Characterization and functional analysis of the 5′-flanking region of the mouse 20α-hydroxysteroid dehydrogenase gene

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INTRODUCTION

20α-Hydroxysteroid dehydrogenase (20α-HSD), which metabolizes progesterone to an inactive steroid in the corpus luteum of mice and rats but not of humans, is thought to play a crucial role in shortening the oestrous cycles in these rodent species. We determined the nucleotide sequence of the 5′-flanking region of the mouse 20α-HSD gene, and examined its promoter activity using a rat luteinized granulosa cell culture. A reporter assay, using reporter constructs of various lengths of the 5′-flanking region, revealed that the region between −83 and 60 bp upstream of the transcription start site was essential for transcriptional activity. Furthermore, mutational analysis demonstrated that a putative Sp1 site in this region was critical to the expression of the reporter gene. Electrophoretic mobility-shift assays showed that the interaction of proteins in a nuclear extract from rat luteinized granulosa cells with this region was inhibited by a competitor having the wild-type Sp1 sequence in its promoter, but not a mutated Sp1 sequence. Supershift analysis confirmed that Sp1 and Sp3 were present in the nuclear extract of these cells, and that these factors bound to the element. Finally, promoter activity was elevated by the cotransfection of an Sp1 expression vector, and, to a lesser extent, by an Sp3 expression vector, supporting further the involvement of these factors in the expression of the 20α-HSD gene.

Key words: 20α-hydroxysteroid dehydrogenase, ovary, promoter, Sp1, Sp3.

MATERIALS AND METHODS

Reagents

Restriction enzymes, T4 DNA ligase and Klenow enzyme were purchased from Takara (Shiga, Japan). Oligonucleotides were obtained from Sigma Genosys Japan (Hokkaido, Japan). DpnI was obtained from New England Biolabs (Beverly, MA, U.S.A.). PMSG (pregnant mare’s serum gonadotropin) and hCG (human chorionic gonadotropin) were purchased from Teikoku Zoki (Tokyo, Japan). DMEM (Dulbecco’s modified Eagle’s medium)/F-12 (1:1, v/v), FBS (fetal bovine serum), penicillin–streptomycin and Fungizone were obtained from Invitrogen (Carlsbad, CA, U.S.A.). Heparin and Mops were purchased from Dojin Chemicals (Kumamoto, Japan). Protease inhibitor cocktail was purchased from Sigma (St Louis, MO, U.S.A.). Poly(di-dc) was obtained from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Antibodies against Sp1 (PEP2) and Sp3 (D-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). pCMV-Sp1 and pCMV-Sp3 expression vectors were kindly provided by Dr G. Suske (University of Marburg, Germany). The other reagents were purchased from Wako (Osaka, Japan).

Determination of the sequence of the mouse 20α-HSD gene 5′-flanking region

The λFIX II mouse genomic library was screened, and 20α-HSD genomic clones containing the 5′-flanking region of the gene were...
isolation as described previously [11]. The 5'-flanking region of the mouse 20α-HSD gene was excised by restriction enzymes and subcloned into pBluescript SK plasmid (Stratagene, La Jolla, CA, U.S.A.). The nucleotide sequences were determined by digesting further with the appropriate restriction enzymes, subcloning into plasmids, and then sequencing.

DNA sequence analysis

The 5'-flanking sequences of the mouse 20α-HSD gene were compared with those of the rat 20α-HSD gene (accession no. AF084365) with DNASeis software (Hitachi, Tokyo, Japan). Transcription Element Search Software (TESS; http://www.cbil.upenn.edu/tess/) was used to analyse the 5'-flanking region of the mouse 20α-HSD gene.

Construction of luciferase reporter vectors

DNA fragments of sizes 2.6, 2.2, 1.7, 1.1 and 0.6 kb were excised from the pBluescript SK plasmid with EcoRV for the 3' end, and with appropriate enzymes (KpnI, SalI, BamHI, BglII or SalI respectively) for the 5' end. They were ligated to a pGL3-Basic luciferase reporter vector (Promega, Madison, WI, U.S.A.) and digested with NcoI, followed by a fill-in reaction and appropriate enzymes (KpnI, XhoI or BglII). DNA fragments of nt −255/+58, −132/+58, −103/+58, −92/+58, −83/+58, −60/+58 and −35/+58 were amplified by PCR using a 0.6 kb construct as a template, with primers as shown in Table 1. They were subcloned into pGEM-T vector (Promega), sequenced, digested with SalI and EcoRI and ligated to the vectors prepared from the 0.6 kb construct.

Introduction of mutations in a putative Sp1-binding site

Mutations of a putative Sp1-binding site were introduced by PCR using the primers shown in Table 1. PCR was performed with Pfu Turbo DNA polymerase (Stratagene) and pGEM-T, into which the DNA fragment of nt −255/+58 was subcloned as a template. For construction of the deletion mutant, primers were phosphorylated at the 5' ends with T4 polynucleotide kinase before the reaction, and the PCR product was self-ligated with T4 DNA ligase. After reaction with DpnI for digestion of the template plasmid, the solution was used for transformation and the mutant plasmids were obtained. They were sequenced and subcloned into the luciferase vector, as described above.

Rat luteinized granulosa cell culture

Twenty-day-old immature female Wistar–Imamichi rats housed in our laboratory were injected subcutaneously with 50 units of PMSG, and 42 h later intraperitoneally with 10 units of hCG. After 6 h, the rats were killed by decapitation, and the ovaries were isolated and collected in DMEM/F12 (1:1, v/v) without FBS. In a clean bench, the follicles were punctured with a 27-gauge needle, and the luteinized granulosa cells were recovered. The cells were mechanically dispersed by pipetting, filtered through a nylon mesh, centrifuged and suspended in DMEM/F12 (1:1, v/v) with 5% (v/v) FBS. Luteinized granulosa cells were cultured on a collagen type I-coated dish (Iwaki, Chiba, Japan) at a density of 3 × 10⁶ cells/ml. After a 24 h incubation, the medium was changed and the cells were cultured for an appropriate time [24, 48 and 84 h for Northern hybridization, 48 h for transfection, and 24–48 h for EMSA (electrophoretic mobility-shift assay) experiments respectively]. Cells (6 × 10⁵ cells/well in 6-well plates, 1.5 × 10⁶ cells/well in 24-well plates and 10⁵ cells/well in 60 mm plates) were used for Northern hybridization, transfection and EMSA respectively.

Northern hybridization

Cells were washed with PBS, and total RNA was extracted with TRIzol® Reagent (Invitrogen), according to the manufacturer’s instructions. RNA was precipitated with 2-propanol and dissolved in diethylypyrocarbonate-treated water. RNA (5–10 µg) was fractionated by electrophoresis in a 1% (w/v) agarose gel with Mops/formalin, and stained with ethidium bromide to confirm that equal amounts of RNA were loaded in each lane. After the gels were washed with distilled water, RNA was transferred to a Biodyne B nylon membrane (Pall, East Hills, NY, U.S.A.). Full-length rat 20α-HSD cDNA [12] was labelled with [α-³²P]dCTP using a Random Primed Labeling Kit (Roche, Mannheim, Germany) and purified with a ProbeQuant G-50, allowed to proceed in 5 × Denhardt’s solution (where 1 × Denhardt’s solution is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% BSA), 6 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.5%
SDS, 50% formamide and 100 μg/ml denatured fragmented salmon sperm DNA containing [α-32P]dCTP-labelled probe (1 × 10^6 c.p.m./ml) at 42 °C overnight. Membranes were washed with 2 × SSC/0.1% SDS at 55 °C, and exposed to an X-ray film (Kodak, Tokyo, Japan).

Transfection experiments

Samples (0.75 μg) of each experimental vector plus 0.15 μg of pRL-TK control vector (Promega) were mixed with 3 μl of FuGENE6 Transfection Reagent (Roche) in 100 μl of Opti-MEM (Invitrogen) and incubated at room temperature (≈25 °C) for 15 min, after which the FuGENE6–DNA complex was added to each well containing cells that were pre-incubated at 37 °C for 24 h followed by exchange of medium with 500 μl of Opti-MEM. Following incubation at 37 °C for 3 h, the medium was changed to DMEM/F12 (1:1) containing 5% fetal bovine serum (FBS). Cells were washed with PBS, cells were collected with a cell scraper, and the pellet was resuspended in 200 μl of binding buffer [10 mM Hepes/KOH (pH 7.8)/250 mM KCl/5 mM EDTA/25 mM MgCl2/50% (v/v) glycerol], followed by incubation for 30 min at 4 °C, with vortex-mixing every 10 min. After centrifugation at 17 400 g (15 000 rev./min) for 15 min at 4 °C, the supernatant was recovered and then used for EMSA.

Preparation of nuclear extracts

After washing with PBS, cells were collected with a cell scraper, recovered in a microtube and pelleted by centrifugation. Cells were suspended in 400 μl of buffer A [10 mM Hepes/KOH (pH 7.8)/10 mM KCl/0.1 mM EDTA/0.1% Nonidet P40/1 mM dithiothreitol/protease inhibitor cocktail (1:1000, v/v)], vortex-mixed and centrifuged at 1900 g (5000 rev./min) for 1 min at 4 °C. The supernatant was removed, and the pellet was resuspended in 50 μl of buffer B [50 mM Hepes/KOH (pH 7.8)/420 mM KCl/0.1 mM EDTA/5 mM MgCl2/2% (v/v) glycerol/1 mM dithiothreitol/protease inhibitor cocktail (1:1000, v/v)], followed by incubation for 30 min at 4 °C, with vortex-mixing every 10 min. After centrifugation at 17 400 g (15 000 rev./min) for 15 min at 4 °C, the supernatant was recovered and then used for EMSA.

EMSA

Sets of complementary oligonucleotides for probe, wild-type and mutant competitors (Table 1) were annealed in Tris/EDTA buffer by boiling for 5 min, followed by gradual cooling. The oligonucleotides for the probe were designed to have 5'- cohesive ends when annealed. The oligonucleotides for probe were radiolabelled with [α-32P]dCTP and Klenow enzyme, followed by purification with a ProbeQuant G-50 Micro Column (Amersham Biosciences). A DNA–protein-binding reaction was allowed to proceed in a volume of 25 μl, consisting of 5 μl of 5× binding buffer [50 mM Hepes/KOH (pH 7.8)/250 mM KCl/5 mM EDTA/25 mM MgCl2/50% (v/v) glycerol], 1.5 μl of 2 μg/μl poly(dI-dC), 5 μl of nuclear extract and 1 μl of the labelled probe (10^4 c.p.m./μl; specific radioactivity, 10^6 c.p.m./nmol) with or without 2 μl of 4 μM wild-type or mutant competitor for 15 min at room temperature. For supershift analysis, 2 μg of rabbit polyclonal antibodies against Sp1 and/or Sp3 was added and incubated for 1 h at 4 °C, before the addition of the probe. The reaction mixtures were then run on a 4% Tris/borate/EDTA polyacrylamide gel, which was then exposed to X-ray film at −80 °C.

Data analysis

All experiments were repeated at least three times and representative results are shown in the Figures. For the luciferase assay, results are expressed as means ± S.E.M. Differences in luciferase activity were tested by a one-way analysis of variance followed by Sheffe’s multiple comparison test, with STATVIEW 4.0 (Abacus Concepts, Berkeley, CA, U.S.A.), and differences were considered to be significant at P < 0.05.

RESULTS

Determination of the sequences and analysis of putative transcription-factor-binding sites of the mouse 20α-HSD gene 5'-flanking region

Although the draft sequences of the mouse genome have been already reported, to exclude ambiguity of the sequence, the sequences of the 5'-flanking region of the mouse 20α-HSD gene were determined for up to approx. 4.2 kb using the λFIX II genomic clones containing the mouse 20α-HSD gene. The sequence identity between that of the present study and the mouse genome databases was 99.8%. Computer analysis using TESS revealed putative transcription-factor-binding sites, including a single STAT6 (signal transducers and activators of transcription protein 6) binding site at −3 101 to −3 093 bp, three GREs (glucocorticoid-response elements) at −2 710 to −2 692, −673 to −664 and −333 to −322 bp, a single CRE (cAMP-response element) at −2 612 to −2 605 bp, two PRe(s) (progesterone-response elements) at −1 806 to −1 792 and −1 117 to −1 163 bp, and single NF-I (nuclear factor-1) and Sp1-binding sites at −123 to −109 and −75 to −63 bp respectively (Figure 1).

Promoter activities of various lengths of the mouse 20α-HSD gene 5'-flanking region

We used primary cultured rat luteinized granulosa cells to study the mechanisms of 20α-HSD expression, because this system expresses endogenous 20α-HSD mRNA and successfully reproduces the regulation of the gene by PRL and PGE2 [8,9]. As shown in Figure 2, the amount of 20α-HSD mRNA increased spontaneously in a time-dependent manner in these cells, which seems to reflect the spontaneous rise in 20α-HSD expression in the luteal tissue of rats during the oestrous cycle. Both an inhibition and a stimulation of 20α-HSD mRNA expression by PRL
Expression of the endogenous 20α-HSD mRNA in rat luteinized granulosa cells

After 24 h of culture, the medium was changed and incubated further for 12, 24, 48 and 84 h. Total RNA was extracted and used for Northern hybridization with rat 20α-HSD cDNA as a probe.

Promoter activities of the serially deleted 5′-flanking region of the mouse 20α-HSD gene

DNA segments [−2.6, −2.2, −1.7, −1.1, −0.6 and −0.25 kb (A)] and −255, −132, −92, −83, −60 and −35 kb (B)] of the 5′-flanking region of the mouse 20α-HSD gene were fused to a luciferase reporter gene (luc), and rat luteinized granulosa cells were transfected with the constructs. Results (means ± S.E.M. for triplicate wells) are presented as a percentage of relative luciferase activity of −2.6 k (A) or −255 k (B) construct. Promoter activity of pGL3-Basic is also shown. *P < 0.05 compared with the −255-luc construct.

Promoter activities of the serially deleted 5′-flanking region of the mouse 20α-HSD gene

As the putative Sp1 element was found between −83 and −61 bp, we examined the effect of deleted (−255(delSp1)-luc) or substituted (−255(mutSp1)-luc) mutations of the element on promoter activity in the next experiment (Figure 4A). Compared with the wild-type −255 bp construct, mutant constructs showed markedly reduced promoter activities, similar to those of pGL3-Basic used as a negative control (Figure 4B). To confirm binding of certain nuclear factors to wild-type, but not mutated, Sp1 elements, EMSA was performed with nuclear extracts prepared from rat luteinized granulosa cells. There were three bands in the presence of the nuclear extract, the patterns of which were similar to those in other reports [13]. Addition of an unlabelled wild-type competitor resulted in the disappearance of the bands, whereas addition of an unlabelled mutant competitor had no effect (Figure 5, left panel). To confirm further that the shifted bands contained Sp1 family molecules, a supershift experiment with antibodies against Sp1 and Sp3 was carried out. As shown in Figure 5 (right panel), pre-incubation of the antibody against both Sp1 and Sp3 produced a supershift band, in accordance with disappearance of the uppermost band and the two lower bands respectively. Almost all of the three bands disappeared upon addition of antibodies against Sp1 and Sp3 in combination, and a dense supershifted band appeared.

Effects of co-transfection with Sp1 and/or Sp3 expression vectors on promoter activity

Finally, the involvement of Sp1 and Sp3 in the expression of the 20α-HSD gene was examined by co-transfection experiments. Rat luteinized granulosa cells were co-transfected with −255-luc constructs together with expression vectors pCMV-Sp1 and/or pCMV-Sp3, and the effect on promoter activity was assessed.
The present study demonstrated that the expression of the 20α-HSD gene in the luteal cells required Sp1 and/or Sp3, which are known as ubiquitous transcription factors that bind to the GC/GT-box element found in 5′-flanking regions of many constitutively expressing and inducible genes [14]. Considering that 20α-HSD is not expressed in undifferentiated rat granulosa cells, in which Sp1 and Sp3 are expressed [13], it seems that the tissue specificity of 20α-HSD expression is not determined by these factors, but by other transcription factors and/or specific chromatin structures suitable for transcriptional activation in luteal cells, which may be achieved during the process of luteinization. Whatever the mechanism of the tissue specificity of 20α-HSD, our present results clearly indicate that either Sp1 or Sp3 is indispensable for the regulation of 20α-HSD gene expression.

The Sp1 family consists of four members: Sp1, Sp2, Sp3, and Sp4. Sp1 and Sp3 have been extensively studied, and are known to be co-expressed in several tissues and to interact with an identical consensus sequence. There are reports that Sp1 is phosphorylated [15] and glycosylated [16], whereas Sp3 is acetylated at a site known as an inhibitory domain [17]. Although the general role of these modifications in Sp1- or Sp3-mediated transcriptional regulation is not clear, it seems reasonable to consider the involvement of such modifications in the hormonal regulation of the 20α-HSD gene.

Another possibility is that hormones regulate the expression of the 20α-HSD gene by modifying the expression level of Sp1 and Sp3. For example, it has been reported that the level of the Sp3 protein is reduced in the interferon-γ-mediated suppression of macrophage lipoprotein lipase gene transcription. In contrast, the DNA-binding activity of Sp1 is decreased without affecting the protein levels by the cytokine [18]. It would be necessary to examine whether the level of Sp1 or Sp3 is regulated by hormones such as PGF2α, PRL or other factors which regulate the expression of the 20α-HSD gene.

In the present study, both Sp1 and Sp3 elevated the promoter activity of the 5′-flanking region of the mouse 20α-HSD gene. The role of Sp3 is complicated by the fact that Sp3 functions as a transcriptional repressor, as well being as an activator of Sp1-mediated transcription [19,20]. The role of Sp3 seems to depend on the promoter sequence or on cellular context. The present results indicate that Sp3 is a transcriptional activator for the 20α-HSD gene in luteal cells, but a role as a transcriptional repressor due to its relatively weak effect on transcriptional activity is also possible by increasing the expression ratio of Sp3 to Sp1. Further investigations are necessary to clarify the precise role of Sp1 and Sp3 in the hormonal regulation of the 20α-HSD gene.

In conclusion, the present study has demonstrated that Sp1 family molecules play a significant role in the expression of the 20α-HSD gene in luteal cells. Further characterization of the 5′-flanking region of the 20α-HSD gene, including comparative analyses between species, will be useful in understanding the general mechanisms of tissue-specific expression, and for providing an insight into the molecular evolution, as well as the regulatory mechanisms, of 20α-HSD gene expression. The present study is a prerequisite for such comparative analyses.

REFERENCES


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