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Gonadotropin-releasing hormone induction of c-Jun gene expression by phosphorylation of ATF-2

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Publication Date  
2011

Peer reviewed|Thesis/dissertation
Gonadotropin-Releasing Hormone Induction of c-Jun Gene

Expression by Phosphorylation of ATF-2

A thesis submitted in partial satisfaction of the requirements for the Degree Master of Science

in

Biology

by

Lacey Lee Lindaman

Committee in Charge:
Professor Pamela L. Mellon, Chair
Professor William McGinnis, Co-Chair
Professor Mandy Butler

2011
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Co-Chair

Chair

University of California, San Diego

2011
This work is dedicated to my family for all their support and love, to my friends that have stood by me and helped me laugh along the way, and to my love who has been my rock and my partner on this journey.
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<table>
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<tbody>
<tr>
<td>α-GSU</td>
<td>alpha glycoprotein subunit</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AP-1</td>
<td>activating protein 1</td>
</tr>
<tr>
<td>ATF</td>
<td>activating transcription factor</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>HPG</td>
<td>hypothalamic pituitary gonadal</td>
</tr>
<tr>
<td>JDP2</td>
<td>jun dimerizing protein 2</td>
</tr>
<tr>
<td>JNK</td>
<td>jun N-terminal kinase</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NFY</td>
<td>nuclear factor Y</td>
</tr>
<tr>
<td>PCOS</td>
<td>polycystic ovary syndrome</td>
</tr>
<tr>
<td>PIP2</td>
<td>phoshpatidylinositol 4, 5 bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PRL</td>
<td>prolactin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>TPA</td>
<td>tetradecanoyl-phorbol acetate</td>
</tr>
<tr>
<td>TRE</td>
<td>TPA response element</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

First, I would like to thank my committee members for taking the time to evaluate my thesis. I would like to thank Dr. Pamela Mellon for her guidance and support during this process. She has opened up countless opportunities for me and has furthered my scientific career. I am very blessed to have Dr. Djurdjica Coss as my mentor. She took me into her lab with no previous research experience, spent many hours training me, and was very patient. I would like to thank her for making our lab feel like a family and helping me attain scholarships, fellowships, and my degree. Her guidance has shaped me into the scientist that I am today. Additionally I would like to thank the members of the Coss, Mellon, and Lawson lab for their help and support.

Furthermore, I would like to acknowledge my family who has been a constant source of love and encouragement. They have given me the means and the motivation to become who I am today. I would like to thank my friends for bringing balance to my life and for helping me on this journey. I would especially like to thank the following people: Ted and Debbie Lindaman, Ryan Roybal, Patricia Pepa, and Arpi Hambarchyan.

Finally, I would like to acknowledge the Howell Foundation and the Endocrine Society for supporting my research and expanding my scientific horizons. These fellowships have allowed me to focus on my research and complete my degree.
The only transcription factors induced by gonadotropin-releasing hormone known to
directly regulate FSHβ gene expression are members of the AP-1 family. AP-1 is composed
of heterodimers of homodimers of Jun and Fos family members, and of these c-Jun is the most
potent transcriptional activator. Thus, understanding the molecular mechanisms of c-Jun
induction by GnRH will further elucidate GnRH regulation of FSHβ gene expression and
GnRH gonadotrope signaling pathways. GnRH can induce c-Jun protein and mRNA and
GnRH induction of the c-Jun promoter maps between -70 and -61 region of the c-Jun
Promoter, a region that contains a cAMP response element. The CRE site is necessary and
sufficient for GnRH induction of c-Jun, and the CRE binding proteins activating factor 2
(ATF-2), phosphorylated ATF-2, and activating factor 3 (ATF-3) can bind to the c-Jun promoter. GnRH treatment induces ATF-3 and phosphorylation of ATF-2 in LβT2 cells, and ATF-2 subsequently induces the c-Jun promoter and CRE element. A dominant-negative ATF-2 reduces basal expression, induction of c-Jun promoter by wild type ATF-2, and GnRH induction, therefore ATF-2 is necessary for c-Jun induction by GnRH. The known repressor ATF-3 is able to act as a repressor for c-Jun induction with ATF-2 over-expression, but it is not able to repress c-Jun induction by GnRH. However, Jun Dimerizing Protein 2 (JPD2) is able to repress ATF-2 over-expression and well as reduce GnRH induction of c-Jun thus identifying a novel player that is able to negatively feedback on GnRH regulation of c-Jun.
Introduction

Hypothalamic-Pituitary-Gonadal Axis

The hypothalamus, anterior pituitary, and gonads form the hypothalamic-pituitary-gonadal (HPG) axis, which is essential for mammalian development and regulation of the reproductive cycle. The anterior pituitary is the critical integrator of signals received from the hypothalamus such as gonadotropin-releasing hormone (GnRH). GnRH neurons originate in the nose and migrate into the brain where most localize to the pre-optic area of the hypothalamus and innervate the median eminence [1]. GnRH is a decapeptide secreted into the hypophyseal portal system in a pulsatile manner that binds to its seven transmembrane G-protein coupled receptor on the anterior pituitary gonadotrope cells. Consequently, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are released from the gonadotropes and act on the gonads, regulating gametogenesis, steroidogenesis, and ovulation [2]. The frequency of GnRH pulses secreted from the hypothalamus determines the ratio of FSH and LH produced. Studies have shown that a decrease in GnRH pulse frequency favors FSH production, while a more rapid pulse frequency favors LH production, and LH gene transcription is more sensitive to GnRH pulse frequency than FSH[3]. Producing the gonadotropins in the correct proportions is critical for normal fertility. Disproportionate amount of LH and FSH can lead to disorders such as polycystic ovarian syndrome, a disorder that affects about 10% of women of childbearing age (PCOS).
FSH and LH are heterodimeric glycoproteins that are composed of a common α-subunit and a unique β-subunit that confers biological specificity [4]. The α-subunit gene is produced in excess and therefore is not regarded as a major determinant of the biosynthetic rate. However, the β subunit is not produced in excess and its production is tightly controlled, making it a rate-limiting step in the production of mature hormones [5]. FSH regulates follicular growth and ovary development in females and sperm production in the testes in males. LH is responsible for ovulation and steroidogenesis by mature follicles in females, and testosterone production in male Leydig cells [6]. The gonadal steroid hormones such as estrogen, testosterone, and progesterone, and peptide hormones such as activin and inhibin are able to feedback to the anterior pituitary and hypothalamus and regulate production of FSH and LH [7]. Humans with inactivating mutations in LHβ lack pubertal development and are infertile [8]. Mutations in FSHβ result in women with absent or incomplete pubertal development, men with essentially normal pubertal development, but azoospermia, and infertility of both sexes [9]. Therefore, proper functioning of the HPG axis and regulation of FSH and LH production is crucial for reproductive health [2].

**Anterior Pituitary**

Considered the master gland of the endocrine system, the anterior pituitary serves as a critical link between the nervous system and target organs such as the adrenal gland, thyroid gland, ovaries, testes, liver, adipose tissue, and mammary gland by integrating various signals from the brain and synthesizing hormones necessary for growth, development, metabolism and reproduction. The anterior pituitary, adenohypophysis, develops from cells in Rathke’s pouch, an invagination of the oral ectoderm, and contains five morphologically distinct endocrine cell types and folliculostellate cells. The five endocrine cell types: corticotropes, thyrotropes, gonadotropes, somatotropes, and lactotropes arise in sequential order during
development and each produces a specific hormone. Corticotropes produce adrenocorticotropic hormone (ACTH), thyrotropes make thyroid-stimulating hormone (TSH), gonadotropes produce FSH and LH, somatotropes make growth hormone (GH), and lactotropes produce prolactin (PRL) [10]. These hormones are crucial for the proper functioning of other endocrine glands and peripheral target tissues as follows: ACTH acts on the adrenal gland stimulating glucocorticoid secretion, TSH acts on the thyroid gland stimulating hormone release, FSH and LH act on the ovaries and testes and influence reproductive functions, GH acts on liver and adipose tissues and stimulates growth and protein and carbohydrate metabolism, and PRL stimulates milk secretion from the mammary glands.

The gonadotrope cells only account for five to fifteen percent of the cells in the anterior pituitary, therefore, in vivo studies of the regulation of gonadotropin synthesis are difficult [10]. Our laboratory solved this problem by the creation of two immortalized gonadotrope cell lines using transgenic mice that developed pituitary tumors created with SV40 T-antigen linked to the 5’ regulatory regions of alpha glycoprotein subunit (α-GSU) or LHβ[11, 12]. An immature gonadotrope cell line, αT3-1 cells, is committed to gonadotrope lineage since it expresses GnRH receptor and α-GSU, and a gonadotrope cell line, LβT2, that expresses LHβ and FSHβ, GnRH receptor, α-GSU, is model of a mature, fully differentiated gonadotrope cells that responds to hormonal signals by secreting LH and FSH[2]. Therefore, the creation of LβT2 cells has allowed for further characterization of the hormonal signals that regulate gonadotropes.

**Regulation of FSH**

FSH is a heterodimeric gonadotropin composed of a common α-subunit and a unique β- subunit. The α-subunit is identical among FSH, LH, TSH and chorionic gonadotropin
hormone and is produced in excess. Therefore the unique β- subunit confers biological specificity and is the rate limiting step for mature FSH production [13]. In females, FSH causes follicular growth by binding receptors in the ovaries, which then secrete inhibin that can negatively feedback on FSH production. In males, FSH regulates sperm production in the testes and binds to receptors in Sertoli cells. Multiple studies in mice and humans have shown that FSH is required for pubertal development in females and fertility in both sexes. Kumar, et al., have shown that female mice lacking FSHβ have problems with folliculogenesis at the pre-antral stage and male mice have reduced reproductive function due to a decrease in testes size and reduction of epididymal sperm count [14]. Humans with mutations in FSHβ are infertile. The women have absent or incomplete pubertal development, while the men have relatively normal pubertal development but with reduced spermatogenesis [9]. Therefore, understanding the mechanism by which FSH is regulated is crucial for understanding mammalian fertility and reproduction.

The anterior pituitary synthesizes and secretes FSH in response to GnRH, activin, inhibin, follistatin, and steroid hormone signaling, and upon secretion, FSH travels to the gonads via the bloodstream. Activin, follistatin and inhibin are all expressed in the mature pituitary gonadotropes and can act in an autocrine manner, in addition to their secretion from the gonads. Activin is a dimer of activin/inhibin β subunits, inhibin is a heterodimer of activin/inhibin β subunits and α subunit and follistatin is a single chain glycoprotein [15]. Activin signals through Smad proteins to up-regulate FSH production, while inhibin and follistatin antagonize the effect of activin [16]. Progestins and androgens have also been shown to induce FSHβ gene expression. Progesterone and testosterone treatment in LβT2 cells up-regulates the FSHβ promoter and mRNA levels [17, 18].
**GnRH Regulation of the FSHβ Gene**

GnRH is a major regulator of FSHβ expression and does so by binding to its 7-transmembrane receptors expressed specifically on the gonadotrope cells. GnRH binding to the GnRH receptor causes a signal cascade by activating G proteins, specifically Gαq/Gα11, that activate phospholipase Cβ, which in turn cleaves PIP2, promoting the creation of diacylglycerol and activation of protein kinase C (PKC). PKC triggers the mitogen-activated protein kinase (MAPK) cascade causing the activation of extracellular signal-regulated kinase (ERK 1/2), jun-N-terminal kinase (JNK) and p38. Studies have shown that both ERK 1/2 and p38 are necessary for GnRH regulation of FSHβ though an activator protein-1 site (AP-1), but the JNK pathway has been suggested as an activator of ovine FSHβ through this site as well [19, 20].

GnRH induces FSHβ through the immediate early genes of the Fos (c-Fos, FosB, Fra-1 and Fra-2) and Jun families (c-Jun, JunB and JunD), which compose activator protein-1 (AP-1) transcription factor. Most of the Fos and Jun isoforms are induced by GnRH. Studies in the ovine FSHβ promoter in JAR placental cells have identified four putative AP-1 like elements in the -215/+1 bp region of the promoter, and two of these sites, -120 and -83, are bound by AP-1 in vitro. Also, FSHβ transcription was stimulated by c-Jun and c-Fos proteins [20]. Studies in the murine FSHβ promoter found that the proximal 398 bp of the promoter is sufficient for response to GnRH and this response localizes to an AP-1 half site (-72/-69) adjacent to a CCAAT box, which nuclear factor-Y (NFY) can bind and is responsible for basal transcription of FSHβ. Mutation or deletion of this AP-1 site reduced GnRH induction of FSHβ and its mutation eliminated FSHβ induction by c-Jun and c-Fos, indicating that it is the only active AP-1 site within the promoter[21]. The human FSHβ promoter has been shown to
be regulated by AP-1 proteins at the homologous site [22]. AP-1 is the only transcription factor known to convey GnRH responsiveness to the FSHβ promoter.

**Activating Protein 1 (AP-1)**

AP-1 is an important transcription factor that regulates multiple cell functions including proliferation, apoptosis, and organ development. Fos and Jun family proteins function as dimers and bind to AP-1 regulatory elements in promoter regions of many mammalian genes. Jun members form homodimers or heterodimers with Fos or activating transcription factor (ATF) members. c-Jun is necessary for DNA binding since a dimer of c-Jun can bind DNA, while Fos members form heterodimers with Jun members to bind to DNA. The heptanucleotide recognition sequence TGA(C/G)TCA of the AP-1 site has the highest affinity for binding of Fos-Jun dimers, but these dimers also bind the cAMP response element (CRE) site TGACGTCA with lower affinity [23]. Fos-Jun proteins are members of the bZIP family of transcription factors that contain a highly conserved basic DNA binding region and a heptad repeat of leucine residues that is required for dimerization. AP-1 regulation occurs through changes in jun and fos gene transcription, mRNA turnover, protein turnover and post-translational modifications of Jun and Fos proteins. Studies have shown that c-Jun is the most potent transcriptional activator in its group, and that c-Jun is a positive regulator of cell proliferation in various cell types [Shaulian, 2001 #5140; 24]. In AP-1 knockout mice, deletion of c-Jun has been shown to cause embryonic lethality demonstrating that c-Jun is a critical component of AP-1 function.

**c-Jun Regulation**

The proto-oncogene c-Jun is an immediate early gene, which is a group of genes that is rapidly but transiently induced directly by intracellular signaling cascades and yields
regulators of transcription. A component of the AP-1 transcription factor, c-Jun is essential for proper regulation of FSHβ by GnRH, however, little is known about how c-Jun gene transcription is regulated. Multiple studies have shown that activating transcription factor 2 (ATF-2) and c-Jun heterodimerize and up-regulate c-Jun gene transcription (Van Dam, 1995 #2276; [25]. ATF-2 is continuously shuttled between the nucleus and cytoplasm, dimerization with c-Jun in the nucleus prevents the export of ATF-2, and this dimerization is critical for c-Jun promoter activation. In F9 mouse embryonic carcinoma cells, ATF-2 localization to the nucleus occurs during retinoic acid-induced differentiation [25] and the transcription of the c-Jun gene is induced [26]. Kawasaki et al. found that ATF-2 and p300 interact in vivo and in vitro and are prerequisites for activation of the c-Jun promoter in F9 cells [Kawasaki, 1998 #2182]. A stress activating protein kinase, p38 kinase, can increases c-Jun gene expression by phosphorylating the ATF-2 transactivation domain, but not through direct phosphorylation of c-Jun [27]. In HeLa cells, an immortal human cell line, epidermal growth factor (EGF), and tissue plasminogen activator (TPA) were both able to induce the c-Jun promoter at -72 base pairs. EGF acts through a Ras-Rac-MEKK pathway and TPA stimulates ATF-1-CREB heterodimers to bind to the c-Jun promoter [28, 29].

In contrast, ATF-2 does not up-regulate c-Jun in mouse primary chondrocytes. Xinying, et al., showed, in transient transfections, that ATF-2 over-expression had no effect on c-Jun promoter activity in these cells, and, in ChIP, that ATF-2 does not interact with the c-Jun promoter [30]. Activating transcription factor 3 (ATF-3), a bZIP protein like c-Jun and ATF-2, has been reported to activate transcription as a heterodimer with c-Jun, while repress transcription as a homodimer. ATF-3 is expressed in multiple cell types including pituitary gonadotropes. In αT3-1 cells, ATF-3 functions as a transcriptional repressor, negatively regulating its own promoter and the chromogranin B gene [31], and represses the glycoprotein
hormone α– subunit gene though interaction with CRE sites [32]. In HeLa cells, ATF-3 homodimerizes and auto-represses its own promoter as well [33]. ATF-3 co-transfection attenuates the activation of the osteocalcin promoter by fibroblast growth factor and forskolin in calvarial osteoblastic cells and represses activation of an artificial promoter by c-Fos and JunB in liver cells [34]. Taub, et al., have demonstrated that co-transfection with c-Jun activates the proenkaphalin promoter. In contrast, ATF-3 has been reported to activate transcription of the c-Jun promoter when it heterodimerizes with c-Jun in NIH3T3 fibroblast cells.

Recently, a novel repressor of AP-1, jun dimerization protein 2 (JDP2), has been identified that may be able to repress c-Jun gene transcription. JDP2 is a ubiquitously expressed bZIP repressor protein that homodimerizes and heterodimerizes with c-Jun and ATF-2 proteins. In fibroblast cells, JDP2 over-expression resulted in the inhibition of AP-1 transcriptional activity [35]. JDP2 has also been identified as a repressor of ATF-2, and ATF-2/p300 induction of c-Jun by recruiting histone deacetylase 3 complex [36]. JDP2 is closely related to ATF-3 and the proteins exhibit 90% homology of their bZIP domain. Studies have shown that ATF-3 induction is dependent on JDP2 expression level and JDP2 regulates ATF-3 transcription by binding to the ATF-3 promoter region used for auto-regulation [37]. It is well established that JDP2 efficiently inhibits AP-1 transcriptional activity and known regulators of c-Jun, thus JDP2 may also interfere with c-Jun transcription directly.

**Summary**

The hypothalamic-pituitary-gonadal axis is critical for regulation of mammalian development and the reproductive cycle, with the anterior pituitary as the major integrator of
neuroendocrine and feedback signals. The anterior pituitary synthesizes and secretes the
gonadotropins, LH and FSH, in response to GnRH signaling from the hypothalamus.

Studies in humans and mice have shown that FSH is required for fertility and
complete pubertal development, and, therefore, the mechanism by which FSHβ is regulated is
of particular interest. AP-1, a dimer of c-Jun or c-Fos members, or c-Jun alone, is the only
transcription factor known to convey GnRH responsiveness to the FSHβ promoter. Fos and
Jun family proteins function as dimers and bind to AP-1 regulatory elements in promoter
regions of many mammalian genes. c-Jun has been shown to be a potent transcriptional
activator and in AP-1 knockout mice, and deletion of c-Jun has been shown to cause
embryonic lethality, demonstrating that c-Jun is a critical for development.

Contrary to c-Fos, which has been a focus of numerous studies, the mechanism of
regulation of c-Jun transcription is not yet known, although some proteins have been identified
that play roles in c-Jun gene expression. Studies have shown that ATF-2 is able to induce c-
Jun expression and that p38 through regulation of ATF-2 can regulate c-Jun. ATF-3 has been
identified as a repressor in multiple cell types and in various genes, however, it does cause
activation in particular cell types. Recently, JDP2 has been identified as a novel repressor of
ATF-2 and AP-1 transcriptional activity, therefore JDP2 has the potential to modulate
signaling pathways that regulate GnRH induction of c-Jun and transcription factors that are
involved in c-Jun expression. This study aims to characterize the mechanism by which GnRH
induces c-Jun and, thus, reveal the regulation of FSHβ gene in gonadotrope LβT2 cells.
Material and Methods

Plasmid Constructs

The 1kb of the murine c-Jun promoter linked to luciferase-reporter in pGL3 plasmid and the CRE-multimer in pGL3 plasmid were generously provided by Dr. Djurdjica Coss. Expression vectors of ATF2 and C2/ATF2 were kindly provided by Dr. Gerald Thiel (Homberg, Germany). The expression vectors A-ATF2 and dominant-negative ATF2 were generously provided by Dr. Charles Vinson (NIH). ATF3 expression vector was kindly provided by Dr. Chunhong Yan and Dr. Tsonwin Hai (Ohio State University). Dr. Richard Goodman kindly provided the expression vectors of constitutively active CREB DIEL and wild type CREB (Portland, Oregon). CREB Y134F and CREB M1 expression vectors were generously provided by Dr. Marc Montminy (La Jolla, CA).

Culture

An immortalized LβT2 cell line was cultured in 10cm plates in DMEM (Dulbecco’s Modification of Eagles Medium from Mediatech Inc., Herndon, VA) with 10% FBS (Fetal Bovine Serum from Gemini Bio-Products, West Sacramento, CA) and penicillin/streptomycin antibiotics (Gibco / Invitrogen, Grand Island, N.Y.) at 37°C. Cells were split using 1X Trypsin- EDTA (Sigma-Aldrich, St. Louis, MO).
**Hormones and Inhibitors**

Gonadotropin-releasing hormone, also known as luteinizing hormone releasing hormone, was purchased from Sigma-Aldrich (St. Louis, MO). Phorbol-12-myristate-13 acetate (TPA) (PKC activator), and inhibitors bisindolylmaleimide I hydrochloride (BIM) (PKC), KN-93 (CAMKII), H-89 (PKA), UO123 (ERK 1/2), SB 202190 hydrochloride (p38 MAPK), and JNK Inhibitor II were all obtained from Calbiochem (La Jolla, CA).

**Plasmid Sub-cloning**

5’ truncations of the c-Jun 1kb promoter were made by sub-cloning the 1kb murine luciferase c-Jun reporter plasmid. Primers used in the subcloning are listed in Table 1. The 100µl PCR reaction contained 100ng of 1kb c-Jun plasmid template, 1X HiFi Taq buffer, 0.2mM each dNTP, 0.2µM of forward and reverse primers, 2mM MgSO₄, and 1 unit Platinum Taq HiFi (Invitrogen, Carlsbad, CA). The PCR conditions were 95°C for 5 min. with a hot lid followed by 40 cycles of 95°C for 1 min., 54°C for 1 min. and 68°C for 2 min., with an additional step at 68°C for 10 min. 5% µl of each PCR product was run on a 1% gel to determine if products had the correct length. The products were phenol/chloroform extracted and ethanol precipitated. Purified samples and the pGL3 vector were double digested with KpnI and HindIII for 2 hours at 37°C. The QIAquick Gel Extraction Kit (Qiagen, Maryland) was used to gel purify the digested PCR products. The PCR products and pGL3 reporter vector were ligated overnight with Amersham’s Ready-To-Go T4 DNA Ligase (Piscataway, NJ). Ligations were transformed into DH5α Supercompetent cells (Invitrogen, Carlsbad, CA) and DNA was analyzed for the insert by digestion, prior to sequencing through the UCSD Cancer Center.
**Mutagenesis**

Mutagenesis was performed using the Quickchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions. The mutagenesis of the murine c-Jun- luciferase reporter used the PCR conditions as follows: 95°C for 30 sec., 95°C for 30 sec., 55°C for 1 min., 68°C for 14 min., then repeat steps 2-4 for 18 cycles followed by 37°C for 1 hour of Dpn treatment. The PCR products were transformed using (50μl) XL1 Supercompetent Cells (Stratagene, La Jolla, CA) and bacteria were grown on LB plates containing Ampicillin to obtain resistant clones. Mutations were confirmed by dideoxyribonucleotide sequencing performed by the DNA Sequencing Shared Resources, UCSD Cancer Center. The oligonucleotides used to make internal deletions in the wild type promoter are described in Table 2. The c-Jun wild type promoter was mutated with the oligonucleotides described in Table 3.

**Transient Transfections**

Transfection was performed one day after the cells were plated into 12-well plates in DMEM with 10% FBS. FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN). transfection reagent was used to transfect the cells following the manufacturer’s instructions. Each well was transfected with 0.5 μg of a luciferase-reporter plasmid and 0.1 μg of TK β-galactosidase, a reporter plasmid driven by a Herpes virus thymidine kinase (TK) promoter, used as a control for transfection efficiency. The night before harvesting, the cells were starved in serum-free DMEM with 0.1% BSA and penicillin / streptomycin antibiotics. 5 hours before lysing, the cells were treated with 10nM GnRH or 100nM TPA, or vehicle. In selected experiments, cells were also treated with inhibitors for 10 minutes before GnRH treatment.
**Luciferase and β-galactosidase Assay**

Forty-eight hours after transfection, cells were washed with 1X PBS and lysed with 100nM K-PO₄ buffer containing 0.2% Triton X-100. 96-well Nunc plates were loaded with 20μl of each lysate and luciferase activity was measured on a luminometer (Veritas Microplate luminometer from Turner Biosystems) by injecting 100 µl of buffer containing 25mM Tris pH 7.8, 15mM MgSO₄, 10mM ATP, and 65µM luciferin per well. The Tropix Galacto-light β-galactosidase assay (Applied Biosystems, Foster City, CA) was used to measure β-galactosidase activity on the luminometer following the manufacturer’s instructions. Transfections were performed in triplicate and repeated 3 times. Statistical analysis was performed using JMP software and ANOVA followed by the Tukey's posthoc test was used to determine significance.

**Nuclear Extract**

LβT2 cells were starved in serum free DMEM with 0.1% BSA and treated with 100nM GnRH for 15 min., 30 min, 1hr., and 2hrs. The cells were rinsed with 1X PBS and hypotonic buffer (20mM Tris pH 7.4, 10mM NaCl, 1mM MgCl₂, 1mM PMSF, protease inhibitor cocktail from Sigma (Sigma-Aldrich), 10mM NaF, 0.5mM EDTA, 0.1mM EGTA) was added. After swelling the cells were passed 3 times though a 25 G needle. The nuclear material was spun down and the pellet was re-suspended using hypertonic buffer (20mM Hepes pH 7.8, 20% glycerol, 420 mM KCl, 1.5mM MgCl₂, 1mM PMSF, protease inhibitor cocktail (Sigma-Aldrich), 10mM NaF, 0.5mM EDTA, 0.1mM EGTA). After incubating for 20 minutes, the samples were centrifuged and supernatant was aliquoted. The Bradford reagent (Bio-Rad, Hercules, CA) was used for protein determination that was calculated on a basis of a standard curve performed each time.
**Whole Cell Extract**

The LβT2 cells were starved overnight in serum-free DMEM with 0.1% BSA. Some cells were treated with inhibitors for 15 minutes prior to treatment with hormone. The cells were treated with 100nM GnRH for 30 minutes or 2 hours, and then rinsed with 1x PBS and lysed. The lysis buffer contains: 20mM Tris pH 7.4, 140mM NaCl, protease inhibitors (Sigma), 1 mM PMSF, 10mM NaF, 1% NP-40, 0.5mM EDTA, and 1mM EGTA. Bradford reagent was used for determining protein concentrations and the concentrations were calculated using a standard curve.

**Western Blot**

Equal amounts of protein from whole cell extracts were loaded with 4x sample buffer into an SDS-PAGE gel: 4% stacking gel and a 10% or 12.5% separating gel. The proteins were resolved in the gel using electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane with blocked with 10% milk in wash buffer (20mM Tris 7.4, .1% tween, 150mM NaCl, and .5% BSA) and then probed with antibodies to: ATF-2 (Santa Cruz/sc-242), phospho-ATF-2 (Santa Cruz/sc-7982), CREB (Santa Cruz/sc-271), phospho-CREB (Upstate biotechnology), TBP (Abcam/63766-100), cJun (Santa Cruz/sc-1694), and ATF-3 (Santa Cruz/sc-188). Bands were detected with a secondary antibody to rabbit or mouse IgG linked to horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA.). Enhanced Chemiluminescence (ECL) Western Blotting Detection Reagent was used to detect bands on the film (GE Healthcare).
EMSA

Oligonucleotides for probes were obtained from Integrated DNA Technologies and annealed, then labeled with \( {\gamma}^{32} \)P ATP using T4 Polynucleotide Kinase (New England Biolabs, Inc., Beverly, MA) and column purified using Micro Bio-Spin Chromatography Columns (Bio-Rad Laboratories, Inc., Hercules, CA); both reagents were used according to manufacturers protocol. Binding reactions were 20\( \mu \)l and contained 2\( \mu \)g of nuclear extract, 10mM Hepes pH 7.8, 50mM KCl, 0.5mM MgCl\(_2\), 10% glycerol, 0.1% NP-40, 0.25\( \mu \)g dIdC, 5mM DTT, and 5fmol of probe. In competition or antibody shift assays, 1nM of unlabeled oligonucleotide or 1\( \mu \)g antibody was added to the binding reaction (probe and competitor oligonucleotide sequences in tables 4-1 and 4-2). Electrophoresis was carried out on a 5% nondenaturing polyacrylamide gel. Gels were run at 250V/cm\(^2\), dried and autoradiography was performed to identify complexes.

Statistical Analysis

All experiments were performed a minimum of three times with transfections run in triplicate. Transfection efficiency was controlled by dividing the luciferase values by \( \beta \)-galactosidase and individual experiments were normalized by dividing the luciferase/\( \beta \)-galactosidase ratio by control vector pGL3-luciferase / \( \beta \)-galactosidase ratios. All normalized luciferase/\( \beta \)-galactosidase values were averaged from three experiments and ANOVA statistical analysis was performed using the JMP7 program with significance set at \( p<0.05 \). Values from western blot quantifications were also normalized and three experiments averaged and presented as fold induction from control-treated cells and (*) represents values that are statistically significantly different as analyzed by one-way ANOVA.
### Table 1: Truncation primers for c-Jun used in subcloning.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>-800</td>
<td>5’ –CGG GGT ACC ACT CTC AAA CCC GCT CAA -3’</td>
</tr>
<tr>
<td>-700</td>
<td>5’ –CGG GGT ACC GTT GCG GAG CCA GCT TAC -3’</td>
</tr>
<tr>
<td>-600</td>
<td>5’ –CGG GGT ACC GTT GCA ACC TTC ACT CCC- 3’</td>
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</tr>
<tr>
<td>-200</td>
<td>5’ –CGG GGT ACC CCT CAT CCC GTG AGC CTT-3’</td>
</tr>
<tr>
<td>-100</td>
<td>5’ –CGG GGT ACC TGG GAA AGC CTC GGG GTG-3’</td>
</tr>
<tr>
<td>-100B</td>
<td>5’ –CGG GGT ACC ACT CTC AAA CCC GCT CAA-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ –CCC AAG CTT GGA CTC TCC AAA TGC TCC -3’</td>
</tr>
</tbody>
</table>

### Table 2: Primers for mouse c-Jun internal deletions.

Only forward primers are listed.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>Δ-90/-80</td>
<td>5’-CCC CCT GAG AAC GAC GCA AGA GCC TCG GGG TCA CAT CAT C -3’</td>
</tr>
<tr>
<td>Δ-80/-70</td>
<td>5’-ACG ACG CAA GCC AAT GGG AAT CAC ATC ATG GGC TAT TTT T -3’</td>
</tr>
<tr>
<td>Δ-70/-60</td>
<td>5’-CCA ATG GGA AAG CCT CGG GGG GCT ATT TTT AGG GAT TCA C -3’</td>
</tr>
<tr>
<td>Δ-60/-50</td>
<td>5’-AGC CTC GGG CTC ACA TCA TGA GGG ATT GAC TGG TAG CAG A-3’</td>
</tr>
<tr>
<td>Δ-50/-40</td>
<td>5’-TGA CAT CAT GGG CTA TTT TTT CTT AGC AGA TAA GTG TTG A-3’</td>
</tr>
<tr>
<td>Δ-40/-30</td>
<td>5’-GGC TAT TTT TAG GGA TTG ACT AAG TGT TGA GCT CAG GCT G -3’</td>
</tr>
<tr>
<td>Δ-30/-20</td>
<td>5’-AGG GAT TGA CTC GTA GCA GAG CTC AGG CTG GAT AAG GAC T-3’</td>
</tr>
<tr>
<td>Δ-20/-10</td>
<td>5’-TGG TAG CAG ATA AGT GTT GAG ATA AGG ACT CAG ACT TGC A-3’</td>
</tr>
<tr>
<td>Δ-10/-1</td>
<td>5’-TAA GTG TTG AGC TCA GGC TGC AGA GTT GCA CTG AGT GRG -3’</td>
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</table>
Table 3: Mutated c-Jun promoter sequence.
Bold and underlined bases represent a change from wild type.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
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<td>WT</td>
<td>-105- TCGAAAACGGCGCAAGCCATGGGAA-80</td>
</tr>
<tr>
<td>NF-Y mut 1</td>
<td>-105- TCGAAAACGGCGCAAGGGAATGGGAA-80</td>
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<tr>
<td>NF-Y mut 2</td>
<td>-105- TCGAAAACGGCGCAAGCCCAAAGAA-80</td>
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<tr>
<td>WT 2</td>
<td>-105- ACGACGCAAGCCAATGGGAAAGGCT-80</td>
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<tr>
<td>NF-Y mut 3</td>
<td>-105- ACGACGCAAGCTTTTGGGAAAGGCT-80</td>
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<tr>
<td>CRE WT</td>
<td>-105- AAGCCTCGGGGTTAGCTGCGCGGT-80</td>
</tr>
<tr>
<td>CRE mut 1</td>
<td>-105- AAGCCTCGGGGTTTTTTGCTGCGCGGT-80</td>
</tr>
<tr>
<td>CRE mut 2</td>
<td>-105- AAGCCTCGGGGTTTTTTGCTGCGCGGT-80</td>
</tr>
<tr>
<td>Double mutant</td>
<td>-105-TGAGAAACGACGCAAGGGAATGGGAAAGGCTTCGGGGATCCAAATGGGAT</td>
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</tbody>
</table>

Table 4-1: Probe and Competitor Sequences used in EMSA
Mutated nucleotides in the competitors are shown in bold underline.

<table>
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<tr>
<th>Probe</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>-80 WT c-Jun</td>
<td>AGCCTCGGGG TTACGTATG GGCTATTTTT</td>
</tr>
<tr>
<td>CRE</td>
<td>AGCCTCGGGG TCCAAAATG GGCTATTTTT</td>
</tr>
<tr>
<td>-105 WT</td>
<td>TGAAGACGCA GCAAGCAAT GGGAAAGCCT</td>
</tr>
<tr>
<td>NFY mut</td>
<td>TGAAGACGCA GCAAGCAAT GGGAAAGCCT</td>
</tr>
</tbody>
</table>

Table 4-2: Scanning mutations used as competitors in EMSA
Mutated nucleotides in the competitors are shown in bold underline.

<table>
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<th>Probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>-80 WT c-Jun</td>
<td>AGCCTCGGGG TTACGTATG GGCTATTTTT</td>
</tr>
<tr>
<td>A</td>
<td>AGCCTCGGGG TTACGTATG GGCTATTTTT</td>
</tr>
<tr>
<td>B</td>
<td>AGCCTCGGGG TTACGTATG GGCTATTTTT</td>
</tr>
<tr>
<td>C</td>
<td>AGCCTCGGGG TTACGTATG GGCTATTTTT</td>
</tr>
<tr>
<td>D</td>
<td>AGCCTCGGGG TTACGTATG GGCTATTTTT</td>
</tr>
<tr>
<td>E</td>
<td>AGCCTCGGGG TTACGTATG GGCTATTTTT</td>
</tr>
<tr>
<td>F</td>
<td>AGCCTCGGGG TTACGTATG GGCTATTTTT</td>
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<tr>
<td>G</td>
<td>AGCCTCGGGG TTACGTATG GGCTATTTTT</td>
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<td>H</td>
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<td>I</td>
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<td>J</td>
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<td>K</td>
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<tr>
<td>L</td>
<td>AGCCTCGGGG TTACGTATG GGCTATTTTT</td>
</tr>
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</table>
III

Results

**GnRH Induces c-Jun and the Response Maps to the -86/+1 Region of the c-Jun Promoter**

GnRH regulation of FSHβ gene occurs through an AP-1 site found in the proximal promoter at -76 base pairs from the transcriptional start site [21]. The AP-1 protein is a heterodimer of c-Jun and c-Fos or a homodimer of the c-Jun protein, and therefore by examining how c-Jun induction is regulated, we can further understand GnRH regulation of FSHβ. Studies in primary gonadotropes have shown that mRNA and protein levels of c-Jun are induced by GnRH [38], but the scarcity of gonadotropes in the pituitary prevents detailed molecular studies using primary cells. Thus, to analyze the molecular mechanism of GnRH induction of the c-Jun gene LβT2 cells, an immortalized pituitary gonadotrope cell line, were used. We have shown c-Jun protein is induced by GnRH treatment in the LβT2 cells (Debra Yeh, Coss Lab, personal communication). Therefore to investigate if the c-Jun promoter is sufficient for induction by GnRH, LβT2 cells were transfected with the -1000/+200 base pair (bp) murine c-Jun promoter fused to a pGL3 luciferase reporter and thymidine kinase-β-galactosidase (β-gal), then treated with 5 hours of GnRH. Luciferase and β-gal expression were measured and the level of GnRH induction was analyzed as described in the Methods and Materials section. The luciferase expression was divided by β-gal expression levels to normalize for transfection efficiency, and this ratio was normalized to pGL3 to account for any expression of the empty vector and to normalize three experiments. The experiments were performed three times in triplicate. GnRH treatment increased expression 3.4 fold compared to vehicle (Figure 1A). To ensure that the +1/+200 bp sequences do not contain a site that is
important for GnRH induction of c-Jun, a -800/+200 bp c-Jun promoter and a -800/-1 bp promoter were created and used in transient transfections. The luciferase to β-gal ratio was normalized to pGL3, and to eliminate the effect of basal expression and to more easily observe the effect of GnRH. Results were presented as fold induction, where GnRH treatment for each reporter was divided with vehicle control. We determined that the +1/+200 sequence does not have any effect on fold induction, because there is no significant difference between the -800/+200 and -800/-1 bp reporters (Figure 2B).

To identify regions of the c-Jun promoter critical for the induction by GnRH, truncations consisting of different promoter lengths were created and analyzed in transient transfection assay. For these experiments, 0.5 µg of -1000, -900,-800,-700, -600, -400, -200, or -86 bp c-Jun promoter luciferase-reporter plasmid along with 0.1 µg of thymidine kinase-β-gal control plasmid were transfected into LβT2 cells. Cells were treated with vehicle control or 100 nM GnRH for five hours prior to harvest. Basal expression of the promoter was reduced significantly when the promoter was truncated from -200 to -86 base pairs but fold induction is not significantly changed, while GnRH induction is maintained in the most proximal region (Figure 2). Thus, GnRH activity on the c-Jun promoter maps to the region -86 bp from the transcriptional start site.

To further limit the region of responsiveness, site directed mutagenesis in the -1000 bp c-Jun reporter plasmid was performed to make consecutive 10 base-pair internal deletions between -100 and the start site of transcription. LβT2 cells were transfected with the specified 10 bp internal deletions fused with luciferase reporter gene and treated with vehicle or GnRH (Figure 3). Basal expression was decreased significantly with -90/-71 deletions, while GnRH induction decreased with the -70/-61 deletions. These experiments determined that -70/-61
region contains elements that convey responsiveness to GnRH, while upstream regions between -90/-71 contain elements critical for basal expression.

Upon in silico analysis, we determined that the -90/-71 region contains a CAATT element that could be bound by NFY transcription factor. The -70/-61 region contains a CRE element that is one base pair different from the consensus CRE site, TGACGTCA, which can be bound by members of CREB, ATF and JUN families of transcription factors. To determine the functional significance of these sites, NFY and CRE, point mutations were created in the two putative transcription factor binding sites using site directed mutagenesis. Two different mutations were created for the CRE element, while three were created for the NFY element. The mutations were analyzed using transient transfection assays. Basal expression of the c-Jun promoter was significantly lower with mutations in the NFY site, while induction by GnRH was significantly decreased with the CRE mutations indicating its important role in GnRH induction (Figure 4). Thus, mutation of either of these elements consistent with their deletion, have functional significance in the regulation of c-Jun expression.

**The CRE Site is Necessary and Sufficient for GnRH Induction**

The CRE site was determined to be critical for GnRH induction of the c-Jun promoter by both deletion (Figure 3) and mutation (Figure 4), but whether it was sufficient was not yet known. To assess the sufficiency of the CRE site, a multimer was created consisting of four copies of the canonical CRE site linked to the minimal heterologous TK promoter on the luciferase reporter. The TPA response element (TRE) multimer contains four AP-1 binding sites and was used as a positive control, since previous studies have shown TRE is responsive and sufficient for GnRH induction [21]. Using transient transfection of the CRE multimer or TRE multimer as a positive control, with a control β-gal plasmid, LβT2 cells were treated with
vehicle or 100 nM GnRH for 5 hr. GnRH induced the TRE multimer by 12.6 fold and the CRE multimer by 5.6 fold when compared to the TK-luciferase reporter vector demonstrating that the CRE site is sufficient for GnRH responsiveness (Figure 5).

**GnRH Treatment Phosphorylates ATF-2 and Induces ATF-3**

Knowing that the CRE site was critical for c-Jun induction, the next step was to determine if GnRH treatment would induce any CRE binding proteins. Our lab has performed westerns with whole cell extracts from LβT2 cells treated with GnRH at different time points from 0 to 4 hours, resolved on an SDS-PAGE gel and transferred to an to a polyvinylidene fluoride membrane. The membranes were blotted with antibodies for CREB, phospho-CREB, ATF-1, ATF-2, phospho-ATF-2, and ATF-3. CREB, ATF-1, and ATF-2 do not show any significant change in protein amount upon treatment with GnRH. ATF-3 is induced by GnRH at about one hour with a maximum at 2 hours, as has been previously shown [32]. Blotting for phospho-CREB surprisingly yielded multiple bands. Upon checking the molecular weight carefully and stripping and realigning the protein, the upper band was determined to be CREB and it was not changed by GnRH treatment. The company was contacted and it was disclosed that the antibody can cross react with phospho-ATF-1 as well as phospho-CREB and by using the molecular weight the lower band was determined to be phospho-ATF-1. Previous studies using αT3-1 cells found that CREB is phosphorylated by GnRH [39] however, in LβT2 cells, CREB is already basally phosphorylated and it does not change with GnRH treatment. On the other hand, GnRH induced phosphorylation of ATF-2 at thirty minutes which is maintained up to four hours. Thus, GnRH induces phosphorylation of ATF-2 at thirty minutes and ATF-3 protein at 1 hour.
Figure 1: GnRH induces c-Jun expression.
A) The LβT2 cells were transfected with -1000/+200 base pair (bp) murine c-Jun promoter fused to a pGL3 luciferase reporter and treated with vehicle or GnRH. GnRH treatment increases expression 3.4 fold compared to vehicle. The luciferase expression was divided by β-galactosidase (β-gal) expression levels and this ratio was normalized to luciferase / β-gal ratio for pGL3, to eliminate GnRH effect on the empty vector and to normalize 3 repeats. The values represent the mean of three experiments performed in triplicates and * indicates statistically significant induction as determined by JMP software and student's T-test.

B) LβT2 cells were transfected with c-Jun promoter reporters indicated underneath the bar and treated with vehicle or GnRH. The luciferase to β-gal ratio was normalized to pGL3, and to determine fold induction, GnRH treatment was normalized to vehicle control for each reporter. The result illustrate that +1/+200 sequence does not have any effect on fold induction by GnRH.
LβT2 cells were transfected with specified murine c-Jun promoter truncations linked to a pGL3 luciferase reporter or with control pGL3 reporter and treated with vehicle or GnRH. GnRH activity on the c-Jun promoter maps to the region -86 from the transcription start site. Luciferase/β-gal ratio was normalized to pGL3 and values represent mean of three experiments done in triplicates. (*) indicates a statistically significant reduction in GnRH induction between two reporters. (#) indicates a decrease in basal expression.

**Figure 2: GnRH Response Maps to -86/+1 Region of the c-Jun Promoter.**
Figure 3: 10 base pair internal deletions in the c-Jun promoter determined sites of responsiveness to GnRH.
LβT2 cells were transfected with the specified 10 bp internal deletions of mouse -1000 bp c-Jun promoter fused with luciferase reporter gene and treated with vehicle or GnRH. These experiments determined that the -70 to -61 region contains elements that convey responsiveness to GnRH, while an upstream regions between -90/-81 and -80/-71 contain elements critical for basal expression. Luciferase/β-gal ratio was normalized to pGL3 and values represent mean of three experiments performed in triplicates. (*) indicates a statistically significant reduction in GnRH induction from wild type. (#) indicates a decrease in basal expression.
Figure 4: CRE and NFY sites have functional significance in the regulation of c-Jun expression.

Prior to treatment, mutations of the -1000 bp mouse c-Jun promoter were transfected into LβT2 cells. The NFY mutations elicit a significant reduction in basal expression from wild-type, while CRE sites have a role in GnRH response. Luciferase/β-gal ratio was normalized to pGL3 and the average of three experiments performed in triplicate is represented. (*) indicates a statistically significant reduction in GnRH induction from wild type. (#) indicates a reduction in basal expression.
Figure 5: CRE element is sufficient for GnRH responsiveness.

LβT2 cells were transfected with CRE (cAMP response element), TRE (TPA response element) multimers (four copies of the canonical CRE or TRE site linked to the minimal heterologous TK promoter on the luciferase reporter), minimal TK-Luciferase reporter, served as a control, and treated with vehicle or GnRH. GnRH fold induction is increased with the CRE multimer in comparison to TK-luciferase heterologous promoter showing that the CRE element is sufficient for GnRH responsiveness, while previous studies have shown that TRE is responsive and sufficient. The luciferase to β-gal ratio was normalized to pGL3, and GnRH treatment was divided with vehicle control and the average of three experiments done in triplicate is represented. (*) represent significant difference from control.
**ATF-2, Phosphorylated ATF-2, and ATF-3 Bind to the c-Jun Promoter**

To identify which of these protein complexes may bind the c-Jun promoter and are involved in GnRH induction and basal expression, electrophoretic mobility shift assays (EMSAs) were performed. Cells were treated with GnRH for 1 hour, and nuclear extracts were obtained and incubated with the probe. Using -105 to -76 region of the c-Jun promoter as probe containing the CAATT element, LβT2 cells nuclear extracts were analyzed for binding. There are a number of complexes that bind this region regardless of GnRH treatment. To determine if NFY is one of these complexes, we included anti-NFY (αNFY) antibody, and to ensure that our protein of interest is binding specifically to the probe, competitors were included. The wild-type competitor is non-labeled -105/-76 probe that is added in excess and if the protein binding is specific, proteins would bind to this excess probe and not appear on the gel. Mutant competitor is non-labeled -105/-76 c-Jun with a mutated CAATT site that is added in excess, and if the binding of NFY is specific for this site then NFY will not be able to bind the mutant competitor and will appear on the gel. Upon inclusion of αNFY there is a supershift of the NFY band in GnRH treated and untreated lanes. The WT competitor competed successfully for the NFY containing complex, indicating specificity of binding to this probe, while competitor that included mutation in the NFY site was not able to compete for the complex, indicating that the NFY indeed binds this CAATT element (Figure 6).

To determine the proteins that play roles in GnRH induction of c-Jun by potentially binding to the identified CRE element, we performed additional EMSA analysis. Using the -80/-51 region of the c-Jun promoter as a probe, containing CRE binding domains, LβT2 cells nuclear extracts were analyzed for binding complexes. Previous studies have shown that CRE sites are mainly bound by the CREB transcription factor, but members of the ATF family
of transcription factors may also bind [40]. EMSAs determined that at least three complexes differ in their binding to DNA following incubation of the probe with nuclear extract from GnRH treated gonadotrope cells compared to controls, indicating that GnRH induced protein complexes to bind this region. To determine the identity of the proteins in these complexes, we included antibodies for CREB and ATF family members in the binding reaction (Figure 7). Upon addition of antibody for CREB and ATF-1, no supershifts were detected, but with the addition of antibody for ATF-2 there is a supershift in GnRH treated and non-treated LβT2 cells indicating ATF-2 binds to the c-Jun promoter in a constitutive manner. Addition of the ATF-3 antibody caused a supershift in the GnRH treated lane, indicating that ATF-3 binds the c-Jun promoter upon its induction by GnRH. This is consistent with the western blot results that show ATF-2 is present in the cells regardless of GnRH treatment and that GnRH induces ATF-3.

To evaluate which base pairs are needed for the identified complexes’ interaction with the c-Jun promoter, we used 2 base pair scanning mutations, as competitors, in 200 fold excess in EMSA. Nuclear extracts were collected from LβT2 cells and incubated in the binding reaction, with the non-labeled scanning mutation competitors and the 32P-labeled wild-type probe. The scanning mutations made within the -80/-50 wild-type c-Jun promoter sequence were labeled A-L (Figure 8A). WT competitor was able to compete for binding of all the complexes. When the core CRE sequence is mutated, as in lanes containing competitors D-H, the oligonucleotides are not able to compete for the 3 GnRH induced complexes (Figure 8B). These experiments illustrate that all complexes induced by GnRH treatment require the CRE site for binding to DNA, since mutating the core residues eliminates binding. This indicates that GnRH activates several proteins that can interact with the same sequence on the c-Jun promoter.
Since there are three different complexes that are induced by GnRH, to analyze whether the time points at which they bind the c-Jun promoter change, EMSAs were performed with nuclear extracts that were made from LβT2 cells treated at different time points with GnRH: 0 min. to 120 min. and incubated with oligonucleotides encompassing the -80/-51 region of the c-Jun promoter labeled with $^{32}$P. GnRH again caused various proteins to change binding to the probe, but at different time points (Figure 9). Upon the addition of anti-ATF-2 antibody, the ATF-2 containing complex is supershifted at all time points, indicating that ATF-2 binds c-Jun promoter in the constitutive manner (Figure 9). However, there is an increase in binding affinity at 15 and 30 min. and decrease at 120 minutes. With the addition of antibody specific for phosphorylated-ATF-2, there is a supershift and increase in the intensity beginning at 15 min. to 30 min., while returning to basal level by the 2 hours of GnRH treatment. We conclude that GnRH induces phosphorylation of ATF-2 at 15 min. of treatment and that is consistent with observation in the western blotting and likely contributes to higher affinity binding at those time points. The ATF-3-containing band is observed following 60 min. of treatment, which is consistent with ATF-3 protein induction following the 1-hour GnRH treatment as observed in western blots. ATF-3 is supershifted beginning at 60 min. and through 120 min. of GnRH treatment, as seen with the addition of αATF-3 (Figure 9). Since ATF-3 and ATF-2 bind the same sequence, the CRE site, we postulate that the induction of ATF-3 at 1 and 2 hours of treatment contributes to the decreased ATF-2 binding observed at those time points, due to ATF-3 replacement of ATF-2 at the CRE site. Thus, GnRH rapidly phosphorylates ATF-2 at 15 min. of treatment, which contributes to its higher affinity binding, and subsequently induces ATF-3 protein at 1 hour of treatment, which replaces ATF-2 at CRE sites.
Figure 6: NFY proteins bind to the -105/-75 region in the c-Jun promoter
Nuclear extracts of LβT2 cells, treated with GnRH or vehicle control, were incubated with the -105/-75 probe and an NFY antibody or unlabeled nucleotides used in 200 fold excess. The wild type competitor is unlabeled -105 to -76 probe that is added in excess and mutant competitor is unlabeled -105/-76 c-Jun with a mutated CAATT site. Arrows indicate the supershift with αNFY and the complex containing NFY protein.
Figure 7: ATF-2 and ATF-3 proteins bind to the -80/-51 region of the c-Jun promoter. EMSA was used to detect complexes binding to the -80/-51 region of the c-Jun promoter. Nuclear extracts were collected from LβT2 cells treated with vehicle control or two hour GnRH treatment. The binding reactions included antibodies for CREB, ATF-1, ATF-2, ATF-3, and IgG control. Arrows indicate proteins of interest that bind the probe. ATF-2 protein binds the probe in a constitutive manner, while ATF-3 binds the probe only following GnRH treatment.
Figure 8: All complexes induced by GnRH require the CRE site for binding.
Nuclear extracts from LβT2 cells treated with GnRH and unlabeled competitors in 200 fold excess were included in the binding reaction with -80/-51 probe in the EMSA. A) The wild type -80/-51 sequence is shown and aligned to competitors A-L that were used in B; the base pairs that are mutated in the competitor are shown. B) The competitors can compete for binding except when the core CRE site is mutated; D-H, oligonucleotides are not able to compete for the GnRH induced complexes.
Figure 9: GnRH treatment induces ATF-3 and the phosphorylation of ATF-2. EMSAs were performed using nuclear extracts from LβT2 cells treated with GnRH for various time periods, 0 to 120 minutes, to detect complexes binding to the -80/-51 region of c-Jun promoter. Antibodies for control IgG, ATF-2, phospho-ATF-2 and ATF-3 were added to the binding reactions, as indicated above corresponding lanes. Increase in phosphorylation of ATF-2 by GnRH can be observed starting at 15 to 30 min and returns to basal at 120 min. ATF-3 was detected beginning at 1 hour of GnRH treatment as indicated both by appearance of the ATF-3 containing band in IgG lane samples and by appearance of the supershift in samples where ATF-3 antibody was included. Arrows indicate proteins of interest that bind the probe.
**ATF-2 Induces through the c-Jun Promoter and CRE element**

After establishing that ATF-2 and ATF-3 proteins are induced by GnRH treatment and bind the c-Jun promoter, we determined whether the CRE site binding proteins have functional significance in induction of c-Jun promoter. We obtained expression vectors that were over-expressed with a c-Jun luciferase reporter to determine which one of these proteins is functional. The CRE multimer linked to a TK promoter on the luciferase reporter was used in transient transfections to determine if the CRE site was sufficient and if the proteins in question work solely through the CRE element. LβT2 cells were transfected with 0.5 µg of multimer or -1000/+200 c-Jun promoter and 0.1 µg of ATF-1, ATF-2, ATF-3, or CREB expression vector. The luciferase to β-gal ratio was normalized to empty vector control. These results demonstrate that ATF-1 does not significantly induce either the CRE multimer or the c-Jun promoter (Figure 10), while over-expression of ATF-2 causes significant induction of both reporters. ATF-3 represses the CRE site by 65% (Figure 10A) but has no effect on c-Jun promoter expression (Figure 10B). Surprisingly, over-expression of CREB does not induce the CRE multimer (Figure 10A), although it is functional on the c-Jun promoter (Figure 10B). Although CREB over-expression led to a higher expression of the c-Jun promoter, we do not think that endogenous CREB protein plays a role, since it was not regulated by GnRH as shown either in the western blot or in EMSA analysis. GnRH does not change the phosphorylation of CREB nor the amount of CREB protein, nor does CREB bind c-Jun promoter in EMSA. ATF-3 is induced by GnRH (Debra Yeh, Coss lab, personal communication) and it binds c-Jun promoter following GnRH treatment (Figure 7); however, over-expression of ATF-3 did not reduce c-Jun expression, while it behaved as a repressor on the CRE multimer. ATF-3 was previously reported to function primarily as a repressor [31] and thus, the lack of effect on c-Jun promoter was surprising. Differential effects of ATF-3 are
most likely context dependent and interaction with another protein, such as NFY, which is found binding to the c-Jun promoter, may change its function.

**ATF-2 is Necessary for c-Jun Induction by GnRH**

After establishing that ATF-2 induces the c-Jun promoter, we continued our analysis of ATF-2, and overexpressed a dominant-negative ATF-2, in which the DNA-binding domain was mutated. LβT2 cells transfected with -1000/+200 c-Jun promoter and expression vectors for empty vector control, ATF-2, and dominant-negative ATF-2 were treated with vehicle or GnRH (Figure 11). The dominant-negative ATF-2 reduces basal expression by 35%, induction of c-Jun promoter by wild type ATF-2 by 48% and induction with GnRH treatment by 53%. Therefore, ATF-2 is necessary for c-Jun induction by GnRH.
Figure 10: ATF-2 Induces the c-Jun Promoter and CRE element.

LβT2 cells were transfected with 0.5µg of CRE multimer (A) or -1000/+200 c-Jun promoter (B) and 0.1 µg of either ATF-1, ATF-2, ATF-3 or CREB expression vector. Overexpression of ATF-1 does not significantly change expression of either the CRE multimer or c-Jun promoter, but overexpression of ATF-2 causes significant induction of both reporters. ATF-3 represses CRE site expression by 65% but has not effect on expression of c-Jun promoter. CREB does not induce the CRE multimer (A) although it is functional in the c-Jun promoter (B). The results were presented as fold induction from the empty vector control. Experiments were performed three times in triplicate. (*) indicates a statistically significant induction, while (#) indicates a decrease in expression.
Figure 11: ATF-2 is necessary for induction of the c-Jun promoter by either ATF-2 overexpression or GnRH.
LβT2 cells were transfected with -1000/+200 bp c-Jun promoter luciferase-reporter plasmid and control expression vectors or wild-type ATF-2 or dominant-negative ATF-2, where DNA binding domain is mutated. The cells were treated with vehicle or 5 hours of GnRH where indicated. The dominant-negative ATF-2 reduces basal expression by 35%, induction of c-Jun promoter by wild-type ATF-2 by 48% and induction with GnRH treatment by 53%. (#) indicates a decrease in expression.
**ATF-3 Represses ATF-2 Induction of c-Jun**

Having established that ATF-2 is necessary for GnRH induction of c-Jun, and due to ATF-3’s function as a repressor, we analyzed whether ATF-3 can function as a repressor of c-Jun induction. c-Jun is an immediate early gene with a very transient expression profile and we postulated that there is a protein that actively switches off transcription of c-Jun, to allow for timely regulation of this important transcription factor. Activation of ATF-2 is rapid, at 30 min. (Coss, personal communication and Figure 9), and, thus, corresponds to its function in rapid induction of c-Jun. ATF-3 is induced at 1 hour of treatment and binds the c-Jun promoter after 1 hour (Figure 9), which corresponds to the start of decline in c-Jun mRNA (Coss, personal communication). Thus, because of its function as a repressor and the time course of its induction, we postulated that ATF-3 may serve to replace ATF-2 on the promoter to rapidly shut off c-Jun transcription. LβT2 cells were transfected with CRE multimer (A, B) or c-Jun promoter (C, D) linked to luciferase reporter. The cells were co-transfected with expression vectors, ATF-2 and/or ATF-3, (Figure 12 A and C). ATF-2 significantly increases expression of both the CRE multimer and c-Jun, as demonstrated before (Figure 10), but ATF-3 only reduces basal expression in the CRE multimer. ATF-3 repressed ATF-2 induction of both c-Jun promoter and the CRE multimer.

The cells were treated with vehicle or GnRH (Figure 12 B and D), and ATF-3 expression vector significantly reduced basal expression, as well as GnRH induction, of the CRE multimer. However, c-Jun promoter expression was not significantly reduced with ATF-3 over-expression, demonstrating that ATF-3 alone is not able to repress GnRH induction of c-Jun. Therefore ATF-3 is able to act as a repressor for c-Jun induction with ATF-2 over-expression, but it is not able to repress c-Jun induction by GnRH.
**JDP2 Represses GnRH and ATF-2 induction of c-Jun**

Because ATF-3 is not inhibitory to GnRH induction of c-Jun, we postulated that JDP2 (Jun Dimerizing Protein 2), a previously reported repressor of AP-1 and ATF-2 [35], may repress c-Jun transcription. LβT2 cells were transfected with CRE multimer (A, B) or c-Jun promoter (C, D) linked to luciferase reporter. The cells were co-transfected with expression vectors, ATF-2 and/or JDP2, (Figure 13 A and C). ATF-2 significantly increases expression of both the CRE multimer and c-Jun, as demonstrated before (Figures 10 and 12), while JDP2 reduces basal expression and represses ATF-2 induction of both the c-Jun promoter and the CRE multimer.

The cells were treated with vehicle or GnRH (Figure 13 B and D), and JDP2 expression vector significantly reduced basal expression, as well as GnRH induction of the CRE multimer. The c-Jun promoter induction by GnRH was significantly reduced with JDP2 over-expression, therefore JDP2 is able to repress induction by ATF-2 over-expression as well as GnRH induction of c-Jun. Thus, the ability of JDP2 to repress the induction of c-Jun with ATF-2 and GnRH makes it a critical regulator of c-Jun expression and a novel player in the GnRH signaling pathway.

This study identified the molecular mechanism of GnRH induction of the c-Jun promoter through the phosphorylation of ATF-2. Although ATF-3 was not identified as the repressor for c-Jun expression, we identified a novel player, JDP2, which is able to negatively feedback on GnRH regulation of c-Jun.
Figure 12: ATF-3 Represses ATF-2 Induction of c-Jun.
LβT2 cells were transfected with CRE multimer (A, B) or WT c-Jun promoter (C, D) linked to luciferase reporter and control or ATF-2 or ATF-3 expression vector, and treated with vehicle or GnRH. A) ATF-2 induction and basal expression of CRE multimer was inhibited with overexpression of ATF-3. B) ATF-3 expression vector significantly reduced basal expression as well as GnRH induction of the CRE multimer. C) ATF-2 induction of the c-Jun promoter was also inhibited with overexpression of ATF-3. D) Surprisingly, GnRH induction of c-Jun was not inhibited by overexpression of ATF-3.
Figure 13: JDP2 Represses GnRH and ATF-2 Induction of c-Jun.
LβT2 cells were transfected with CRE multimer (A, B) or WT c-Jun promoter (C, D) linked to luciferase reporter and control, ATF-2 or JDP2 expression vector, and treated with vehicle or GnRH. A) JDP2 over-expression inhibited basal and ATF-2 induction of the CRE multimer. B) JDP2 expression vector significantly reduced basal expression and GnRH induction of the CRE multimer. C) Over-expression of JDP2 inhibited ATF-2 induction of the c-Jun promoter. D) C-Jun induction by GnRH was inhibited by over-expression of JDP2.
Follicle-stimulating hormone is crucial for mammalian development and regulation of the reproductive cycle. Released from gonadotropes in the anterior pituitary, FSH acts on the gonads and is responsible for folliculogenesis in females and sperm production in the testes in males. FSH is a heterodimeric glycoprotein composed of a common α-subunit and a unique β-subunit. The β-subunit is the rate-limiting step in production of mature FSH, as well as the subunit that confers biological specificity. Women with FSHβ mutations undergo absent or incomplete pubertal development, while males have essentially normal pubertal development, but azoospermia, and infertility of both sexes [9] Thus, understanding the mechanisms that regulate FSHβ gene transcription is important for reproductive health.

Gonadotropin-releasing hormone is an important regulator of FSHβ gene expression, and AP-1 is the only transcription factor known to convey GnRH responsiveness to the FSHβ promoter. The FSHβ promoter contains an AP-1 half site (-72/-69) and deletion or mutation this AP-1 site reduces GnRH induction of FSHβ [21]. A component of the AP-1 transcription factor, c-Jun is essential for proper regulation of FSHβ by GnRH. Studies in primary gonadotropes have shown that c-Jun mRNA and protein are induced by GnRH [38], however, the mechanisms of regulating c-Jun gene transcription are not well established. Therefore, the purpose of this study was to investigate the mechanism of GnRH induction of c-Jun.

We identified two elements important for c-Jun expression: an NFY site, important for basal expression and a CRE site, critical for GnRH induction. Previous studies identified
AP-1, SP-1, MEF-2, and GAGCCTC elements to be important for basal expression of c-Jun in HeLa and HEK293 cells [41, 42]; however, the major regulator of basal expression in the gonadotropes seems to be NFY. NFY was already shown to be important for basal expression of other genes in the gonadotrope: rodent and human FSHβ[43] and the bovine LHβ gene [44]. Our study identified an NFY site at -90/-71 base pairs in the c-Jun promoter. Although there are likely additional elements important for basal expression 5’ to the NFY site, this NFY site appears to integrate hormonal induction and basal expression of gonadotrope genes.

C-Jun is induced in numerous cells by variety of stimuli, hormones, growth factors, and cytokines. In the human hepatoma cell line, HepG2, IL-1 uses CRE and TRE sites, -71 and -191 upstream of the transcriptional start site, for induction[45], while in MEF cells epidermal growth factor (EGF) uses CRE and MEF2 sites, -72 and -59 upstream of the transcriptional start site, to induce c-Jun [46]. GnRH, however, uses only the CRE site for c-Jun induction, and we identified a CRE element at -70/-61 of the murine c-Jun promoter that is one base pair different from the consensus CRE site, TGACGTCA. It is likely that GnRH acts specifically through the CRE site due to interactions of CRE site binding elements and NFY elements in the regulation of the c-Jun promoter.

Multiple studies have affirmed that CRE sites are bound by CREB and ATF family members. Glycoprotein hormone alpha subunit (α-GSU), the common α-subunit in FSH, LH, TSH, and chorionic gonadotropin hormone, is comprised of two tandem CRE sites. In thyrotrope cells, the α-GSU CRE sites are bound by CREB [47], while in gonadotrope cells, studies have identified multiple factors that bind. In αT3-1 cells, Xie et al., showed that the CRE sites can be bound by ATF-3[32], while Fowkes, et al., found that ATF-2 and phosphorylated CREB (p-CREB) can bind to the CRE sites in αT3-1 and LβT2 cells[48]. GnRH has been shown to phosphorylate ATF-2 and CREB in αT3-1 and LβT2 cells, and
GnRH treatment enhanced the binding of CREB and ATF-2 and other CREB-related proteins to the CRE element of the α-GSU promoter [48]. Using western blots, we identified two proteins in LβT2 cells that were affected by GnRH: phosphorylation of ATF-2 protein at 30 minutes and induction of ATF-3 at 1 hour, and using EMSAs we found that both phospho-ATF-2 and ATF-3 are able to bind the CRE site on the c-Jun promoter. ATF-2 over-expression significantly increases expression of both the CRE multimer and the c-Jun promoter and GnRH induces phosphorylated ATF-2. Therefore, we believe that GnRH induces c-Jun through ATF-2 binding to the CRE site.

Although previous studies have identified CREB and phosphorylated CREB as proteins that are able to bind the CRE site in gonadotropes, we do not believe that endogenous CREB protein plays a role, since it was not regulated by GnRH, as shown either in the western blot or in EMSA analysis. GnRH does not alter the phosphorylation of CREB nor the amount of CREB protein, and CREB does not bind to the c-Jun promoter in EMSAs. As we stated, the antibody for phospho-CREB can cross react with phospho-ATF-1, because the same kinases regulate these proteins and the antibody recognition site is not specific, making it difficult to properly distinguish them.

Since ATF-3 and ATF-2 bind the same sequence, the CRE site, we postulated that the induction of ATF-3 at 1 and 2 hours of GnRH treatment contributes to the decreased ATF-2 binding observed at those time points, due to ATF-3 replacement of ATF-2 at the CRE site. However, although we saw an induction of ATF-3, we do not believe that it plays a role in c-Jun regulation because GnRH induction of c-Jun promoter expression was not significantly reduced with ATF-3 over-expression. Therefore, ATF-3 is able to act as a repressor for c-Jun induction with ATF-2 over-expression, but, alone, it is not able to repress c-Jun induction by GnRH. Although previous studies identified ATF-3 as a repressor in gonadotropes, we do not
see ATF-3 repression of GnRH induction (Xie J Mol. Endo 2005). ATF-3 has been shown to form dimers with other proteins to act as a functional repressor. Therefore ATF-3 may need to interact with other factors to bind and repress c-Jun induction [31].

Since ATF-3 is not able to inhibit GnRH induction of c-Jun, we wanted to identify another possible player that could repress c-Jun. We postulated that JDP2 (Jun Dimerizing Protein 2) may repress c-Jun transcription because JDP2 is closely related to ATF-3 and the proteins exhibit 90% homology of their bZIP domain. Previous studies also reported JDP2 as a repressor of AP-1 and ATF-2[35, 36]. In our study, JDP2 overexpression was able to significantly reduce GnRH induction of the c-Jun promoter, as well as induction by ATF-2 over-expression. Our study agrees with previous studies’ findings that identify JDP2 as a repressor of transcription and further defines JDP2 proteins role as a repressor expanding its role in gonadotrope cells.

This study is the first to identify JDP2 as a critical regulator of c-Jun expression and a novel player in the GnRH signaling pathway in LβT2 cells. c-Jun is an immediate early gene with a very transient expression profile and we have identified a possible feedback mechanism for c-Jun regulation by GnRH. GnRH is able to phosphorylate ATF-2, which can bind to the CRE site in the c-Jun promoter and we postulate that JDP2 can turn the transcription of c-Jun off, to allow for timely regulation of this important transcription factor. c-Jun is a critical component of the AP-1 transcription factor, and is therefore essential for proper regulation of FSHβ by GnRH.
References


