A Steroidogenic Factor-1 Binding Site Is Required for Activity of the Luteinizing Hormone β Subunit Promoter in Gonadotropes of Transgenic Mice*

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Analysis of luteinizing hormone (LH) β subunit promoters from a broad range of species including teleosts and humans revealed strict conservation of a sequence homologous to the steroidogenic factor-1 (SF-1) regulatory element of cytochrome P-450 steroid hydroxylase genes. Interaction between SF-1 and this putative response element in the bovine LHβ promoter was confirmed by electrophoretic mobility shift assays. Furthermore, co-transfection of αT3-1 cells with an expression vector encoding SF-1 induced binding site-dependent transcription from the bovine LHβ promoter. Physiological significance of the LHβ SF-1 consensus sequence was established using transgenic mice containing either the wild type bovine promoter or a promoter with a site-specific mutation of this site. Mutation of the SF-1 binding site nearly eliminated promoter activity, and the mutant transgene remained inactive following induction of gonadotropin-releasing hormone accomplished by castrating male and female mice. Thus, increases of gonadotropin-releasing hormone within a physiological range did not compensate for the loss of the SF-1 binding site. Together, these findings indicate that the SF-1 binding site is a key regulator of LHβ promoter activity in vivo and implicate SF-1 as at least one of the transcription factors that acts through this site.

Gonadotropes within the anterior pituitary are defined, in part, by their unique ability to synthesize and secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These glycoprotein hormones contain an identical α subunit that heterodimerizes with unique β subunits (1). Expression of gonadotropin α and β subunit genes depends on secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus (2). GnRH responsiveness, in turn, requires that gonadotropes also express GnRH receptors (3). Thus, functional integrity and physiological control of mature gonadotropes relies on regulated expression of at least four genes: α, LHβ, FSHβ, and GnRH receptor.

While a complex array of regulatory elements controls transcription of the α subunit gene in gonadotropes (4), the elements regulating the genes encoding LHβ, FSHβ, or GnRH receptor remain undefined. This lag is due partly to weak activity of LHβ and FSHβ promoters when examined in cell culture models (5, 6). Moreover, the promoter of the GnRH receptor gene has only recently been cloned (7). Nevertheless, there is a possibility that a single transcription factor may contribute to regulated expression of all four genes.

One such candidate was revealed by gene-targeting studies directed at the Fushi tarazu factor 1 (FTZ-F1) gene that encodes steroidogenic factor-1 (SF-1) and embryonal long terminal repeat-binding protein (ELP) (8–10). SF-1 is an orphan member of the nuclear receptor superfamily initially identified as a transcription factor that controls expression of the cytochrome P-450 steroid hydroxylase genes in gonads and adrenal cortex (8). Targeted disruption of FTZ-F1 results in an absence of the adrenal cortex (9), ovary (9), testes (9), ventromedial hypothalamic nucleus (11), and gonadotropes (10). This result was later attributed solely to the loss of the SF-1 encoding portion of the FTZ-F1 gene (12).

Although all members of the hypothalamic/pituitary/gonadal axis are influenced by removal of the SF-1 gene, the mechanism responsible for gonadotrope deficiency remains unclear. This could be explained by a direct effect of SF-1 on gonadotrope development. Alternatively, if absence of the VMH attenuates secretion of GnRH, then gonadotropes would fail to develop (11). A direct gonadotrope site of action of SF-1 is suggested by studies with the α subunit gene. All mammalian α subunit promoter regions examined to date contain a gonadotrope-specific element (GSE) (13) that has strong homology to SF-1 binding sites in steroid hydroxylase genes. This element in the human α promoter binds to SF-1 (14) and accounts for approximately 50% of its activity when analyzed after transfection in the gonadotrope-lineage cell line, αT3-1 (4, 13). In contrast to evidence suggesting a direct pituitary effect of SF-1, an indirect hypothalamic site of action is supported by the recovery of detectable gonadotropin gene expression in GnRH-treated, SF-1 “knock-out” mice (11). The latter finding implies that restoration of GnRH secretion may compensate for loss of SF-1 in gonadotropes. Thus, the exact role SF-1 plays in regulating expression of the genes that define the functional properties of gonadotropes remains to be determined.

Herein, we address the functional significance of the SF-1 binding site found within the proximal 776-bp promoter-regulatory region of the bovine LHβ gene. This promoter directs high level expression of reporter genes specifically to gonadotropes in transgenic mice, is fully penetrant, and is appropriately regulated by GnRH and gonadal steroids (5).

EXPERIMENTAL PROCEDURES

Materials—Acetyl coenzyme A and the GnRH antagonist, antide, were from Sigma. Radiolabeled nucleotides were obtained from DuPont NEN. DNA-modifying enzymes were from either Boehringer Mannheim or Life Technologies, Inc., and Sequenase version 2.0 was from U. S.
Biochemical Corp. Antiserum directed against mouse SF-1 (8) was the generous gift of Keith L. Parker.

Plasmids—The vectors CMVSF-1 (8), BSK*-776/+10bLHβCAT (5), and pGL2 Control (Stratagene) have been described previously. CMVGH contains the BamHI/EcoR1 fragment of the bovine growth hormone gene linked to the CMV promoter. Generation of BSK*-776/+10bLHβCAT was accomplished by using the polymerase chain reaction with Taq polymerase and the following primers: 5’-AGGAGCCTAATACATGC TCTTTTGCAGCT-3’ and 5’-GGTTCCTCCGGGGCGAGAGGGAGGCAGGACT-3’, with the mutation highlighted. The resulting 514-bp product was cleaved with SalI and subcloned into BSK*-776/+10bLHβCAT replacing the analogous wild type sequence. Sequencing confirmed polymerase chain reaction fidelity. Plasmids (776/+10bLHβCAC and (776/+10)GSEβHβlac were made by subcloning the HindIII promoter fragments from the CAT vectors into this site of pBasic (Stratagene).

Cell Culture—Transient transfection assays of αT3-1 cells were performed essentially as described (4), except activation vectors (CMVGH or CMVSF-1) were also included at a concentration of 60 ng/35-mm well. All wells included a RSVβ-gal vector (0.42 μg/well) to control for transfection efficiency. Cells were harvested 48 h post-transfection in 150 μl of reporter lysis buffer (Promega). Luciferase and β-galactosidase assays were performed as reported with 15 μl of extract (4). Electrophoretic Mobility Shift Assays—Nuclear extracts from αT3-1 cells were prepared as described (4). Electrophoretic mobility shift assays (EMSA) were performed with the following double-stranded oligodeoxynucleotides: wild type GSE, 5’-CCCTTCCTCGACCTGTCTTGGTGCCTCT-3’; GSE, 5’-CCCTTCCTGGCCATGGTCCTCGCTCT-3’, with the mutation highlighted. Nuclear extract (8.3 μg) was incubated with unlabeled oligodeoxynucleotide competitors in a final volume of 20 μl of binding buffer (12.5 mM HEPES (pH 7.8), 25 mM KCl, 1.5 mM MgCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 1.5 μg of poly(dI-dC), 0.2 μg of single-stranded DNA, and 0.2 μg of Escherichia coli DNA). Following 5 min at 4 °C, 25 fmol of end-labeled probe was added for an additional 15 min at room temperature; 2 μl of rabbit anti-mouse SF-1 or nonimmune serum were then added. After 15 min, the samples were placed on ice and run on 5% polyacrylamide gels at 4 °C.

Transgenic Mice—Mice containing the wild type bLHβ promoter linked to CAT have been characterized previously (5). Liberation of the (776/+10)bLHβGSEβHβCAT transgene fragment was accomplished with SalI/BamHI. Transgenic mouse production, identification, and characterization of tissue-specific expression were done as reported (5). CAT assays were performed as described with 10 μg of protein for 1 h (5). Each assay included control tissues from mice containing the wild type promoter.

The intact/castrate/antiestrogen treatment paradigm was accomplished as follows. Male and female mice (6–11 weeks old) were divided into three groups for each sex. One group remained intact, received injections of antide vehicle (20% propylene glycol in normal saline) every 48 h, and animals were housed individually. The other two groups were gonadectomized under avertin anesthesia and treated with vehicle or 60 μg of antide every 48 h for 10 days (5). On day 10, mice were killed and pituitaries and venular blood collected. CAT assays of 17.5 h duration were performed as above. All animal studies were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

RESULTS AND DISCUSSION

A Consensus SF-1 Binding Site Resides within the 5′ Flanking Regions of the Genes Encoding α, LHβ, and GnRH Receptor—Sequence analysis of LHβ promoters from multiple species including teleosts and humans revealed conservation of a core sequence highly related to a canonical SF-1 binding site (Fig. 1). In some species, including bovine, the SF-1 homolog was identical to the GSE found in the human α subunit gene (13). This homolog was also present in the GnRH receptor gene, suggesting that the element may be functionally significant for genes uniquely expressed in gonadotropes. Although the GSE in the human α subunit gene binds SF-1 and thus is not gonadotrope-specific, the identity between this sequence and that in the LHβ promoter led us to retain the GSE nomenclature.

The bovine LHβ GSE Binds SF-1—EMSA were performed with nuclear extracts from SF-1-containing αT3-1 cells (14) to determine whether SF-1 binds the bovine LHβ GSE. Three complexes were detected with the wild type probe (Fig. 2). Each was eliminated by inclusion of 100-fold molar excess of homologous competitor, but not with 500-fold molar excess of a competitor containing an 8-bp mutation disrupting the SF-1 consensus (μGSE). Incubation with SF-1 antisera resulted in loss of the highest mobility band (indicated by the arrow), suggesting the presence of SF-1. Nonimmune serum had no effect, indicating that disruption of the complex was specific to the SF-1 antisera. A radiolabeled μGSE probe failed to detect the highest mobility band. Together, these data establish that the bovine GSE selectively binds SF-1.

Overexpression of SF-1 Activates the LHβ Promoter in αT3-1 Cells—The endogenous LHβ gene is silent in αT3-1 cells, even though they have GnRH receptors and express the endogenous α subunit gene (21). The LHβ promoter is also inactive in transfection assays with this cell line (5), which is possibly due to a lack, or diminished concentration, of critical transcription factors. If SF-1 actually regulates the LHβ promoter, its overexpression might lead to activation of the promoter in these cells. Therefore, to assess functional significance of SF-1 binding to the putative LHβ GSE, cotransfection assays were performed in αT3-1 cells with the 776-bp bLHβ promoter linked to luciferase and a vector containing the CMV promoter directing expression of SF-1. Duplicate cotransfections with a CMV-growth hormone expression vector were used to establish specificity of an SF-1 response.

As expected, activities of the wild type LHβ promoter (bLHβ) and a promoterless control were indistinguishable (data not shown). In contrast, overexpression of SF-1 resulted in ~5-fold induction of bLHβ promoter activity relative to that obtained in the presence of the GH control (Fig. 3). Greater induction (~8-fold; data not shown) was observed following addition of a constitutively active form of SF-1 (22). Although the bLHβ promoter containing an 8-bp GSE mutation was 2-fold more
active than the wild type promoter in the presence of GH, its response to SF-1 was negligible and equivalent to that observed with the heterologous SV40 promoter. Thus, much of the stimulatory effect of SF-1 is mediated through the GSE.

While SF-1 stimulated LHβ promoter activity only 5-fold, this was sufficient to establish a functional and statistically significant correlation between binding of SF-1 and transcriptional activation. A site-dependent effect of full-length SF-1 on other promoters has been difficult to establish. For example, Shen et al. (22) only observed activation of the Mullerian inhibiting substance promoter with a truncated form of SF-1 devoid of the putative ligand binding domain. Similarly, the human α subunit promoter with an intact SF-1 binding site is refractory to overexpression of both full-length and truncated SF-1 (data not shown), and ablation of the SF-1 binding site in the human α subunit promoter reduces basal activity by only 50% (4, 13). This suggests that transcription effects of SF-1 may be dependent on promoter context. The level of SF-1-dependent activation observed with the LHβ promoter is also significant because overexpression of other proteins potentially involved in the GnRH signal transduction pathway, including constitutively active forms of Ras (23) or Goα (24), had no effect (data not shown). Thus, these transfection assays are the first to identify both a discrete promoter element and a transcription factor that regulate activity of the LHβ promoter.

The GSE Is Essential for LHβ Promoter Activity in Transgenic Mice—We previously reported that the 776-bp bovine LHβ promoter conferred pituitary-specific expression to a CAT reporter gene in transgenic mice (5). Activity of this promoter in mice was much stronger than that observed in transfection assays. Being approximately 160-fold more active than that of the 1500-bp human α subunit promoter (5). Moreover, activity was completely penetrant (observed in all mice), occurred solely in gonadotropes, and was appropriately regulated by hormones, including GnRH, 17β-estradiol, and testosterone, which effect expression of the endogenous LHβ gene.

Transgenic mice were made with the 776-bp LHβ promoter, containing the same GSE mutation used in the EMSA and transfection assays, linked to CAT. Integrity of the transgene in seven founders was confirmed by Southern blot analysis (data not shown), and lines were derived from five to obviate integration site effects. Activity of the μGSE-LHβ promoter in pituitary was reduced at least 10-fold when compared to activity in two lines of mice containing the wild type promoter (Fig. 4). No ectopic expression was observed in nine other tissues (data not shown).

Activity of the wild type promoter tended to be higher in females than in males (Fig. 4). This trend continued with the mutant promoter, such that the occasional mouse having detectable CAT was always female. Thus, while mutation of the GSE has a substantial effect on activity of the LHβ promoter in both sexes, it may be even greater in male mice. From these results, we conclude that the SF-1 binding site (GSE) is required for full bLHβ promoter activity in pituitaries of transgenic mice.

GnRH Cannot Compensate for Loss of the GSE—Qualitative rescue of LHβ mRNA results from GnRH treatment of SF-1-deficient mice maintained with exogenous adrenal steroids (11). This suggests that the loss of LHβ gene expression in those mice is due to an effect on GnRH secretion rather than a direct effect of SF-1 in gonadotropes. To determine if an increase in GnRH could rescue activity of the mutant bLHβ promoter, male and female transgenic mice were gonadectomized to promote a post-castration rise in GnRH that lies within a physiological range (2). An additional group were treated with the GnRH antagonist antide to confirm the efficacy of gonadectomy. To enhance detection of CAT, duration of the enzymatic assay was extended significantly from 1 to 17.5 h. This extended assay allowed detection of CAT activity expressed as percent conversion/μg of protein/h. Mouse pituitaries containing the wild type or μGSE-LHβ transgenes were assayed in parallel.
SF-1 Regulation of LHβ Gene Expression

Fig. 5. GnRH cannot compensate for loss of the GSE. Female (A) and male (B) mice from line 1 containing the bLHβGSE CAT transgene remained intact or were gonadectomized. The intact animals and a subset of gonadectomized mice were treated with vehicle, while another subset received antide. At the conclusion of the experiment, blood and pituitaries were collected for LH and CAT assays, respectively. All CAT assays were performed with 10 μg of protein for 17.5 h. CAT activity is expressed as percent conversion/100 μg of protein/h. Values are means ± S.E. for 5–6 animals/group. The shaded region in the lower portion of the figure represents the mean activity of multiple liver samples included in this assay (1.14 ± 0.14). Any values that approach this level are considered non-expressing. The activity of wild type mouse pituitaries was 49.8 ± 3.0.

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