Sp1 Binds to the Rat Luteinizing Hormone β (LHβ) Gene Promoter and Mediates Gonadotropin-releasing Hormone-stimulated Expression of the LHβ Subunit Gene*

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Ursula B. Kaiser‡, Elena Sabbagh, Marian T. Chen, William W. Chin, and Brian D. Saunders
From the Division of Genetics, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts 02115

The hypothalamic hormone gonadotropin-releasing hormone (GnRH) plays a critical role in reproductive function by regulating the biosynthesis and secretion of the pituitary gonadotropins. Although it is known that GnRH induces luteinizing hormone β (LHβ) gene transcription, the mechanisms by which this occurs remain to be elucidated. We have shown previously that GH3 cells transfected with the rat GnRH receptor cDNA (GGRH-1) support the expression of a cotransfected fusion gene composed of 797 base pairs of rat LHβ gene 5′-flanking sequence and the first 5 base pairs of the 5′-untranslated region fused to a luciferase reporter (−797/+5 LHβ/LUC) and respond to a GnRH agonist with a 10-fold stimulation of activity. Furthermore, we have shown that DNA sequences at −490/−352 confer GnRH responsiveness to the rat LHβ gene. We have now identified two putative binding sites for Sp1, a three-zinc-finger transcription factor, within this region. Using electrophoretic mobility shift assay, DNase I footprinting, and methylation interference assays, we demonstrate that Sp1 can bind to these sites and that Sp1 is responsible for DNA-protein complexes formed using GGH3-1 and αT3-1 nuclear extracts. Mutations of the Sp1 binding sites, which block binding of Sp1, blunt the stimulation of the LHβ gene promoter by GnRH. These data define GnRH-responsive elements in the LHβ 5′-flanking sequence and suggest that Sp1 plays an important role in conferring GnRH responsiveness to the LHβ subunit gene.

The pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) play integral roles in the regulation of normal reproductive development and function. The biosynthesis and secretion of these pituitary glycoproteins are controlled by the complex interaction of multiple factors, among the most important of which is gonadotropin-releasing hormone (GnRH). Pulsatile GnRH stimulates the secretion of LH and FSH as well as transcription, steady-state mRNA levels, and biosynthesis of the gonadotropin subunits α, LHβ, and FSHβ (1–4). This regulation is dependent on GnRH pulse amplitude and frequency, which varies with physiologic state, during puberty, during the rat estrous and human menstrual cycles, and during menopause (5, 6). An understanding of the mechanisms of regulation of LHβ gene expression is an important first step in elucidating the mechanisms of physiologic differential regulation of LH and FSH by GnRH.

Studies of the gonadotropin α-subunit gene have identified a number of DNA elements in the 5′-flanking region that mediate tissue-specific and regulated expression and their cognate binding factors. Recently, two transcription factors, steroidogenic factor-1 (SF-1) and early growth response-1 (Egr-1), have been recognized to be involved in expression of the LHβ gene (7–9). Nevertheless, relatively little is known about transcription factors that direct gonadotrope-specific or hormonally regulated expression of the LHβ and FSHβ subunit genes. A systematic approach to identifying mechanisms of hormonal regulation of LHβ and FSHβ subunit gene expression has been hampered by the lack of available cell lines that express either the endogenous or transfected LHβ and FSHβ genes in a regulated manner. In our studies, we have used GH3 cells, a well-characterized rat pituitary somatolactotrop cell line, as a model for the analysis of cis-regulatory elements in the rat LHβ gene. We have demonstrated previously that GH3 cells, when transfected with rat GnRH cDNA, bind and respond to GnRH (10–13). Cotransfection with the 5′-flanking region of the α, LHβ, or FSHβ subunit gene fused to a luciferase reporter results in the expression of luciferase and a stimulation of luciferase activity in response to GnRH (14). Characterization of this cell model has demonstrated many similarities in the GnRH response compared with that in primary pituitary cells, including the specific intracellular signal transduction pathways activated, the degree of stimulation of the gonadotropin subunit promoter activities, and the presence of differential regulation of LHβ and FSHβ gene promoter activities by GnRH. GH3 cells thus appear to be a useful model for the study of the regulation of expression of the gonadotropin subunit genes by GnRH.

Using this cell model, we have recently identified two elements in the rat LHβ gene promoter that contribute to the stimulation of LHβ gene expression by GnRH (15). These two elements lie at positions −490/−352 (referred to as region A) and −207/−82 (region B), relative to the transcriptional start site. A protein(s) present in GH3 and αT3–1 nuclear extracts binds to DNA sequences within region A. In this study, we characterize the DNA sequences within region A to which...
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protein binding occurs. These sequences have homology to the consensus binding site for the three-zinc-finger transcription factor, Sp1, which binds to GC-rich sequences. We demonstrate that Sp1 binds to several sites within region A of the rat LHβ gene promoter. Mutations of these sequences abrogate Sp1 binding and blunt the responsiveness of the LHβ gene promoter to stimulation by GnRH. These data suggest that Sp1 plays an important role in conferring GnRH responsiveness to the LHβ subunit gene.

**EXPERIMENTAL PROCEDURES**

**Materials—**The GnRH agonist des-Gly10-[d-Ala6]GnRH ethylamide (GnRHag) was purchased from Sigma. Human Sp1 protein was purchased from Promega (Madison, WI). Anti-Sp1, anti-Sp3, and anti-Egr-1 polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-follistatin polyclonal antibody was generated as described previously (16).

**Reporter Plasmids and Expression Vectors—**An expression vector encoding the rat GnRH was prepared by subcloning the rat GnRH cdNA sequence into pcDNA1 (Invitrogen, San Diego, CA), as described previously (10). An expression vector expressing β-galactosidase driven by the Rous sarcoma virus promoter (RSV-β-galactosidase) was used as an internal standard and control (17). The reporter constructs used had sequences of the rat LHβ gene promoter cloned into the pXP2 luciferase reporter vector (14, 18). The rat LHβ gene promoter was sequenced from rat genomic DNA by dye sequencing. The nucleotide sequence of the rat LHβ gene promoter used in these studies is based on our findings, with position 1 assigned to the nucleotide immediately 5′ of the transcriptional start site. The GHS-0-pXP1 construct was prepared by subcloning the rat growth hormone gene minimal promoter into BglII/XbaI polylinker restriction sites in pXP1 (7, 18, 19). All reporter constructs were confirmed by dye sequencing.

The DNA sequences between −490 and −344 of the rat LHβ gene promoter were arbitrarily subdivided into five subregions, A1–A5 (see Fig. 1A). Oligonucleotides corresponding to sense and antisense strands of each of these sites were synthesized, as described previously (16). Binding sites for Sp1 are indicated.

**Luciferase Assays—**Luciferase activity was normalized for expression of RSV-β-galactosidase. β-Galactosidase activity was assayed colorimetrically by standard protocols (17).

**Preparation of Nuclear Extracts—**GHH-1′ and αt3–1 cells were grown to approximately 70% confluence and treated with GnRHag 100 nM or vehicle for varying time intervals; then, cells were harvested, and nuclear extracts were prepared by the method of Andrews and Faller (20). Cell Culture and Transfection—

Electrophoretic Mobility Shift Assay (EMSA)—DNA sequences encompassing LHβ region A (LHβ) (−490/−334) were amplified by polymerase chain reaction and subcloned into pBluescript KS+ (LHβ-pBS) as described previously (15). 32P-End-labeled LHA was prepared by digesting LHA-pBS with XhoI and XhoI and 3′-end labeling with [γ-32P]ATP by T4 polynucleotide kinase and purified over a Nick column (Amersham Pharmacia Biotech).

The binding reaction for EMSA was performed by incubating 50,000 cpn of DNA probe with 5 μg of nuclear extract and 2 μg of salmon sperm DNA in reaction buffer (20 mM HEPES, pH 7.9, 60 mM KC1, 5 μM MgCl2, 10 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, 1 mg/ml bovine serum albumin, and 5% (v/v) glycerol) for 30 min at 4 °C. Nuclear fraction studies, expressing unlabeled DNA was added 5 min prior to the addition of probe. Protein-DNA complexes were resolved on 4% low ionic strength nondenaturing polyacrylamide gel electrophoresis in 0.5× Tris borate-EDTA buffer in 1× Tris borate-EDTA buffer. The gels were then dried and subjected to autoradiography. Antibody-supershift experiments were performed using anti-Sp1, anti-Sp3, anti-Egr-1, and anti-follistatin antibodies.

Antibody (1 μl) was added to the EMSA reaction samples after 30 min and incubated at 4 °C for an additional 2 h prior to gel electrophoresis.

**DNase I Footprinting Assay—**DNase I footprinting assays were performed as described by the method of Fried (22). Briefly, LHA-pBS was 3′-end labeled with either sense or antisense strand as described above. 50,000 cpn of end-labeled DNA fragments were incubated with 1 footprinting unit of Sp1 in a binding reaction mixture prepared as for EMSA. After 30 min at room temperature, samples were treated with DNase I (4 μl of 0.05–0.1 units/ml; Sigma) for 2 min. Reactions were stopped by the addition of 4 μl of formamide loading buffer to inhibit the DNase I. Samples were then immediately transferred to a 95 °C water bath and incubated for 4–5 min. Denatured samples (1 μl) were subjected to electrophoresis in 0.3% agarose containing polyacrylamide gels. The untreated end-labeled DNA fragments were also subjected to Maxam and Gilbert sequence reactions for G, A, G, C, and C+T to serve as markers on the gels (23). Gels were dried and subjected to autoradiography.

**Methylation Interference Assay—**Methylation interference assays were performed essentially according to the method of Ikeda et al. (24). Briefly, LHA-pBS was 3′-end labeled on either the sense or antisense strand as described above. 2 × 106 cpn of end-labeled DNA fragments were then partially methylated with dimethyl sulfate. Preparative EMSAs were performed as described above, using 25 μg of nuclear extract or 1–2 footprinting units of Sp1 and 250,000 cpn of methylated probe. Specifically complexed and free DNAs were visualized by autoradiography of the wet gel at 4 °C for 4 h and excised. The DNA was eluted, purified, cleaved with 1 μl piperidine for 30 min at 90 °C, lyophilized, and resuspended in formamide loading buffer at a concentration of 1000 cpm/μl. The samples (6 μl) were subjected to electrophoresis in 6% denaturing polyacrylamide gels. Gels were dried and subjected to autoradiography.

**Statistical Analysis—**Transfections were performed in triplicate and repeated multiple times. Data in each experiment were normalized to the basal levels of activity of −797/+5LHβLUC or GHH50-pXP1. Data were then combined across experiments to give a mean ± S.E. for basal and GnRH-stimulated activities for each construct, and fold stimulation in response to GnRH was calculated. One-way analysis of variance, followed by post hoc comparisons with Fisher’s protected least significant difference test, was used to assess whether changes in GnRH responsiveness among different LHβ promoter-luciferase reporter constructs were significant. Significant differences were established as p < 0.05.
RESULTS

Protein Binding to Region A of the LHβ Gene Involves Subregions A2 (−451/−428) and A4 (−411/−386)—We have demonstrated previously that proteins in GGHH-1 and α-T3 nuclear extracts bind specifically to region A of the rat LHβ gene promoter (15). We were interested in determining the DNA sequence(s) within region A that was responsible for this protein binding activity. In order to define more precisely the binding sequence, we divided region A into five subregions, A1–A5 (Fig. 1A). Using oligonucleotides corresponding to A1–A5, we performed EMSA to ascertain whether GGHH-1 nuclear proteins were able to bind to these sequences (Fig. 1B). A DNA fragment encompassing region A (LHA; −490/−334) of the LHβ 5′-flanking sequence was 3′-end-labeled and incubated with 5 μg of GGHH-1 nuclear extract. A1–A5 were used in 200-fold excess as unlabeled competitor DNAs. Subregions A2 and A4 were able to compete for binding to GGHH-1 nuclear proteins, as indicated by the decreased formation of a specific DNA-protein complex on the labeled LHA probe, whereas subregions A1, A3, and A5 did not compete significantly. LHA, used as a positive control, was also able to compete successfully for binding to the labeled LHA probe. A sequence containing the Pit-1 binding site, used as a negative control, was not able to prevent binding of GGHH-1 nuclear proteins to LHA. For additional analysis of these five subregions, the A1–A5 oligonucleotides were 5′-end-labeled and used directly as probes in an EMSA (Fig. 1C). Complexes similar to those seen using LHA were seen using sequences A2 (−451/−428) and A4 (−411/−386). A similar complex was also formed using A3 (−427/−399) as the DNA probe, albeit with lower abundance, suggesting a lower affinity for the DNA-binding protein. The predominant DNA-protein complexes formed using A2 and A4 as probes migrated at similar rates in the EMSA. This was confirmed by demonstrating that a 200-fold excess of unlabeled A2 could prevent the formation of the major DNA-protein complex on A4 and, conversely, that a 200-fold excess of unlabeled A4 could prevent the formation of the major DNA-protein complex on A2 (data not shown). A 200-fold excess of unlabeled A3 (−427/−399) was also able to decrease formation of protein-DNA complexes formed on either A2 or A4, consistent with a weak affinity for the same DNA-binding protein(s). LHA could also prevent binding of GGHH-1 nuclear proteins to both A2 and A4 when used as an unlabeled competitor DNA. The formation of additional, specific DNA-protein complexes of lower abundance was observed when A2 and A4

![DNA-protein interactions of GGHH-1' nuclear extract proteins with rat LHβ gene promoter region A](image)

FIG. 1. DNA-protein interactions of GGHH-1' nuclear extract proteins with rat LHβ gene promoter region A and its subregions by electrophoretic mobility shift assay. A, rat LHβ gene promoter region A (−490/−334) subdivided into five subregions, A1–A5. A1, −490/−452; A2, −451/−428; A3, −427/−399; A4, −411/−386; A5, −385/−344. All numbering is relative to the transcriptional start site of the rat LHβ gene. B, LHβA, a DNA fragment corresponding to region A (−490/−334) of the rat LHβ gene 5′-flanking sequence was end-labeled and incubated with 5 μg of GGHH-1' nuclear extract in an electrophoretic mobility shift assay. The five subregions of region A, A1–A5, were used in 200-fold excess as unlabeled competitor DNAs, as indicated. The three arrows indicate three specific DNA-protein complexes formed. The full region A was used as a competitor as a positive control, and a sequence containing the Pit-1 binding site was used as a negative control, as indicated. C, oligonucleotides corresponding to DNA sequences of subregions A1–A5 of the rat LHβ gene promoter region A (LHA; −490/−334) subdi- lated into five subregions, A1–A5 (Fig. 1A). Using oligonucleotides corresponding to A1–A5, we performed EMSA to ascertain whether GGHH-1 nuclear proteins were able to bind to these sequences (Fig. 1B). A DNA fragment encompassing region A (LHA; −490/−334) of the LHβ 5′-flanking sequence was 3′-end-labeled and incubated with 5 μg of GGHH-1' nuclear extract. A1–A5 were used in 200-fold excess as unlabeled competitor DNAs. Subregions A2 and A4 were able to compete for binding to GGHH-1' nuclear proteins, as indicated by the decreased formation of a specific DNA-protein complex on the labeled LHA probe, whereas subregions A1, A3, and A5 did not compete significantly. LHA, used as a positive control, was also able to compete successfully for binding to the labeled LHA probe. A sequence containing the Pit-1 binding site, used as a negative control, was not able to prevent binding of GGHH-1' nuclear proteins to LHA. For additional analysis of these five subregions, the A1–A5 oligonucleotides were 5′-end-labeled and used directly as probes in an EMSA (Fig. 1C). Complexes similar to those seen using LHA were seen using sequences A2 (−451/−428) and A4 (−411/−386). A similar complex was also formed using A3 (−427/−399) as the DNA probe, albeit with lower abundance, suggesting a lower affinity for the DNA-binding protein. The predominant DNA-protein complexes formed using A2 and A4 as probes migrated at similar rates in the EMSA, suggesting that the same protein(s) may bind to both of these DNA elements. This was confirmed by demonstrating that a 200-fold excess of unlabeled A2 could prevent the formation of the major DNA-protein complex on A4 and, conversely, that a 200-fold excess of unlabeled A4 could prevent the formation of the major DNA-protein complex on A2 (data not shown). A 200-fold excess of unlabeled A3 (−427/−399) was also able to decrease formation of protein-DNA complexes formed on either A2 or A4, consistent with a weak affinity for the same DNA-binding protein(s). LHA could also prevent binding of GGHH-1' nuclear proteins to both A2 and A4 when used as an unlabeled competitor DNA. The formation of additional, specific DNA-protein complexes of lower abundance was observed when A2 and A4
were used as probes. The additional minor complexes formed using A3 and A5 were nonspecific (data not shown). Simpler patterns of DNA-protein complexes were formed on A1–A5 using αT3 nuclear extracts instead of GGH3–1 nuclear extracts (data not shown). This suggests that the protein(s) binding to these DNA sequences is common to both cell lines.

**DNA Sequences A2, A3, and A4 Confer GnRH Responsiveness to a Heterologous Promoter**—We have demonstrated previously that region A of the rat Lβ gene promoter can confer GnRH responsiveness to the rat growth hormone (GH) minimal promoter (GH50), a heterologous gene promoter (15). GH is normally expressed in the GH3 cell line and is not GnRH-responsive or thyrotropin-releasing hormone-responsive. We were interested in defining more precisely the DNA sequences within region A that were responsible for this functional GnRH response. In particular, we wished to correlate functional GnRH responsiveness with the presence of DNA-protein interactions noted by EMSA using A2 and A4, and to a lesser extent A3. We therefore subcloned each subregion A1–A5, two copies in tandem, upstream of the rat GH50 minimal promoter in a luciferase reporter plasmid (LHA12GH50LUC–LHA52GH50LUC). We tested these fusion constructs in a transient transfection assay to determine whether they were GnRH-responsive. Each of these constructs, or -797/+5LHβLUC or LHAGH50LUC for comparison, or GH50-pXP1 or pXP1 as controls, was transfected into GGH3–1 cells by electroporation. The cells were harvested, and luciferase activity was measured 48 h after transfection and after stimulation with 100 nM GnRHAg for 6 h immediately prior to harvesting. -797/+5LHβLUC (which uses the native Lβ gene promoter) and LHAGH50LUC (Lβ region A fused upstream of GH50) were used as positive controls for comparison of the degree of response to GnRHAg. GH50-pXP1 and pXP1 were used as negative controls. Levels of luciferase activity were then measured 48 h after transfection and after stimulation with 100 nM GnRHAg or vehicle for 6 h immediately prior to harvesting (Fig. 2). Levels of expression of pXP1 and GH50-pXP1 were very low, and a small but significant response to GnRHAg was observed. All subsequent analyses of GnRH responsiveness were hence made in comparison to GH50-pXP1. As noted in our previous studies (15), LHAGH50LUC increased basal luciferase activity substantially, in this case by 25-fold, compared with GH50-pXP1, and Lβ region A conferred a 4.0 ± 0.6-fold GnRH response to the rat growth hormone minimal promoter. This was somewhat less than the degree of stimulation by GnRHAg of -797/+5LHβLUC (6.6 ± 0.4-fold), which contains the native rat Lβ gene minimal promoter and additional regions of the rat Lβ gene 5′-flanking sequence. None of the five subregions of LHA were able to confer full GnRH responsiveness to the level observed with the entire Lβ region A upstream of GH50 or to the level observed using the native rat Lβ gene promoter. However, LHA22GH50LUC and LHA42GH50LUC increased basal luciferase activity and also conferred partial GnRH responsiveness, to a significantly greater level than that observed with GH50-pXP1. These reporter genes contain subregions A2 and A4, respectively, corresponding to the oligonucleotides that bind nuclear proteins present in GGH3–1 and αT3 cells. In contrast, LHA12GH50LUC and LHA52GH50LUC had low basal activity and little stimulation of activity in response to GnRH, and A1 and A5 oligonucleotides did not bind GGH3–1 and αT3 nuclear proteins. LHA32GH50LUC increased basal activity but did not confer a significant GnRH response compared with GH50-pXP1. Thus, there appears to be a correlation between the presence of DNA-protein interactions noted by EMSA and functional GnRH responsiveness for DNA sequences within Lβ region A. Furthermore, the sequences that confer GnRH responsiveness are also able to enhance basal transcriptional activity. 

**Sp1 Can Bind to Subregions A2 and A4 and Is Responsible for the Major DNA-Protein Complexes Observed Using These DNA Sequences with GGH3-1 Nuclear Extracts**—Analysis of the DNA sequences within subregions A2 and A4 revealed the presence of GC-rich sequences within both of these elements. These sequences have homology with the consensus binding site for Sp1, a three-zinc-finger transcription factor (Fig. 3). We therefore sought to test the hypothesis that Sp1 was able to bind to both A2 and A4 and that the DNA-protein complexes observed by EMSA on A2, A4, and LHA using GGH3–1 nuclear extracts contained Sp1 protein. EMSA studies demonstrated that a 100-fold excess of oligonucleotide containing the consensus DNA-protein complex.
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**Consensus Sp1 Sequence:**

\[(G/T)GGCCGG(G/A)(G/A)\]

**LHβ Region A:**

![DNA sequences with homology to the consensus DNA binding sequence for Sp1, a three-zinc-finger transcription factor, are indicated in subregions A2 (in the antisense orientation) and A4 within region A of the rat LHβ gene promoter.](image)

Sus Sp1 binding site was able to compete successfully for GGH₃₋₁' nuclear extract binding to A2, A4, or LHA (Fig. 4A). Oligonucleotides containing the consensus binding sites for AP1 or AP2 were also tested because these transcription factors are both activated by protein kinase C, which is, in turn, activated by GnRH. Hence, these are also putative mediators of transcriptional responses to GnRH. However, in contrast to the results observed using the Sp1 oligonucleotide, oligonucleotides containing the consensus binding sites for AP1 or AP2 did not compete for binding to A2, A4, or LHA when present in 100-fold molar excess. Purified human Sp1 is able to bind to LHA, giving a binding pattern similar to that observed using GGH₃₋₁' nuclear extracts (Fig. 4B). Similarly, Sp1 is able to bind to A2 and A4 (data not shown). Co-incubation of Sp1 antibody with GGH₃₋₁' nuclear extract and LHA, A2, or A4 DNA probe in an antibody supershift assay resulted in the formation of a more slowly migrating “supershifted” complex (Fig. 4B), whereas no supershift was observed using an anti-follistatin antibody as a negative control (Fig. 4B) or using an anti-Sp3 antibody or an anti-Egr-1 antibody (data not shown).

Similarly, the Sp1 antibody was also able to supershift protein-DNA complexes observed using αT3 nuclear extracts and LHA, A2, or A4 probes (data not shown). Taken together, these studies indicate that Sp1 or an antigenically related protein is present in GGH₃₋₁' and αT3 nuclear extracts and binds to regions A2 (~451/~428) and A4 (~411/~386) of the LHβ gene promoter.

**DNase I Footprinting Studies Demonstrate That Sp1 Binds to GC-rich Sequences in Subregion A2**—Our EMSA studies demonstrated that Sp1 could bind to DNA sequences in A2 and A4 as well as to LHβ region A. Based on homology to the Sp1 consensus DNA binding sequence, we hypothesized that Sp1 binding occurred to GC-rich sequences within these elements. In order to test this hypothesis and define the Sp1 binding sites more precisely, we performed DNase I footprinting experiments, using human Sp1 and 3'-end-labeled LHA (Fig. 5). These studies demonstrated that Sp1 could bind to ~456/~442 of the rat LHβ gene promoter, using either the sense or antisense labeled DNA fragment as the probe, resulting in protection from DNase I digestion. Binding was reduced using a 100-fold excess of Sp1 oligonucleotide, LHA, A2, or A4 as an unlabeled DNA competitor but not using an oligonucleotide containing the Pit-1 binding sequence, used as a negative control (25), confirming the specificity of the Sp1-DNA interaction. Bovine serum albumin was not able to protect the DNA fragment from DNase I digestion and therefore did not generate a footprint. We were unable to observe a footprint on the GC-rich DNA element corresponding to A4 (~411/~386); however, this sequence was able to compete for Sp1 binding to ~456/~442 (Fig. 5B). Therefore, the lack of a footprint on this region likely represents a limitation of the assay rather than an absence of Sp1 binding to this sequence. Due to the high GC content of this sequence, secondary structure may have interfered with resolution of this region on denaturing polyacrylamide gels, possibly obscuring the presence of a footprint.

**Methylation Interference Studies Demonstrate That Sp1 and a Protein(s) in GGH₃₋₁' and αT3-1 Nuclear Extracts Bind to GC-rich Sequences in Subregion A2**—In order to define more precisely the base pairs contacted by Sp1 and to compare the pattern of binding of Sp1 with that observed using GGH₃₋₁' or αT3 nuclear extracts, we performed methylation interference studies with gel retardation, using 3'-end-labeled LHA. As shown in Fig. 6, methylation of the guanine nucleotides at positions ~450/~443 on the noncoding strand interferes with human Sp1 binding, as well as with GGH₃₋₁' nuclear extract binding. The identical pattern was observed using αT3-1 nuclear extract (data not shown). No methylation interference pattern was observed using the coding strand of LHA (data not shown); however, there are no guanine residues in this region of the coding strand. These methylation interference assay data, taken together with the DNase I footprinting studies and EMSA analyses, suggest that Sp1 can bind to sequences at positions ~456/~442 and ~411/~386 and that the binding observed using GGH₃₋₁' and αT3 nuclear extracts is due to Sp1 or a protein antigenically related to Sp1 and able to recognize and bind to the same sequences as Sp1.

**Point Mutations in Subregions A2 and A4 Abolish Binding of Sp1 and GGH₃₋₁' Nuclear Proteins**—In order to study the functional role of Sp1 binding to sequences in LHβ region A in mediating the stimulation of LHβ gene promoter activity by GnRH, we wanted to abolish binding of Sp1 to this region. Oligonucleotides containing point mutations in the putative Sp1 binding sites were generated and are referred to as M1 and M2 (Fig. 7A). When these two mutant oligonucleotides were 5'-end-labeled and used in EMSA studies, GGH₃₋₁' nuclear extract and Sp1 were no longer able to bind to these DNA sequences (Fig. 7B). Furthermore, these oligonucleotides were unable to prevent DNA-protein interactions between the A2 or
A4 oligonucleotides and GGH$_3$-1' nuclear extracts, when used in 200-fold excess as unlabeled competitors, whereas the wild-type sequences were able to reduce such DNA-protein interactions (Fig. 7B). These studies confirm that Sp1 binding occurred to the GC-rich sequences identified in LH$\beta$ region A as having homology to the Sp1 consensus binding site and that the point mutations abolished Sp1 binding.

**Mutations That Abrogate Sp1 Binding to Sequences in Re-**
FIG. 6. Methylation interference assays were performed using LHA 3′-end-labeled on the antisense strand as a probe and GGHH-1′ nuclear extract or human Sp1, as described under “Experimental Procedures.” The sequence shown corresponds to the sense strand in this region. F, free; B, bound.

region A Blunt the Stimulation of the LHβ Gene Promoter by GnRH—The EMSA, DNase I protection, and methylation interference studies defined two DNA elements within the LHβ gene promoter that bind Sp1 or an Sp1-like protein and that lie within a GnRH-response element. We therefore hypothesized that this protein-DNA interaction was necessary to mediate stimulation of the LHβ promoter activity by GnRH. In order to test this hypothesis, we used site-directed mutagenesis to introduce point mutations into these two Sp1 binding sites in -797/+5LHβLUC, which would abolish Sp1 binding as demonstrated by EMSA analysis (LHβSp1M1LUC, LHβSp1M2LUC, and LHβSp1M1M2LUC). We then used these mutant constructs in transient transfection assays in order to compare the stimulation of luciferase activity by GnRH of the wild-type -797/+5LHβLUC to that using the Sp1 binding site mutants (Fig. 8). Mutation of either of the Sp1 binding sequences decreased the stimulation of the LHβ gene promoter by GnRH (wild-type, 5.4 ± 0.3-fold compared with vehicle-treated controls; mutation of 5′ Sp1 site (M1), 3.7 ± 0.4-fold response to GnRH (p < 0.005); mutation of 3′ Sp1 site (M2), 3.1 ± 0.5-fold response to GnRH (p < 0.005)). Mutation of both Sp1 sites causes a slight but not statistically significant further decrease in the GnRH response (2.7 ± 0.5-fold; p = NS). Mutations in either or both of the Sp1 binding sites also decreased basal luciferase activity to 45–50% of the wild-type promoter. Thus, Sp1 both binds to and mediates GnRH stimulation of the rat LHβ gene promoter. These data suggest that Sp1 or an Sp1-like protein not only binds to the LHβ gene promoter at positions -456/-442 and -411/-386 but also plays an important role in conferring GnRH responsiveness to the LHβ subunit gene.

DISCUSSION

The gonadotropins play an integral role in the regulation of reproductive development and function, responding to GnRH and other modulatory influences with changes in production and secretion that, in turn, act on the gonads to regulate gametogenesis and gonadal hormone production. LH and FSH secretion are known to be tightly regulated throughout development, at puberty, menopause, and particularly during the menstrual or estrous cycle. LH and FSH production are also highly regulated, and much of this regulation occurs at the level of gene transcription. Although studies of the α-subunit gene have led to some understanding of the molecular mechanisms involved in this transcriptional regulation, little is known about the mechanisms involved in the regulation of the LHβ and FSHβ subunit genes. In this study, we have demonstrated that two GC-rich DNA elements, which have homology to the consensus binding site for Sp1 and are able to bind Sp1, are necessary and sufficient to mediate stimulation of LHβ gene expression by GnRH. Mutations in these elements that prevent Sp1 binding also decrease the stimulation of LHβ gene transcription by GnRH.

Sp1 is a member of a family of three-zinc-finger transcription factors, binding to GC-rich elements found in a wide variety of cellular and viral promoters (26, 27). Transcriptional activation is thought to occur through interaction with co-activator proteins that, in turn, interact with the basal transcriptional machinery (28, 29). Sp1 is posttranslationally modified by multiple N- and O-linked glycosylations as well as by phosphorylation by a DNA-dependent protein kinase (30). Sp1...
Sp1 Binder to a GnRH Response Element in the LHβ Promoter

is widely expressed and is often involved in transcriptional activation of genes in a non-hormonally regulated manner. In recent years, it has also been shown that Sp1 is involved in the hormonal regulation of gene expression for a variety of genes by a number of different mechanisms. Two Sp1 binding sites mediate cAMP-induced transcription of the CYP11A gene by adrenocorticotropic hormone (31). Regulation of the low density lipoprotein receptor gene promoter by cholesterol requires a sterol regulatory element (SRE) and an adjacent binding site for Sp1 (32). The SRE functions as a conditionally positive element, binding its cognate factor, SRE-binding protein and activating expression only when sterol levels are low. It cannot function efficiently by itself but requires binding of Sp1 to an adjacent site. It is hypothesized that the concerted action of Sp1- and SRE-binding protein results in synergistic activation of the low density lipoprotein receptor gene by coupling two different co-activator pathways together. Thyroid hormone suppression of the epidermal growth factor receptor promoter is mediated by overlapping Sp1 and thyroid hormone receptor-retinoid X receptor complex binding sites (33). In contrast, when a thyroid hormone response element is separated from Sp1 binding sites, synergism is observed. The induction of the cathepsin D and heat shock protein 27 genes by estrogen depends on the presence of both estrogen receptor and Sp1 binding sites in the gene promoters; mutation of either binding site prevents induction by estradiol (34, 35).

The DNA binding of Sp1 to the LHβ gene promoter is not regulated by GnRH at either of the two binding sites that we have identified. Sp1 binding patterns on A2 and A4 by EMSA were the same using nuclear extracts prepared from GGH3-1 cells that had been treated with GnRH for varying time intervals ranging from 10 min to 6 h, as they were for untreated or vehicle-treated cells (data not shown). The mechanism by which Sp1 binding to the LHβ gene promoter activates transcription in response to GnRH is unclear. The stimulation of cells by GnRH may lead to posttranslational modification of Sp1 that results not in changes in DNA binding but in changes in transcriptional activity by altering the interaction of Sp1 with the basal transcriptional machinery, either directly or indirectly through a coactivator(s). Another possible mechanism is by interaction with a protein(s) binding to the LHβ gene promoter at sites adjacent to the Sp1 binding sites. These proteins may be tissue-specific and/or specific to the LHβ gene promoter, and their DNA binding or transcriptional activation functions may be regulated by GnRH but require interaction with Sp1 for full DNA binding or transcriptional activity. This would be analogous to the cathepsin D and heat shock protein genes, which are dependent on Sp1-estrogen receptor interactions, or to the sterol-dependent regulation of the low density lipoprotein receptor gene by SRE-binding protein and Sp1 (32, 34, 35). Alternatively, Sp1 bound to subregions A2 and A4 may interact with proteins bound to other regions of the LHβ gene promoter to regulate gene expression. We have shown previously that full GnRH responsiveness of the LHβ gene promoter requires sequences at positions −207/−82 (region B) in addition to the DNA sequences in region A (15). Region B includes sequences with homology to the SF-1 consensus binding site and has been shown to bind SF-1 and activate expression of the rat LHβ gene (7). It is possible that Sp1 bound to region A interacts with SF-1 bound to region B to mediate stimulation by GnRH. Indeed, it has been shown that Sp1 and SF-1 can associate in vivo in a mammalian two-hybrid system and function cooperatively in the transactivation of the bovine CYP11A gene promoter (36).

We have demonstrated that DNA sequences with homology to the Sp1 consensus binding site function as GnRH response elements in the rat LHβ gene promoter and that purified human Sp1 can bind to these sites. Using anti-Sp1 antibodies, we show that Sp1 is present in the complexes formed between A2 or A4 and nuclear extracts from GGH3-1 or αT3 cells. The possibility remains that a protein antigenically related to Sp1, rather than Sp1 itself, binds to these elements to mediate the GnRH effect. Using antibodies, we have shown that Egr-1 and Sp3 are not responsible for the DNA binding we observed (data not shown). However, we have not ruled out all members of this family of transcription factors. Overexpression of Sp1 in GGH3-1 cells does not augment the GnRH response (data not shown), but this is most likely explained by the presence of adequate levels of Sp1 occurring endogenously in the cells. Other investigators have used the Drosophila Schneider cell line for such studies because these cells lack Sp1 (28), but these cells would not be expected to be GnRH-responsive and therefore would not be suitable for our studies.

Anti-Sp1 antibody failed to supershift all of the protein-DNA complexes observed using LHβ region A or A2 as the DNA probe, as indicated in Fig. 4B. This suggests that an additional...
protein(s) present in GGH3 cell nuclear extracts (and αT3-1 nuclear extracts, not shown) binds to these DNA sequences. The identity of these additional proteins has not yet been determined. It is possible that these proteins may bind to the LHβ gene promoter adjacent to the Sp1 binding sites and interact with Sp1 to mediate the GnRH response, as outlined above.

Sp1 binding sites close to the transcriptional start site are known to result in high levels of transcriptional activity (37). This likely accounts for the high levels of basal activity observed using LHA22GH50LUC, LHA32GH50LUC, and LHA42GH50LUC, in which the Sp1 binding sites have been moved immediately upstream of the transcriptional start site. Mutation of the Sp1 binding sites in the context of the native rat LHβ gene, relative to the transcriptional start site, resulted in a 50% decrease in basal activity of the promoter in addition to decreasing stimulation by GnRH, suggesting that Sp1 binding to LHβ region A also contributes to basal activity of the rat LHβ gene.

Although the GGH3 cell line is pituitary in origin, it is not gonadotrope-derived and does not express the LHβ gene. Therefore, at best, it can be used as a model for the study of LHβ gene expression and regulation, and results need to be confirmed in other, more physiologic systems. Nevertheless, we have shown previously that the regulation of the gonadotropin subunit promoter activities by GnRH in this cell line, when transfected with the GnRHR, closely reflects the regulation observed in primary pituitary cells (14). Furthermore, the DNA-protein complexes observed using GH3 nuclear extracts are identical to those observed using nuclear extracts from the gonadotrope-derived αT3 cell line. The role of Sp1 in the regulation of LHβ gene expression by GnRH provides some insight into this effect. Sp1 is widely expressed in all mammalian cells. Therefore, the stimulation of LHβ promoter activity by GnRH could be predicted to occur in any cell expressing the GnRHR on its cell surface, because Sp1 would be present. However, full activity of the LHβ gene likely requires additional factors. Indeed, other studies have demonstrated roles for SF-1 and Egr-1 in basal and possibly GnRH-stimulated activity of the LHβ gene (7–9, 38, 39). These two factors are also not gonadotrope-specific, and it is likely that additional, gonadotrope-specific factors are necessary for full LHβ promoter activity. The recent development of a gonadotrope-derived cell line, LβT2 cells, may be useful for such studies (40, 41).

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