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The Rat 17β-Hydroxysteroid Dehydrogenase Type III: Molecular Cloning and Gonadotropin Regulation

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ABSTRACT

17β-Hydroxysteroid dehydrogenase (17βHSD), the enzyme that catalyzes the final step of testosterone biosynthesis in the testis, was cloned from a rat Leydig cell complementary DNA library to gain insights into the functional requirements, activation mechanisms, and molecular regulation. The 17βHSD complementary DNA encoded 306 amino acids (molecular mass of 33.7 kDa) and displayed 75% and 85% amino acid sequence homology to the human and mouse 17βHSD type III enzymes, respectively. Northern analysis revealed a single 1.4-kb messenger RNA (mRNA) species in rat Leydig cells, whereas ovarian mRNA was detected only by RT-PCR amplification. The cloned 17βHSD expressed in mammalian cell lines specifically catalyzed the reductive reaction in androgen formation with androstenedione as the preferred substrate. This reaction was significantly reduced in the absence of glucose. Expression of the endogenous 17βHSD gene in rat Leydig cells was inhibited by a single dose of hCG *in vivo*, with maximum reduction of steady state mRNA levels at 24 h and recovery at 9 days. Such agonist-induced down-regulation of 17βHSD expression, which preceded the marked reduction of LH receptors, resulted from changes at the transcriptional level and was accompanied by loss of enzymatic activity. These studies have demonstrated a glucose requirement for optimal activity of the enzyme *in vitro* and for a role of gonadotropin in regulating the expression of 17βHSD gene *in vivo*. Cloning of the 17βHSD type III enzyme from rat Leydig cells will facilitate further investigation of the molecular regulation of its activity in the testis. (*Endocrinology* **140:** 3534–3542, 1999)

17β-HYDROXYSTEROID dehydrogenase (17βHSD) is one of the essential enzymes involved in the regulation of intracellular levels of biologically active androgens and estrogens in gonadal and extragonadal tissues (1). It catalyzes the reversible interconversion of estrone and estradiol, androstenedione and testosterone, and dehydroepiandrosterone and Δ^5-androstene-3β,17β-diol. Seven distinct 17βHSD isoenzymes have been cloned and characterized from various tissues and are designated types I, II, III, IV (revieved in Refs. 2–4); V (5); VI (6); and VII (7). Types I-IV, VI, and VII are members of the short chain alcohol dehydrogenase super family (2–4, 6, 7). However, type V belongs to the aldo-keto reductase family (5). The 17βHSD isoenzymes differ in their tissue/cellular localization, substrate specificities, cofactor requirements, and preference for oxidation or reduction reactions. Three of the isoforms, types I, III, and VII, catalyze the reductive reaction, whereas the other four preferentially catalyze the oxidation reaction.

The type III isoform was isolated from the human and mouse testes (8, 9). The human type III enzyme appears to be testis specific (8), whereas the mouse type III 17βHSD, in addition to its strong expression in the testis, is weakly expressed in the ovary, uterus, seminal vesicle, and prostate, as determined by PCR amplification (9). The human type III enzyme is predominantly associated with the production of testosterone by testicular Leydig cells. Mutations of the human testicular type III 17βHSD that involve either spliced junction abnormalities or single base substitutions result in nonexpression or severely compromised enzyme activity, respectively, and cause male pseudohermaphroditism (8, 10, 11). In the rat Leydig cell, 17βHSD activity is under ATP control, and intracellular glucose plays an important role in the regulation of the enzyme. The contribution of the glycolytic pathway to meet optimal provision of functional ATP for 17βHSD activity has been recently demonstrated (12). However, hormonal regulation of 17βHSD type III activity and its gene expression in the gonads have not been investigated. Gonadotropins exhibit dual control of testicular Leydig cell function (reviewed in Ref. 13). Low doses of LH/hCG maintain LH/hCG receptors and steroidogenic enzymes in the up-regulated state, whereas high doses cause receptor down-regulation and desensitization of steroidogenic enzymes. Previous studies have demonstrated a blockade at the site of conversion of progesterone to androgens and a consequent reduction of testosterone production that is partly attributable to a decrease of CYP17 (17-hydroxylase/lyase) expression (14, 15). More recently, we have demonstrated rapid gonadotropin-induced negative transcriptional control of types I and II 3β-hydroxysteroid dehydrogenase genes in rat Leydig cells (16). However, the participation of 17βHSD in this desensitizing process remains to be elucidated.

To further understand the functional requirements of the enzyme, the mechanism of its activation, and molecular regulation, we have cloned the 17βHSD type III enzyme from rat Leydig cells in this study. The rat type III enzyme encodes a single 1.4-kb messenger RNA (mRNA) species with high similarity to the human and mouse type III isoform. A glucose requirement for 17βHSD optimal activity was observed

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in cells transiently transfected with 17βHSD complementary DNA (cDNA). Moreover, 17βHSD was down-regulated *in vivo* by hCG in a dose- and a time-dependent manner. These studies provide a basis for further investigation of the metabolic and molecular regulation of this enzyme.

Materials and Methods

Cloning of 17βHSD type III cDNA

mRNA was isolated from rat Leydig cells purified by centrifugal elutriation (17) using the Invitrogen isolation method (Carlsgad, CA). mRNA was reverse transcribed using a random primer and SuperScript reverse transcriptase (Life Technologies Inc., Gaithersburg, MD). Subsequently, PCR was carried out using specific oligonucleotides based on the published sequence of human 17βHSD type III (8) to generate a specific probe for screening of a rat Leydig cell ZAP library (18). The set of primers 5-ATGTTGTCCTTATTA GCCGGACGC-3(270–293 nucleotides) and 5-AAATGCGCACACAAACGCCTTGGAAGCTGAGTA-3 (673–641 nucleotides) generated the expected size product of 403 bp. This PCR product was subcloned into TA cloning vector (Invitrogen) and subjected to sequence analysis. This fragment showed 82% similarity with the 17βHSD human type III DNA and was used for screening the rat Leydig cell cDNA library. Approximately 5×10^5 clones were screened with a randomly labeled rat 17βHSD fragment by standard colony hybridization (19). One of four positively identified clones with a 1087-bp insert was sequenced by the dideoxy chain termination method (20) using Sequenase version 2.0 kit (U. S. Biochemical Corp., Cleveland, OH). The sequence analysis revealed 75% similarity with the human type III sequence and had two missing bases of the initiation codon (AT). A fragment containing the missing 5′-end bases was obtained by rapid amplification of cDNA 5′-ends analysis essentially as previously described (Life Technologies, Gaithersburg, MD) using mRNA isolated from rat Leydig cells (21). First strand cDNA was synthesized using a primer oligonucleotide corresponding to nucleotides 326–347 of rat 17βHSD (ATTTTACCAGAGAAGACATCTA) by reverse transcriptase and 3′-end tailed with deoxy-CTP using terminal deoxynucleotidyltransferase, followed by PCR with reverse primer 288–309 nucleotides of the rat 17βHSD (GAGGACCACTGGAAGCCGTGTG) and forward dG adaptor primer (GGCCACGCGTCGACTAG-TACGGGIIGGGIIGGGIIG). The PCR product of 309 bp was cloned into TA cloning vector (Invitrogen, San Diego, CA) and sequenced. Thereafter, to obtain a full-length cDNA, a fragment was isolated by digestion with *Eco*RI (restriction site at the TA vector) and *Sau*96I (within insert) and ligated to a 820-bp *Sau*96I site of the initial isolated 17βHSD cDNA clone. The nucleotide sequence of the full-length cDNA was verified by sequence analysis. The full-length 17βHSD in pBK expression plasmid driven by cytomegalovirus promoter (Stratagene, La Jolla, CA) was used in expression studies.

Animal treatment and Leydig cell preparation

Adult male rats (200–250 g; Charles River Laboratories, Inc., Wilmington, MA) were housed in pathogen-free, temperature- and light-controlled conditions (20 C; alternating 14-h light, 10-h dark cycles). All animal studies were approved by the NICHHD animal and care and use committee (protocols 97–039 and 97–041). The male adult rats were given single sc injections of various doses of Pregnyl (Organon, West Orange, NJ): 0.1, 1, and 25 IU, equivalent to 0.01, 0.1, and 2.5 μg purified hCG, in 100 μl of Dulbecco's PBS. hCG international units (IU) are equivalent to hCG USP (United States Pharmacopea unit). Control animals were injected with vehicle alone. Animals were killed by asphyxiation with CO_2 and decapitated 24 h after hCG treatment unless otherwise indicated. Leydig cells were prepared by collagenase dispersion and purified by centrifugal elutriation, as previously described (17). These cells were immediately frozen on dry ice and maintained at −70 C until extraction of RNA. *In vivo* ovarian luteinization and down-regulation of LH receptor (LHR) and LHR mRNA was induced by sequential PMSG/hCG treatment of 21-day-old female rats (Sprague Dawley, Charles River Laboratories, Inc., Wilmington, MA) following established protocols (22). The animals received a sc injection of 50 IU PMSG, a preparation rich in FSH activity that produces follicular maturation, followed 65 h later by injection of 25 IU hCG (superovulation/luteinization). Six days later, the animals received a second injection of 25 IU hCG (LHR down-regulation, steroidogenic desensitization). Animals were killed, and their ovaries were removed, rapidly frozen, and preserved at −70 C until RNA extraction.

Ribonuclease (RNase) protection analysis

The RNase protection assay was performed by established methodology (23). The 17βHSD complementary RNA (cRNA) probes were generated by PCR followed by subcloning. Primers F2 (5-ATGTTGTACTTA TCAGCCGGACAC-3) and R5 (5-AAATGTGCACACAAAAGCCTTGGAAGCTGAGTA-3) were used to amplify the fragment complementary to the coding region from 230–633 nucleotides relative to the translational start site of 17βHSD. Primers of 5-GCTACAGCTTCACCAC CACA-3 and 5-GGTCTTTACGGATGTCAACG-3, located at exons 4 and 5, respectively, of the rat β-actin gene (24), were employed to amplify the β-actin fragment used as an internal control for the RNase protection assay. The fragments were cloned into pGEM vector (Promega Corp., Madison, WI) and verified by sequencing. The constructs were linearized by the digestion with *Spe*I. The cRNA probes were produced by *in vitro* transcription with RNA polymerase T7 (Life Technologies) following the manufacturer's protocol and labeled by [^{32}P]UTP (800 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, CA). Ten micrograms of total RNA samples were applied for hybridization. Ribonuclease T1 (Life Technologies) and ribonuclease A (Amersham, Arlington Heights, IL) were used for the digestion of unhybridized RNA and cRNA probe. The protected fragments were resolved on a 6% sequencing gel, dried, and autoradiographed for visual evaluation only. Bands were quantified by phosphorimage analysis using PhosphorImager Scanner model Storm 860 from Molecular Dynamics, Inc. (Sunnyvale, CA).

Northern blot and mRNA half-life analysis

mRNA was extracted from rat Leydig cells and from various male and female rat tissues using the Invitrogen isolation method (Carlsbad, CA). The mRNA samples (10 μg for each lane) were resolved on 1% agarose gels and transferred onto a Gene Screen membrane (Biotechnology System NEN Research Products, Boston, MA). The fixed membrane was prehybridized with 0.5 M phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.5% BSA, and 7% SDS at 50 C for 3 h and hybridized with a ^{32}P-labeled PCR-generated 403-bp fragment (230–633 bp) at 50 C overnight. Thereafter, the membrane was washed twice in 2 × SSC (standard saline citrate)-0.1% SDS at 50 C for 15 min each and twice in 0.5 × SSC-0.1% SDS for 10 min each. In experiments investigating the half-life of the 17βHSD mRNA, cells were incubated with 10 μg/ml actinomycin D for 0, 1, 3, 6, and 10 h in medium 199/0.1% BSA. Ten micrograms of mRNA samples were resolved as described above. Hybridization was recorded by radioautography and quantified by phosphorimage analysis. The half-life of mRNA was calculated by linear regression analysis.

The expression of 17βHSD type III in the ovary was followed by RT-PCR analysis using total RNA of ovaries at different times after treatment of animals with two doses of hCG (2.5 μg). RNA (1 μg) was reverse transcribed using random hexamers and Superscript II RNase H reverse transcriptase. Paired primers corresponding to nucleotide positions 61–81 and 311–331 of 17βHSD cDNA were used to amplify the 270-bp fragment. Also, primers corresponding to exons 4 and 5 of β-actin (24) were used to amplify a 290-bp fragment for use as an internal control. PCR products were analyzed in 3% agarose gels.

Nuclear run-off assay

Nuclei were isolated from batches of 50×10^6 rat Leydig cells from rats injected with or without 2.5 μg hCG were prepared as previously described and suspended in 50 mM Tris-HCl (pH 8.3), 40% glycerol, and 5 mM $MgCl_2$ (16). Nuclear RNA was labeled by *in vitro* transcription as previously described (25). Two hundred microliters of Leydig cell nuclear extracts (30×10^6 nuclei) were incubated with 20 μl [α-^{32}P]UTP (3000 Ci/mmol; 10 mCi/ml; from DuPont NEN) and 200 μl buffer containing 10 mM Tris-HCl (pH 8); 5 mM $MgCl_2$; 0.3 M KCl; and 1 mM each of unlabeled ATP, CTP, and GTP for 30 min at 30 C. After deoxyribonuclease I and proteinase K treatments, the labeled transcripts were extracted by phenol-chloroform-isoamyl alcohol (25:24:1) and subsequently subjected to chromatography on G-25 columns to remove free nucleotides. The linearized complementary DNA fragments of 17βHSD type III, β-actin, and pGEM vector were immobilized to the nitrocellu-

lose membranes using a slot blot apparatus. The labeled RNA (3×10^6 cpm) were then hybridized to the membranes in 2 ml hybridization buffer (10 mM *N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 10 mM EDTA, 0.2% SDS, and 0.3 M NaCl) for 20 h at 60 C. The membranes were washed with 2 × SSC and exposed to Kodak x-ray film (Eastman Kodak Co., Rochester, NY) at −70 C with intensifier screens. The densities of the bands were quantified by phosphorimage analysis.

Transient expression of rat 17βHSD type III in mammalian cells

Expression studies were performed in COS-1 and 293 cells (American Type Tissue Collection, Manassas, VA). The cells were plated into six-well plates in DMEM supplemented with 10% FBS, penicillin (100 IU/ml), and streptomycin (100 IU/ml) and were transfected at approximately 70–80% confluence using DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium methyl sulfate) following the manufacturer's procedure (Boehringer Mannheim, Indianapolis, IN). Briefly, empty vector (pBK) or pBK-17HSD (2 μg/well each) were incubated for 15 min with 15 μg/well DOTAP in 140 μl HBS (20 mM HEPES containing 150 mM NaCl, pH 7.4). After incubation, the reaction mixture was added to wells containing 2 ml OPTI-MEM I (Life Technologies, Inc.). Transfection was carried out for 5 h; subsequently, OPTI-MEM I was replaced by fresh DMEM, and cells were further cultured for 48 h.

Measurement of 17βHSD activity

Both reductive and oxidative activities of 17βHSD were measured essentially as previously described (4, 12) in Leydig cells and in transiently transfected COS-1 and 293 cells with 17βHSD cDNA (see above). Enzyme activity assays were performed by addition to cell suspensions (Leydig cells) and cell cultures (COS-1 and 293 cells) of 2 μM ^{14}C-labeled substrate in the presence of 5 μM of the respective unlabeled substrates: androstenedione (53.86 mCi/mmol), testosterone (mCi/mmol), dehydroepiandrosterone (54 mCi/mmol), estrone (57 mCi/mmol), and estradiol (54 mCi/mmol), purchased from DuPont NEN (Boston, MA). Forty-eight hours after transfection, cell cultures were washed three times with serum and glucose-free DMEM-0.1% BSA. Enzyme activity was determined in 1 ml DMEM-0.1% BSA in the presence or absence of 5.8 mM glucose. The Leydig cells were first incubated with 100 μg/ml aminoglutethimide (Sigma Chemical Co., St. Louis, MO) for 15 min to block steroid metabolism early (cholesterol side-chain cleavage) and distally (aromatase) in the pathway (26). This was followed by incubation with ^{14}C-labeled androstenedione (2 μM) and unlabeled substrate (5 μM) in HEPES buffer (5 mM; pH 7.4) containing 4 mM KCl, 140 mM NaCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5.8 mM glucose, and 0.1% BSA with the addition of substrate(s) (see above). After incubation for the designated times, the media were collected, and steroids were extracted with ethyl acetate and separated on silica-coated TLC plates with chloroform and ethyl acetate (3:1, vol/vol) as previously described (12). Plates were exposed to x-ray films for visual record only. The radioactivities corresponding to unconverted steroid substrates and products in the TLC plate were quantified by phosphorimage analysis.

The LHR binding studies included in Fig. 5 are taken from our recent report (16) and are presented for comparison with the changes observed in 17βHSD gene expression. The materials obtained for analysis were derived from the same experiments. LH receptor binding presented in Fig. 4 was performed as previously described (16).

All experiments were performed three times in triplicate unless specified. Results are the mean ± SE. Statistical significance was evaluated by ANOVA followed by Duncan's multiple range test.

Results

Molecular cloning of rat 17βHSD type III cDNA

A full-length cDNA (1111 bp) of 17βHSD was cloned from a rat Leydig cell cDNA library. It contains 21 bases of 5′-untranslated sequence, an open reading frame of 918 nucleotides encoding an hydrophobic protein of 306 amino acids with an apparent molecular mass of 33.7 kDa, and 172 bases of 3′-untranslated region (Fig. 1A; GenBank accession no. AF035156). The sequence identity between the rat type III 17βHSD and previously cloned human and mouse type III 17βHSD is shown in Fig. 1B. Pairwise comparison of rat type III to human and mouse displays an overall 75% and 85% amino acid similarity, respectively (Fig. 1B). The encoded protein contains a region of homology to the short chain alcohol dehydrogenase super family (amino acids 174–225) with the conserved YXXXK structure (amino acids 194) found in all members of short chain alcohol dehydrogenase/reductase family. The N-terminal of 17βHSD contains a putative secretory signal sequence of 20 amino acids. Two putative transmembrane helixes are predicted at amino acid position 2–21 close to the N-terminus, and at amino acid position 255–277 close to the C-terminus. The primary structure also shows the presence of four potential *N*-linked glycosylation sites (amino acids 153, 178, 236, and 302), four potential PKC sites (amino acids 170, 237, 251, and 287), three casein kinase II sites (amino acids 104, 142, and 243), and at least three potential sites for phosphorylation by cAMP-dependent protein kinase and cGMP-dependent protein kinase (27) at amino acids 61, 256, and 296. A consensus NADPH binding motif (GXXXGXG) is located at amino acids 51–57 (PC Gene PROSITE program).

Tissue distribution of 17βHSD type III

Northern analysis revealed the presence of a single mRNA transcript of 1.4 kb only in rat Leydig cells (Fig. 2). The molecular size of the mRNA is slightly higher than the 1.1-kb 17βHSD cDNA isolated from the rat Leydig cell library (Fig. 2A). This difference may be due to the presence of additional sequences at the 5′- and/or 3′-end in the mRNA. Only using RT-PCR amplification, a minor band of 17βHSD mRNA could be detected in rat ovaries at 9 and 24 h after PMSG administration and after hCG treatment in rats with superovulated/luteinized ovaries (Fig. 2B).

Transient expression of 17βHSD in COS-1 cells

The cloned 17βHSD enzyme significantly converted androstenedione to testosterone in transfected COS-1 cells (Fig. 3). The time-course study revealed significant 17βHSD activity within 15 min of substrate addition in the presence of glucose in the medium compared with that in cells incubated in the absence of glucose or cells transfected with vector only. A further increase in activity was observed in the presence of glucose at the 60 min point and reached a plateau at 120 min. In the absence of glucose, the enzyme activity was undetectable at 15 and 30 min and increased at 60 and 240 min to 29% and 65%, respectively, of the activity observed in the presence of glucose. Overall, the 17βHSD activity in COS-1 cells was significantly increased by the addition of glucose in the medium throughout the time examined ($P < 0.001$; Fig. 3).

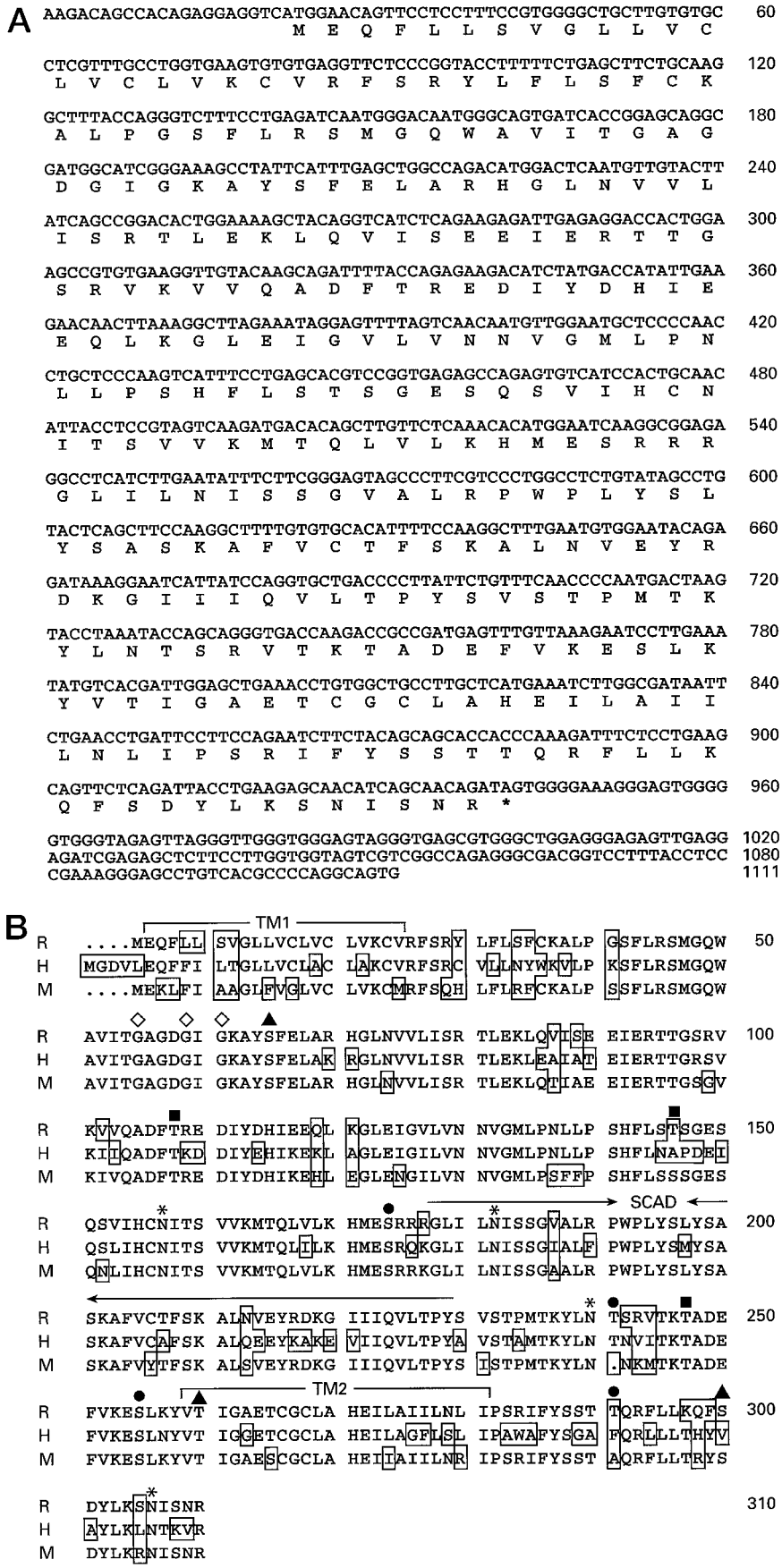

FIG. 1. Nucleotide sequence of cDNA encoding rat 17βHSD type III and deduced amino acid sequence. A, Nucleotides are numbered at the right with position 1 assigned to the first nucleotide in the cloned cDNA. Initiation codon ATG is at nucleotide position 22. *, Stop codon. B, Amino acid alignment of rat (R), human (H), and mouse (M) 17βHSD type III. Differences in the sequences are boxed. SCAD, The conserved region characteristic of members of the short chain alcohol dehydrogenase superfamily. ◇, NADPH binding motif. TM1 and -2, Putative transmembrane region. *, Potential *N*-glycosylation site. ●, Potential protein kinase C. ■, Casein kinase II. ▲, Sites for cAMP- and cGMP-dependent protein kinases.

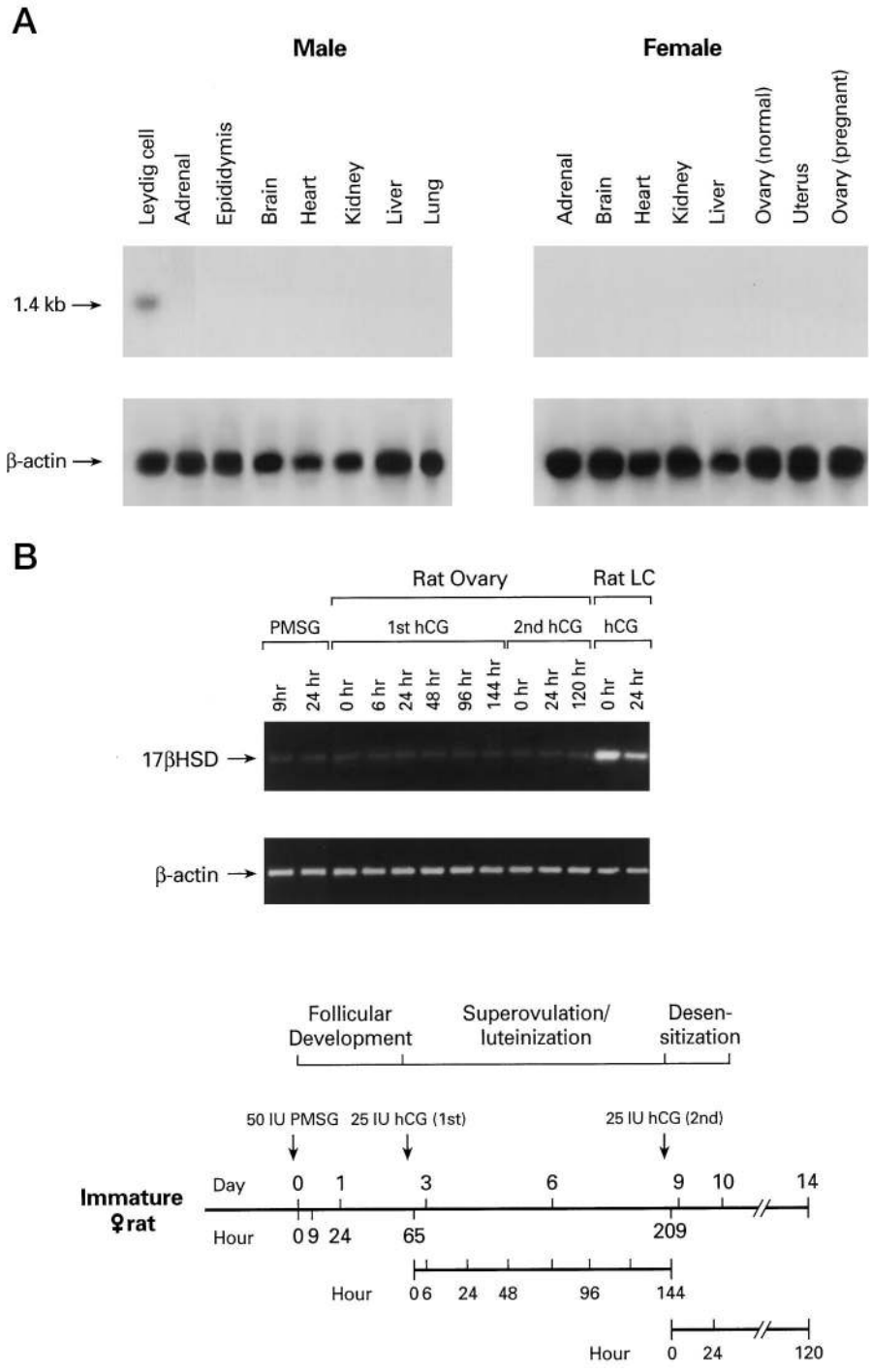

FIG. 2. Tissue distribution of 17βHSD type III mRNA. A, Polyadenylated RNA (10 μg) from several tissues of adult male and female rat was subjected to Northern blot analysis. The loading of the RNA was monitored by hybridization of β-actin. B, RT-PCR analysis in rat ovary and Leydig cells. RNA samples extracted from rat Leydig cells and from ovaries of rat treated with hormones (see *Materials and Methods* and diagram in this figure) were reverse transcribed as described in *Materials and Methods*. RT-PCR transcripts of Leydig cell RNA was used as a positive control.

Substrate specificity for cloned 17βHSD cDNA

To assess the catalytic properties of the enzyme, the substrate specificity for the cloned 17βHSD expressed in different mammalian cells was evaluated. ^{14}C-labeled steroids (androstenedione, testosterone, dihydroepiandrosterone, estrone, and estradiol) were used as substrates (Table 1). The rat type III isozyme catalyzed the reductive reaction almost exclusively with substrate preference for androstenedione over dihydroepiandrosterone in both cell types employed. Estrogen failed to serve as a substrate for the cloned 17βHSD enzyme.

In vivo regulation of 17βHSD activity by hCG in rat testicular Leydig cells

To gain insights into gonadotropin regulation of 17βHSD gene expression, we initially examined the activity of the enzyme in Leydig cells 24 h after single sc injections of various doses of hCG to adult rats. Administration of 0.01–

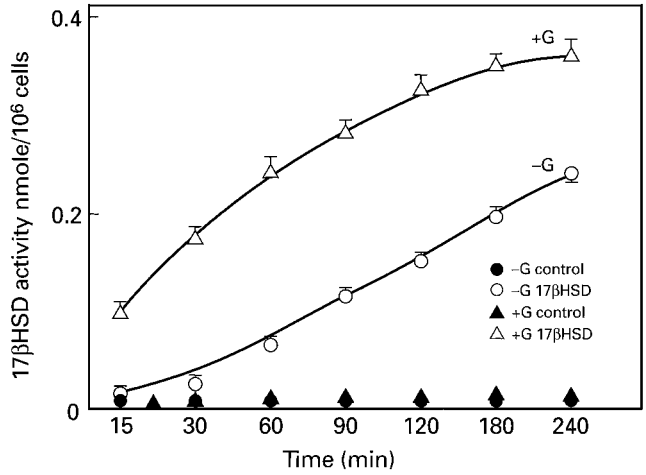

FIG. 3. Activity of 17βHSD in transiently transfected mammalian COS-1 cells. Cells were transfected with pBK (control) and pBK-17βHSD for 48 h. Subsequently, the 17βHSD enzyme activity was measured in culture cells incubated for 0–4 h in DMEM-0.1% BSA medium with or without 5.8 mM glucose at different time intervals. [^{14}C]Androstenedione (A; 2 μM) was used as substrate. The substrate [^{14}C]androstenedione and product [^{14}C]testosterone (T) were separated by TLC, and radioactivity from product and substrate were quantified by phosphorimager analysis. Values are the mean ± SE of triplicate determinations from a representative experiment. Three separate experiments were performed. G, Glucose.

TABLE 1. Substrate specificity for rat 17βHSD type III expressed in mammalian cells

Cell lines	17βHSD activity (pmol/10^6 cells · 2 h)				
	A → T	T → A	DHEA → Diol	$E_1 \rightarrow E_2$	$E_2 \rightarrow E_1$
COS-1	400 ± 30	ND	140 ± 9	ND	ND
293	420 ± 20	14 ± 0.7	15 ± 2	ND	ND

Androstendione (A), testosterone (T), dihydroepiandrosterone (DHEA), Δ^5-androstenediol (Diol), estrone (E_1), and estradiol (E_2) (2 nmol/ml) were used as substrates for the enzyme activity in COS-1 and 293 cells transiently transfected with pBK-17βHSDcDNA (described in *Materials and Methods*). After 2 h of incubation in the presence of glucose, the amounts of respective products in the medium were analyzed by TLC and quantitated by phosphorimager. The results are expressed as the mean ± SE of three independent experiments performed in triplicate.

2.5 μg hCG caused dose-dependent inhibition of 17βHSD mRNA steady state levels (Fig. 4). A small, but significant, inhibition of 17βHSD mRNA levels was observed even at low doses of hCG (by 30%; $P < 0.007$ at 0.01 μg hCG; $P < 0.003$ at 0.1 μg hCG), and marked down-regulation was induced by the high dose of hCG (2.5 μg; by ~80%; $P < 0.001$). Temporal analysis of 17βHSD gene expression using the high dose hCG (2.5 μg) showed no significant changes in 17βHSD mRNA levels at 1 and 4 h ($P > 0.05$), whereas these were significantly inhibited at 12 and 24 h ($P < 0.001$) and recovered to control levels on day 9 post-hCG treatment (Fig. 5B). It is important to note that the significant reduction of 17βHSD gene expression (Fig. 5B) at 12 h preceded the major down-regulation of LH/hCG receptors observed at 24 h (Fig. 5A). This result suggests that regulation of 17βHSD gene transcription is not related to the loss of hormone-binding sites. Furthermore, the 17βHSD enzyme activity was inhibited at 12 h by 30% ($P < 0.001$; Fig. 6), and a further reduction

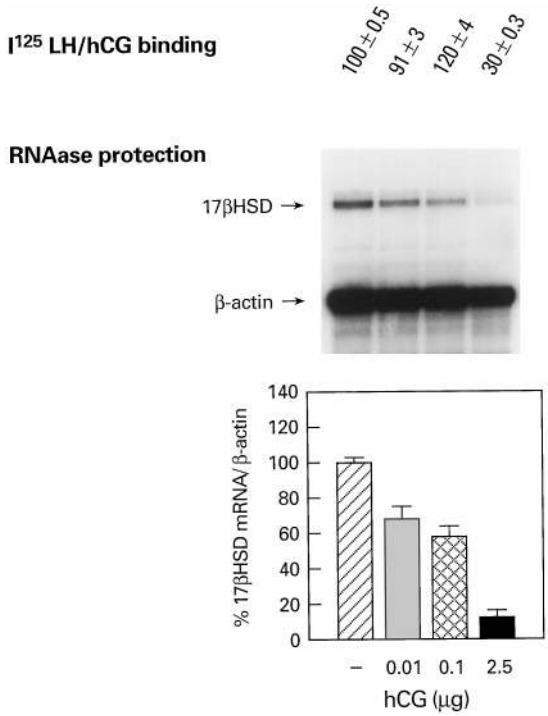

FIG. 4. Dose-dependent hCG regulation of 17βHSD gene expression by RNase protection assay. RNA samples were prepared from Leydig cells of rats 24 h after treatment with a single sc injection of different doses of hCG (0.01, 0.1, and 2.5 μg) or vehicle (control). RNA samples (10 μg) were hybridized to the ^{32}P-labeled 17βHSD probe and β-actin probe (control). The protected fragment was resolved in a denaturing sequencing gel, recorded by autoradiography by visual display (*middle panel*), and quantified by phosphorimager (*lower panel*). Data are presented as the mean ± SE relative to the saline control of triplicate determinations and are from one of three individual experiments. Counts of 17βHSD were normalized to β-actin. LHR binding activity was determined by binding of [^{125}I]hCG to intact Leydig cells in suspension and expressed as a percentage (mean ± SE) relative to the control value (*upper panel*).

was observed at 24 h (to 25% of controls; $P < 0.001$). Although major reductions in 17βHSD mRNA levels were observed at 12 and 24 h, the enzyme activity was only moderately reduced at 12 h, whereas a marked decrease was observed at 24 h (Fig. 5).

Transcriptional regulation of 17βHSD activity by hCG

We further investigated whether a transcriptional or post-transcriptional mechanism was involved in the hCG-induced down-regulation of 17βHSD gene expression in Leydig cells (Fig. 7). To determine whether the gonadotropin-induced decline in steady state levels of 17βHSD mRNA was due to increased mRNA degradation, mRNA stability was evaluated after the addition of actinomycin D to control and hCG-treated groups. Significant reduction of 17βHSD gene expression by hCG was observed at all times after actinomycin D treatment ($P < 0.001$). The rates of degradation were similar for the two groups, with half-lives of 3.8 and 3.5 h for the control and hCG-treated groups, respectively (Fig. 7A).

Nuclear run-off assays showed that newly synthesized mRNA was significantly reduced ($P < 0.01$) in hCG-treated Leydig cells compared with control cells (Fig. 7B).

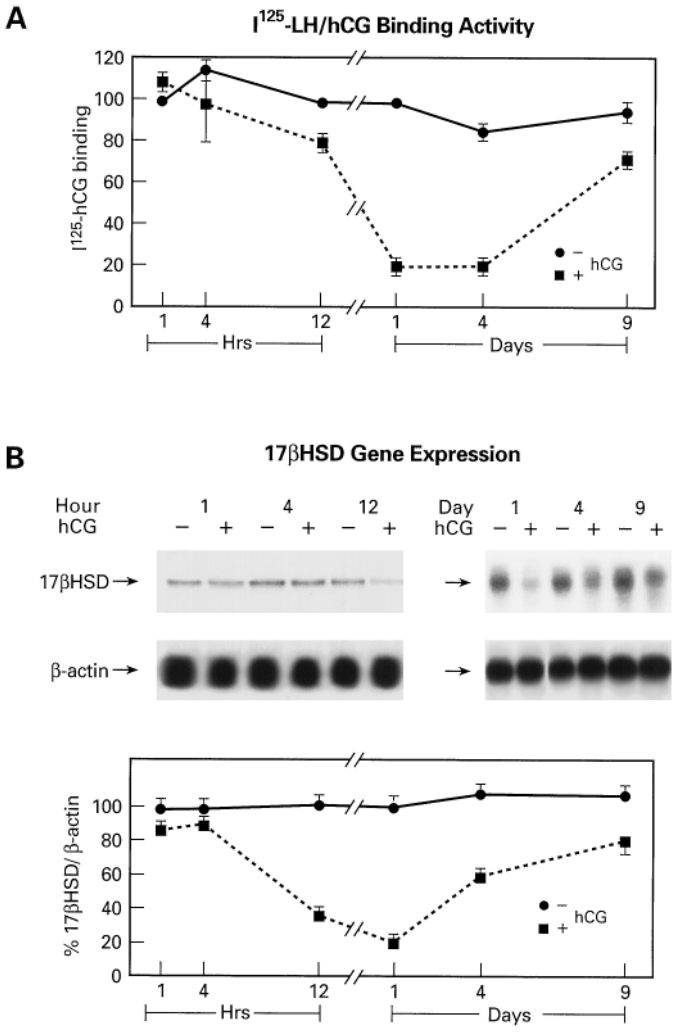

FIG. 5. Temporal analysis of 17βHSD gene expression by hCG in rat Leydig cells. Leydig cells were obtained and purified from male rat at different times after sc injection of vehicles (control) or 2.5 μg hCG at different times. A, [125I]LH/hCG binding activity in rat Leydig cells was measured after releasing endogenous bound hCG with acid-glycine treatment. LHR binding activity was expressed as a percentage (mean ± SE) relative to the control value. A is taken from Ref. 16 and has been placed here for comparative purposes. B, 17βHSD mRNA level was determined by RNase protection (403 bp) at 1, 4, and 12 h after *in vivo* hCG treatment and by Northern analysis (1.4 kb) on days 1, 4, and 9. Results were normalized by β-actin gene expression and are expressed as a percentage (mean ± SE) relative to the control value. The representative data shown are from one of three individual experiments, each performed in triplicate.

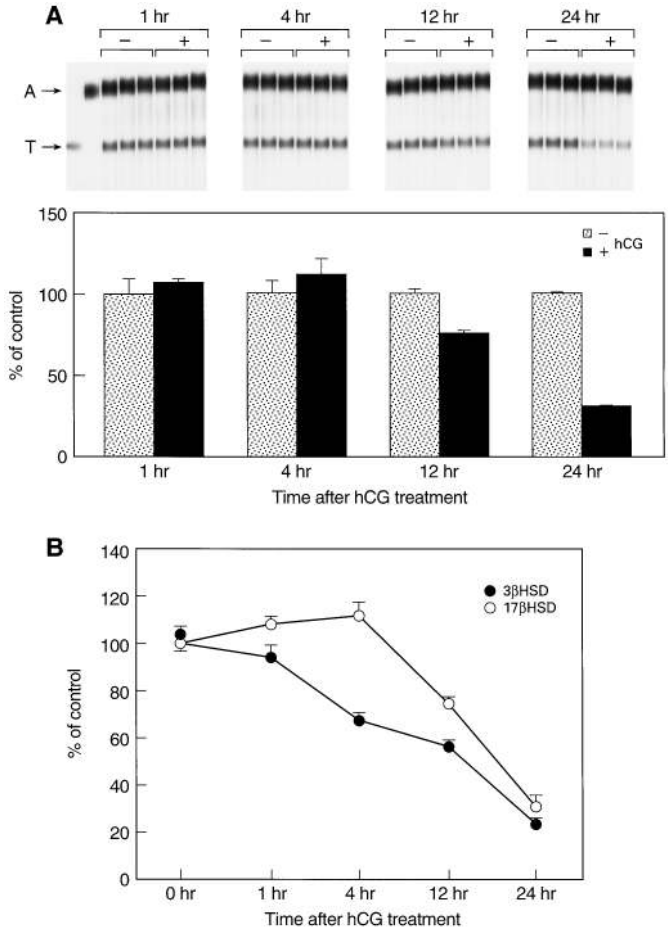

FIG. 6. A, Down-regulation of enzymatic activity of 17βHSD by hCG in rat Leydig cells. Leydig cells were obtained from male rats injected sc with vehicle (control) or 2.5 μg hCG and obtained at different times (1, 4, 12, and 24 h) after treatment. Intact cells (1×10^6 cells) after preincubation with aminoglutethimide (100 μg) were incubated with 2 μM [14C]androstenedione (A) and 5 μM unlabeled A for 60 min in the presence of 5.8 mM glucose. The substrate [14C]A and product [14C]-testosterone (T) were separated by TLC, and radioactivity from product and substrate were recorded by autoradiography for visual display (*upper panel*). TLCs were quantified by phosphorimager analysis (*lower panel*). Results represent the percent conversion to testosterone from androstenedione relative to the control value. A representative experiment (mean ± SE) of triplicates is shown. Three separate such experiments were performed. B, Comparative profiles of type III 17βHSD and 3ββHSD enzyme activities after administration of a single dose of hCG (2.5 μg). Results for 3βHSD are from our previous study (16) and used for comparative purpose in the *Discussion*.

Discussion

These studies have provided information about the primary structure, tissue distribution, and characterization of the rat testicular type III 17βHSD enzyme. Furthermore, relevant aspects of the hormonal regulation of type III 17βHSD gene expression in Leydig cell were elucidated. The amino acid sequence of the cloned rat type III 17βHSD displays a high degree of overall homology to those reported for the human and mouse enzymes (8, 9). This includes the sequence common to the short chain alcohol dehydrogenase family, and other motifs such as Tyr-X (3)-Lys (amino acid position 194–198) and Gly-X (3)-Gly-X-Gly (amino acid position 51–57), which have been proposed to participate in the catalytic reaction and to be associated with cofactor binding, respectively (28). However, the rat type III 17βHSD and other isoforms (I II, IV, VI, and VII) share little amino acid similarity except for the common short chain alcohol dehydrogenase sequence (2–4, 6, 7, 28, 29).

The highest expression of rat 17βHSD type III mRNA was observed in the Leydig cells, in contrast to the ovary, where it could only be detected by RT-PCR amplification. This is consistent with a previous report that the human type III 17βHSD was only detected in the testis (8). However, the mouse type III form detected by RT-PCR was reported to be

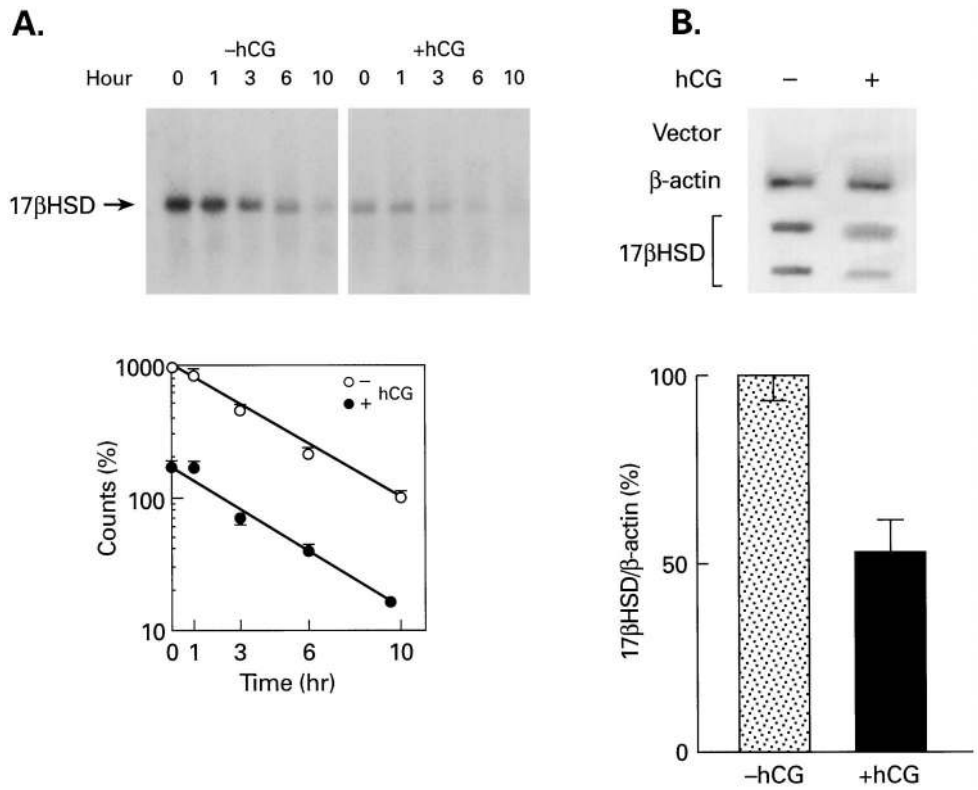

FIG. 7. Determination of the effect of hCG on 17βHSD mRNA stability (A) and transcription rate (B) in rat Leydig cells. A, Leydig cells were obtained from male rats 12 h after sc injection of vehicle (control) or 2.5 μg hCG. Cells were incubated with 10 μg/ml actinomycin D for 0, 1, 3, 6, and 10 h in medium 199 with 0.1% BSA. The mRNA samples were isolated and analyzed by Northern analysis. Results were recorded by autoradiography for visual display (*upper panel*) and quantified by phosphorimage analysis (*lower panel*). B, Run-off transcription assays of nuclei obtained from rat Leydig cells at 24 h after *in vivo* vehicle (control) or 2.5 μg hCG treatment. The *in vitro* transcribed [^{32}P]UTP-labeled RNA sample were hybridized to immobilized cDNA corresponding to 10 and 20 μg 17βHSD, β-actin, and vector. The corresponding hybridization results are shown in the *upper panel*. Results were quantified by phosphorimager, normalized by β-actin, and expressed as a percentage of the control value (*lower panel*). Values in A and B are the mean ± SE of triplicate determinations from a representative experiment of three experiments performed.

ubiquitously expressed in gonadal and nongonadal tissues, with highest expression in the testis (9). The low level of mRNA expression observed in murine nongonadal tissues indicates their potential capacity for conversion of 17-ketosteroids to 17-hydroxysteroids. The apparent difference in tissue distribution patterns between species is in part attributable to the level of sensitivity of the analysis employed (Northern blot *vs.* RT-PCR amplification). In the gonads (ovary *vs.* testis), the catalytic activity of 17βHSD is dependent on the presence of the specific isoform and the level of its expression. Previous expression studies (8, 30, 31) have shown that the various isoforms will catalyze the conversion of both C_{19} and C_{18} steroids with different affinities. The type I isoform displays high affinity for the reduction of C_{18} steroids, whereas the type III enzyme reduces C_{19} steroids with high affinity. In this study, the rat type III isozyme was mainly responsible for the synthesis of testosterone. This enzyme prefers androstenedione (C_{19}) as a substrate, and its failure to convert C_{18} steroids (*e.g.* estrone to estradiol or *vice versa*) demonstrated that the cloned rat type III 17βHSD possesses catalytic activity only for androgen formation (Table 1). The findings of the present study, which have demonstrated that the main function of the rat type III enzyme is the testicular production of testosterone, and those of previous reports (8, 30, 31), indicating that the type I isoform is expressed mainly in the ovary and catalyzes the conversion of estrone to estradiol, are consistent with the low expression of the type III enzyme found in the ovary.

When transiently expressed in COS-1 cells, type III 17βHSD displayed catalytic activity that was markedly stimulated by the presence of glucose in the medium. This finding indicated that the cloned enzyme had similar requirements to the endogenous Leydig cell enzyme, where the integrity of the glucose transport system and the viability of the glycolytic pathway for ATP generation are necessary for optimal 17βHSD activity at the final step of androgen pathway (12). However, no such requirement was observed for other enzymes of the steroidogenic pathway (12). Thus, further investigation with the cloned enzyme should reveal the extent to which posttranslational events, such as phosphorylation or ATP binding, are of relevance to the control of 17βHSD activity in the Leydig cell.

Hormonal regulation of 17βHSD gene expression in rat Leydig cells became evident during our investigation of hCG action on 17βHSD mRNA expression and activity as components of steroidogenic desensitization. These studies demonstrated a gonadotropin-induced steroidogenic block at the level of conversion of androstenedione to testosterone by

17βHSD in rat Leydig cells, in addition to the impairment of other steroidogenic enzymes, including 3βHSD (16) and 17α-hydroxylase/lyase (14, 15). In intact animals, treatment with hCG inhibited 17βHSD type III gene expression in a dose-dependent manner. This reduction was observed even with low doses of the hormone 24 h after treatment (0.01 and 0.1 μg/hCG; by 40%). This is in contrast with the lack of effect of low doses of hormone on 3β-hydroxysteroid dehydrogenase type II gene expression (16). The high dose of hCG (2.5 μg) reduced 17βHSD mRNA levels significantly after 12 h, and a further decrease was observed at 24 h followed by recovery over several days. The reduction at 12 h preceded the major decrease in LH receptors induced by the hormone, which occurred at 24 h, indicating that the former change was not related to receptor down-regulation. Moreover, with the lower doses of the hormone (0.01 and 0.1 μg/hCG) the changes in 17βHSD mRNA levels were not accompanied by changes on the LHR, further confirming the lack of dependence between these parameters. The hormone-induced reduction of 17βHSD gene expression resulted in decreased 17βHSD activity and probably contributed to the *in vitro* decrease in testosterone responses to hCG in rat Leydig cells (13). Although major reductions in 17βHSD mRNA levels (by 60%) were observed at 12 h, the activity of the enzyme was only modestly reduced (by 30%). However, at 24 h the major decrease in mRNA (by 80%) was consistent with the changes in enzyme activity. Overall, the changes observed in 17βHSD mRNA and enzyme activity were noted at later times than those in 3βHSD (12 *vs.* 4 h).

These studies have shown that hCG treatment decreased the incorporation of [α-^{32}P]UTP into 17βHSD transcripts in total nuclear RNA and had no effect on the apparent half-life of the enzyme, indicating that the loss of 17βHSD type III gene expression results from an inhibitory action of hCG at the transcriptional level. It is possible that the consequences of desensitization by hCG include changes in transcriptional regulation of the steroidogenic enzymes through a common control mechanism. The promoter domain and 5′-flanking region of 17βHSD type III gene and the mechanisms of its regulation have not been determined. However, the control of this enzyme could be exerted directly or indirectly by the actions of steroid products or second messengers elicited by the hormonal stimulus.

In conclusion, the present study describes the cloning and characterization of the rat type III 17βHSD. Furthermore, we have demonstrated that the Leydig cell enzyme is down-regulated at the transcriptional level by gonadotropin. The cloning of the rat type III 17βHSD will permit further elucidation of the structural requirements that influence the enzymatic activity of the gene product.

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