We report characterization of a novel member of the short chain dehydrogenase/reductase superfamily. The 1513-base pair cDNA encodes a 319-amino acid protein. The corresponding gene spans over 26 kilobase pairs on chromosome 2 and contains five exons. The recombinant protein produced using the baculovirus system is localized in the microsomal fraction of SF9 cells and is an integral membrane protein with cytosolic orientation of its catalytic domain. The enzyme exhibits an oxidoreductase activity toward hydroxysteroids with NAD\(^+\) and NADH as the preferred cofactors. The enzyme is most efficient as a 3α-hydroxydehydrogenase, converting 3α-tetrahydroprogesterone (allopregnanolone) to dihydroprogesterone and 3α-androstanediol to dihydrotestosterone with similar catalytic efficiency (V\(_{\text{max}}\) values of 13–14 nmol/min/mg microsomal protein and K\(_{\text{m}}\) values of 5–7 \(\mu\)M). Despite \(~44–47\%\) sequence identity with retinol/3α-hydroxysteroid dehydrogenases, the enzyme is not active toward retinols. The corresponding message is abundant in human trachea and is present at lower levels in the spinal cord, bone marrow, brain, heart, colon, testis, placenta, lung, and lymph node. Thus, the new short chain dehydrogenase represents a novel type of microsomal NAD\(^+\)-dependent 3α-hydroxysteroid dehydrogenase with unique catalytic properties and tissue distribution.

Oxidation and reduction of hydroxyl and ketone groups in position 3 on naturally occurring steroids play an important role in regulation of intracellular levels of biologically active steroid hormones. For example, in gonads, 3α-hydroxysteroid oxidoreductase activity is responsible for maintaining the balance of a potent androgen, 5α-dihydrotestosterone, with a ketone group in position 3 and a weak androgen, 3α-androstanediol, which has a hydroxyl group in the same position (Fig. 1; reviewed in Ref. 1). In the central nervous system, 3α-hydroxysteroid oxidoreductase activity controls the amount of a potent neurosteroid, 3α-tetrahydroprogesterone (also called allopregnanolone), which serves as an allosteric regulator of all \(\gamma\)-aminobutyric acid type A receptors and potentiates \(\gamma\)-aminobutyric acid mediated chloride conductance (1). 3α-Hydroxysteroid oxidoreductase activity has been described in the cytosolic and microsomal fractions of a number of human and animal tissues (2–11). In addition to the different subcellular localization, cytosolic and microsomal enzymes appear to have a distinctively different cofactor preference. Cytosolic 3α-hydroxysteroid oxidoreductases exhibit a preference for the phosphorylated nucleotides as cofactors (NADP\(^+\)/NADPH), whereas microsomal enzymes prefer NAD\(^+\)/NADH (2–11). Because the predominant forms of nucleotide cofactors in the cells are NAD\(^+\) and NADPH, it is generally believed that the NAD\(^+\)-dependent dehydrogenases function mainly in the oxidative direction, whereas the NADPH-dependent enzymes function as reductases (12). Thus, the balance between the oxidative and reductive activities will determine the local concentrations of bioactive compounds in specific tissues. To date, at least four types of cytosolic 3α-hydroxysteroid dehydrogenases (HSDs)\(^1\) have been identified in humans (1, 13, 14). All four cytosolic enzymes, named AKR1C1-AKR1C4, are members of the aldo-keto reductase gene superfamily and prefer NADPH as cofactor (13). Liver is the only tissue that contains all four isoforms at similar levels (13). Extrahepatic tissues such as lung, prostate, uterus, mammary gland, brain, small intestine, and testis vary significantly in their composition and relative amounts of individual isoforms (15, 18).

The identity of the enzymes responsible for the NAD\(^+\)-dependent 3α-hydroxydehydrogenase activity found in microsomes has remained elusive until recently, when several newly discovered members of the short chain dehydrogenase/reductase superfamily were shown to stereospecifically oxidize the 3α-hydroxy group on 3α-androstanediol (15–20) and allopregnanolone (21, 22). In contrast to cytosolic 3α-HSDs, these membrane-bound short chain dehydrogenases exhibit a strong preference for NAD\(^+\) as cofactor. Previously, we characterized the properties of two human microsomal short chain dehydrogenases that are capable of oxidizing 3α-androstanediol to 5α-dihydrotestosterone and allopregnanolone to 5α-dihydroprogesterone (20, 21). The range of potential physiological substrates for these enzymes (RoDH-4 and RoDH-like 3α-HSD) is not limited to 3α-hydroxysteroids because they can also oxidize all-trans-retinol and might contribute to the biosynthesis of a potent morphogen, all-trans-retinoic acid (20, 21). Besides RoDH-4 and RoDH-like 3α-HSD, human cis-retinol

\(^1\) The abbreviations used are: HSD, hydroxysteroid dehydrogenase; CHAPS, 3-(N-cholamidepropyl)dimethylammonion)-1-propane-sulfonate; HPLC, high performance liquid chromatography; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; RoDH, retinol dehydrogenase.
dehydrogenase Rdh5 was also shown to oxidize 3α-hydroxysteroids, although with lower catalytic efficiency than cis-retinoids (16). Rdh5 was found to localize on the luminal side of the endoplasmic reticulum (23) and was implicated in the biosynthesis of 11-cis-retinal (24), the cofactor for vision, and 9-cis-retinoic acid, the activating ligand for retinoid X receptors (25). The three human retinol/sterol dehydrogenases share ~50% amino acid sequence identity, similar genomic structure, and chromosomal localization on chromosome 12 (26–28). Individual enzymes exhibit distinctively different tissue distribution patterns. RoDH-4 is primarily expressed in human liver and skin (20, 29). RoDH-like 3α-HSD is present in liver, lung, prostate, testis, spleen (15), spinal cord (21), and various areas of human brain (21). Rdh5 appears to be ubiquitously expressed in a wide variety of tissues: liver, mammary gland, colon, thymus, small intestine, kidney, and others. (16, 25). Remarkably, liver contains the highest levels of mRNAs for all three enzymes. Here, we report molecular cloning and characterization of a novel human 3α-hydroxysteroid dehydrogenase. We show that, in contrast to the previously identified enzymes, this human 3α-hydroxy-steroid dehydrogenase is not active toward retinoids and exhibits different membrane topology as well as different tissue distribution.

**EXPERIMENTAL PROCEDURES**

**Cloning of the Full-length cDNA**—cDNA clone W17165 was identified in the expressed sequence tag data base of GenBank based on its similarity to human RoDH-4 cDNA. Sequencing of the clone obtained from American Type Culture Collection revealed that it lacked the 5′-end (started at nucleotide 346 in Fig. 2). The missing part was obtained by rapid amplification of cDNA ends (RACE) using 5′-RACE-ready human liver cDNA (CLONTECH) as template. The internal gene-specific primers (TGACATTTCTGGGTGTCGTC and TCCTCAGCCACTGGCGAGTC; both antisense; indicated by *arrows* in Fig. 2) were designed based on the 5′-end sequence of clone W17165. The gene-specific primers were paired with the anchor primer (CLONTECH), and the cDNA was amplified in two sequential reactions using *Taq* polymerase (PerkinElmer Life Scienes). The amplifications were performed for 30 cycles as follows: denaturing at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 5 min. The ~400-bp pair cDNA product was subcloned into M13mp18 and sequenced. This cDNA fragment encoded amino acids 1–90 and contained 153 base pairs of the 5′-untranslated region (Fig. 2). The complete nucleotide sequence was submitted to GenBank with accession number AF543729.

To obtain the full-length cDNA, human liver and heart mRNAs were amplified by PCR using *Pfu* polymerase (Stratagene Cloning Systems, La Jolla, CA) and primers GGGGGATCCATGCTCTTTTGGGTGCTAGG (sense primer; the *Bam*HI site is underlined) and TTAGATTCTCAGCTGGGATAG (antisense primer; the EcoRI site is underlined). Thirty cycles of amplification were performed as follows: denaturing at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 6 min. The amplified cDNAs were subcloned into *Bam*HI/EcoRI restriction sites of pVL1393 transfer vector (PharMingen, San Diego, CA). The transfer vectors were sequenced to verify the cDNA sequence of the gene of interest. The sequences of the cDNAs obtained from liver and heart were identical. The structure of the corresponding gene was determined by searching Human Genome GenBank data base using the full-length cDNA sequence and the BLAST 2.0 homology search tool.

**Northern Blot Analysis**—The following multiple tissue mRNA blots (MTNs) were used for Northern blot analysis: Human 12-lane MTN Blot, Human 12-lane MTN Blot II, and Human Endocrine System MTN Blot from CLONTECH, which contained a minimum of 1 μg of polyadenylated RNA per lane. The blots were hybridized with ~1.0-kilobase pair 32P-labeled cDNA probe in ExpressHyb hybridization solution (CLONTECH) according to the manufacturer’s instructions. Briefly, the blots were prehybridized in ExpressHyb solution for 30 min at 68 °C and transferred to a fresh solution containing 2 × 106 cpm/ml denatured radiolabeled cDNA. The hybridization was performed at 68 °C for 1 h. The blots were rinsed in 2 × SSC, 0.05% SDS several times at room temperature and washed in 0.1 × SSC, 0.1% SDS for 10 min at 50 °C. The mRNA bands were visualized by exposure to x-ray film at ~70 °C with two intensifying screens for 1–10 days.

**Expression in SF9 Cells**—Expression of the cDNA in SF9 cells was performed essentially as described previously (20). To produce recombinant protein, SF9 cells were infected at a virus/cell ratio of 10:1. Cells were collected after 3 days of incubation at 27 °C, resuspended in 0.01 M potassium phosphate, pH 7.4, 0.25 M sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors (1.5 μg/ml aprotinin, 1.5 μg/ml leupeptin, and 1.0 μg/ml pepstatin A), and homogenized using a French pressure cell. The unbroken cells, cellular debris, and nuclei were removed by centrifugation at 1000 × g for 10 min, and then mitochondria were removed by centrifugation at 10,000 × g for 30 min. Microsomes were pelleted by centrifugation at 105,000 × g for 1 h through a 0.6 M sucrose cushion and resuspended in 90 mM potassium phosphate, pH 7.4, 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol. Protein concentration was determined by the method of Lowry et al. (30) using bovine serum albumin as a standard.
Alkaline Extraction and Western Blot Analysis—Five-
ml aliquots of microsomes (1.8 μg/m1) containing the recombinant protein were incu-
bated with 100 μl of 100 mM sodium carbonate and 25 mM potassium acetate (extraction buffer) or with phosphate-buffered saline (PBS), pH 7.4, or with 1–10% Triton X-100 in PBS for 30 min on ice. After incubation, samples were loaded onto 100-
ml cushions of 0.5 M sucrose prepared in extraction buffer, PBS, or Triton/PBS and centrifuged for 1 h at 200,000 g. Pellets were dissolved in 20 μl of SDS-polyacryl-
amide gel electrophoresis sample buffer. Supernatants were precipi-
tated with an equal volume of ice-cold 50% trichloroacetic acid for 30
min on ice and centrifuged for 3 min at 12,000 g. The resulting pellets
were washed twice with ethyl ether, dried, and dissolved in 20 μlo f
SDS-polyacrylamide gel electrophoresis sample buffer. After separation
in a 15% denaturing polyacrylamide gel, samples were transferred to
Hybond-P membrane (Amersham Pharmacia Biotech). Protein was de-
tected using the ECL Western blotting analysis system (Amersham
Pharmacia Biotech) according to the manufacturer’s instructions. Rab-
pbit antibodies raised against the N-terminal fragment of RoDH-like
3α-HSD were used as primary antibodies at a 1:3000 dilution in 3%
bovine serum albumin, 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1%
Tween 20. Visualization was performed using horseradish peroxidase-
conjugated anti-rabbit antibodies (at a 1:10,000 dilution) and ECL
Western blotting detection reagents (Amersham Pharmacia Biotech).

Identification of Reaction Products and Determination of Kinetic
Constants—All reactions were performed in 90 mM potassium phos-
phate, pH 7.4, and 40 mM KCl at 37 °C (reaction buffer) in siliconized
glass tubes as described previously (20). Commercially available radio-
labeled steroids (PerkinElmer Life Sciences, pH 7.4, or with 1–10% Triton X-100 in PBS for 30 min on ice. After incubation, samples were loaded onto 100-
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Western blotting detection reagents (Amersham Pharmacia Biotech).
New Type of Human Microsomal 3α-HSD

RESULTS

Characterization of the Primary Structure and Genomic Organization—The 1513-base pair cDNA shown in Fig. 2 was constructed from the overlapping sequences of the original clone W17165 (nucleotides 346–1513) and the 5'-RACE PCR product (nucleotides 1–449) obtained in this study (see "Experimental Procedures"). The open reading frame starts at nucleotide 154 and ends in a stop codon at nucleotide 1113, resulting in a 519-amino acid-long polypeptide (Fig. 2). A search of the GenBankTM data base for homologous sequences revealed that three unpublished sequences exhibit similarity to the cDNA reported here: (a) human retinol dehydrogenase homolog gene (AF067174), (b) Homo sapiens retinol dehydrogenase homolog isoform-1 mRNA (AF240698), and (c) H. sapiens retinol dehydrogenase homolog isoform-2 mRNA (AF240697). The first cDNA is identical to segments 147–603 and 667–1513 in the cDNA shown in Fig. 2 but is missing a segment coding for 21 amino acids between Pro-150 and Val-172. Instead, in this position, it contains nucleotides 1–45 of the 5'-untranslated sequence joined with an unidentified 51-base pair fragment. Retinol dehydrogenase homolog isoform-1 lacks 120 base pairs coding for 40 amino acids between Glu-204 and Ser-246, whereas isoform-2 has an insertion of 119 nucleotides between positions 94 and 95 in the 5'-untranslated region (Fig. 2) as well as four mismatched nucleotides and one missing nucleotide that cause a frameshift.

To obtain the full-length cDNA and further confirm the cDNA and deduced protein sequence, we performed PCR amplification of mRNA isolated from two human tissues, liver and heart, using gene-specific primers flanking the coding region. Sequencing of the PCR products showed that they were identical with the sequence in Fig. 2. Furthermore, a BLAST 2.0 search of the Human Genome database revealed that the cDNA sequence determined in this study (AF343729) is 100% identical to five consecutive segments in the NCBI-assembled contig NT 005343 assigned to chromosome 2q31.1 (Fig. 3A).

The corresponding gene spans over 26 kilobase pairs and does not appear to have pseudogenes. The proposed exon-intron boundaries follow the canonical GT/AG rule and are summarized in Table I.

The deduced protein sequence of the novel protein contains the signature cofactor binding motif G(X)XG at Gly-36 and the active site consensus sequence X(Y)K at Tyr-176 characteristic of the short chain dehydrogenases/reductases. The amino acid sequence is most closely related to retinol/sterol dehydrogenases of the short chain dehydrogenases/reductases superfamily: (a) RdH-4, 47% identity; (b) RdH-like 3α-HSD, 43% identity; and (c) Rdh5, 44% identity. Furthermore, the positions of exon-intron junctions in the translated region of the novel gene and the sizes of exons 3 and 4 are identical to those in the genes for RdH-4, RdH-like 3α-HSD, and Rdh5 (Fig. 3B). However, the new gene is present on chromosomes 2q31.1, whereas retinol/sterol dehydrogenases are clustered on chromosome 12q13 (28).

Tissue Distribution—Tissue distribution of the corresponding message was examined by Northern blot analysis. Hybridization of pre-made Northern blots with radiolabeled cDNA revealed that the message for the putative short chain dehydrogenase is present in a number of human tissues (Fig. 4). The most intense signal was observed in trachea. Relatively strong signals were detected in spinal cord, bone marrow, heart, and colon. Weaker bands were also present in lymph node, brain, lung, placenta, testis, prostate, and mammary gland. A total of three different-size mRNA species were detected, which exhibited tissue-specific expression. The longest mRNA species were detected in trachea, testis, and lung. An intermediate size mRNA was present in trachea, colon, placenta, lung, bone marrow, and lymph node, with trace amounts seen in mammary gland, prostate, and stomach. The shortest mRNA was detected in spinal cord, lymph node, brain, bone marrow, and heart.

Expression and Characterization of the Recombinant Enzyme—To establish whether the novel cDNA codes for a functional enzyme, we expressed the corresponding protein using the baculovirus expression system. Similar to human retinol/sterol dehydrogenases, the recombinant protein was present in the microsomal fraction of SF9 cells as indicated by Western blot analysis using antibodies against the conserved N-terminal cofactor-binding domain of retinol/sterol dehydrogenases. Under the same conditions, no protein bands were detected in microsomes isolated from SF9 cells infected with wild-type virus, which did not express the recombinant protein (data not shown). To determine whether the new short chain dehydrogenase is an integral membrane protein, microsomal fraction from SF9 cells that expressed the recombinant protein was subjected to alkaline and detergent extractions. Equal amounts of microsomes were incubated with either sodium carbonate buffer, pH 11.5 (alkaline extraction), Triton X-100/PBS (detergent extraction), or PBS, pH 7.4 (control) (Fig. 5). Extracted proteins were separated from membrane-bound proteins by ultracentrifugation and analyzed by Western blotting. As shown in Fig. 5, the recombinant protein remained membrane-bound after alkaline extraction but was partially solubilized by increasing concentration of a detergent, consistent with the behavior of an integral membrane protein.

Next, we tested whether the putative short chain dehydrogenase is enzymatically active. Because its amino acid sequence showed the closest similarity to retinol/sterol dehydrogenases, we examined whether it possessed a hydroxysteroid dehydrogenase activity using reaction conditions established previously for RdH-4 (20) and RdH-like 3α-HSD (21). Microsomes were incubated with a number of tritiated steroid compounds for 1 h at 37 °C in the presence of 1 mM NAD+ or NADH. The reaction products were extracted, separated by
TLC, and visualized using a PhosphorImager. At a 5 mM concentration of each substrate, the highest percentage of conversion in the oxidative direction in the presence of NAD\(^1\) was observed with 3\(\alpha\)-hydroxysteroids, 3\(\alpha\)-androstanediol and allo-pregnanolone (Fig. 6A). 3\(\alpha\)-Androstanediol contains two hydroxyl groups in positions 3\(\alpha\) and 17\(\beta\) (Fig. 1)). The enzyme possessed both 3\(\alpha\)-HSD and 17\(\beta\)-HSD activity, as evidenced by the appearance of androstanedione with ketone groups in positions 3 and 17 (Fig. 6A). To estimate the relative efficiency of the two activities, we used substrates that have only one hydroxyl group: 3\(\alpha\)-hydroxyl (androsterone) or 17\(\beta\)-hydroxyl (dihydrotestosterone) (Fig. 1). The 3\(\alpha\)-hydroxyl group was oxidized much more efficiently (androsterone to androstanedione) than the 17\(\beta\)-hydroxyl group (dihydrotestosterone to androstanedione). To evaluate the 3\(\beta\)-HSD activity of the enzyme, we used dehydroepiandrosterone as substrate. As seen in Fig. 6A, no oxidation products of dehydroepiandrosterone were detected by autoradiography, indicating that the rate of conversion was not sufficient to detect the respective oxidation product.

One of the best substrates for the novel 3\(\alpha\)-HSD in the oxidative direction was allopregnanolone (3\(\alpha\)-tetrahydroprogestrone) (Fig. 6A). Interestingly, besides a significant amount of dihydroprogesterone with ketone group in position 3, an additional product, isopregnanolone (3\(\beta\)-tetrahydroprogestrone), appeared in this reaction. This implied that the ketone group on carbon 3 of dihydroprogesterone could be reduced to either a 3\(\alpha\) or 3\(\beta\)-hydroxyl group. This type of activity was observed previously during prolonged incubations of RoDH-like 3\(\alpha\)-HSD with allopregnanolone and NAD\(^1\) (22).

Analysis of the reaction products in the reductive direction in the presence of NADH showed that ketone group on carbon 3 was reduced to hydroxyl group with the highest catalytic effi-
Fig. 4. Northern blot analysis of 3α-HSD distribution in human tissues. Human multiple tissue Northern blots (CLONTECH) containing a minimum of 1 μg polyadenylated RNA/lane were hybridized with human radiolabeled 3α-HSD cDNA at high stringency conditions. The blots were exposed to x-ray film for 1 day (the panel with uterus and trachea on the for left) and then re-exposed for 10 days (all other panels). The hybridized blots were stripped of residual radioactivity, reprobed with β-actin cDNA, and exposed for 3 h (bottom panel). Positions of the size standards are indicated on the left.

Fig. 5. Alkaline extraction and Western blot analysis of recombinant 3α-HSD. Microsomal membranes containing 3α-HSD were extracted with 10% Triton X-100 in PBS (1% Tx-100), 1% Triton X-100 in PBS (1% Tx-100), sodium carbonate buffer, pH 11.5 (alkaline extraction), or PBS, pH 7.4 (PBS). Solubilized proteins (S) were separated from integral membrane proteins (P) by ultracentrifugation. Distribution of 3α-HSD between the soluble and the membrane-bound fractions was analyzed by Western blotting as described under “Experimental Procedures.”

Fig. 6. Analysis of the reaction products produced by 3α-HSD in the presence of NAD⁺ (A) or NADH (B). Control reactions performed in the absence of cofactor (−NAD⁺/NADH) are shown next to the sample with the same substrate in the presence of cofactor. All reactions were allowed to proceed for 1 h at 37 °C. Substrates were used at a concentration of 5 μM. The microsomes were used at concentration of 25 μg/ml in the reaction volume. C, analysis of the endogenous activity of SF9 microsomes isolated from cells infected with wild-type baculovirus toward dihydrotestosterone in the presence of NAD⁺ (+ NAD⁺) (17β-dehydrogenase activity) or NADH (+ NADH) (3 keto-reductase activity). ADT, androsterone; DHT, dihydrotestosterone; ADIOL, 3α-androstanediol; DHEA, dehydroepiandrosterone; ALLO-P, allopregnanolone; ISO-P, isopregnanolone; DHP, dihydroprogesterone; DIONE, androstanedione.

iciency (dihydrotestosterone to 3α/3β-androstanediol) (Fig. 6B). The 17-ketone group on androsterone was also reduced, albeit at a much slower rate. To ensure that the observed 3β- and 17β-hydroxysteroid dehydrogenase activity was not due to endogenous activity of insect cell microsomes, we determined the activity of microsomes isolated from SF9 cells that were infected with wild-type virus and did not express 3α-HSD. At a 5 μM substrate concentration, 0.2 pmol of androstenedione and 0.08 pmol of androstenedione were formed by 3 μg of control microsomes from dihydrotestosterone and dehydroepiandrosterone, respectively, in the presence of NAD⁺. In contrast, the same amount of microsomes that contained 3α-HSD produced 199 pmol of androstenedione and 28 pmol of androstenedione. The endogenous hydroxysteroid activity of SF9 microsomes from the cells infected with wild-type virus was also tested using [14C]dihydrotestosterone in the presence of NAD⁺ (17β-hydroxysteroid dehydrogenase activity) or NADH (3-keto reductase activity). As shown in Fig. 6C, no products were detected in the oxidative or the reductive direction. Based on this analysis, we concluded that all of the observed steroid conversions were catalyzed by the novel 3α-HSD.

Before measuring kinetic constants for oxidation and reduction of steroids, we determined whether NAD⁺ and NADH were, in fact, the preferred cofactors. In the oxidative direction, using 5 μM alloprogesterone as substrate, the reaction rate was 44-fold higher in the presence of 1 mM NAD⁺ than in the presence of 1 mM NADH. In the reductive direction, using 10 μM dihydrotestosterone as substrate, the rate was 9-fold higher with 1 mM NADH than it was with 1 mM NADPH. Thus, NAD⁺ and NADH were the preferred cofactors. The apparent Kₘ value for NAD⁺ determined with 20 μM alloprogesterone was 72.0 ± 5.0 μM. The apparent Kₘ value for NADH determined with 25 μM dihydrotestosterone was 9.0 ± 0.5 μM.

Considering that multiple products are formed by the enzyme over long periods of incubation (or with high enzyme concentrations), we established conditions under which only one reaction product was formed. These conditions were used to determine kinetic constants for steroid substrates. Specifically, the apparent Kₘ and Vₘax values for alloprogesterone and 3α-androstanediol were measured when there was no detectable formation of secondary products (isopregnanolone and androstanedione, respectively). Consistent with autoradiography analysis of the reaction products, alloprogesterone and androstanediol were the best substrates in the oxidative direction with the apparent Kₘ values of 5–7 μM (Table II). The apparent Kₘ value for androsterone was ~5-fold higher (Table II). Kinetic analysis also showed that the enzyme was capable of binding 3β-hydroxysteroid dehydroepiandrosterone; however, the rate of conversion was about 20-fold lower compared with that of alloprogesterone (Table II), which explains why the corresponding product could not be visualized using a PhosphorImager (Fig. 6A). Dihydrotestosterone (3-ketone group)
Kinetic constants for steroid substrates

| Substrate           | \( K_m \) | \( V_{max} \) |
|---------------------|--------|----------|
| Allopregnanolone    | 5.0 ± 0.7 | 13.0 ± 0.7 |
| 3α-Androstanediol   | 7.5 ± 0.4 | 14.0 ± 0.3 |
| Androsterone        | 24.0 ± 1.0 | 14.6 ± 0.4 |
| Dehydroepiandrosterone | 14.0 ± 1.0 | 0.6 ± 0.1 |
| Dihydrotestosterone | 12.0 ± 0.4 | 31.0 ± 0.5 |

was reduced to androstanediol with a catalytic efficiency similar to that for the oxidation of 3α-hydroxyl group (Table II).

To determine whether the new enzyme was active toward retinoids, we incubated 30–300 \( \mu \)g of microsomal membranes containing the recombinant protein with 10–50 \( \mu \)M all-trans-retinol or 13-cis-retinol in the presence of 1 \( \mu \)M NAD\(^+\). After a 30-min incubation at 37 °C, the reaction products were extracted and analyzed by HPLC as described previously (21). The amount of retinaldehyde formed was calculated from the calibration curve of the amount of pure retinal injected onto the column. Based on these measurements, 71 \( \mu \)mol were produced by 30 \( \mu \)g of microsomal protein over the 30-min incubation time from 10 \( \mu \)M retinol. For comparison, 9600 \( \mu \)mol of dihydropregnanolone would have been produced from 10 \( \mu \)M allopregnanolone by the same amount of protein under the identical conditions. Considering that the rate of retinol oxidation is at least 100 times lower than that of allopregnanolone, the new enzyme is not efficient as retinol dehydrogenase.

Transmembrane Topology of 3α-HSD—Because we have established that the novel 3α-HSD is an integral membrane protein, we were interested in determining its transmembrane orientation. Analysis of its protein sequence for signature sites and motifs indicated that there is a potential N-terminal transmembrane segment (amino acids 2–17) as well as three potential N-glycosylation sites at Asn-161, Asn-187, and Asn-253. Because N-glycosylation is carried out exclusively on the luminal side of the endoplasmic reticulum, the appearance of protein species with higher subunit molecular weight in the presence of microsomes would indicate that they become glycosylated and were therefore exposed to the lumen.

To investigate the transmembrane topology of 3α-HSD, we used a coupled in vitro transcription/translation system and canine pancreatic microsomal membranes. 11β-HSD1, a protein with known luminal orientation and three glycosylation sites (32), served as a positive control for glycosylation. 3α-HSD characterized in the present study exhibits less than 50% amino acid sequence identity to any known protein. It is most closely related to retinol/sterol dehydrogenases of the short chain dehydrogenase/reductase superfamily.

**DISCUSSION**

The novel 3α-HSD characterized in the present study exhibits less than 50% amino acid sequence identity to any known protein. It is most closely related to retinol/sterol dehydrogenases of the short chain dehydrogenase/reductase superfamily.
Furthermore, the gene for 3α-HSD shares similar structural organization with the genes for retinol/sterol dehydrogenases (28). Some variation is observed in the length of exon 5, which is 1 amino acid longer that the corresponding exon in the Rdh5 gene and 2 amino acids longer than that in the RoDH-4 and RoDH-like 3α-HSD genes. Interestingly, 3α-HSD gene is found on a different chromosome and does not appear to have satellite pseudogenes like RoDH-4 and RoDH-like 3α-HSD genes (28), which might indicate that it appeared later in evolution.

Analysis of the sequences deposited in the GenBank™ expressed sequence tag data base showed that cDNA fragments identical to different segments of 3α-HSD cDNA were isolated from multiple sources: lymph node (nine reports), testis (three reports), normal colon and colon adenocarcinoma (three reports), cervical tumor (one report), breast (one report), uterus and endometrial adenocarcinoma (four reports), fetal lung (one report), pancreas adenocarcinoma (two reports), and lymphoma (one report). Thus, 3α-HSD appears to exhibit wide tissue distribution. Indeed, Northern blot analysis of human tissues revealed that 3α-HSD mRNA is present in the central nervous system and in a number of peripheral endocrine tissues. Interestingly, the size of the predominant mRNA species varied depending on the tissue source, suggesting that there might be a tissue-specific splicing of 3α-HSD mRNA. In fact, we found that the 113-nucleotide segment inserted in the 5'-untranslated sequence of a nearly identical clone submitted under the name of retinol dehydrogenase homolog isof orm-2 (AF240697) is located within the 11,805-base pair sequence of putative intron 1. This observation suggests that alternatively spliced products may exist.

The most striking difference in the distribution pattern of the novel 3α-HSD compared with that of retinol/sterol dehydrogenases is that the 3α-HSD message is practically absent in liver. The lack of hybridization with the liver mRNA serves as a confirmation that the cDNA probe does not cross-hybridize with either RoDH-4 or RoDH-like 3α-HSD mRNA, both of which are very abundant in the liver.

The presence of 3α-HSD in the brain is consistent with its activity toward allopregnanolone, which serves as a potent allosteric regulator of γ-aminobutyric acid type A receptors. Allopregnanolone is likely to be produced in the brain by cytosolic 3α-HSDs, AKR1C1 and AKR1C2, which catalyze the reduction of the 3-ketone group on dihydropregesterone (13). The back-oxidation of allopregnanolone to dihydropregesterone is thought to be catalyzed by an unidentified membrane-bound NAD+ -dependent 3α-HSD (4, 5). Previously, we have reported that RoDH-like 3α-HSD is capable of oxidizing allopregnanolone and is expressed in various regions of the human brain and in the spinal cord (21), suggesting that it may function as allopregnanolone dehydrogenase in the central nervous system. This study shows that, in addition to RoDH-like 3α-HSD, the central nervous system contains a second isoform of microsomal oxidative 3α-HSD active toward allopregnanolone. Interestingly, similar to RoDH-like 3α-HSD (21, 22), the newly characterized enzyme exhibits “epimerase” activity toward allopregnanolone, slowly converting it to 3β-hydroxyandrostanediol in the presence of NAD+. We have previously proposed that NADH, which is produced during oxidation of allopregnanolone, might be retained in the active site and trigger the nonstereospecific reduction of the 3-ketone group on dihydropregesterone to both a 3α-hydroxy and a 3β-hydroxy group. Consistent with this hypothesis, the new 3α-HSD exhibits a higher affinity for NADH than for NAD+ (the apparent Km value is 9 versus 72 μM), similar to RoDH-like 3α-HSD (0.18 versus 1.9 μM) (21). At the same time, RoDH-4, which has almost identical Km values for NAD+ (0.13 μM) and NADH (0.1 μM), does not seem to possess the epimerase activity.2 It should also be noted that, in addition to allopregnanolone, RoDH-like 3α-HSD epimerized androsterone into epiandrosterone. However, we did not observe epimerization of androsterone with the new 3α-HSD, possibly due to the 5-fold higher apparent Km value of the enzyme for androsterone compared with allopregnanolone.

Besides utilizing allopregnanolone as substrate, the new 3α-HSD is equally efficient at converting 3α-androstanediol into a potent androgen, dihydrotestosterone. This is consistent with the expression of the enzyme in testis and prostate. Previously, Biswas and Russell (15) proposed that RoDH-like 3α-HSD, which is expressed in prostate and testis, contributes to the back-oxidation of 3α-androstanediol to dihydrotestosterone in these tissues. This study shows that, in addition to RoDH-like 3α-HSD, dihydrotestosterone can be produced in testis and prostate by the novel isoform of 3α-HSD.

Similar to retinol/sterol dehydrogenases, 3α-HSD is an integral membrane protein. Therefore, depending on its transmembrane topology, the active site of the enzyme may be exposed either to the cytosol or to the lumen of the endoplasmic reticulum. This, in turn, may affect its access to the substrates and cofactors. Previous analysis of the transmembrane topology of Rdh5, which was proposed to catalyze the biosynthesis of 11-cis-retinaldehyde in retinal pigment epithelium, showed that Rdh5 is anchored to the membranes of smooth endoplasmic reticulum by two hydrophobic peptide segments (23). The catalytic domain of this enzyme is confined to the luminal compartment, suggesting that generation of 11-cis-retinaldehyde occurs in the lumen of the endoplasmic reticulum (23). In contrast to Rdh5, 3α-HSD appears to be facing the cytosolic side of the membrane, indicating that it will utilize the cytosolic pool of steroids and nucleotides. In the liver cytosol, and presumably in other tissues, the NAD+ :NADH ratio is about 1000, whereas the NADP+ :NADPH ratio is about 0.01 (33). This suggests that the NAD+ -preferring oxidoreductases, which face the cytosol like the new 3α-HSD, will function in the oxidative direction. The available substrate and cofactor pool for the lumennally oriented Rdh5, which also prefers NADP+ over NADP+ as cofactor (34), is less clear.

The existence of enzymes that share similar substrates but exhibit different transmembrane orientation is not unusual. For example, two other members of the short chain dehydrogenase/reductase superfamily, 11β-HSD type 1 and type 2, which catalyze the interconversion between a potent glucocorticoid cortisol and a weak glucocorticoid cortisone, exhibit opposite transmembrane orientation: 11β-HSD type 1 is a lumennally oriented glycoprotein, whereas 11β-HSD type 2 faces the cytosol (32). Interestingly, these two enzymes exhibit a different cofactor preference: 11β-HSD type 1 catalyzes oxidation and reduction using NADP(H) as cofactor, whereas 11β-HSD type 2 is NAD+ -specific and catalyzes only 11β-dehydrogenation. This is in contrast to Rdh5 and 3α-HSD, which both appear to exhibit a cofactor preference for NAD+.

Besides the difference in transmembrane topology and tissue distribution, 3α-HSD exhibits different substrate specificity compared with Rdh5 and the all-trans-retinol-oxidizing microsomal dehydrogenases, RoDH-4 and RoDH-like 3α-HSD. Rdh5 has similar affinity for retinoids (Km of 6.3–6.6 μM for 9-cis and 11-cis-retinol) and 3α-androstanediol (Km of 6.4 μM) (16) and is about two times more efficient as retinol dehydrogenase than as a steroid dehydrogenase. RoDH-4 and RoDH-like 3α-HSD occupy an intermediate position and are more efficient as 3α-hydroxysteroid dehydrogenases with apparent

2 S. V. Chetyrkin, and N. Y. Kedishvili, unpublished observations.
transmembrane topology, and catalytic properties.

Acknowledgment—We thank Dr. Kirill Popov (Division of Molecular Biology and Biochemistry, University of Missouri-Kansas City, Kansas City, MO) for helpful discussions and critical reading of the manuscript.

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Characterization of a Novel Type of Human Microsomal 3α-Hydroxysteroid Dehydrogenase: UNIQUE TISSUE DISTRIBUTION AND CATALYTIC PROPERTIES
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J. Biol. Chem. 2001, 276:22278-22286.
doi: 10.1074/jbc.M102076200 originally published online April 9, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102076200

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