A Novel AP-1 Site Is Critical for Maximal Induction of the Follicle-stimulating Hormone β Gene by Gonadotropin-releasing Hormone*

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Regulation of follicle-stimulating hormone (FSH) synthesis is a central point of convergence for signals controlling reproduction. The FSHβ subunit is primarily regulated by gonadotropin-releasing hormone (GnRH), gonadal steroids, and activin. Here, we identify elements in the mouse FSHβ promoter responsible for GnRH-mediated induction utilizing the LβT2 cell line that endogenously expresses FSH. The proximal 398 bp of the mouse FSHβ promoter is sufficient for response to GnRH. This response localizes primarily to an AP-1 half-site (−72/−69) juxtaposed to a CCAAT box, which binds nuclear factor-Y. Both elements are required for AP-1 binding, creating a novel AP-1 site. Multimers of this site confer GnRH induction, and mutation or internal deletion of this site reduces GnRH induction by 35%. The same reduction was achieved using a dominant negative Fos protein. This is the only functional AP-1 site identified in the proximal 398 bp, since its mutation eliminates GnRH induction by c-Fos and c-Jun. GnRH regulation of the FSHβ gene occurs through induction of multiple Fos and Jun isoforms, forming at least four different AP-1 molecules, all of which bind to this site. Mitogen-activated protein kinase activity is required for induction of FSHβ and JunB protein. Finally, AP-1 interacts with nuclear factor-Y, which occupies its overlapping site in vivo.

FSH is a heterodimeric glycoprotein hormone consisting of two subunits: an α-subunit, which is common to LH, thyroid-stimulating hormone, and chorionic gonadotropin (CG), and a unique β-subunit that confers specific biological activity (2). Expression of the β-subunit gene is the limiting factor in FSH synthesis, and its transcription is regulated primarily by gonadotropin-releasing hormone (GnRH), gonadal steroids, and the activin-inhibin-follistatin system (3–5).

GnRH is a decapeptide neurohormone, released by a subset of hypothalamic neurons into the hypophysial portal system, where it binds its receptor on the pituitary gonadotrope membrane. The GnRH receptor belongs to the G protein-coupled receptors and, upon ligand binding, activates the protein kinase C and mitogen-activated protein kinase (MAPK) signaling pathways (6). GnRH administration, either to cells in culture or to hypogonadotropic animals, induces transcription of the early response genes, c-fos, c-jun, and eg-r-1 (7–9). The transcription factor AP-1, which is composed of Jun/Jun homodimers or Jun/Fos heterodimers, has been implicated in GnRH induction of the FSHβ gene. Previous studies reported that nuclear proteins from GnRH-treated cells bind an AP-1 consensus sequence (10), that purified c-Jun protein binds putative AP-1 sites in the ovine FSHβ promoter (11), and that mutation of putative AP-1 sites in this promoter reduces GnRH induction in heterologous HeLa cells (10). However, in mice carrying a transgene of the ovine FSHβ 5′-flanking region linked to luciferase in which the same AP-1 sites were mutated, transgene response to GnRH did not differ from the wild-type ovine FSHβ promoter (12). Furthermore, one of these AP-1 sites is not conserved in the mouse, rat, or human promoters. Therefore, there is a need to examine FSHβ regulation of the mouse gene, especially in light of the fact that there is a high degree of conservation between mouse and human FSHβ genes and that targeted disruption of the FSHβ gene in mice has a phenotype similar to loss-of-function mutations in humans (1).

Until recently, no FSHβ-producing cell lines were available. Models of GnRH action using reconstitution of GnRH receptor in non-gonadotrope-derived cell lines may lack signaling molecules or transcription factors necessary for appropriate induction of gonadotrope-specific genes, whereas primary pituitary cell cultures contain only about 5% gonadotropes and are difficult to manipulate in vitro. The gonadotrope-derived LβT2 cell line expresses FSHβ endogenously (13) and secretes FSH in response to activin (14). These cells also express other markers of pituitary gonadotropes, most notably α-subunit, GnRH receptor, LHβ, and all of the components of the activin system autocrine loop: activin, follistatin, and activin receptor (13) as well as inhibin and inhibin receptor (15). Therefore, the LβT2 cell line is an excellent model in which to directly study regulation of FSHβ gene expression.

Indeed, these cells have been used to investigate GnRH signal transduction (16) and, more recently, the molecular ba-
sis for cell-specific expression of FSHβ, by comparison with the non-FSH-producing gonadotrope-derived cell line, aT3-1 (17). In the latter study, we identified specific promoter elements binding steroidogenic factor-1, an orphan nuclear receptor that is specifically expressed in the gonadotrope population and regulates gonadotrope-specific genes within the pituitary. We also identified a conserved binding site in the proximal region of the promoter for nuclear factor-Y (NF-Y), a ubiquitously expressed heterotrimeric transcription factor (18), and showed a role for both NF-Y and steroidogenic factor-1 in gonadotrope-specific gene expression of the mouse FSHβ promoter (17).

The goal of the present study is to gain an understanding of the molecular mechanisms by which GnRH induces FSHβ gene expression, using the mouse LβT2 gonadotrope cell model. We demonstrate that regulation by GnRH is mediated in part by induction of multiple AP-1 isoforms. These AP-1 isoforms bind a novel site that overlaps the element that binds the basal transcription factor NF-Y. This novel site consists of a half-site of the AP-1 consensus binding sequence and an adjacent CCAAT box. Furthermore, NF-Y and AP-1 physically interact and, following GnRH stimulation, co-occupy this site.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transient Transfection**—LβT2 cells were plated on 6-well plates 1 day prior to transfection. Transfection was performed in Dulbecco's modified Eagle's medium with 10% fetal bovine serum using FuGene 6 reagent (Roche Applied Science) following the manufacturer's instructions. Cells were rinsed with phosphate-buffered saline and lysed with lysis buffer (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.5% Nonidet P-40, 0.5 mM EDTA, with protease inhibitors: aprotonin, pepstatin, and leupeptin at 10 μg/ml each and 1 mM phenylmethylsulfonyl fluoride. Protein concentration was determined with Bradford reagent (Bio-Rad), and, following GnRH stimulation, co-occupy this site.

**GnRH Induces FSHβ through a Novel AP-1 Site**—After GnRH treatment, cells were scrapped in hypotonic buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1 mM MgCl₂, with the same protease inhibitors as mentioned above for the lysis buffer) and allowed to swell on ice. Cells were lysed by passing through a 20-gauge needle, and proteins were pelleted by centrifugation. Nuclear proteins were extracted in hypertonic buffer (250 mM Hepes, pH 7.8, 50 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol, 0.1% bovine serum albumin, 0.1% Nonidet P-40 with 0.5 μg/ml poly(dI-dC) and 2 fmol per reaction of end-labeled probe). Oligonucleotides were labeled with [γ-32P]ATP using T4 kinase. In the competition experiments, competitor oligonucleotide was added 10 min prior to the addition of the probe, as were the antibodies in the supershift assays. The Fos, Jun, and NF-Y antibodies used are the same as in the Western blot, whereas the nonspecific IgG is from Santa Cruz Biotechnology. The reaction was loaded on a 5% acrylamide gel in 0.25% TBE and electrophoresed at 1-V/cm constant voltage. After drying, gels were exposed to autograph film.

**Mutagenesis**—Mutagenesis and deletion of the FSHβ-luc plasmid were performed using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The oligonucleotide used to mutate the AP-1 half-site (GTCA) was 5′-CAGCACGGTTTATGGTATTGCTGCTGGTTAACACCCAC-3′ (top strand; mutated nucleotides are underlined), and the oligonucleotide used to mutate the NF-Y site (ATTGG) was 5′-CAGCACGGTTTATGGTTGTTAACACCCACAGCTAGATACCTACC-3′ (top strand; again, underlined nucleotides indicate a change from the wild-type sequence). The PCR product was digested with KpnI and BglII restriction enzymes and ligated into the corresponding sites in pGL3 vector. The PCR product was ligated with KpnI and BglII restriction enzymes and ligated into the corresponding sites in pGL3. The PCR product was digested with KpnI and BglII restriction enzymes and ligated into the corresponding sites in pGL3.

An expression plasmid containing β-galactosidase driven by the Herpesvirus thymidine kinase promoter was co-transfected with mFSHβ-luc and used as an internal control. Sixteen h after transfection, the luciferase activity was measured using the Galacto-light assay (Tropix, Bedford, MA) following the manufacturer's instructions. All transfection experiments were performed in triplicate and repeated at least three times. Luciferase values from reporter gene-transfected cells were consistently at least 100 times higher than values from control-transfected cells. Results are presented as the mean ± S.E. of all samples analyzed. An asterisk marks a statistically significant difference from the control-treated cells, determined by analysis of variance followed by Tukey-Kramer HSD post hoc multiple range test for individual comparison with p < 0.05.

**Western Blot**—Following overnight starvation and GnRH treatment, LβT2 cells were rinsed with phosphate-buffered saline and lysed with lysis buffer (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.5% Nonidet P-40, 0.5 mM EDTA, with protease inhibitors: aprotonin, pepstatin, and leupeptin at 10 μg/ml each and 1 mM phenylmethylsulfonyl fluoride. Protein concentration was determined with Bradford reagent (Bio-Rad), and, following GnRH stimulation, co-occupy this site.

**Electrophoretic Mobility Shift Assay (EMSA)**—After GnRH treatment, cells were scrapped in hypotonic buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1 mM MgCl₂, with the same protease inhibitors as mentioned above for the lysis buffer) and allowed to swell on ice. Cells were lysed by passing through a 20-gauge needle, and proteins were pelleted by centrifugation. Nuclear proteins were extracted in hypertonic buffer (250 mM Hepes, pH 7.8, 50 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol, 0.1% bovine serum albumin, 0.1% Nonidet P-40 with 0.5 μg/ml poly(dI-dC) and 2 fmol per reaction of end-labeled probe). Oligonucleotides were labeled with [γ-32P]ATP using T4 kinase. In the competition experiments, competitor oligonucleotide was added 10 min prior to the addition of the probe, as were the antibodies in the supershift assays. The Fos, Jun, and NF-Y antibodies used are the same as in the Western blot, whereas the nonspecific IgG is from Santa Cruz Biotechnology. The reaction was loaded on a 5% acrylamide gel in 0.25% TBE and electrophoresed at 1-V/cm constant voltage. After drying, gels were exposed to autograph film.
35S-Labeled proteins were produced using the TNT coupled reticulocyte lysate system (Promega). Bacteria transformed with the pGEX vectors were grown to an OD of 0.6, upon which protein expression was induced by the addition of 0.25 mM isopropyl-β-D-thiogalactosidase. Bacterial pellets were sonicated in phosphate-buffered saline with 5 mM EDTA and 0.1% Triton X-100 and centrifuged, and the supernatant was bound to glutathione-Sepharose beads (Amersham Biosciences). Beads were washed four times with sonication buffer, followed by equilibration in the binding buffer (below), and split equally between different samples and the control. 35S-Labeled proteins were added to the beads and bound for 1 h at 4 °C in 20 μl Hepes (pH 7.5), with 50 μM NaCl, 10 mg/ml bovine serum albumin, 0.1% Nonidet P-40, and 5 mM dithiothreitol. After extensive washing, samples were eluted from the beads by boiling in Laemmli sample buffer and subjected to SDS-PAGE. Afterward, the gels were dried and autoradiographed.

RESULTS

GnRH Induces FSHβ through Proximal Regulatory Sequences—Since GnRH is a major regulator of FSHβ synthesis, we sought to determine the molecular mechanisms by which GnRH induces FSHβ gene expression. A plasmid containing the proximal 398 bp of the mouse FSHβ 5’ regulatory region linked to a luciferase reporter gene (mFSHβ-luc) was transiently transfected into LβT2 cells. We chose to study the mouse FSHβ regulatory sequence transfected into this FSH-expressing, murine pituitary gonadotrope cell line to provide a homologous model for analysis of gene expression. This region of the mouse gene is highly conserved with ovine, rat, and human FSHβ genes. We have previously shown that 398 bp of the 5’ regulatory region of the mouse FSHβ gene is sufficient to provide gonadotrope-specific expression (17). LβT2 cells transiently transfected with mFSHβ-luc were treated with increasing concentrations of GnRH to test whether this region of the mouse FSHβ gene is also sufficient for GnRH responsiveness. Stimulation with GnRH over a range of doses and time periods revealed that this short regulatory region contains elements that allow response to GnRH in a time- and dose-dependent manner. Maximal induction is observed after 6 h of GnRH treatment, and expression returns to basal level within 24 h of GnRH treatment (Fig. 1A). Increasing doses of GnRH result in increasing activity of the mouse FSHβ promoter. In the following experiments, cells were treated for 6 h with 10 nM GnRH, a concentration closer to the physiological range of GnRH during the estrous cycle.

To identify which promoter elements convey GnRH responsiveness, we mapped regions of the mouse FSHβ gene promoter that confer GnRH response using truncation deletion analysis. LβT2 cells were transiently transfected with a series of truncations of the mouse FSHβ gene 5’-flanking region, ranging in length from 398 to 95 bp upstream of the transcription start site, and the ability of GnRH to induce transcription was assayed (Fig. 1B). Fig. 1B displays the GnRH regulation as -fold induction over vehicle-treated for each of the truncated promoter regions. As we have previously shown, the basal level of expression is not significantly changed by truncation through the region from −398 to −95 in LβT2 cells (17). However, here we show that GnRH induction is significantly reduced by sequential deletion of either of two regions of the mouse FSHβ promoter. Significant decreases in GnRH responsiveness were found when the promoter was truncated from −304 to −230 bp and in the most proximal region between −95 bp and the start site of transcription. The reduction due to truncation from −304 to −230 is minor, and an apparent increase in responsiveness from −127 to −95 is not statistically significant, but it leads to the finding that no statistically significant decrease in responsiveness exists between truncations −304 and −95. Indeed, a substantial level of induction is retained in the 95-bp most proximal region, and this is the focus of the following investigation.

An AP-1 Half-site Overlapping the CCAAT Box Is Bound by AP-1 following GnRH Stimulation and Is Essential for Maximal Induction of FSHβ by GnRH—Because the proximal 95-bp region retains 2.6-fold induction by GnRH, whereas the entire 398 bp of the regulatory region is induced 3.4-fold, we focused on this proximal region to determine what transcription factors confer GnRH induction. Using the TransFac® data base (25), the proximal region of the promoter was analyzed for putative transcription factor binding sites. This search revealed a half-
site for the AP-1 transcription factor adjacent to the CCAAT box, a binding site for the AP-1 half-site were identified in the mouse FSHβ promoter using the Transfac® data base. Alignment of the sequence from −99 to −65 of the mouse FSHβ gene regulatory region reveals that the NF-Y site (underline) and the AP-1 half-site (dashed underline) are conserved in human and rodent species but are absent from the ovine and bovine promoters. Consensus binding sites are noted below the alignment. 

| Species  | Sequence          |
|----------|-------------------|
| Mouse    | CTTCACGAGCTTTATGGTTGATTGCCATAGT |
| Rat      | CTTCACGAGCTTTATGGTTGATTGCCATAGT |
| Human    | CTTCACGAGCTTTATGGTTGATTGCCATAGT |
| Ovine    | CTTCACGAGCTTTATGGTTGATTGCCATAGT |
| Bovine   | CTTCACGAGCTTTATGGTTGATTGCCATAGT |

NF-Y consensus
ATTG
TAAC

AP-1 consensus
tgaGTCA
actCAGT

Next, EMSA was utilized to determine whether NF-Y and/or AP-1 bind to the identified elements and, if so, whether binding is affected by GnRH treatment. For that purpose, a radiolabeled 35-bp sequence from −99 to −65 bp, which spans these sites in the mouse gene, was incubated with nuclear extracts from LβT2 cells treated with vehicle (0 h) or 10 nM GnRH (0.5, 2, or 6 h), using the −99−65 sequence as a probe, are shown. The length of GnRH treatment in hours is indicated above each lane, and the antibodies used in supershift assay are marked above corresponding lanes. IgG represents a nonspecific antibody used as a control. The supershifted bands are indicated with ss, whereas 1 and 2 designate complexes that change following the treatment.

Fig. 2. The −99−65 region of the mouse FSHβ gene contains binding sites for NF-Y and AP-1. A, an NF-Y site and an adjacent AP-1 half-site were identified in the mouse FSHβ promoter using the Transfac® data base. Alignment of the sequence from −99 to −65 of the mouse FSHβ gene regulatory region reveals that the NF-Y site (underline) and the AP-1 half-site (dashed underline) are conserved in human and rodent species but are absent from the ovine and bovine promoters. Consensus binding sites are noted below the alignment. B, EMSA analysis of nuclear extracts from LβT2 cells treated with vehicle (0 h) or 10 nM GnRH (0.5, 2, or 6 h), using the −99−65 sequence as a probe, are shown. The length of GnRH treatment in hours is indicated above each lane, and the antibodies used in supershift assay are marked above corresponding lanes. IgG represents a nonspecific antibody used as a control. The supershifted bands are indicated with ss, whereas 1 and 2 designate complexes that change following the treatment.

GnRH Induces FSHβ through a Novel AP-1 Site
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To determine which nucleotides are needed for NF-Y and AP-1 binding, competitions with unlabeled wild-type probe, mutated oligonucleotides were utilized (Fig. 3). Two base-pair mutations (mutants A–J) in the NF-Y element (mutant K), were introduced into the wild-type probe. Mutated oligonucleotides were used as competitors in a 100-fold excess in EMSA experiments in B–D, whereas wild-type sequence (WT) was used as a probe. The NF-Y binding site is underlined with a solid line in the wild-type sequence, whereas the AP-1 half-site is underlined with a dashed line.

B. nuclear extracts from control cells were subjected to EMSA with radiolabeled wild-type probe. Mutated oligonucleotides were used as competitors in a 100-fold excess in the corresponding lanes. In the lane labeled AP-1, the AP-1 consensus sequence was used as a competitor in the same manner. C. nuclear extracts from cells treated with GnRH for 6 h were subjected to EMSA with the wild-type probe, and the same oligonucleotides with mutations as above were used as competitors. D. NF-Y antibody was added to nuclear extracts from the cells treated with 10 nM GnRH for 6 h, and competition EMSA was performed with the wild-type probe and competitor oligonucleotides indicated above the lanes.

To assess the contribution of these sites to FSHβ induction by GnRH, we introduced selective mutations into mFSHβ-luc. We chose mutations that either inhibit both AP-1 and NF-Y binding in vitro (mutant F in EMSA) or inhibit AP-1 binding only (mutant I in EMSA). These mutations reduce GnRH induction of luciferase activity by 25 and 35%, respectively, compared with the wild-type mFSHβ-luc plasmid response (Fig. 4A). Approximately the same level of reduction in the response to GnRH was achieved when putative AP-1 sites were mutated in the ovine FSHβ promoter (10). However, mutation of the NF-Y site/AP-1 half-site to an AP-1 consensus sequence containing the novel AP-1 box is required to bind AP-1. Together, these two regulatory elements create a novel AP-1 site.

To address whether the AP-1 element contributes independently to GnRH induction, we introduced a 9-bp deletion, elim-
result indicates that, in addition to being essential for maximal induction by GnRH, this site is sufficient for GnRH response.

**Multiple AP-1 Isoforms Are Induced by GnRH and Bind to the Novel AP-1 Site**—When we identified AP-1 binding to this novel site by EMSA (Fig. 2B), we identified two complexes that changed following GnRH treatment. Both were supershifted with antibodies against Fos and/or Jun proteins. To determine whether these complexes differ in their protein components, we first tested whether one of these bands is AP-1 protein in complex with another factor, using in vitro transcribed/translated c-Jun and c-Fos proteins in EMSA. We found that AP-1 composed only of c-Jun and c-Fos binds DNA directly at the −99/−65 region as a single band (lane 4), not present in the retnilocyte lysate control (lane 1, Fig. 5A). This c-Jun/c-Fos complex co-migrates with complex 1 from nuclear extracts treated with GnRH (lane 3), indicating that this upper complex is indeed AP-1 factor alone. Furthermore, this result confirms that AP-1 can bind directly and does not serve only as a co-factor; nor does it need to be in complex with NF-Y to bind DNA in vitro.

Since AP-1 does not need another factor to bind to the −99/−65 region, we postulated that the two different complexes observed following GnRH treatment are composed of different AP-1 isoforms. To determine which AP-1 isoforms are induced in LβT2 cells following stimulation with GnRH, Western blots of whole cell lysates with and without GnRH treatment were performed. GnRH selectively induces c-Fos, c-Jun, FosB, and JunB but not JunD in LβT2 cells (Fig. 5B). As expected from the EMSA results in which the NF-Y complex intensity did not change (observable after Fos was supershifted), the amount of NF-YA in the cells does not change with GnRH treatment.

To examine which of these GnRH-induced AP-1 isoforms binds to the FSHβ promoter, isoform-specific antibodies were used in EMSA (Fig. 5C). Inclusion of an antibody to c-Fos induces a supershift in the upper, slower migrating AP-1 complex (complex 1), whereas an antibody to FosB shifts the lower, faster migrating complex (complex 2). Non-isoform-specific antibodies, which recognize all of the Fos or all of the Jun isoforms (labeled ns in Fig. 5), supershifted both complexes. Both of the AP-1 bands appear to contain c-Jun and, to a lesser degree, JunB, since both bands were diminished upon inclusion of antibodies specific for c-Jun and JunB. Thus, the upper band (complex 1) contains c-Fos/Jun heterodimers, whereas the lower AP-1 band (complex 2) contains FosB/Jun heterodimers binding the AP-1 half-site in the mouse FSHβ promoter.

**AP-1 Is Necessary and Sufficient for Maximal Induction of FSHβ**—To test the role of AP-1 in GnRH induction of the mouse FSHβ gene, the dominant negative form of c-Fos, named A-Fos (27), was co-transfected with mFSHβ-luc into LβT2 cells treated with vehicle (control) or GnRH. A-Fos has an acidic extension on the N terminus of the Fos leucine zipper, which physically interacts with the Jun basic region, thus preventing the basic region of the heterodimer from binding DNA (27). Introduction of A-Fos with mFSHβ-luc into LβT2 cells treated with vehicle (control) or GnRH. A-Fos induces a supershift in the upper, slower migrating AP-1 complex (complex 1), whereas an antibody to FosB supershifts the lower, faster migrating complex (complex 2). Non-isoform-specific antibodies, which recognize all of the Fos or all of the Jun isoforms (labeled ns in Fig. 5), supershifted both complexes. Both of the AP-1 bands appear to contain c-Jun and, to a lesser degree, JunB, since both bands were diminished upon inclusion of antibodies specific for c-Jun and JunB. Thus, the upper band (complex 1) contains c-Fos/Jun heterodimers, whereas the lower AP-1 band (complex 2) contains FosB/Jun heterodimers binding the AP-1 half-site in the mouse FSHβ promoter.
Additionally, c-Fos and c-Jun are sufficient to induce mouse FSHβ promoter activity. c-Jun and c-Fos expression vectors, co-transfected with wild-type mFSHβ-luc into LβT2 cells, induce mFSHβ-luc greater than 6-fold over the vector control-transfected cells (Fig. 6B). In contrast, c-Jun and c-Fos expression vectors do not significantly increase luciferase expression when the AP-1 site mutant I is used as a reporter. This again confirms that the novel AP-1 site we identified is the only site for AP-1 binding within the 398-bp regulatory sequence. Furthermore, it suggests that the GnRH responsiveness remaining in the −398 FSHβ promoter after mutation of the AP-1 site is due to an activity unrelated to induction of AP-1.

MAPK Is Involved in FSHβ Induction by GnRH through JunB and c-Fos—MAPK is acutely activated following GnRH treatment of LβT2 cells, and this activation is involved in the induction of the ovine FSHβ promoter (16). To test whether MAPK plays the same role in the induction of the mouse FSHβ promoter by GnRH, we treated LβT2 cells with the MEK inhibitor, UO126, for 30 min prior to and during GnRH treatment. We first established a dose response for MEK inhibition by UO126 in LβT2 cells, by Western blotting for phospho-MAPK in whole cell lysates following the treatment (data not shown). The minimal concentration of the inhibitor needed to completely inhibit phosphorylation of MAPK by GnRH (1 μM) was then used in our experiments. As expected, MAPK signaling plays a role in induction of the mouse FSHβ promoter by GnRH, since this induction is reduced by 46% in the presence of the inhibitor (Fig. 7A).

To test whether levels of AP-1 proteins in LβT2 cells were altered by inhibition of the MAPK pathway, Western blotting was performed following GnRH treatment. MEK inhibition prevents maximal JunB induction by GnRH (Fig. 7B); however, it does not reduce the induction of the c-Jun or FosB isoforms found to be induced by GnRH in Fig. 5B (data not shown). In accordance with previously published results (6), c-Fos levels are also reduced with MAPK inhibition (data not shown). Therefore, we conclude that the MAPK pathway is involved in GnRH induction of the FSHβ gene, in part through the induction of JunB and c-Fos proteins.

AP-1 and NF-Y Interact and Co-occupy the Site in Vivo following GnRH Stimulation—Since the AP-1 binding site overlaps the NF-Y binding site, as demonstrated in competition EMSA in Fig. 3, we hypothesized that there are two possible ways that this region can accommodate these transcription factors. One possibility is that AP-1 displaces NF-Y on this site in vivo following Fos and Jun induction by GnRH. This was tested with a ChIP assay, which allows examination of the proteins binding to this gene region in vivo with and without GnRH treatment. After overnight serum starvation, the LβT2 cells were treated with GnRH for 3 h. Then, after lysis and
sonication, chromatin was precipitated with antibodies specific to NF-YA, Fos, or Jun. DNA was extracted and subjected to PCR analysis, amplifying the FSHβ gene regulatory sequence. The ChIP assay shows that Fos and Jun proteins bind the mouse FSHβ promoter sequence more intensely following GnRH treatment, consistent with their induction by GnRH, whereas NF-Y binding to this region does not change (Fig. 8).

This site is the only CAATT box identified in the region amplified with the primers, and, to our knowledge, there are no other CAATT boxes in the proximity of this site. This assay assesses binding in a cell population, so if NF-Y were dislodged by AP-1 in even a portion of the cells following GnRH treatment, we would expect the NF-Y antibody-precipitated band to be diminished. Therefore, AP-1 does not appear to displace NF-Y on the mouse FSHβ promoter.

An alternative mechanism by which these two transcription factors could occupy the same site is if AP-1 and NF-Y physically interact. We tested this hypothesis using GST pull-down assays in which in vitro transcribed and translated c-Fos and c-Jun proteins were tested for their ability to interact with NF-YA-GST fusion protein. In this assay, c-Jun interacts with the NF-YA subunit (Fig. 9, top panel), whereas NF-YB and NF-YC are not (data not shown). No interactions were observed using GST alone; nor did labeled NF-YA protein precipitate with glutathione beads through an interaction with GST-NF-YA. Additionally, we performed the reverse experiment in which NF-Y proteins were labeled and synthesized in vitro and then tested for interaction with c-Jun-GST fusion protein. Labeled NF-YA is retained in the precipitate by the interaction with GST-c-Jun (Fig. 9, bottom left panel), whereas NF-YB and NF-YC are not (data not shown). No interactions were observed using GST alone; nor did labeled green florescent protein, which serves as a control, interact with any of the used GST fusion proteins (Fig. 9, bottom right panel). Thus, NF-Y and AP-1 form heteromeric complexes in vitro, through protein-protein interaction between Jun and NF-YA.
GnRH Induces FSHβ through a Novel AP-1 Site

FIG. 8. ChIP reveals that NF-Y binds DNA in the proximal mouse FSHβ promoter in both control and cells treated with GnRH for 3 h, whereas AP-1 binds DNA following GnRH treatment. A, chromatin was isolated from LβT2 cells treated with vehicle or 10 nM GnRH for 3 h and cross-linked with formaldehyde. After sonication, sheared chromatin was precipitated with the antibodies indicated above the lanes. The precipitated and purified DNA is then amplified in the PCR. The antibody specific for NF-Y precipitates the DNA specific for the sequence in the proximal mouse FSHβ promoter, in both control and GnRH-treated cells. Fos and Jun, on the other hand, bind DNA in vivo only following the GnRH treatment. In the first two lanes, chromatin was precipitated with protein A beads only serving as controls. B, chromatin prior to precipitation serves as the control for the amount of chromatin used for precipitation in the untreated and GnRH-treated samples. A serial dilution of the chromatin was performed and then used in PCR together with precipitated samples. C, four independent experiments were quantified using a PhosphorImager and then normalized to intensity in the control sample precipitated with protein A beads only to normalize for any difference in the activity of the [α-32P]dATP used in PCRs. The solid bars represent chromatin immunoprecipitation from GnRH-treated cells, whereas open bars represent control samples.

DISCUSSION

GnRH is a key regulator of FSHβ gene expression and therefore FSH synthesis. Previous studies were limited by a lack of available cell lines that express both FSH subunit genes and respond to GnRH. The scarce number of gonadotropes in the pituitary precludes performing these studies in primary cells. The genesis of the LβT2 cell line that endogenously expresses FSHβ, allows for the dissection of molecular pathways governing its expression (13, 28). In the current study, we have focused on delineating the mechanisms of GnRH induction of the mouse FSHβ gene.

FSH regulates gonadal development in mammals and is required for folliculogenesis (1). Tight regulation of FSH levels is crucial for the menstrual or estrous cycle. Both FSH protein in circulation and β-subunit mRNA in the pituitary normally fluctuate 4-fold during the cycle (26, 29). In mice lacking GnRH, serum FSH levels are 60–90% lower (30). One pulse of GnRH administered to castrated, testosterone-replaced rats (with low endogenous GnRH) increased FSHβ transcription 4-fold (31). This level is comparable with the induction observed in our studies using the LβT2 cell line.

We report here that the AP-1 transcription factor, induced by GnRH, can bind a half-site of its consensus sequence, GTCA, when this half-site is juxtaposed to a site involved in basal expression, in this case a CCAAT box binding NF-Y. Further, we show that this AP-1/NF-Y site is necessary for maximal GnRH induction of the mouse FSHβ gene. As shown in Fig. 4, the half-site is, as expected, a low affinity site for AP-1. Specifically, when we mutate the NF-Y site/AP-1 half-site in the FSHβ promoter to create a full AP-1 consensus site in this position, luciferase expression after GnRH treatment is greater than 15-fold higher than the untreated control. This is 6-fold higher than GnRH induction of the wild-type promoter. Further, in gel shift assays, an AP-1 consensus competed more effectively for AP-1 bands than the nonlabeled wild-type sequence, and, when used as a probe, the AP-1 consensus requires one-tenth of the protein as wild-type probe to observe AP-1 binding (data not shown). Half-sites may have important physiological roles despite, or perhaps because of, their low affinity. Genes such as FSHβ fluctuate only 4-fold, but this relatively modest change in expression during the estrous cycle is crucial for normal egg development and selection. A full consensus sequence might bring forth an unnecessarily high induction.

In a recent report describing the regulation of GnRH receptor expression by GnRH and activin in LβT2 cells, the promoter element studied, GTCCTAGTCAC, was of special interest (32). The authors conclude that AP-1 binds a novel 6-bp site, AGTCAGTCA, instead of its usual 7-bp site, whereas activin-regulated Smad 4 binds a 2-bp site. However, the competition EMSA experiments shown in that report suggest the alternative explanation that Smad 4 binds a Smad half-site GTCT, and AP-1 binds its half-site GTCA, which is separated from the Smad half-site by only one nucleotide. Thus, in the GnRH receptor gene, in light of our findings, it is possible that AP-1 and Smad 4 are stabilized on their respective half-sites by mutual interaction, and this is the reason both sites are needed for response to either activin or GnRH. It would be of interest to examine whether half-sites or low affinity sites are commonly involved.
in the induction of gonadotrope-specific genes that usually have a low level of induction but are very tightly regulated throughout the menstrual/estrous cycle.

Another interesting characteristic of the FSHβ promoter element is that the AP-1 half-site overlaps an NF-Y site involved in basal expression. Recently, overlapping NF-Y and YY1 sites have been identified in the promoter of the Hoxd4 gene (33); however, this element was in a specialized intronic site able to bind either factor in a mutually exclusive manner. In the FSHβ promoter, AP-1 and NF-Y occupy this element concurrently. In EMSA experiments with control extracts, NF-Y binds this site, and the complex is completely supershifted with antibodies to NF-YA. Competition EMSA in Fig. 3B also shows that a CCAAT box is needed for NF-Y to bind. The ChIP assay indicates that NF-Y is present in the complex at the same level before and after GnRH treatment in live cells. AP-1 can also bind this site in vitro, without other proteins present. In vitro transcribed and translated AP-1 binds this site, and it co-migrates with the AP-1 complex from GnRH-treated cells. Thus, the FSHβ promoter element can be bound by both NF-Y and AP-1.

AP-1 binding to this low affinity site may be stabilized by protein-protein interactions with the basal transcription factor NF-Y. Our attempts to observe a higher order complex in EMSA were unsuccessful; this is probably due to its expected size of 220 kDa, which would be too large to migrate into the gel. Further, the higher order complex would consist of five different proteins (NF-YA, NF-YB, and NF-YC, which are all necessary for NF-Y to bind, and Jun and Fos, which form AP-1) and therefore is difficult to reconstitute from recombinant proteins. However, we have established that this is a low affinity site, compared with the AP-1 consensus sequence, and that it is bound by NF-Y prior to and during GnRH treatment, which leads us to speculate that NF-Y may stabilize AP-1 binding. At least three such examples have been reported: SP1, SREBP1, and RF-X. In such cases, NF-Y considerably increases the affinity of the neighboring factor for DNA, making these complexes more stable on the DNA (18). In addition to physical interaction with many transcription factors (21, 34, 35), NF-Y interacts with several components of the basal transcriptional machinery (36–38). Therefore, through direct contact with induced and/or activated transcription factors and the basal machinery, NF-Y may serve as a transcriptional coordinator or integrator.

We determined that AP-1 interacts with NF-Y through direct protein-protein contact between c-Jun and NF-YA. This is not surprising, since there is mounting evidence that NF-YA is the regulatory subunit of the trimeric NF-Y complex (18). c-Jun protein has also been reported to physically interact with other transcription factors, such as ERF (39), Cbfa1 (40), and Smads (41). How AP-1 is able to circumvent the spatial constraints of binding to a half-site directly adjacent to a NF-Y-occupied CAAT box, without a single nucleotide space, remains to be determined. AP-1 has been shown to bind a weak binding site when it cooperatively associates with transcription factors on juxtaposed sites (42); however, in that case, there were two base pairs between the binding sites. Fos and Jun heterodimers form a flexible fork, which might permit binding of other transcription factors at adjacent sites on the DNA (43). Since the mouse FSHβ promoter contains an AP-1 half-site, it is possible that only one member of the heterodimer, in this case Fos, directly binds the DNA and that Jun binds to NF-Y, as we have demonstrated in vitro, as well as to Fos, but not directly to the DNA. Alternatively, it is possible that both Fos and Jun proteins contact the DNA. We determined that residues TAATGT-GTCA are needed for AP-1 to bind. Conserved residues in the AP-1 consensus TCGAGTCA, are printed in boldface type and/or underlined for easier observation. The CCAAT element (opposite strand: ATTGG) is bound by NF-Y. However, one member of the AP-1 heterodimer can bind an underlined T, which is conserved in our novel site and the AP-1 consensus, and G (in boldface type), which both Fos and Jun bind according to the crystal structure (43). The other partner can bind the GTCA half of the consensus (in boldface type). Fos and Jun bind their site in the major groove, and only four amino acid residues contact the DNA (43). Vast portions of either protein are found perpendicular to the DNA. That conformation and the twist of the DNA helix may allow enough space for NF-Y to bind. NF-Y, on the other hand, contacts the DNA in the minor groove (44). From our studies, it appears that the NF-Y site has to be present for AP-1 to bind, and since NF-Y occupies the site in vivo, NF-Y may stabilize low affinity AP-1 DNA interactions.

We used 398 bp of the mouse FSHβ regulatory sequence in these experiments, and this relatively short region has both higher expression and greater response to GnRH than the much longer ovine regulatory sequence used in previous studies (13, 16). Thus, important species-specific differences in FSH regulation exist. The NF-Y/AP-1 site identified comprises sequences from −76 to −69 in the mouse FSHβ promoter and is not conserved in the ovine or bovine promoters, although it is conserved in the human and rat. Two potential AP-1 sites previously identified in the ovine promoter correspond to −69/−63 and −106/−100 sequences in the mouse FSHβ promoter (10). When we used oligonucleotides spanning those sites as probes in EMSA, we did not detect AP-1 binding or any change in binding complexes following GnRH treatment (data not shown). The reports describing those AP-1 sites used purified proteins to detect binding to these sites (11) or detected AP-1 from extracts of GnRH-treated HeLa cells binding to the AP-1 consensus sequence (10). However, this is not surprising, since GnRH induces Fos and Jun isoforms. Further, mutations in these sites do not affect appropriate regulation of FSH by gonadectomy or GnRH antagonist in transgenic animals (12).

Two AP-1 consensus sites exist within the 398 bp of the mouse regulatory region, at −10/−4 and −181/−175. As expected, AP-1 from GnRH-treated nuclear extracts can bind the AP-1 consensus sequence. However, based on the result of our transfection experiments, we conclude that those sites are not functional in the context of the promoter. Namely, when we used a reporter containing a mutation in the AP-1 half-site, mutant I, the induction by GnRH decreased by about the same amount as when we co-transfected the dominant negative A-Fos in Fig. 6. Notably, dominant negative Fos cannot reduce the induction by either GnRH or overexpression of c-Jun and c-Fos when mutant I is used instead of the wild-type promoter. This finding strongly suggests that this site is required for AP-1 action in this regulatory region. From the truncation analysis, we determined that there is another region of the promoter between −230 and −304 responsive to GnRH to a lesser degree, since there was a statistically significant drop in the induction level upon truncation of that region. Furthermore, it is possible that another GnRH-responsive site exists in the region proximal to the start site, since the −95 bp truncation maintains two-thirds of the response, whereas the AP-1 site we identified is responsible for one-third of the induction. We are currently investigating the elements in those regions for their role in GnRH response.

The GnRH receptor belongs to the class of G protein-coupled receptors and, upon ligand binding, activates protein kinase C and downstream MAPK signaling pathways (6). Our results showing the role of MAPK in mouse FSHβ induction are in agreement with previously published reports that MAPK is
involved in ovine FSHβ up-regulation and c-Fos induction by GnRH. Our data extend those findings and demonstrate that MAPK is involved in JunB induction by GnRH but not in c-Jun or FosB induction. However, mutation of the AP-1 site or introduction of the dominant negative Fos reduces induction of FSHβ by 35 and 30%, respectively, whereas the inhibition of the MAPK pathway causes 46% reduction in the FSHβ response to GnRH. Thus, the MAPK pathway probably regulates other transcription factor(s) in addition to AP-1, either through activation by phosphorylation or by induction of their gene expression.

In this report, we demonstrate that AP-1 binds a novel site composed of an AP-1 half-site and a CCAAT box. This site is involved in GnRH induction of the mouse FSH

transcriptional machinery.

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GnRH Induces FSHβ through a Novel AP-1 Site