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An NF-Y Binding Site Is Important for Basal, but Not Gonadotropin-releasing Hormone-stimulated, Expression of the Luteinizing Hormone β Subunit Gene*

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Regulated synthesis of luteinizing hormone (LH) requires coordinated transcriptional control of the α and LHβ subunits in pituitary gonadotropes. Several *cis*-acting elements and *trans*-acting factors have been defined for control of the LHβ promoter through heterologous cell culture models. In this report, we describe the identification of bipartite NF-Y (CBF/CP1) binding sites within the proximal bovine LHβ promoter. When multimerized, one of these sites activates the heterologous, minimal HSV thymidine kinase promoter in the gonadotrope-derived cell line αT3-1. The functional role of the promoter-distal site in regulating the full-length bovine LHβ promoter was assessed *in vivo* using transgenic mice harboring a mutant promoter linked to the chloramphenicol acetyltransferase reporter gene. While this element is important for conferring high level activity of the LHβ promoter in pituitary, it does not appear to be essential for mediating gonadotropin-releasing hormone (GnRH) regulation. This is the first characterization of a *cis*-acting element within this GnRH-dependent promoter that is restricted to regulating basal expression and not GnRH-induced activity.

Stimulation of gametogenesis and synthesis of sex steroids by luteinizing hormone (LH)[1] is essential for reproduction in all mammalian species. Thus, understanding the regulated synthesis and secretion of this hormone from the gonadotropes in the pituitary is paramount to understanding the reproductive process.

LH is a member of the glycoprotein hormone family whose members are composed of a shared α subunit that combines with unique, hormone-defining β subunits. Synthesis and secretion of LH requires coordinated, gonadotrope-specific expression of the genes encoding both the α and LHβ subunits (1). Gonadotrope-specific expression of the α subunit gene requires a number of *cis*-acting elements located within the promoter-proximal 400 bp of the 5′-flanking region of the gene (2–7). Discernment of the essential *cis*-acting elements involved in expression of the LHβ subunit has lagged behind that made for the α subunit due to the lack of readily accessible, appropriate model systems.

Five models have been used to uncover regulatory elements within the LHβ promoter. These include cell lines corresponding to kidney fibroblasts (CV-1) (8, 9), modified somatotropes (GGH_3-1′) (10, 11), primordial gonadotropes (αT3-1) (12–14), and differentiated gonadotropes (LβT2) (15, 16) as well as transgenic mice (17–21). Using these approaches, four transcription factors have been identified that regulate expression of the LHβ subunit gene through direct binding to its promoter (see Fig. 1*A*). These include the orphan nuclear receptor, SF-1 (8, 15, 21), an early growth response protein, Egr-1 (22, 23), a *bicoid*-related homeodomain protein, Pitx1 (9), and the ubiquitous transcription factor, Sp1 (11). There are two Egr-1 and SF-1 binding sites that exist in a pairwise conformation interrupted by a Pitx1 binding site within the promoter-proximal 150 bp (24). This configuration is conserved across many, if not all, mammalian LHβ promoters. Numerous recent studies have shown that SF-1 and Egr-1 functionally cooperate to activate transcription (16, 24, 25) of the LHβ subunit gene. In addition, Pitx1 further potentiates the activation mediated by these two proteins; however, this does not appear to require direct binding of Pitx1 to DNA (25). Rather, Pitx1 can interact directly with SF-1 and Egr-1 to induce expression (25). Sp1 regulates the rat LHβ promoter by binding two elements that reside further upstream in the promoter at (−451/−428) and (−411/−386) (11). This region is less conserved across species than the promoter-proximal sequences, and its importance in other species remains to be determined.

The relative importance of Egr-1, SF-1, and Pitx1 in regulating LHβ gene expression has been confirmed with targeted disruption of each gene in mice. Mice deficient in Egr-1 exhibit a specific loss of LHβ gene expression, while the α and follicle-stimulating hormone β genes remain functional (22, 23). This suggests that Egr-1 is a selective transcriptional regulator of the LHβ gene and may mediate differential regulation of LH and follicle-stimulating hormone within the same cell. In contrast, mice deficient in SF-1 have a reduced gonadotrope population accompanied by attenuated expression of all three gonadotropin genes (26). Whether this is due solely to a defect within the gonadotrope (21) or to an additional impact in the hypothalamus (27) remains to be determined. Targeted disruption of the Pitx1 gene leads to gross defects in limb development (28, 29) as well as multiple pituitary effects (28). Although gonadotrope number and expression of the gonadotropin genes is significantly reduced in these animals, there is not an ablation of either (28). Thus, while Pitx1 is important for LHβ gene expression and gonadotrope development, it does not appear to be essential.

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[1] The abbreviations used are: LH, luteinizing hormone; SF-1, steroidogenic factor-1: GnRH, gonadotropin-releasing hormone; CAT, chloramphenicol acetyltransferase; HSVtk, herpes simplex virus thymidine kinase; EMSA, electrophoretic mobility shift assay; GSE, gonadotrope-specific element; bp, base pair(s).

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While earlier work focused on evaluating basal expression of the LHβ subunit gene, this rapidly evolved into analyses of the effects of gonadotropin-releasing hormone (GnRH). This reflects the exquisite dependence of transcriptional activity of the LHβ gene on pulsatile release of GnRH from the hypothalamus, exemplified by the *hpg*/*hpg* mouse (30) that harbors a spontaneous deletion of this gene (31). Mice harboring this mutation express the LHβ subunit gene at only 5–10% of normal levels (32), suggesting that "basal" activity of this gene requires activation of GnRH-induced signaling cascades. Indeed, all of the factors described to date as being important for LHβ gene expression also contribute to GnRH induction (11, 13, 14, 16, 21, 24, 25). In the current manuscript, we report the identification of an additional pairwise *cis*-acting element that binds NF-Y (CBF/CP1). In contrast to the previously described *cis*-acting elements and their cognate factors characterized for this gene, these sites are the first to be described that solely affect basal expression of this gene.

EXPERIMENTAL PROCEDURES

Materials—Radiolabeled nucleotides and chloramphenicol were obtained from NEN Life Science Products. Acetyl coenzyme A and the GnRH antagonist, antide, were from Sigma. DNA-modifying enzymes were from either Roche Molecular Biochemicals or Life Technologies, Inc. Sequenase version 2.0 was from Amersham Pharmacia Biotech. Poly(dI-dC) was purchased from Roche Molecular Biochemicals. Sp1 antibody was obtained from Santa Cruz Biotechnology, Inc., and NF-Y (A subunit-specific) antibody was from Rockland Immunochemicals.

Plasmid Constructs—The HSVtk-luc vector was constructed by inserting an *Xho*I/*Sal*I fragment containing the HSVtk minimal promoter (−105/+5) from BLCAT2 (33) into the *Xho*I site of pGL2-Basic (Promega). Kinased, double-stranded oligodeoxynucleotides corresponding to Lβ1 or Lβ2 (see below) were ligated into the *Sac*I site 5′ to the thymidine kinase promoter. The number and orientation of elements was then determined by dideoxynucleotide sequencing.

A transversion mutation of the Lβ1 site within the context of the full-length (−776/+10)bLHβ promoter was accomplished using an oligodeoxynucleotide replacement approach in the previously described (−776/+10)bLHβCAT vector (18). Oligodeoxynucleotides corresponding to the region to be mutated were 5′-**CCGGGAACCGACAACGATTCCCCGGT**TCAGAGGGGCGGTGCTGCA-3′ and 5′-GCACCGCCCCTCTGA**ACCGGGGAATCGTTGTCGGTTCCCGG**-3′, with the mutant sequence indicated with boldface letters. The annealed oligodeoxynucleotide has a 5′ blunt end and a 3′ *Pst*I overhang. The annealed, double-stranded oligodeoxynucleotide was inserted into the *Pst*I sites at −397 and −355, relative to the start site of transcription, that flank the Lβ1 consensus site. This required conversion of the 5′ *Pst*I site into a blunt end prior to ligation with the mutant oligonucleotide. The composition of the resultant clone was confirmed by sequencing the region containing the mutation and restriction digests confirming the remainder of the expression cassette. Isolation of the transgene was accomplished by restriction digestion with *Sal*I and *Bam*HI.

Cell Culture/Transient Transfections—Transient transfection assays of αT3-1 and MCF-7 cells were performed essentially as described (7) using 1.2 μg of test vector and 0.42 μg of RSVβ-gal per 35-mm^2 well containing 110,000–170,000 cells. Cells were harvested 48 h post-transfection in 150 μl of reporter lysis buffer (Promega). Luciferase and β-galactosidase assays were performed as previously reported (7).

Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts from αT3-1, MCF-7, and BeWo cells were prepared as described previously (7). EMSAs were performed essentially as described (21) except with 5–10 μg of nuclear protein. The following oligodeoxynucleotides were used (lowercase type indicates *Sac*I sites added for cloning purposes): Lβ1(+), 5′-cTGCAGCCAATCACCATCGGAAAATTGagct-3′; Lβ1(−), 5′-CAATTTTCCGATGGTGATTGGCTGCAgagct-3′; Lβ1 μ6Δ10(+), 5′-cTGCAGTCAACACCATCGGAAAATTGagct-3′; Lβ1 μ6Δ10(−), 5′-CAATTTTCCGATGGTGTTGACTGCAgagct-3′; Lβ1Δ10 μ18(+), 5′-cTGCAGCCAACACCATCTGAAAATTGagct-3′; Lβ1Δ10 μ18(−), 5′-CAATTTTCAGATGGTGTTGGCTGCAgagct-3′; Lβ1 μ18/25(+), 5′-cTGCAGCCAATCACCATCTGAAAATGGagct-3′; Lβ1 μ18/25(−), 5′-CCATTTTCAGATGGTGATTGGCTGCAgagct-3′; Lβ1 μ6(+), 5′-cTGCAGTCAATCACCATCGGAAAATTGagct-3′; Lβ1 μ6(−), 5′-CAATTTCGATGGTGATTGACTGCAgagct-3′; Lβ2(+), 5′-cTGTGAAGTCACCTTCTCCTGGGTGagct-3′; Lβ2(−), 5′-CACCCAAGGAGAAGGTGACTTCACAgagct-3′; Bα1(+), 5′-CTGCAGTCAACACCATCTGAAAATGGA-3′; Bα1(−), 5′-CCATTTTCAGATGGTGTTGACTGCAGA-3′ and an Sp1 consensus oligodeoxynucleotide obtained from Promega Corp. Double-stranded oligodeoxynucleotides were end-labeled with [γ-^{32}P]ATP using polynucleotide kinase. For the antibody supershift experiments, binding reactions were performed with 5–10 μg of αT3-1 nuclear extract and 1 μg of poly(dI-dC) in 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 4% glycerol in a total volume of 15 μl. After a 30-min incubation on ice, 5×10^4 cpm of labeled probe was added with an additional 30-min incubation on ice. For reactions containing antibody, 1 μl of anti-Sp1 or anti-NF-Y was added during the initial 30-min incubation.

Transgenic Mice—All mice were housed in microisolator-plus units under pathogen-free conditions. Food and water were given *ad libitum*, and animals were subjected to a 12-h light/dark cycle. Mice harboring the (−776/+10)bLHβCAT construct have been previously described (18). For this work, we regenerated this transgenic strain to utilize mice that were as few generations from the founder animals as possible for direct comparison with mice harboring the μLβ1 construct.

To generate mice with the (−776/+10)μLβ1bLHβCAT construct, the 2.4-kilobase pair expression cassette was liberated with *Sal*I plus *Bam*HI. Transgenic mouse production, identification, and characterization of tissue-specific expression were done as reported (18). Chloramphenicol acetyltransferase (CAT) assays were performed as described (18) with 10–25 μg of cellular lysate for 1 or 4 h. Each assay contained control tissues from mice containing the wild type promoter. The amount of lysate used in each CAT assay was identical for each sample within that assay.

The ovariectomy/antide treatment paradigm was performed as follows. Female mice harboring either the wild type or mutant promoter were ovariectomized under avertin anesthesia. Following surgery, randomized mice were separated into two groups: those receiving vehicle (20% propylene glycol in saline) injections and those receiving antide injections. 60 μg of antide was given as a subcutaneous injection every 48 h for 10 days. On day 10, mice were killed by asphyxiation in a CO_2 chamber, and cardiac blood, pituitaries, and livers were collected. All animals used were adults and age-matched (within 1 month) within individual experiments. All animal studies were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

RESULTS

Alignment of the Bovine α and LHβ Promoters Reveals Three Conserved Regions—Production of LH requires coordinated expression of both the α and LHβ genes. These genes must be expressed concurrently in a temporal and spatial pattern to form an intact heterodimer. In addition, regulation of their expression by GnRH and gonadal steroids may involve similar mechanisms for the two genes. Thus, we speculated that specific *cis*-acting elements may be shared between the two gonadotropin subunit genes as a means to confer similar signals for regulated expression. To begin to identify targets for functional analysis of the LHβ promoter in transgenic animals, we examined the bovine gonadotropin promoters for regions of apparent conservation. Three regions with greater than 80% homology were identified (Fig. 1*B*). One of these, the GSE, was previously shown to be important for activity of both the α (5) and LHβ (8, 21) genes. The other two elements located at positions −399/−372 (element Lβ1) and −228/−204 (element Lβ2) relative to the start site of transcription of the bovine LHβ gene, were subject to further analyses to determine their role, if any, in activating the LHβ promoter. A diagrammatic sketch of the positions of these elements as well as others identified for the bovine or rat genes is shown in Fig. 1*A*.

Lβ1, but Not Lβ2, Stimulates Transcriptional Activity of a Minimal Thymidine Kinase Promoter—In the absence of GnRH, the bovine LHβ promoter has low activity in transiently transfected cells (18) that precludes functional analyses of the Lβ1 and Lβ2 elements in the context of the full-length promoter. Therefore, functional activity was initially assessed by determining whether multimerized Lβ1 or Lβ2 elements could stimulate expression of the HSVtk minimal promoter when linked to luciferase. The resulting constructs were transiently

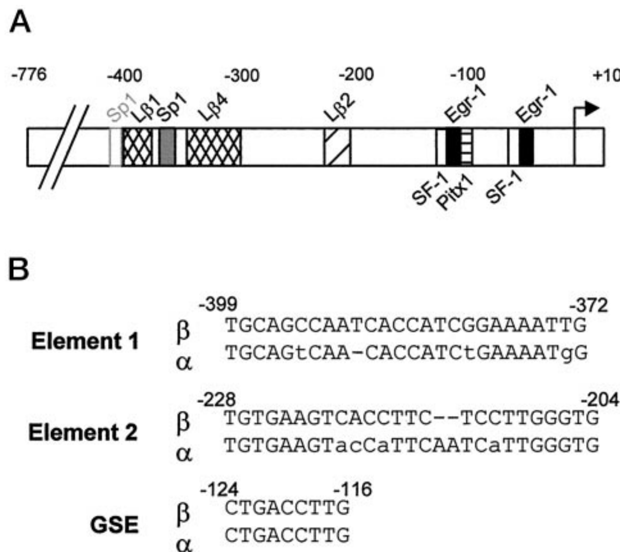

FIG. 1. **Alignment of the bovine α and LHβ promoters reveals three conserved regions.** *A*, a schematic diagram of the bovine LHβ promoter with the position of Lβ1 and Lβ2 is shown. Binding sites for SF-1 (GSE), Egr-1, and Pitx1 are indicated. The positions of Sp1 binding sites that are present within the rat LHβ promoter are also shown. Lβ4 harbors a sequence that binds the same protein as Lβ1. The Sp1 site that is 5′ to the Lβ1 site is outlined in *gray* to reflect the lack of an Sp1 consensus at this site in the bovine promoter. *B*, the bovine α and LHβ subunits were scanned for any regions of conservation between the two promoters. The sequences of three conserved elements are indicated. The GSE has previously been described (5, 8, 21). Element 1 and element 2 are designated Lβ1 and Lβ2 when referring to the elements within the LHβ promoter.

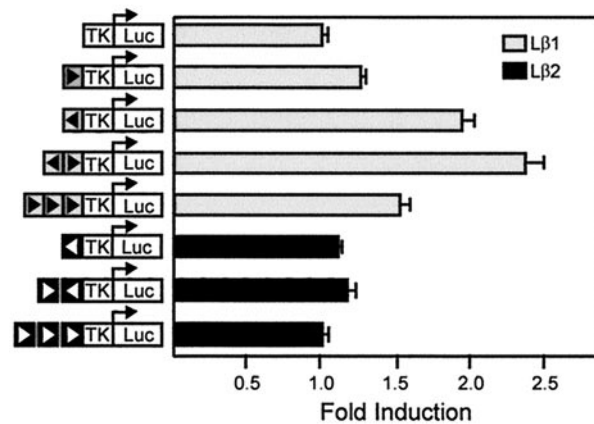

FIG. 2. **Lβ1, but not Lβ2, stimulates transcriptional activity of a minimal thymidine kinase promoter.** Oligodeoxynucleotides corresponding to Lβ1 or Lβ2 were multimerized and ligated upstream of the HSVtk minimal promoter linked to luciferase. A *shaded* (Lβ1) or *black* (Lβ2) *box* indicates the presence and orientation (*arrowheads*) of each element. αT3-1 cells were transiently transfected with each construct. Values are expressed relative to HSVtk-luc activity and represent the mean ± S.E. of three independent experiments each with three replicates.

transfected into αT3-1 cells. As shown in Fig. 2, the Lβ1 element was capable of activating the HSVtk promoter 2–3-fold with inverted repeats of this sequence resulting in the greatest induction. In contrast, Lβ2 was incapable of activating the heterologous promoter. Similar results were obtained in the breast cancer cell line, MCF-7 (data not shown). The ability of Lβ1 to function independently of cell lineage suggested that it might bind a factor that is not gonadotrope-specific.

Lβ1, but Not Lβ2, Binds a Nuclear Factor That Is Common to a Variety of Cell Lines—To determine if Lβ1 and Lβ2 elements bind nuclear proteins, EMSAs were performed with αT3-1 nuclear extracts. This assay revealed that Lβ1 could bind a single complex with high specificity and affinity (Fig. 3*A*). While only a 50–100-fold molar excess of homologous competitor was necessary to eliminate binding of the factor to the probe, a 100-fold molar excess of heterologous competitor (LHβ GSE (21)) had no impact on protein binding. A monophasic Scatchard analysis revealed a high affinity interaction between the nuclear protein and the Lβ1 site ($K_D = 1.2 \times 10^{-10}$ M, data not shown) that was well within the known range for eukaryotic transcription factors that bind to DNA (34). In contrast to Lβ1, incubation of the Lβ2 probe with αT3-1 nuclear extract failed to reveal any high affinity complexes (Fig. 3*A*). This supports data obtained from the transient transfection studies and suggests that Lβ2 is nonfunctional in these cells. Given the transcriptional activity and protein binding function of Lβ1, we chose to focus on further characterization of this element. Whether Lβ2 has a transcriptional function in mature gonadotropes remains to be determined. Studies aimed at addressing this issue will probably involve complex experimental paradigms such as transgenic mice and preparation of nuclear extracts from mature gonadotrope cells.

To address whether the Lβ1 protein-binding complex was unique to gonadotrope origin cell lines, we performed additional EMSA studies using nuclear extracts from MCF-7 and BeWo cells. Both extracts exhibited a complex with the same mobility and competition profile as that observed with αT3-1 nuclear extracts (Fig. 3*B*). An additional complex displaying a faster migration pattern was also observed in both heterologous extracts. This interaction was considered nonspecific due to the increase in intensity that corresponded with increasing concentrations of unlabeled competitors. From these results, we conclude that the protein(s) that bind to LB1 are not cell-specific and are likely to be ubiquitous given their distribution in gonadotrope-, mammary-, and choriocarcinoma-derived cell lines.

The Lβ1 Element Contains a CCAAT Motif That Is Essential for Protein Binding—To begin to discern whether the Lβ1 element was functionally conserved in the bovine α subunit promoter, we performed EMSA with its Lβ1 homolog, designated Bα1. Interestingly, although homology analysis was utilized to originally identify Lβ1, the Bα1 element did not bind any proteins with high affinity in EMSA with αT3-1 nuclear extracts (data not shown). While function of the Bα1 element within the context of the full-length α subunit promoter was not addressed, lack of protein binding in gonadotrope-derived cells suggests that this region was not important for transcriptional function of the bovine α subunit gene promoter.

Only four nucleotide differences exist between the Lβ1 and Bα1 elements. Thus, the lack of high affinity binding of a nuclear protein to Bα1 could be used to rapidly discern the specific nucleotides required for protein binding to Lβ1. Mutations in the Lβ1 element were made to mimic those nucleotide differences observed in Bα1. Fig. 4*A* outlines the mutant oligodeoxynucleotides utilized in EMSA. Two nucleotide changes were within a CCAAT motif, while two additional differences were external to this motif. The importance of these particular nucleotides in protein binding was addressed using pairwise mutations and competition analysis in EMSA utilizing the wild type Lβ1 probe. As shown in Fig. 4*B*, mutation of nucleotides at positions 6 and 10 within Lβ1 eliminated the ability of this oligonucleotide to compete for binding to the wild type Lβ1 probe. Similar to the 6/10 mutation, mutation of nucleotides 10 and 18 also resulted in loss of Lβ1 protein binding. In contrast, pairwise mutation of the two nucleotides outside the CCAAT motif at positions 18 and 24 resulted in no change in affinity. These results implicate the position 10 nucleotide (the T of CCAAT) in protein binding and minimized the likelihood that

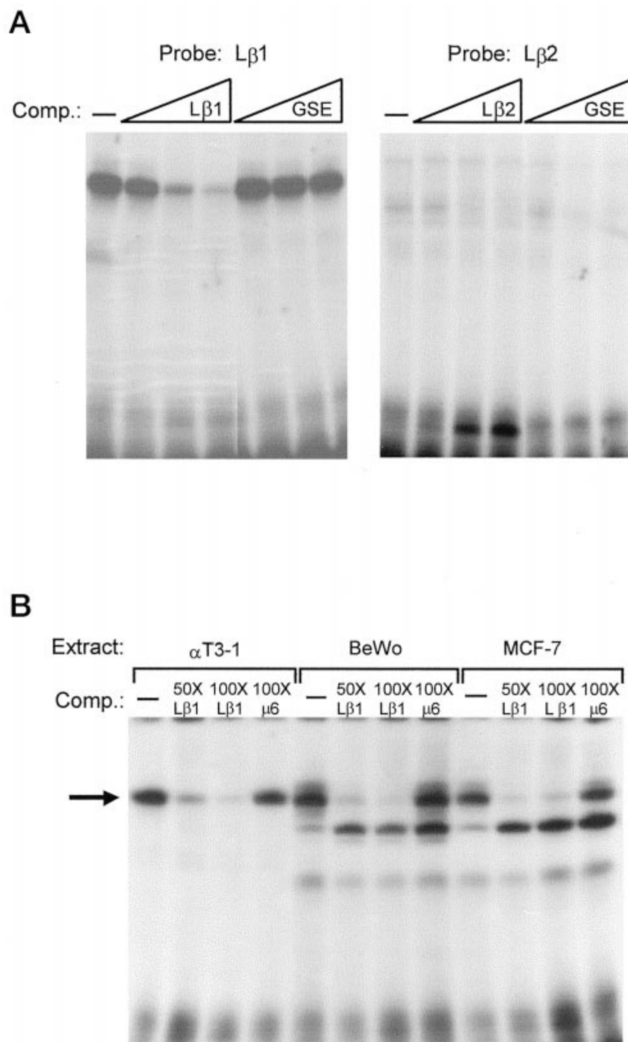

FIG. 3. **Lβ1 binds a nuclear factor that is common to a variety of cell lines.** *A*, EMSA were performed with either the Lβ1 or Lβ2 probes and αT3-1 nuclear extracts. A single complex was observed with the Lβ1 probe. To assess binding specificity, competition analyses were performed by adding 10-, 50-, or 100-fold molar excess of unlabeled, homologous Lβ1 or Lβ2 (as indicated) or heterologous GSE (21). Increasing concentrations of added oligodeoxynucleotides are indicated by *triangles*. *B*, EMSA were accomplished with nuclear extracts obtained from cell lines derived from gonadotrope (αT3-1), choriocarcinoma (BeWo), or mammary epithelium (MCF-7). The Lβ1 band is indicated by an *arrow*. Competition analyses were performed with unlabeled homologous Lβ1 or an Lβ1 mutant harboring a C/T transition at position 6 (see Fig. 4*A* for the Lβ1 and mutant sequence).

binding involved specific sequences eight nucleotides downstream of the CCAAT motif. To directly assess the importance of the C at position 6 of Lβ1, an additional point mutant was used in EMSA. This mutant was incapable of competing with protein binding to the wild type Lβ1 probe (Fig. 3*B*). Thus, both the initial C and the T of the CCAAT motif are essential for protein binding to Lβ1.

NF-Y Is the Lβ1-binding Protein—The requirement for sequences corresponding to a CCAAT motif suggested that the Lβ1 element might bind to a known CCAAT box-binding factor. To begin to determine the nature of the CCAAT-binding protein that interacts with Lβ1, we performed a variety of competition EMSA studies with consensus oligodeoxynucleotides and antisera to known CCAAT box-binding factors. Antiserum directed against NF-Y was capable of "supershifting" the Lβ1 protein band (Fig. 5*A*). NF-Y is a ubiquitous transcription factor that binds to CCAAT motifs. Thus, the protein that

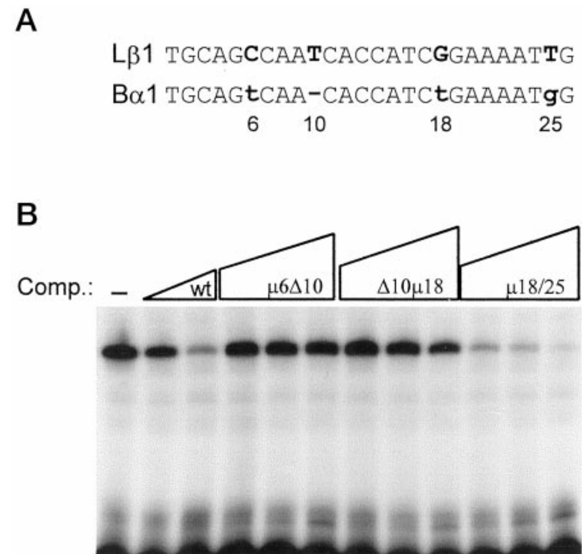

FIG. 4. **The Lβ1 element contains a CCAAT motif that is essential for protein binding.** *A*, alignment of the element 1 sequences from the bovine α (Bα1) and LHβ (Lβ1) promoters reveals only four nucleotide differences between the two that must dictate binding activity. These differences are indicated by *boldface letters*, and the position of each is denoted. *B*, EMSA with αT3-1 nuclear extracts and Lβ1 probe. Unlabeled Lβ1 competitor (*i.e.* wild type) was added at 10- and 50-fold molar excess. Unlabeled mutant Lβ1 oligonucleotide competitors were added at 50-, 100-, and 300-fold molar excess. The mutations within the Lβ1 oligodeoxynucleotides are indicated by the position of the mutation. At each of these positions, the sequence was converted from the LHβ promoter sequence to that present within the α promoter.

interacts with Lβ1 is either NF-Y or a highly related protein. Within the rat LHβ promoter, an Sp1 site that resides just 5′ to the NF-Y binding site was shown to be important for mediating GnRH regulation. No Sp1 consensus binding sequence exists in the bovine promoter at this site; however, to determine if Sp1 could also bind to the Lβ1 element, we performed additional EMSA. Antiserum to the transcription factor Sp1 did not affect binding of nuclear factors to the Lβ1 element but did retard the mobility of a complex formed with an oligodeoxynucleotide corresponding to a consensus Sp1 element (Fig. 5*A*). Thus, we conclude that NF-Y, but not Sp1, binds to this site in the bovine LHβ promoter.

The Lβ1 Element Exists as an Inverted Repeat in the Bovine LHβ Promoter—Subsequent analysis of the bovine LHβ promoter for additional CCAAT motifs surprisingly indicated that no motif was present in the region surrounding −80 bp relative to the start site of transcription. In contrast, as shown in Fig. 5*B*, an additional imperfect motif (CCTAT) was observed in the opposing orientation of the LHβ promoter at positions −332 to −328. This sequence, within the context of a larger oligodeoxynucleotide (designated Lβ4), was analyzed for its ability to bind a nuclear protein in EMSA (Fig. 5*A*). Interestingly, Lβ4 bound the same protein as Lβ1 as shown by complete cross-competition and antibody supershift experiments. However, Lβ4 bound this protein with significantly lower affinity. While only a 100-fold molar excess of unlabeled Lβ1 is required to compete for binding to the Lβ1 probe, over 500-fold molar excess of unlabeled Lβ4 is required for a similar level of competition (data not shown). It is intriguing that the inverted orientation of the Lβ4 and Lβ1 elements within the LHβ promoter is identical to the most active multimerized Lβ1 elements in transient transfection (Fig. 2). Inverted repeats of NF-Y sites have been identified in multiple genes, suggesting that these sequences may cooperate to lead to full expression of the LHβ promoter (35).

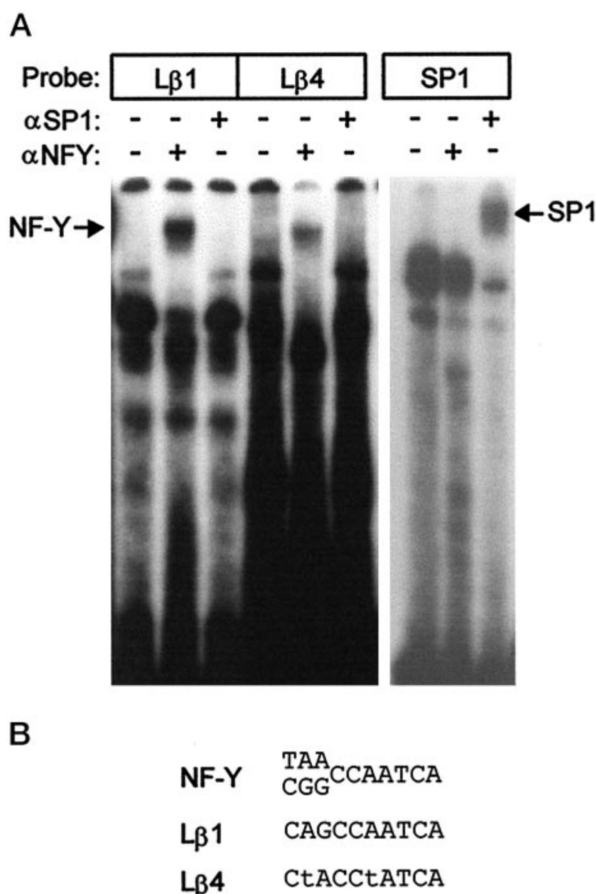

FIG. 5. **NF-Y is the Lβ1- and Lβ4-binding protein.** *A*, EMSA were performed with αT3-1 nuclear extracts and either Lβ1-, Lβ4-, or Sp1-labeled probes. Supershift assays were performed with antisera directed against NF-Y or Sp1 to assess the nature of the protein complexes associated with these oligodeoxynucleotide probes. Supershifted complexes containing either NF-Y or Sp1 are indicated with *arrows*. *B*, the Lβ1 and Lβ2 core CCAAT elements are aligned with the NF-Y consensus sequence (36). The Lβ1 site harbors an exact consensus sequence for NF-Y. *Lowercase letters* indicates mismatches from the consensus. The full sequences of the probes used in the EMSA are delineated under "Experimental Procedures."

The Lβ1 Element Regulates Pituitary-specific Expression of the Bovine LHβ Promoter—To determine the functional importance of the Lβ1 element in the context of a full-length promoter in an appropriate physiological context, transgenic mice were made. These mice harbored the 776-bp bovine LHβ promoter containing a transversion mutation throughout the entire 26-bp region of the Lβ1 element linked to CAT. Pituitary CAT activity in these mice was compared with that observed in mice harboring the wild type promoter linked to CAT. Previous studies have shown that the full-length, wild type promoter is highly active only in gonadotropes of transgenic mice (18). In addition, the promoter responds appropriately to GnRH and gonadal steroids (18). Pituitary CAT activity from six independent lines of mice harboring the mutant LHβ promoter was compared with activity observed in three independent lines of mice harboring the wild type promoter. Mutation of the Lβ1 element reduced activity of the LHβ promoter to approximately 14% of the wild type promoter's activity (Fig. 6). While this reduction was substantial, it is important to note that the Lβ1 mutant promoter had detectable activity in a number of mice. In particular, mutant lines 1, 3, and 4 displayed higher activity than the other lines of mice harboring the mutation. This suggests that while Lβ1 is important for high level expression of the LHβ promoter, some integration sites permit low level

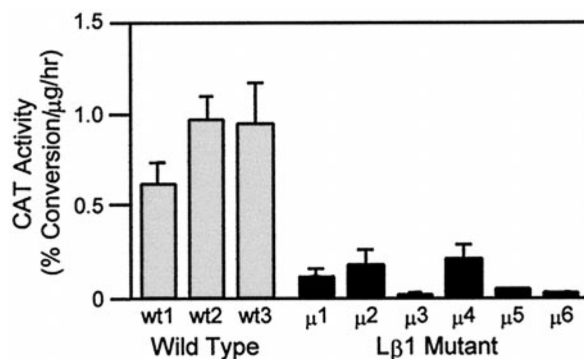

FIG. 6. **The Lβ1 element regulates pituitary-specific expression of the bovine LHβ promoter.** Transgenic mice harboring either the wild type (*wt*, *gray bars*) or an Lβ1 mutant (μ, *black bars*) LHβ promoter linked to CAT were made. Pituitary CAT activity was measured in three lines of mice with the wild type promoter and six lines of mice harboring the mutant promoter. Values are the mean ± S.E. The following numbers of animals were used: 29 (wt1), 44 (wt2), 11 (wt3), 12 (μ1), 5 (μ2), 8 (μ3), 6 (μ4), 1 (μ5), and 1 (μ6). The μ5 and μ6 animals were founder transgenics. In all animals in which liver activity was examined, it was below detection and similar to the levels of activity observed in μ3, μ5, and μ6 animals. Expression of the wild type transgene *versus* the mutant transgene was significantly different ($p \leq 0.0001$, two-tailed Student's *t* test assuming unequal variances).

expression from this attenuated promoter. Thus, the remaining *cis*-acting elements must compensate to some degree for the loss of the distal NF-Y binding site.

Lβ1 Is Unnecessary for GnRH Regulation of the LHβ Promoter—Numerous *cis*-acting elements have been described for the LHβ promoter, and most, if not all, mediate GnRH stimulation (11, 14, 16, 21, 24, 25). To determine the role of the Lβ1 element in conveying GnRH regulation, female transgenic mice harboring either the wild type promoter or the Lβ1 mutant promoter were subjected to an ovariectomy/antide treatment paradigm. Ovariectomy removes negative feedback from ovarian steroids and causes an increase in GnRH synthesis and secretion from the hypothalamus that further results in increased LHβ gene expression (1). Treatment with the GnRH-specific antagonist, antide, can block this increase (18). Thus, the degree of repression of transgene activity following antide administration is an indicator of the degree of promoter responsiveness to GnRH. To assess the impact of the Lβ1 mutation, mice were ovariectomized and treated with either vehicle or antide for 10 days. As previously shown (18, 21), antide treatment lead to a 38–63% reduction in wild type promoter activity in two independent lines of mice (Fig. 7). Similarly, antide treatment of ovariectomized mice harboring the Lβ1 mutation resulted in 50% reduction in promoter function. This indicates that while the basal function of the LHβ promoter is significantly attenuated by mutation of the Lβ1 element, this element is not necessary for mediating GnRH-regulated expression. Whether NF-Y can mediate GnRH regulation through the intact Lβ4 site or additional, uncharacterized, binding sites is unknown and warrants further study.

DISCUSSION

Expression of luteinizing hormone in the gonadotrope of the pituitary requires the coordinated expression of the α and LHβ subunits. Both subunit genes respond to GnRH and sex steroids to promote synthesis and secretion of appropriate levels of the intact heterodimer (1). Several elements have been defined for transcriptional control of both the α and LHβ subunit genes. However, a complete picture of LHβ gene regulation has not been resolved. This has largely been due to the lack of appropriate cell culture models to study expression of this gene.

Using transgenic mice, we and others have shown that the

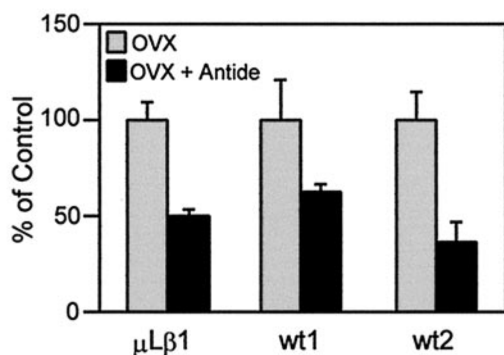

FIG. 7. **Lβ1 is unnecessary for GnRH regulation of the LHβ promoter.** Transgenic mice harboring either the wild type (*wt*) or mutant (*µLβ1*) (−776/+10) LHβ promoter were subjected to a GnRH regulation assay. The mutant line used in this study corresponds to µ1 in Fig. 6. Mice were ovariectomized and treated with either vehicle (*OVX*, *gray bars*) or 60 µg antide (*OVX + antide*, *black bars*) every 48 h. for 10 days. Pituitaries were collected, and CAT enzymatic activity was measured. Values are expressed relative to the OVX control for each line of mice and represent the mean ± S.E., $n = 5$ animals per group. In each case, antide treatment resulted in CAT activity that was significantly different from OVX animals ($p \leq 0.05$, one-tailed Student's t test assuming unequal variances).

LHβ promoter-proximal region confers high level expression that is confined to gonadotropes and responds appropriately to GnRH and gonadal steroids (17–20). In this report, we describe an inverted repeat element that binds to NF-Y and is important for full activity of the LHβ promoter in transgenic mice. In contrast to the other elements that have been described thus far for this promoter, the distal NF-Y binding site is not required for mediating GnRH responsiveness. Thus, two different classes of *cis*-active elements exist for the LHβ promoter. These include basal and GnRH-responsive sites, both of which contribute to transcriptional tone. Since expression of the LHβ gene is very low in the absence of GnRH (32), the so-called basal elements may play a role in amplifying a signal that must be initiated by GnRH acting through the promoter-proximal SF-1, Pitx1, and Egr-1 binding sites, as well as potential Sp1 sites.

NF-Y is a ubiquitous CCAAT box-binding factor that is composed of three subunits (36). It regulates both TATA-containing and TATA-less promoters (35). Functional NF-Y sites within promoters are usually closely associated with the start site of transcription, occurring within the promoter-proximal 100 bp (36). However, there are several genes that contain NF-Y binding sites that are over 1 kilobase pairs 5′ to the promoter (35). Both the Lβ1 and Lβ4 sites are located more distally than the typical NF-Y site. In addition, this factor displays an almost strict requirement for the pentanucleotide CCAAT motif that can occur on either the coding or noncoding strand (35, 36). While Lβ1 harbors an exact consensus for NF-Y, the Lβ4 site has a CCTAT motif on the opposite strand from Lβ1. Although the Lβ4 site also binds NF-Y, it does so with lower affinity than Lβ1 (data not shown). The Lβ4 motif has been observed in the *cdc25* gene (37) as well as the MHC Class II *DPA* gene (38). Interestingly, these sequences, like Lβ4, all occurred in the reverse orientation on the coding strand. Unlike the LHβ subunit gene, however, both of these genes are TATA-less.

There are numerous examples of NF-Y cooperativity with other transcription factors (39–41). It is particularly interesting to note that NF-Y can synergize with the transcription factor Sp1 (39, 41). An Sp1 site has been characterized within the rat LHβ promoter that is immediately adjacent to a conserved NF-Y sequence (11). Whether these two proteins synergize to activate the rat promoter remains to be determined. With regard to LHβ promoters from other species, the Sp1 sequence is not conserved at this site, although the adjacent NF-Y sequence is. Within the bovine LHβ promoter, we have shown that an oligodeoxynucleotide that encompasses the NF-Y site fails to bind Sp1. Thus, the transcriptional regulation that occurs through this *cis*-acting element cannot be attributed to Sp1. It will be important to determine if Sp1 consensus sequences located within other regions of this promoter can bind to Sp1 and potentially synergize with the NF-Y binding sites to activate transcription.

Sp1 and the other factors that define transcriptional activity of the LHβ promoter have largely been characterized using heterologous cell culture systems. These include the use of kidney fibroblasts (CV-1) (8, 9, 22), somatotropes (GH3 and GGH3–1′) (10, 11, 24), and immature gonadotropes (αT3-1) (13–15). None of these cell lines express the endogenous LHβ subunit gene, and they are probably devoid of essential factors for activating its transcription. While these models have allowed ascertainment of the ability of specific transcription factors to induce the LHβ promoter, most depended upon overexpression of those factors. Thus, the relative importance of these factors in defining the transcriptional tone of the LHβ promoter within the mature gonadotrope is unknown. The recently derived LβT2 cell line, which does express the endogenous LHβ subunit gene (42), will probably present an ideal avenue to confirm studies previously performed in these alternative models. Prior to development of this cell line, however, the only mature gonadotrope model effectively used for analyses of the LHβ promoter was the pituitary of transgenic mice. Even with this cell line, the transgenic mouse presents the most physiologically relevant system to assess important factors in regulating expression of the LHβ gene. In this regard, we have characterized a new site that is important for basal expression of this gene but not GnRH induction. The function of this element contrasts with the previously defined GSE that was shown to be essential for pituitary activity as well as GnRH activation of this promoter in transgenic mice (21). While previous *in vivo* and *in vitro* studies have focused specifically on the promoter-proximal 150 bp, the studies presented herein indicate that additional sequences involved in regulation of promoter activity reside upstream of this region. Thus, any attempts to study the proximal promoter in isolation will probably result in a limited picture of the transcription control of this gene.

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