Identification of regulatory elements in the *Cyp19* proximal promoter in rat luteal cells

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Abstract

The cytochrome P450 aromatase (*Cyp19*) gene encodes an enzyme of crucial importance in the synthesis of estradiol. Estradiol is luteotropic in the rat. In this species, luteal *Cyp19* expression increases progressively during pregnancy and falls before parturition. The mechanisms that control these changes are unknown. Using gel shift assays, we sought to identify the promoter regions that control *Cyp19* expression in the rat corpus luteum (CL). The *Cyp19* promoter contains a cAMP response element-like sequence (CLS), two nuclear receptor elements half sites (NREs), a GATA binding site, a Yin Yang-1 (YY1) response element, and an activation protein 3 (AP3) binding site. Nuclear extracts were obtained from CL of rats on days 4, 15, and 23 of pregnancy and from the ovaries of immature rats treated with vehicle or a hormone that induces *Cyp19* expression in the follicles. CLS was active in immature ovaries but inactive in the CL of pregnant rats, whereas binding to NREs and GATA was observed in both tissues. YY1 was inactive in all samples tested. In the CL, AP3 binding was higher on day 15 of pregnancy when compared with day 4 and day 23 but it was absent in ovaries of immature rats, whereas luteinization increased AP3 binding activity. Mutation of the AP3 site blunted the stimulation of *Cyp19* promoter activity in granulosa cells. Our results indicate that CLS is active only in follicles; whereas in the CL, binding to the GATA, NRE, and AP3 sites associates with changes in *Cyp19* expression, suggesting that they control *Cyp19* promoter activity in luteal cells.

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Introduction

In rats, luteal function is sustained in part by the luteotropic action of locally produced estradiol (Stocco *et al.* 2007). Estradiol biosynthesis depends on the expression of the aromatase enzyme. This enzyme is encoded by the *Cyp19* gene. During pregnancy, *Cyp19* expression is low on day 4, increases progressively to reach maximal expression from days 15 to 19, and decreases from day 20 to reach undetectable levels on day 23 (Akinola *et al.* 1997, Hickey *et al.* 1988, Stocco 2004). The mechanisms that control the expression of this gene, and therefore estradiol production, in the rat corpus luteum (CL) remain to be determined.

The genomic region located immediately upstream of the coding region (also known as the proximal promoter) drives *Cyp19* expression in rat luteal and granulosa cells (GC; Fitzpatrick *et al.* 1997, Stocco 2004). Within the proximal *Cyp19* promoter, a cAMP response element-like sequence (CLS; Hickey *et al.* 1990), two nuclear receptor elements (NREa and NREb; Fitzpatrick & Richards 1993a,b, Lynch *et al.* 1993, Carlone & Richards 1997b, Michael *et al.* 1997, Hinshelwood *et al.* 2003), and a GATA binding element (Jin *et al.* 2000, Stocco 2004, Kwintkiewicz *et al.* 2007) have been found.

CLS is essential for the expression of *Cyp19* in GCs (Fitzpatrick & Richards 1994). CLS is recognized by the cAMP response element-binding protein (CREB; Fitzpatrick & Richards 1994), whereas NRE is recognized by steroidogenic factor-1 (Lynch *et al.* 1993) and liver receptor homolog-1 (LRH1; Falender *et al.* 2003, Pezzi *et al.* 2004). The GATA binding site is recognized by GATA4 in luteal and GCs (Stocco 2004, Kwintkiewicz *et al.* 2007). GATA4 binding to the *Cyp19* promoter is stimulated *in vitro* and *in vivo* by follicle-stimulating hormone (FSH) and contributes to the stimulation of *Cyp19* expression in GCs (Kwintkiewicz *et al.* 2007). Transcription factors that bind to the CLS, NRE, and probably to GATA interact in an additive manner to increase *Cyp19* expression in rat GCs (Carlone & Richards 1997a,b, Kwintkiewicz *et al.* 2007).

The aromatase promoter contains, in addition to the CLS, NRE, and GATA, a binding site for the Yin Yang 1 (YY1) transcription factor and an AP3 binding site (Fig. 1). YY1 is known to have a fundamental role in normal biologic processes such as embryogenesis, differentiation, replication, and cellular proliferation (Gordon *et al.* 2006). However, the transcription factors that recognize the AP3 binding site are unknown. The AP3 binding site was first identified as a part of the viral

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-184 CAGAACGTTGACTCTCAATTGAGTATGCACGTCACTCTACCCACT
                                CLS
-139 CAAGGGCAAGATGATAAGGTTCTATCAGACCAACCGCTGAACAGG
      NRE B      GATA
-94  ACCTGAGTCTCCCAAGGTCATCCTTGTTTTGACTTGTAACCACAA
                        NRE A      YY1      AP3
-49  ATTTGTCTTGCCTTGTCACTTATAAAACATCTGTCCATTCCAGCAC
                                TATA

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Figure 1 Rat *Cyp19* proximal promoter. Transcription factor binding sites are underlined. The numbers on the left indicate the location in relation to the transcription initiation site +1 (Fitzpatrick & Richards 1993a).

SV40 enhancer, which is composed of multiple sequence motifs acting synergistically to stimulate gene expression (Mercurio *et al.* 1992). AP3 is essential for maximal SV40 enhancer activity and viral replication (Weiher *et al.* 1983). Moreover, the AP3 binding site which acts as a tumor-promoter response element (Chiu *et al.* 1987), is implicated in the up-regulation of *IL-2* and *IL-2R* genes (Emmel *et al.* 1989, Granelli-Piperno & Nolan 1991), and activates mRNA transcription during hematopoietic differentiation induced by phorbol esters (Adler & Kraft 1995). Despite the proven capacity of the AP3 binding site to stimulate transcription, efforts to clone the factors that recognize AP3 have not been successful. Summarizing, in contrast to the established role of the CLS, NRE, and GATA in *Cyp19* expression in the ovary, it is not known whether the YY1 and AP3 binding sites are important for *Cyp19* expression.

The aim of this investigation was to evaluate the participation of the following binding sites: CLS, GATA, NRE, YY1, and AP3 in the expression of *Cyp19* in the rat CL. The capacity of luteal proteins obtained at different stages of pregnancy to bind these sites was evaluated using gel shift analyses. The results indicate that CLS and YY1 are inactive in luteal cells, whereas the GATA, NRE, and AP3 binding sites are recognized by luteal nuclear proteins. Changes in binding to GATA, NRE, and AP3 are associated with changes in *Cyp19* expression, suggesting that these sites may regulate *Cyp19* expression in luteal cells.

Materials and methods

Animals and cell culture

The following protocols were approved by the Yale Animal Resources Center. Sprague–Dawley rats of 26 days of age or timed-pregnant rats of the same strain were obtained from Charles River Laboratories Inc., (Wilmington, MA, USA). Ovaries were collected from 26-day-old immature rats (day 26) treated with pregnant mare serum gonadotropin (PMSG, 15 IU/rat) or vehicle for 48 h. Ovaries were also collected from rats

sequentially treated with PMSG for 48 h and with human chorionic gonadotropin (hCG, 15 IU/rat) for 24 h. Corpora lutea were dissected from the ovaries of rats on days 4, 15, and 23 of pregnancy (p4, p15 and p23). Samples were stored at -80°C until nuclear extract isolation. Three animals were included in the experimental groups – d26 and p4, whereas four animals were included in the experimental groups – PMSG, PMSG/hCG, and p15 and p23.

Undifferentiated GC were obtained from immature rats treated subcutaneously with estradiol (1.5 mg/day) for 3 days, whereas luteinized GCs were obtained from immature rats treated with PMSG (15 IU) for 48 h followed by treatment with hCG (15 IU) for 6 h. In both cases, the ovaries were trimmed to remove the bursa, fat, and oviducts, and incubated for 15 min at 37°C in 6 mM EDTA in Dulbecco's modified Eagle media/Ham's F-12 media (DMEM/F-12). The ovaries were then incubated for 10 min in 0.6 M sucrose in DMEM/F-12. The GCs were extracted by puncturing and squeezing the follicles with a 30-gauge needle. The cells were plated on 24-well plates coated with laminin at a density of 9×10^4 cells/well in DMEM/F-12 serum-free medium supplemented with insulin (10 mg/l), transferrin (5.5 mg/l), selenium (5 $\mu\text{g/l}$), BSA (0.5 mg/ml), penicillin G (100 units/ml), streptomycin (100 $\mu\text{g/ml}$), and amphotericin B (250 ng/ml).

RNA isolation and quantitative real-time PCR analysis

Total RNA from ovaries of immature rats treated with PMSG or from corpora lutea was isolated using TRIzol®-Reagent (Invitrogen) following the manufacturer's instructions. For mRNA analysis by RT-PCR, 1 μg of the total RNA was reverse-transcribed at 42°C using Advantage RT-for-PCR kit (Promega) and later diluted to a final volume of 100 μl .

To generate standard curves for rat *Cyp19* or *L19*, the cDNA of these genes were cloned into pCR 2.1 vector (Invitrogen), sequenced, and excised by restriction enzyme. Purified cDNA was diluted to concentrations ranging from 10^3 to 6×10^6 copies/ μl . Standard cDNA

or sample cDNA of 5 µl aliquots were combined with SYBR Green I (Bio-Rad), specific primers for rat *Cyp19* or L19, and water to 50 µl final volume. The following intron spanning primers were used to amplify *Cyp19*: ctgctgatcatgggctct and ctcacaggtcgggtgtt and L19: ctgaaggtaaaagggaatgtg and ggacagagtcttgatgatctc. Real-time quantification of the PCR product in each cycle was carried out in an iQcycler Real-time PCR machine (Bio-Rad) with the following cycling conditions: pre-incubation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 10 s, and extension at 72 °C for 20 s. The melting peak of each sample was routinely determined by melting curve analysis in order to ascertain that only the expected products had been generated. The minimal number of cycles sufficient to produce detectable levels of fluorescence (Ct) was calculated using the MyiQ software (Bio-Rad). The number of *Cyp19* or L19 mRNA molecules present in each sample was calculated using a standard curve and expressed as copies per nanogram of total RNA. The results are expressed as the ratio between the copies number per nanogram of total RNA of *Cyp19* and ribosomal L19 mRNA.

Transient transfection and luciferase assay

Undifferentiated or luteinized GCs were transfected with reporter constructs containing 600 or 245 bp of the rat *Cyp19* promoter upstream of the firefly luciferase cDNA (600*Cyp19*-Luc and 245*Cyp19*-Luc; Cai *et al.* 2007) or the same promoter carrying a mutation on the AP3 binding site using FuGene 6 transfection reagent (Roche). Transfection efficiency was normalized by co-transfection of the *pCMV-renilla* luciferase expression vector (Promega). After 30 h of transfection, cells were treated with either FSH (50 ng/ml) or vehicle for 6 h. Cell lysates were prepared using 100 µl of passive lysis buffer (Promega). Luciferase activity was determined in 50 µl of lysate/sample using the Dual Luciferase Reporter Assay (Promega) according to the protocol supplied by the manufacturer. Results are expressed as relative luciferase units normalized to renilla activity.

Electrophoresis mobility shift assay (EMSA)

Nuclear protein extracts from ovaries or corpora lutea were prepared by extracting nuclei with the following buffer: 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol, 25% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 2 µg/ml pepstatin A, 1 µg/ml aprotinin, and pH 7.9. Protein concentration was determined using the BCA assay (Pierce). Complementary oligonucleotides spanning the regions -164 to -143 (CLS), -145 to -125 (NREb), -134 to -116 (GATA), -84 to -71 (NREa),

-76 to -58 (YY1), and AP3 -65 to -47 (Fig. 1) were annealed and labeled with ³²P using T4 kinase. The numbers indicate the location in relation to the transcription initiation site +1 (Fitzpatrick & Richards 1993a). Nuclear extracts (5 µg) were incubated for 20 min at room temperature in binding buffer (20 mM HEPES, pH 7.6; 60 mM KCl, 0.01 mM ZnSO₄, 0.1 mM EDTA, 0.035 mM BSA, 1 mM dithiothreitol, and 6% glycerol (v/v)) in the presence of 1 µg of salmon sperm DNA and 50 000 c.p.m. of radiolabeled double-stranded oligonucleotides. Following incubation, protein-DNA complexes were resolved by electrophoresis in 6% non-denaturing acrylamide gels. Competitor oligonucleotides were added 10 min prior to the addition of the probe. Antibodies against nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NF-ATc1) (sc-13033; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or nuclear factor NF-kappa-B p50 subunit (NFkB p50) (Cat# 1616737; Geneka Biotechnology, Montreal, Canada) were added 10–40 min prior to the addition of the probe. EMSA assays were performed for each individual animal. Films were scanned and the intensity of band shifts was determined with ImageJ (NIH, Bethesda, MA, USA). The results are expressed as fold increase versus PMSG in Fig. 2 or versus day 4 (p4) in Fig. 3. Three to four animals were used for each group. Representative results are shown.

Protein-DNA crosslinking

Luteal nuclear proteins were incubated in the presence of ³²P-labeled AP3 probes for 30 min. Reactions containing a 50-fold excess of wild type or mutant D (Fig. 4B) unlabeled oligonucleotides were also performed. The reactions were exposed to ultraviolet light (300 nm, 50 W) for 20 min at 4 °C. The samples were denatured with Laemmli sample buffer and run, along with prestained protein molecular markers (Bio-Rad), through 12% SDS-PAGE gels. Dried gels were developed by autoradiography.

Statistical analysis

Band shift intensities showed a normal Gaussian distribution. Results are expressed as means ± S.E.M. and significance was determined using ANOVA I followed by Tukey tests for multiple group comparisons. Significant differences were indicated by *P* < 0.05.

Results

Binding of luteal nuclear proteins to the *Cyp19* promoter during pregnancy

To evaluate the participation of the CLS, GATA, NRE, YY1, and AP3 sites on the expression of *Cyp19* in the rat

CL, the capacity of luteal nuclear extracts to bind these response elements was investigated. Luteal nuclear extracts were obtained from rats on days 4, 15, and 23 of pregnancy. These days represent maximal (day 15) and minimal (days 4 and 23) levels of *Cyp19* expression during pregnancy (Fig. 2A). We also evaluated the binding capacity of nuclear extracts from ovaries of 26-day-old immature rats that do not express *Cyp19* and ovaries of immature rats treated with PMSG, a hormone that increases *Cyp19* expression in the follicles.

CLS binding activity was high in 26-day-old immature rats and in PMSG-treated rats. However, low CLS binding activity was observed in luteal nuclear extracts from rats on days 4, 15, or 23 of pregnancy (Fig. 2B). Band shift quantification of three independent experiments demonstrated a significant decrease in CLS binding in CL samples when compared with ovarian samples from 26-day-old immature rats or PMSG-treated rats (Fig. 2B).

Binding to the NREa, NREb, and GATA response elements was significantly higher in PMSG-treated rats when compared with 26-day-old rats (Fig. 2C–E). Little or no binding to these elements was detected in luteal nuclear extracts from rats on days 4 and 23 of pregnancy. Binding to NREa, NREb, and GATA was significantly higher in rats on day 15 of pregnancy when compared with days 4 and 23 (Fig. 2C–E).

The addition of a 50-fold excess of unlabeled CLS, NREa, NREb, or GATA oligonucleotides completely prevented the formation of their respective band shifts, whereas a 50-fold excess of mutant oligonucleotides had no effect (data not shown).

No YY1 binding activity was detected in nuclear extracts obtained from ovaries of immature rats or from corpora lutea of pregnant rats (data not shown). On the other hand, nuclear extracts obtained from corpora lutea of pregnant rats showed high AP3 binding activity. As shown in Fig. 3 top panel, low AP3 binding activity was observed in luteal nuclear extracts from rats on day 4 of pregnancy. AP3 binding increased significantly ($P < 0.01$) on day 15 of pregnancy and decreased toward the end of pregnancy. No binding to the AP3 binding site was observed before or after PMSG treatment of immature rats. Taken together, these results indicate that the CLS and YY1 binding sites are not active in luteal cells, whereas activation of NRE,

GATA, and AP3 in the CL seems to be developmentally regulated.

AP3 binding increases after the induction of luteinization

Our results thus far suggest that AP3 binding activity appears only in luteal cells. To examine this hypothesis we examined the effect of the administration of hCG, a hormone that induces ovulation and luteinization, to immature rats pretreated with PMSG for 48 h. As shown in Fig. 4A, ovarian extracts of 26-day-old immature rat or PMSG-treated rats did not exhibit AP3 binding activity. In contrast, high AP3 binding activity was observed in ovarian extracts of PMSG/hCG treated animals.

Next, we investigated whether AP3 binding sites are present in the human and mouse proximal promoters. Like rats, humans and mice express *Cyp19* in the CL under the control of the proximal promoter (Means *et al.* 1991, Foyouzi *et al.* 2005). We also examined the bovine *Cyp19* proximal promoter, which is not active in luteal cells (Lenz *et al.* 2004). AP3 binding sites were found in the human and mouse *Cyp19* promoters (Fig. 4B). However, no AP3 sequences were found in the bovine proximal promoter.

Characterization of the luteal AP3 binding protein

To determine which nucleotides are needed for AP3 binding, two base-pair scanning mutations were introduced into the AP3 oligonucleotide (Fig. 5A). Nuclear extracts from corpora lutea of rats on day 15 of pregnancy were used in these assays. The addition of 50-fold excess of the wild-type oligonucleotide A or of the mutant oligonucleotides B and G prevented AP3 band shift formation (Fig. 5B, lanes 2, 3, and 8). However, oligonucleotides with mutations in the AP3 site (mutant oligonucleotides C, D, E, and F) did not affect the formation of the AP3 band shift (Fig. 5B, lanes 4 to 7). Next, we performed competitions with increasing concentrations (5, 10, 50, 100, and 200×) of a consensus AP3 oligonucleotide (Mercurio *et al.* 1992). As shown in Fig. 5C, the AP3 consensus oligonucleotide prevented the formation of the AP3 band shift in a concentration-dependent manner, whereas similar concentrations (50, 100, and 200×) of mutant D did not have an effect.

Figure 2 Binding of luteal nuclear proteins to the CLS, NRE, and GATA response elements. Samples were obtained from ovaries of 26-day-old immature rats (d26), immature rats treated with PMSG, and from corpora lutea of rats on days 4 (p4), 15 (p15), or 23 (p23) of pregnancy. (A) Relative *Cyp19* expression levels. *Cyp19* and L19 mRNA levels were determined by real-time PCR. Results are expressed as the ratio between the copies number per nanograms of total RNA of *Cyp19* and ribosomal L19. (B) Gel shift analyses were performed using oligonucleotides spanning the CLS, (C and D) the nuclear receptor elements a and b (NREa and NREb), or (E) the GATA binding sites found in the proximal *Cyp19* promoter (Fig. 1). Three to four animals were included in each group. EMSA assays were performed for each individual animal, representative results are shown. The intensity of each band shift (arrow head) was quantified and expressed as fold increase versus PMSG. Columns with different letters differ significantly ($P < 0.05$; ANOVA I–Tukey test).

Figure 3 Binding of luteal proteins to the AP3 binding site. (A) Gel shift analysis was performed using oligonucleotides spanning the AP3 region found in the *Cyp19* promoter. Three to four animals were used for each experimental group. EMSA assays were performed for each individual animal, representative results are shown. (B) Band shifts (arrow) were quantified and expressed as fold increase versus p4. Columns with different letters differ significantly ($P < 0.05$; ANOVA I–Tukey test).

The consensus binding sequence of AP3 is similar to the binding sites of NF κ B and NFATc1 (McCaffrey *et al.* 1992, Lee *et al.* 2003). Therefore, the possibility arises that the AP3 binding activity found in the rat luteal cells could be due to NF κ B or NFATc1. To test this possibility, we added antibodies against these factors to the gel shift reaction. No supershift bands were observed with either an anti-NF κ B or an anti-NFATc1 antibody, suggesting that these transcription factors do not form part of the AP3 complex (Fig. 5D).

The molecular mass of the luteal protein that binds to the AP3 site was determined using DNA/protein crosslink assays followed by denaturing electrophoresis. In good agreement with gel shift assays, a single DNA/protein complex was observed (Fig. 6). The molecular mass of this complex is ~ 45 kDa. The addition to the binding reaction of a 50-fold excess of a wild-type oligonucleotide prior to the crosslink step decreased the formation of this complex (Fig. 6, lane 2), whereas 50-fold excess of a mutant oligonucleotide (TAACCACA to TAgtCACA) had no effect (Fig. 6, lane 3). This result suggests that a single

protein with an apparent molecular mass of ~ 45 kDa binds to the *Cyp19* promoter at the AP3 site.

Mutation of the AP3 binding site decreases *Cyp19* promoter activity in GCs

Next, we examined whether AP3 had any effect on the activity of the *Cyp19* promoter. For this purpose, we used luteinized GCs. We have previously used these cells to study the regulation of Nur77 and 20 α -hydroxysteroid dehydrogenase promoters (Stocco *et al.* 2002). Luteinized GCs were transfected with the 600*Cyp19*-Luc or the 245*Cyp19*-Luc reporter vectors. As a control, cells were transfected with the pCMV-renilla reporter construct. Although the activity of the pCMV-renilla construct was high in all experiments performed, the activity of the 600*Cyp19*-Luc or the 245*Cyp19*-Luc constructs was not detectable in luteinized GCs (data not shown).

In GCs, *Cyp19* expression and promoter activity are stimulated by FSH (Kwintkiewicz *et al.* 2007). Interestingly, we observed that FSH treatment of GCs increases

Figure 4 AP3 binding increases after the induction of luteinization. Samples were obtained from ovaries of 26-day-old immature rats (d26), immature rats treated with PMSG for 48 h, or from immature rats sequentially treated with PMSG for 48 h and with hCG (15 IU/rat) for 24 h. (A) Gel shift analyses were performed using oligonucleotides spanning the AP3 region found in the *Cyp19* promoter. Three animals were used in each group. EMSA assays were performed for each individual animal, representative results are shown. (B) Alignment of the *Cyp19* gene regulatory region reveals that the AP3 binding site is conserved in human and rodent species, but is absent from the bovine promoter. The consensus AP3 binding site is noted below the alignment.

binding to the AP3 site. Thus, as shown in Fig. 7A, treatment of GCs with FSH for 48 h increased AP3 binding. Therefore, we thought to examine whether mutation of the AP3 binding site affects the FSH-induced increase in *Cyp19* promoter activity. For this purpose, GCs were transfected with the 245*Cyp19*Luc reporter vector or with the mAP3-245*Cyp19*Luc construct that contains a mutation in the AP3 site (TAACCACA to TAgtCACA). After transfection, cells were treated with FSH (50 ng/ml) or vehicle. As expected, FSH treatment of undifferentiated GCs increased *Cyp19* promoter activity (Fig. 7B). Mutation of the AP3 binding site significantly decreased the stimulatory effect of FSH, suggesting that AP3 contributes at least in part to the increase in *Cyp19* promoter activity induced by this hormone.

Discussion

In GCs, the CLS is essential for *Cyp19* expression (Fitzpatrick & Richards 1994). However, *Cyp19* expression in the rat CL does not correlate with binding to CLS. In luteal cells, *Cyp19* expression is affected by cAMP only from days 10 to 12 of gestation (Hickey *et al.* 1988, 1989). Moreover, in these cells, phospho-CREB resides in the cytoplasmic region, not in the nucleus (Gonzalez-Robayna *et al.* 1999). This evidence suggests that the cAMP–protein kinase A–CREB pathway is not involved in the regulation of luteal *Cyp19* expression and further supports our finding showing that the CLS present in the *Cyp19* promoter is not active in luteal cells.

Our results suggest that luteal *Cyp19* expression depends on the activation of GATA, NREs, and AP3 elements. We have previously demonstrated that GATA4 binds to the GATA response element in luteal and GCs (Stocco 2004, Kwintkiewicz *et al.* 2007). However, the identity of the proteins binding to NRE and AP3 in the CL remains to be determined. NRE is recognized by transcription factors such SF1 (Lynch *et al.* 1993) and LRH1 (Falender *et al.* 2003, Pezzi *et al.* 2004). SF1 is expressed in the CL at levels that are at least one order of magnitude lower than LRH1 (Mendelson *et al.* 2005). Unfortunately, antibodies against SF1 (Santa Cruz sc-10976) or against LRH1 (Santa Cruz sc-5995 and sc-5997) failed to supershift NRE band shift (data not shown). Whether SF1 or LRH1 is the main factor involved in the regulation of aromatase in the ovary remains to be determined. Both LRH1 and SF1 have been shown to physically interact with GATA4 to synergistically activate the 3βHSD2 promoter (Martin *et al.* 2005) and the MIS promoter (Tremblay & Viger 1999), suggesting that the interaction between GATA4 and LRH1/SF1 may also be important in the regulation of *Cyp19* expression in luteal cells.

Binding to NRE and GATA in early pregnancy is significantly lower when compared with preovulatory follicles or CL of rats on day 15 of pregnancy. This finding is supported by previous studies showing that LRH1, SF1, and GATA4 expression decreases during luteinization and increases later in pregnancy (Carlone & Richards 1997b, Fitzpatrick *et al.* 1997, Falender *et al.* 2003, Lavoie *et al.* 2004, Kwintkiewicz *et al.* 2007). Administration of prolactin, the main luteotropic hormone in rodents, stimulates the expression of LRH1 in the CL (Falender *et al.* 2003), suggesting that prolactin may control the expression of LRH1 and *Cyp19* in luteal cells. Prolactin regulates *Cyp19* expression in the CL of pregnant rats (Hickey *et al.* 1989, Krasnow *et al.* 1990). However, prolactin exerts inhibitory or stimulatory effects depending on the stage of pregnancy. For instance, prolactin inhibits *Cyp19* expression when administered during the first week of

Figure 5 Characterization of luteal AP3 binding protein. (A) Mutant AP3 oligonucleotides used in competition assays. (B) Top panel: Competitions with a 50-fold excess of unlabeled mutant oligonucleotides of AP3 binding activity observed in rats on day 15 of pregnancy. Bottom panel: Quantification of band shift in upper panel. (C) Competition of AP3 binding with increasing concentrations of unlabeled consensus AP3 oligonucleotides (5, 10, 50, 100, and 200 $\times$) or a mutant oligonucleotide (TAACCACA to TAGtCACA; 50, 100, and 200 $\times$). (D) Supershift assays: NF-ATc1 and NFkB antibodies were added to nuclear extracts from corpora lutea of rats on day 15 of pregnancy. Samples were also compared with a wild-type unlabeled probe (Cold Ex.).

pregnancy (Krasnow *et al.* 1990), but increases aromatase expression after day 12 of gestation (Hickey *et al.* 1989). The molecular basis for this shift is not known. This differential effect of prolactin could be related to its ability to activate or stimulate the expression of transcription factors such as LRH1 and/or GATA4. Further experiments are needed to examine this hypothesis.

We provide evidence indicating that the CL of pregnant rats contains a protein that binds to the AP3 binding site found in the *Cyp19* promoter. The AP3 binding site seems to be recognized by a protein with a molecular mass of 48 kDa, termed activation protein 3 (Mercurio *et al.* 1992). Our crosslink experiments revealed that in luteal cells a protein with an apparent molecular mass of ~ 45 kDa binds to the AP3 binding site. Moreover, gene reporter experiments suggest a role for the AP3 binding site in the activity of the *Cyp19* promoter. These results suggest that activation protein 3 is expressed in rat luteal cells and that it may play a role in the regulation of *Cyp19* expression.

However, purification and cloning of activation protein 3 are necessary to further examine and confirm its capacity to stimulate *Cyp19* expression.

AP3 binding sites are present in the *Cyp19* promoter of rats, humans, and mice. These species express high levels of *Cyp19* in the CL (Hickey *et al.* 1988, Foyouzi *et al.* 2005). Interestingly, AP3 binding sites were not found in the promoter of the bovine *Cyp19* gene. *Cyp19* expression in the bovine CL is low when compared with follicles and is controlled by a weak distal promoter (Lenz *et al.* 2004) instead of a more strong proximal promoter, as in rat, mouse, and human luteal cells (Means *et al.* 1991, Stocco 2004). AP3 binding site interacts with luteal nuclear extracts from pregnant rats but not with ovarian extracts of immature rats or immature rats treated with PMSG. Moreover, administration of hCG to PMSG-treated rats increases AP3 binding activity suggesting that *in vivo* this AP3 binding protein is active only in luteal cells. However, we observed that *in vitro* treatment with FSH increases

Figure 6 Determination of the molecular mass of the luteal AP3 binding factor. Binding of luteal nuclear extracts from rats on day 15 of pregnancy was carried out as detailed in Material and methods. Binding reactions, including 50-fold excess of wild type (AP3) or a mutant (mAP3: TAACCACA to TAgTCAACA) unlabeled oligonucleotide, were also performed. After exposure to ultraviolet light, the binding reactions were subjected to SDS-PAGE analysis. The gels were then visualized by autoradiography. This experiment was repeated three times with similar results.

AP3 binding activity in GCs. We speculate that this occurs because differentiated GCs luteinize spontaneously *in vitro* (Luck *et al.* 1990, Zhao & Luck 1996, Wehrenberg & Rune 2000). In PMSG-treated rats, preovulatory GCs are fully differentiated; however, they are prevented from luteinizing prematurely, probably due to inhibitory factors found in the follicular fluid (Ledwitz-Rigby *et al.* 1977, Channing *et al.* 1980, Rigby *et al.* 1980, Bar-Ami & Channing 1988). *In vitro* preantral GCs are transformed into preovulatory GCs by the action of FSH (Hunzicker-Dunn & Maizels 2006). This then allows preovulatory GCs to spontaneously luteinize with the consequent increase in AP3 binding activity.

In conclusion, this is the first report describing the regions in the *Cyp19* promoter that could control the expression of this gene in the rat CL. Our results indicate that there are differences between granulosa and luteal cells in the control of the *Cyp19* promoter. Thus, whereas CLS is essential for maximal *Cyp19* expression in GCs (Fitzpatrick & Richards 1994), this binding site is not active in luteal cells. On the other hand, transcription factors that bind to NREa, NREb, and GATA seem to participate in the regulation of *Cyp19* in both granulosa and luteal cells. We have also

Figure 7 AP3 mutation decreases *Cyp19* promoter activity. (A) Gel shift assays were performed with nuclear extracts from undifferentiated granulosa cells treated with FSH (50 ng/ml) or vehicle (C) for 48 h ($n=3$). (B) Undifferentiated granulosa cells were transfected with the 245 bp *Cyp19* reporter construct (*Cyp19Luc*) or the same construct carrying a mutation (TAACCACA to TAgTCAACA) in the AP3 binding site (*Cyp19Luc mAP3*). After transfection, cells were treated with FSH (50 ng/ml) or vehicle. Columns with different letters differ significantly ($P<0.01$: a–b, b–c, and a–c; ANOVA I–Tukey test; $n=4$).

demonstrated that luteal cells express a protein that binds to the AP3 binding site found in the proximal *Cyp19* promoter.

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