Molecular cloning of equine 17 β -hydroxysteroid dehydrogenase type 1 and its downregulation during follicular luteinization *in vivo*

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Abstract

The type 1 form of 17 β -hydroxysteroid dehydrogenase (17 β HSD1) was the first isoform to be identified and is capable of converting estrone to 17 β -estradiol. This study was aimed at characterizing the molecular structure of the equine 17 β HSD1 gene and cDNA, as well as its molecular regulation during human chorionic gonadotropin (hCG)-induced follicular luteinization/ovulation *in vivo*. The equine 17 β HSD1 gene was cloned from an equine genomic library and shown to have a conserved genomic structure composed of six exons. Its cDNA sequence was also identified and coded for a 308 amino acid protein, 72.1–74.5% homologous to other mammalian orthologs. RT-PCR/Southern blot analyses were performed to study the regulation of the 17 β HSD1 transcript in equine preovulatory follicles isolated between 0 and 39 h after hCG treatment. Results demonstrated the presence of high 17 β HSD1 mRNA expression prior to hCG treatment with a marked decrease observed 12 h after hCG ($P < 0.05$). Analyses on isolated preparations of granulosa and theca interna cells identified the granulosa cell layer as the site of 17 β HSD1 transcript expression and downregulation ($P < 0.05$). A 1412 bp fragment of the equine 17 β HSD1 proximal promoter was sequenced and shown to contain many putative transcription factor binding sites. Electromobility shift assays (EMSA) using a fragment of the proximal promoter (–230/–30) and nuclear extracts prepared from granulosa cells isolated prior to hCG (0 h post-hCG) revealed the presence of a major complex, and results from competition assays suggest that steroidogenic factor-1 (SF-1), NF κ B, GATA, and Sp1 *cis*-elements are involved. Supershift assays using an antibody against the p65 subunit of NF κ B led to the displacement of the binding nuclear proteins to the DNA probe, whereas the use of an anti-equine SF-1 antibody demonstrated the clear formation of a DNA–protein–antibody complex, confirming the potential role of these transcription factors in EMSA results. Interestingly, a notable decrease in DNA binding was observed when granulosa cell nuclear extracts isolated 30 h post-hCG were used, which paralleled the decrease in 17 β HSD1 transcript after hCG treatment. Thus, this study is the first to report the gonadotropin-dependent downregulation of 17 β HSD1 transcript expression in a monoovulatory species, the presence and regulation of protein/DNA interactions in the proximal region of the 17 β HSD1 promoter during gonadotropin treatment, and the characterization of the primary structure of equine 17 β HSD1 cDNA and gene.

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Introduction

The biosynthesis of ovarian steroid hormones requires a complex enzymatic cascade that ultimately involves the enzyme, 17 β -hydroxysteroid dehydrogenase (17 β HSD), in the production of both androgen and estrogen secretory products. Twelve types of 17 β HSDs have thus far been described, and numbered in the order in which their DNA sequences were determined. They are all members of the short-chain dehydrogenase/reductase superfamily, except for type 5, which belongs to the aldo–keto reductase superfamily (Adamski & Jakob 2001, Baker 2001, Luu-The 2001, Luu-The *et al.* 2005). These 17 β HSDs catalyze the interconversion between less active 17-ketosteroids (i.e. low receptor affinity) and more active 17 β -hydroxysteroids (i.e. high receptor affinity) such as estrone and

17 β -estradiol respectively (Penning 1997, Peltoketo *et al.* 1999). The occurrence of different substrate specificities, cofactor preference, subcellular localizations, and tissue distributions allows 17 β HSDs to dictate the biological potency of androgens and estrogens in mammals.

Type 1 17 β HSD (17 β HSD1) was the first 17 β HSD to be characterized. Its cDNA was first cloned from human placenta and was shown to encode a cytosolic protein of 327 amino acids (Peltoketo *et al.* 1988, Luu The *et al.* 1989). It has been shown to preferentially catalyze the reduction of estrone to 17 β -estradiol using NADP(H) as a cofactor in humans (Dumont *et al.* 1992, Lin *et al.* 1992), whereas its substrate specificity in rodents is broader as it includes androstenedione (Nokelainen *et al.* 1996, Mustonen *et al.* 1997). Tissue distribution analyses revealed 17 β HSD1 transcript expression in the ovary,

placenta, breast, endometrium, prostate, skin, and adipose tissue (Peltoketo *et al.* 1988, Dumont *et al.* 1992).

In mammals, the preovulatory surge in luteinizing hormone is responsible for the process of follicular luteinization, which is accompanied by dramatic changes in follicular steroidogenesis, including the decreased biosynthesis of 17 β -estradiol (Fortune 1994, Zeleznik 1994, Murphy 2000). The marked decrease in expression of cytochrome P450 enzymes, such as P450 aromatase (CYP19A1) and P450 17 α -hydroxylase/C17-20 lyase (CYP17A1), has been used to explain this loss in 17 β -estradiol biosynthetic capacity (Fortune 1994, Richards 1994, Liu *et al.* 1999). To date, no attempts have been made to elucidate the regulation of enzymes required for the activation of these estrogens, such as 17 β HSD1, during the luteinization process in mono-ovulatory species. The present study uses the equine preovulatory follicle as a model to investigate the regulation of 17 β HSD1 during gonadotropin-induced ovulation/luteinization. The specific objectives were to clone the equine 17 β HSD1 gene and cDNA, and determine the regulation of its mRNA in preovulatory follicles following human chorionic gonadotropin (hCG) treatment, as well as begin characterizing interactions at the level of the 17 β HSD1 promoter.

Materials and methods

Materials

The QuickHyb hybridization solution and the equine genomic library were obtained from Stratagene Cloning Systems (LaJolla, CA, USA); [α -³²P]dCTP was purchased from Perkin-Elmer Canada, Inc. (Woodbridge, Ontario, Canada); the Prime-a-Gene labeling system and pGEM-T Easy Vector System I were obtained from Promega Corp.; the Expand High Fidelity DNA polymerase was purchased from Roche Diagnostics (Laval, Québec, Canada); the plasmid pcDNA3.1, SuperScript II reverse transcriptase, TRIzol total RNA isolation reagent, 1 kb DNA ladder, 5'-rapid amplification of cDNA ends (RACE) system (version 2.0), and synthetic oligonucleotides were obtained from Invitrogen Life Technologies; the Qiagen OneStep RT-PCR System was purchased from Qiagen, Inc.; Biotrans nylon membranes (0.2 μ m) were obtained from ICN Pharmaceuticals, Inc. (Montréal, Québec, Canada); Bio-Rad Protein Assay and all electrophoretic reagents were obtained from Bio-Rad Laboratories; hCG was purchased from The Butler Co. (Columbus, OH, USA); and poly (dI/dC) was obtained from Amersham Pharmacia Biotech. Polyclonal antibodies against p65 nuclear factor (NF) κ B (catalog number sc-372X), p50 NF κ B (sc-7178X), and GATA-4 (sc-9053x) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA,

USA). The equine-specific SF-1 protein was produced by the Sheldon Biotechnology Center, McGill University (Montreal, PQ, Canada).

Characterization of the equine 17 β HSD1 cDNA and gene

The equine 17 β HSD1 cDNA was characterized by a combination of RT-PCR, 5'-rapid amplification of cDNA ends (5'-RACE) and genomic cloning. A cDNA fragment was first isolated by RT-PCR using sense primer 1 and anti-sense primer 2 designed by sequence alignments of 17 β HSD1 homologs from other species, 100 ng RNA obtained from a preovulatory follicle isolated prior to hCG administration (0 h; see below), and the Qiagen OneStep RT-PCR System as directed by the manufacturer (Fig. 1A; RT-PCR1). As a second approach, the 5' end of equine 17 β HSD1 was characterized using the 5'-RACE system version 2.0 (Invitrogen Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed using anti-sense primer 3 (Fig. 1A; 5'-RACE) and 3 μ g RNA from a preovulatory follicle isolated prior to hCG administration. The first 5'-RACE/PCR was accomplished with sense abridged anchor primer 4 (Invitrogen Life Technologies) and anti-sense primer 5, whereas the second 5'-RACE/PCR employed the sense abridged universal amplification primer 6 (Invitrogen Life Technologies) and anti-sense primer 7 (Fig. 1A; 5'-RACE). PCRs consisted of 35 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min. RT-PCR and 5'-RACE cDNA products were subcloned into the pGEM-T Easy plasmid vector (Promega), and sequenced by the Service de Séquençage de l'Université Laval (Québec, Canada).

Despite numerous attempts to clone the remaining 3' end of the open reading frame, they remained unsuccessful. As a second approach, an equine genomic library (Stratagene) was screened with the equine RT-PCR 17 β HSD1 cDNA fragment, as previously described (Liu *et al.* 1999). The probe was labeled with [α -³²P]dCTP using the Prime-a-Gene labeling system (Promega) to a final specific activity greater than 1×10^8 c.p.m./ μ g DNA, and hybridization was performed at 68 °C with QuickHyb hybridization solution (Stratagene). Positive clones were plaque purified through secondary and tertiary screenings, and DNA sequencing was performed commercially as described earlier. The equine 17 β HSD1 genomic sequence was used to design an oligonucleotide 3' to the coding region. This primer, as well as another oligonucleotide designed from the 5' end of the cDNA (primers 8 and 9; Fig. 1A; 5'-RACE), allowed RT-PCR amplification of the complete open reading frame, thereby confirming the contiguous cDNA sequence presented herein (Fig. 1A; RT-PCR2). The complete equine genomic sequence was obtained after a

Figure 1 Cloning strategy for equine 17 β HSD1. (A) The open reading frame (ORF) of the equine 17 β HSD1 cDNA is depicted as an open box, whereas the 5'- and 3'-untranslated regions (UTR) are shown as lines; the size in base pairs of each element is given in parenthesis. Equine 17 β HSD1 was characterized by a combination of RT-PCR, 5'-RACE, and a second round of RT-PCR with an anti-sense primer obtained through genomic cloning and encompassing the entire ORF, as described in Materials and Methods; arrows and numbers show the relative position, orientation, and identity of oligonucleotides used in each cloning procedure. (B) List of oligonucleotides used for equine 17 β HSD1 cloning. The abridged anchor primer (4) and abridged universal amplification primer (6) are components of the 5'-RACE system (Invitrogen Life Technologies). (C) Sequence of 17 β HSD1 cDNA. 5'- and 3'-UTRs are shown in lowercase letters, whereas the ORF is presented in uppercase letters with the start (ATG) and stop (TGA) codons in bold. The complete nucleotide sequence of the equine 17 β HSD1 cDNA was submitted to GenBank (Accession number DQ418451).

number of sequencing reactions using equine 17 β HSD1-specific primers. A comparison of the cDNA and the resulting genomic DNA sequences was used to determine the gene structure of equine 17 β HSD1 as well as the exon-intron junctions (Fig. 2).

Equine tissues

Equine preovulatory follicles and corpora lutea were isolated at specific stages of the estrous cycle from Standardbred and Thoroughbred mares, 3–10 years old and weighing approximately 375–450 kg, as previously

Figure 2 Equine 17 β HSD1 gene structure. (A) Schematic representation of the equine 17 β HSD1 gene structure. Exons are shown as boxes and introns are presented as lines. All elements are drawn to scale. Roman numerals indicate the exon number, whereas numbers in parentheses show the number of nucleotides in the exon. Numbers next to asterisk, represent numbers of nucleotides in the exon within the open reading frame. (B) Exon/intron boundaries of the equine 17 β HSD1 gene. Exonic sequences at each splice junction are presented in uppercase letters, whereas intronic sequences are shown in lowercase letters. Numbers in parentheses represent the exact size of the intron. The complete nucleotide sequence of the equine 17 β HSD1 gene was submitted to GenBank (Accession number DQ418450).

described (Sirois & Doré 1997). Briefly, when preovulatory follicles reached 35 mm in diameter during estrus, the ovulatory process was initiated by injection of hCG (2500 IU, i.v.). Ovariectomies were then performed via colpotomy using an ovariometer at 0, 12, 24, 30, 33, 36, or 39 h post-hCG ($n=4-6$ mares/time point). Follicles were dissected into preparations of follicle wall (theca interna with attached granulosa cells) or further dissected into separate isolates of granulosa cells and theca interna, as previously described (Sirois *et al.* 1991). Ovariectomies were also performed on day 8 of the estrous cycle (day 0 = day of ovulation) to obtain corpora lutea ($n=3$ mares). Testicular tissues were obtained from the Large Animal Hospital of the Faculté de Médecine Vétérinaire (Université de Montréal) following a routine castration, whereas other non-ovarian tissues were collected at a local slaughterhouse. All animal procedures were approved by the Institutional Animal Use and Care Committee.

RNA extraction and semi-quantitative RT-PCR/Southern analysis

Total RNA was isolated from tissues with TRIzol reagent (Invitrogen Canada, Inc.), according to manufacturer's instructions using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Montréal, Canada). The OneStep RT-PCR System (Qiagen) was used for semi-quantitative analysis of 17 β HSD1 and rpl7a mRNA levels (control gene) in equine tissues. Reactions were performed as directed by the manufacturer, using sense (5'-ACCTTGCAGTTGGACGTGAGAGA-3') and anti-sense (5'-TCGCGGTACATCTGCTCGCAGT-3') primers specific for equine 17 β HSD1. Sense (5'-ACAGGACATC-CAGCCCAAACG-3') and anti-sense (5'-GCTCCTTTGT-CTTCCGAGTTG-3') primers specific for equine rpl7a were designed from a published sequence deposited in GenBank (Accession no. AF508309). These reactions resulted in the production of 17 β HSD1 and rpl7a DNA fragments of 503 and 516 bp respectively. Each reaction was performed using 100 ng total RNA, and cycling conditions were one cycle of 50 °C for 30 min and 95 °C for 15 min, followed by a variable number of cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The number of cycles used was optimized for each gene to fall within the linear range of PCR amplification, and were 26 and 18 cycles for 17 β HSD1 and rpl7a respectively. Following PCR amplification, samples were subjected to electrophoresis on 2% tris-acetate EDTA (TAE)-agarose gels, transferred to nylon membranes, and hybridized with corresponding radiolabeled 17 β HSD1 and rpl7a cDNA fragments using QuikHyb hybridization solution (Stratagene). Membranes were exposed to a phosphor screen, and signals were quantified by means of a Storm imaging system using the ImageQuant software version 1.1 (Molecular Dynamics, Amersham Biosciences).

Granulosa cell nuclear extracts and electrophoretic mobility shift assays (EMSAs)

Equine granulosa cells were obtained from preovulatory follicles isolated at 0 and 30 h post-hCG and nuclear extracts were prepared as described (Sirois *et al.* 1993, Liu *et al.* 1999). Protein concentration in each extract was determined by the method of Bradford (1976). EMSAs were performed as described (Sirois *et al.* 1993, Liu *et al.* 1999), with minor modifications. Briefly, extracts of nuclear proteins (0.5 μ g/reaction) were incubated with 40 000 c.p.m. of end-labeled -230/-30 17 β HSD1 promoter fragment and 1 μ g poly(dI/dC) (Amersham Biosciences) in a final volume of 20 μ l binding buffer containing 15 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol, and 12% (v/v) glycerol. Cold oligonucleotide pairs, both wild type and mutated, were used in 50 times molar excess in order to determine the identity of bound sequence. When antibodies (Santa Cruz Biotechnology) were used in supershift EMSAs, the nuclear extract was first incubated for 1 h on ice with the antiserum prior to the addition of other reagents. Binding complexes were resolved by 5% acrylamide, 0.5 $\times$ tris-borate EDTA (TBE) gel electrophoresis.

Protein extracts, anti-equine SF-1 antibody, and immunoblot analysis

Preovulatory follicle extracts were prepared as previously described (Filion *et al.* 2001). Briefly, tissue was homogenized and sonicated on ice in TED buffer (20 mM Tris (pH 8.0), 50 mM EDTA, and 0.1 mM diethyldithiocarbamic acid) containing 1.0% Tween. The sonicate was centrifuged at 16 000 *g* for 15 min at 4 °C. The recovered supernatant (whole cell extract) was stored at -80 °C until electrophoretic analyses were performed. Protein concentration was determined by the method of Bradford (1976; Bio-Rad protein assay). Samples (50 μ g proteins) were resolved by one-dimensional SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Filion *et al.* 2001). The equine-specific anti-SF-1 polyclonal antibody was generated as previously described (Brown *et al.* 2004) using a peptide fragment encompassing amino acids Cys²⁴⁸ to Ser²⁶² (Sheldon Biotechnology Center). Membranes were incubated with the polyclonal anti-equine SF-1 antibody (1:1000) and immunoreactive proteins were visualized on Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY, USA) after incubation with the horseradish peroxidase-linked donkey anti-rabbit secondary antibody (1:10 000 dilution) and the enhanced chemiluminescence system (ECL Plus), following the manufacturer's protocol (Amersham Pharmacia Biotech).

Statistical analysis

One-way ANOVA was used to test the effect of time after hCG administration on levels of 17βHSD1 mRNA in samples of follicle wall, corpora lutea, theca interna, and granulosa cells. 17βHSD1 transcript levels were normalized with the control gene rpl7a before analysis. When ANOVAs indicated significant differences ($P < 0.05$), Dunnett's test was used for multiple comparisons of individual means. Statistical analyses were performed using JMP software (SAS Institute, Inc., Cary, NC, USA).

Results

Characterization of the equine 17βHSD1 cDNA, gene, and protein

To clone the equine 17βHSD1 transcript, RT-PCR was performed on ovarian RNA using oligonucleotide primers designed by sequence alignment of 17βHSD1 homologs in other species. The resulting cDNA fragment (Fig. 1A; RT-PCR1) was sequenced and found to be highly homologous to 17βHSD1 transcripts identified thus far. The 5'-RACE reactions yielded a cDNA product corresponding to the remaining 5' end coding regions, as well as the 5'-untranslated region (Fig. 1A; 5'-RACE). An equine genomic library was screened with a cDNA probe obtained by RT-PCR. The genomic sequence of equine 17βHSD1 was determined by performing several sequencing reactions (GenBank Accession number DQ418450) and used to derive the 3' end of the equine 17βHSD1 cDNA, as well as the

genomic structure. It was determined to have six exons (Fig. 2A), identical to what is observed for the human and mouse genes. The exon-intron junctions were also shown to be conserved (Fig. 2B). After sequencing of the 17βHSD1 gene, equine-specific primers were designed and used to amplify a RT-PCR product that extended the entire length of the cDNA open reading frame, thereby confirming that all RT-PCR products were derived from the same transcript (Fig. 1A; RT-PCR2). The deduced 982 bp primary transcript encoded a 924 bp open reading frame (Fig. 1A; GenBank Accession number DQ418451), which predicted a protein of 308 amino acids.

The predicted protein is highly conserved when compared with human (NP_000404), marmoset (AAG01115), rat (AAH86365), and mouse (CAA61770) 17βHSD1 proteins (Fig. 3). Equine 17βHSD1 has 73.3% identity at the amino acid level and 81.2% identity at the nucleic acid level relative to human 17βHSD1 (NM_000413). Homology was lost when the carboxy terminus of the proteins was examined and the proteins also exhibited variability in sizes; the equine 17βHSD1 is 20 amino acids shorter than human 17βHSD1, which is 16 amino acids shorter than both rodent proteins presented (Fig. 3). The marmoset protein may be incomplete in its amino terminus.

Tissue distribution of equine 17βHSD1 mRNA

RT-PCR/Southern blot analyses were used to evaluate the expression of equine 17βHSD1 mRNA in various tissues. High levels of 17βHSD1 transcript were detected in a preovulatory follicle isolated prior to

equ	MDRTIVLITGCGSSGIGLHLALRLASDPSSR	FKVYATLRDLTAQGPLWEMARARGCPPGSL	ETLQLDVRDADSVAAARARVTEGRVDVLC	90
hum	.A..V.....V.....Q.	KT..R..A..LA.....	SK.....E.....	90
mar	-----Q.	M..KT..Q..A..Q..RD..	SK.....N.Q.C.....I..	68
rat	.S.V.....V.....R.Q.	KS.....LEA..Q.....	.I.E.....SE.....C.....	90
mou	.P.V.....M..V.....R.Q.	K.....L.A..TQ.....	.I.E.....SK.....Q.C.....	90
equ	NAGRGLLGPLELHPAGAVASVLDANVAGTV	RTIQAFPLPDMKRRRSGRILVTGSMGGLMGL	PFNAIYCASKFAIEGLCESLAVLLQPFVGH	180
hum	...L.....ALGED.....V..V....	.ML.....G.....V.....V.....	...DV.....L.....L.....	180
mar	...Q.....ALGED..G.....V.....	.ML.....E.....G.....V.....	...DV.....L.....P.....	158
rat	F.....A.ELN..GA..V..L..I	.ML.....H.....V..A.V.....	..HEV.....L.....I.....PL....	180
mou	F.....A.ELN..GA..V..L..I	.ML.....H.....V..A.V.....	..HEV.....L.....I.....PL....	180
equ	VSLIECGPVHTAFQEKLEGGPGGALDGADA	DTRDLFSRYLRHCEQMYREVAQDPEAVTEV	FLTALRAPRPAALRYFSTERFLPLVQLRLSD	270
hum	L.....M..VL.S.EEV..RTDI	H.FHR.YQ..A.SK.VF..A..N..E.A..	K.T.....T.....LRM..D.	270
mar	P.MQ.VL.....WM..RT.T	R..R..HQ..Q.NKEIF..A..H..E.V..	K.....T.....Q.M..D.	248
rat	A.....H.....ER...	Q..H..AH.Q.GY..ALS..-.....E...	M.....Q.....N.....ARM.TE.	269
mou	A.....Y..V.....ER...	Q..H..AH.Q.GY..ALS..-.....E..L	M.....Q.....N.....ARM.TE.	269
equ	PSGRSYVAAMHSAVFADEPAQDPDGARAEA	R-----DG-----EAGEP-----	-----	308
hum	...SN..T...RE..G.V..KAEA..E.GG	GAGPGAEDAGRSVAGDP..L.D.PAAPQ--	-----	73,3% 328
mar	...S.....RN..PK.....EAEA..G.GG	-----V-----DP.L.DTLAAPQ--	-----	74,5% 292
rat	...S...E...REA.S.LQV.EGAK.G.QV	SGDPDTPPRALICLPECAIPRVTAELGWSA	SDKPGQNKSCYQQKI	72,1% 344
mou	...S.....QEA.SNLQT.ENAK.G.QV	PGVSDTASSALICLPECAIPRVASELGWSA	SDKPGQDNQSCYQQKI	72,5% 344

Figure 3 Equine 17βHSD1 predicted amino acid sequence. (A) The amino acid sequence of equine (equ) 17βHSD1 is aligned with the human (hum), marmoset (mar), rat, and mouse (mou) homologs. Identical residues are marked with a printed period, hyphens indicate gaps in protein sequences created to optimize alignment, and numbers on the right refer to the last amino acid on that line. The percentage presented at the end of each sequence represents the sequence's homology to the equine protein.

hCG and in a placenta sample; whereas the message was very low or absent in all other tissues examined (Fig. 4A). Abundance of the control gene rpL7a remained constant in all the tissues studied (Fig. 4B).

Regulation of 17 β HSD1 mRNA in preovulatory follicles

The regulation of equine 17 β HSD1 mRNA in preovulatory follicles isolated during estrus between 0 and 36 h after hCG treatment and in corpora lutea on day 8 of the estrous cycle was examined by RT-PCR/Southern blot. The results clearly demonstrated a dramatic decrease in 17 β HSD1 transcript expression in equine follicles during the hCG-induced ovulatory/luteinization process. Elevated levels of 17 β HSD1 mRNA were observed prior to hCG (0 h) with a pronounced downregulation observed 12 h post-hCG. Levels remained low, almost undetectable, in samples isolated between 24 and 36 h post-hCG, as well as in day 8 corpus luteum (Fig. 5A). No variation was observed in levels of rpL7a transcript in follicle wall and corpora lutea preparations (Fig. 5B). When results from multiple follicles and corpora lutea were expressed as ratios of 17 β HSD1 to rpL7a, a significant decrease in 17 β HSD1 transcript was observed in follicles between 12 and 36 h after hCG treatment and in corpora lutea ($P < 0.05$; Fig. 5C).

In order to determine the contributions of the different steroidogenic cell types that make up the

Figure 4 Expression of 17 β HSD transcript in equine tissues. RNA extracts were prepared from various equine tissues, and samples (100 ng) were analyzed for 17 β HSD1 and rpL7a (control gene) by semi-quantitative RT-PCR/Southern blotting, as described in Materials and methods. (A) Expression of 17 β HSD1 mRNA in equine tissues. (B) Expression of rpL7a mRNA in equine tissues. The number of PCR cycles for each gene was within the linear range of amplification, and they represented 26 and 18 cycles for 17 β HSD1 and rpL7a respectively. The follicle wall extract was prepared from a preovulatory follicle obtained prior to (i.e. 0 h) hCG treatment. Numbers on the right indicate the size of the PCR fragment.

Figure 5 Downregulation of 17 β HSD1 mRNA by hCG in equine preovulatory follicles. RNA extracts were prepared from the wall of preovulatory follicles isolated between 0 and 36 h after hCG and from corpora lutea (CL) isolated on day 8 of the estrous cycle. RNA samples (100 ng) were analyzed for 17 β HSD1 and rpL7a by semi-quantitative RT-PCR/Southern blotting, as described in Materials and Methods. (A) Regulation of 17 β HSD1 mRNA in equine follicles (one representative follicle per time point). (B) Constitutive expression of rpL7a mRNA in the same follicles. Numbers on the right depict the size of the PCR fragment. (C) Relative changes in 17 β HSD1 mRNA in equine follicles after hCG treatment. The 17 β HSD1 signal was normalized with the control gene rpL7a, and results are presented as a ratio of 17 β HSD1 to rpL7a ($n = 5-6$ distinct follicles (i.e. animals) per time point, and $n = 3$ corpora lutea). Bars marked with an asterisk are significantly different from 0 h post-hCG ($P < 0.05$).

follicle wall, granulosa and theca interna cells were isolated from follicles obtained between 0 and 39 h after hCG treatment (Fig. 6). The results revealed that the granulosa cell layer was the sole contributor of 17 β HSD1 transcript expression. In this cell type, a significant decrease in 17 β HSD1 mRNA was observed 12–39 h after hCG ($P < 0.05$; Fig. 6A). 17 β HSD1 mRNA expression was low to absent in all theca interna samples examined (Fig. 6B).

Binding activity of nuclear extract proteins to the 17 β HSD1 proximal promoter

Genomic cloning led to the characterization of a 1.4 kb fragment of 5'-flanking DNA region (Fig. 7). Use of transcription start site prediction software (www.fruitfly.org/seq_tools/promoter.html) identified a putative

Figure 6 Cell type-dependent expression and regulation of 17 β HSD1 mRNA in equine preovulatory follicles. RNA extracts were prepared from granulosa cells (A) and theca interna (B) isolated from equine preovulatory follicles between 0 and 39 h post-hCG, and samples (100 ng) were analyzed for 17 β HSD1 and rpL7a by semi-quantitative RT-PCR/Southern blotting, as described in Materials and Methods. Autoradiograms show representative results of 17 β HSD1 and rpL7a mRNA levels (one sample per time point). The 17 β HSD1 signal was normalized with rpL7a, and results are presented as a ratio of 17 β HSD1 to rpL7a (mean $\pm$ S.E.M.; $n=4$ samples (i.e. mares) per time point). Bars marked with an asterisk are significantly different from 0 h post-hCG ($P<0.05$).

transcription start site 23 bp upstream of the ATG start codon (Fig. 7). Putative *cis*-acting elements located within 300 bp upstream of the transcription start site were identified with the TRANSFAC database (<http://motif.genome.jp/>) and included a C/EBP, an AP-2, two GATA, an SF-1, a NF κ B, two Sp1, and a cAMP response element (CRE) element. To determine whether hCG affected the binding of putative transcriptional regulators to the $-230/-30$ fragment (+1 representing the transcriptional start site) of the 17 β HSD1 promoter, nuclear extracts from granulosa cells isolated from preovulatory follicles obtained at 0 and 30 h post-hCG were used in EMSA. Results demonstrated that a major protein/DNA complex was formed with nuclear extracts at 0 h post-hCG (Fig. 8A, lane 2). The use of 30 h post-hCG nuclear extracts did not affect the migration of the band, but resulted in a net decrease in protein binding (Fig. 8A, lane 3). To characterize the specificity of protein/DNA interactions, EMSAs were performed using molar excess of unlabeled oligonucleotides (Fig. 8D) containing various putative transcription factor-binding sites present within the $-230/-30$ fragment (Fig. 8A, lanes 4–7). Results showed that competitors containing Sp1-binding sites reduced protein–promoter complex formation (Fig. 8A, lane 5), whereas no effect was observed

when competitors containing the first GATA or C/EBP and AP-2 elements were used (Fig. 8A, lanes 4 and 6). Interestingly, the signal was completely abolished when an oligonucleotide containing both SF-1/NF κ B and the second GATA binding sites was used as a competitor (Fig. 8A, lane 7).

To further confirm the specificity of binding to the putative binding sites, individual transcription factor-specific regions were mutated and again used as competitors. The previously observed SF-1/NF κ B/GATA competition was greatly reduced when either sites were mutated, indicating a putative and collaborative binding instance (Fig. 8B, lanes 4 and 5). To investigate the potential presence of NF κ B, SF-1, and GATA protein in the binding complex, supershift EMSAs were performed using antibodies specific to the p50 and p65 subunits of NF κ B, as well as antibodies specific for GATA-4 and equine SF-1. The intensity of the major band decreased when the anti-p65 antibody was used (Fig. 8B, lane 7 vs lane 2, band (a)), and a clear supershift band appeared when the anti-equine SF-1 antibody was used (Fig. 8C, lane 3 vs lane 2, band (b)). No change in intensity was discernable when the anti-p50 or anti-GATA4 antibodies were used (Fig. 8B, lanes 6 and 8 vs lane 2, band (a)).

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-1412          GTTTTTAGCTAGTGACAGCC
-1400 GCTTCCTGCTGGGCAGCTGGCTGAA GCAGGCCCGGAGATGGCGGTGAGT
-1350 GAGGCTGAGGCCCATTTCTATGAAC AGAACAGTTGCTACCAGCTGACCCCT
-1300 GTGGGGGCCCGAGGGCAACATCCTA GACTACGCCAACAGCAGCTGGCGG
-1250 GCCTGGTGGCTGACTACTACACGCC CCGCTGGCAGCTCTTTGGGAGATG
-1200 CTGGTTCAGAGCCCTGGCCCAAGCGC TCCCCTTCCAACAGCAACAGTTTGA
-1150 CAAGAACCCTTTCGAGTTGAGAGAG GCCTTTGTCTTCAGACACGGAGAT
-1100 ATCCTAGCCAGCCCAAGGTGACAC CGTGGACCTGGCCCAAGAAGTTCTTT
-1050 CTCAAATATTACCCCGGTGGGTGG CTGGCTCTTTGTGACAGATTGACCA
-1000 CCCCTGGGCCATGTTCTCCCCAGAC GCCAACTCCAGGCCCTGGGCAGGTTT
-950 TCGGGGCCCTAGAGCTGGACAGGCAT CACAGAAGGTCCCAGGCCCTAGGTGA
-900 AGGAAGTCAAGACCTATTTGGTGGG ATCCAGCATGGGTGGACCATGCGGA
-850 GGGAGATAGACTGCCCCCCCCCCCCC CCCCCGCCCGGCCAAAGTTTGGGAT
-800 CAAAGTACTGTTTAAATACGCGTTA ATAAACTGATGAATCACCTGGGTCT
-750 GTCTGTGAGAAATGCTACTGCCACAA TGCTGGGAGGACTCAGGGGAATAG
-700 CGTGGACACTGGTGTGGGGCCAGTT TCCCTGCCCCCTTTGCCCTCAGCCTC
-650 CCCATTTTTTTGATGTGCATCTGAG CTAACAACTGTTACCAATCTTTT
-600 TTTTCTGCTTTTCTCTCCCAAGC CCCCCTACATGGTTGTATATTTTA
-550 GTTGTGGGTCTCTTAGTTTGCGCA TGTAGGACGCTGCCTCAACGTGGCC
-500 TGATGAGCGGTGCCATGTCGTCGCC CAGGATCCGAACCAAGTAAACCTG
-450 GGCCGCGGAAGCAGAGCGCAAGAAC TTAACCACTCGCCATAGGGCGGCG
-400 CCAACCTCCCATTTTAACTACTAAC CCTTGGCCTTTGAAGTGAAGTGGC
-350 CTCGAAAAGCATTTACTTTTACAC CCTTCAAAACTGTAATTGTCCGTG
-300 TGAGACAGAGCATGCTCAGGGGAA GAGCCATCCAGGGCACTCATTTGCC
-250 ACAGGGGTGCCACACCTTGCTTGC AAAGCTTTACCTTCCCTCTGACCAA
      oligo 3
-200 CTACTGAGGAGCCCGCAGGGTGCCA GCATTGGCCTCAGCTCTGCCTTGAC
      oligo 1
-150 CCAGAGCACTGTCCGGAGATCAGGC CAGGCAGCASCAGAAGGACTAGCC
      oligo 4      oligo 2
-100 TTGAGATTGTGTGGCAACACAACA AGGGGTGGGGACCTGGAGGGCAGG
      oligo 3
-50 CGCTGGTGAGCACCTATATCCAGC CCAGAGACCAGAGCCAGCACTCCC
+1 AGCCTCTCCTGGACCGCGCCTCAT GGACCGCACCATCGTGCTCATCAC

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Figure 7 Isolation and characterization of the equine 17 β HSD1 promoter. Numbering is relative to the putative transcription start site (+1) and the ATG translation start codon is in bold. The oligos used in competition assays are boxed. Putative *cis*-acting elements located within the 300 bp upstream of the start site are as follows: oligo 1, GATA (italic); oligo 2, Sp1 (italic); oligo 3, C/EBP (bold) and AP-2 (italic); and oligo 4, NF κ B/SF-1 (bold) and GATA (italic). The probe used in binding assays is double underlined. The nucleotide sequence of the 1.4 kb promoter fragment is part of the genomic sequence deposited to GenBank (Accession number DQ418450).

Anti-equine SF-1 antibody specificity and expression of SF-1 protein in equine preovulatory follicles

The SF-1 antibody specificity and the hCG-dependent downregulation of the SF-1 protein were demonstrated at the protein level by immunoblot in follicles at 0 and 39 h post-hCG. As shown, the antibody raised in rabbit recognized the equine SF-1 protein from preovulatory follicle cell extracts, with two bands appearing at approximately 52 kDa (Fig. 8E). Gonadotropin treatment resulted in a marked decrease in SF-1 protein expression (Fig. 8E).

Discussion

This is the first study to identify hCG as a negative regulator of 17 β HSD1 mRNA expression during the follicular luteinization in granulosa cells of a mono-ovulatory species. Follicular luteinization/ovulation has previously been associated with dramatic changes in

steroidogenic enzyme expression. Enzymes responsible for androgen and estrogen biosynthesis have been shown to be downregulated, whereas the expression of those responsible for enhanced progesterone synthesis is upregulated (Fortune 1994, Richards 1994, Ronen-Fuhrmann *et al.* 1998, Sandhoff *et al.* 1998). This study thereby provides an additional molecular basis for the decrease in 17 β -estradiol production. Previous investigations of 17 β HSD1 in the ovary have included its detection by northern blot in the rat (Ghersevich *et al.* 1994), RT-PCR in humans (Nelson *et al.* 2001), *in situ* hybridization, and Southern blotting in mice (Sha *et al.* 1997, Pelletier *et al.* 2004), as well as in human corpora lutea by immunohistochemistry (Vaskivuo *et al.* 2002). It has been detected in the granulosa cells of developing follicles in immature and mature rats (Akinola *et al.* 1997), and its regulation during the ovulatory process has been examined in rodents; however, it was limited by the use of immature hypophysectomized rats (Ghersevich *et al.* 1994).

This study characterizes the hCG-dependent downregulation of 17 β HSD1 transcript expression in a series of preovulatory follicles from a monoovulatory species. Previous reports using immature hypophysectomized rats have demonstrated that recombinant follicle stimulating hormone (FSH) had a stimulatory effect on 17 β HSD1 transcript and protein expression, and that further treatment with hCG resulted in a downregulation of 17 β HSD1 mRNA, visible after 1 day of treatment (Ghersevich *et al.* 1994). The present study shows that this downregulation is already visible 12 h after hCG treatment in the equine preovulatory follicle. Therefore, the results presented herein are consistent with the previous report and further establish the rapidity of 17 β HSD1 transcript downregulation.

The molecular control of the 17 β HSD1 gene in granulosa cells has remained largely uncharacterized. It has been shown, however, that AP-2 can interfere with Sp1 binding, and that GATA-3 can prevent transcription of constructs containing the 17 β HSD1 proximal promoter in choriocarcinoma cells (Piao *et al.* 1997). Further, retinoic acids and activin-A have been demonstrated to induce 17 β HSD1 mRNA in human JEG-3 cells and cultured rat granulosa cells respectively (Piao *et al.* 1997, Ghersevich *et al.* 2000, Zhu *et al.* 2002). The present study demonstrates for the first time the gonadotropin-dependent decrease in nuclear extract binding to the 17 β HSD1 proximal promoter and identifies SF-1 and NF κ B as putative *cis*-acting elements in 17 β HSD1 transcriptional regulation. Interestingly, the transcript for SF-1 has previously been shown to be downregulated after hCG treatment in these same follicles (Boerboom *et al.* 2000), thereby supporting the present observation of SF-1 protein downregulation after hCG. Moreover, the inverted SF-1-binding site identified in this study is identical to that reported for

Figure 8 Gonadotropin-dependent regulation of DNA-binding activities in equine granulosa cell nuclear extracts. (A) Nuclear protein extracts were prepared from granulosa cells of follicles isolated before (0 h) and after (30 h) hCG treatment (lanes 2 and 3), as described in Materials and methods. Extracts were incubated with 32 P-labeled 17 β HSD1 promoter fragment –230/–30, and protein–DNA interactions were studied by EMSAs. For reference purposes, the major protein–DNA complex is designated as *band a*. Competitive EMSAs were performed in the presence of various unlabeled competitor DNA (lanes 4–7). (B) Competitive EMSAs and supershift assays were performed in the presence of nuclear extracts prepared from granulosa cells from follicles isolated prior to hCG-treatment, 32 P-labeled oligo 4 and various unlabeled competitor wild-type and mutated DNA, as well as with NF κ B and GATA-specific antibodies. (C) Supershift assay performed in the presence of nuclear extracts prepared from granulosa cells from follicles isolated prior to hCG-treatment, 32 P-labeled oligo 4 and an SF-1-specific antibody. (D) Sequences of competitor wild-type and mutated (Δ) oligonucleotides. (E) SF-1 antibody specificity and regulation of SF-1 protein by hCG in equine preovulatory follicles. Protein extracts were prepared from preovulatory follicles isolated 0 and 36 h after hCG treatment ($n=2$ samples (i.e. mares) per time point) and were analyzed by one-dimensional SDS-PAGE and immunoblotting using a specific polyclonal antibody raised against a fragment of the equine SF-1 protein, as described in Materials and Methods. Results from protein extracts (50 μ g/lane) are shown. The marker on the left indicates the expected position of the SF-1 protein.

the bovine *CYP11A* gene, encoding the cytochrome P450 side-chain cleavage enzyme, and this sequence has been demonstrated to bind the SF-1 protein (Liu & Simpson 1997). Even though incubation of the anti-p65 NF κ B antibody with the nuclear extract did not lead to the formation of an antibody–protein–DNA complex, it did lead to the displacement of binding of nuclear proteins to the oligonucleotide, and the level of displacement may in part be due to the antibody raised against the human protein. Nonetheless, the decrease

in complex formation observed using this antibody is indicative that this transcription factor may be involved in promoter regulation. Interestingly, NF κ B has been demonstrated to activate transcription of the *CYP19A1* gene (Fan *et al.* 2005). *CYP19A1* encodes the cytochrome P450 enzyme aromatase, whose role in estrogen biosynthesis has largely been characterized (Simpson *et al.* 2005). In that study, it was shown that the activation of NF κ B resulted in an upregulation of *CYP19A1*'s promoter II activity and that this may be due

to the direct interaction of the p65 subunit of NF κ B with the CYP19A1 promoter as identified by chromatin immunoprecipitation (Fan *et al.* 2005). In addition, peroxisome proliferator-activated receptor- γ and retinoid X receptor were speculated of downregulating aromatase expression, when stimulated simultaneously by disrupting the p65-promoter interaction (Mu *et al.* 2001, Fan *et al.* 2005). Considering that the regulation of CYP19A1, transcript levels of which are high in equine preovulatory follicles at 0 h and drop dramatically after hCG (Boerboom *et al.* 1999), is identical to the regulation observed for 17 β HSD1, further studies will be needed to unravel whether similar or distinct transcriptional mechanisms are involved.

The 17 β HSD1 cDNA has previously been cloned in various species, including human (Peltoketo *et al.* 1988), rat (Ghersevich *et al.* 1994), and mouse (Nokelainen *et al.* 1996). This study presents the equine cDNA and gene structure. Its genomic structure is consistent with that of other species, as they all have been demonstrated to have six exons and five introns. A pseudogene present upstream of the 17 β HSD1 gene has been identified in humans, orang utan, chimpanzees, and gibbons; however, it is not conserved in all species (Keller *et al.* 2005). It will be interesting to determine if this is also the case in horses. In addition, the sizes of introns 3 and 4 vary slightly between species and the 3' end of exon 6 has been shown to exhibit considerable variability from one species to the next (Keller *et al.* 2005), as is the case in the mare. This variability is discernable when examining the amino acid sequence of the carboxy terminus and the length of the 17 β HSD1 proteins. A putative TATA-box motif was identified approximately 30 bp upstream of the transcription start site of the equine gene. An initiator (Inr) sequence ((C/T)₂C-A-(C/T)₅), located at the transcription start site, also appears to be present (Lewin 2000). Notwithstanding, these sequences are not exact matches to traditional TATA and Inr sequences, and their relevance in transcriptional activation remains to be elucidated.

This study also investigates the expression of 17 β HSD1 mRNA in equine tissues and establishes high levels of 17 β HSD1 transcript in preovulatory follicles prior to hCG. While high levels of equine 17 β HSD1 mRNA were also observed in a placenta sample, which is consistent with the high 17 β HSD1 mRNA and protein observed in placenta of humans and non-human primates (Lin *et al.* 1992, Castagnetta *et al.* 1997, Schwabe *et al.* 2001), this is not the case for rodents, however (Akinola *et al.* 1997). It has also been detected by *in situ* hybridization in mice in granulosa cells of growing follicles, the intermediate lobe melanotrophs of the pituitary, in epithelial cells of the prostate, and in germ cells of the testis (Pelletier *et al.* 2004). It is further found in the epithelium of normal and cancerous breast tissue of women by *in situ* hybridization (Soderqvist *et al.* 1998, Miettinen *et al.* 1999, Oduwole *et al.* 2004).

In summary, this study is the first to characterize the primary structure of the equine 17 β HSD1 cDNA and gene, to demonstrate the regulation of this gene during follicular luteinization in a monoovulatory species, to identify the preovulatory gonadotropin signal as a negative regulator of equine 17 β HSD1 mRNA expression, and to propose NF κ B and SF-1 as putative *cis*-acting elements in 17 β HSD1 promoter activation. Considering the estrogen-activating activity of 17 β HSD1, its gonadotropin-dependent downregulation provides an additional molecular basis for the decrease in 17 β -estradiol biosynthetic capacity observed during the process of ovulation/luteinization.

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