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Isolation of a New Mouse 3β-Hydroxysteroid Dehydrogenase Isoform, 3β-HSD VI, Expressed During Early Pregnancy*

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ABSTRACT

The enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) is a key enzyme in the biosynthesis of steroid hormones. To date, this laboratory has isolated and characterized five distinct 3β-HSD complementary DNAs (cDNAs) in the mouse (3β-HSD I through V). These different forms are expressed in a tissue- and developmentally-specific manner and fall into two functionally distinct enzymes. 3β-HSD I and III, and most likely II, function as dehydrogenase/isomerases, whereas 3β-HSD IV and V function as 3-ketosteroid reductases. This study describes the isolation, characterization, and tissue-specific expression of a sixth member of this gene family, 3β-HSD VI. This new isoform functions as an NAD^+-dependent dehydrogenase/isomerase exhibiting very low Michaelis-Menten constant (K_m) values for pregnenolone (~0.035 μM) and dehydroepiandrosterone (~0.12 μM). 3β-HSD VI is the earliest isoform to be expressed during embryogenesis in cells of embryonic origin at 7 and 9.5 days postcoitum (pc), and is the major isoform expressed in uterine tissue at the time of implantation (4.5 days pc) and continues to be expressed in uterine tissue at 6.5, 7.5, and 9.5 days pc. 3β-HSD VI is expressed in giant trophoblasts at 9.5 days pc and is expressed in the placenta through day 15.5 pc. In the adult mouse, 3β-HSD VI appears to be the only isoform expressed in the skin and also is expressed in the testis, but to a lesser extent than 3β-HSD I. Mouse 3β-HSD VI cDNA is orthologous to human 3β-HSD I cDNA. Human type I 3β-HSD has been shown to be the only isoform expressed in the placenta and skin. The demonstration that mouse 3β-HSD VI functions as a dehydrogenase/isomerase and is the predominant isoform expressed during the first half of pregnancy in uterine tissue and in embryonic cells suggests that this isoform may be involved in local production of progesterone, which is needed for successful implantation of the blastocyst and/or maintenance of early pregnancy. (*Endocrinology* **138:** 1392–1399, 1997)

ALL STEROID hormones are derived from cholesterol. A key enzyme in this pathway is 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD). This enzyme catalyzes the conversion of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids, a reaction that is essential for the biosynthesis of all active steroid hormones, including the adrenal steroid hormones, cortisol, corticosterone, and aldosterone, as well as the gonadal steroid hormones progesterone, testosterone, and estradiol.

Recent reports have provided evidence for the expression of multiple isoforms in humans (1–3) and rodents (4–7). For a classification of the human, mouse, and rat isoforms see Clarke *et al.* (8). These isoforms are products of distinct genes and are expressed in a tissue-specific manner. Our laboratory has studied the isoforms expressed in the mouse. To date, we have isolated and characterized five distinct but highly homologous mouse 3β-HSD complementary DNAs (cDNAs), which are indicated by roman numerals (I-V) in the chronological order in which they have been isolated. The genes encoding the different isoforms are found closely linked on mouse chromosome 3 (9). The five isoforms not only are expressed in a tissue-specific manner, but also fall into two distinct functional groups. One group, as represented by 3β-HSD I and III (and most likely II), functions as dehydrogenase/isomerases (10) and is essential for the biosynthesis of active steroid hormones, whereas the other group, represented by IV and V, functions as 3-ketosteroid reductases and appears to be involved in the inactivation of active steroid hormones (6, 7). 3β-HSD I in the adult mouse is expressed in the gonads and adrenal glands (5). 3β-HSD II and III are expressed in the liver and kidney (5), with much greater expression of III in the liver than in the kidney. The major site of expression of 3β-HSD IV is in the proximal tubules of the kidney of male and female mice (6), with minor expression in the testis (7). The expression of 3β-HSD V appears to occur only in the liver of the male mouse, with expression starting during the latter half of pubertal development (7, 11). This paper describes the isolation and tissue-specific expression of a new isoform, 3β-HSD VI, which is the major isoform expressed during the first half of pregnancy in cells of embryonic origin and in uterine tissue. 3β-HSD VI appears to be the only isoform expressed in skin. It is expressed in Leydig cells

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of the adult testis but to a lesser extent than 3β-HSD I. Mouse 3β-HSD VI appears to be orthologous to human 3β-HSD I, which is expressed in the placenta and skin (3). The temporal expression of 3β-HSD VI during the first half of pregnancy suggests that this isoform may serve an important role in the local production of progesterone needed either for implantation of the embryo and/or maintenance of early pregnancy.

Materials and Methods

Materials

Restriction endonucleases *Hin*dIII and *Ssp*I, Taq polymerase, and RNAsin were purchased from Promega Corp. (Madison, WI). *Acc*I and *Ava*II restriction enzymes were obtained from GIBCO BRL (Gaithersburg, MD), and *Nde*I and Sfu I restriction enzymes and AMV reverse transcriptase from Boehringer Mannheim Biochemicals (Indianapolis, IN). The SpinBind DNA Recovery System was purchased from FMC BioProducts (Rockland, ME), and the Enhanced ChemiLuminescent detection kit was purchased from Amersham Corp. (Arlington Heights, IL). RNAzol B was obtained from Cinna Biotex (Houston, TX). Diethylaminoethyl-dextran was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden; [7-N-^{3}H]pregnenolone (10 Ci/mmol), [1,2-N-^{3}H]dehydroepiandrosterone (50 Ci/mmol), [4-^{14}C]progesterone (50 mCi/mmol), [4-^{14}C] androstenedione (50 mCi/mmol) were purchased from Dupont (Wilmington, DE). Nonradioactive dehydroepiandrosterone and androstenedione were purchased from Steraloids (Wilmington, NH), and nonradioactive pregnenolone and progesterone were purchased from Sigma (St. Louis, MO). ITLCA SA sheets were purchased from Gelman Sciences, (Ann Arbor, MI). Mouse 7-day embryo cDNA pooled from Swiss-Webster/NIH embryos was purchased from Clontech Laboratories (Palo Alto, CA).

Animals and tissue preparation

A variety of mice were used: from Jackson Laboratory (Bar Harbor, ME), C57BLxSJL andC57BL/6J male and female mice, as well as pregnant mothers, were delivered to the University of Michigan on day 7.5 postcoitum (pc). All procedures were within the Guideline of Responsible Animal Care at the University of Michigan and had the approval of the Institutional Committee on Animal Care and Use at the University of Michigan. C57xBalb/C male and female mice were obtained from Harlan Laboratories Ltd. (Jerusalem, Israel). All experiments performed in Israel were reviewed by the Institutional Committee on Animal Care and Use of the Faculty of Science, The Institute of Life Sciences, The Hebrew University of Jerusalem and found to be compatible with the standards for care and use of laboratory animals. The C57BLxSJL or the C57xBalb/C mice were mated and the next morning examined for vaginal plugs. 1200 h was designated day 0.5 pc.

Adult mice were killed by cervical dislocation, and the appropriate tissues were removed and immediately frozen on dry ice and stored at −70 C until needed for RNA or protein extraction. Pregnant mothers were killed in the same manner at indicated times.

9.5- and 10.5-day embryos. Uterine horns were removed and placed in PBS on ice. The embryos were separated from the uterus. The 9.5-day embryos include the yolk sac and placenta. The 10.5-day embryos were separated from the yolk sac and placenta, and only the embryos were used for RNA extraction. Each embryo was frozen individually on dry ice and stored at −70 C.

Uterine tissue and giant trophoblast cells. At 4.5 days pc, uterine horns were flushed with PBS to validate the presence of blastocysts, and the entire horn was used for RNA extraction; at 6.5 days pc the isolated implantation sites were trimmed free of the muscle layers, and the stromal and decidual tissue, including the embryonic tissue, was used for RNA extraction; at 7.5 and 9.5 days pc, the implantation sites were trimmed free of the muscle layers and the embryo, and its sacs were removed. RNA was extracted from the inner layers of the giant trophoblast cells, which were carefully scraped from hemi sites and separated from the decidua basalis and decidua capsularis tissues. The latter tissues were used for RNA extraction of the typical 9.5-day decidua preparation. The giant trophoblast cells were used separately from the decidua basalis and the decidua capsularis tissue for RNA extraction.

Placentas and yolk sacs. Placentas and yolk sacs were removed from 13.5-, 15.5-, and 17.5-day embryos. Placentas were used for extraction of RNA, and yolk sacs were used for extraction of DNA for determination of gender.

RT-PCR amplification

Total RNA from the 9.5- and 10.5-day embryos and from the adult tissues was isolated using the acid-guanidinium-phenol-chloroform method (12). RNA from uterine tissues and trophoblast cells was extracted by homogenization of these tissues in RNAzol according to the manufacturer's instruction. The concentration and purity of total RNA was determined by measuring the optical density at 260 and 280 nm. Aliquots of total RNA (at amounts indicated in the figure legends) from each mouse tissue were reverse transcribed using 25 U of AMV reverse transcriptase in 20 μl total reaction. RT was performed as described in the protocol from Perkin-Elmer (Norwalk, CT), using random hexamers. Following the RT reaction, each sample (20 μl) was diluted with 80 μl PCR mixture (final concentrations: 2 mM $MgCl_2$, 0.15 μM of the appropriate primers and 2.5 U Taq polymerase) and incubated at 70 C for 5 min before PCR. Sequential cycles of amplification were performed using a Perkin-Elmer thermocycler for 35 cycles with each cycle consisting of 1 min at 95 C and 1 min at 60 C. The primer pairs (5′–>3′): for amplification of all forms of mouse 3β-HSD: P1 (629 bp amplified product), sense CAGACCATCCTAGATGT +283 to +300, antisense AGGAAGCTCACAGTTTCCA +893 to +911; for complete coding region of 3β-HSD II and VI, P2 (1137 bp amplified product), sense TTCCTGTGTTGACCATG −14 to +3, antisense ATCACTGAGACGTTGTG +1107 to +1123; P3 (c-abl 230 bp amplified product) proto-oncogene (13), sense TTTATGGGGCAGCAGCCTGGAAAAGTACTTGGG, antisense TCACTGGGTCCAGCGAGAAGGTTTTCCTTGGAGTT. Each primer pair spans introns permitting amplified fragments of cDNA to be distinguished from any fragments derived from genomic DNA.

Identification of the RT-PCR products was established by subjecting equal aliquots of the amplified 3β-HSD to digestion with one of five specific restriction enzymes, and the resultant fragments separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. A few representative samples were subjected to Southern analysis using a ^{32}P-labeled 3β-HSD I cDNA probe. Each analysis represents results from at least two or three separate tissue samples. Table 1 describes the size of the fragment that is diagnostic for each of the 3β-HSD cDNAs with the indicated specific restriction enzyme. In some of the figures only the larger diagnostic fragment described in Table 1 is seen, the smaller fragment resulting from the enzyme digestion being below the sensitivity of the assay. *Ava*II digests both 3β-HSD IV and V.

Southern blot analysis

RT-PCR was performed on RNA from adult skin, 9.5-day embryos, and 7.5-day pc uterine tissues. Equal aliquots of the amplified 3β-HSD cDNA were subjected to restriction enzyme analysis as described above. The resulting DNA fragments were transferred to nylon membranes (Hybond-N+, Amersham). The 3β-HSD I cDNA with the polyA tail removed (5) was labeled with [α-^{32}P] deoxycytidine 5′-triphosphate (New England Nuclear Radiochemicals, Boston, MA) by random hexanucleotide primer method to a specific activity of 1×10^{8} to 10^{9} cpm/μg. Hybridization and washing procedures were performed as described previously (8).

TABLE 1. Specific restriction sites in 3β-HSD isoforms

3β-HSD	Restriction enzyme	Diagnostic fragment (bp)
I	*Acc*I	455
II	*Hin*dIII	507
III	*Sfu*I	439
IV and V	*Ava*II	334
VI	*Nde*I	461

DNA sequence analysis

For sequencing, the 1137-bp PCR product (obtained from four separate RT-PCR reactions with testis RNA using the P2 primers) was separated on 1% low melt agarose gel, eluted, and purified using the SpinBind DNA Recovery System. Both strands of the purified fragment were sequenced in the DNA Sequencing Core of the Biomedical Research Core Facilities, University of Michigan, by automated fluorescent cycle sequencing using dye labeled terminators and Applied Biosystems (Foster City, CA) instrumentation. The primers used for sequencing were synthetic oligonucleotides derived from highly conserved regions of 3β-HSD cDNAs.

The 5′ untranslated region of 3β-HSD VI cDNA was deduced from a genomic clone containing the 3β-HSD VI gene, including exons 1 and 2, which was isolated from a 129 Sv/J genomic library (Abbaszade and Payne, unpublished observations). The 5′ region of the 3β-HSD gene was amplified by PCR, as described above, using a T7 primer from the vector and a 3β-HSD VI-specific primer: 5′-GGAGCTGCCTGGTGAC-3′ from +11 to +26. Both strands of the 300-bp PCR product using the same primers were sequenced. A 132-bp exonic sequence for 5′ untranslated region of 3β-HSD VI cDNA was identified by homology to the sequence of the 3β-HSD I gene (5) and identification of the consensus intron/exon splice sequences. The 3′ untranslated sequence was determined by the 3′ rapid amplification of cDNA ends (RACE) method (14). Pregnant mouse uterine RNA was reversed transcribed with the adapter primer 5′-ACGGGCAAATTCTCCACAGCC-3′ with added 17 dT residues. The cDNA pool was then amplified using the 3β-HSD VI specific primer 5′-ACGGGCAAATTCTCCACAGCC-3′ (+643 to +663) as the forward primer and the adapter primer without the poly dT. Both strands of the PCR product were sequenced from two independent clones using the same two primers. The sequence of the coding region and the 3′ and 5′ untranslated region obtained as described above is presented in Fig. 1. Sequencing of the 5′ and 3′ untranslated sequences was performed at the DNA Sequencing Core (Protein and Nucleic Acid Biotech Facility of Stanford University) by automated dye labeled terminator sequencing.

Construction of a 3β-HSD VI cDNA expression vector

The 1137-bp RT-PCR product (see above) obtained from testis RNA that contains the 1122-bp open reading frame of 3β-HSD VI cDNA was subcloned into the pCR II vector using the TA Cloning Kit (Invitrogen, San Diego, CA). The resulting construct was digested with *Hin*dIII and *Xba*I, and the restriction fragment containing the entire coding region was inserted into identically cleaved sites in the p cytomegalovirus (CMV) 5 expression vector to yield pCMV5.3β-HSD VI. The structure of the recombinant plasmid was verified by restriction enzyme analysis. Plasmids were purified before transfection by using the Qiagen Plasmid Purification Kit (Qiagen, Chartsworth, CA).

Transient expression of mouse 3β-HSD cDNAs

Monkey kidney tumor (COS-7) cells were grown in DMEM (GIBCO, Grand Island, NY) supplemented with 10% calf bovine serum (Hyclone, Logan, VT). One day before transfection, cells were plated at about $1.5–2 \times 10^6$ cells/100 mm. After 24 h incubation, medium was removed, and cells were transfected either with the pCMV5 parent vector or with pCMV5.3β-HSD VI, I, III, IV, or V containing recombinant plasmid by the dimethylaminoethyl-dextran method. The latter four expression plasmid-transfected cells were used only for the Western blot analysis illustrated in Fig. 7. Sixty-six hours after transfection, cells were harvested, and homogenates were prepared as previously described (3).

Measurement of 3β-HSD activity

Enzyme activity was determined by measuring the conversion of [^{3}H]pregnenolone to [^{3}H]progesterone and [^{3}H]dehydroepiandrosterone (DHEA) to [^{3}H]androstenedione. Cell-free homogenates of 3β-HSD-transfected COS cells were incubated at 37 C for 10 min in 50 mM potassium phosphate buffer (pH 7.4) with increasing concentrations of the appropriate ^{3}H-labeled substrate and 0.5 mM NAD^+ in a total volume of 1 ml. Incubations were stopped by the addition of 0.1 ml 1 M

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                                Exon I / Exon II            GCTCGATCGATCTGAGGGCTGAGGAGACCAGCATCCAGACTC  -91
TCCCATCTGACTTTTAACAATTTAACAGCCCTCTTCAGGGTCACCCTACATCACACCTGCTCCCACTGTGATCTGTTTCCTGTGTTGACC  -1
                          10                  20                  30
MetProGlyTrpSerCysLeuValThrGlyAlaGlyGlyPheLeuGlyGlnArgIleValGlnLeuLeuMetGlnGluLysAspLeuGlu
ATGCCTGGGTGGAGCTGCCTGGTGACTGGAGCAGGAGGGTTTTTGGGCCAGAGGATTGTCCAGTTGTTGATGCAGGAGAAAGATCTGGAG   90
                          40                  50                  60
GluIleArgValLeuAspLysPhePheArgProGluThrArgGluGlnPhePheAsnLeuAspThrAsnIleLysValThrValLeuGlu
GAGATCAGGGTCCTGGACAAGTTCTTCAGACCAGAAACCAGGGAGCAATTCTTCAACCTAGATACAAACATCAAGGTGACAGTGTTGGAA   180
                          70                  80                  90
GlyAspIleLeuAspThrGlnTyrLeuArgLysAlaCysGlnGlyIleSerValValIleHisThrAlaAlaValIleAspValThrGly
GGAGACATTCTTGACACCCAGTACCTGAGGAAAGCCTGCCAGGGCATCTCTGTTGTTATCCACACTGCAGCTGTCATTGATGTCACAGGT   270
                          100                 110                 120
ValIleProArgGlnThrIleLeuAspValAsnLeuLysGlyThrGlnAsnLeuLeuGluAlaCysIleGlnAlaSerValProAlaPhe
GTCATTCCCAGGCAGACCATCCTAGATGTCAATCTGAAAGGTACCCAGAACTTATTGGAGGCCTGTATCCAAGCCAGTGTTCCAGCCTTC   360
                          130                 140                 150
IlePheSerSerSerValAspValAlaGlyProAsnSerTyrLysGluIleIleLeuAsnGlyAsnGluGluGluHisHisGluSerIle
ATCTTCTCCAGCTCAGTTGATGTTGCAGGGCCCAACTCATACAAGGAGATAATCCTGAATGGCAACGAGGAAGAGCATCATGAAAGCATA   450
                          160                 170                 180
TrpSerAspProTyrProTyrSerLysLysMetAlaGluLysAlaValLeuAlaAlaAsnGlySerMetLeuLysIleGlyGlyThrLeu
TGGTCTGATCCATACCCATACAGCAAAAAGATGGCTGAGAAGGCAGTGCTGGCAGCCAATGGGAGCATGCTGAAAATTGGTGGCACTTTG   540
                          190                 200                 210
HisThrCysAlaLeuArgProMetPheIleTyrGlyGluArgSerProIleSerAsnThrIleIleThrAlaLeuLysAsnLysAsn
CATACTTGTGCATTAAGGCCCATGTTTATTTATGGGGAGAGAAGTCCATTCATTTCTAACACAATAATTACGGCCCTCAAAAATAAGAAT   630
                          220                 230                 240
IleLeuGlyCysThrGlyLysPheSerThrAlaAsnProValTyrValGlyAsnValAlaTrpAlaHisIleLeuAlaAlaArgGlyLeu
ATTCTCGGTTGTACGGGCAAATTCTCCACAGCCAACCCAGTATATGTGGGTAATGTAGCCTGGGCACACATTCTGGCTGCCAGGGGCCTT   720
                          250                 260                 270
ArgAspProLysLysSerProAsnIleGlnGlyGluPheTyrTyrIleSerAspAspThrProHisGlnSerTyrAspAspLeuAsnTyr
CGAGACCCCAAAAAGTCACCAAATATCCAAGGAGAGTTCTACTACATCTCAGATGACACCCCTCACCAAAGCTATGATGATTTAAATTAC   810
                          280                 290                 300
ThrLeuSerLysGluTrpGlyPheCysProAspSerSerTrpSerLeuProValProLeuLeuTyrTrpLeuAlaPheMetLeuGluThr
ACCCTGAGCAAGGAGTGGGGCTTCTGCCCTGATTCCAGCTGGAGCCTTCCTGTGCCCCTACTGTACTGGCTTGCATTCATGCTGGAAACT   900
                          310                 320                 330
ValSerPheLeuLeuSerProIleTyrArgPheIleProProPheAsnArgHisLeuValThrLeuThrGlySerThrPheThrPheSer
GTGAGCTTCCTGCTGAGTCCAATCTACAGATTTATACCTCCCTTTAACCGCCACTTGGTCACACTGACAGGTAGCACGTTCACTTTCTCC   990
                          340                 350                 360
TyrLysLysAlaGlnArgAspLeuGlyTyrGluProLeuValSerTrpGluGluAlaLysGlnLysThrSerGluTrpIleGlyThrLeu
TACAAGAAAGCTCAGCGAGATCTGGGCTATGAGCCACTTGTCAGCTGGGAGGAAGCCAAGCAGAAAACCTCAGAGTGGATCGGGACACTA  1080
                          370
ValGluGlnHisArgAlaThrLeuAspThrThrSerGln*End*
GTGGAGCAGCACAGGGCGACACTGGACACAACGTCTCAGTGATGGGAAGAGGGTGGAGACATGGCTCTGGGTGTTACCAGGTCCTCCAGT  1170
AAGGACAAAAGCACAACACAGGTGCTGCTGCCTTCTTTTGACACAGAGGCCAATTTAGTGCCTTAATCAAGTCACCAAAGCCTTGCCAGT  1260
CACTGATCCAGTCACAAGCCTTCTTTCTGAAATACCTCCCCAAAGACACACAGGCATCTGTGCCCCAGCTCCTGGGTCCAAGCCTCTCAG  1350
CACCTCTTGTACCTCAGAGCTCTCTCCATTATTTTCTCTCCCCATTCAGAGCATGTATAGCCTTCAGAAAATTCTACTTGCTTTATGAAA  1440
CCCAATGGAAGAAACAATAAATACTTGTCAATACCTCAAAAAAAAAAAAAAAAAAAA                                  1497
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FIG. 1. Nucleotide and deduced amino acid sequence of coding region of mouse 3β-HSD VI cDNA. Number of last nucleotide in each row is indicated at *right side* of row; amino acid sequence is numbered *above* sequence. Nucleotides 5′ of ATG initiation codon are designated by negative numbers. Exon 1/exon 2 junction in 5′ untranslated region is noted *above* nucleotide sequence. Putative polyadenylation signal is *underlined*.

NaOH. Before extractions of steroids, unlabeled substrate and product (150 μg each) were added as carrier, and ^{14}C-labeled (~500 cpm) product was added to each incubation tube to monitor product recovery. Steroids were extracted and separated by ITLC SA sheets in chloroform-diethyl ether (70:3) and quantified as previously described (7). The assays were performed in duplicate in two separate experiments from two different preparations of COS-transfected cells. Enzyme activity was determined as picomoles of product formed per min per μg protein. Michaelis-Menten constant (K_m) values were determined by the enzyme Kinetics program (version 1.0c, 1989), a Macintosh Hypercard stack by D. C. Gilberts (Biology Department, Indiana University, Bloomington, IN).

Western blot analysis

Proteins were extracted from transfected COS-7 cells, 9.5-day embryos, or testes from 50-day old mice and subjected to Western blot analysis as described previously (5–7). 3β-HSD immunoreactive proteins were detected with a rabbit antiserum raised against the human placental 3β-HSD and horseradish peroxidase-labeled secondary antibody using the Enhanced ChemiLuminescence kit.

Results

Isolation and identification of a new 3β-HSD cDNA

RT-PCRs using oligonucleotide primers (P1; see *Materials and Methods*) designed to amplify a 629-bp fragment of all known mouse forms of 3β-HSD cDNAs were used to determine the expression of 3β-HSD messenger RNA (mRNA) in a number of mouse nonsteroidogenic tissues (prostate, seminal vesicles, epididymis, sebaceous glands, skin, and nonpregnant and pregnant uterus) that had not previously been examined. Among the tissues surveyed, an amplified fragment was observed only in adult skin and in the uterus during the early stages of pregnancy. To identify which of the five previously characterized 3β-HSD cDNAs was expressed in these tissues, the cDNAs were digested with restriction enzymes specific to each of the known mouse forms. Treatment with the different enzymes did not result in smaller fragments. This finding suggested that the 3β-HSD mRNA expressed in these tissues was the product of a new member of the mouse 3β-HSD gene family. From the preliminary sequence of the amplified cDNA fragment, two restriction sites, *Nde*I and *Ssp*I, were identified that were not found in any of the previously characterized mouse 3β-HSD cDNAs. To obtain a fragment comprising the entire coding region of this presumed new form of 3β-HSD cDNA, primers were designed, based on the sequences of the five known 3β-HSD cDNAs, that would amplify the complete open reading frame. Total RNA from skin and from several steroidogenic tissues was subjected to RT-PCR with these primers. One particular set of primers (P2; see *Materials and Methods*) was found to amplify an 1137-bp fragment both from skin and testis RNA, which when examined by restriction enzyme digestion demonstrated that these 1137-bp fragments contained both an *Nde*I and an *Ssp*I restriction site, but contained none of the restriction sites shown to be unique for each of the five previously identified 3β-HSD cDNAs. The RT-PCR product amplified from testis RNA was purified and further analyzed by restriction enzyme digestion and subjected to sequencing. The 3′ and 5′ untranslated sequences were obtained as described in *Materials and Methods*. The nucleotide sequence of this newly isolated cDNA, 3β-HSD VI, as well as the deduced amino acid sequence is presented in Fig. 1. The sequence includes 132 bp of 5′ untranslated sequences, 1122 bp of open reading frame encoding a protein of 373 amino acids identical to the predicted number of amino acids of the other five characterized mouse 3β-HSD cDNAs, and the complete 375 bp 3′ untranslated sequences including 20 bp of polyadenylated tail. The A in the first in frame ATG codon is designated as position +1. The ATG codon is flanked by the eukaryotic consensus sequence for translation initiation, including the invariant purine, preferably A, in position −3 (15). Table 2 compares the percent identity of the predicted amino acid sequence of mouse 3β-HSD VI to the predicted amino acid sequence of the other five mouse forms (7). Data in this table show that the predicted amino acid sequence of 3β-HSD VI is most closely related to 3β-HSD III and II and somewhat less to 3β-HSD I. Amino acid identity of 3β-HSD VI to 3β-HSD IV and V, which belong to the 3-ketosteroid reductase functional group, is considerably less.

RT-PCR was used to determine tissue-specific expression of 3β-HSD VI mRNA using the primers described above, which yield a 629-bp fragment of all known mouse 3β-HSD cDNAs. The identity of the amplified fragment was established by specific restriction enzyme digestion and separation of the fragments by agarose gel electrophoresis (see Table 1). Figure 2 indicates that the only isoform observed in skin from a male adult mouse is 3β-HSD VI. Southern blot analysis of the gel using a ^{32}P-labeled 3β-HSD I cDNA (see *Materials and Methods*) also did not detect any digested DNA fragments with any of the restriction enzymes tested except with the 3β-HSD VI specific enzyme, *Nde*I (data not shown). We also observed expression of 3β-HSD VI mRNA in skin of female mice and in mice homozygous for the hairless mutation h^{tg} (16) (data not shown).

Figure 3 illustrates the temporal expression of 3β-HSD VI and I in embryonic cells during the first half of pregnancy. The only isoform observed in 7- and 9.5-day embryos was 3β-HSD VI (Fig. 3, A and B). These embryo samples include the yolk sac and embryonic membranes. The 10.5-day embryos that were free of placenta and yolk sac expressed 3β-HSD VI and I (Fig. 3, C and D). Figure 3, C and D represent

TABLE 2. Percent amino acid sequence identities between murine 3β-HSD isoforms

	3β-HSD II	3β-HSD III	3β-HSD IV	3β-HSD V	3β-HSD VI
3β-HSD I	84	83	77	74	**84**
3β-HSD II		93	75	72	**91**
3β-HSD III			73	72	**91**
3β-HSD IV				93	**74**
3β-HSD V					**72**

Data for mouse 3β-HSD I and III were taken from Ref. 5; data for 3β-HSD II were taken from Refs. 5 and 7; data for 3β-HSD IV were taken from Ref. 6; data for 3β-HSD V were taken from Ref. 7.

10.5-day embryos from two different strains of mice. Figure 3C represents two different embryos from the same litter of a C57BL/6J mother; Fig. 3D is a representative gel of one of several embryos examined from a F_2 litter of C57BL/6J×SJL/J parents. Expression of both 3β-HSD VI and I was observed in 10.5-day embryos of either sex. These data indicate that 3β-HSD VI is the earliest isoform expressed in the embryo and/or embryonic membranes, with the expression of 3β-HSD I occurring in the embryo between days 9.5–10.5 pc.

FIG. 2. Expression of 3β-HSD VI mRNA in adult mouse skin. One hundred nanogram of skin RNA from an approximately 50-day-old male C57BL/6J mouse was subjected to RT-PCR using P1 primers (see *Materials and Methods*). To identify type of 3β-HSD mRNA expressed, equal aliquots of RT-PCR product were digested with restriction enzymes specific for the different forms listed in Table 1. U, Undigested; *roman numerals*, specific isoforms of 3β-HSD. The specific restriction enzyme used for identification is indicated following the isoform number: I, *Acc*I; II, *Hin*dIII; III, *Sfu*I; IV, *Ava*II; VI, *Nde*I. Resulting fragments were analyzed by agarose gel electrophoresis. *Ava*II digests both IV and V.

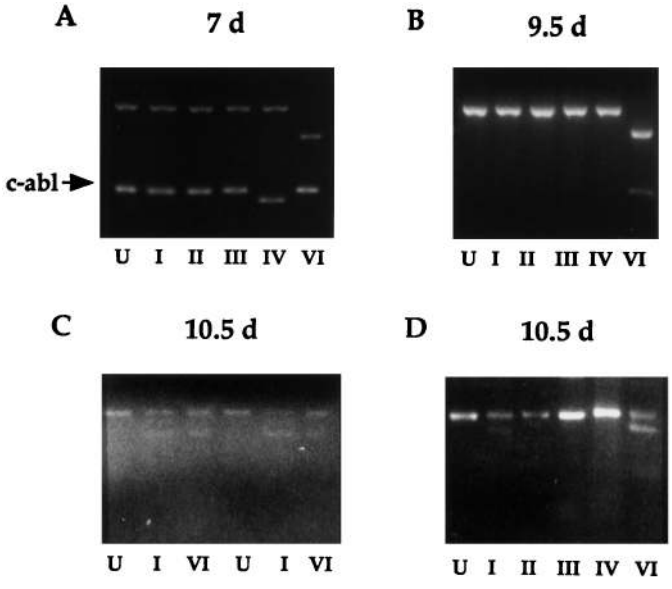

FIG. 3. Expression of 3β-HSD I and VI mRNA in developing mouse embryos. A, Two nanograms of 7-day mouse embryo cDNA (Clontech) were amplified using P1 and P3 primers (see *Materials and Methods*). PCR products were analyzed by enzyme digestion as described for Fig. 2. B, Total RNA (200 ng) from 9.5-day C57BL/6J mouse embryos was analyzed as described for Fig. 2. C, Total RNA (200 ng) from two different 10.5-day C57BL/6J mouse embryos from same litter were analyzed as described for Fig. 2. Results represent two separate embryos. D, Total RNA (200 ng) from a 10.5-day mouse embryo (from F_2 litter of C57BL/6J×SJL/J) was analyzed as described for Fig. 2.

The expression of 3β-HSD isoforms in uterine tissue was examined at days 4.5, 6.5, and 9.5 pc and in isolated giant trophoblast cells at 9.5 days pc. As illustrated in Fig. 4A, 3β-HSD VI is expressed as early as 4.5 days pc in the pregnant uterus, which is the time of implantation, and continues to be expressed at 9.5 days pc (Fig. 4, B and C). No 3β-HSD isoforms were detected in the 3.5-day pc pregnant uterus (data not shown). Illustrated in Fig. 4D is the expression of 3β-HSD VI in giant trophoblasts at 9.5 days pc.

To ascertain whether 3β-HSD VI is the only or the major isoform expressed in the pregnant uterus and in embryos before 10.5 days, RNA from representative samples was subjected to RT-PCR and examined by Southern blot analysis using a ^{32}P-labeled 3β-HSD I cDNA as a probe (see *Materials and Methods*). Figure 5 shows that 3β-HSD VI is the predominant isoform of 3β-HSD expressed in the 9.5-day embryo (Fig. 5A) and in uterine tissue (Fig. 5B) during the first half of mouse pregnancy with minimal expression of 3β-HSD I.

The expression of 3β-HSD isoforms in mouse placenta was studied at 13.5, 15.5, and 17.5 days pc. A representative gel is shown in Fig. 6. Placentas from 13.5-day-old embryos express both 3β-HSD VI and I, the expression of VI decreases in placentas from 15.5-day-old embryos and is no longer observed in placentas from 17.5-day-old embryos, which appear (by ethidium bromide staining) to express only 3β-HSD I. Placentas from several embryos were examined, and the sex of the embryo was determined by PCR amplification of the male-specific gene *Sry* (17). The temporal expression of the two isoforms in placenta was identical whether it came from a female or a male fetus.

Because 3β-HSD VI was first identified in skin and testes

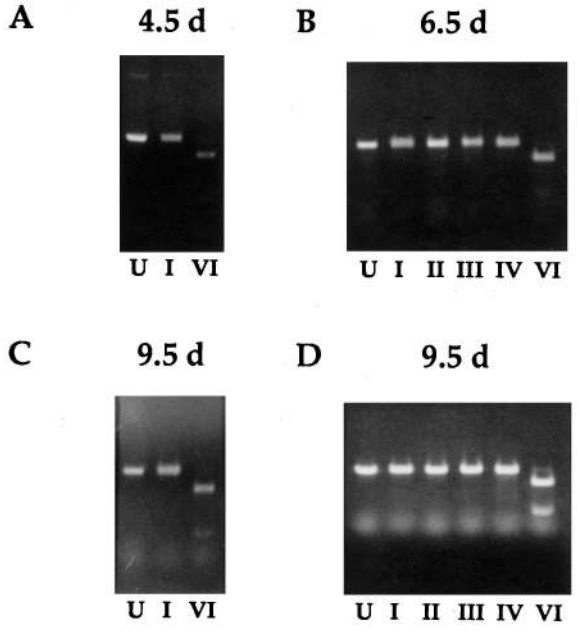

FIG. 4. Expression of 3β-HSD I and VI mRNA in pregnant mouse uterus and trophoblast cells. Total RNA (500 ng) from each tissue (see *Materials and Methods*) was analyzed as described for Fig. 2. A, Pregnant mouse uterus at 4.5 days. B, Implantation sites of 6.5-day pregnant mouse uterus. C, Decidual tissue of 9.5-day pregnant uterus. D, Giant trophoblast cells from 9.5-day embryonic membranes.

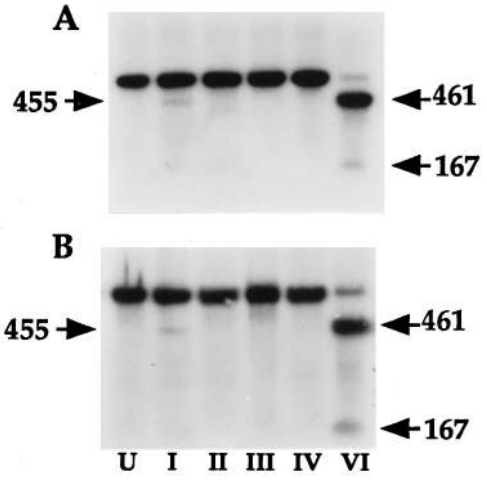

FIG. 5. Southern blot analysis of expression of 3β-HSD isoforms in 9.5-day embryo (A) and 7.5-day pc pregnant uterus (B). Total RNA from 9.5-day mouse embryos (500 ng) and from 7.5-day pregnant uterus (700 ng) was analyzed as described for Fig. 2. DNA fragments were detected by Southern blot analysis as described in *Materials and Methods*. *Arrows*, Predicted major fragment for 3β-HSD I (455 bp) and predicted fragments for 3β-HSD VI (461 bp and 167 bp).

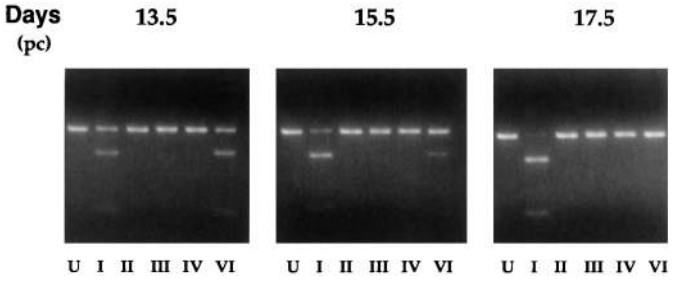

FIG. 6. Expression of 3β-HSD I and VI mRNA in mouse placentas. Total RNA (100 ng) from a 13.5-, 5.5-, and 17.5-day mouse placenta was analyzed as described for Fig. 2.

from adult mice, we reexamined other tissues from adult mice for expression of 3β-HSD VI as well as the other forms. Previously, using RNase protection analysis, we had observed only 3β-HSD I mRNA in the classical steroidogenic tissues, the gonads, and adrenal glands (5). Figure 7 indicates that 3β-HSD I is the only form of 3β-HSD expressed in the adrenals of both sexes and in the ovary, whereas in the testis (using the more sensitive assay of RT-PCR) the presence of 3β-HSD VI mRNA was detected as well as 3β-HSD I mRNA. Examination of livers and kidneys from mature mice, which previously have been shown to express different isoforms of 3β-HSD (5, 6, 7, 11), indicated the absence of 3β-HSD VI expression in the adult liver and kidney of both sexes (data not shown).

To characterize the protein encoded by 3β-HSD VI cDNA, COS-7 cells were transiently transfected with pCMV5.3β-HSD VI, an expression vector containing the coding region of 3β-HSD VI cDNA. The apparent molecular weight of the expressed protein was compared by Western blot analysis to transiently expressed 3β-HSD I, III, IV, and V, as well as the endogenous isoforms expressed in the mature testis and the 9.5-day embryo. Figure 8 shows that the mobility of 3β-HSD VI (~44 kDa) is identical to, or very similar to, the mobility of 3β-HSD III, but differs from the mobility of 3β-HSD I, IV, and V. Figure 8 also shows that a protein of identical mobility as VI is expressed in the 9.5-day embryo and also in the testis. In the testis, as previously reported, the major expressed protein is 3β-HSD I (~42 kDa). No 3β-HSD immunoreactive protein was observed in COS-7 cells transfected with the pCMV5 parent vector.

To characterize the enzymatic characteristics of the protein encoded by 3β-HSD VI cDNA, cell-free homogenates of COS-7 pCMV5.3β-HSD VI-transfected cells were first incubated with a range (0.05–5.0 μM) of the substrates [^{3}H]pregnenolone or [^{3}H]DHEA in the presence of NAD^+ or $NADP^+$. These preliminary studies indicated that very little if any activity was observed when $NADP^+$ was used as the cofactor (data not shown). Enzymatic activity was observed with either substrate in the presence of NAD^+. To determine the apparent K_m values of expressed 3β-HSD VI for preg-

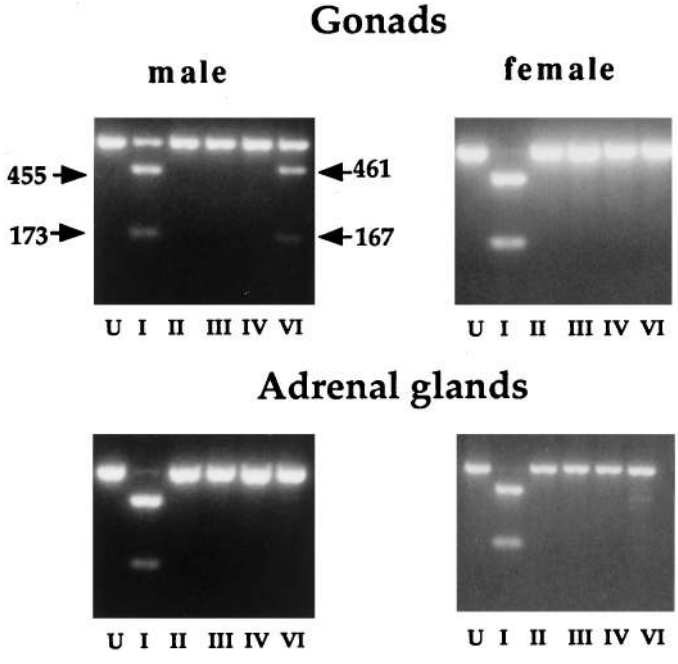

FIG. 7. Expression of 3β-HSD isoforms in adult mouse gonads and adrenal glands. Total RNA (100 ng) from male and female mouse gonads and adrenal glands were analyzed as described for Fig. 2. *Arrows* for male gonad indicate size of predicted fragments for 3β-HSD I (455 bp and 173 bp) for 3β-HSD VI (461 bp and 167 bp) after restriction enzyme digestion.

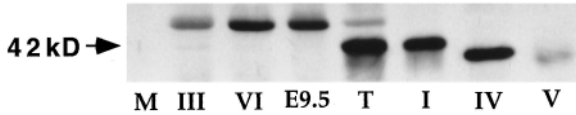

FIG. 8. Western blot analysis of 3β-HSD proteins in transfected cells and in 9.5-day mouse embryo (E9.5) and mouse testis (T). COS-7 cells were transiently transfected as described in *Materials and Methods*. M, pCMV5-(vector only) transfected cells (30 μg); III, pCMV5.3β-HSD III-transfected cells (30 μg protein); VI, pCMV5.3β-HSD VI-transfected cells (30 μg protein); E9.5, 9.5-day embryo (50 μg protein); T, testis from an approximately 50-day-old mouse (75 μg protein); I, pCMV5.3β-HSD I-transfected cells (5 μg protein); IV, pCMV5.3β-HSD IV-transfected cells (10 μg protein); V, pCMV5.3β-HSD V-transfected cells (10 μg protein). Proteins were subjected to SDS-PAGE and Western blot analysis as described in *Materials and Methods*.

nenolone and DHEA, increasing concentrations of [^{3}H]pregnenolone (0.023–2.0 μM) or [^{3}H]DHEA (0.023–5.0 μM) in the presence of NAD^+ were incubated with cell-free homogenates of 3β-HSD VI-transfected cells as described in *Materials and Methods*. No activity was observed in COS-7 cells transfected with the parent vector only. The apparent K_m values of 3β-HSD VI in two series of experiments using cell-free homogenates from two separate transfections were: for pregnenolone, 0.03 ± 0.008 μM and 0.04 ± 0.013 μM and for DHEA, 0.103 ± 0.027 μM and 0.138 ± 0.015 μM. These data demonstrate that 3β-HSD VI functions as an NAD^+-dependent dehydrogenase/isomerase exhibiting very low K_m values for pregnenolone.

Discussion

In this study we identified a new form of mouse 3β-HSD, 3β-HSD VI, which functions as an NAD^+-dependent dehydrogenase/isomerase. The predicted amino acid sequence 3β-HSD VI is most closely related to the amino acid sequence of mouse 3β-HSD III and II and somewhat less to to the amino acid sequence of 3β-HSD I (Table 2). Both 3β-HSD III and I have previously been shown to function as NAD^+-requiring dehydrogenase/isomerases (10). 3β-HSD VI exhibits very low K_m values for pregnenolone (~0.035 μM), somewhat lower than the K_m value reported for 3β-HSD I, and markedly lower than the value reported for 3β-HSD III (Table 3). 3β-HSD VI is the earliest and predominant mouse 3β-HSD isoform expressed during the first half of pregnancy in cells of embryonic origin, and in the pregnant uterus at the time of implantation. 3β-HSD VI continues to be expressed in placenta from 13.5-day embryos, but decreases by day 15.5, and is no longer observed at 17.5 days. Previous studies reported expression of P450 side chain cleavage (scc) mRNA at the time of implantation, and intrauterine administration of oil can mimic this effect of implantation (18). Furthermore, P450scc (18) or 3β-HSD mRNA (19) are not expressed before implantation. Thus, decidual expression of P450scc and 3β-HSD VI mRNA at this time suggests that these two enzymes may be needed for local biosynthesis of progesterone during the first half of pregnancy. 3β-HSD I mRNA was first detected in 9.5-day embryos. 3β-HSD I is the major or only isoform expressed in gonads and adrenal glands of the mature mouse (this study; Ref. 5). In a previous study we reported the expression of 3β-HSD I in fetal testes as early as embryonic day 13.5 (17) and in adrenal glands of both sexes from embryonic day 15.5 and throughout development (unpublished observations). The previous studies on 3β-HSD mRNA expression in embryonic tissues were carried out before the identification of 3β-HSD VI. The complete digestion of the RT-PCR product from fetal testes and adrenal glands of both sexes by 3β-HSD I-specific restriction enzymes indicates that 3β-HSD I is the only isoform expressed in these fetal tissues.

TABLE 3. Comparison of K_m values for the dehydrogenase/isomerase isoforms

3β-HSD Isoform	Pregnenolone	DHEA
	K_m (μM)	
I[a]	0.076	0.14
III[a]	1.030	0.49
VI[b]	0.035	0.12

[a] Data taken from Clarke *et al*. (10).
[b] Data represents average of the K_m values from two separate experiments as described in text.

The mouse 3β-HSD VI cDNA appears to correspond to human 3β-HSD I (3) and rat 3β-HSD IV (4). These are the only isoforms in each species that are expressed in the skin. In addition, these isoforms are expressed in the placenta (3, 4). The demonstration in this study that mouse 3β-HSD VI is the major isoform expressed during the first half of pregnancy through day 9.5 in the uterus and embryonic cells suggests a possible role for this isoform in the local production of progesterone needed for implantation of the blastocyst and/or maintenance of early pregnancy. The failure to identify human patients lacking the orthologous enzyme, 3β-HSD I, is consistent with the hypothesis that these forms of 3β-HSD are required for implantation and/or maintenance of pregnancy in both rodents and humans.

The expression of 3β-HSD VI mRNA and protein in the 50-day-old testis was unexpected. Previous reports from our laboratory examining immunoreactive proteins in Leydig cells and testes from CD-1 mice by Western analysis indicated the presence of only one immunoreactive protein with the mobility of 3β-HSD I (5). On over exposure of these immunoblots we did observe an immunoreactive protein with a lower mobility than 3β-HSD I (unpublished observations). In the current study, a number of testicular samples from a variety of inbred strains of mice (~50–60 days old) were examined for expression of the 3β-HSD immunoreactive protein with lower mobility. When larger amounts of testicular protein (50 μg or greater) were analyzed, this protein was detected. As shown in Fig. 8, expression of this protein is considerably less than expression of the 3β-HSD I protein. We also examined 3β-HSD mRNA isoforms in MA-10 Leydig tumor cells. Both 3β-HSD I and VI mRNA were expressed in the MA-10 Leydig cells (data not shown). This observation indicates that Leydig cells are the cell of the testis in which both 3β-HSD VI and I are expressed. The function served by 3β-HSD VI in the testis remains to be established. Expression of 3β-HSD VI appears to be male specific in the gonad, because this isoform was not detected in ovaries (Fig. 7). Recently, the expression of human 3β-HSD type I transcripts were observed in human testes (20). The observation that human testes as well as mouse testes express these forms of 3β-HSD provides additional evidence that human type I and mouse type VI are orthologous. Keeney *et al.* (21) reported the expression in mouse testes of an immunoreactive protein with a lower mobility relative to mouse 3β-HSD I. These authors reported that this lower mobility protein was male specific in the gonad and first appeared early during pubertal development. From Northern analysis using an oligonucleotide based on the partial sequence of the 3β-HSD II cDNA published by our laboratory (21), Keeney *et al.* concluded that the lower mobility immunoreactive protein represented 3β-HSD II. The oligonucleotide used by these authors exhibits only one mismatch out of 25 bases when compared with 3β-HSD VI. Therefore, this oligonucleotide would not discriminate between 3β-HSD II and VI. From the current study (Figs. 7 and 8) it can be concluded that

this testicular 3β-HSD isoform represents 3β-HSD VI and not 3β-HSD II as reported (21).

In summary, a sixth form of the mouse 3β-HSD multigene family, the orthologous form to human I, has been identified. The demonstration in this study that this form is expressed in the uterus at the time of implantation and in embryonic cells during the first half of pregnancy predicts a similar role for human 3β-HSD I, and suggests that these isoforms of 3β-HSD may be involved in the local production of progesterone that is needed for successful implantation of the blastocyst and/or maintenance of early pregnancy. The mouse is an ideal model for testing the functional role of 3β-HSD VI by analysis of targeted mutations of mouse 3β-HSD VI.

Acknowledgments

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