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Complementary Deoxyribonucleic Acid Cloning and Enzymatic Characterization of a Novel 17β/3α-Hydroxysteroid/Retinoid Short Chain Dehydrogenase/Reductase*

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ABSTRACT

17β-Hydroxysteroid dehydrogenases (17βHSDs) convert androgens and estrogens between their active and inactive forms, whereas retinol dehydrogenases catalyze the conversion between retinol and retinal. Retinol dehydrogenases function in the visual cycle, in the generation of the hormone retinoic acid, and some also act on androgens. Here we report cloning and expression of a complementary DNA that encodes a new mouse liver microsomal member of the short chain dehydrogenase/reductase (SDR) superfamily and its enzymatic characterization, *i.e.* 17βHSD9. Although 17βHSD9 shares 88% amino acid identity with rat 17βHSD6, its closest homolog, the two differ in substrate specificity. In contrast to other 17βHSD, 17βHSD9 has nearly equivalent activities as a 17βHSD (with estradiol ≃ adiol) and as a 3αHSD (with adiol ≃ androsterone). It also recognizes retinol as substrate and represents in part the NAD^+-dependent liver microsomal dehydrogenase that uses unbound retinol, but not retinol complexed with cellular retinol-binding protein. Thus, this enzyme has catalytic properties that overlap with two subgroups of SDR, 17βHSD and retinol dehydrogenases. Inactivation of estrogen and a variety of androgens seems to be its most probable function. Because of its apparent inability to access retinol bound with cellular retinol-binding protein, a function in the pathway of retinoic acid biosynthesis seems less obvious. These data provide additional insight into the enzymology of estrogen, androgen, and retinoid metabolism and illustrate how closely related members of the SDR superfamily can have strikingly different substrate specificities. (*Endocrinology* **140:** 5275–5284, 1999)

17-β-HYDROXYSTEROID dehydrogenases (17βHSDs) compose a group of at least eight distinct enzymes that interconvert androgens or estrogens between their active and relatively inactive forms (1–16). These enzymes have unique tissue distribution patterns and serve as either dehydrogenases or reductases, but usually not as both. Some act predominantly with estrogens (17βHSD1, -4, and -7), others act predominantly with androgens (17βHSD3 and -5), whereas others metabolize both estrogens and androgens (17βHSD2, 17βHSD6, and Ke6). The substrate specificity and expression loci of several suggest specific functions in modifying sex hormone activity. Females express 17βHSD1, for example, which acts as a reductase to activate estrone into estradiol in the human ovary, placenta, and breast (1, 2, 9). Males express 17βHSD3, which functions as a reductase in the testis to activate androstenedione into testosterone (4). Both males and females express 17βHSD2, which functions as a dehydrogenase in liver, placenta, prostate, and other tissues, but not in testis, to inactive estradiol and testosterone into estrone and androstenedione, respectively, with about equivalent efficiency (3, 10). Rat liver and prostate express 17βHSD6, which further inactivates 5α-androstan-3α,17β-diol (adiol) into androsterone (5). With the exception of 17βHSD4, which seems to have only a minor function in steroid metabolism (7, 13), the precise contributions to sex steroid metabolism of most of the others require further study. The very expression of so many 17βHSDs, however, indicates the precise control mechanisms required for steroid hormone metabolism in different spatial and perhaps temporal patterns.

Most 17βHSD enzymes belong to the superfamily of short chain dehydrogenase/reductase (SDRs), but 17βHSD5 belongs to the aldo-keto reductase superfamily (8), and the peroxisome-localized 17βHSD4 consists of a unique fusion of an SDR, an acyl-coenzyme A dehydrogenase, and sterol carrier protein-2 (7, 13). The SDR superfamily consists of approximately 100 different members in animals, bacteria, and plants that function in steroid, PG, and retinoid metabolism (17, 18). The members of the SDR superfamily share relatively little amino acid sequence similarity and have only about 20 strictly conserved residues. Conservation resides in the N-terminal placement of the cofactor-binding residues, catalytic and cofactor-binding residues, the sequence NNAG, and tertiary structures. As alluded to above, SDR frequently act multifunctionally, catalyzing dehydrogenations and/or reductions of seemingly disparate substrates. A single SDR, 17βHSD2 for example, serves as a 17βHSD with estrogen and multiple androgen substrates and as a 20αHSD with 20α-dihydroprogesterone (3, 10). Others, human retinol dehydrogenase (RoDH) for example, have activity with retinoids and as 3αHSD and 17βHSD (14). Despite the multiple catalytic functions of SDR, some tend to cluster in major

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substrate-oriented groups, such as 17βHSD and the several [RoDH1, -2, and -3; *cis*-retinol/androgen dehydogenase-1 (CRAD1) and -2; retSDR1; RDH4; and 11-*cis*-RoDH) that catalyze retinoid metabolism (19–31).

The SDR that serve as retinol dehydrogenases function in the pathway of retinoic acid biosynthesis by catalyzing the first step in the conversion of retinol (vitamin A) into the hormone retinoic acid (32, 33). Other SDR/retinol dehydrogenases function in the visual cycle by interconverting either 11-*cis*-retinol into 11-*cis*-retinal or all-*trans*-retinal into all-*trans*-retinol (22, 23, 28, 31). Rodent liver microsomes express several retinol dehydrogenases active in the biosynthesis of retinoic acid (34–36). One of these, which can use either $NADP^+$ or NAD^+ *in vitro*, recognizes retinol complexed with cellular retinol-binding protein (CRBP) and unbound retinol as substrates to produce retinal, the intermediate in all-*trans*-retinoic acid biosynthesis. At least three complementary DNAs (cDNAs) have been cloned (RoDH1–3) that appear to encode this activity (19–21). Others use NAD^+ *in vitro* and seem to recognize only unbound retinol as substrate.

Here we report cloning of a cDNA that encodes a new mouse SDR, *i.e.* 17βHSD9, and its enzymatic characterization. This enzyme shares 88% amino acid identity with rat 17βHSD6, its closest SDR homolog, but the two seem not to represent interspecies homologs. First, PCR with specific primers identified expression of a homolog of rat 17βHSD6 in mouse, but did not amplify mouse 17βHSD9. Moreover, 17βHSD9 and 17βHSD6 differ in substrate specificity. Mouse 17βHSD9 has roughly equivalent activities as a 17βHSD (with estradiol ≃ adiol) and as a 3αHSD (with adiol ≃ androsterone), whereas rat 17βHSD6 has 10-fold greater 17βHSD activity with adiol than it does with estradiol and has low 3αHSD with androsterone. 17βHSD9 also recognizes retinol as substrate and contributes to the NAD^+-dependent liver microsomal dehydrogenase that recognizes unbound retinol. These data provide additional insight into the enzymology of estrogen, androgen, and retinoid metabolism and illustrate how closely related SDR can have quite strikingly different substrate specificity.

Materials and Methods

cDNA isolation

A mouse liver λgt10 cDNA library (CLONTECH Laboratories, Inc., Palo Alto, CA) was screened under low stringency conditions (hybridization at 40 C and final wash at 60 C) with a ^{32}P-labeled probe consisting of rat RoDH1 nucleotides 298–673 (19). The first round of screening identified 189 positive plaques from approximately 3.6×10^5. The second round of screening was performed by PCR at an annealing temperature of 65 C with the sense primer TTCTAGTGCGCTGTCATC (nucleotides 197–214 of the final cDNA) designed from a partial cDNA sequence in the EST database (GenBank access no. AA239724, nucleotides 12–517) and a degenerate antisense primer GAAGA(A/G)CTT(A/G)GCATCCCA (nucleotides 1088–1105 of the final cDNA) designed from a conserved C-terminal region in the cDNAs that encodes the RoDH/CRAD family (19–21, 24, 26). Sixteen plaques were identified with the 909-bp size fragment anticipated from the primers used. To determine insert sizes in the *Eco*RI site of the λgt10 phages, PCR was performed with a pair of primers designed to bind to the two phage arms near the cDNA insert (left arm primer, AGCAAGTTCAGCCTGGTTAAGT; right arm primer, TTATGAGTATTTCTTCCAGGG).

The PCR products were resolved with an agarose gel, and all 16 cDNA inserts were digested with *Eco*RI and ligated into pBluescript II SK^+. All 16 were sequenced in both directions by nested deletion. The longest (no. 55) was designated pBSK/17βHSD9.

Expression of 17βHSD9

The cDNA insert of pBSK/17βHSD9 was digested from λgt10 with *Eco*RI and ligated into pcDNA3 to produce pcDNA3/17βHSD9. COS cells were transfected using Lipofectamine with pcDNA3/17βHSD9 or with pcDNA3 (mock transfection). Assays were conducted with intact plated cells for 2 h, beginning 24 h after transfection. Alternatively, cell pellets were suspended in 10 mM HEPES and 10% sucrose (pH 7.5), and homogenized with a Virsonic 60 ultrasonic wave homogenizer (Virtis, Gardener, Inc.). The homogenate was centrifuged at $800 \times g$ for 10 min. The supernatant protein was used for enzymatic assays, unless noted otherwise. Protein concentrations were determined by the method of Bradford (37).

Enzyme assays

Incubations and analyses of products have been described in detail previously (24, 26). Assays were run for 30 min at 37 C in either 0.25 ml 10 mM cyclohexlaminoethanesulfonic acid (Ches) (pH 9) or 10 mM succinic acid (pH 5), 150 mM KCl, 2 mM EDTA, and 1.6 mM NAD^+ or 2 mM NADH with the $800 \times g$ supernatant of mock- or pcDNA3/17βHSD9-transfected cells, unless noted otherwise. The NADH-regenerating system used in some experiments consisted of 5 U sorbitol dehydrogenase, 20 mM sorbitol, and 2 mM NADH incubated in the reaction mixture for 10 min before substrate addition. Retinoid dehydrogenase assays were quenched with 0.1 ml 0.1 M *O*-ethylhydroxylamine and 0.35 ml methanol, incubated at room temperature for 10 min, and extracted with 2.5 ml hexane. The retinoids in the hexane extract were quantified by normal phase HPLC with a detection limit of approximately 1 pmol (20, 21). Steroid dehydrogenase assays were performed with [^3H]steroids (40–101 Ci/mmol, 20,000 dpm/reaction). Incubates were extracted with methylene chloride (4 ml), and the extracts were analyzed by TLC. ^3H-Labeled steroids were detected by autoradiography. The radioactive zones were excised and counted with a liquid scintillation counter. Kinetic data were obtained under initial velocity conditions and were analyzed with Enzfitter (38).

Northern blotting

Northern blots were performed with the mouse Multiple Tissue Northern blot, which provides 2 μg poly(A)$^+$ RNA/lane on a Nylon membrane (CLONTECH Laboratories, Inc.). The probe was a 97-base chemically synthesized oligo consisting of nucleotides 125–221 of the cDNA labeled with ^{32}P by random priming. Prehybridization was performed in 10 ml hybridization solution (50% formamide, 5 × Denhardt's, 0.1% SDS, 100 μg/μl denatured salmon sperm DNA, and 5 × SSPE) (SSPE = 3 M sodium chloride, 0.2 M sodium phosphate, 0.02 EDTA, pH 7) at 40 C for 4 h. Hybridization was performed overnight in the same solution containing 2×10^6 cpm probe. The final wash was performed at 55 C with 1 × SSC-0.1% SDS. Signals were visualized with a Bio-Rad Laboratories, Inc. GS-505 Molecular Imager System (Hercules, CA).

Ribonuclease (RNase) protection assays

A 17βHSD9-specific probe was generated by digesting pBSK/17βHSD9 with *Bst*XI. The 3′-protruding ends were blunted with mung bean nuclease. The 347-bp nucleotide cDNA fragment (nucleotides 380–726 of the cDNA) was recovered from an agarose gel, subcloned into the *Eco*RV site of pBluescript II SK^+, and linearized with *Hin*dIII. A ^{32}P-labeled antisense probe was transcribed with T3 RNA polymerase (Ambion, Inc., Austin, TX) for 1 h at 37 C in 10 mM dithiothreitol; 0.5 mM each of ATP, CTP, and GTP; and 50 μCi of UTP (800 Ci/mmol). The 280-nucleotide antisense β-actin complementary RNA probe (nucleotides 79–358) used as an internal standard was transcribed from pTRI mouse β-actin template (Ambion, Inc.) under the same conditions. DNA templates were removed by deoxyribonuclease I digestion. Transcripts were purified with 5% polyacrylamide and 8 M urea gels. RNase protection assays were performed with the Hybspeed RPA kit (Ambion, Inc.) following the manufacturer's directions. Briefly, total RNA (50 μg) was extracted from mouse tissues with guanidinium thiocyanate-phenol-

chloroform and coprecipitated with the complementary RNA probes (1×10^5 cpm 17βHSD9; 5×10^4 cpm β-actin). Pellets were resuspended in 10 μl hybridization buffer (Ambion, Inc.) by four alternating 15-sec periods of vigorous vortexing and incubated at 95 C for 3 min and then at 68 C for 10 min. A 100-μl aliquot of RNase A/T1 mixture (diluted 1:100) was allowed to digest the unhybridized probes and RNA for 30 min at 37 C. Inactivation/precipitation mixture (150 μl) was added to precipitate the undigested RNA. After centrifugation, the supernatants were removed, and the pellets were dissolved in 40 μl gel loading buffer by heating at 95 C for 4 min. The samples were loaded onto 5% polyacrylamide-8 M urea gels and run at about 180 V for 3–4 h. Quantitative analysis was performed with a Molecular Imager system (Bio-Rad Laboratories, Inc.).

Results

cDNA and amino acid sequences

Although multiple microsomal RoDH/CRAD cDNAs have been isolated, several more enzymatic activities occur (35, 39–41). To determine whether any of the partially characterized activities are related to known isozymes, a mouse liver cDNA library was screened initially with a probe from the 5′-end of RoDH1, highly conserved among RoDH1–3 and CRAD1 and 2 (19–21, 24, 26). The second round of screening by PCR relied on a sense primer from an EST library representing an unknown SDR, but with 89% identity to RoDH1 in the first 94 amino acid residues. (These residues are highly conserved among RoDH1–3 and CRAD1 and CRAD2.) A degenerate antisense primer was designed from conserved amino acid residues (WDAKFF) in the C-termini of RoDH1–3 and CRAD1 and CRAD2. This process identified 16 clones. Four were identical. Of the 12 that differed, all had identical nucleotide sequences in their coding regions; 8 had identical nucleotide sequences in their 5′-untranslated regions (5′-UTRs) in regions of overlap, and 9 had identical nucleotide sequences in their 3′-UTRs. One had no complete coding region, and 1 was fused with another gene in its 3′-end. Five different sequences occurred, outside of the coding region, because of differences in the sequences of 5′- and/or 3′-UTRs (Table 1). These differences included either a 38-bp insertion and/or a 5-bp deletion in the 5′-UTRs. The longest cDNA (no. 55), designated pBSK/17βHSD9, differed totally from the others in its 3′-UTR sequence.

The protein encoded by pBSK/17βHSD9 has a deduced amino acid sequence that includes the 25 conserved signature SDR residues (Fig. 1). Notably, the cofactor binding residues G^{36}XXXGXG occur in the identical loci with those in RoDH1–3 and CRAD1 and CRAD2, as do the catalytic residues S^{164}, Y^{176}, and K^{180}. The latter group, however, has a catalytic area sequence of GGGYCISK compared with GGFYSCSK for 17βHSD9, perhaps contributing to differences in substrate specificity. The first 114 amino acid residues of 17βHSD9 showed only 10 differences compared with RoDH1–3 and CRAD1 and CRAD2, which differ among each other by 7 or less residues in this area. As in other SDR that catalyze retinoid dehydrogenation, the first 18 amino acid residues of 17βHSD9 provide a hydrophobic area sufficient to span a membrane and are adjacent to 4 hydrophilic amino acid residues, in this case RERQ, suggestive of a membrane insertion motif.

The sequence of the pBSK/17βHSD9 insert was identical with three partial cDNA sequences in the GenBank EST database: AA239724, nucleotides 12–517; AA239189, nucleotides 900-1309; and AA239249, nucleotides 907-1087. Not only were none complete, but there were no data suggesting the 5′-most fragment and the two 3′-end fragments represented different pieces of the same cDNA.

Mouse 17βHSD9 has closest amino acid identity with rat 17βHSD6. Therefore, expression of a mouse liver 17βHSD6 was demonstrated by PCR to distinguish the expression of two different genes in the mouse, 17βHSD6 and 17βHSD9. PCR was performed with a sense primer specific for 17βHSD6 and a degenerate antisense primer. The primer pair amplified the expected 640-bp signal for 17βHSD6 from both rat and mouse liver cDNA, but did not amplify segments from a mixture of rat RoDH1, -2, and -3; CRAD1 and -2; and mouse 17βHSD9 cDNAs (Fig. 2).

17βHSD9 has less homology with RoDH1–3 and CRAD1 and -2 (Table 2). Even further away in homology are a second subgroup of retinoid-associated SDR, bovine and human 11-*cis*-RDH/9-*cis*-RDH and mouse RDH4 (22, 23, 25, 31). The various enzymes denoted as 17βHSD (types 1–5), other than 17βHSD6, have the lowest amino acid identity with 17βHSD9.

Tissue loci of messenger RNA (mRNA) expression

Liver expressed 17βHSD9 as a 1.6-kb mRNA transcript (Fig. 3). 17βHSD9 mRNA was not detected in any other tissue screened by either Northern analysis or the more sensitive RNase protection assay technique.

TABLE 1. mRNA isoforms of 17βHSD9

Clone no.[a]	Start (nucleotide no. relative to #55)	5′-UTR insertion[b]	5′-UTR nucleotides 107–111	Length (nucleotides)
55[c] (17βHSD9)	1	−	+	2189
2, 5, 9, 48, 65, 140	−2 (CA), 1, 1, 8, 1, 35	−	+	1493, 1499, 1491, 1504, 1396, 1454
22	6	+	−	1542
104, 171	1, 75	−	−	1497, 1083
110	1	+	+	1548
182	187	NA	NA	1269

[a] Clones grouped together have identical sequences in areas of overlap, but differ in the extent of 5′-UTR and/or 3′-UTR. Clones 5, 28, 84, and 165 are identical. Clone 60 is not listed because it is fused with another gene in its 3′-end.

[b] A 38-nucleotide 5′-UTR insertion, GTAAGCCTGTCCTGAGAGTTCTCAAACTCTACAATTAT, occurs between nucleotides 106 and 107 for the clones designated.

[c] All clones have part or all of the sequence AGAGCCATGTAAGAACAAAAAAAAAAAA starting with nucleotide 1487 in the 3′-UTR, except clone 55. Clones 65, 171, and 182 end before nucleotide position 1487.

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  GCAATGTAGGACTCACCCTGTGCCTCCCTGGATCCTTACTCTGAGGGTTATATGATCAGTTTCATTGGATCTGAAGA     77
CCTGGACTTGGACTGAGACCTCAATTACACATAGACTGAAGGAAGACCCGAGGCTCTCCAGGCATCCTCAAGGCTTTCC    156
AGGACAGACTGAGATTGCTGAGGTGTTTGGCACCGAGTCTTTCTAGTGCGCTGTCATCACAGACAGACATTTCTTCATA    235

ATG TGG TTC TAC CTG GTA ACT CTT GTG GGC CTT TAC CAC CTT CTG CGT TGG TAT CGT GAG     295
Met Trp Phe Tyr Leu Val Thr Leu Val Gly Leu Tyr His Leu Leu Arg Trp Tyr Arg Glu     20

AGG CAG GTG GTG AGC CAT CTC CAA GAC AAG TAT GTC TTC ATC ACG GGC TGT GAC TCT GGC     355
Arg Gln Val Val Ser His Leu Gln Asp Lys Tyr Val Phe Ile Thr Gly Cys Asp Ser Gly     40

TTT GGG AAC CTG CTG GCC AGA CAG CTG GAC AGG AGA GGC ATG AGG GTA TTG GCT GCA TGT     415
Phe Gly Asn Leu Leu Ala Arg Gln Leu Asp Arg Arg Gly Met Arg Val Leu Ala Ala Cys     60

CTG ACG GAG AAG GGA GCC GAG GAG CTG AGG AAC AAG ACA TCT GAC AGG CTG GAG ACA GTG     475
Leu Thr Glu Lys Gly Ala Glu Glu Leu Arg Asn Lys Thr Ser Asp Arg Leu Glu Thr Val     80

ATC CTG GAT GTC ACC AAG ACA GAG AGT ATT GTG GCA GCC ACT CAG TGG GTG AAG GAG CGT     535
Ile Leu Asp Val Thr Lys Thr Glu Ser Ile Val Ala Ala Thr Gln Trp Val Lys Glu Arg    100

GTT GGA GAC AGA GGA CTC TGG GGT TTG GTT AAT AAT GCA GGT GTG TTA CAA CCA TTT GCC     595
Val Gly Asp Arg Gly Leu Trp Gly Leu Val Asn Asn Ala Gly Val Leu Gln Pro Phe Ala    120

TAC ATT GAA TGG TAC AGA CCA GAG GAC TAC ATG CCT ATC TTT CAA GTG AAC CTC ATT GGT     655
Tyr Ile Glu Trp Tyr Arg Pro Glu Asp Tyr Met Pro Ile Phe Gln Val Asn Leu Ile Gly    140

TTG ACC CAG GTG ACT ATA AGC ATG CTT TTC CTG GTA AAG AAG GCT CGG GGC AGG ATC GTC     715
Leu Thr Gln Val Thr Ile Ser Met Leu Phe Leu Val Lys Lys Ala Arg Gly Arg Ile Val    160

AAT GTC TCC AGT GCT TTG GGA AGA GTT GCA TTG TTT GGA GGA TTC TAC AGT TGC TCC AAG     775
Asn Val Ser Ser Ala Leu Gly Arg Val Ala Leu Phe Gly Gly Phe Tyr Ser Cys Ser Lys    180

TAT GGG GTT GAG GCA TTT TCA GAT GTG CTA AGG CAT GAG GTT CAA GAT TTC GGG GTG AAA     835
Tyr Gly Val Glu Ala Phe Ser Asp Val Leu Arg His Glu Val Gln Asp Phe Gly Val Lys    200

GTC AGC ATA ATT GAA CCT GGG AGC TTC AAG ACG GAA ATG ACA GAT GCA GAG TTA ACC ATT     895
Val Ser Ile Ile Glu Pro Gly Ser Phe Lys Thr Glu Met Thr Asp Ala Glu Leu Thr Ile    220

GAG AGA ACT AAG AAA GTC TGG GAA GCT GCC CCT GAG CAC ATC AAG GAA TCC TAC GGA CAG     955
Glu Arg Thr Lys Lys Val Trp Glu Ala Ala Pro Glu His Ile Lys Glu Ser Tyr Gly Gln    240

CAG TTT TTT GAC GAC TTT TGC AGC ACC ACC AAA CGA GAG TTG ATG AAG TGT AGC AGG AAC    1015
Gln Phe Phe Asp Asp Phe Cys Ser Thr Thr Lys Arg Glu Leu Met Lys Cys Ser Arg Asn    260

CTG AGC CTA GTC ACG GAC TGC ATG GAG CAC GCC CTG ACC TCC ACG CAT CCT CGC ACC CGG    1075
Leu Ser Leu Val Thr Asp Cys Met Glu His Ala Leu Thr Ser Thr His Pro Arg Thr Arg    280

TAC TCG GCT GGC TGG GAT GCC AAG TTT TTC TTC ATC CCT CTA TCT TAT TTG CCT GCG TCA    1135
Tyr Ser Ala Gly Trp Asp Ala Lys Phe Phe Phe Ile Pro Leu Ser Tyr Leu Pro Ala Ser    300

CTG GTA GAC TAC TTA TTG GCC ATA TCT AGG GGC AAG CCA GCT CAA GCA GCC TGA AGGATCC    1196
Leu Val Asp Tyr Leu Leu Ala Ile Ser Arg Gly Lys Pro Ala Gln Ala Ala  *           317

CGGATTGGTGGCTGTTGGAATGAAGAAATGACTCAGTTATTCCCAACACTAGCTATCTGCGCAGACACGCTCTCTTTCC    1275
GAAACCACTCAAGTGGCGGTCTTTTCCTTTCCCTCCACCCCCAGAGACATCTGGTCTCACTCTATAACCCTGGCTGGCA    1354
GCAACTCATAGAAGTCTGCCTCTCTCTCAATCAAGGTGTTCCCATTCCAGGTCTATACAATTTATTACTATCTTAACCA    1433
CTTTTTCCTGGGAATATTTCCATAGTATATCATTCTTTCTTGTCCTATTAAACCCTAAATTCTAAATGTACAAATAAAT    1512
TAACTCAGCACGTGAGACCACCCAACCTATTCCACGATATCCCACCTAAGGAAGAAACATTCCCTGGGCTCAAGATCAA    1591
TGGCTGTTCATTTTCTTCACCTAGAAATCAGTCACTTTCACGGGTTTTGCGGGCGACTTCTTTTCCTGAAAAAGTGATT    1670
TATTAAAGAAGGGACTTCTTTACTGTCTCTTCCCTCTGGAATACTTCTGCACTGAAGAAGGCCACGGGATAGGAGTCTT    1749
GTCTGCAATTTCATTGTTAGTTTTAAATCAGTTGATGAAATTCTCTTCAATTTTGAAGGGCCATTTCTCACCTAGATAT    1828
CAGACAACTTCAAGTATTTGAATTGATAGTAATGTGTTGAATTTACAAATTGTTTTTTATCTTGATTAAAAAGGAGAGT    1907
ATGTTCATTTAACATAATTATAGTTTTCAGCTCTGTTAATAAGAAAAATAGCCTTTTATGTGGTTGTATGAGGCGAAGA    1986
GAGAAAAAGGTGGGCAGTACCAGCTACACAAATCACTGAGCATGTGGCACACGCCTGCGACCCTAGCGCTTGGGTGGTA    2065
GAAGCAGAGGCTGAGACTTTGAAGGCTCAGCTACAGAGGGATTCAGGGTCAGCCGTAACTGAAAAGAGCAGTAGTAATC    2144
TAAAAGTCCAAGGAGTCATGTCCCTGTGTCTGTACTTTCCATTGT                                      2189
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FIG. 1. Nucleotide and deduced amino acid sequences of 17βHSD9. *Boldface type* in the translated region denotes the amino acids conserved in many, if not most, SDR family members (18). The *underlined* nucleotide sequences denote the primers used in the second round of screening by PCR.

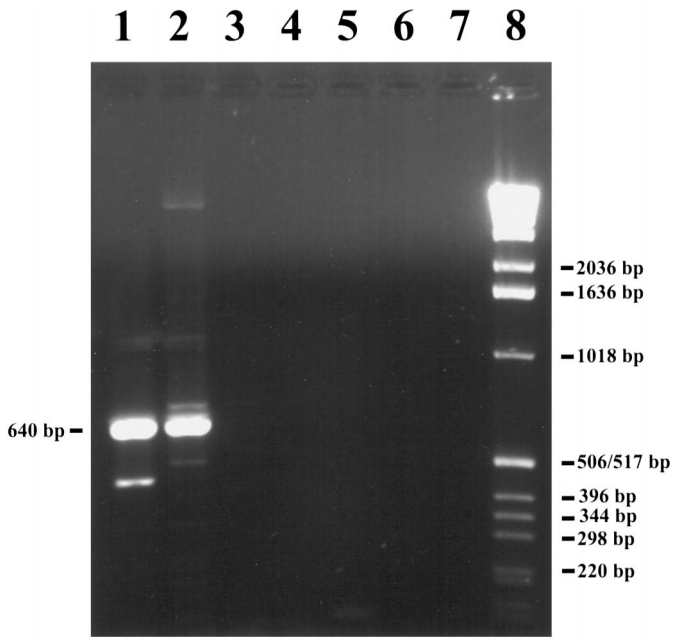

FIG. 2. Expression of 17βHSD6 in rat and mouse liver. PCR was performed at an annealing temperature of 65 C with a sense primer specific for 17βHSD6 (5′-GGAGACAGTGATCCTTGAC) and a degenerate antisense primer [5′-GAAGA(A/G)CTT(A/G)GCATCCCA]. The templates and primers used were: 1) a rat liver λgt11 cDNA library (CLONTECH Laboratories, Inc.) and both primers; 2) a mouse liver λgt10 cDNA library (CLONTECH Laboratories, Inc.) and both primers; 3) cDNAs of rat RoDH1, -2, and -3; mouse CRAD1 and -2; mouse 17βHSD9; and both primers; 4) the rat liver cDNA library and antisense primer only; 5) the rat liver cDNA library and sense primer only; 6) the mouse liver cDNA library and antisense primer only; 7) the mouse liver cDNA library and sense primer only; and 8) the DNA ladder.

Enzymatic activity

Assayed with its most efficient retinoid (all-*trans*-retinol) and steroid (estradiol) substrates (see below), 17βHSD9 had much greater activity with NAD^+ than with $NADP^+$ as a dehydrogenase (Table 3). In fact, $NADP^+$ supported no detectable activity with estradiol, but did support retinol dehydrogenase activity, albeit at a rate 23-fold lower than that supported by NAD^+. As a reductase, 17βHSD9 had similar activity with either NADH or NADPH with both all-*trans*-retinal and a representative steroidal ketone, dihydrotestosterone.

Recombinant 17βHSD9 had a pH optimum for dehydrogenation of all-*trans*-retinol from 9–9.5. The pH optimum for estrogen dehydrogenation was broader and included the range from 8.5 to at least 9.5. Rates of reactions with both substrates were optimum with Ches buffer and were diminished at most pH values in the presence of HEPES. The rates of reduction of all-*trans*-retinal and dihydrotestosterone were highest at a pH of 5 with succinate buffer. Mock-transfected COS cells showed little or no activity under a variety of conditions (buffer, pH, substrate, cofactor), with one exception. At pH 5 with all-*trans*-retinal and NADPH, mock-transfected cells produced 241 ± 56 pmol/assay retinol, indicating the presence of an NADPH-dependent retinal dehydrogenase distinct from 17βHSD9.

Of the retinol isomers tested, 17βHSD9 showed the most activity with all-*trans*-retinol (Table 4). No activity was detected with either 9-*cis*- or 13-*cis*-retinol with lower amounts of protein. At relatively high protein levels, activity was observed with both 9-*cis*- and 13-*cis*-retinol, but the rates were approximately 30- and 50-fold less, respectively, than the rate with all-*trans*-retinol at the lower protein level.[1] 17βHSD9 also was less active with 11-*cis*-retinol than with all-*trans*-retinol. Although 17βHSD9 converted free all-*trans*-retinol into all-*trans*-retinal, it did not covert all-*trans*-retinol bound with CRBP, *i.e.* 5 μM holo-CRBP into all-*trans*-retinal, in the presence of either NAD^+ or $NADP^+$ under standard dehydrogenation conditions.

17βHSD9 showed the highest steroid activity as a 17βHSD with estradiol and was approximately 14- and 50-fold less active as a 17βHSD with dihydrotestosterone and testosterone (see Footnote 1), respectively (Table 4). 17βHSD9 showed no activity as an 11βHSD with corticosterone and was about 2-fold less active as a 3αHSD with androsterone compared with its 17βHSD activity with estradiol. The actions of 17βHSD9 with 3α-adiol were complex. 3α-Adiol has both 3α-hydroxyl and 17β-hydroxyl groups capable of undergoing dehydrogenation (Fig. 4). Indeed, the 17βHSD9-catalyzed reaction with 3α-adiol produced three products in proportions that changed with enzyme concentration. At the lower enzyme concentration, 17βHSD9 recognized each of the hydroxyl groups with similar efficiencies, producing the 3α- and 17β-dehydrogenation products (dihydrotestosterone and androsterone, respectively) in nearly equivalent amounts and only a small amount of the 3α,17β-dione product, androstandione. At the higher enzyme concentration, the major product became 3α,17β-dione.

The route to androstandione from 3α-adiol at the higher 17βHSD9 concentration probably involved 17βHSD activity, *i.e.* production of androsterone, followed by 3αHSD activity, *i.e.* conversion of androsterone into androstandione, rather than conversion of dihydrotestosterone into the dione. The following observations support this conclusion. With 3α-adiol as substrate, 17βHSD9 acts equally efficiently as a 17βHSD or a 3αHSD (at the lower protein concentration), but the activity of 17βHSD9 as a 3αHSD with androsterone exceeds its activity as a 17βHSD with dihydrotestosterone at the higher protein concentrations. This would result in accumulation of dihydrotestosterone but conversion of the androsterone produced from 3α-adiol into androstandione.

17βHSD9 displayed Michaelis-Menten kinetics with all-*trans*-retinol, but cooperative kinetics with estradiol, androsterone, and 3α-adiol (Fig. 5). Enzymatic efficiency was highest with the steroid substrates and was markedly lower with all-*trans*-retinol (Table 5).

17βHSD9 had lower reductase activity than dehydrogenase activity (compare the data in Table 4 with those in Table 6). Moreover, no major differences were noted with 17βHSD9 as a retinoid, 17-oxo-steroid, or 3-oxo-steroid reductase. 17βHSD9 had no detectable reductase activity with

[1] The higher protein levels used for all-*trans*-retinol and estradiol were out of the linear rate range and therefore were not used to compare the all-*trans*- with the *cis*-retinol activities of 17βHSD9.

TABLE 2. Comparison of amino acid sequences of 17βHSD9 with related SDR

Species	SDR	Amino acid homology (%)		Accession no.	Ref. no.
		Identity	Similarity		
Mouse	17βHSD9	100	100	AF103797	—
Rat	17βHSD6	88	92	U89280	14
Human	RoDH	69	75	U89281	14
Mouse	CRAD2	68	76	AF056194	26
Rat	RoDH1	67	74	S75875	19
Rat	RoDH3	67	74	U33501	21
Mouse	CRAD1	66	74	AF030513	24
Rat	RoDH2	63	71	U33500	20
Human	RoDH4	62	70	AF057034	29
Bovine	11-*cis*-RDH	48	57	X82262	22
Mouse	RDH4	47	57	AF013288	25
Human	11-*cis*-RDH/9-*cis*-RDH	47/45	56/54	U43559/U89717	31/27
Mouse	17βHSD2	34	42	Y09517	11
Mouse	17βHSD3	22	31	U66827	12
Mouse	17βHSD4	25	25	X89998	7
Human	17βHSD3	19	29	U05659	4
Mouse	Ke6(17βHSD8)	19	27	U34072	16
Mouse	17βHSD5	17	17	D45850	8
Mouse	17βHSD1	12	18	X89627	9
Mouse	17βHSD7	0	0	Y15733	15

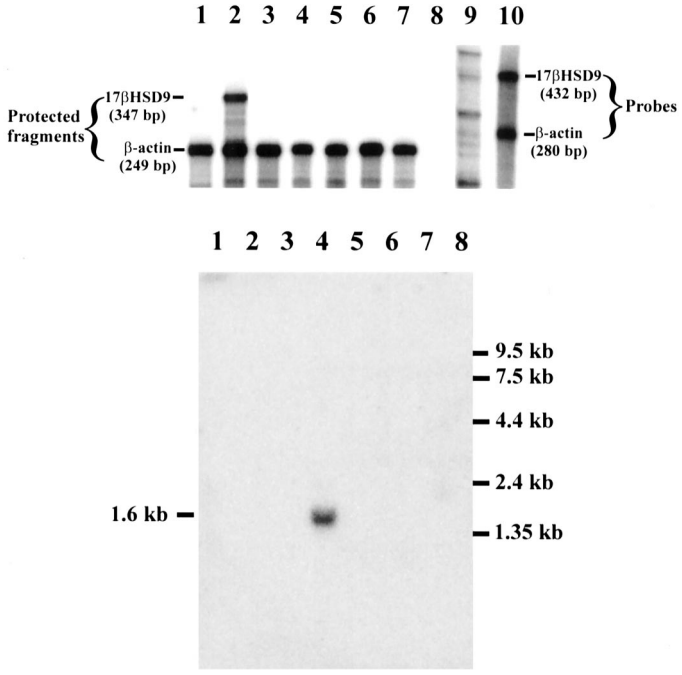

FIG. 3. mRNA expression of 17βHSD9 in mouse tissues. *Top panel*, RNase protection assays were performed with RNA from 2-month-old male BALB/C mice. Lane 1, Testis; lane 2, liver; lane 3, lung; lane 4, kidney; lane 5, heart; lane 6, eye; lane 7, brain; lane 8, yeast RNA; lane 9, DNA markers (200, 300, 400, and 500 bp); lane 10, probes. *Bottom panel,* Northern hybridization was performed as described in *Materials and Methods* with a commercially available mouse multiple tissue blot: 1, testis; 2, kidney; 3, skeletal muscle; 4, liver; 5, lung; 6, spleen; 7, brain; and 8, heart.

androstenedione, consistent with its inability to dehydrogenate testosterone into androstenedione.

To demonstrate the multifunctional nature of 17βHSD9 *in vivo*, 5 μM all-*trans*-retinol, 3α-adiol, or estradiol were added individually to pcDNA3/17βHSD9-transfected and mock-transfected COS cells. All-*trans*-retinol supported retinal formation in intact cells at a net rate of 180 ± 8 pmol/mg protein/2 h (mean ± SD; n = 3 plates). 3α-Adiol supported dihydrotestosterone, androsterone, and androstenedione formation at an overall net rate of 5980 ± 34 pmol/mg protein·2 h. Estradiol supported estrone formation at a net rate of 6160 ± 450 pmol/mg protein·2 h. Because these rates were not necessarily in the linear ranges, they should not be compared for relative enzyme efficiencies.

TABLE 3. Cofactor use by 17βHSD9

Substrate	Cofactor		
Dehydrogenation	None	NAD^+	$NADP^+$
All-*trans*-retinol	ND[a]	678 ± 98	30 ± 20
Estradiol (→ estrone)[b]	ND	375 ± 16	ND
Reduction	None	NADH	NADPH
All-*trans*-retinal	ND	530 ± 7	281 ± 52[c]
Dihydrotestosterone (→ 3α-adiol)	ND	307 ± 18	283 ± 5

Reactions were run at pH 9 (dehydrogenations) or pH 5 (reductions) with 5 μM substrate and 100 μg protein and 2 mM cofactor. Data are the mean net picomoles of product per assay ± SD of triplicate determinations.

[a] ND, Product not detected in amounts significantly greater than in mock-transfected cells.

[b] Assay was performed with 10 rather than 100 μg protein.

[c] Net above a background of 241 ± 56 pmol/assay in mock-transfected cells in the presence of NADPH. In the absence of cofactor or in the presence of NADH, mock-transfected cells did not convert all-*trans*-retinal into all-*trans*-retinol.

Inhibitors of 17βHSD9 activity

Carbenoxolone and phenyl arsenoxide inhibited 17βHSD9 activity potently (IC_{50} values of 5 and 20 μM, respectively; Fig. 6). Carbenoxolone represents a prototypical inhibitor of SDR, including RoDH and CRAD isozymes (19, 20, 24, 26). Its activity with 17βHSD9, therefore, was anticipated. The sulfhydryl cross-linking agent phenyl arsenoxide also inhibits RoDH1 and -2 and CRAD1 and -2 potently. 4-Methylpyrazole inhibited 17βHSD9 with an IC_{50} of 5.2 mM and has an

TABLE 4. Dehydrogenase activity of 17βHSD9

Substrate	Product	Activity	pmol assay	
			200 μg	10 μg
All-*trans*-retinol	All-*trans*-retinal	RoDH	793 ± 81	170 ± 25
11-*cis*-retinol	11-*cis*-retinal	RoDH	325 ± 52	54 ± 12
13-*cis*-retinol	13-*cis*-retinal	RoDH	111 ± 11	ND[a]
9-*cis*-retinol	9-*cis*-retinal	RoDH	66 ± 1	ND
3α-Adiol	Total	17β/3αHSD	1104 ± 33	693 ± 43
	Dihydrotestosterone	3αHSD	277 ± 9	319 ± 25
	Androsterone	17βHSD	6 ± 10	350 ± 21
	Androstandione	17β/3αHSD	821 ± 16	24 ± 4
Estradiol	Estrone	17βHSD	1297 ± 47	469 ± 32
Androsterone	Androstanedione	3αHSD	899 ± 16	227 ± 13
Dihydrotestosterone	Androstanedione	17βHSD	321 ± 26	33 ± 3
Testosterone	Androstenedione	17βHSD	187 ± 9	ND
Corticosterone	11-Deoxycorticosterone	11βHSD	ND	ND

Reactions were run with 5 μM substrate at pH 9 with the indicated amount of protein in the presence of 1.6 mM NAD^+. Data are the mean ± SD of triplicate determinations.

[a] ND, Product not detected in amounts significantly greater than in mock-transfected cells.

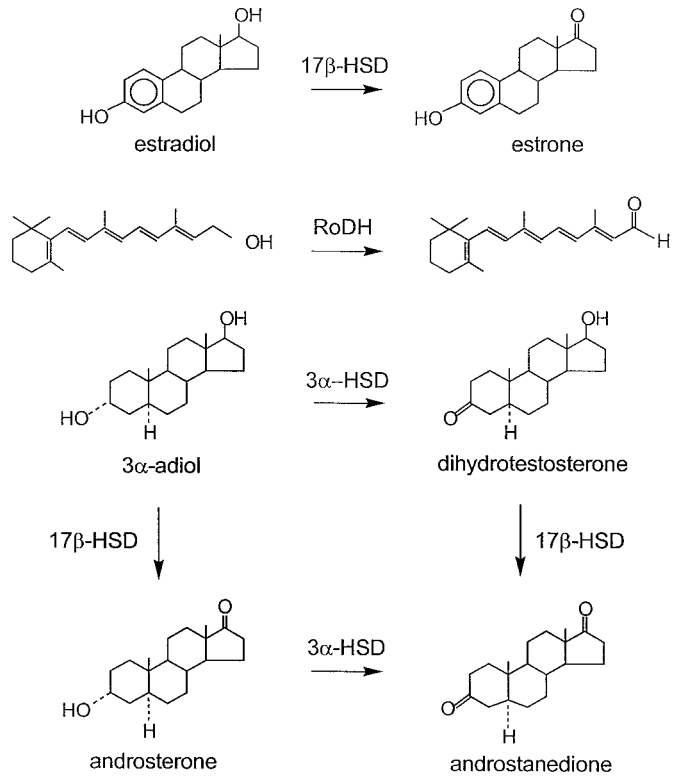

FIG. 4. Reactions catalyzed by 17βHSD9. 17βHSD9 shows three dehydrogenase activities: RoDH, 17βHSD, and 3αHSD.

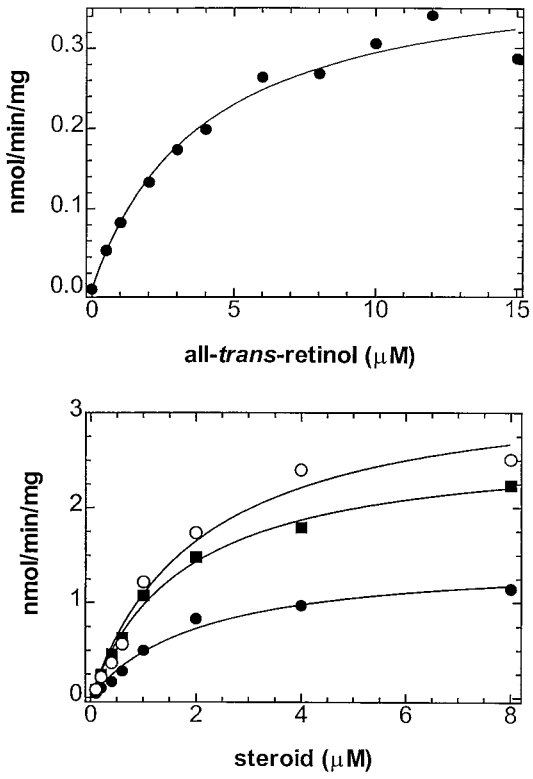

FIG. 5. Kinetics of recombinant 17βHSD9 with all-*trans*-retinol and sterol substrates. Reactions were run with all-*trans*-retinol (*top panel*; 40 μg protein) or with steroids (*bottom panel*; *open circles*, 3α-adiol; *filled squares*, estradiol; *filled circles*, androsterone; 6 μg protein) as described in *Materials and Methods*.

IC_{50} of 5 mM with CRAD2, but does not inhibit RoDH isozymes even at 500 mM (19, 20, 24, 26). 4-Methylpyrazole has been associated closely with inhibiting the class I, II, and IV alcohol dehydrogenases, with K_i values ranging from micromolar to high millimolar depending on both isozyme and species.

Subcellular locus of 17βHSD9

Centrifugation of the 800 × *g* supernatant of transfected COS cells at 10,000 × *g* for 30 min partitioned 77% of the activity assayed with estradiol into the supernatant. Centrifugation of the supernatant at 100,000 × *g* for 2 h partitioned 98% of the recovered activity into the microsomal pellet.

Discussion

This work identifies a new microsomal SDR, 17βHSD9, with unique properties compared with the distinct subgroups of SDR known to date to catalyze either steroid 17β-hydroxyl dehydrogenation or androgen 3α-hydroxyl/retin-

TABLE 5. Kinetic constants of 17βHSD9 activity with steroid and retinoid substrates

Substrate	$K_{0.5}$ (μM)	V (nmol/min·mg)	H[a]	$V/K_{0.5}$
Estradiol	1.5	2.3	1.1	1.5
3α-Adiol[b]	1.4	2.8	1.4	2
Androsterone	1.3	1.1	1.3	0.85
All-*trans*-retinol	3.2	0.4		0.13

[a] H, Hill coefficient.
[b] Products were formed via both 3α- and 17β-dehydrogenase activities.

TABLE 6. Reductase activity of 17βHSD9

Substrate	Product	Activity	pmol assay	
			200 μg	10 μg
All-*trans*-retinal	All-*trans*-retinol	RoDH	834 ± 75	51 ± 2
Estrone	Estradiol	17βHSD	631 ± 29	51 ± 1
Androsterone	3α-Adiol	17βHSD	603 ± 40	50 ± 14
Androstenedione	Testosterone	17βHSD	ND[a]	ND
Dihydrotestosterone	3α-Adiol	3αHSD	866 ± 52	50 ± 4

Reactions were run with 5 μM substrate at pH 5 with the indicated amount of protein in the presence of 2 mM NADH and an NADH-regenerating system. Data are the mean ± SD of triplicate determinations.
[a] ND, Product not detected in amounts significantly greater than in mock-transfected cells.

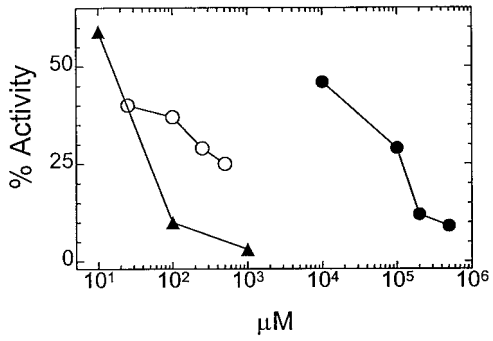

FIG. 6. Inhibitors of 17βHSD9. The effects of carbenoxolone (*open circles*), phenyl arsenoxide (*filled triangles*), and 4-methylpyrazole (*filled circles*) were tested on 17βHSD9 dehydrogenation activity with 5 μM estradiol at pH 9 in the presence of NAD^+. Values are the means of three replicate determinations.

oid dehydrogenation (RoDH1–3, CRAD1,2, retSDR1, RDH4, 11-*cis*-RoDH, and human RoDH). Unique properties of 17βHSD9 include its combination of 17βHSD, 3αHSD, and retinoid activities. Although several oxidative 17βHSD are known (*e.g.* 17βHSD2, -4, and -6), oxidative 3αHSD have been rare among steroid-metabolizing enzymes; the first example was the 3αHSD activity (14) of RoDH1 (19). The combination of 3αHSD and 17βHSD activity suggests that 17βHSD9 would be relevant to both potential sites of androgen inactivation (3α/17β-hydroxyl groups) as well to estrogen inactivation. 17βHSD9 also presents the only enzyme related to the retinoid and 3α-hydroxyl androgen dehydrogenation SDR subgroup that has appreciable 17βHSD activity, and it is the only one related to the retinoid subgroup known to recognize estrogen as a substrate. A major function of 17βHSD9, given its expression locus and its kinetic characteristics, could be inactivation of circulating estrogen and a broad spectrum of androgens, including 3α-adiol, dihydrotestosterone, and androsterone. 17βHSD9 also seems to represent (at least in part) the NAD^+-dependent microsomal dehydrogenase activity that converts all-*trans*-retinol into all-*trans*-retinal *in vitro*, but does not recognize efficiently the CRBP-retinol complex (35, 36). Early evidence for the occurrence of a retinol dehydrogenase that recognized holo-CRBP as substrate included higher rates of retinal formation with $NADP^+$ *vs.* NAD^+ for liver microsomal retinol dehydrogenation when retinol was presented bound with CRBP. On the other hand, NAD^+ supported the higher rate of retinal formation when unbound retinol was added to the incubation medium. These data suggested the expression of at least two enzymes, one that preferred $NADP^+$ *in vitro* and recognized holo-CRBP and another that preferred NAD^+ *in vitro* and used free retinol as substrate. Cloning and expression of the cDNA encoding 17βHSD9 and characterization of its catalytic characteristics confirm this observation and extend it to demonstrate that retinoid activity represents only one aspect of its properties.

A function for 17βHSD9 in liver all-*trans*-retinol metabolism seems somewhat uncertain because of its apparent inability to access retinol bound to CRBP. Rodent liver has a retinol concentration of approximately 5 μM, but a CRBP concentration of approximately 7 μM (42). Remeasurement under equilibrium conditions has provided a K_d of about 0.1 nM for CRBP binding with retinol (43). Under these conditions, the concentration of the non-CRBP-associated retinol would be approximately 0.25 nM, 20,000-fold lower than the total retinol concentration. Not only does the equilibrium lie overwhelmingly in favor of the CRBP-bound state, retinol apparently has a slow off-rate *in vivo*. Two well known facts support this idea. 1) Retinol isolates from liver homogenates bound with CRBP despite the capacity of biological membranes to sequester far more retinol than occurs *in vivo* (44). 2) Routine purification of holo-CRBP by size-exclusion chromatography takes hours through a matrix that readily binds free retinol, yet the CRBP emerges in the holo form (45). Thus, holo-CRBP represents the major physiological form of retinol in liver and free retinol has an exceedingly low concentration. Because microsomal RoDH isozymes occur that recognize both holo-CRBP and free retinol as substrates, a function seems problematic for the several dehydrogenases that recognize only free retinol, such as 17βHSD9. This concern also

pertains to soluble enzymes that do not access retinol bound with holo-CRBP, especially those with relatively high K_m values for retinol, such as ADH isozymes.

Would an NAD^+-dependent RoDH contribute more to retinoic acid biosynthesis than an $NADP^+$-preferring RoDH because the *ratio* $NAD^+/NADH$ reportedly nears approximately 1000 compared with approximately 0.01 for $NADP^+/NADPH$ (46)? Not necessarily, because these ratios were measured in liver and may not pertain to other tissues, nor should these ratios remain fixed under all dietary/metabolic conditions or in all subcellular compartments. Perhaps more importantly, net directions taken by reversible metabolic reactions depend on complex input. The concentrations and affinity constants of both the substrate/product pair and reduced/oxidized forms of cofactors, not the oxidized/reduced cofactor ratio, determine the net direction of a reversible dehydrogenation. Generally, reversible metabolic reactions are driven in the direction of the final product by thermodynamically favored (irreversible) reactions that affect the concentrations of one or more of the reactants/products. The biosynthesis of retinoic acid from retinol includes such a step, the irreversible conversion of the intermediate retinal into retinoic acid by retinal dehydrogenases. This more complete appraisal of factors that influence the net flux of reversible reactions and recognition of the physiological substrate (holo-CRBP) positions the $NADP^+$-preferring RoDHs in the pathway of retinoic acid biosynthesis. Additionally, the $NADP^+$-preferring RoDHs do use NAD^+ *in vitro*, albeit less efficiently than $NADP^+$. Nevertheless, use of both cofactors *in vitro* introduces uncertainty about the cofactor used *in vivo*; it would depend on the concentrations of $NADP^+$ and NAD^+ and their relative K_d values.

In summary, this work reports a new mouse SDR with a primary amino acid sequence closest to that of rat 17βHSD6, but with catalytic properties that overlap two subgroups of SDR, the steroid-metabolizing 17βHSD and the androgen/retinoid-metabolizing RoDH and CRAD enzymes. Inactivation of estrogen and a variety of androgens represents the most probable function of 17βHSD9. Because of its apparent inability to access retinol bound to CRBP, a role for 17βHSD9 in the pathway of retinoic acid biosynthesis seems less certain.

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