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Cloning and Characterization of a 5′ Regulatory Region of the Prolactin Receptor-Associated Protein/17β Hydroxysteroid Dehydrogenase 7 Gene

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Prolactin receptor-associated protein (PRAP) originally cloned in our laboratory was shown to be a novel, luteal isoform of 17β hydroxysteroid dehydrogenase 7 (17βHSD7). In this study, we cloned the promoter region of rat PRAP/17βHSD7 and investigated the mechanisms regulating both basal activity and LH-induced repression of this promoter. Truncated and site-specific mutants of PRAP/17βHSD7 promoter identified two enhancer regions that contained highly conserved Sp1 binding site and bound Sp1 from nuclear extracts of both corpora lutea and a rat luteal cell line. Repression of PRAP/17βHSD7 expression and promoter activity by human chorionic gonadotropin/forskolin was localized to a −52-bp proximal segment of the promoter. This region contained a conserved CCAAT site and bound nuclear factor Y; binding of this transcription factor was inhibited by human chorionic gonadotropin *in vivo*. Furthermore, mutation of the nuclear factor Y site in the −52-bp promoter-reporter construct abolished forskolin-mediated inhibition of the promoter in a rat luteal cell line. In summary, we have identified the promoter elements involved in the basal expression of PRAP/17βHSD7. We have also found that LH-mediated repression of this gene is at the level of transcription and involves inhibition of nuclear factor YA binding to the CCAAT site within the proximal promoter. (*Endocrinology* 146: 2807–2816, 2005)

THE CORPUS LUTEUM (CL) is the sole source of progesterone throughout gestation in rodents and thus is critical to the maintenance of pregnancy. Survival of this gland is dependent on two hormones: prolactin (PRL) (reviewed in Ref. 1) and estradiol (2, 3). PRL and rat placental lactogens, which act through the PRL receptor, are provided by the anterior pituitary and placenta, respectively (4–6), whereas estradiol is generated by the CL of pregnancy itself (3, 7–9). In early pregnancy, rodent CL can synthesize estradiol *de novo*; however, during the second half of pregnancy, androstenedione from the placenta serves because a substrate for luteal estradiol production (10, 11). The production of estradiol from androstenedione is accomplished by the activity of two enzymes: P450 aromatase and 17β hydroxysteroid dehydrogenase (17βHSD). The 17βHSD isozyme involved in estradiol production by the follicle is 17βHSD1. This enzyme has dual activities (12, 13). It converts androstenedione to testosterone, which is then transformed into estradiol by P450 aromatase. It can also directly convert estrone to estradiol. It is highly expressed in the granulosa cells but disappears totally from these cells after luteinization and remains undetectable in the CL (13). The absence of 17βHSD1 in the CL was puzzling because rodent luteal cells produce estradiol (12, 13). Recently, the 17βHSD isozyme involved in estradiol production by the CL was found to be a protein first identified in our laboratory as PRL receptor-associated protein (PRAP) (14, 15).

PRAP is a 32-kDa protein that associates with the cytoplasmic domain of the short form of the PRL receptor (16) and is expressed at high levels in the CL in the rat (17, 18), specifically in the large luteal cells (19). PRAP has been identified as a novel isoform of 17βHSD (PRAP/17βHSD7). This enzyme is a potent converter of estrone to estradiol (14, 15) but, in contrast to 17βHSD1, it does not convert androstenedione to testosterone. PRAP/17βHSD7 has been found in CL of every species investigated to date (18) and has been cloned from the rabbit (21), marmoset monkey (22), and human (23), in addition to the mouse (14) and rat. The presence of this enzyme in the CL reveals the novel possibility that the CL in general has the capacity to convert estrone and to synthesize estradiol. To date, 11 different isozymes of 17βHSD have been cloned; however, the only isozymes known to metabolize estrone to estradiol are 17βHSD1 and PRAP/17βHSD7.

Abundant expression of PRAP/17βHSD7 by the rat CL begins around mid-pregnancy (17, 18), coincident with a drop in LH levels (5). Experimentally, LH has been shown to decrease PRAP/17βHSD7 expression, whereas estradiol has been shown to increase PRAP/17βHSD7 mRNA levels (17, 24, 25). Down-regulation of PRAP/17βHSD7 expression in

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Abbreviations: 17βHSD, 17β Hydroxysteroid dehydrogenase; CHO, Chinese hamster ovary; CL, corpus luteum; DMSO, dimethylsulfoxide; ERE, estrogen response element; hCG, human chorionic gonadotropin; NF-Y, nuclear factor Y; PRAP, prolactin receptor-associated protein; PRL, prolactin.

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the CL by LH is in marked contrast to the stimulatory effect of this hormone on transcription of 17βHSD1 in granulosa cells (13) and the drop in LH at mid-pregnancy thus results in a change in the primary ovarian source of estradiol, from follicular granulosa to luteal cells. Tight regulation of estradiol production during pregnancy is essential, as fetal survival in the rodent is negatively affected by high levels of estradiol (26, 27). Increased expression of PRAP/17βHSD7 also coincides with the transition from ovarian-derived androgens to placental-derived androstenedione as the substrate for luteal estradiol production (10, 11). At the end of pregnancy, beginning around d 18 of gestation, LH levels rise (5) and PRAP/17βHSD7 mRNA expression drops (17, 24).

The consistent luteal expression of PRAP/17βHSD7 gene across species, and its tight regulation by hormones critical to luteal function, led us to examine transcriptional elements involved in basal and hormonal-regulated expression of this gene. We initially determined the 5′ untranslated region of the PRAP mRNA transcript and cloned a 1.2-kb fragment of genomic DNA 5′ to the transcription start site. Sequence comparison between the rat and human 5′ genomic regions of PRAP/17βHSD7 along with EMSA and promoter-reporter studies led to the discovery of conserved enhancer regions in this promoter, as well as the DNA binding site mediating down-regulation by LH.

Materials and Methods

Animal model and tissue preparation

Timed pregnant Sprague Dawley rats were obtained from Sasco Animal Labs (Wilmington, MA) and kept under conditions of controlled light (0500–1900 h) and temperature (22–24 C) with free access to standard rat chow and water. Rats were injected ip with either saline or 40 IU/0.5 ml human chorionic gonadotropin (hCG) in saline twice per day (0800 and 1800 h) on d 12 and 13 and at 0800 on d 14 of pregnancy. Rats were killed at 1500 h on d 14, and CL were dissected from the ovaries under a stereoscopic microscope. CL were frozen in liquid nitrogen and stored at −80 C until processing for RNA or protein extraction. All experiments were conducted in accordance with the principles and procedures of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

5′ Rapid amplification of cDNA ends (5′ RACE)

RACE was performed using the 5′ RACE System, Version 2.0 (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 1 μg of total mRNA isolated from the corpora lutea of d 15 pregnant rats was converted to cDNA through reverse transcription. This cDNA was then capped at its 5′ end with poly-C using terminal deoxytransferase. A primer that recognizes a portion of the PRAP cDNA (GAGGTTTAGCTGGGGGTTAGG) was used in a PCR with a poly-G primer to isolate the region of PRAP cDNA 5′ to nucleotide 303 of the coding portion. This PCR product was then cloned and sequenced to determine the PRAP 5′ UTR sequence

Primer extension

Primer extension was performed using the avian myeloblastosis virus reverse transcriptase primer extension system (Promega, Madison, WI). Once again, RNA from d 15 pregnant rat CL was used. A PRAP-specific primer (GAGGTTTAGCTGGGGGTTAGG) was labeled with ^{32}P via a T4 polynucleotide kinase and then purified and analyzed for labeling efficiency. This radiolabeled primer was then hybridized to luteal RNA and extended using reverse transcriptase. The size of this product was compared with sequencing products of known size using SDS-PAGE and autoradiography.

PRAP/17βHSD7 promoter cloning

The region of genomic DNA 5′ to the transcription start site was cloned using the Promoter Finder DNA walking kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol. Two primers specific to the 5′ untranslated region of PRAP/17βHSD7 were used in a two-step nested PCR with two primers to the cap region provided by the kit. The largest piece, about 1.2 kb in size, was cloned and sequenced (sequence shown is consensus of three independent clones). The promoter clone (−1166 to +73) was subcloned from the sequencing vector into the *Kpn*I and *Bgl*II sites of the pGL3 basic luciferase reporter vector (Promega). The −369-bp promoter was created by digesting the initial construct with *Hin*dIII and cloning the resulting truncation into the *Hin*dIII site in the pGL3 basic luciferase reporter vector.

The −368, −185, −115, and −52 PRAP promoter constructs were generated via PCR using the Advantage PCR kit (Clontech), primers adding a *Bgl*II site (5′) and a *Hin*dIII site (3′) (Table 1; 10 pmol of each primer per reaction), and the full-length PRAP promoter as a template (100 ng per reaction). The reaction underwent 4 cycles of 25 sec at 94 C and 4 min at 72 C, followed by 16 cycles of 25 sec at 94 C and 4 min at 65 C, and last, 67 C for 4 min. The PCR products were extracted from a 0.7% agarose gel using the GeneClean kit (Q.BIOgene, Irvine, CA), and digested with *Bgl*II and *Hin*dIII. The digested products were again purified from a 0.7% agarose gel using the GeneClean kit and ligated into pGL3 basic plasmid digested with *Bgl*II and *Hin*dIII, using T4 DNA ligase.

The −1165 to −369 and −1165 to −804 constructs were made using the *Kpn*I site in the sequencing vector along with an internal *Hin*dIII site or *Hin*cII site, respectively, and were cloned into the pGL3 promoter vector using the *Kpn*I and *Hin*dIII or *Sma*I sites, respectively. The −369 to −109 pGL3 promoter construct was generated by PCR as described above; the primers used to create this construct were: 5′-CTG CTG GCT AGC AAG CTT TAG AAA AGG ATA GGA-3′ and 5′-CTG CTG GAG TCT CGC CCA CAC CGG ATG AGG TGA-3′. *Nhe*I and *Bgl*II sites were added at the 5′ and 3′ end of the PCR product, respectively. All constructs were confirmed by restriction enzyme digestion and DNA sequencing.

Cell culture and transfection

RCLP cells, a cell line derived from a primary culture of rat luteal cells isolated at d 16 of pregnancy, were cultured in medium 199 (Cellgro/Mediatech, Herndon, VA) containing 10% FBS (Hyclone Labs, Logan, UT) and 2× antibiotic/antimycotic solution (Mediatech). Chinese hamster ovary (CHO) cells were cultured in DMEM/F12 (Sigma-Aldrich, St. Louis, MO) containing 10% FBS and 2× antibiotic/antimycotic solution. COS1 cells were cultured in DMEM (Sigma-Aldrich) containing 10% FBS and 2× antibiotic/antimycotic solution. For transfection, cells were plated at 40–60% confluency in six-well plates the afternoon before transfection. Cells were transfected using LIPOFECTAMINE or LIPOFECTAMINE 2000 (Life Technologies) according to the manufacturer's protocol. The cells were transfected with the appropriate PRAP/17βHSD7 promoter constructs and β-galactosidase expression vector to control for transfection efficiency, each at 0.5 μg per well. Treatments

TABLE 1. Sequence of the oligonucleotides used in gel shift analysis

Probe name	Probe sequence
−156 to −123	GTGTGGCTCCGCCCCTCGGCCTATCTCACCTCAT
mGATA (−156 to −123)	GTGTGGCTCCGCCCCTCGGCCTAAGTCACCTCAT
mSp1 (−156 to −123)	GTGTGGCTCCGTGCCTCGGCCTATCTCACCTCAT
−68 to −35	GGAACGGATGACGCAGGAGAGGTGGGGCCTGGTT
mSp1 (−68 to −35)	GGAACGGATGACGCAGGAGAAATGGGGCCTGGTT
−40 to −11	CTGGTTGAGCCTTGATTGGTCGGGGTGCGC
mPbx-1 (−40 to −11)	CTGGTTGAGCCCTCATTGGTCGGGGTGCGC
mCAAT (−14 to −11; gel shift)	CTGGTTGAGCCTTGATTGATGGGGGTGCGC
mCAAT (−14 to −11; mutagenesis)	CTGGTTGAGCCTTGCGCAGTCGGGGTGCGC

with dimethylsulfoxide (DMSO) or forskolin (100 μM; Sigma-Aldrich) began immediately after transfection, for 6 h.

Luciferase activity

Luciferase and β-galactosidase activities were measured in cell lysates prepared in 1× reporter lysis buffer (Promega) using a luminometer (Lumat LB 9507 luminometer; EG&G Berthold, Oak Ridge, TN). Luciferase activity was measured using a firefly luciferase assay system (Promega) and β-galactosidase activity was measured using a luminescent β-galactosidase system (Clontech), according to the respective manufacturer instructions. Luciferase activity was normalized to β-galactosidase activity for each lysate.

EMSA

Nuclear extracts from both luteal tissue and cultured cells were prepared as described by Dignam *et al.* (28) with modifications. Cells or tissue were homogenized in approximately five packed cell volumes of solution A (10 mM HEPES-KOH, pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.5 mM dithiothreitol) with a Dounce homogenizer and the nuclear pellet obtained by centrifugation at 12,000 × *g* for 30 sec. The pellet was then resuspended in 50–100 μl of solution B (20 mM HEPES-KOH, pH 7.9; 0.42 mM NaCl; 1.5 mM $MgCl_2$; 25% glycerol; 0.2 mM EDTA; 0.5 mM phenylmethylsulfonylfluoride; 0.5 mM dithiothreitol) and rocked vigorously for 20 min at 4 C. This was then centrifuged for 20 min at 14,000 × *g* and the supernatant containing the nuclear extract was then dialyzed in 50 vol of 1× binding buffer (20 mM HEPES-KOH, pH 7.9; 0.1 M KCl; 20% glycerol; 0.2 mM EDTA; 0.5 mM phenylmethylsulfonylfluoride; 0.5 mM dithiothreitol) for 5 h. Protein concentration in nuclear extracts was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Five picomoles of each annealed oligonucleotide probe (Table 1) were labeled using 10 U of T4 polynucleotide kinase (Life Technologies) and 25 μCi of γ-labeled ^{32}P ATP (Amersham, Piscataway, NJ) to a specific activity of more than 8000 cpm/fmol. Five micrograms of nuclear extract were incubated with 1 μg of poly(dI-dC) and 50 fmol of probe in 1× binding buffer on ice for 30 min. Cold competitor probes were added to a final concentration of 2.5 pmol, and antibodies in supershift studies were used according to the manufacturer's protocol. Antibodies to Sp1 (sc-59, sc-420), GATA-6 (sc-7244), and nuclear factor YA (NF-YA)/CCAAT-binding factor-B (sc-7712) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Samples were run on a 4% nondenaturing polyacrylamide gel in 0.5× Tris borate EDTA buffer at 200 V for 2–3 h. The gels were then dried and analyzed by autoradiography.

Mutation of PRAP/17βHSD7 promoter

Mutations to the promoter were made via PCR using the QuikChange or QuikChange II site-directed mutagenesis kits (Stratagene, La Jolla, CA) according to the manufacturer's protocol. All mutations were made using the probes shown in Table 1 as primers in the PCR or as indicated, and the −185 or −52 PRAP/17βHSD7 promoter in pGL3 basic was used as the template. The presence of correct mutations was confirmed through DNA sequencing.

RNA extraction and semiquantitative PCR

RNA was extracted using TRIzol reagent (Life Technologies) following the manufacturer's protocol. Luteal RNA was transformed into cDNA via reverse transcription. Custom oligonucleotide primers based on the ribosomal L19 cDNA sequence (5′-CTG AAG GTC AAA GGG AAT GTG-3′, 5′-GGA CAG AGT CTT GAT GAT CTC-3′) and the PRAP cDNA sequence (5′-AAT TAT GTC AAG GGC CAA AAG ATG-3′, 5′-CCT CGC TGG GAC TAA AAG AAG ATT-3′) were obtained from Life Technologies and used to amplify the appropriate cDNA templates by PCR. Conditions for each template were optimized so that signals were in the linear range of detection. The PCR products with DNA loading buffer were then separated by gel electrophoresis on a 0.7% agarose gel. L19 concentrations were used as internal control for comparison.

Statistical analysis

One-way (Figs. 2, 3, and 5) or two-way (Figs. 6 and 9) ANOVA was performed on each data set, followed by the Bonferroni multiple comparisons post test.

Results

Cloning and sequencing of the PRAP/17βHSD7 promoter

To clone the luteal promoter for PRAP/17βHSD7, the 5′ end of exon 1 of PRAP/17βHSD7 was determined by two methods, 5′ RACE and primer extension. The RACE clones sequenced were of two different sizes, 64 and 67 bases, with the sequences being identical in all except for the addition of three bases found in only a portion of the clones [5′-(ATC) GTG TTC TGG CTG AGA GCT GCT GAG AAC CGT GGA CGG GGT GGA ACT CCG CTG CAA GGC GTG AAT G-3′]. This suggested two neighboring transcription start sites, and this fact as well as the length of the 5′ UTR was confirmed by primer extension.

The sequence obtained by 5′ RACE was used to make primers and clone an 1165-bp fragment 5′ to the distal PRAP/17βHSD7 transcription start site via a PCR-based method, and this sequence is shown in Fig. 1. This promoter does not contain a classical TATA box; however, the sequence surrounding the more distal start site resembles an initiator sequence (29), which may be involved in transcription initiation. Furthermore, sequence analysis found a number of putative transcription factor binding sites; however, it contained neither a consensus estrogen response element (ERE) nor a typical cAMP response element, which are the sites most frequently associated with estrogen and LH signaling, respectively. It did, however, find three ER half sites, which may allow for estrogen signaling in association with other transcription factors, such as Sp1 (30) or AP-1 (31). This sequence has been submitted to GenBank (accession no. AY390340).

PRAP/17βHSD7 promoter analysis in culture

We have previously demonstrated that PRAP/17βHSD7 is expressed almost exclusively in the CL in the rat. LH inhibits the expression of PRAP/17βHSD7 in the rat CL during early pregnancy and removal of LH inhibition at approximately d 11 of pregnancy, when circulating LH becomes undetectable, results in production of large amounts of PRAP/17βHSD7 by luteal cells (17, 18). Indeed, during the second half of pregnancy, PRAP/17βHSD7 becomes the most abundant protein in the rat CL (19). Therefore, we set out to determine the promoter elements involved in basal expression as well as LH-mediated repression of PRAP/17βHSD7. The full-length 1166-bp PRAP/β17HSD7 promoter was cloned into a luciferase reporter plasmid. A shorter promoter-reporter construct was generated using an internal *Hin*dIII site at −369. These constructs were transfected into RCLP cells, a rat luteal cell line, and analyzed for promoter activity (Fig. 2A). In RCLP cells, the −1166 and −369 promoter constructs contained similar high basal levels of transcriptional activity indicating that elements responsible for basal activity of the promoter were proximal to −369.

Due to the lack of convenient restriction sites, smaller PRAP promoter constructs were generated through PCR-

-1166 CGACGGCCCG GGCTGGTAAA AAGCGAGCAT TTTTTCCGCT
-1126 GCTCTTTTGC AGCCACTTC**T GACC**TTCCAG GGCTTAAAAG
-1086 GATCATCTGG GCATGGAATT CTCAAGGTGG AGGATTCTTT
-1046 ATTTTTCACT CCAAGGCTTT TAGGAAATGT GTTGTTACCA
-1006 TCTTTGACGA ACAGCTGAAA TGATCAGTTT TCTTAAGTTT
-966 TAGCAGTTCG TTGGTGTCAT TAATCGAAAC ATATCGATTA
-926 AAGTCCAGAA AAGGTGTTAT CAGTCATTTA GGATCACAGC
-886 TATTCATCGT TAAGTACAGC TTTGAAAGAG GCATAAAGTT
-846 TGTTCAGTCA CCCTATGTAT TAAGTTCGTT CACTCAA**GGT**
-806 **CA**ACTGTAAG TAGCGAAAGC TGGAATAACG ATTAACAGCT
-766 GTGCGGGATA CTTTTATGTG AAGTGAATTT AATGAAAATT
-726 GGGAGTTTTG TATGCACTTG CCCTTCCCTC TCTCAATACC
-686 TAGCCTCACC CCTTCGGAAG CCTTTATCTG GGATAGTTTG
-646 GCGTTCCCCC CCCCCACCAA ACCAAACCAA AACGAAAAAC
-606 AACAACAACA ACAACAACAA CAACAACAAA AACCAAAAAA
-566 ATAAAACAAA AAGCAACCAA CCAAACAAAA AACATTACAG
-526 CAGCCTGATA GGCACTGAAG GAGATGACAG TTCCCATTCC
-486 TTTGGAGTTT TGAGATTCTG GTTTTCATTC CACACTCAGA
-446 GACTGAAATT GCTAATTTAC CAGTGACTCG ACATAAATAA
-406 AAGTCAATGA AATCCACGTT CTCTTTCTCA AAGACGAAGC
-366 TTTAGAAAAG GATAGGAAGG TGGATCTACA CGCCCCAAGG
-326 AGCCACCTTT GACTTGGATT CCTGCAGCCC AGCCTCTCCC
-286 TGGGAAAACC CGGCTGAGCC AGGCTCGCCT CACTCAGAAC
-246 AAAGACTCCG CCCTACAGTG ACACCCACTG CCCGCTGGCT
-206 TGAGCAGACT GGGGGCGGGG ACTTCCTGGA TTGGCTAGGG
-166 CTCCTGGGTC GTGTGGCTCC GCCCCTCGGC CTATCTCACC
-126 TCATCCGGTG TGGGCGTGGA GAGAAGACGC CGAGTTGCGA
-86 TTGGCGGGCC CAGCGCGGGG AACGGATGAC GCAGGAGAGG
-46 TGGGGCCTGG TTGAGCCTTG ATTGGTCGGG GTGCGCGT**GG**
-6 **TCA**GGG
+1 ATCGTGTTCT GGCTGAGAGC TGCTGAGAAC CGTGGACGGG
+41 GTGGAACTCC GCTGCAAGGC GTGAATGATG

FIG. 1. Nucleotide sequence of the PRAP/17βHSD7 promoter. The Sp1 sites at −151 to −142 and −48 to −43 and the NY site at −26 to −21 are *underlined*. The three estrogen receptor half sites discussed in the text are shown in *bold*.

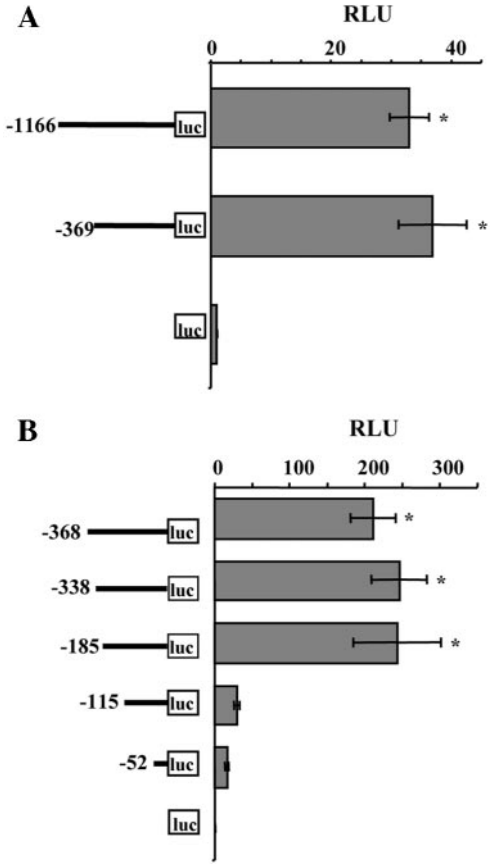

FIG. 2. RCLP cells were transfected with full-length or truncated forms of the PRAP promoter, linked to a luciferase reporter, or with empty luciferase vector. Luciferase activity of each sample was normalized to concurrently transfected β-galactosidase activity and expressed as relative luciferase activity (RLU). Results are mean ± SEM for three independent experiments performed in triplicate. A, Basal activity of the PRAP promoter is not affected by truncation to −369 bp. Cells were harvested 6 h after transfection. *Asterisks* denote $P < 0.01$ relative to pGL3 basic. B, Basal activity of the PRAP promoter is mediated through an enhancer region between −185 and −115 bp. Cells were harvested 24 h after transfection. *Asterisks* denote $P < 0.01$ relative to −115 PRAP promoter.

based cloning, and these constructs were transfected into RCLP cells. These shorter constructs had higher promoter activity (compare −369 in Fig. 2A to −368 in Fig. 2B) presumably resulting from the loss of the translation start codon during PCR derivation. As shown in Fig. 2B, PRAP/17βHSD7 promoter activity was greatly diminished with the deletion of the region between −185 and −115, demonstrating the existence of a major enhancer(s) of basal (in the absence of hormonal stimulation) activity in this region.

To examine the specificity of this enhancer, segments of the PRAP/17βHSD7 were placed in the pGL3 promoter vector, in which luciferase expression is under control of an SV40 promoter. These constructs were transfected into different cell types to examine their effect on transcription of a heterologous promoter. Two segments upstream of the putative enhancer region were generated, −1166 to −369 and −1166 to −804, whereas a third segment, −368 to −110, contained the putative enhancer region. In RCLP cells, the sequence from −368 to −110 possessed enhancer activity, whereas more 5′ segments of the promoter did not (Fig. 3A). These constructs were then transfected into another ovarian cell line (CHO) as well as a kidney cell line (COS) to determine whether this enhancer activity was specific to luteal cells (Fig. 3, B and C). Although the stimulation was not quite as high in these two cell lines, the −368 to −110 region still caused 2.5- to 3-fold greater activity in these cells relative to the SV40 promoter alone. The other constructs again had no enhancer activity in these cells. In summary, these experiments revealed that the identified enhancer region in the PRAP/17βHSD7 promoter mediates enhancer activity in a variety of cell types, although the increased activity in RCLP cells (~4.5-fold over promoters not containing the region) *vs.* the other cell types (2- to 3-fold increase) suggests some regulatory component specific to luteal cells.

Analysis of the enhancer region by EMSA

To determine the transcription factor(s) involved in the enhancer activity, we first identified a highly conserved region within the −185- to −115-bp fragment. Comparative

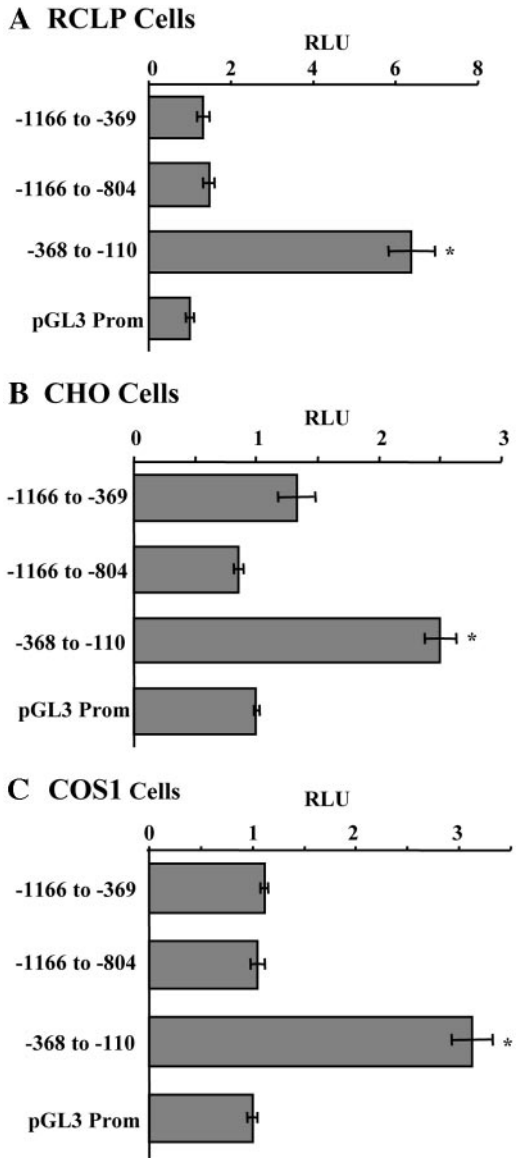

FIG. 3. Characterization of the enhancer region of the PRAP promoter. A, RCLP cells were transfected with different PRAP promoter constructs or pGL3 promoter vector and β-galactosidase expression vector. Relative luciferase activity (RLU) was determined by normalizing luciferase activity to β-galactosidase activity for each sample, and the results are the mean + SEM for three independent experiments performed in triplicate. B and C, Cells were transfected and analyzed as in A except in CHO cells and COS1 cells, respectively. *Asterisks* in A–C indicate $P < 0.001$ relative to pGL3 promoter.

analysis demonstrated that a region from −156 to −123 within this fragment of the rat promoter is conserved between the rat and the human 17βHSD7 promoters (23). This 33-bp piece contains putative response elements for both the GATA and Sp1 families of transcription factors.

Because PRAP/17βHSD7 protein becomes abundant around the time of mid-pregnancy (17), nuclear extracts were made from the CL of d 14 pregnant rats and from RCLP cells and were analyzed via EMSA with a probe consisting of the −156- to −123-bp promoter fragment. The results for these two cell types were identical, and only results for RCLP cells are shown in Fig. 4A. Both CL and RCLP cell nuclear extracts contained protein(s) that bound to the −156 to −123-bp sequence, forming three specific bands (Fig. 4A, lane 2). Binding was competed with wild-type cold probe (lane 3) and with a probe mutated at the putative GATA site (lane 4), but not with a probe mutated at the Sp1 site (lane 5). Addition of an Sp1 antibody to the probe/nuclear-extract mix (lane 8) led to the formation of a supershift (*vs.* lane 7), and this supershift mainly detracted from the intensity to the uppermost band. In contrast, an antibody to GATA-6 failed to supershift the DNA/protein bands (data not shown).

Sp1 is a common transcription factor that has been shown to regulate transcription of steroidogenic enzymes (32, 33) and this prompted us to look for other sites of regulation by Sp1 in the rat PRAP/17βHSD7 promoter. Comparison of the rat promoter to the human 17βHSD7 promoter revealed another portion, from −68 to −35, that was conserved between the two species and that also contains a proximal Sp1 response element. An EMSA probe was made of this region of the rat PRAP/17βHSD7 including the putative proximal Sp1 site and analyzed for binding activity with RCLP and CL nuclear extracts. Again, the results for these two cell types were identical and the results for RCLP cells only are shown in Fig. 4B. Nuclear extracts of RCLP cells formed complexes with the −68 to −35 probe similar to those formed using the −156 to −123 probe and these were supershifted with Sp1 antibody (lane 6).

Effect of Sp1 mutations on PRAP/17βHSD7 promoter activity

To assess the effect of the two identified Sp1 binding sites on basal promoter activity, mutations were made of either the proximal, distal, or both Sp1 binding sites in the −185-luc promoter construct and these constructs were then transfected into RCLP cells (Fig. 5). Mutation of the distal Sp1 binding site alone led to a 50% decrease in basal promoter activity. Whereas mutation of the proximal Sp1 site had a minor effect on basal activity, only mutation of the distal Sp1 or both Sp1 sites resulted in a clear inhibition of the promoter. These results demonstrate an important role for Sp1 in enhancing the basal promoter activity of PRAP/17βHSD7 and indicate that the distal Sp1 site is a critical mediator of this effect.

Regulation of the PRAP/17βHSD7 promoter by LH

Previous studies in our laboratory demonstrated that LH has an important role in inhibiting the expression of PRAP/17βHSD7 in early pregnancy, and that the drop in LH at midpregnancy allows PRAP/17βHSD7 mRNA expression in the rat CL (17, 25). To look at possible mechanisms of repression by LH, we transfected RCLP cells with the −368, −115, and −52 PRAP/17βHSD7 promoter constructs. Because RCLP cells do not respond to LH/hCG, we used forskolin, which directly activates adenylyl cyclase, to mimic the increase in cAMP caused by LH. As shown in Fig. 6, all PRAP/17βHSD7 promoter-reporter constructs were inhibited by forskolin, whereas the pGL3 basic construct was not. This indicates that the necessary element for response to cAMP-mediated inhibition is contained in the proximal 52 bp of the PRAP promoter.

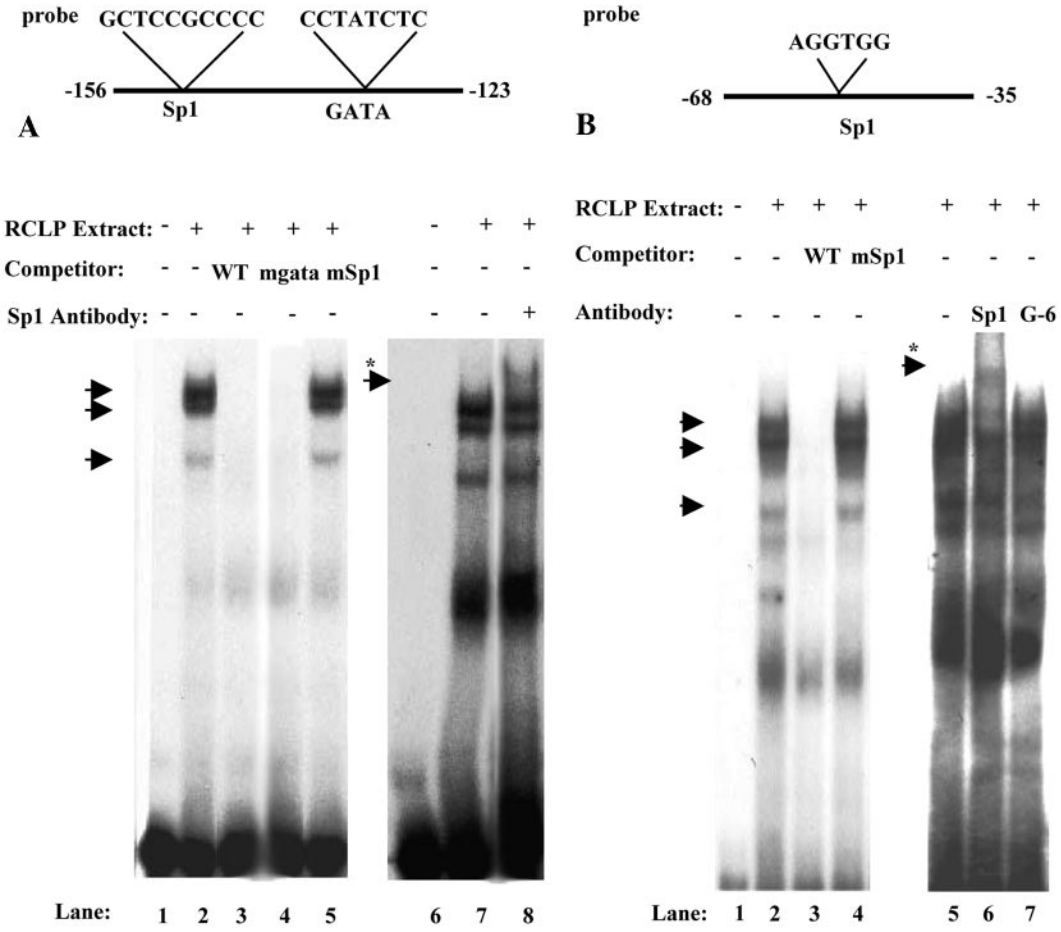

FIG. 4. EMSA was performed using nuclear extracts from RCLP cells. Lanes 1 and 6 (A) and 1 and 5 (B) represent free probe. *Arrows* designate complexes of interest (see text); *asterisk* designates complex formed by antibody. Gels are representative of three independent experiments. A, Sp1 binds the −156 to −123 region of the PRAP/17βHSD7 promoter. Formation of DNA-protein complexes is illustrated in lane 2. Competition with 50× cold wild-type probe (lane 3) and 50× cold probe with a mutated putative GATA binding site (lane 4) eliminated these complex, but they were not eliminated by competition with 50× cold probe containing a mutated Sp1 site (lane 5). Furthermore, a portion of this complex (lane 7) was supershifted with an Sp1 antibody (lane 8). B, Sp1 binds the −68 to −35 region of the PRAP/17βHSD7 promoter. Formation of DNA-protein complexes is shown in lane 2. These complexes were eliminated by competition with 50× cold wild-type probe (lane 3) but not by competition with 50× cold probe containing a mutated Sp1 site (lane 4). A portion of this complex was supershifted with antibody to Sp1 (lane 6), but not by antibody to GATA-6 (lane 7).

Analysis of the conserved inverted CCAAT sequence

The proximal 52 bp of the PRAP promoter was examined for potential conserved transcription factor binding sites, and this analysis yielded a region from −40 to −11 highly conserved between the human, mouse, and rat 17βHSD7 promoters that contains an inverted CCAAT sequence as well as

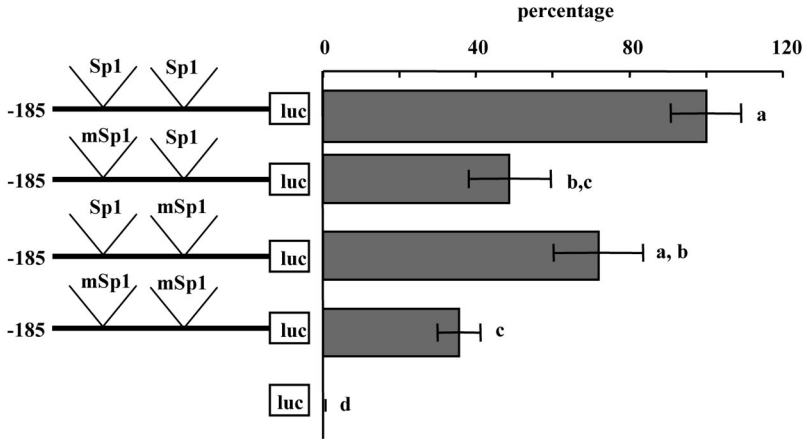

FIG. 5. Effect of mutation of the putative Sp1 binding sites on PRAP/17βHSD7 promoter activity. PRAP/17βHSD7 promoter-reporter constructs containing intact (Sp1) or mutated (mSp1) Sp1 binding sites were transfected into RCLP cells, as was the empty vector pGL3 basic, and harvested after 6 h. Luciferase activity of each sample was normalized to the activity of concurrently transfected β-galactosidase expression vector and converted to a percentage of the average for the −185 promoter. Results are the mean ± SEM for three independent experiments performed in triplicate. *Differing letters* indicate significant differences ($P < 0.05$).

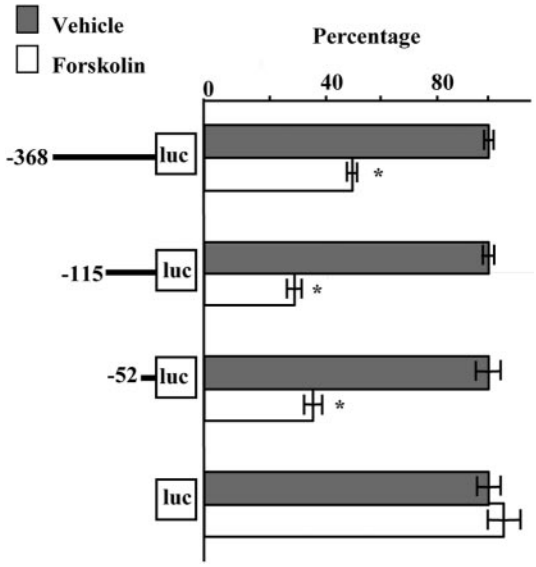

FIG. 6. Regulation of PRAP promoter activity by forskolin. PRAP promoter constructs of various sizes or the pGL3 basic plasmid were transfected along with β-galactosidase expression vector into RCLP cells. After transfection, cells were treated for 6 h with either DMSO (*dark bars*) or 100 μM forskolin (*light bars*). Luciferase activity of each sample was normalized to β-galactosidase activity. The results are presented as percentage of the DMSO-treated control for each promoter (mean ± SEM) for three independent experiments performed in triplicate. *Asterisks* denote $P < 0.05$ relative to control for each promoter.

a putative binding site for Pbx-1. CCAAT boxes have been described in a number of systems to dynamically regulate transcription of a number of diverse genes, in part due to the wide variety of transcription factors that bind to sites containing the "CCAAT" sequence (reviewed in Ref. 34). This includes such diverse factors as the CCAAT/enhancer binding proteins, nuclear factor 1, CCAAT displacement protein, and CCAAT binding protein/NF-Y (35–37). Whereas many of these may have a CCAAT sequence within their binding site, NF-Y is the only one that requires this sequence (38). Pbx-1 has been shown to mediate a cAMP response in the promoter of another steroidogenic enzyme, P450c17 (39–42), and can cause either transcriptional activation or repression depending on cell and promoter context (43).

To determine whether transcription factors are indeed binding to this sequence in the CL, we performed EMSA analysis using the rat PRAP/17βHSD7 DNA sequence from −40 to −11 to probe luteal nuclear extracts for binding activity. As mentioned earlier, we have shown that the normal increase in PRAP/17βHSD7 expression seen at mid-pregnancy can be inhibited by administration of LH/hCG (25). Using the same model, nuclear extracts were obtained from CL of rats on d 14 of pregnancy that had been injected with either PBS or hCG for 3 d. In addition, the RNA was isolated from these same CL and analyzed by semiquantitative RT-PCR for PRAP/17βHSD7 expression (Fig. 7). As previously demonstrated (25), hCG treatment leads to a decrease in PRAP/17βHSD7 mRNA expression. The nuclear extracts obtained from these same animals were used in EMSA studies with the −40 to −11 promoter region. As shown in Fig. 8, luteal nuclear extracts from control rats possessed binding

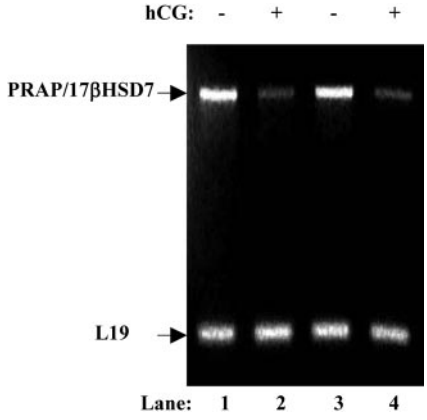

FIG. 7. hCG effectively decreases PRAP/17βHSD7 expression in the rat CL of pregnancy. Total RNA was isolated from CL from d 14 pregnant rats that had received ip injections of saline (lanes 1 and 3) or hCG (lanes 2 and 4). The RNA was analyzed for levels of PRAP/17βHSD7 and ribosomal protein L19 by RT-PCR, and the product was separated by agarose gel electrophoresis. Each lane represents the set of CL harvested from a single rat.

activity for this probe, and this activity was not found in luteal nuclear extracts from hCG-treated rats. This binding could be blocked by cold wild-type probe as well as a cold probe mutated at a putative Pbx-1 binding site but not by cold probe mutated at the putative CCAAT site. This suggests that a luteal factor binds to this CCAAT site, and that this binding is prevented by hCG. To identify this protein, antibody to NF-YA was coincubated with the luteal nuclear extracts and the −40 to −11 probe. As shown in Fig. 8, antibody to NF-YA could supershift this complex formed by the control luteal extracts. No supershift was observed when nuclear extracts from hCG-treated rats were used.

To determine whether this NF-YA site was involved in the response to forskolin observed in RCLP cells, the NF-Y binding site was mutated in the −52-bp promoter and mutant and wild-type promoter constructs were transfected into RCLP cells. Mutation of the putative NF-Y binding site in the −52-bp PRAP/17βHSD7 promoter prevented inhibition of the promoter by forskolin, indicating that this factor plays an important role in regulating PRAP/17βHSD7 promoter activity in luteal cells (Fig. 9).

Discussion

In these studies, we have determined the length and sequence of the 5′ UTR of the rat PRAP/17βHSD7 transcript and revealed the existence of two transcription start sites by both 5′ RACE and primer extension. We have also cloned and sequenced an 1166-bp promoter fragment 5′ to the distal transcription start site, which contains a number of putative response elements, although lacking a classical TATA box. It does contain GC-rich regions as well as a proximal inverted CCAAT box, both of which may regulate appropriate initiation of transcription. In fact, the inversion and proximal location of the CCAAT box (−22) is more characteristic of TATA-less promoters (44). We have previously shown that LH and estradiol regulate PRAP expression; however, a palindromic ERE and a cAMP response element are both notably absent from this 1166-bp proximal PRAP promoter

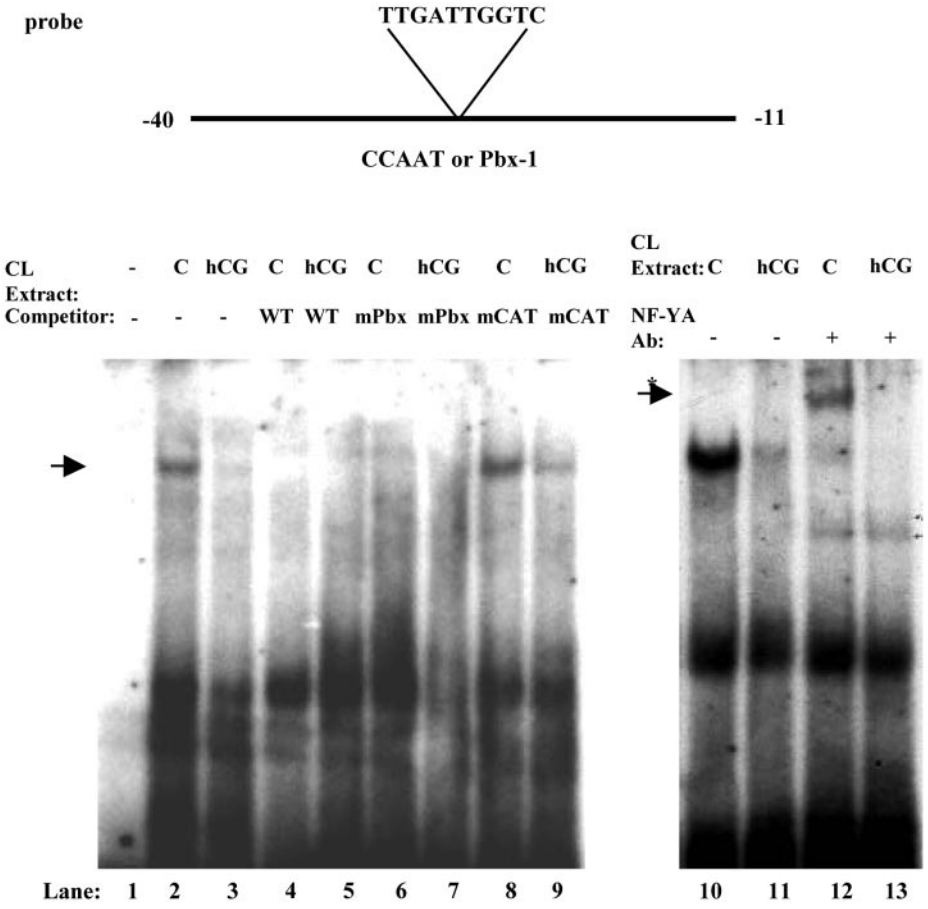

FIG. 8. hCG eliminates the binding of luteal nuclear protein to the −40 to −11 region of the PRAP/17βHSD7 promoter. EMSA was performed with a probe consisting of the −40 to −11 region of the PRAP/17βHSD7 promoter, which contains putative, overlapping binding sites for CCAAT and Pbx-1, and with probes in which either the Pbx-1 or CCAAT binding sites was mutated. The results shown are representative of three independent experiments. Nuclear extracts from rats injected with saline (C) bound to the wild-type probe (lane 1), but this binding was virtually absent in nuclear extracts from rats injected with hCG (lane 2). Coincubation with 50× cold wild-type probe (lanes 4 and 5) or 50× cold probe with a mutated Pbx-1 site (lanes 6 and 7) eliminated binding; however, a 50× cold probe with a mutated CCAAT site (lanes 8 and 9) could not compete with the wild-type probe. Furthermore, the protein-DNA complex was supershifted by an antibody to NF-YA (lane 12).

sequence. The cloned promoter sequence does contain three ERE half sites.

Analysis of promoter activity in culture revealed the presence of a strong enhancer between −185 and −115, and this enhancer was active in the presence of a heterologous promoter in all cell lines tested, although it was most active in the rat luteal cell line RCLP. Sequence analysis of this promoter fragment and comparison to the human and mouse promoter sequences identified a region containing two Sp1 binding site that was conserved between species and binding of Sp1 to this site was confirmed by EMSA in both RCLP cells and luteal extracts. An additional proximal Sp1 binding site was also confirmed by EMSA. These two Sp1 sites were mutated individually and in combination, and the results revealed the distal Sp1 site to be a potent enhancer, consistent with the location of the major enhancer element revealed by truncation studies. Interestingly, the proximal site also enhanced basal activity to a lesser extent. It is worth noting that this proximal Sp1 site is physically close to the CCAAT or NF-Y binding site also identified in this promoter. Numerous

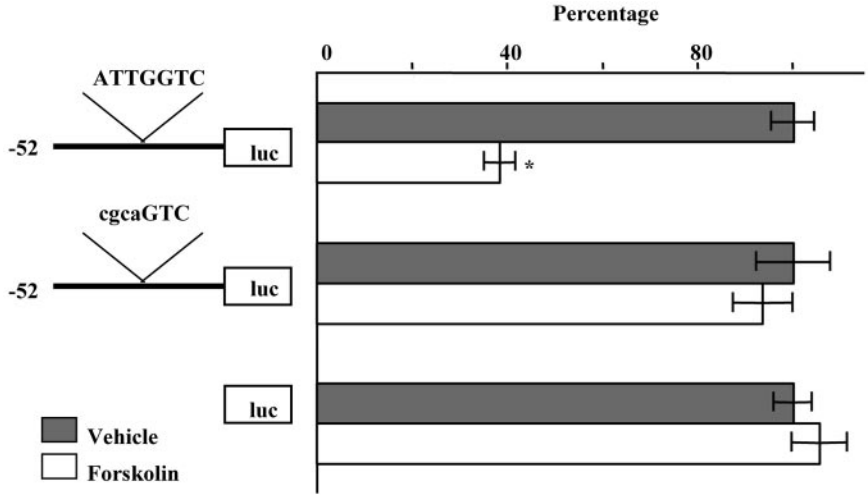

FIG. 9. Mutation of the NF-Y binding site in the 52-bp PRAP/17βHSD7 promoter eliminates LH-induced inhibition of the promoter. PRAP/17βHSD7 promoter constructs consisting of the proximal 52 bp of the promoter, containing either the wild-type NF-Y binding site (ATTGGTC) or a mutated binding site (cgcaGTC) were transfected into RCLP cells, and cells were treated for 6 h with either DMSO or 100 μM forskolin. Luciferase activity of each sample was normalized to the activity of concurrently transfected β-galactosidase expression vector and the results are presented as expressed as percentages of DMSO-treated control (mean ± SEM) for three independent experiments performed in triplicate. *Asterisks* denote $P < 0.05$ relative to control for each promoter construct.

studies have shown that NF-Y and Sp1 interact to affect transcription in various promoters (45–48). The two factors have been shown to cooperatively bind DNA and synergistically activate transcription. NF-Y and Sp1 have also been shown to interact in the absence of DNA through glutathione S-transferase pulldown assay and coimmunoprecipitation (49).

In addition, binding of the PRAP promoter by Sp1 may serve as a conduit for estrogen regulation of PRAP promoter activity. A number of laboratories have shown Sp1 to mediate stimulation in response to estrogen in a variety of genes via differing mechanisms, including cooperative binding with an ER half site (30). The presence of three ER half sites within the PRAP/17βHSD7 promoter raises the possibility of interaction between these factors, which, although beyond the scope of the current study, is fertile ground for future investigation.

In the present study, we explored the mechanisms by which LH inhibits expression of PRAP/17βHSD7. PRAP/17βHSD7 expression has been shown by our laboratory to be decreased in response to LH in the rat CL, and in RCLP cells we found that forskolin could effectively decrease PRAP/17βHSD7 promoter activity in all constructs, including the smallest construct (−52 bp). This minimum construct contains a conserved CCAAT box and binds NF-Y in nuclear extracts from both RCLP cells (data not shown) and CL in gel mobility shift assays. The NF-Y complex is also known as CCAAT-binding factor and is a heterotrimeric transcription factor that consists of three conserved subunits, /A, NF-YB, and NF-YC (50, 51) that must be associated together to bind to DNA. In TATA-less promoters, which, like the PRAP/17βHSD7 promoter, contain only one or two *cis*-acting elements, the CCAAT box is absolutely required for regulating gene transcription (34, 52). Treatment with hCG abolished the CCAAT binding activity of luteal nuclear extracts, suggesting that loss of this CCAAT binding activity may play a role in the inhibition of PRAP expression by LH *in vivo*. cAMP has been suggested to mediate inhibition of the fatty acid synthase gene through NF-Y (53, 54); and this is similar to our observation that hCG inhibits NF-Y binding to the CCAAT site in the PRAP/17βHSD7 promoter. Mutation of this CCAAT box within the context of the 52-bp promoter blocked forskolin-mediated inhibition of PRAP promoter activity, further confirming the crucial role of NF-Y in regulation of this promoter.

The inhibition of PRAP/17βHSD7 by LH is a crucial element in control of estradiol production during pregnancy in the rat. The two isoforms of 17βHSD found in the ovary, type 1 and type 7, are the only isoforms of this enzyme capable of converting the weak estrogen estrone to the strong estrogen estradiol, which is essential for pregnancy in the rodent (55). As the other hormones in the steroidogenic pathway are active in ovarian tissues throughout pregnancy, the presence of two, differently regulated isoforms of 17βHSD provides an efficient mechanism for regulating estradiol production. In the initial stages of pregnancy, high levels of LH stimulate transcription of 17βHSD1 in granulosa cells (13), the primary source of estradiol. When circulating LH drops at midpregnancy (5), production of 17βHSD7 begins in the CL (17, 18). This new enzyme replaces the activity of follicular 17βHSD1, maintaining estradiol production in the absence of high levels of LH. Furthermore, by preventing concomitant follicular and luteal production of estradiol, this dual regulation prevents overproduction of estradiol, excessive amounts of which are detrimental to fetal survival in the rodent (26, 27).

In addition to its critical role in maintaining adequate ovarian estradiol production, 17βHSD7 may carry out other important functions during pregnancy. PRAP/17βHSD7 has been identified in the decidua of the mouse (56). A role for this enzyme in decidual development or maintenance has not yet been identified; however, it is interesting to speculate on the necessity for maintaining adequate local estradiol production in this tissue. One known action of estradiol in the rodent decidua is the inhibition of IL-6 production (57). As IL-6 can lead to abortion (20, 58), the presence of locally produced estradiol may be a crucial factor in the success of pregnancy.

In conclusion, in this study we have successfully cloned the PRAP/17βHSD7 5′ flanking region and characterized elements involved in its regulation. An enhancer region was identified and found to bind Sp1 from the nuclear extracts of a luteal cell line and the CL of pregnancy. A critical Sp1 site within this enhancer was identified by mutation analysis. A second Sp1 site in a more proximal region of the promoter also had an impact on basal activity of the promoter. In addition, a binding site for NF-Y was found in the proximal promoter region, which is essential for forskolin-induced inhibition of the promoter. Because binding of NF-Y to this region of the promoter is inhibited by hCG, this is a likely mechanism by which LH acts to repress the transcriptional expression of luteal PRAP/17βHSD7 gene during early and late pregnancy.

Acknowledgments

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