contain the sequences depicted in Fig. 2 (SeqWright, Houston, TX). Construct 3 contained a mutation that destroyed the putative Runx1 enhancer located between -171 and -163 bp of the ovine FSHB promoter. This construct was transiently expressed in L β T2 cells and also was used as a transgene in mice. The transgene made from construct No. 3 was named mut-oFSHBLuc in this report.

Our laboratory has produced and reported on 12 transgenic mouse lines that contained the intact wild-type promoter (11, 12), but these mice were all terminated by the time this study began. These former transgenic mouse lines were replaced by seven new lines that expressed luciferase as well as the original wild-type transgenes. These new lines contained distal 5' deletions (deletions 5' to -2817 bp), but all expressed luciferase in the same range as the original wild-type transgenic mice. Furthermore, luciferase expression was also regulated by activin in a similar manner. One of these lines (Lj) was chosen as the wild-type “standard” for this study because it produced average amounts of luciferase activity (average luciferase activity with regard to all previous wild-type transgenic lines) and was regulated by activin in a representative manner. The wtLj-oFSHBLuc transgene that was used in this study lacked sequences from -4736 to -3980 bp and -3398 to -2817 bp.

All the transgenes used or referred to in this study were cut from their parent plasmid by digestion with BamHI/KpnI, which left less than 100-bp plasmid sequence on each end of the transgene. As with all previous transgenes produced in this laboratory [wt-oFSHBLuc (11) and mut-oFSHBLuc- Δ AP1 (12)], wtLj-oFSHBLuc and mut-oFSHBLuc reported here were expressed in every founder line that carried the respective transgene.

Animal care: sheep and transgenic mice

Ewes and transgenic mice were maintained and studied with the approval and oversight of the Institutional Animal Care and Use Committee at the University of North Carolina Chapel Hill or North Carolina State University. Mice containing wild-type (wtLj-oFSHBLuc) or mutant (mut-oFSHBLuc) transgenes were produced in B6SJL mice as described earlier (11), but at the transgenic mouse facility of the University of North Carolina Chapel Hill. Mice were bred and cared for at the Biological Resource Facility of North Carolina State University. Retired breeder ewes were kept at a North Carolina State University farm. Testing mice for the presence of a transgene and measuring luciferase activity in tissues was performed as previously reported (11, 12).

Cell cultures

Primary cells from ewe pituitaries were dispersed and cultured in 24-multiwell tissue culture plates with 200,000 cells per well, as described previously (13). Primary cell cultures from mouse pituitaries were also prepared as described (11, 12) and incubated in 96-well tissue culture plates at 30,000–60,000 cells per well in media 199 plus 10% charcoal-treated sheep serum. Cells were allowed to attach and adjust to culture conditions for at least 1 d before treatment. Purified gonadotropes were obtained from 8-wk-old crossbred male mice hemizygous for wt-oFSHB-H2K⁸ (14) and mut-oFSHBLuc. Purification was as described (14), except the biotin anti-H-2K⁸ antibody (BD Pharmingen, San Diego, CA) was doubled to increase gonadotrope yield to approximately 16,000 per pituitary (>90% recovery of 95% pure gonadotropes).

Luciferase data in Fig. 5 were obtained from cultures treated on the afternoon of d 2 with or without 250 ng/ml follistatin for 16 h. Media containing follistatin were removed on the morning of d 3, cultures were washed once with fresh media, and then incubated in medium 199 with 1% serum instead of 10% serum (to restrain autocrine/paracrine production of activin) and the following hormones: follistatin (250 ng/ml), activin (50 ng/ml), GnRH (1 nM), or activin plus GnRH. After 6 h, cells were assayed for luciferase activity.

Primary sheep pituitary cultures are known to express robustly FSH for weeks without a decline in production (15), so studies with ewe cultures extended over 5 d. By contrast, mouse pituitary cultures express FSH best on d 2 and 3, after which FSH expression declines sharply and unpredictably (11). Thus, experiments with mouse cultures were completed during the d 2–3 timeframe after pituitary dispersion. Either female or male mouse pituitaries were used because preliminary studies showed that there was no sex difference in response to activin, follistatin, or GnRH in tissue culture (data not shown).

Transformed gonadotropes (L β T2 cells) were cultured at 37 C in DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% FBS (HyClone Laboratories, Inc., Logan, UT) and plated at a density of 30,000 cells per well in 96-well culture plates. Cells were transfected 24 h later in triplicate or quadruplicate with 50 ng plasmid as described (16). Briefly, 50 ng wild-type or mutant ovine FSHB plasmids were transfected into each well. Cells were also cotransfected with 5 ng/well pRL-TK, a plasmid used to express Renilla as an internal standard for transient expression assays (Promega Corp., Madison, WI). Transfections were performed with Fugene 6 (Roche, Indianapolis, IN) using 165 ng/well. Plasmids and Fugene 6 were incubated in serum-free DMEM for 15 min at RT, and then 50 μ l was added to each well. After 24 h, cells were treated with or without 50 ng/ml activin for 22 h and then assayed for luciferase activity.

Induction of transgenes during the reproductive cycle

Female mice (4 months old) were housed in cages containing male bedding for 2 wk before testing to promote estrous activity. Littermates (six to eight) were paired with each other for these studies with equal numbers being assigned to either diestrus or estrus groups; with odd-numbered litters, the extra mouse was assigned to the estrus group. Reproductive stages were determined using an Estrous Cycle Monitor (ECM) (Model EC40; Fine Science Tools, Foster City, CA), followed by cytological inspection of vaginal exfoliative cells as reported earlier (11, 12). Diestrus was characterized by an ECM reading of 2.5–3.5 and a preponderance of small round leukocytes. Estrus was characterized by an ECM reading of 9–11 and the presence of large nucleated cells plus

some leukocytes. After determining the reproductive status of each mouse (between 0900 and 1100 h), pituitaries were immediately removed, homogenized, and assayed for luciferase activity and protein content as described earlier (11, 12). Data from littermates were compared to determine “fold-induction at estrus.”

Firefly and Renilla luciferase assays

Firefly luciferase was measured to detect expression of the wt-*oFSHBLuc* or mut-*oFSHBLuc* transgenes in mouse pituitary cultures or mouse tissues. For primary pituitary cultures, cells were lysed in 96-well plates with 50 μ l Passive Lysis solution (Promega Corp.), followed by analyzing 35 μ l with a Luciferase Assay System (Promega Corp.). Luminescence for data in Table 1 and Fig. 3 was obtained using a Monolight 2000 as previously reported (11, 12, 16), and data are reported as relative light units (RLUs). L β T2 cells (Figs. 2, 4, and 5) were lysed with 25 μ l Passive Lysis Buffer (Promega Corp.), and 10 μ l was assayed for luciferase activity using an automated 1420 Victor-Light micro plate luminometer (PerkinElmer, Waltham, MA). The Victor-Light luminometer was five times more sensitive than the Monolight luminometer, but all experiments in this study were done with either one or the other, and no comparisons were necessary between instruments.

Firefly luciferase was measured in whole tissue (pituitary, liver, gonads, spleen, lung, forebrain) that was excised within 5 min of death and immediately frozen in liquid nitrogen until the assay, which occurred within 24 h of tissue extraction. Pituitary tissue (~1 mg wet weight) and nonpituitary tissue (~30 mg) were homogenized in 100 μ l Passive Lysis buffer, and 20 μ l of each sample was assayed in duplicate. Protein concentrations were determined using bicinchoninic acid (Pierce Chemical Co., Rockford, IL).

Renilla luciferase expression was used as an internal standard when the activities of mutant ovine *FSHBLuc* constructs were compared in L β T2 cells. The *Renilla* construct (pRL-TK; 5 ng/well) was cotransfected with 50 ng/well of ovine *FSHBLuc* construct (Fig. 2). Both Firefly and *Renilla* luciferase activities were sequentially measured in the same samples using the Dual-Luciferase Assay System (Promega Corp.). For the data in Fig. 2, L β T2 cells in 96-well plates were lysed with 25 μ l Passive Lysis solution, followed by analysis of 10 μ l lysate.

Quantifying transgene copy number per cell

Transgene copy number was quantified in transgenic mouse lines using real-time PCR performed in an iCycler (Bio-Rad, Inc., Hercules, CA) with TaqMan technology. The luciferase gene was quantified, and 11 luciferase standards were prepared (0.016–17 pg/ μ l) using our pGL3-based original wild-type ovine *FSHBLuc* plasmid (10,345 bp) (11). This plasmid was quantified using a Qubit Fluorometer (Invitrogen, Eugene, OR) following their recommended protocol and Quant-iT dsDNA BR assay kit (Invitrogen). Samples were then diluted in distilled water containing 10 μ g/ml salmon sperm DNA as carrier (Invitrogen). Two microliters of DNA were assayed per sample starting at 95 C for 3 min, and then for 40 complete cycles (95 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec). There was a final extension step of 72 C for 3 min. Threshold cycle values were determined with Bio-Rad software and plotted *vs.* log of plasmid DNA, and ultimately *vs.* log of copy number based on the amount of chromosomal DNA assayed per sample (20 ng). The standard curve had a threshold cycle axis with values from 19–30 and an x-axis

from 0.1–3.0 genomic copies per cell ($r^2 = 0.999$). The forward oligonucleotide was 5'-GAACTGTGTGTGAGAGGTCCTATG-3', the reverse oligonucleotide was 5'-GCTATGTCTCCAGAATGTAGC-3', and the FAM probe was 5'-ACCAACGCCTTGATTGACAAG-3'. All oligonucleotides were complementary to sequences within the luciferase portion of the transgene.

Statistical analysis

Statistical calculations were performed using Prism version 4 (GraphPad software, Inc., San Diego, CA). The Student's *t* test was used when comparing two means. When more than two means were compared, one-way ANOVA was used with Tukey's multiple comparison test. Data are reported as means \pm SEM throughout the paper.

Results

Expression of FSH and its inhibition by follistatin in ovine pituitary cultures

The data in Fig. 1 show that FSH was expressed in primary ovine pituitary cultures and that follistatin (25, 75, 225, and 675 ng/ml) blocked FSH production with an IC₅₀ near 20 ng/ml during the first 24 h. By d 2, 225 and 675 ng/ml inhibited FSH production by 95% and 99%, respectively. These data created a normal saturation curve predicted by an IC₅₀ of approximately 20 ng/ml. Ovine cultures were maintained 5 d, and the media were collected every 24 h with new media replacing the old. Therefore, each FSH value represented hormone that had accumulated during the previous 24-h period. Because activin action is not halted immediately by follistatin (there is typically a 4-h delay before inhibition by either inhibin or follistatin; Miller, W. L., unpublished data), the small amount of FSH observed after 1-d treatment with 225 or 675 ng/ml follistatin (~14% compared with control) was probably secreted before follistatin became fully effective against activin action.

Activin induction of wild-type and mutant ovine *FSHBLuc* constructs in L β T2 cells

The data in Fig. 2 show that expression of wt-*oFSHBLuc* increased 7.1-fold after a 22-h treatment with activin in L β T2 cells (Fig. 2, *wt*). Activin also routinely increased the expression of *Renilla* by 50–75% (data not shown), and all luciferase data were corrected for alterations in expression of *Renilla* with or without activin. Mutations from –174 to –143 bp revealed an activin sensitive region between –168 and –157 bp. Mutations at the edges of this region (constructs 1, 2, 10, 11, and 12) all showed induction of 6.9 ± 0.3 , which was not different from wild-type induction. Mutations within the

TABLE 1. Luciferase activity in mut-*oFSHBLuc* transgenic mouse tissues

Founder	Sex	Pituitary	Liver	Gonad	Spleen	Lung	Brain
M248	M (5)	28 \pm 3	1.0 \pm 0.10	0.8 \pm 0.04	0.1 \pm 0.06	0.3 \pm 0.04	7.5 \pm 1.1
	F (5)	38 \pm 2	0.6 \pm 0.01	1.0 \pm 0.05	ND	ND	8.4 \pm 0.05
M157	M (5)	8 \pm 1	0.33 \pm 0.01	0.31 \pm 0.02	0.07 \pm 0.04	0.16 \pm 0.02	1.30 \pm 0.04
	F (5)	6.4 \pm 0.6	0.19 \pm 0.01	0.22 \pm 0.01	ND	ND	1.02 \pm 0.11
M230	M (4)	5.2 \pm 0.2	0.23 \pm 0.02	0.08 \pm 0.00	ND	ND	0.42 \pm 0.02
	F (5)	7.1 \pm 0.4	0.21 \pm 0.02	0.11 \pm 0.01	ND	ND	0.72 \pm 0.04
M251	M (6)	5.8 \pm 0.8	0.18 \pm 0.03	0.18 \pm 0.02	ND	ND	0.93 \pm 0.13
	F (6)	6.2 \pm 0.4	0.15 \pm 0.01	0.15 \pm 0.01	ND	ND	0.83 \pm 0.05

Expression of the mut-*oFSHBLuc* transgene in tissues. Tissues were harvested from mice between 7 and 20 weeks old, and lysates were assayed for luciferase activity. Values are luciferase activity expressed as RLUs $\times 10^{-3}$ /mg protein. The number of animals used is designated in parentheses according to “sex,” and values represent the mean \pm SEM for each group of mice. M, Male; F, female; ND, not determined.

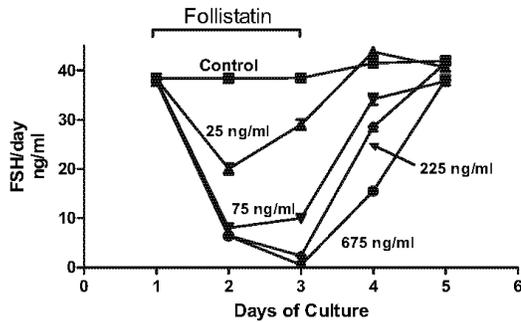


FIG. 1. Follistatin inhibited FSH production in primary ovine pituitary cultures. Ewe pituitary cultures were prepared and treated with 25, 75, 225, or 675 ng/ml follistatin on d 1, and media were collected for RIA every 24 h thereafter through d 5. Follistatin treatment ended on d 3, after which cultures were washed once with fresh media, and then new media were added and assayed/changed every 24 h as previously described. Inhibition was $99 \pm 1\%$ on d 3 with 675 ng/ml follistatin. Expression from all treatments was statistically the same on d 5. To avoid normalizing data, results are the mean \pm SEM from a representative sheep (ewe) pituitary culture, and each data point was obtained from triplicate treatments each assayed in duplicate.

sensitive region (constructs 3–9) showed an average induction of only 2.2 ± 0.1 . Thus, mutations within the sensitive region reduced induction by $68 \pm 6\%$. Statistically, there was no difference among the responses of the following fully responsive constructs: 1, 2, 10, 11, and 12. Likewise, there were no statistical differences among the responses of constructs that showed reduced induction by activin (constructs 3–9). There were no significant changes in the basal expression of any construct compared with wild-type basal expression.

Low expression of *mut-oFSHBLuc* (construct 3 in Fig. 2) in transgenic mice

Figure 3 shows the specific activities of luciferase expression in male pituitaries from 10 transgenic mouse lines that expressed *mut-oFSHBLuc* in a hemizygous manner. Luciferase expression for all founder lines in Fig. 3 (except M248 and M231) averaged $9.0 \times 10^3 \pm 3 \times 10^3$ RLU/mg protein. Expression for M248 and M231 was $34 \times 10^3 \pm 2 \times 10^3$ RLU/mg protein and $99 \times 10^3 \pm 8 \times 10^3$ RLU/mg protein, respectively. Because several transgenes can insert into a single chromosomal locus, the number of transgenes present in each cell for each transgenic line was quantified. All mice shown in Fig. 3 contained 12–23 copies of the transgene per cell except M231, which contained 283 copies per cell (data not shown). Two transgenic lines were excluded because they contained ≥ 3000 copies of the transgene, although these two lines did not express luciferase significantly differently from the average low expression of 9×10^3 RLU/mg protein.

Pituitary expression of the wild-type transgene, *wtLj-oFSHBLuc*, was $13,100 \times 10^3 \pm 120 \times 10^3$ RLU/mg protein, which is nearly 1500 times greater than the average of all *mut-oFSHBLuc* lines in Fig. 3, excluding M248 and M231. The wild-type mice expressing *wtLj-oFSHBLuc* contained only two transgene copies per cell. Finally, it should be noted that

even the highest expressing mutant line (M231) did not express *mut-oFSHBLuc* at a level higher than 0.7% of the wild-type construct, *wtLj-oFSHBLuc*.

Tissue-specific expression of *mut-oFSHBLuc* in transgenic mice

Table 1 shows luciferase expression in the pituitaries, livers, gonads (testis and ovaries), and forebrain of mice from four founder lines harboring *mut-oFSHBLuc*. Luciferase activities were also determined for the spleen and lung in two of these lines (M248 and M157). In essentially all cases, expression of the transgene in nonpituitary tissue was $\leq 3\%$ of that found in pituitary tissue, except for the forebrain region, where expression averaged 16% of that found in the pituitary.

Our laboratory previously showed that the *wt-oFSHBLuc* transgene is expressed almost exclusively in pituitary gonadotropes, which comprise only 3–5% the male mouse pituitary (14). To determine gonadotrope-specific expression of *mut-oFSHBLuc*, only gonadotropes were isolated from male transgenic mice (M248 line only), and their expression was compared with that of unpurified dispersed pituitary cells in the presence of 50 ng/ml activin (same conditions as for wild-type in Ref. 14). In three independent experiments, the gonadotrope fraction showed 23 ± 3 -fold more luciferase activity than unpurified dispersed cells. These data are consistent with gonadotropes containing all the activity for *mut-oFSHBLuc*. Therefore, specific activities in the pituitary column of Table 1 could be justifiably multiplied by 23-fold to represent only gonadotrope tissue because gonadotropes accounted for only 4.3% (1/23rd) of pituitary tissue on average. Using these new calculations, gonadotrope-specific expression was 644:1 (liver), 805:1 (gonads), 6440:1 (spleen), 2147:1 (lung), and 86:1 (brain) for male mice from the M248 transgenic line.

Expression of *mut-oFSHBLuc* did not increase at estrus

The data in Fig. 4 show that expression of *wtLj-oFSHBLuc* was increased at the time of estrus by 3- to 25-fold, with an average value of 10.4 ± 6.8 . This widely variant value was similar to that found for 10 of the wild-type constructs previously reported (10.2 ± 2) (11, 12). The data in Fig. 4 show that no increase in expression occurred for *mut-oFSHBLuc* during estrus compared with diestrus. The mean ratio \pm SEM for *mut-oFSHBLuc* was 0.7 ± 0.2 , which indicates no increase in expression at estrus.

Activin did not induce *mut-oFSHBLuc* in primary pituitary cultures

The data in Fig. 5A (*WT Mouse FSH*) are similar to those in Fig. 1, except that mouse pituitary cultures were used instead of ovine pituitary cultures. Follistatin (250 ng/ml) decreased FSH secretion by an average of 71%, indicating that follistatin-sensitive factors, presumably activins, were stimulating the majority of endogenous FSH expression/secretion in mouse pituitary cultures. FSH was measured by RIA. Data in Fig. 5B (*WT-Luc*) show similar inhibition by follistatin of the *wtLj-oFSHBLuc* transgene, which is known

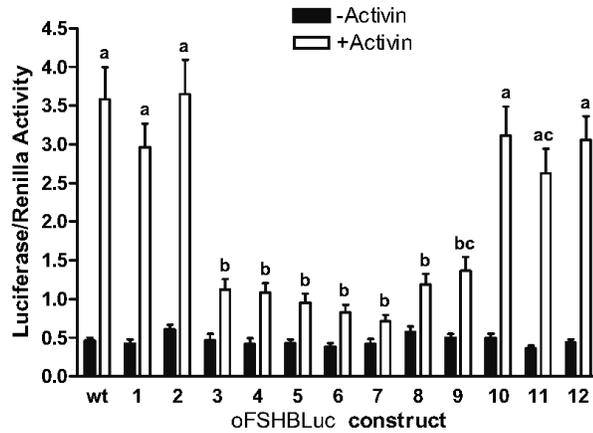


FIG. 2. Basal and activin-induced expression of *oFSHBLuc* constructs in $L\beta T2$ cells. Cells were transiently cotransfected with an internal standard (*Renilla*) plus wild-type (wt) *oFSHBLuc* or 12 different mutant *oFSHBLuc* constructs. Cultures were treated with or without 50 ng/ml activin A for 22 h, and assayed for luciferase and *Renilla* activities. The luciferase/*Renilla* ratios of activities are reported as the mean \pm SEM of three independent experiments each assayed in triplicate. Bars with different letters are significantly different ($P < 0.05$). Bars with the same letter are not different from each other ($P > 0.05$). The wild-type sequence appears at the top of the table, and all 12 mutations (bold letters) appear below it. The putative Runx1 and SBE sites are labeled.

	Runx1												SBE		
wt	-175	ATC	TAC	TGC	GTT	TAG	ACT	ACT	TTA	GTA	AGG	CTT	-143		
1		AAG	ATC	TGC	GTT	TAG	ACT	ACT	TTA	GTA	AGG	CTT			
2		AAC	GCG	TGC	GTT	TAG	ACT	ACT	TTA	GTA	AGG	CTT			
3		ATC	TAC	TCG	AGT	TAG	ACT	ACT	TTA	GTA	AGG	CTT			
4		AAG	ATC	TGC	TGT	TAG	ACT	ACT	TTA	GTA	AGG	CTT			
5		AAG	ATC	TGC	GTG	GAG	ACT	ACT	TTA	GTA	AGG	CTT			
6		ATC	TAC	TGC	GTT	AGA	TCT	ACT	TTA	GTA	AGG	CTT			
7		ATC	TAC	TGC	GTT	TCT	AGA	ACT	TTA	GTA	AGG	CTT			
8		ATC	TAC	TGC	GTT	TAG	ATA	TCT	TTA	GTA	AGG	CTT			
9		ATC	TAC	TGC	GTT	TAG	ACG	CCT	TTA	GTA	AGA	TCT			
10		ATC	TAC	TGC	GTT	TAG	ACT	AAG	TTA	GTA	AGA	TCT			
11		ATC	TAC	TGC	GTT	TAG	ACT	ACT	TTT	TTA	AGA	TCT			
12		ATC	TAC	TGC	GTT	TAG	ACT	ACT	TTA	GTA	AGA	TCT			

bp # | -175 to -170 | -169 to -160 | -159 to -150 | -149 to -143

to be induced by activin and inhibited by follistatin. Data in Fig. 5, C and D, show relatively low expression of mut-*oFSHBLuc* in pituitary cultures from M231 or M248 transgenic mouse lines. Expression was not inhibited by follistatin, suggesting that they are not induced by activin.

Finally, the data in Fig. 5, C and D, show that neither activin A nor GnRH individually induced expression of mut-*oFSHBLuc* in primary pituitary cultures previously treated with follistatin for 16 h (*i.e.* deprived of activin-like paracrine stimulation). The combined effects of both activin and GnRH significantly increased expression of mut-*oFSHBLuc* by approximately 90% within the 6-h treatment period.

Discussion

Previous studies in rats showed that iv injections of either follistatin (80 μ g) or inhibin (50 μ g) decreased serum FSH by 60% (3). These studies showed that FSH production was significantly dependent on activins *in vivo* but were not designed to determine the full extent of this dependency. More recent studies focused on induction of FSH by activin and pulsatile GnRH with perfused rat pituitary cultures (Fig. 1 of Ref. 17) or activin alone with transformed $L\beta T2$ gonadotropes (Fig. 2 of Ref. 16). These studies indicate that expres-

sion of rodent FSH is heavily dependent on activin, perhaps 95–99% dependent, regardless of GnRH treatment.

Figure 1 of this study reinforces the aforementioned concept, especially for sheep because follistatin (225 or 675 ng/ml) suppressed FSH expression by 95 or 99%, respectively, in primary ewe pituitary cultures. It was considered important to show this level of inhibition for ovine FSH because it was the ovine *FSHB* gene studied here. It is not fully known what factors are produced in static cultures of mixed pituitary cells to stimulate expression of FSH, but evidence suggests that highly potent activin B (2) and less potent bone morphogenetic proteins (16) are made in gonadotropes to induce FSH in an autocrine manner. A recent report indicates that activin A and bone morphogenetic protein 2 can synergistically stimulate *FSHB* transcription (18). Stimulation from autocrine sources implies that high levels of follistatin would be necessary to rapidly intercept autocrine inducers just as they leave the plasma membrane to bind their cognate receptors. These high levels of follistatin can only be achieved *in vitro*. In this study high levels of follistatin were used to inactivate activins or other TGF β family members, which blocked 99% FSH expression in primary ewe pituitary cultures.

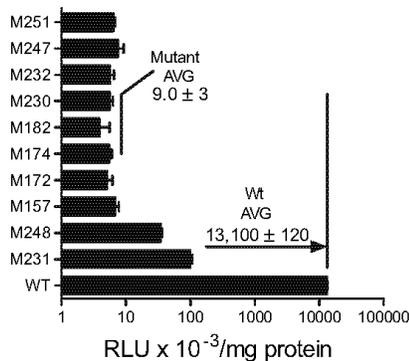


FIG. 3. Pituitary expression of mut-oFSHBLuc (see mutant 3 in Fig. 2) in 10 transgenic mouse lines. Expression of luciferase was measured in male pituitaries and is reported as the mean \pm SEM of five to 10 mice taken from different litters (data for seven lines came from nine mice each). Pituitary tissue was harvested from mice between 8 and 20 wk old. The most active mut-oFSHBLuc lines (M231 and M248) were selected for further study (see Figs. 4 and 5). AVG, Average; WT, wild type.

It could be argued that such supraphysiological levels of follistatin inhibit FSH expression through a nonspecific toxic mechanism. However, there are no reports showing that follistatin at 250 ng/ml is toxic to gonadotropes, and this concentration has been used routinely to block FSH expression in L β T2 cells (19). Furthermore, follistatin produced a normal saturation-type inhibition curve ($IC_{50} = \sim 20$ ng/ml; Fig. 1). The dose-response curve for follistatin-mediated inhibition that was constructed from Fig. 1 (data not shown) contained no discontinuities to suggest the presence of nonspecific toxic effects. In addition, we showed that the effects of all concentrations of follistatin were fully reversed within 24 h after follistatin withdrawal. Nevertheless, all levels of follistatin used in this study were supraphysiological, and it is possible that some effects were due to rapidly reversible toxicity.

The data in Fig. 3 show that changing four nucleotides in 4741 bp of the ovine FSHB promoter decreased luciferase expression *in vivo* to an average of 9×10^3 RLU/mg protein from $13,100 \times 10^3$ RLU/mg protein for wtLj-oFSHBLuc. In addition, low expression of the mutant transgene should be compared with previously reported data from 10 wild-type transgenes ($16,100 \times 10^3$ RLU/mg protein) (11, 12). Therefore, this small mutation caused a 99.93% reduction in luciferase expression. Because this mutation caused no change in basal expression of mut-oFSHBLuc in L β T2 cells (Fig. 2), there was no evidence to link any of this decrease to a change in basal expression, which suggested that the entire decrease was due to a complete and absolute withdrawal of activin like stimulation. These data are consistent with the results shown in Fig. 1, in which follistatin inhibited culture-mediated induction of FSH by 95–99%.

The almost absolute blockade of FSHB expression caused by mutating the -169 to -165 -bp site seems so large, however, that it raises a question about this site being involved in basal expression as well as activin induction. It could even

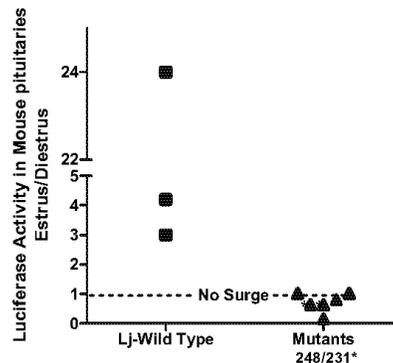
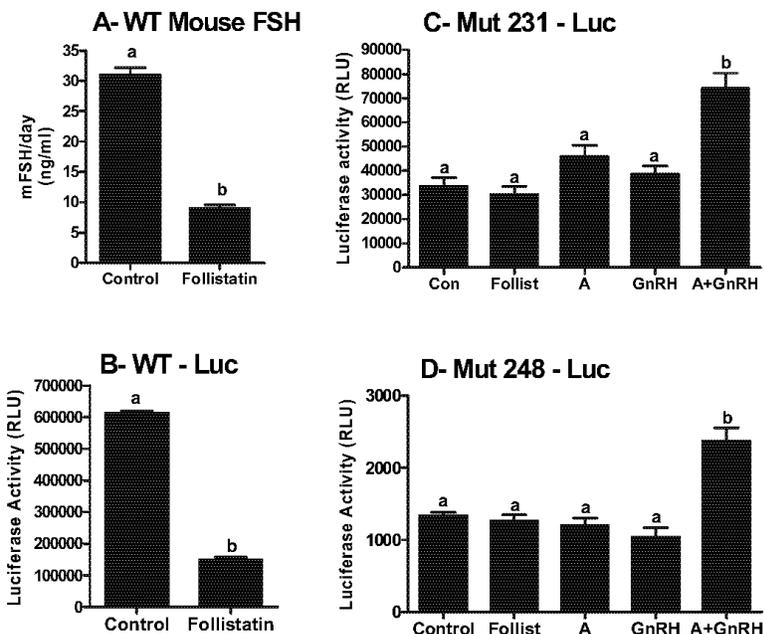


FIG. 4. Expression of mut-oFSHBLuc- Δ Runx1 did not increase at estrus as did wild-type transgenes. Each triangle shown comprised results from six to eight littermates. A total of 46 mut-oFSHBLuc- Δ Runx1 mice were used (M231 = 30 mice; M248 = 16 mice), and 24 wtLj-oFSHBLuc mice were used with triplicate values for both luciferase and protein in all cases. Because diestrus values varied significantly between litters, results were always normalized by reporting the ratio of estrus-diestrus for each wild-type or mutant litter. Student's *t* test was used to show that there was a significant difference between the wild-type and mutant responses ($P < 0.01$).

be possible that the mutation itself created an inhibitory site on the FSHB promoter that artificially decreased expression. However, the Consite computer program (7) did not associate any protein (activator or inhibitor) with the mutated site. Furthermore, Runt or forkhead family members have never been associated with basal expression before. Nevertheless, it is possible that this particular site participates in both basal and activin-stimulated FSHB expression. This could explain the inhibition caused by Smad3 small interfering RNAs at both basal and activin-induced levels at the putative SBE site (20). An analysis of the proteins that bind this sequence and the putative SBE site juxtaposed to it will be needed to reveal the full nature and importance of these sites.

Mutating sequences from -169 to -165 bp (the putative Runx1 binding site) prevented mut-oFSHBLuc from being induced during the estrous surge. This induction during estrus was observed for wtLj-oFSHBLuc and all other wild-type transgenes studied to date in our laboratory (11, 12). For example, the increase in luciferase activity at estrus for wtLj-oFSHBLuc was 10 ± 7 -fold (range 3–24), which was similar to that previously reported for seven similar wild-type transgenic lines (10 ± 2 -fold increase with a range from 3–19). This increase mimicked that of endogenous mouse FSH (21–23), which is associated with the recruitment of follicles for development during subsequent reproductive cycles (24, 25). Studies in rats have concluded that activin B drives this FSH surge at estrus (26–28). If mutating the putative Runx1 site destroyed basal expression of FSHB, the data in Fig. 4 simply reflect the fact that induction cannot occur in a gene that is transcriptionally inactive. However, if basal expression remained unchanged, the data presented here support the idea that activin, working through the -169 to -165 bp site, helps create the secondary FSH surge.

FIG. 5. Regulation of endogenous FSH, wtLj-*oFSHBLuc*, or mut-*oFSHBLuc* in mouse pituitary cultures by follistatin (Follist), activin, and/or GnRH. A, Mouse pituitary cultures were treated with 250 ng/ml follistatin on d 2 (0900 h), and culture media were collected on d 3 (0900 h) and assayed for FSH by RIA. Follistatin decreased FSH by $71 \pm 3\%$. B, Pituitary cultures were prepared from mice carrying the wtLj-*oFSHBLuc* transgene, cultured 2 d, and then treated with or without follistatin for 24 h before being assayed for luciferase (Luc) activity. C, Pituitaries from mice carrying the mut-*oFSHBLuc* transgene (M231 line) were cultured 1.5 d and treated without (control) or with 250 ng/ml follistatin for 16 h to deprive them of activin stimulation from auto-crine/paracrine factors made in pituitary culture. They were then treated with follistatin again (follist), activin (50 ng/ml), GnRH (1 nM), or activin plus GnRH for another 6 h before being assayed for luciferase activity. D, Same as panel C except pituitaries from M248 transgenic mice were used. The results represent the mean \pm SEM of three replicate experiments each assayed in triplicate. Means that are significantly different from each other are labeled with different letters (a or b) where $P < 0.05$. Con, Control.



The putative Runx1 binding site was discovered experimentally while studying deletion and point mutants of ovine *FSHBLuc* constructs in L β T2 cells (Fig. 2). Computer analysis of transcription binding sites, an imprecise science still in its infancy, identified the region from -171 to -163 bp as a likely Runx1 binding site (81%; score = 6.0) (7). This was useful information because Runx1 is known to interact specifically and strongly with Smad3 to promote the proper growth and differentiation of hematopoietic cells (8). This linkage suggested that ovine FSHB might be induced by activin working through a similar complex containing a Runt family member plus Smad3. Furthermore, the putative Runx1 binding site is highly conserved for sheep, human, pig, buffalo, and cow (29). There is one nucleotide change in the pig *FSHB* promoter, but this natural mutation creates an even better Runx1 binding site according to the ConSite analysis.

Interestingly, the single mutation in the pig *FSHB* promoter also creates a putative binding site for FREAC-4, a forkhead protein that may also interact with Smads, although this has not been proven for this particular forkhead protein at this specific site. Another interesting finding is that mutating GC to TA at positions -168 and -167 bp created a putative forkhead-1 binding site. This construct significantly increased responsiveness to activin (48-fold induction), while barely altering basal expression in L β T2 cells (Su, P., unpublished data). In addition, a single mutation from T to C at position -164 bp created a palindromic Smad binding site that permitted activin to induce this construct 38-fold in L β T2 cells, but basal expression was increased approxi-

mately 20-fold (Su, P., unpublished data). Therefore, depending on the nature of DNA sequences and protein factors surrounding the putative Runx1/SBE binding sites, this promoter region might accommodate several forkhead-related proteins known to interact with Smads and might even act as a palindromic SBE element like the one found in rodent promoters for *FSHB* at -266 bp (29). As noted previously, further analysis of the proteins that bind this region will be required to determine the true nature of transcription factor interactions that help drive activin-mediated induction, and possibly basal expression, of the ovine *FSHB* gene.

It might appear that identifying the transcription factors that bind to sequences between -171 and -158 bp on the ovine *FSHB* promoter would be relatively easy using EMSAs, but this perception would be incorrect. No laboratory has yet demonstrated activin-induced binding of any protein to either the putative SBE or its adjacent site identified here. The entire 24-h time course of activin induction has been analyzed without success (data from our laboratory). There is one report of a protein-DNA complex with this promoter region, but this interaction was not induced by activin (5). Nevertheless, the *in vitro* and *in vivo* data in this report about the putative Runx1 binding site plus data from others (SBE site) indicate that the ovine *FSHB* promoter region from -171 to -158 bp is critical for *FSHB* expression in sheep and presumably in humans and other mammals that contain this highly conserved promoter sequence.

Cell-specific expression is important for every gene. It is possible in many cases for proximal enhancers to play a significant role in cell-specific expression and that appears to

be partly true for the putative Runx1 site. Pituitary specific expression for mut-o*FSHBLuc* appears to be 66% less than for wt-o*FSHBLuc* (11) or wtLj-o*FSHBLuc*. Expression of wt-o*FSHBLuc* (11) or wtLj-o*FSHBLuc* (unpublished data from our laboratory) is less than 1% pituitary expression in the liver, gonads, spleen, or lung, but expression of mut-o*FSHBLuc* was approximately 3% pituitary expression in the liver and gonads; however, expression in the spleen and lung were still $\leq 1\%$ compared with pituitary expression. For the liver and gonads this represents a 66% decline in gonadotrope-specific expression suggesting that the putative Runx1 site has some effect on cell-specific expression. Nevertheless, data from purified gonadotropes expressing mut-o*FSHBLuc* show that gonadotrope-specific expression is still favored 644:1 (see *Results*) when comparing gonadotrope expression to expression in either the liver or gonads. Pituitary specific expression is even higher when considering the spleen (6440:1) or lung (2147:1). Expression of ovine *FSHBLuc* constructs in brain tissue is another matter entirely. The original wild-type construct (wt-o*FSHBLuc*) was often expressed in brain tissue at high levels (pituitary-brain = 100:1, 100:30 100:270) (11). The mutant transgene, mut-o*FSHBLuc*, was also expressed at significant levels in the brain (pituitary-brain = 100:8 or 100:22). In this regard, expression of mut-o*FSHBLuc* appeared even more specific compared with the original wild-type transgenes with regard to brain tissue. These data comparing pituitary and brain expression are difficult to comprehend and have not been helpful in understanding cell-specific expression of the ovine *FSHB* gene. The overall results indicate that the putative Runx1 site has some influence over gonadotrope-specific expression, at least in the liver and gonads, but it seems to play only a minor role overall.

Finally, GnRH and activin often interact at a molecular level to alter *FSHB* expression, but these interactions are not well understood. Our laboratory previously reported that isolating primary gonadotropes from activin for 17 h (follicle-stimulating hormone treatment) permits subsequent treatment with activin and GnRH to synergistically induce wt-o*FSHBLuc* during a 4-h period (12). Moreover, recent studies with the mouse *FSHB* promoter show that GnRH and activin can cooperate synergistically when critical AP-1 and Smad binding sites are multimerized (30).

The data in Fig. 5 show that GnRH and activin can synergistically increase expression of mut-o*FSHBLuc*, but this occurred in a construct that lacked the putative Runx1 site and was, presumably, incapable of responding to activin directly (Figs. 2–4). These data suggest that GnRH and activin cooperated either through the one remaining Smad binding site or in a general way that did not involve specific interactions on the ovine *FSHB* promoter. Recent evidence indicates that activin dramatically alters the responsiveness of L β T2 gonadotropes to GnRH in a global sense (31), so it is possible that global effects of GnRH and activin caused the synergistic induction of mut-o*FSHBLuc* shown in Fig. 5. These data are not meant to imply that activin and GnRH do not cooperate with each other through signaling pathways that complement each other directly at the ovine *FSHB* promoter, but it does indicate there are ways for activin and GnRH to cooperate through global actions in gonadotropes.

In summary, this study presents data that are consistent

with the concept that 99.9% of ovine *FSHB* expression *in vivo* depends on sequences between –171 and –165 bp on the promoter of ovine *FSHB* promoter/reporter gene mutants in L β T2 gonadotropes. It is shown here that this element was essential *in vivo* and was required for the estrous surge of FSH in mice. It seems to have had little effect on pituitary specific expression. Finally, it was shown that activin and GnRH cooperatively increased *FSHB* expression, even in the absence of a functional activin response element on the ovine *FSHB* promoter.

Acknowledgments

We thank Dr. Frank French for collaborating with us for the production of transgenic mice carrying the following transgenes: mut-o*FSHBLuc* and wtLj-o*FSHBLuc*. We also thank B. J. Welker, Linda Hester, and their co-workers at the North Carolina State University Biological Resources Facility for their careful and caring work with our mice. We thank Dr. P. L. Mellon for the generous gift of L β T2 cells for our *in vitro* studies.

Received December 8, 2006. Accepted June 1, 2007.

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Our work was supported by North Carolina State University Agricultural Research Service and National Institutes of Health Grant R01-HD-04529.

Disclosure Statement: The authors have nothing to declare.

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Appendix C

A Conserved PITX1/2 Binding Site and Adjacent Promoter Region are Important for Expression of Ovine Follicle Stimulating Hormone Beta-Subunit *in vivo*

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Being resubmitted.

A conserved PITX1/2 binding site and adjacent promoter region are important for expression of ovine follicle stimulating hormone beta-subunit *in vivo*.

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Key words: FSH, PITX1/2, RUNX, Transgenic, tissue-specific

This work was supported by the North Carolina State University Agricultural Research Service and National Institutes of Health Grant HD 042459

ABSTRACT

Follicle stimulating hormone (FSH) stimulates egg/sperm maturation. Its β -subunit (FSHB) is rate-limiting for FSH production and strongly induced by activins. We recently reported that a site juxtaposed to a minimal Smad binding element (-169/-158 bp) in the ovine *Fshb* promoter is necessary for activin A induction in L β T2 cells and transgenic mice. In mice this site is required for $\geq 99\%$ of transgenic expression. The study reported here focuses on another highly conserved site comprised of 5 bp and a PITX1/2 binding element between -68/-58 bp of the ovine *Fshb* promoter. When ovine *Fshb* promoter-Luc constructs were studied in L β T2 cells, this region seemed as important as the region noted above for induction by activin A, but also for basal expression. Mice expressing an ovine *Fshb* promoter-luc transgene with a mutated PITX1/2 site showed 97-99% less expression than the wild type transgene. When pituitaries from these transgenic mice were dispersed and cultured, the response to activin A was normal although expression was low. Adjacent to, and overlapping the PITX1/2 site, is a RUNX-like binding site. Mutation of this region did not significantly affect expression of ovine *Fshb*-Luc transgenes *in vivo*, but did alter gonadotrope targeting. We conclude that the PITX1/2 site (-68/-63 bp) in the ovine *Fshb* promoter is required for 97-99% of Fshb expression *in vivo*, but is unlikely to participate in induction of Fshb by activins. In addition, the region adjacent to the PITX1/2 binding element is involved in gonadotrope-specific expression of ovine Fshb.

Introduction

Follicle stimulating hormone (FSH) is produced in the gonadotropes of all vertebrate pituitaries and is required for egg maturation and optimal sperm performance (1-3). FSH is a α/β heterodimer, and its β -subunit (FSHB) is rate-limiting for overall FSH production. To fully understand the factors that control human fertility, it is important to identify the signaling pathways and transcriptional regulators that control FSHB expression.

Activins A, B or AB are the most potent and most probable inducers of *FSHB* transcription in

vivo (4-6). One important element in the ovine *Fshb* promoter which also appears in the human *FSHB* promoter, is the sequence between -169/-158 bp (7, 8). This site contains a minimal Smad binding site (7) juxtaposed to sequences that might bind a Smad partner (8), recently reported to be FoxL2 for the genes encoding human and porcine FSHB/Fshb (9). Transgenic studies indicate this second site is required for induction of ovine Fshb by activins and $\geq 99\%$ of ovine Fshb expression *in vivo*. This site was originally located by functionally testing many 5' promoter mutants in L β T2 cells (8).

Further analysis of the ovine promoter led us to study a region between -68/-58 bp. This region is conserved in all mammals studied to date and part of the region has already been investigated as a PITX1/2 binding element (-68/-63 bp) (10, 11). One study focused on this PITX1/2 binding site as being important for induction of rat Fshb by GnRH (gonadotropin releasing hormone) (10). Most recently, PITX1/2 and PITX2C were shown to interact with SMAD3 and this site was found to be important for both basal and activin A-induced expression of murine Fshb (11).

In the studies reported here, we focus on this PITX1/2 binding site, but also on its overlapping companion site that appears critical for expression and activin A induction of ovine *Fshb* transcription in L β T2 cells. This report shows the importance of this entire region (-68/-58 bp) for expression in L β T2 cells and also in transgenic mice.

Materials and Methods

Reagents

Recombinant human activin A and recombinant mouse follistatin 288 were purchased from R&D systems (Minneapolis, MN). FuGeneTM6 transfection reagent was purchased from Roche Applied Science (Indianapolis, IN). QuikChange[®] Site-Directed Mutagenesis kit was purchased from Agilent Technologies Co. (La Jolla, CA). Taq DNA polymerase, yeast extract, Tryptone, Agar, Tris, and Boric acid were purchased from Fisher Scientific Inc. (Pittsburgh, PA). Restriction enzymes including BglII, KpnI, AgeI, XhoI, T4

DNA ligase, Dual luciferase assay kit, Luciferase assay kit, and Passive lysis buffer were purchased from Promega (San Luis Obispo, CA). TaqMan Universal PCR mixer for real-time PCR was purchased from Applied Biosystems (Foster City, CA). Quanti-iT^M Protein assay kit, Dulbecco's modified eagle medium (DMEM), Fetal bovine serum, and Hanks' Balanced Salt Solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). Steptomycin, collagenase, and Pancreatin as well as all primers for point mutations in this study were purchased from or synthesized by Sigma-Aldrich Co. (St. Louis, MO). Mutant plasmids and PCR products from transgenic mice were sequenced by SeqWright Technology (Houston, TX). Immortalized murine gonadotrope LβT2 cells were provided by Dr. Pamela Mellon (University of California, San Diego, CA).

Plasmid constructs

The wild type ovine *Fshb* promoter/reporter plasmid (ovine *Fshb*-Luc) was described previously (12, 13). Briefly, ovine *Fshb*-Luc contains 4741 bp of 5' ovine *Fshb* promoter plus intron 1 driving expression of a luciferase (Luc) gene in the pGL3 basic vector. Mutant 1A was constructed by replacing sequences between -90/-39 bp with synthesized sequences where A/C and T/G inter-conversions were made. Mutants 6, 7, 8 and 9 were prepared using direct point mutations at specific sites. Mutants 2-5, 1B and 1C were made by replacing sequences with synthesized oligonucleotides inserted between the AgeI site at -90 and BglII site at -39. M10 was constructed the same way as M2-5, but the sequences were selected from the human *FSHB* promoter between -82/-37 bp. All mutations were shown to be correct by sequencing.

Transgenic mice

Transgenic mice were maintained and studied with the approval and oversight of the Institutional Animal Care and Use Committee at North Carolina State University and the University of North Carolina at Chapel Hill, NC.

Wild type (WT) and 3 different mutants (M7, M8, and M9) were chosen to be used as transgenes. For the generation of transgenic mice, ovine *Fshb*-Luc constructs (-4741 bp to +2738 bp) were cut from plasmid backbone using KpnI and BamHI and mice containing these transgenes were produced in B6/SJL mice as described earlier (8, 13). Transgenic mice were produced at the transgenic mouse facility of the University of North Carolina at Chapel Hill, NC and then bred and cared for at the Biological Resource Facility of North Carolina State University. Testing mice for the presence of a transgene was performed as noted below, and measuring luciferase activity in tissues was performed as previously reported (8).

Real-time PCR and PCR

Determining the presence of transgenes in mice was performed using real-time PCR (RT-PCR) on DNA extracted from mouse tails with primers to the luciferase gene: forward primer = 5'-TGGGCTCACTGAGACTACATCA-3' and reverse primer = 5'-CGCGCCCGGTTTATCATC-3'. To confirm the presence of 5'-ovine *Fshb* in transgenic mice, the forward primer bound between -4644/-4615 bp = 5'-CCTCCATCATCCCCCTTCTCCTCCTGCCTTC-3', and the reverse primer bound between -2531/-2504 bp, 5'-TCTCTCCTAACGTCAAGCCTCTTAGAGC-3'. For confirming the presence of 3' sequences of the ovine *Fshb* transgene and also producing DNA for sequencing mutated sequences, the forward primer bound between -1740/-1713bp = 5'-CAAAGGAAGTCTCAAGCCAAGGGTGTC-3', and the reverse primer bound between +831/+857 bp = 5'-CGCCGGGCCTTTCTTTATGTTTTGGCG-3', of the ovine *Fshb* promoter. The regions internal to these latter primers included 1740 bp of the 5'-region, 1st exon, 1st intron, 53 bp of 2nd exon of ovine *Fshb* plus 38 bp of the luciferase coding sequence. The selected regions were amplified by PCR and sequenced to determine the correctness of mutations between -73/-53 bp.

L β T2 cell culture

Immortalized murine gonadotropes (L β T2 cells) were maintained in complete DMEM containing 10% (v/v) fetal bovine serum plus 100 μ g/ml streptomycin and 100 U/ml of penicillin at 37°C under 5% CO₂: 95% air. For experiments, cells were plated at a density of 30,000 cells with 50 μ l of complete media per well in 96-well tissue culture plates. Cells were transfected 24 h after plating in quadruplicate by adding 50 μ l of serum-free media containing 50 ng of plasmid plus 0.15 μ l Fugene6. Cells were also co-transfected with 5 ng of renilla luciferase expression vector (pRL-TK), as an internal control since its production of renilla luciferase was not altered by activin A. After 24 h of transfection, media were removed and cells were treated with or without 25 ng/ml activin A for 22 h prior to harvesting with passive lysis buffer. Firefly and renilla luciferase were quantified sequentially using the Dual Luciferase assay kit following the manufacture's directions in an automated 1420 Victor-Light micro plate luminometer (PerkinElmer, Waltham, MA)

Tissue specific-expression of transgenic mice

Transgenic mice (2-4 months old) were sacrificed, and their pituitary, brain, heart, lung, liver, spleen, kidney and gonads were taken within 5 minutes of death, frozen on dry ice in 0.5 ml tubes and stored at -80 C for no more than 24 h before assay. Just prior to assay, samples were homogenized in 100 μ l of passive lysis buffer, and centrifuged for 10 minutes at 12,000 rpm in a microfuge at 4 C. All samples were assayed within 30 minutes of thawing. Luciferase activity was normalized for protein measured with a Qubet™ Fluorometer (Invitrogen, Carlsbad, CA).

Primary cell Culture

Primary pituitary cells from transgenic mice were prepared as described previously (8, 13). Briefly, transgenic mice (2-4 months old) were sacrificed and pituitaries were dissociated using

collagenase for 2 h at 36° C, followed by Pancreatin for an additional 15 minutes. Cells (50,000) were placed in each well, and incubated for 48 h at 37 C under 95% air/5% CO₂ in a humidified incubator. Cells were treated with activin A (50 ng/ml) or follistatin (50 ng/ml). After 24 h, the cells were harvested with passive lysis buffer, and luciferase activity was measured as with L β T2 cells described above.

Results

Importance of the PITX1/2 binding element and downstream 5 bp for expression of ovine *Fshb*-Luc in L β T2 cells

An 11 bp sequence from -68/-58 bp comprises a PITX1/2 binding site which overlaps a putative binding site for a RUNX transcription factor (Fig 1). Mutation of all nucleotides in this region (mutant 1A) decreased basal expression by 55% and eliminated activin A induction entirely. Mutants 1B, 1C, 2 and 5 were within the PITX1/2 binding site, but 2 and 5 were also within the putative RUNX binding element. All were effective in blocking activin A induction and decreasing basal expression. Mutant 2 seemed particularly effective although only 1 nucleotide was mutated; its basal expression was decreased by 80% and induction by activin A was zero. Mutants 6-8 were partly in the PITX1/2 binding region, but also in the exclusive 5 bp domain of possible RUNX binding. Mutations 6 and 8 showed the lowest basal expression (denoted by "c"). Mutation 9 contained 3 mutations totally outside the PITX1 binding region at the far end of the putative RUNX binding domain; its basal and activin A-induced expressions were not significantly different from the other mutants, however. Finally, mutant 10 (human sequence -82/-37 bp) substituted for the same ovine sequence, gave results similar to WT ovine *Fshb*-Luc in L β T2 cells. Neither basal expression nor activin A-induced expression was significantly different from WT ovine *Fshb*-Luc expression. Based on L β T2 cell analysis, mutations at any site between -68/-58 bp depressed basal expression and also inhibited activin A induction of ovine *Fshb*-Luc constructs.

Expression of WT and mutant ovine *Fshb*-Luc transgenes

Mutants 7-9 contained mutations spaced throughout the region of interest (-68/-58 bp). Statistically, all showed equal reduction in basal expression and low induction by activin. Mutant 7 contained 4 bp mutations primarily in the PITX1/2 binding site although a fifth bp was mutated downstream of this site. Its expression as a transgene was reduced 97 % (females) to 99 % (males) compared to WT transgene expression (Table 1). This result with the mutant 7 transgene was predicted by L β T2 studies. Mutants 8 and 9 covered separate regions of the putative Runx1 binding site, and, surprisingly, showed no major difference in pituitary expression in transgenic mice. These results were not predicted by studies with L β T2 cells.

Mutant 7 was expressed poorly in pituitary tissue, but also minimally in non-pituitary tissues where its expression equaled that of WT ovine *Fshb*-Luc except in the brain where expression was somewhat elevated (~ 4-fold). Interestingly, according to the Student's t test, luciferase expression in the brain and pituitary were equal for both males and females of mutant 7; Table 1). Mutants 8 and 9, however, showed significantly more expression in non-pituitary tissues, especially in the brain and gonads. Expression in the forebrain was 50x higher in the males of mutant 9 compared to WT brain expression. Mutant transgenes 8 and 9 were also expressed 9x and 18x higher in the testes compared to WT testicular expression. Nevertheless, expression in both mutants 8 and 9 was focused primarily in the pituitary (Table 1).

All mutant transgenes were induced by activin A in primary pituitary culture

Activin A maximizes *Fshb* expression in mouse pituitary cultures whereas follistatin inactivates endogenously made activins and depresses *Fshb* expression to its lowest level (8, 13). Therefore, the comparison between activin A and follistatin treatments indicates how much activins can induce ovine *Fshb*-Luc expression in primary murine pituitary cultures. The data in Figure 2 indicate that WT and all transgene

expression was responsive to activin A. In fact, it appears that Mutant 7 was actually the most responsive to activin A in primary tissue culture. This is the same mutant that showed absolutely no induction by activin A in L β T2 cells (Figure 1).

Discussion.

The PITX1/2 binding site at -68/-63 in the ovine *Fshb* promoter is highly conserved in mammals and has been studied previously in *Fshb*-reporter constructs of the rat (10) and mouse (11) using transformed cells in culture. In these studies, the PITX1/2 binding element was shown to be essential for high level expression, but the regions upstream or downstream were not investigated.

This study shows that nucleotides 5 bp downstream of the PITX1/2 site in the ovine *Fshb* promoter are also important for expression and activin A induction in L β T2 cells which are used routinely for understanding regulation of murine, rat, ovine, porcine and human *Fshb*/FSHB in vitro.

It is instructive to note that L β T2 cells accurately predicted that the -68/-63 PITX1 site is very important for FSHB expression in vivo in transgenic mice. However, the results from L β T2 cells incorrectly predicted that five adjacent 3' nucleotides were important for activin action and expression in vivo. Also data from L β T2 cells predicted that activin-induction of FSHB was dependent on the PITX1 site which was not observed in transgenic mice. It is not clear why these differences occurred, but possible reasons are discussed in the last paragraph of this section.

Studies in the Bernard laboratory (11) recently showed that PITX1 or 2 can bind to the conserved PITX1/2 binding site on the mouse *Fshb* promoter and that two nucleotide changes anywhere within this region disrupts binding as detected by electrophoretic mobility shift assays (EMSA) (11). Mutations outside this region had no effect on PITX1/2 binding, however. Our data suggest that "another" transcription factor binds the sequences adjacent to, and perhaps, overlapping the PITX1/2 binding site and are important for FSHB expression in L β T2 cells.

Using the on-line ConSite program for predicting transcription binding sites, RUNX1 was suggested as an appropriate binding protein for the site shown in Figure 1 (ConSite, <http://asp.iu.uib.no:8090/cgi-bin/CONSITE/consite/>) (14). Attempts to show RUNX1 binding to the -68/-58 region using EMSA analysis with L β T2 nuclear extracts were not successful. In fact, no specific binding was observed at this site, but under identical conditions, RUNX1 from nuclear extracts of L β T2 cells did bind a consensus RUNX1 site (data not shown). Therefore, RUNX1 is unlikely to bind this region with high affinity. Furthermore, there is no functional *in vitro* assay that can precisely determine the DNA sequence between -68/-58 bp needed for proper function. Studies with L β T2 cells exaggerated the importance of this site. Only transgenic studies showed this site has some effect on gonadotrope-specific expression. Thus, it will be very difficult to identify factors that bind this region.

The -68/-63 PITX1/2 binding site is conserved in all mammals studied to date, but other parts of the rodent promoter differ significantly from the ovine and human *Fshb/FSHB* promoters. Data from the Bernard laboratory using L β T2 cells strongly supports the concept that the PITX1/2 site aids induction of FSHB by activin A (just as our L β T2 data support it), and this may be true in the mouse *in vivo* as well.

By contrast, the transgenic data shown here indicate that activin A induction of ovine *Fshb*-Luc occurred robustly without the PITX1/2 binding site. Evidence strongly suggests that the important activin A-inducible site in sheep resides between -167/-158 bp (8) and this site is conserved in humans, but not in rodents. Rodents have a different palindromic Smad binding element at -269/-263 bp which can be shown to bind Smads (11). Bernard's group showed that PITX1/2 can bind Smad3 and suggested that proteins which bind the mouse PITX1/2 binding element interact with proteins at the -269/-263. Therefore, there is the possibility that the PITX1/2 binding site helps activin A induction in rodents. It seems that a similar helper function does not exist for the ovine *Fshb* gene.

Replacement of the human for sheep sequences between -82/-37 bp in WT ovine *Fshb*-Luc (mutant 10) did not significantly alter either basal expression or activin A induction. The human sequence is a faithful reproduction of the ovine sequence between -68/-58 bp, but there are minor changes in the flanking regions. These data suggest that the human *FSHB* promoter sequence may work much like the ovine *Fshb* promoter in this region of the promoter.

Finally, it is important to know how expression of *Fshb/FSHB* is targeted specifically to gonadotropes. The wild type ovine *Fshb*-Luc transgene is expressed 100-1,000x more in pituitary gonadotropes than the next most active tissue (brain) and about 5,000-10,000 more than any other tissue tested (2, 8). This specificity is very high, and ovine *Fshb*-Luc contains all the sequences required for this specificity.

A nearly complete mutation of the PITX1/2 binding decreased expression of mutant 7 ovine *Fshb*-Luc by 97-99 % when it was tested as a transgene, but did not raise the level of expression in any other tissue except the brain where expression increased only slightly by a factor of ~4 compared to the wild type construct. Mutant 8 showed even more expression in the brain and Mutant 9 showed expression in the brain that was about 10 % of the really high expression found in the pituitary. In the testis, expression in Mutants 7 and 8 was > 36-fold and >114-fold higher than for WT ovine *Fshb*-Luc, respectively. These changes in specificity of expression strongly suggest that sequences between -63/-58 bp have some influence over gonadotrope-specific expression of ovine *Fshb* *in vivo*.

It is impossible at this time to determine how gonadotrope-specific expression is altered by mutants 8 and 9. Perhaps a new site was created by mutation that attracts transcriptional inducers prevalent in the brain or gonadal tissues. Mutation could also have prevented the binding of a critical gonadal factor that suppresses expression in the brain or gonadal tissues. It seems, however, that the effect is relatively minor so it is unlikely to be a major site that controls gonadotrope or non-gonadotrope-specific expression.

It is instructive to compare transgenic and L β T2 results. Studies with L β T2 cells indicated that each transgenic construct should have been expressed very poorly in mice and not induced by activin A, but this prediction was not realized. One construct was expressed very poorly as a transgene as predicted by L β T2 cell expression (mut 7), but the others (mutants 8 and 9) showed essentially no difference in transgene expression [Note: male-female differences often occur, but not always, and vary with different founder lines (13)]. Mutants 8 and 9, however, showed another kind of change in terms of pituitary-specific expression.

The data presented here suggest that L β T2 cells are useful for analyzing *Fshb/FSHB* promoter function, but may not reveal all the important information and may also give false information. It is not clear why data from L β T2 cell cultures and transgenic mice were different in terms of activin induction and basal expression, but it may be due to the fact that L β T2 cells are transformed embryonic mouse gonadotropes. Transformation, itself, involves the expression of many genes not normally expressed in primary cells, and the difference in expression of transcription factors and signal transduction pathways between embryonic and adult gonadotropes is likely to be significant,

although not yet documented. Moreover, gonadotropes in vivo are exposed to GnRH and paracrine factors that cannot be reproduced in L β T2 cell culture. Finally, promoter/reporter genes are presented in plasmid form in L β T2 cells whereas they are incorporated into chromatin in transgenic mice. There can be differences in how promoters are regulated in plasmid and chromatin forms (15).

IN SUMMARY, the data presented here show that the PITX1/2 site at -68/-63 bp is required for 97-99 % of ovine FSHB expression in vivo. This was not proven before. The data fail to show any effect of the PITX1/2 site on activin A induction for the ovine *FSHB* when tested in vivo using transgenes. Finally, it shows that sequences 5 bps downstream of the PITX1/2 site are required for high level gonadotrope-specific expression in vivo.

Acknowledgements

The authors thank Dr. Pamela Mellon (University of California) for her gift of L β T2 cells. This work was supported by North Carolina State University Agricultural Research Service and National Institutes of Health Grant R01-HD-042459.

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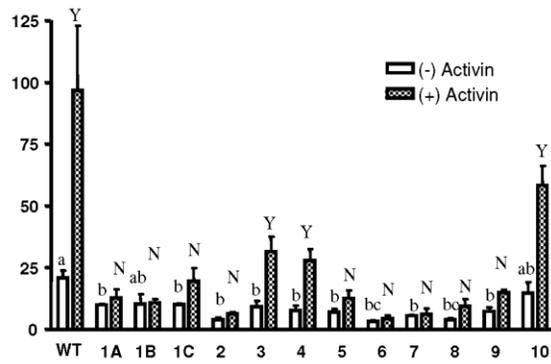
Table 1

Table-1- Expression of wild type (WT) and -68/-58 bp mutants of ovine *Fshb*-Luc transgenes in mouse tissues. Tissues were harvested within 3 minutes of death from mice 2-4 months old, and assayed for luciferase activity. Values represent relative light units of firefly luciferase activity (RLU) times 10^{-5} /mg protein. All values represent the mean \pm sem of results from at least five mice. Because of unequal variances, the data were transformed into logarithmic format and then analyzed by ANOVA followed by Tukeys multiple comparison test.

Figure legends

Figure 1 - Expression of WT and -68/-58 bp mutants of ovine *Fshb*-Luc in L β T2 cells \pm activin A. The boundaries and overlap of putative RUNX and PITX1/2 binding sites are shown. Expression of firefly luciferase was normalized to renilla luciferase activity; renilla activity did not vary more than \pm 10 % with activin A treatment or between mutants tested. Bars represent the mean \pm sem of results from quadruplicate cultures from a single but complete experiment representative of three independent experiments. Means with identical lower case letters are not significantly different from each other for basal expression ($P < 0.05$). Means with different symbols are significantly different from each other at $P < 0.05$. ANOVA and Tukey's multiple comparison test were used to determine significant differences for basal (a, b, c) and activin A-induced expression (N, Y). Fold-induction of wild type (WT) or mutant 10 (human sequence between -82/-37 bp) was statistically equal \sim 430 %. Activin A induction and basal expression of all other mutants were not significantly different from each other, but were different from the wild type and mutant 10 ovine *Fshb*-Luc expression.

Figure 2 - Activin A-induction of wild type (WT) and mutant ovine *Fshb*-Luc transgenes in mouse pituitary culture. The differential expression between treatments with follistatin (FS) or activin A (Act) represents induction due to activin A action. Mouse pituitary cells were incubated 48 h after dispersion, treated with 25 ng/ml activin A or 125 ng/ml follistatin for 24 h and then assayed for luciferase activity. The bars represent the mean \pm sem of triplicate cultures. Means with different letters are significantly different. A one-tailed unpaired t-test with Welch's correction was used to determine significance between means using $P < 0.05$.



	PITX1			RUNX1				
WT	-73CC	AAC	AAA	TCC	ACA	AGG	TTT	A-53
1A	-73AA	CCA	CCC	GGA	CAC	CTT	GGG	C-53
1B	-73AA	CCA	CCA	TCC	ACA	AGG	TTT	A-53
1C	-73CC	AAA	CCA	TCC	ACA	AGG	TTT	A-53
2	-73CC	AAC	AAC	TCC	ACA	AGG	TTT	A-53
3	-73CC	AAC	AAA	GCC	ACA	AGG	TTT	A-53
4	-73CC	AAC	AAA	ACC	ACA	AAG	TTT	A-53
5	-73CC	AAC	AAA	TAC	ACA	AGG	TTT	A-53
6	-73CC	AAC	AAA	GGT	ACC	AGG	TTT	A-53
7	-73CC	AAC	AAC	GGA	ACC	AGG	TTT	A-53
8	-73CC	AAC	AAA	TCT	AGA	AGG	TTT	A-53
9	-73CC	AAC	AAA	TCC	ACG	CGT	TTT	A-53
10	-69CC	AAC	AAA	TCC	ACA	AGG	TGT	T-49

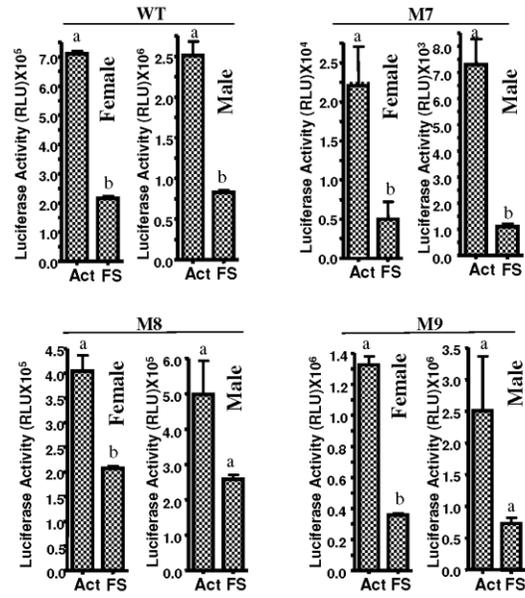


Table 1. Tissue-specific expression

		RLUX10 ⁻⁵ /mg protein							
WT or Mutants	Sex	Pituitary	Brain	Lung	Heart	Liver	Spleen	Kidney	Gonad
WT	F	348±153	4±2	<0.1	<0.1	<0.1	<0.5	<0.05	<0.5
	M	5485±1896	2±1	<0.5	<0.5	<0.05	<0.5	<0.1	<0.5
M2	F	26±16	16±2	<0.1	<0.1	<0.05	<0.05	<0.05	<0.1
	M	47±20	13±4	<0.1	<0.1	<0.05	<0.05	<0.05	<0.5
M3	F	500±81	132±5	<0.5	<0.5	<0.5	<0.5	<0.1	5±2
	M	2761±600	62±21	<0.5	<0.5	<0.5	<0.5	<0.05	18±5
M4	F	872±326	73±42	1±0	4±2	1±0	2±1	2±1	5±2
	M	729±139	99±40	1±0	3±2	1±0	1±0	2±0	57±12