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cAMP-dependent regulation of *CYP19* gene in rabbit preovulatory granulosa cells and corpus luteum

Thomas Andrieu[a,1], Vincenzo Pezzi[b], Rosa Sirianni[b], Raluca Le Bas[a,2], Colette Feral[a], Annie Benhaim[a,c], Hervé Mittre[a,d,*]

[a] *Laboratoire, EA 2608, INRA, USC 2006, Universite, Esplanade de la Paix, F-14000 Caen, France*
[b] *Department Pharmaco-Biology, University of Calabria 87036 Arcavacata di Rende (CS), Italy*
[c] *CHU de Caen, Laboratoire de Fécondation In Vitro, Service de Gynécologie Obstétrique, F-14033 Caen cedex, France*
[d] *CHU de Caen, Laboratoire de Génétique Moléculaire, F14033 Caen cedex, France*

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ABSTRACT

Transcription of the *CYP19* gene encoding the aromatase P450 enzyme in ovarian cells is under the control of the two gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), via modulation of intracellular cyclic 3′,5′-adenosine monophosphate (cAMP) levels. Primary cultures of rabbit ovarian cells were used to identify the functional regions of the ovarian promoter (PII) that are responsive to the gonadotropic secondary messenger and to estradiol. Transfection experiments in granulosa and luteal cells with deleted constructs of the PII promoter show that the region between −274 and −193 bp is critical for cAMP-dependent transcriptional activity. A comparison of PII activities in granulosa and small luteal cells highlights a 50% decrease consecutive to the LH surge. Sequence analysis of the above mentioned region revealed the presence of a cAMP responsive element like sequence (CLS) and of a nuclear receptor element A (NREA). Binding of CREB to CLS has been shown using granulosa and luteal cells nuclear extracts. In addition, we identified the expression of NR5A1 (Steroidogenic Factor 1) and NR5A2 (Liver Receptor Homologue 1) in granulosa and luteal cells. However, the binding to NREA is observed only with granulosa cells nuclear extracts. Data suggest that the NR5A factors are not the main regulators of *CYP19* gene, in contrast with the others genes of streroidogenesis enzymes, and additional sites may play an important role during the post-LH surge down-regulation of *CYP19* transcription.

1. Introduction

Luteinization of follicular granulosa cells is achieved by luteinizing hormone (LH), which increases progesterone and decreases estradiol secretions. Functions of the corpus luteum are under the control of LH, but also of prolactin and estradiol. In rabbits, estradiol is considered to be the primary luteotropic hormone [1].

Differentiation of follicular cells into high progesterone producing cells occurs by (i) an increased expression of enzymes necessary for the conversion of cholesterol into progesterone (cholesterol side-chain cleavage cytochrome P450 complex (P450 SCC) and 3β-hydroxysteroid dehydrogenase Δ5,Δ4 isomerase (3β-HSD) and (ii) a decreased expression of enzymes that convert progesterone into estrogens (17α-hydroxylase cytochrome P450 and aromatase) [1].

Aromatase is a microsomal enzymatic complex consisting of a specific cytochrome P450 aromatase and a ubiquitous non-specific flavoprotein, NADPH cytochrome P450 reductase. In rabbits, aromatase is encoded by the *CYP19* gene, which is expressed in a tissue-specific manner by activation or repression of three promoters, PI.1, PI.r and PII. In rabbit granulosa and luteal cells, *CYP19* transcription is mainly driven by PII [2]. The ovulatory dose of hCG/LH induces the disappearance of 90% of the PII-derived transcripts of *CYP19* gene starting from 6 h before ovulation followed by a gradual decrease during pseudopregnancy [3]. The mechanism causing the rapid fall in *CYP19* expression after the LH peak is unknown. Estradiol has been found to down-regulate *CYP19* expression in the corpus luteum of Day 3 pseudopregnant rabbits, but not to affect expression in preovulatory granulosa cells [3].

In granulosa cells, *CYP19* is transcriptionally regulated by follicle stimulating hormone (FSH) and LH. Gonadotropin action is mainly mediated by an increase in intracellular cyclic 3′,5′-adenosine monophosphate (cAMP) levels and by activation of the cAMP-dependent protein kinase (PKA). In rats and humans, CREB and NR5A1/NR5A2 binding regions appear to be key elements conferring cAMP-dependent regulation of PII activity [4,5]. In rabbit, the

* Corresponding author. Tel.: +33 231272314; fax: +33 231565120.
E-mail address: mittre-h@chu-caen.fr (H. Mittre).
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sequence of the NREA (nuclear receptor element A) site is not conserved, and this modification induces a decrease in affinity for the NR5A factors. So, this site seems to be non-functional in rabbits [6]. In this recent study, we have shown that PII cAMP sensitivity in rabbit granulosa cells requires the involvement also the cAMP responsive element like sequence (CLS). Moreover, our data are in accordance with previous studies [4,5], which propose that additional ovarian-specific regulatory regions may also participate in this cAMP sensitivity [6].

Several study indicate that there are differences between granulosa and luteal cells in the control of *CYP19* promoter. In bovine luteal cells both binding sites CLS and NREA are recruited for cAMP-dependent transcription [7]. CREB expression in the corpus luteum has been identified in bovine and rat luteal cells [7–9] but has not been confirmed in primates [10] until a recent study reported such activity in the corpus luteum of bonnet monkeys [11]. In rats, an ovulatory dose of hCG decreases NR5A1 binding to PII and decreases NR5A1 mRNA in luteinized granulosa cells [12]. In mice, the NR5A1 transcript is not observed in the corpus luteum. NR5A2 transcripts, highly abundant in granulosa cells from preovulatory follicles, tend to be lower in Day 7 pregnant corpus luteum [13]. The role of CLS, NRE sites and their binding proteins in the aromatase expression regulation by cAMP in corpus luteum cells is not clear and seem species specific. Indeed, recently, in rat, Stocco et al. [14] indicate that CLS is active only in follicles, whereas in the corpus luteum, the change in *CYP19* expression is dependant of NRE, GATA and AP3 sites. Moreover, in these coprus luteum cells, phospho-CREB resides in the cytoplasmic region, not in the nucleus [15].

In this study we analyzed the functionality of CLS and NREA sites and monitored the expression of CREB, NR5A1 and NR5A2 factors in granulosa and luteal cells. The results indicate the persistence of CLS functionality in luteal cells but with a lower efficiency that in granulosa cells. This result could suggest the presence of an inhibitor factor in the luteal cells that prevent the NR5A1/NR5A2 binding on the NREA site.

2. Materials and methods

2.1. Reagents

Minimum Essential Medium (MEM) with Earle's salts, penicillin–streptomycin and Taq II polymerase were purchased from Eurobio (Les Ulis, France). Foetal Calf Serum (FCS) was purchased from BioWhittaker (Emerainville, France). Equine Chorionic Gonadotropin (eCG) was purchased from Intervet (Paris, France), human Chorionic Gonadotropin (hCG) was purchased from Organon (Puteaux, France) and Embutramide T61 was purchased from Distrivet (Angers, France). Accutase was obtained from PAA laboratories (Les Mureaux, France). Dibutyryl cAMP, EDTA, ethidium bromide, Hepes, NaCl, Tris base, protease inhibitor cocktail, type IV collagenase and type I DNAse were purchased from Sigma-Chimie (L'Isle d'Abeau, France). Primers were purchased from Invitrogen Life Technologies (Cergy Pontoise, France). The Easy-A high-fidelity PCR cloning enzyme was purchased from Stratagene (Amsterdam, Netherlands). PGEM-T Easy Vector System I, pGL3 basic vector, pSV β-galactosidase Control Vector, reporter lysis buffer, Luciferase Assay System, EcoRI, T4 DNA ligase, T4 polynucleotide kinase, Wizard Plus Maxiprep DNA Purification System, TNT T7 Quick Coupled Transcription/Translation System were provided by Promega (Charbonnières, France). Hind III and Xho I were obtained from QBiogen (Illkirch, France). Galacto Light Plus β-galactosidase Reporter Gene Assay System and ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit were purchased from Applied Biosystems (Courtaboeuf, France). [γ^{32}P] ATP was purchased from Perkin Elmer and poly (dI-dC) was obtained by Roche.

2.2. Animals

HY female white rabbits (Elevage La Bazirée, Cerisy Belle Etoile, France), were housed individually for about 2 weeks on a 14 h light (from 06.00 h to 20.00 h) and 10 h dark schedule with standard rabbit food and water *ad libitum*. Animals were brought up under standard conditions according to the instructions of Ministère de l'Agriculture et de la Pêche-Service Santé Animale (France). Development of numerous preovulatory follicles was induced in 12 weeks old rabbits by i.m. injection of 200 IU eCG daily for 2 days. Corpus luteum development was induced in 18 weeks old rabbits by i.m. injection of 200 IU hCG 4 days after the eCG stimulation. The animals were killed by intracardiac injection of 3 ml of Embutramide T61, either 4 days after the first injection of eCG or 9 days after the injection of hCG and ovaries were excised.

2.3. Cell culture and transient transfection

Gene reporter constructs were prepared as previously described [6]. Briefly, the promoter II sequences were subcloned into the pGEM-T Easy vector and next subcloned into the Hind III and Xho I sites of a pGL3 basic vector.

Granulosa and luteal cells were collected as previously described [16,17]. Briefly, luteal cells were obtained after dissociation of corpus luteum at 37 °C for 35 min with magnetic agitation in MEM containing 0.1% BSA, 0.3% collagenase IV (125 U/mg) and 0.05% DNAse. Undigested tissue was removed by filtration through a 80 μm nylon mesh. The filtrate was washed three times with fresh MEM medium containing 0.1% BSA and centrifugated at 50 × *g* for 10 min. Next, small luteal cells were prepared by equilibrium centrifugation on a stepwise gradient of Percoll as previously described [17]. Cells were cultured in MEM containing 20 mmol Hepes l^{-1}, 50 IU penicillin ml^{-1}, 50 μg streptomycin ml^{-1}, supplemented with 5% of FCS in an humidified atmosphere containing 5% CO_2/95% air at 37 °C. Cells were seeded at 0.3×10^6 cells/well on 24-well plates. After cell attachment, media was replaced with serum free media for 4 h and cells were transfected. Transient transfection of granulosa cells was performed using the calcium phosphate precipitation method as previously described [6]. 5 μg of promoter luciferase reporter constructs were transfected with 7.5 μg of pSV β-galactosidase vector to normalize the transfection efficiency.

Cells were either treated or not for 6 h with 2.5 mM of db cAMP, 10 nM of estradiol (E_2) or by E_2 plus db cAMP. Each experiment was performed in triplicate and repeated at least three times.

2.4. Luciferase and β-galactosidase assays

Luciferase and β-galactosidase assays were performed according to manufacturer's protocols in a luminometer (Mithras LB 940, Berthold, France). Luciferase data of each sample were normalized on the basis of transfection efficiency measured by β-galactosidase activity. Results are expressed as fold induction by db cAMP ± SEM. Data were analyzed with a Student's *t*-test, or an ANOVA-one way test; differences were considered significant when *P*-value was <0.05.

2.5. Nuclear extract preparation and in vitro NR5A1 and NR5A2 transcription

Adherent cells were detached with 150 μl of accutase for 10 min at 37 °C and were pelleted for 5 min at 900 g. Pellets were stored at −80 °C until protein extraction. Nuclear extracts were prepared from granulosa or luteal cells as previously described [19]. Briefly, cells were resuspended in 800 μl cold buffer A [10 mM HEPES–KOH, pH 7.9, at 4 °C; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM dithiothreitol; 1.5% protease inhibitor cocktail] by flicking the tube. Cells were

allowed to swell on ice for 10 min and vortexed every 2 min. Samples were centrifuged (2 min, 1000 × *g*, 4 °C) and the supernatant fraction was discarded. Pellets were resuspended in 100 μl cold buffer B [20 mM HEPES–KOH, pH 7.9; 25% glycerol; 1.5 mM $MgCl_2$; 420 mM NaCl; 0.2 mM EDTA; 0.5 mM dithiothreitol; 1.5% protease inhibitor cocktail] and incubated in ice for 20 min for high-salt extraction. Cells were vortexed every 5 min. Cellular debris were removed by centrifugation (2 min, 10000 × *g*, 4 °C), and the supernatant fraction (containing DNA binding proteins) was stored at −80 °C. The yield was determined by the Bradford method. In vitro transcribed and translated NR5A2 and NR5A1 proteins were synthesized using T7 polymerase in the rabbit reticulocyte lysate system following the manufacturer protocol (Promega).

2.6. Western blot analysis

Nuclear proteins were separated on a 12% polyacrylamide denaturing gel at 300 V for 3 h using a Tris/glycine/SDS running buffer [25 mM Tris base, 200 mM glycine (pH 8.3), 0.1% SDS] and were blotted onto nitrocellulose membranes (Amersham Biosciences, Orsay, France) using a semi-dry transfer system [1 h, 15 V] (OWL, Illkirch, France). Membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS), pH 7.4, supplemented with 5% non-fat dry milk. Blots were incubated overnight at 4 °C with antibody directed against the amino-terminal region of mouse NR5A2, (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit antiserum to adrenal NR5A1 (1:1000) kindly provided from Dr. Morohashi (National Institute for Basic Biology, Myodaiji-cho, Okazaki, Japan) or incubated for 1 h and 30 min at room temperature with a polyclonal anti-CREB antibody (Serotec, Cergy, France) directed against the carboxy terminal ten amino acids of CREB (1:200) in TBS supplemented with 1% non-fat dry milk. Membranes were then incubated for 1 h at room temperature with either an anti-goat (for NR5A2) or an anti-rabbit IgG (for NR5A1 and for CREB 1:4000) antibody coupled to peroxidase in TBS supplemented with 1% non-fat dry milk and revelation of antibody–protein complexes was carried out using the enhanced chemiluminescence visualization system (ECL Plus Amersham Biosciences). In vitro transcribed and translated NR5A2 and NR5A1 proteins were used as positive controls in the immunoblot and EMSA experiments. Western blot analyses were performed with 30 μg or 100 μg of nuclear proteins respectively for the analysis of CREB and NR5A2 or NR5A1. Blots were stripped and reprobed with lamin B to confirm that we have loaded for each sample the same quantity of total protein. The antibody used for detection of Lamin B was: anti-Lamin B from Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc6216.

2.7. Gel mobility shift assay

Probes were generated by annealing complementary single-stranded oligonucleotides and labeling with [γ^{32}P] ATP and T4 polynucleotide kinase, followed by precipitation with phenol chloroform, isoamyl alcohol (25/24/1). The DNA sequences used as probes or as cold competitors are the following (the nucleotide motifs of interest are *underlined*): CLS probe: 5′-AATTGGGAATGCACGTCACTCTACTCACTC-3′ and NREA probe: 5′-CTGAGATTCTACCAAGGCCAGAAATGCTGC-3′.

For NREA: protein binding reactions were carried out in 20 μl buffer [20 mM HEPES, pH 8; 1 mM EDTA; 50 mM KCl; 10 mM DTT; 10% glycerol; 1 mg/ml BSA] with 50,000 cpm of labeled probe, 20 μg nuclear proteins or 2 μl of transcribed and translated in vitro NR5A1 protein or NR5A2 protein, and 5 μg poly (dI-dC). Mixtures were incubated at 4 °C for 30 min in presence or absence of unlabeled competitor oligonucleotides or in vitro-translated protein. To determine the protein present in the complex antibody directed against NR5A1 or NR5A2 were added to nuclear extracts and incubated at 4 °C for 2 h before the addition of labeled probe. For CLS the reac-

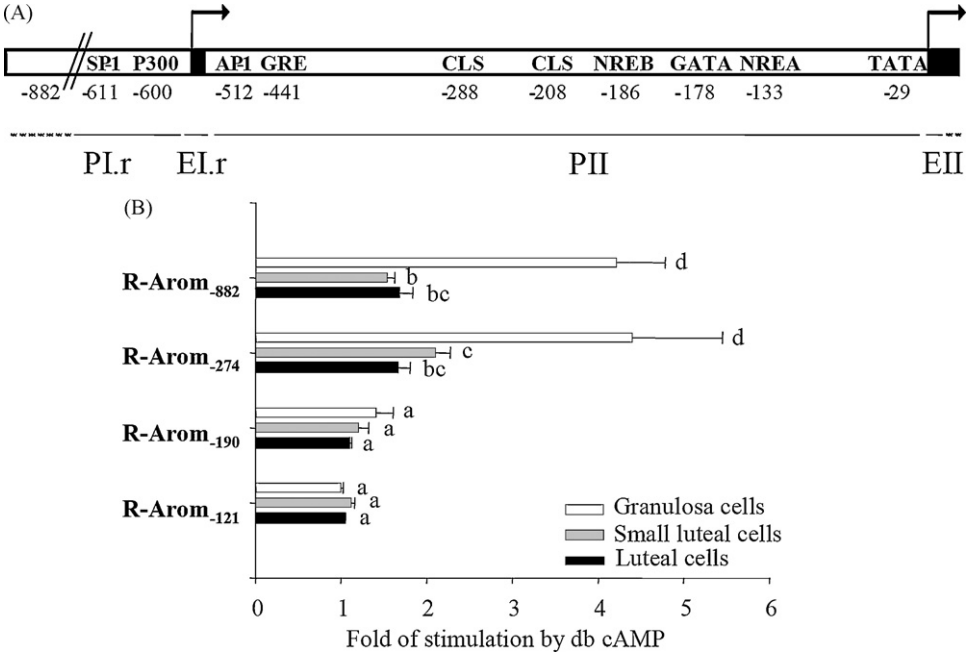

Fig. 1. Transcriptional activity of the 5′-deleted rabbit promoter II fragments of the *CYP19* gene in ovarian cells. (A) Schematic diagram of the rabbit aromatase gene containing the promoter I.r and the promoter II. The arrows indicate the sites of transcription initiation, the black box represents the 5′-untranslated region of the aromatase messenger RNA and the striped box represents the first coding exon transcripted. The location of putative cAMP response elements identified is noted inside of the promoter representation. (B) Progressive deletion of *CYP19* PII from −882 to −121 bp. 5 μg of each construct and 7.5 μg of pSV β-galactosidase vector were transfected as described in Section 2 into luteal and small luteal cells and compared to results obtained in granulosa cells. Cells were incubated with 2.5 mM db cAMP for 6 h. Results are expressed as fold of stimulation by db cAMP (±SEM). Statistical analysis was performed using ANOVA-Fisher test; superscripts letters are different when values are significantly different ($P < 0.05$).

tion mixture was carried out in 50 μl buffer with 10 μg of nuclear extract. Nuclear extracts were incubated with the antibody AHP337 (Serotec) at 4 °C for 45 min before addition of labeled probe. The entire reaction mixture was separated on a 6% polyacrylamide gel in 0.25× Tris borate-EDTA for 3 h at 150 V for NREA and through a 5% polyacrylamide gel in 0.5× Tris borate-EDTA for 2 h at 150 V for CLS. Gels were dried and subjected to autoradiography at −80 °C.

3. Results

3.1. cAMP-dependent transactivation of rabbit CYP19 in ovarian cells

To identify genomic element involved in the cAMP-dependent regulation of P450arom PII in luteal cells, we generated a series of nested 5′-deletion mutations (Fig. 1A), and these promoter fragments were ligated upstream from a luciferase reporter gene. These deleted *CYP19* PII-luciferase constructs were transfected in luteal cells (Fig. 1B). The results indicate that cAMP induced a 1.7 fold increase in the activity of the R-Arom$_{-882}$ and R-Arom$_{-274}$ constructs whereas the smaller constructs R-Arom$_{-190}$ and R-Arom$_{-121}$ were non-responsive. A comparison between granulosa cells and luteal cells underlines that the region of the PII that responds to cAMP is the same. The role of CLS and NR5A sites for the aromatase cAMP response in this region has been previously demonstrated in granulosa cells [6]. However, a 50% decrease of activity is observed. The involvement of small luteal cells was then assessed by comparison with mixed luteal cells. As shown on Fig. 1, the R-Arom$_{-190}$ and R-Arom$_{-121}$ constructs were not responsive in small as in mixed luteal cells. cAMP increased the activity of the R-Arom$_{-882}$ and R-Arom$_{-274}$ constructs but in small luteal cells, the construct R-Arom$_{-274}$ responded significantly more than the R-Arom$_{-882}$ construct (2.1 ± 0.17 fold vs 1.5 ± 0.09 fold; $P < 0.05$). Thus, in mixed as in small luteal cells, the cAMP responsive region is laid from −274 bp to −190 bp, a sequence that contains the CLS site.

3.2. Binding of CREB to the CLS site of the rabbit CYP19 PII

To determine the identity of the nuclear proteins which can bind to the −217/−188 bp region of the *CYP19* PII, which includes CLS (−208/−200 bp), electrophoretic mobility shift assays were performed with granulosa and luteal nuclear extracts (Fig. 2A). Incubation of the labelled fragment with nuclear extracts from fresh granulosa cells (FGC) showed the formation of two protein–DNA complexes (A and B) (lane 2). When the unlabelled fragment was added in excess, the disappearance of both complexes was reported (lane 3). Preincubation of nuclear extracts with an antibody directed against the amino-terminal region of CREB was without effect on the lower complex, (complex B) whereas the upper complex (complex A) was recognised and supershifted (SS, lane 4). When granulosa cells were cultured and treated with db cAMP in vitro, (GC dbc) a third protein–DNA complex (complex C) was formed in addition to complexes A and B (lane 5). Complexes A and C were recognised and supershifted by CREB antiserum (lane 6).

Yet again, when nuclear extracts were incubated with the −217/−188 bp oligonucleotide in fresh luteal cells (FLC) instead of fresh granulosa cells, complex A was detected (lane 7). The mobility patterns observed for fresh luteal cell nuclear extract were almost identical as those found in granulosa cell nuclear extract (lanes 2, 7). The relative abundance of complex B was the only apparent difference observed. Compared to FGC nuclear extract, considerably less complex B was formed in FLC nuclear extract (the complex was barely detected in this luteal extract) (lanes 2, 7). The large complex with the same mobility as complex A was shifted in the presence

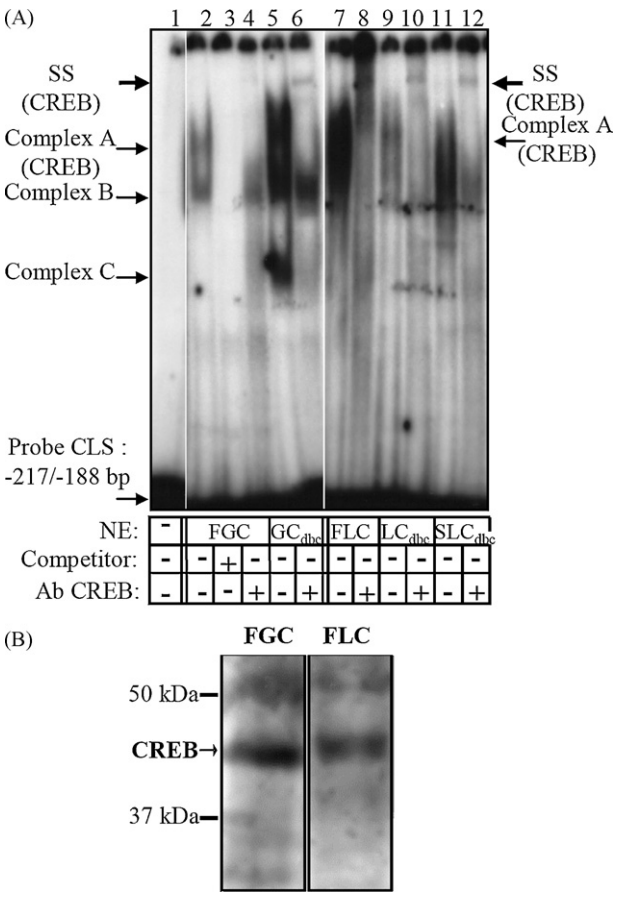

Fig. 2. (A) Nuclear proteins from granulosa and from luteal cells bind to the aromatase CLS site. Nuclear extracts (NE) isolated from fresh granulosa (FGC) and luteal (FLC) cells or from primary cultures of granulosa, luteal and small luteal cells treated with dbc 2.5 mM for 6 h (GC/LC/SLC dbc) were incubated with radiolabeled probe encompassing the CLS site (40,000 cpm) in the presence or absence of non radiolabeled (100×) competitor probe (competitor), in the presence or the absence of antibodies directed against CREB (Ab CREB). The *large solid arrow* indicates the CREB supershift. DNA/protein complexes were separated from free probe by gel electrophoresis. B. Western blot analysis of CREB expression in rabbit ovarian cells. CREB protein was examined by Western analysis using 30 μg of nuclear extracts isolated from granulosa cells (FGC) and luteal cells (FLC).

of a CREB antibody (lane 8). This complex was also observed using mixed luteal cells cultured with db cAMP (LC dbc, lane 9) and fresh small luteal cells (FSLC, lane 11). In these cases too, complex A was shifted in the presence of a CREB antibody (lanes 10, 12).

Western blot analysis was performed to define the size of the CREB factor in rabbits using the same CREB antibody as used in EMSA (Fig. 2B). A band at 42 kDa, was observed in fresh granulosa cells and fresh luteal cells. These data support the hypothesis that granulosa and luteal rabbit cells express CREB protein have some sort of CREB–DNA binding activity.

3.3. Binding of NR5A2 and NR5A1 to the rabbit NREA site of the CYP19 PII.

In luteal cells, the degenerate NREA site in the −144/−115 bp region of the rabbit *CYP19* PII is not involved in the AMPc regulation of aromatase expression as we have observed in granulosa cells. To determine the binding characteristic of NREA site (−133/−126 bp), nuclear extracts from fresh rabbit GC were used in electrophoretic gel mobility shift assays (Fig. 3A). Incubation of the labelled fragment with nuclear extracts from fresh granulosa cells formed one prominent complex (lane 1). An equivalent band was observed

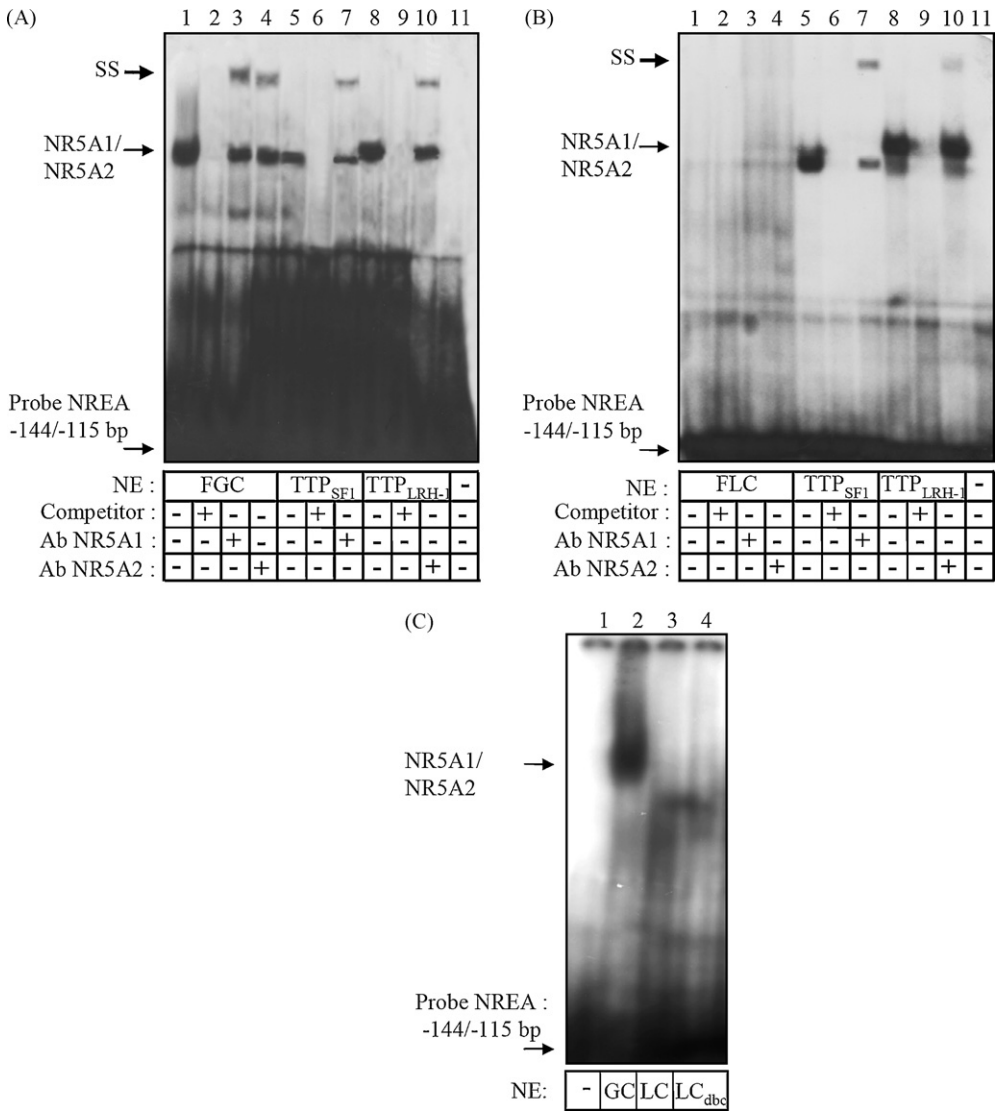

Fig. 3. Binding of nuclear proteins to the NRE site. Fresh granulosa (A) and fresh luteal (B) nuclear extracts or in vitro transcribed/translated mouse NR5A2/NR5A1 (TTP) were incubated with radiolabeled probe encompassing the rabbit NREA site (40,000 cpm) in the presence or absence of non radiolabeled (100×) competitor probe (competitor), in the presence or the absence of antibodies directed against NR5A2 (Ab NR5A2) or NR5A1 (Ab NR5A1). The *large solid arrow* indicates the NR5A1 and NR5A2 supershift. DNA/protein complexes were separated from free probe by gel electrophoresis. (C) In vitro granulosa cells nuclear extract compared with nuclear extracts of luteal cells treated (LCdbc) or not (LC) with db cAMP for 6 h.

using in vitro synthesized NR5A1 (lane 5) and NR5A2 (lane 8). These complexes were competed with unlabelled oligonucleotide containing homologous sequences (lanes 2, 6 and 9). Preincubation of nuclear extracts with an anti-NR5A1 antibody partially abolished the formation of the major complex (lane 3) as well as the complex formed with in vitro synthesized NR5A1 (lane 7). Similarly, preincubation with a NR5A2 antibody abolished in part the formation of the major complex (lane 4) as well as the complex formed with in vitro synthesized NR5A2 (lane 10). Since the prominent complex was supershifted by both anti-NR5A1 and anti-NR5A2 antibodies (Fig. 3A, lanes 3, 4, SS) we can conclude that granulosa cells express NR5A1 and NR5A2 activating transcription factors and present an NR5A1/NR5A2-DNA binding activity on the −133/−126 region of the rabbit *CYP19* PII promoter. However, these data do not exclude the possibility that other factors may also bind to NREA in ovarian cells.

We next performed EMSA experiments using extracts from fresh luteal cells (Fig. 3B). The major complex observed in fresh granulosa cells extracts was not found in fresh luteal cells extracts (lanes 1–4) in spite of the large quantity of nuclear extract used (100 μg). Specific bands were however obtained using the synthetic proteins as positive controls (lanes 5, 8). We confirmed that nuclear proteins do not bind to the −144/−115 bp region of the *CYP19* PII using nuclear proteins extracted from luteal cells that were cultured with db cAMP (Fig. 3C, lane 4).

3.4. NR5A2 and NR5A1 expression in rabbit ovarian cells

Our results showed a binding capacity of the NREA site only in granulosa cells. We investigated whether NR5A2 and NR5A1 protein were expressed in luteal cells. Their expression was monitored by Western blot analysis using nuclear proteins extracted from fresh FGC, FLC and from GC cultured with or without dbcAMP. In vitro-translated NR5A1 and NR5A2 were used as positive control. The two antibodies did not cross react and bands of the expected size were obtained for both in vitro-translated factors. A main band with an apparent molecular mass of 56 kDa was observed when the NR5A2 antiserum was incubated with nuclear proteins extracted from

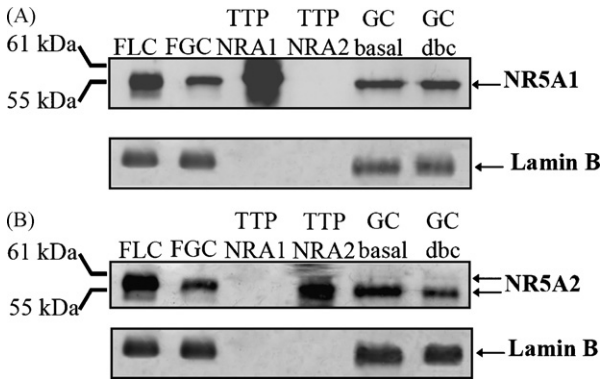

Fig. 4. Western blot analysis of NR5A1 and NR5A2 expression in rabbit ovarian cells. NR5A1 (A) and NR5A2 (B) proteins were examined by Western analysis using 100 μg of nuclear extracts isolated from luteal cells (FLC), granulosa cells (FGC), primary cultures of GC untreated (GC basal) or treated with dbc 2.5 mM for 6 h (GC dbc). In vitro-translated NR5A1/NR5A2 (TTP) served as positive controls. The same nuclear extracts were run on two different gels, transferred to membranes, one probed for NR5A1 and the other for NR5A2. One representative experiment from three independent experiments is shown. Each blot was stripped and riprobed for Lamin B used to normalized protein content. Blots are representative of three independent experiments with similar results.

fresh granulosa cells, fresh luteal cells, and cultured cells (Fig. 4A). Western blot analysis of the NR5A1 protein in fresh/cultured granulosa cell nuclear extracts and in fresh luteal nuclear cell extracts showed the expression of a protein that migrated at the expected size (Fig. 4B). Blots were also stripped and reprobed with a different antibody, directed against lamin B. These experiments confirmed that the same amount of total protein was loaded for each sample. Hence, we established that NR5A1 and NR5A2 are more highly expressed in LC than in GC.

4. Discussion

In this study, we investigated which factors mediate *CYP19* PII regulation by cAMP by comparing luteal and granulosa cells. It is clear that there is a critical participation of CLS and CREB in the cAMP-dependent regulation of PII activity in both granulosa and luteal cells. NR5A2 and NR5A1 are expressed in both cell types, although it is more prominent in luteal cells. The role of both factors in the regulation of *CYP19* PII remains however uncertain. In rabbit, the decrease in PII activity observed during the transition granulosa cells–luteal cells is not a consequence of a cessation in CLS activity.

The present data suggest that CLS, which mediates *CYP19* transcription in granulosa cells, also mediates the cAMP-dependent activation of PII in luteal cells. Studies performed on other species report that CLS is necessary for the cAMP-dependent activation of PII in granulosa cells [4,5,7,14]. In agreement with these studies, our data show that CREB is expressed in rabbit granulosa cells and forms a complex with CLS.

The role of CREB/CLS in the corpus luteum is not as well documented as in granulosa cells. In rat and human luteal cells, as in rabbits, estrogen synthesis is observed [20,21,18]. This does not seem to be the case for ovine and bovine corpora lutea [1,22]. Hinshelwood et al. [5] suggested that this difference may be linked to the lack of consensus CLS in the PII of the latter species. Nevertheless, in rat, Stocco et al. [14] suggest that the control *CYP19* promoter activity in the luteal cells is not dependent of CLS but it seem dependent of GATA, NRE and AP3 sites.

In our studies, we identified that, in luteal cells, as in granulosa cells, an identical region of the PII (−274/−193 bp) is required for cAMP induced transcription. We located CLS (−208/−200 bp), which was found to be the major cAMP responsive element maintained throughout the luteal phase within the PII region. We also showed the expression of CREB in luteal cells and its binding on CLS. In agreement with our study, deletion and mutagenesis experiments performed using human PII showed that CLS is a necessary element for the cAMP-dependent *CYP19* PII activity in luteal cells [7]. Curiously, in Rhesus monkey corpus luteum/corpora lutea, PKA activity is maintained, but the expression of CREB is decreased after ovulation and during luteinization [8,23].

The relative decrease in complex B observed in luteal cells could add to the down-regulation of *CYP19* transcription observed after the LH peak. Since complex B formed in the electrophoretic gel mobility shift assay was not altered by the antibody directed against CREB, it is suggested that other factors differing from CREB family factors must therefore be involved. Previously, we suggested that, as well as CLS, additional sites in the rabbit promoter of aromatase could participate in activating the aromatase gene in granulosa cells, in response to cAMP [6]. We described several potential sites whose CAAT-box is proximal to CLS and within the probe used in EMSA. This site could be involved in granulosa cell *CYP19* regulation by involvement of CCAAT enhancer-binding protein alpha or beta factors (C/EBPalpha/beta factors). All the more so, these factors, in rats, are regulated by FSH in vitro in granulosa cells [24] or by FSH and LH during follicle development [25].

We report in rabbit granulosa cells, the expression of NR5A1 and NR5A2 and their binding on the rabbit NREA site. This binding seems contradictory to the lack in functionality of the rabbit NREA site demonstrated by a gene reporter study [6]. An EMSA performed with a probe containing the human NREA site as the competitor does not show any difference in the ability of the site to bind NR5A1/NR5A2 factors (data not shown). However, cotransfection experiments confirm the weak affinity of the rabbit NREA site for NR5A1 [6]. In the same way, Duggavathi et al. [26] have shown that in mice, NR5A2 may not be directly required for *Cyp19* expression in granulosa cells of growing follicles.

NR5A2 is expressed in several nonsteroidogenic tissues like the pancreas, intestine, and colon [27] as well as in several steroidogenic tissues including ovaries and testes, where NR5A2 plays an important role in the regulation of genes encoding steroidogenic enzymes [10,28,29]. By immunohistology, the expression of NR5A1 and NR5A2 mRNA were reported in the corpus luteum of horse [30], rat [31] and mouse [10] and interestingly, the expression of NR5A2 correlates with *CYP19* expression in the ovary, suggesting a potential role of this factor in the persistence of estrogens secretion by luteal cells. In addition, the functionality of NREA within the human PII has been shown by a gene reporter experiment conducted in bovine luteal cells [7]. However, to date, the binding of NR5A1/NR5A2 to *CYP19* PII has been demonstrated only in rat luteal cells [14]. Our results show that NR5A1/NR5A2 factors, present in luteal cells, do not bind NRE site and so do not modulate rabbit *CYP19* expression in the corpus luteum.

Several hypotheses could explain the lack of binding of *trans* factors to the NREA site in the corpus luteum. In rat preovulatory follicles, hCG down-regulates the binding of NR5A1 to the PII [9]. However, the lack of binding could be explained by the presence of a co-repressor, expressed in luteal cells. DAX-1 has previously been demonstrated to block steroidogenesis at multiple levels [32] including *CYP19* expression (reviewed by [33]). Moreover, in many cases, DAX-1 has been shown to repress NR5A1 and NR5A2-mediated transcriptional activity [31,35,34], an inhibitory effect that could involve direct interaction of NR5A1 [35] or NR5A2 [36]. However, it seems that the direct interaction of DAX-1 with NR5A1 does not interfere with NR5A1 binding to DNA in gel mobility shift assays [32,37]. The chick ovalbumin upstream promoter factor (COUP-TF) decreases NR5A1 induced activation of many genes including aromatase [38]. COUP-TF is expressed in theca cells and luteinized theca cells [39]. In the corpus luteum, COUP-TF down-

regulates transcription of bovine oxytocine [40]. The inhibitory mechanism seems to be due to competition between NR5A1 and COUP-TF for occupancy of the NRE site [38].

Another possible explanation for the observed lack of binding is that NR5A1/NR5A2 proteins are modified by effectors downstream of LH-induced signalling pathways. For example, NR5A1 can be acetylated [41] and both NR5A1 and NR5A2 can be sumoylated [42] or phosphorylated by MAPK [43] or by ERK [44]. Phosphorylation of NR5A1 results in increased interactions with co-regulators [43]. A recent study shows that both NR5A1 and NR5A2 are dependent on activated ERKs in order to maintain full transcriptional activity [44]. This could indicate that changes in the status of these proteins – an increase in acetylation or lack of phosphorylation – could change promoter occupancy in luteal cells.

In conclusion, in rabbit unlike to rat luteal cells, CLS remains functional after the LH surge. The persistence of CLS functionality in luteal cells suggests that additional sites are necessary in the post-LH surge down-regulation of *CYP19* transcription. Moreover, even though the NREA site is involved in the regulation of aromatase expression in rat granulosa and luteal cells, we show that in rabbit granulosa and luteal cells, this site is not necessary to activate the PII in response to cAMP. So, although cAMP is the major regulator of the proximal promoter of aromatase across species, the elements necessary for the expression of aromatase differs between species.

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