Steroidogenic Factor-1 and Early Growth Response Protein 1 Act through Two Composite DNA Binding Sites to Regulate Luteinizing Hormone β-Subunit Gene Expression

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Recent in vivo and in vitro studies have implicated the orphan nuclear receptor, steroidogenic factor-1 (SF-1), and the early growth response protein 1 (Egr-1) in the transcriptional regulation of the luteinizing hormone β-subunit (LHβ) gene. We have previously demonstrated the ability of SF-1 to bind to and transactivate the rat LHβ gene promoter acting at a consensus gonadotrope-specific element (GSE) located at position −127. We have now identified a second functional GSE site at position −59. In addition, based on electrophoretic mobility shift assay, in vitro translated Egr-1 is shown to bind to two putative Egr-1 binding sites (positions −112 and −50), which appear to be paired with the identified GSE sites. By transient transfection assay in pituitary-derived GH3 cells, it was seen that Egr-1 increases promoter activity of region −207/−5 of the rat LHβ gene promoter through action at both Egr-1 sites. Furthermore, LHβ gene promoter activity is markedly augmented in the presence of both factors together relative to activity in the presence of SF-1 or Egr-1 alone (150-fold versus 14-fold and 12-fold, respectively). These data define two composite SF-1-Egr-1 response-elements in the proximal LHβ gene promoter and suggest that SF-1 and Egr-1 act synergistically to increase expression of the LHβ gene in the gonadotrope.

Precise regulation of gonadotropin gene expression is required for normal reproductive function. The pituitary gonadotropins, luteinizing hormone and follicle-stimulating hormone, are composed of a common α-subunit linked to one of two unique β-subunits, LHβ or FSHβ. The common α-subunit can also associate with thyroid-stimulating hormone β-subunit in pituitary thyrotropes or, in humans, with placenta-derived choric gonadotropin β-subunit.

Studies of the α-subunit gene promoter have identified a number of transcription factors and cognate cis-acting DNA elements that provide basal, tissue-specific, and hormonally mediated regulation of gene expression. In particular, a gonadotrope-specific element (GSE) is believed to be important for conferring gonadotrope-specific expression of the α-subunit gene (1, 2).

The GSE, or Ad4 response element, regulates expression of a number of genes with a role in steroidogenesis, sexual differentiation, and adult reproductive function (3). The GSE/Ad4 site has been shown to interact with the transcription factor, steroidogenic factor-1 (SF-1), resulting in transcriptional activation of a variety of genes, including the steroidogenic P450, the Mullerian inhibiting substance, and the aromatase genes, among others (4–6).

SF-1 is a member of the nuclear hormone receptor superfamily. Although it was previously considered to be an orphan member of this family, it has recently been reported that SF-1-dependent transcriptional activity is increased in the presence of cholesterol metabolites, particularly 25-OH-cholesterol (7). It is currently unknown whether this putative ligand is important for SF-1 function in nonsteroidogenic tissues, such as the pituitary gland. SF-1 expression is restricted to the steroidogenic cells of the adrenal gland and gonads and to the gonadotrope subpopulation of the pituitary gland. Thus, the pattern of SF-1 expression suggests that SF-1 may contribute to tissue-specific gene expression.

In contrast with the α-subunit, progress has only recently been made in the identification of transcription factors that regulate the gonadotropin β-subunit genes. Understanding β-subunit regulation at the molecular level is critical because it is the β-subunits that provide the functional specificity that distinguishes luteinizing hormone and follicle-stimulating hormone action.

A number of recent reports have suggested that SF-1 may play a role in the regulation of LHβ gene expression in addition to its effects on the common α-subunit. Targeted disruption of the Ftz-F1 gene encoding SF-1 results in transgenic mice that lack transcripts for the gonadotrope markers LHβ, FSHβ, and gonadotropin-releasing hormone receptor and have greatly diminished levels of α-subunit mRNA (3). Gonadotropin-releasing hormone replacement was able to restore gonadotropin expression in four out of five of these animals, suggesting that cells from the gonadotrope lineage are present but that a specific defect in gonadotropin subunit gene expression exists (8).

By sequence homology, it has been shown that the LHβ gene promoter contains a consensus GSE site at position −127 in the rat and −125 in the cow. In vitro studies demonstrated the
ability of SF-1 to bind to and transactivate the rat LHβ gene promoter through interaction with this putative GSE site (9). The physiologic significance of this site was confirmed using transgenic mice containing either the wild-type bovine LHβ gene promoter or a promoter with a GSE site-specific mutation linked to a CAT reporter vector (10). Introduction of the GSE mutation substantially decreased LHβ gene promoter activity, indicating that the SF-1 binding site is a critical regulator of LHβ gene promoter activity in 
vivo. Of note, however, in both the in vitro and in vivo model systems, mutation of this GSE site failed to eliminate fully LHβ gene expression, suggesting the presence of a second functional SF-1-response element.

Closer inspection of the rat LHβ gene promoter sequence identified a second region with similarity to the consensus GSE site (Fig. 1). We have termed this second putative site the Egr site (Fig. 1). Lee et al. (15) have demonstrated the ability of SF-1 and Egr-1 to interact directly with the Egr site (Fig. 1). Lee et al. (15) have demonstrated that mutation of the Egr site correlates with loss of Egr-1-mediated transactivation. However, the ability of Egr-1 to interact directly with the Egr site and the functional importance of the putative Egr site have not been determined.

In the study reported here, we have therefore verified the ability of in vitro translated Egr-1 to bind to both of the putative Egr-1 binding sites and have shown that both sites confer Egr-1 responsiveness to the rat LHβ gene promoter. In addition, we have demonstrated the ability of SF-1 and Egr-1 to interact with each other and have characterized the functional importance of this interaction to the regulation of LHβ gene expression.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotides—**The oligonucleotides used for mutagenesis, electrophoretic mobility shift assays (EMSA), and polymerase chain reactions are shown in Table I. The nucleotide sequence of the rat LHβ gene promoter is based on newly obtained sequencing data available at GenBank accession number AF020505, which differ slightly from that of Jameson et al. (16). The 5′ GSE oligonucleotide contains additional 5′-BamHI and 3′-BgIII restriction sites. The −207LH-S, −82LH-S, and 5′LH-S primers introduced BamHI restriction sites, whereas the +5LH-AS primer introduced HindIII sites, and the 3′LH-AS primer introduced BglII and XhoI restriction sites (restriction sites not shown).

**In Vitro Translated Proteins and Antisera Used in EMSA—**In vitro translated proteins were generated from plasmids containing 2.1 kilobase pairs of the mouse LHβ 1 cDNA (provided by Dr. K. L. Parker, Northwestern University School of Medicine) or 3.2 kilobase pairs of the mouse Egr-1 cDNA (provided by Dr. D. Nathans, Johns Hopkins University) using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI) (11, 17). The resultant protein was determined to be of appropriate size by comparison with [35S]methionine-labeled protein markers by SDS-polyacrylamide gel electrophoresis (PAGE).

The SF-1 polyclonal antibody, a generous gift of Dr. Parker, was generated in rabbits against a glutathione S-transferase-SF-1 fusion protein (18). The Egr-1 antisera is a rabbit affinity-purified polyclonal antibody raised against the carboxyl terminus of mouse Egr-1 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). The Pit-1 antisera, directed against amino acids 136–150 of rat growth hormone pituitary and rPit-1, was provided by C. Bancroft (Mt. Sinai School of Medicine) (19).

**Electrophoretic Mobility Shift Assays—**Region −141/−44 of the rat LHβ gene promoter (see Fig. 5B) was produced by polymerase chain reaction using primers 5′LH-S and 3′LH-AS and subcloned into the pGEM-T vector (Promega). BamHI and BglII restriction enzymes were used to introduce the insert, which was agarose gel-purified and dephosphorylated. The remainder of the double-stranded oligonucleotides used as probes and in competition experiments were produced by annealing the sense oligonucleotide indicated in Table 1 with the corresponding antisense oligonucleotide (not shown). Probes were created by T4 polynucleotide kinase end-labeling with [γ-32P]ATP followed by purification over a NICK column (Amersham Pharmacia Biotech).

**In vitro translated protein(s) were incubated with 50,000 cpm of oligonucleotide probe in DNA-binding buffer (20 mM HEPES (pH 7.9), 60 mM KCl, 5 mM MgCl2, 10 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, 1 mg/ml bovine serum albumin, and 5% (v/v) glycerol) for 30 min on ice. For competition studies, a 200-fold molar excess of unlabelled oligonucleotide was added 5 min prior to the addition of the radiolabeled probe. After 30 min on ice and then digested with EcoRI, HEPI, and 3′-untranslated region generated by primers SII(+) vector containing the Egr-1 cDNA with either EcoRIII, MscI, or SphI. The Msci-digested cDNA was treated with T4 DNA polymerase to create a blunt end. These vectors were then digested with EcoRI, yielding cDNA fragments having a 5′ EcoRI restriction site and a blunt 3′-end. Next, the pCMX vector (provided by Dr. R. Evans, The Salk Institute) was digested with XhoI, treated with T4 DNA polymerase, and then digested with EcoRI. The EcoRIII, MscI-, and SphI-digested cDNAs were subcloned into the altered pCMX vector to create pCMX Egr-1(1–533), Egr-1 del 486–533, and Egr-1 del 318–533, respectively.

**Plasmids Used in Transfection Studies—**Reporter constructs used for these studies were created by subcloning polymerase chain reaction products containing the LHβ gene promoter sequence into the pXP2 vector using BamHI/HindIII restriction sites, which were introduced by primers (18). The largest construct used for these studies contained 207 base pairs of the 5′-flanking sequence of the rat LHβ gene and the first 5 base pairs of the 5′-untranslated region generated by primers −207LH-S and +5LH-AS. A 5′-truncated construct was obtained by subcloning the polymerase chain reaction product obtained with primers −82LH-S and +5LH-AS.

Mutations in the reporter constructs were created using the Trans-\**
SF-1 and Egr-1 Regulation of LHβ Gene Expression

- **RESULTS**
  - **SF-1 Binds Specifically to the Putative 3′ GSE Site**—We have previously demonstrated the ability of SF-1 to increase rat LHβ gene promoter activity specifically through action at a functional GSE site located at position -127/-119, now designated as the 5′ GSE site (9). These studies were primarily based on transient transfection of the monkey kidney fibroblast cell line, CV-1, with preliminary confirmation in the rat pituitary-derived somatomatotrope cell line, GH3 (9). By Northern analysis, CV-1 cells lack the mRNA that encodes the SF-1 homologue Ad4BP (24). We have also demonstrated that both CV-1 and GH3 nuclear extracts fail to produce specific protein-DNA interactions with the 5′ GSE probe by EMSA (data not shown).
  - In these earlier studies, it was noted that residual SF-1 responsiveness could be observed following mutation of the 5′ GSE (CGG to AA at position −124 and −123). These results suggested two possibilities: 1) the importance of additional base pairs in this GSE site to the SF-1 response, or 2) the presence of additional SF-1-binding sites that contribute to the regulation of LHβ gene promoter activity. Sequence analysis of the rat LHβ gene promoter identified a number of regions that resemble the consensus GSE, including region −59 to −51, which was designated the putative 3′ GSE site (Fig. 1). We therefore tested the ability of SF-1 to bind to this region of the LHβ gene promoter using EMSA. As shown in Fig. 2A, lane 1, in vitro translated SF-1 bound to a 32P-labeled 3′ GSE probe to produce the complex indicated by the arrow. The specificity of this interaction was demonstrated by successful competition with unlabeled 3′ GSE (Fig. 2A, lane 2) but not with the mutated 3′ GSE site (3′ GSEM) (Fig. 2A, lane 3) nor with an unrelated oligonucleotide containing two binding sites for the pituitary transcription factor, Pit-1 (Fig. 2A, lane 4). Unprogrammed reticulocyte lysate failed to bind to this probe (Fig. 2A, lane 5).
  - In order to confirm that the observed band contained SF-1, we investigated the effect of a SF-1-specific antibody on the formation of the identified complex. This antibody has previously been shown to block the ability of SF-1 to bind to the promoter element of a number of genes, including the glycoprotein α-subunit and 21-hydroxylase genes (2, 25). Treatment with this SF-1-specific antibody decreased the intensity of the protein-DNA complex, whereas the addition of an anti-Pit-1 antisera, used as a negative control, had no effect (Fig. 2A, lanes 7–10). The upper band, indicated by an asterisk, was observed in reactions that lacked in vitro translated protein (data not shown). This band is therefore presumed to represent a direct interaction between the antibody and the probe and does not represent supershift of the SF-1-DNA complex.
  - In order to localize further the SF-1-recognition site, a 3-base pair mutation was introduced into the wild-type LHβ oligonucleotide sequence (3′ GSE) to form mutated 3′ GSE. As seen in Fig. 2A, lanes 6 and 7, no additional complexes were observed beyond those produced in the presence of unprogrammed reticulocyte lysate.
  - Parallel EMSA was performed using nuclear extract from the gonadotrope-derived cell line, aT3–1. This cell line has previously been shown to express both SF-1 mRNA and protein (2, 3). Endogenous SF-1 present in aT3–1 nuclear extract was also able to bind to the 3′ GSE region, as demonstrated by successful competition with the SF-1-specific antibody (data not shown). These data firmly establish that the putative 3′ GSE site in the rat LHβ gene promoter is recognized by SF-1, present as either an endogenous protein or an in vitro translated product.
  - The 5′ GSE and 3′ GSE Sites Have Similar Affinity for in Vitro Translated SF-1—We next wished to determine the relative affinities of the two GSE sites that we had identified for SF-1 binding. EMSA was performed using in vitro translated SF-1 and the 3′ GSE oligonucleotide probe with an increasing molar excess of unlabeled 3′ GSE or 5′ GSE oligonucleotides.
Based on this approach, the affinity of SF-1 for either of these sites is essentially identical within the sensitivity of this approach.

The Putative 3' GSE Site Contributes to SF-1-stimulated Increases in LHβ Promoter Activity—In order to determine the functional significance of the interaction between SF-1 and the LHβ gene promoter sequences, transient transfection assays were performed in CV-1 cells, the cell line used in characterization of the 5' GSE site (9). Consistent with our previously published results, cotransfection with the SF-1 expression vector resulted in an 80-fold increase in the luciferase activity of a wild-type reporter construct containing region 2207 to 150 of the rat LHβ gene promoter (Fig. 3A). Mutation of either the 5' GSE or 3' GSE sites eliminated over 90% of the SF-1-response, whereas mutation of both sites decreased the SF-1-response to the level of the empty reporter plasmid, pXP2.

Ideally, we would have pursued these investigations in a gonadotrope-derived cell line. However, at the time these experiments were performed, only the αT3-1 cell line was available for study. Although these cells express the endogenous gonadotropin α-subunit gene, they do not express endogenous or exogenous β-subunit genes at appreciable levels (26). Although we were able to detect a small increase in wild-type -207/+5 LHβ gene promoter activity following cotransfection of expression vectors for SF-1 or Egr-1, the overall low expression of the reporter construct limited the usefulness of this cell line for these studies (data not shown).

More recently, we have attempted to confirm these results using a newly isolated gonadotrope-derived cell line, LβT2, which expresses the endogenous LHβ gene (27). This cell line expresses SF-1 mRNA on Northern analysis and produces SF-1, or an immunologically related protein, based on EMSA studies of LβT2 nuclear extracts (data not shown). In initial investigations, mutation of either the 5' GSE or 3' GSE site in the 2207/+15LHβ-luciferase construct markedly decreased reporter expression, consistent with loss of the ability to respond to endogenous SF-1-stimulation. Furthermore, cotransfection with either the SF-1 or Egr-1 expression vectors increased LHβ gene promoter activity by 2.5-fold and 3-fold, respectively (data not shown). Additional studies in this cell line are currently underway.

With the aim of verifying our results in a pituitary cell line, we repeated these studies in the somatolactotrope cell line, GH3 (Fig. 3B). SF-1 markedly increased -207/+5 LHβ pro-

**Fig. 2. In vitro translated SF-1 binds the putative 5' GSE site and 3' GSE site with similar affinity. A, EMSA binding reactions included in vitro translated SF-1 and, as indicated, either the 32P-labeled 3' GSE or 32P-labeled mutated 3' GSE (3' GSEM) oligonucleotides as a probe. Cold competition with 200-fold molar excess of unlabeled wild-type 3' GSE, mutated 3' GSE (3' GSEM), or Pit-1 binding site oligonucleotides are shown in lanes 2–4. Incubation with antiserum specific to SF-1 or Pit-1 was performed in lanes 9 and 10, respectively. Lanes 5 and 7 contain the unprogrammed reticulocyte lysate used for in vitro translation. The arrowhead indicates the specific binding complex. The asterisk indicates nonspecific binding. B, EMSA was performed using in vitro translated SF-1 and the 32P-labeled 3' GSE oligonucleotide probe. Increasing molar excess of unlabeled 3' GSE or 5' GSE oligonucleotide was added to the reaction mixture, and the intensity of the SF-1-DNA complex formed was quantified by phosphorimaging. The 50% inhibitory concentration was then calculated for each of the GSE sites.
In a recent report, specific loss of pituitary LHβ mRNA expression was described in a transgenic mouse model (15). In these studies, Lee et al. demonstrated that mutation of region -50 to -42 of the rat LHβ gene promoter sequence eliminated the ability of an Egr-1 expression plasmid to increase promoter activity in both a T3-1 and CV-1 cells. On further sequence analysis, we identified a second putative Egr-1 site at position -112/-104 in the proximal rat LHβ gene promoter with homology to the consensus Egr-1 binding site. We have designated these two regions the putative 5'Egr and 3'Egr sites (Fig. 1). Interestingly, these putative Egr-1 elements are paired with the GSE sites, suggesting a functional interaction between the two associated transcription factors.

EMSA studies were initiated to determine whether Egr-1 could bind to either of these putative elements. For these assays, probes were utilized that spanned the paired GSE and Egr sites in either the 3' or 5' regions (Fig. 4, A and B, respectively). In vitro translated SF-1 (Fig. 4, lanes 1 and 2) or Egr-1 (lanes 3 and 4) were added to the wild-type probes to produce the complexes indicated by the labeled arrows. The addition of an SF-1 blocking antibody (lane 2) or an Egr-1 supershifting antibody (lane 4) confirmed the presence of the expected protein in each of the complexes. In order to demonstrate that the Egr-1 was binding to the predicted base pairs in these probes, limited mutations were introduced into the wild-type oligonucleotides within the putative Egr-1 binding sites to form 3’GSE-3’EgrM and 5’GSE-5’EgrM (Table I). When used as probes, these mutations specifically eliminated binding by Egr-1 (Fig. 4, lane 5) while preserving recognition by SF-1 (lane 6). These results clearly support the ability of in vitro translated Egr-1 to bind to both of the identified putative Egr-1 sites.

Affinity for these two sites was determined using an EMSA approach analogous to that used to determine relative affinity.
of the GSE sites (Fig. 2B). The 5′ GSE-5′Egr and 3′ GSE-3′Egr oligonucleotides were used to compete for binding of in vitro translated Egr-1 to the 3′ GSE-3′Egr probe. Based on this assay, the affinity of the 3′ Egr site for Egr-1 is approximately 10-fold greater than the affinity of the 5′ Egr site (data not shown).

**EMSA Fails to Demonstrate an SF-1-Egr-1 Protein-Protein Interaction—Lee et al. (15) reported a synergistic increase in LHβ gene promoter activity following transfection with both SF-1 and Egr-1 expression vectors. We wished to determine whether this observed functional cooperativity could be explained by direct protein-protein interaction between the two transcription factors, particularly in view of the noted pairing of their respective binding sites. EMSA was performed using a probe that spanned the 3′ GSE and 3′Egr sites (Fig. 5A) or a probe containing base pairs −141 to −44 that spanned both GSE sites and both putative Egr-1 binding sites (Fig. 5B). As seen in Fig. 5A and confirmed by antibody treatment, the co-addition of in vitro SF-1 and Egr-1 (lanes 3–6) resulted in the production of two complexes that contained either SF-1 or Egr-1 but failed to demonstrate a higher order complex. Similarly, no evidence of SF-1-Egr-1 heterodimerization was detected using a probe that spanned the 5′ GSE and 3′ Egr-1 sites (Fig. 5B, lane 5). Of note, the intensity of the SF-1 and Egr-1 DNA complexes was the same whether they were produced in the presence of one or both of the transcription factors. Neither the addition of endogenous SF-1 (present in αT3–1 nuclear extracts) nor changes in the binding conditions of the EMSA incubation reaction produced a detectable SF-1-Egr-1 complex (data not shown).

**SF-1 Interacts Directly with Egr-1 in a Protein-Protein Interaction Assay—Because EMSA failed to demonstrate an interaction between SF-1 and Egr-1, we attempted to detect protein-protein binding using an alternative assay. A His6-SF-1 fusion protein linked to Ni-NTA agarose was tested for the ability to bind to Egr-1 that had been radiolabeled with [35S]methionine during in vitro translation. This assay has been used previously to detect an interaction between SF-1 and another gonadotrope-expressed transcription factor, DAX-1 (20). As shown in Fig. 6, Egr-1 bound to the SF-1 fusion protein, whereas no binding was observed to the Ni-NTA beads alone. Relative to the amount of radiolabeled input protein, approximately 6% of the Egr-1 bound to SF-1, similar in magnitude to the interaction between SF-1 and DAX-1 (7% of input). In contrast, no binding was detected between RXR and SF-1. Deletion of the carboxyl terminus of Egr-1 had no effect on, or even increased, binding (9% of input), whereas a more extensive deletion across the Egr-1 DNA binding domain eliminated the observed interaction. The lack of interaction between SF-1 and RXR and the loss of interaction following truncation of Egr-1 both strongly suggest that the binding of SF-1 to full-length Egr-1 is specific.

**Egr-1 Increases LHβ Gene Promoter Activity through Action at Both Putative Egr-1 GSE Sites—in order to verify the functional significance of each of the putative Egr-1 binding sites, transient transfection experiments were performed in GH3 cells. We first tested the effect of introducing mutations into either of the putative Egr-1 sites within the −207/−5 region of the LHβ gene promoter. These mutations corresponded to the mutations that eliminated binding by in vitro translated Egr-1 on EMSA. In order to optimize for detection of the response to the weaker 5′ GSE site, cells were cotransfected with relatively high amounts of Egr-1 expression vector (5 μg/well), whereas lesser amounts were used for subsequent experiments (1 μg/well). As seen in Fig. 7A, LHβ gene promoter activity increased by approximately 12-fold in the presence of Egr-1 in the wild-type construct. Following mutation in the putative 3′Egr or 5′Egr sites, the Egr-1 response decreased to 1.7-fold or 3.5-fold, respectively, but remained significantly greater than control expression. Mutation in both putative Egr sites eliminated Egr-1 responsiveness.

**SF-1 and Egr-1 Act Synergistically to Increase LHβ Gene Promoter Activity—We next investigated whether the observed protein-protein interaction between SF-1 and Egr-1 (Fig. 6) would result in cooperative effects on LHβ gene promoter function. As seen in Fig. 7B, cotransfection with both SF-1 and Egr-1 resulted in marked synergistic stimulation of LHβ gene expression to over 150-fold, consistent with previous reports in CV-1 cells (15).

In order to determine whether both Egr sites are important for this SF-1-Egr-1 interaction, LHβ gene promoter activity was examined.
was measured in the presence of both transcription factors in
−207/+5 LHβ gene reporter constructs containing the various
combination of Egr-1 site mutations (Fig. 8A). The synergistic
response to Egr-1 and SF-1 was maintained, although blunted,
with mutation in either the 3′Egr or 5′Egr site. These data
demonstrate that both of the Egr-1 binding sites in the LHβ
gene promoter contribute independently to the SF-1-Egr-1 syn-
ergistic effect.

SF-1 Interacts Functionally with Egr-1 through Both Adjacent and Spaced Binding Sites—As noted previously, the SF-1
and Egr-1 binding sites appear to be present as pairs in the
LHβ gene promoter, albeit with slightly different spacing be-
tween the paired sites. This localization raised the possibility
that the observed SF-1-Egr-1 functional interaction was de-
pendent on the close proximity of the two binding sites, perhaps
promoting heterodimer formation. Alternatively, as suggested
by the mutually exclusive binding observed on EMSA, the
proximity of the sites might prohibit simultaneous binding by
the two transcription factors. In this situation, SF-1 and Egr-1
would presumably act through interaction between the two
defined regions. Mutation of the 5′GSE site allowed evaluation
of the 3′GSE site with the two Egr-1 binding sites. Additional
mutation of either Egr-1 site decreased, but did not eliminate,
the SF-1-Egr-1 synergistic effect on LHβ gene promoter activ-

**FIG. 5.** Lack of detectable SF-1-Egr-1 interaction by EMSA. *In
vitro* translated SF-1 and Egr-1 were added alone or together to probes
containing both binding sites in an attempt to detect protein-protein
interactions (higher-order complex formation). A, SF-1 alone (lane 1),
Egr-1 alone (lane 2), or both proteins (lanes 3–6) were mixed with a
probe that spans both the 3′GSE and 3′Egr sites. An SF-1 antiserum
(lane 4), an Egr-1 antiserum (lane 5), or both antisera (lane 6) were
added. Lane 7 contains appropriate amounts of the unprogrammed
reticulocyte lysates used to generate the *in vitro* translated proteins. B,
interaction of SF-1 alone (lanes 1 and 2), Egr-1 alone (lanes 3 and 4), or
both proteins (lane 5) with a probe spanning the 5′GSE-3′Egr sites.
Antisera against SF-1 (lane 2) or Egr-1 (lane 4) confirm specificity of
protein-DNA complexes, as indicated. The band indicated by the aster-
isk was observed in unprogrammed reticulocyte lysate (lane 6) and did
not supershift with the Egr-1 antibody (data not shown).

**FIG. 6.** SF-1-Egr-1 interaction demonstrated by protein-pro-
tein interaction assay. [35S]Methionine-labeled *in vitro* translated
proteins for Egr-1, RXR (negative control), or DAX-1 (positive control)
were incubated with 2 μg of His6-SF-1 fusion protein bound to Ni-NTA
agarose or with Ni-NTA agarose in the absence of His6-SF-1. Bound
proteins were analyzed by SDS-PAGE. For each receptor, 10% of the
input radiolabeled protein is shown in the left column.

**FIG. 7.** Both putative Egr-1 sites are functional in the LHβ
gene promoter. GH3 cells were transiently transfected with a lucifer-
ase reporter construct containing region −207/+5 of the rat LHβ gene
promoter present as either the wild-type sequence or with mutation of
one or both of the putative Egr-1 binding sites, as indicated. Cells were
cotransfected with expression vectors for Egr-1 and/or SF-1. The results
were calculated as in Fig. 3, with each point representing the mean ±
S.E. for nine samples from at least three independent experiments. A,
partial loss of Egr-1 responsiveness with mutation in either putative
Egr site. B, interaction of SF-1 and Egr-1 on the wild-type LHβ gene
promoter sequence.
SF-1 and Egr-1 Regulation of LHβ Gene Expression

**DISCUSSION**

The gonadotropins are critical modulators of reproductive development and function, acting on the gonads to stimulate both steroidogenesis and gametogenesis. A wide variety of studies suggest that gonadotropin biosynthesis is tightly controlled and depends, to a large degree, on regulation at the level of gene transcription. Although a number of transcription factors have been identified that modulate expression of the α-subunit gene, only recently have studies begun to identify the molecular mechanisms that regulate β-subunit gene expression.

The results reported here substantially extend our understanding of the transcription factors and DNA response elements that stimulate activity of the LHβ gene promoter. These studies clearly demonstrate the ability of SF-1 to bind to a second region of the rat LHβ gene promoter with homology to the consensus GSE site. This site, located at position −59, acts in conjunction with the previously identified GSE site at position −127 to confer marked SF-1 responsiveness to the LHβ gene. Our studies also demonstrate the ability of *in vitro* translated Egr-1 to bind to and transactivate the LHβ promoter through action at two sites located at positions −112 and −50. In addition, these experiments confirm the previously reported synergistic induction of LHβ gene expression in the presence of both transcription factors, SF-1 and Egr-1 (15). As we now demonstrate, this functionally cooperativity appears to be provided by both Egr-1 binding sites and, at least in the presence of an intact 3′GSE site, occurs through interactions between either proximal or spaced SF-1 and Egr-1 regulatory elements.

In the functional studies reported here, we utilized a heterologous system in which expression vector(s) for SF-1 and/or Egr-1 and a reporter construct containing LHβ gene promoter sequences were transiently transfected into a pituitary-derived somatolactotrope cell line, GH3. Although these studies ideally would have been performed in a gonadotrope-derived cell line, there is precedent for the study of the LHβ gene in GH3 cells. The GH3 cell line has been shown to support transcription initiation from the authentic start site of the LHβ gene and to allow cAMP-mediated increases in LHβ promoter activity (28). These cells have also been utilized to identify an estrogen-responsive element in the LHβ gene promoter (29). When transfected with the gonadotropin-releasing hormone receptor, GH3 cells have been shown to support gonadotropin-releasing hormone-induced regulation of gonadotropin subunit promoter activity, which closely parallels the regulation observed in primary pituitary cells (30). As indicated under “Results,” preliminary studies in the newly available, gonadotropin-derived LjG2 cell line have supported the results obtained in the GH3 cell line.

SF-1 is a member of the nuclear hormone receptor superfamily, which includes the thyroid hormone, estrogen, progesterone, and retinoic acid receptors. Interestingly, the consensus GSE sequence resembles a nuclear receptor binding half-site. Although members of the nuclear hormone receptor family are best known for binding to pairs of recognition half-sites, monomer binding to a single 5′-extended half-site has been described for both SF-1 and another orphan nuclear receptor, NGFI-B (31). Although we have identified two GSE sites in the proximal LHβ promoter, many aspects of our data argue against the need for SF-1 multimer binding to two DNA half-sites. Although DNA bending could allow for close approximation of the two GSE sites, interaction between nuclear hormone binding sites with separations of over 70 base pairs has not been previously described. From a functional standpoint, we have demonstrated that SF-1-mediated increases in LHβ promoter activity can be observed in the presence of a single intact GSE site (Fig. 3).

Our data demonstrate functional cooperativity between SF-1 and Egr-1 on the rat LHβ gene promoter activity and suggest that this effect may be due to direct interaction between these two transcription factors, as demonstrated by use of a His-tagged SF-1 fusion protein. Based on preliminary data using Egr-1 truncation mutants, formation of SF-1-Egr-1 heterodimers requires the presence of the Egr-1 DNA binding domain. Further studies are under way to characterize fully the SF-1 and Egr-1 protein domains that are required for protein-protein interactions and/or functional synergy.

For reasons that remain unclear, SF-1-Egr-1 protein interactions could not be detected on EMSA despite multiple attempts to optimize binding conditions. Although this result could be due to a weak interaction between the two proteins, the amount of Egr-1 bound as a percentage of input was similar to the interactions of RXRα and thyroid hormone receptor β in analogous assays (data not shown). Precedent exists for diffi-
demonstrated that Egr-1 is, in fact, expressed in gonadotropes to increase expression of the LH. This action depends upon the presence of two Egr-1 DNA binding sites in the LHβ gene promoter. Interestingly, in studies of the CYP11A gene promoter, SF-1 has been shown to interact with Sp1, a member of the zinc finger transcription family, which includes Egr-1 (33).

It must be noted that the observed Egr-1 effects on LHβ promoter activity are physiologically relevant only if gonadotropes express endogenous Egr-1. Topilko et al. (14) recently demonstrated that Egr-1 is, in fact, expressed in gonadotropes on the basis of co-localization of LHβ-subunit protein and X-gal staining, which is conferred by a lacZ transgene inserted at the endogenous Egr-1(Krox-24) site (14) and has been confirmed by using untreated nuclear extracts from the gonadotrope-derived cell lines αT3-1 and L6T2 (data not shown).

In studies of nonreproductive systems, it has been observed that levels of Egr-1 mRNA and protein, although low under basal conditions, are rapidly and markedly induced by a number of stimuli, including serum, phorbol 12-myristate 13-acetate, nerve growth factor, and fibroblast growth factor (34). As is well known, gonadotropin gene expression is regulated by a wide variety of hormonal factors arising from the gonads, the hypothalamus, and the pituitary itself, factors that have been postulated to increase Egr-1 levels in the gonadotrope. Experiments are currently underway to define the physiologic stimuli that regulate Egr-1 levels in the gonadotrope and thereby increase expression of the LHβ gene.

In summary, this study has defined two composite GSE/Egr-1 elements in the proximal rat LHβ gene promoter, and it suggests that SF-1 and Egr-1 can act both alone and synergistically to increase expression of the LHβ gene by the gonadotrope.

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