Structure and Expression of a New Complementary DNA Encoding the almost Exclusive 3β-Hydroxysteroid Dehydrogenase/Δ^5-Δ^4-Isomerase in Human Adrenals and Gonads

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The 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3βHSD) enzyme catalyzes the oxidation and isomerization of Δ^5-3β-hydroxysteroid precursors into Δ^4-ketosteroids, thus leading to the formation of all classes of steroid hormones. In addition, 3βHSD catalyzes the interconversion of 3β-hydroxy- and 3-keto-5α-androstane steroids. Clinical observations in patients with 3βHSD deficiency as well as our recent data obtained by Southern blot analysis using a human placental 3βHSD cDNA (type I) as probe suggested the existence of multiple related 3βHSD isoenzymes. We now report the isolation and characterization of a second type of cDNA clone (arbitrarily designated type II) encoding 3βHSD after screening of a human adrenal λgt22A library. The nucleotide sequence of 1676 basepairs of human 3βHSD type II cDNA predicts a protein of 371 amino acids with a calculated molecular mass of 41,921 daltons, which displays 93.5% and 96.2% homology with human placental type I and rhesus macaque ovary 3βHSD deduced proteins, respectively. To characterize and compare the kinetic properties of the two isoenzymes, plasmids derived from pCMV and containing type I or type II 3βHSD full-length cDNA inserts were transiently expressed in HeLa human cervical carcinoma cells. *In vitro* incubation with NAD^+ and ^3H-labeled pregnenolone or dehydroepiandrosterone shows that the type I protein possesses a 3βHSD/Δ^5-Δ^4 isomerase activity higher than type II, with respective K_m values of 0.24 *vs.* 1.2 μM for pregnenolone and 0.18 *vs.* 1.6 μM for dihydroepiandrosterone, while the specific activity of both types is equivalent. Moreover, incubation in the presence of NADH of homogenates from cells transfected with type I or type II 3βHSD indicates that dihydrotestosterone is converted into 5α-androstane-3β,17β-diol, with K_m values of 0.26 and 2.7 μM, respectively. Ribonuclease protection assay using type I- and type II-specific cRNA probes revealed that type II transcripts are the almost exclusive 3βHSD mRNA species in the human adrenal gland, ovary, and testis, while type I transcripts correspond to the almost exclusive 3βHSD mRNA species in the placenta and skin and represent the predominantly expressed species in mammary gland tissue. The present data show for the first time that adrenals and gonads express a type of 3βHSD isoenzyme that is distinct from the type expressed in the placenta. Detection of 3βHSD gene expression in nonclassical steroidogenic tissues such as skin and mammary gland suggests that 3βHSD is likely to play an important role in the intracrine formation of sex steroids in peripheral target tissues. Characterization of type II 3βHSD, in addition to permitting studies of the regulation of its tissue-specific expression, offers the opportunity of elucidating the molecular basis of classical and nonclassical 3βHSD deficiencies, the second most common cause of congenital adrenal hyperplasia and the predicted most common genetic disorder in women with signs of androgen excess, respectively. (Molecular Endocrinology 5: 1147–1157, 1991)

0888-8809/91/1147-1157$03.00/0
Molecular Endocrinology

INTRODUCTION

The membrane-bound enzyme 3β-hydroxysteroid dehydrogenase (EC 1.1.1.145)/Δ^5-Δ^4 isomerase (EC 5.3.3.1), hereafter called 3βHSD, catalyzes the obligatory oxidation and isomerization of Δ^5-3β-hydroxypregnene and Δ^5-3β-hydroxyandrostene steroid precursors into the Δ^4-3-ketosteroids progesterone, 17α-hydroxyprogesterone, Δ^4-androstenedione, and testosterone (1, 2). As recently demonstrated by transient expression of human (1) and rat (2) 3βHSD cDNAs, in addition to being an essential step in the biosynthesis of all classes

of hormonal steroids, 3βHSD is also responsible for the interconversion of 3β-hydroxy- and 3-keto-5α-androstane steroids. 3βHSD is found not only in classical steroidogenic tissues, namely the placenta (1, 3), adrenal cortex (4–6), ovary (2, 6–11), and testis (2, 6, 8, 12), but also in several peripheral tissues, including the skin (2, 13–19), adipose tissue (2), breast (2, 12, 20), lung (21), endometrium (22), prostate (23, 24), liver (2, 8, 25), kidney (2, 8), epididymis (8), and brain (26). The widespread distribution of 3βHSD expression indicates that this enzyme is likely to play an important role in the intracrine (27) formation of sex steroids in peripheral target tissues. Such a high level of extragonadal formation of sex steroids is especially important in the human and some other primates, whose adrenals secrete large amounts of precursor sex steroids, such as dehydroepiandrosterone (DHEA) and especially DHEA sulfate (Refs. 27 and 28 and references therein).

Congenital adrenal hyperplasia is the most frequent cause of ambiguous genitalia and adrenal insufficiency in newborn infants (29–39). This autosomal recessive disease is associated with varying degrees of salt wasting and genital ambiguity in both sexes. With the help of more sensitive and specific steroid measurements, congenital 3βHSD deficiency has become the second most diagnosed form of congenital adrenal hyperplasia (40). On the other hand, signs of nonclassical 3βHSD deficiency can vary from premature puberty and accelerated growth in children, to hirsutism, acne, temporal balding, irregular menses, and infertility (39, 41–45). The incidence of nonclassical 3βHSD among women with signs of androgen excess recently reviewed in the literature is about one in six (39), thus leading to the conclusion that this disease may be more common than the mild form of 21-hydroxylase deficiency that is believed to be the most common autosomal recessive genetic disorder in humans (39). The wide phenotypic heterogeneity of 3βHSD deficiency syndromes and several clinical observations in these patients reporting intact peripheral and/or hepatic Δ^5-3βHSD activity could suggest the existence of multiple isoenzymes and/or tissue-specific regulation of gene expression.

We have recently reported the characterization of a human 3βHSD (hp3βHSD) cDNA isolated from a placental library (1) and the corresponding gene (12) located at the p11-p13 region of chromosome 1 (46), which encodes a deduced protein of 372 amino acids. Homologous proteins of identical size have been predicted for 3βHSD proteins from cDNA clones isolated from macaque (8), bovine (7), and rat (2) ovary libraries. RNA blot analysis of RNA from human adrenals, gonads, placenta, and mammary gland using hp3βHSD cDNA as probe identified a single 1.7-kilobase mRNA species (12). However, the detection of multiple unexpected DNA fragments by Southern blot analysis of human genomic DNA (12) and the heterogeneous clinical picture in 3βHSD-deficient patients suggest the presence of multiple 3βHSDs in the human. The present study describes the molecular cloning and characterization of a new type of 3βHSD cDNA in human adrenals, which is chronologically designated human type II 3βHSD. This new type II 3βHSD cDNA encodes a functional 3βHSD that corresponds to the almost exclusive 3βHSD mRNA species in adrenals and gonads, in contrast to the type I mRNA species, which is the almost exclusive 3βHSD mRNA population in the placenta and skin.

RESULTS

Isolation and Characterization of Human Type II 3βHSD cDNA Clones

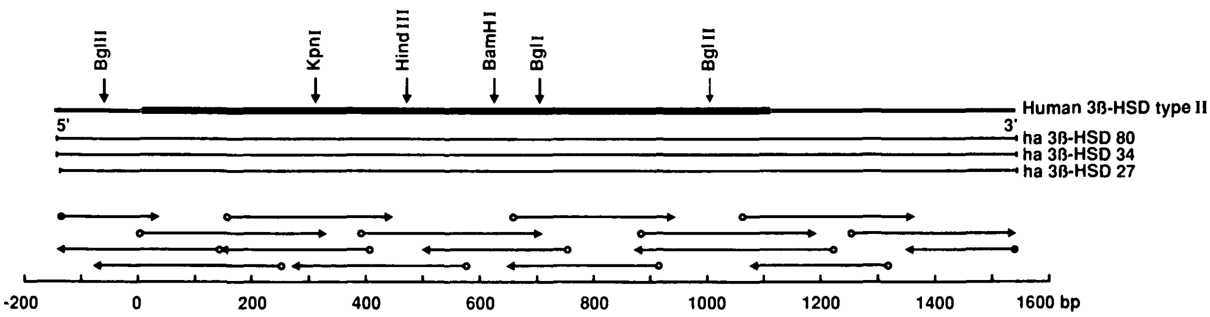
A human adrenal λgt22A cDNA library was screened with human placental cDNA clone hp3βHSD63 (1). Putative positive clones showing a strong hybridization signal were detected at a frequency of approximately $75/10^4$ plaques. One hundred cDNA clones were thus isolated from 400 human adrenal λgt22A recombinants by their ability to hybridize to the ^{32}P-labeled *Eco*RI-*Kpn*I restriction fragment corresponding to the first 318 nucleotides of the 5′ end of hp3βHSD63 cDNA. The 10 longest clones were subcloned into the BSKS vector and sequenced by the dideoxy chain-termination method.

Since all of the 10 selected clones showed identical sequences, only the three longest cDNA clones, namely ha3βHSD80, ha3βHSD34, and ha3βHSD27, were completely sequenced in both orientations (Fig. 1A). The three cDNA clones encoding human type II 3βHSD shared identical sequences in their overlapping regions. The first inframe ATG codon in the nucleotide sequence is designated position 1. The cDNA sequence of type II 3βHSD includes an open reading frame of 1116 nucleotides compared to 1119 nucleotides for type I 3βHSD cDNA. Moreover, the nucleotide sequence of human type II 3βHSD cDNA displays 93.6% similarity with that of human type I 3βHSD (Fig. 1B). The longest cDNA insert of type II (ha3βHSD34) includes a 134-basepair (bp) 5′-untranslated region. Type II cDNA includes a 418-bp 3′-untranslated sequence, which shares 87% and 91% similarity with the corresponding overlapping region of human type I and macaque 3βHSD cDNAs, respectively. The nucleotide sequence of the expected coding region of human type II 3βHSD shares 95.4%, 81.0%, 77.8%, 77.4%, and 75.4% similarity with that of macaque (8), bovine (7), and rat types I, II, and III (2, 25), respectively. The polyadenylation consensus signal AATAAA is located 20 nucleotides up-stream from the poly(A) tail.

Deduced Amino Acid Sequence of Human Type II 3βHSD and Sequence Similarities with Human Type I, Macaque, Bovine, and Rat Type I, II, and III 3βHSD Proteins

The sequence CACGATGG containing the first inframe initiating codon in human type II 3βHSD cDNA corresponds well to the consensus sequence C(A/

A



B

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                                              human 3β-HSD type II              AGGACTTGGACTCC   -120
                                              human 3β-HSD type  I     GAGGTGAGA--T--G-CC----T

II TCTGTCCAGCTTTTAACAATCTAAGTTACGCCCTCTTCTGGGTCACGCTAGAATCAGATCTGCTCTCCAGCATCTTCTGTTTCCTGGCAAGTGTTTCCTGCTACTTTGGATTGGCCACG    -1
 I ----------------------C-A-T-------C-A-------C-----------------C-------------------TG----A---------------*------T-
                                                                                                                   Met

   Met Gly Trp Ser Cys Leu Val Thr Gly Ala Gly Gly Leu Leu Gly Gln Arg Ile Val Arg Leu Leu Val Glu Glu Lys Glu Leu Lys Glu    30
II ATG GGC TGG AGC TGC CTT GTG ACA GGA GCA GGA GGG CTT CTG GGT CAG AGG ATC GTC CGC CTG TTG GTG GAA GAG AAG GAA CTG AAG GAG    90
 I -C- --- --- --- --- --- --- --- --- --- --- --- T-- --- --A --- --- --- A-- --- --C --- --- A-G --- --- --G --- --- ---
   Thr                                           Phe                     Ile                     Lys

   Ile Arg Ala Leu Asp Lys Ala Phe Arg Pro Glu Leu Arg Glu Glu Phe Ser Lys Leu Gln Asn Arg Thr Lys Leu Thr Val Leu Glu Gly    60
II ATC AGG GCC TTG GAC AAG GCC TTC AGA CCA GAA TTG AGA GAG GAA TTT TCT AAG CTC CAG AAC AGG ACC AAG CTG ACT GTA CTT GAA GGA   180
 I --- --- -T- --- --- --- --- --- G-- --- --- --- --- --- --- --- --- --A --- --- --- -A- --- --- --- --A --G --G --- ---
           Val                     Gly                                                 Lys

   Asp Ile Leu Asp Glu Pro Phe Leu Lys Arg Ala Cys Gln Asp Val Ser Val Val Ile His Thr Ala Cys Ile Ile Asp Val Phe Gly Val    90
II GAC ATT CTG GAT GAG CCA TTC CTG AAA AGA GCC TGC CAG GAC GTC TCG GTC GTC ATC CAC ACC GCC TGT ATC ATT GAT GTC TTT GGT GTC   270
 I --- --- --- --- --- --- --- --- --G --- --- --- --- --- --- --- --- A-- --- --- --- --- --- --- --- --- --- --C --- ---
                                                                     Ile

   Thr His Arg Glu Ser Ile Met Asn Val Asn Val Lys Gly Thr Gln Leu Leu Leu Glu Ala Cys Val Gln Ala Ser Val Pro Val Phe Ile   120
II ACT CAC AGA GAG TCC ATC ATG AAT GTC AAT GTG AAA GGT ACC CAG CTA CTG TTG GAG GCC TGT GTC CAA GCC AGT GTG CCA GTC TTC ATC   360
 I --- --- --- --- --T --- --- --- --- --- --- --- --- --- --- --C --- --A --- --- --- --- --- --T --- --- --- --- --- ---

   Tyr Thr Ser Ser Ile Glu Val Ala Gly Pro Asn Ser Tyr Lys Glu Ile Ile Gln Asn Gly His Glu Glu Glu Pro Leu Glu Asn Thr Trp   150
II TAC ACC AGT AGC ATA GAG GTA GCC GGG CCC AAC TCC TAC AAG GAA ATC ATC CAG AAC GGC CAC GAA GAA GAG CCT CTG GAA AAC ACA TGG   450
 I --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --T --- --T --- --- --- --- --- --- --- --- ---

   Pro Thr Pro Tyr Pro Tyr Ser Lys Lys Leu Ala Glu Lys Ala Val Leu Ala Ala Asn Gly Trp Asn Leu Lys Asn Gly Asp Thr Leu Tyr   180
II CCC ACT CCA TAC CCG TAC AGC AAA AAG CTT GCT GAG AAG GCT GTG CTG GCG GCT AAT GGG TGG AAT CTA AAA AAT GGT GAT ACC TTG TAC   540
 I --- G-- --- --- --A C-- --- --- --- --- --- --- --- --- --A --- --- --- --C --- --- --- --G --- --C --C -GC --- C-- ---
       Ala             His                                                                                 Gly

   Thr Cys Ala Leu Arg Pro Thr Tyr Ile Tyr Gly Glu Gly Gly Pro Phe Leu Ser Ala Ser Ile Asn Glu Ala Leu Asn Asn Asn Gly Ile   210
II ACT TGT GCG TTA AGA CCC ACA TAT ATC TAT GGG GAA GGA GGC CCA TTC CTT TCT GCC AGT ATA AAT GAG GCC CTG AAC AAC AAT GGG ATC   630
 I --- --- --C --- C-- --- -TG --- --- --- --- --- --- A-- -G- --- --- --- --T --- --- --C --- --- --- --- --- --- --- ---
                           Met                         Ser Arg

   Leu Ser Ser Val Gly Lys Phe Ser Thr Val Asn Pro Val Tyr Val Gly Asn Val Ala Trp Ala His Ile Leu Ala Leu Arg Ala Leu Arg   240
II CTG TCA AGT GTT GGA AAG TTC TCT ACA GTC AAC CCA GTC TAT GTT GGC AAC GTG GCC TGG GCC CAC ATT CTG GCC TTG AGG GCT CTG CGG   720
 I --- --- --- --- --- --- --- --C --T --T --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- --- --- --C --- -A-
                                                                                                                   Gln

   Asp Pro Lys Lys Ala Pro Ser Val Arg Gly Gln Phe Tyr Tyr Ile Ser Asp Asp Thr Pro His Gln Ser Tyr Asp Asn Leu Asn Tyr Ile   270
II GAC CCC AAG AAG GCC CCA AGT GTC CGA GGT CAA TTC TAT TAC ATC TCA GAT GAC ACG CCT CAC CAA AGC TAT GAT AAC CTT AAT TAC ATC   810
 I --- --- --- --- --- --- --C A-- --- --A --G --- --C --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -C-
                               Ile                                                                                 Thr

   Leu Ser Lys Glu Phe Gly Leu Arg Leu Asp Ser Arg Trp Ser Leu Pro Leu Thr Leu Met Tyr Trp Ile Gly Phe Leu Leu Glu Val Val   300
II CTG AGC AAA GAG TTT GGC CTC CGC CTT GAT TCC AGA TGG AGC CTT CCT TTA ACC CTG ATG TAC TGG ATT GGC TTC CTG CTG GAA GTA GTG   900
 I --- --- --- --- --C --- --- --- --- --- --- --- --- --- T-- --- --- T-- --- --- --T --- --- --- --- --- --- --- A-- ---
                                                           Phe         Ser                                         Ile

   Ser Phe Leu Leu Ser Pro Ile Tyr Ser Tyr Gln Pro Pro Phe Asn Arg His Thr Val Thr Leu Ser Asn Ser Val Phe Thr Phe Ser Tyr   330
II AGC TTC CTA CTC AGC CCA ATT TAC TCC TAT CAA CCC CCC TTC AAC CGC CAC ACA GTC ACA TTA TCA AAT AGT GTG TTC ACC TTC TCT TAC   990
 I --- --- --- --- --G --- --- --- A-- --- -G- --G --- --- --- --- --- -T- --- --- --G --- --- --C --A --- --- --- --- --T
                   Arg             Thr     Arg                         Ile

   Lys Lys Ala Gln Arg Asp Leu Ala Tyr Lys Pro Leu Tyr Ser Trp Glu Glu Ala Lys Gln Lys Thr Val Glu Trp Val Gly Ser Leu Val   360
II AAG AAG GCT CAG CGA GAT CTG GCG TAT AAG CCA CTC TAC AGC TGG GAG GAA GCC AAG CAG AAA ACC GTG GAG TGG GTT GGT TCC CTT GTG  1080
 I --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --G --- --- --- --- --- --- --- ---

   Asp Arg His Lys Glu Thr Leu Lys Ser Lys Thr Gln End                                                                     372
II GAC CGG CAC AAG GAG ACC CTG AAG TCC AAG ACT CAG TGA TTTAAGGATGACAGAGATGTGCATGTGGGTATTGTTAGGAAATGTCATCAAACTCCACCCACCTGGCT 1187
 I --- --- --- --- --- --- --- --- --- --- --- --- --- ----------------------------------------G---------G--------T------C

II TCATACAGAAGGCAACAGGGGCACAAGCCCAGGTCCTGCTGCCTCTCTTTCACACAATGCCCAACTTACTGTCTTCTTCATGTCATCAAAATCTGCACAGTCACTGGCCCAACCAGAA*C 1306
 I ----------A-TG---A----------T---------------C------T------G--------T---A---C-------------C----G------T---------A----GG

II TTTCTGTCCTAATCATACACCAGAAGACAAACAATATGATTTGCTGTTACCAAATCTCAGTGGCTGATTCTGAACAA*TTGTGGTCTCTCTTAACTTGAGGTTCTCTTTTGACTAATAGA 1425
 I -----------------T------G--A-G--C--G--G---------------------A---------------T--AG--A----T-----------G--*G---------C---

II GCTCCATTTCCCCTCTTAAATGAGAAAGCATTTCTTTTCTCTTTAATCTCCTATTCCTTCACACAGTTCAACATAAAGAG**CAATAAATGTTTTAATGCTTAACATGGAAAAAAAAAA 1542
 I ----------TA---------------G-----C-----T--------T-C-----------T----**TG-----A--AT-----------G--TG*----A-AA
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Fig. 1. A, Restriction Endonuclease Map of Human 3βHSD Type II cDNA Clones

The protein-coding region is represented by a *black box*, while the flanking 5′- and 3′-noncoding regions are shown as *solid line*. The direction and extent of sequencing, using synthetic oligonucleotide primers (*open circles*) or T3 or T7 BSKS vector primers (*solid circles*), are shown by *arrows*. A scale is shown *below*, where zero corresponds to the ATG initiation codon. B, Nucleotide and predicted amino acid sequences of human type II and human type I 3βHSD cDNAs. Nucleotide and amino acid positions are indicated to the *right* of the sequence. The complete nucleotide sequence of human type II 3βHSD cDNA is shown, while identical and different human type I 3βHSD nucleotides are indicated by *dotted lines* or the corresponding symbol (A, C, T, and G), respectively. The *asterisks* indicate missing nucleotides. The nucleotides corresponding to the putative polyadenylation signal are *underlined by a solid line*.

G)CCAUGG for the initiation of translation by eukaryotic ribosomes, knowing that as long as a purine is located at position −3, deviations from the rest of the consensus sequence surrounding the AUG codon only slightly impair the initiation of translation (47). The human type II 3βHSD cDNA thus predicts a 41,921-dalton protein with 371 amino acid residues (excluding the first methionine; Fig. 1B), while human type I, macaque, bovine, and rat type I, II, and III 3βHSD cDNAs all encode a deduced protein containing 372 amino acids. This difference of one residue results from a substitution of T by C at position −2 and of C by T at position +2, thus deleting and generating an initiation codon, respectively. The deduced amino acid sequence of type II 3βHSD shares 93.5% similarity with that of human type I 3βHSD, which differs by only 23 residues (Figs. 1B and 2). The similarity of the human type II 3βHSD amino acid sequence to that of macaque, bovine, and rat types I, II, and III is 96.2%, 78%, 72%, 71%, and 66.7%, respectively (Fig. 2).

Comparative analysis of the deduced 3βHSD proteins illustrated in Fig. 2 indicates that 211 residues (56.7%) are conserved in all seven amino acid sequences, while conservative changes are found at 37 additional positions, thus leading to an overall similarity of 66.6% across the seven different sequences, which indicates that this isoenzyme family is well conserved throughout the course of evolution.

The 3βHSD enzyme is a well recognized membrane-bound protein located in the endoplasmic reticulum and in mitochondrial membranes. It is of interest to note that computer analysis performed according to the method of Klein *et al.* (48) of the amino acid sequence of human type II 3βHSD predicts two membrane-span-

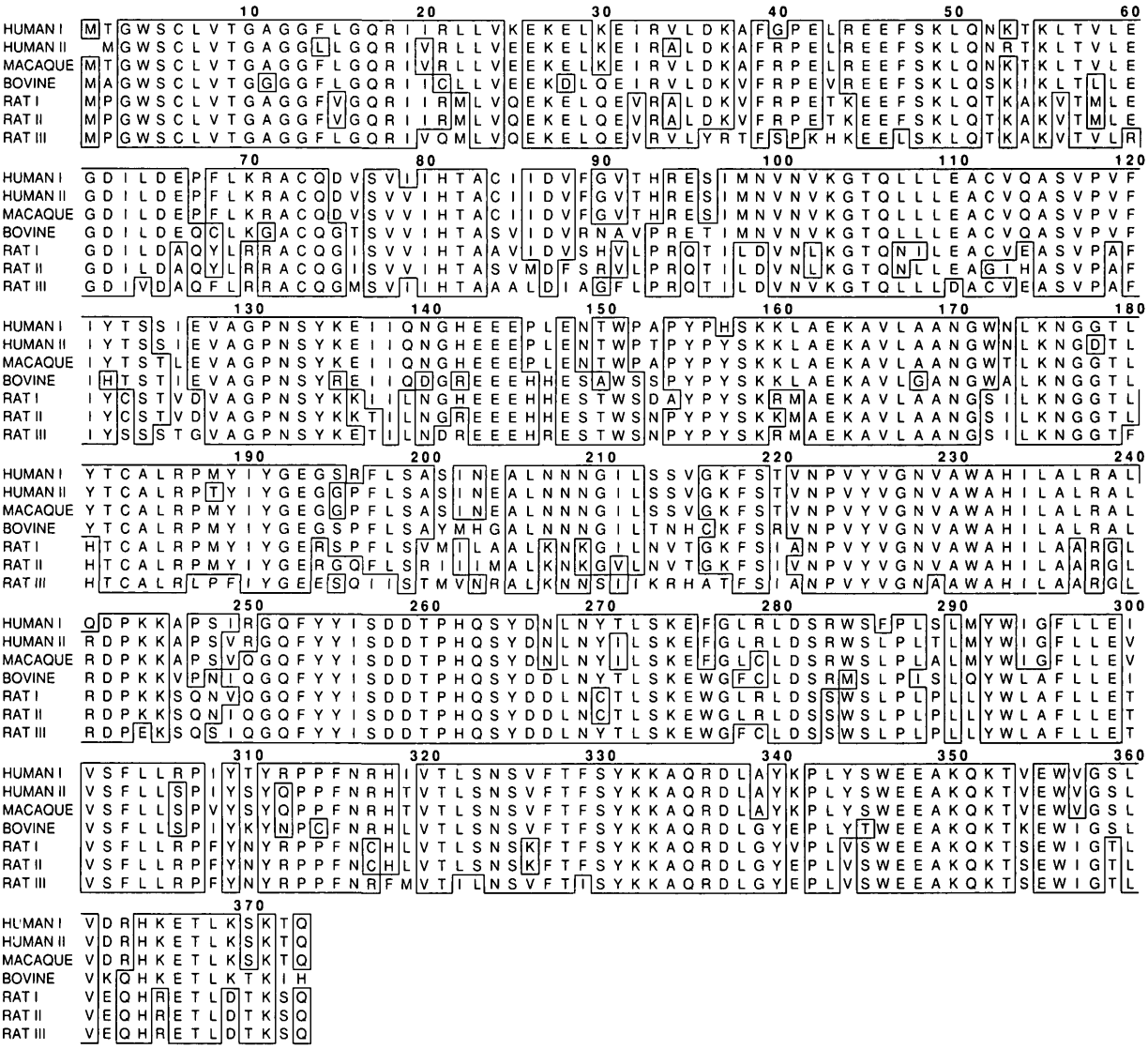

Fig. 2. Comparison of the Deduced Amino Acid Sequences of Human Type I, Human Type II, Macaque, Bovine, and Rat Types I, II, and III 3βHSD Proteins

Amino acid sequences are designated by the universal single letter code. Amino acid residues are numbered relative to the first NH_2-terminal methionine. Residues common to at least four of the seven predicted sequences are *boxed*.

ning domains extending from residues 73–90 and 286–304, with peripheral to integral ratios of 0.057 and 0.074, respectively (data not shown). These two transmembrane segments are also predicted using two other algorythms (49, 50), as previously reported for human type I and macaque and rat type I 3βHSD proteins, while in contrast, only the COOH-terminal membrane-spanning segment (~284–307; depending upon the program used) is predicted in bovine and rat type II 3βHSD proteins (2, 8).

Comparison of the Kinetic Properties of Expressed Human Type I and Type II 3βHSD Proteins

To verify that human type II 3βHSD cDNA encodes a protein that effectively catalyzes 3β-hydroxysteroid dehydrogenation and Δ^5-Δ^4 isomerization and to characterize potential functional differences between the two types, plasmids derived from porcine cytomegalovirus (pCMV) containing either type I (pCMV-type I h3βHSD) or type II (pCMV-type II h3βHSD) 3βHSD cDNA inserts driven by the CMV promoter were transiently expressed in HeLa cells.

As illustrated in Fig. 3, transfection of HeLa cells with either type I (lane 3) or type II (lane 4) h3βHSD cDNA inserts resulted in the production of a single 42-kDa protein that cross-reacts with polyclonal antibodies raised against purified human placental 3βHSD (1, 3) and comigrates with purified human placental 3βHSD (lane 1). Note the absence of the 42-kDa band in mock-transfected HeLa cells that are devoid of endogenous 3βHSD (lane 2) activity. The amounts of type I and type II 3βHSD proteins translated after transient transfection were 0.62 and 0.88 pmol, respectively, as estimated by quantification of the integrated optical intensity of the 42-kDa bands (using a BioImage System, Milligen/Biosearch), knowing that 15.4 pmol purified human placental 3βHSD were loaded onto the gel as a reference. This finding is in agreement with the similar transfection efficiency of these plasmids ascertained by cotransfection of a growth hormone-synthesizing plasmid (data not shown).

In vitro incubation with homogenates from cells transfected with pCMV-type I h3βHSD or pCMV-type II h3βHSD in the presence of 1 mM NAD^+ and ^{3}H-labeled substrates showed that the type I enzyme possesses a 3βHSD/Δ^5-Δ^4 isomerase activity higher than that of type II, with respective K_m values of 0.24 and 1.2 μM for pregnenolone (PREG) and 0.18 and 1.6 μM for DHEA, while the specific activity (V_{max}) of both types was equivalent when standardized for the estimated amount of corresponding translated proteins (Fig. 4). It can also be seen in Fig. 4C that after incubation of cell homogenates in the presence of NADH and ^{3}H-labeled dihydrotestosterone (DHT), the 3β-hydroxysteroid oxidoreductase activity, as measured by the formation of 5α-androstane-3β,17β-diol, was higher for type I than for type II 3βHSD protein, with K_m values of 0.26 and 2.7 μM, respectively. The present data also show that the affinity of the human type II 3βHSD protein is similar

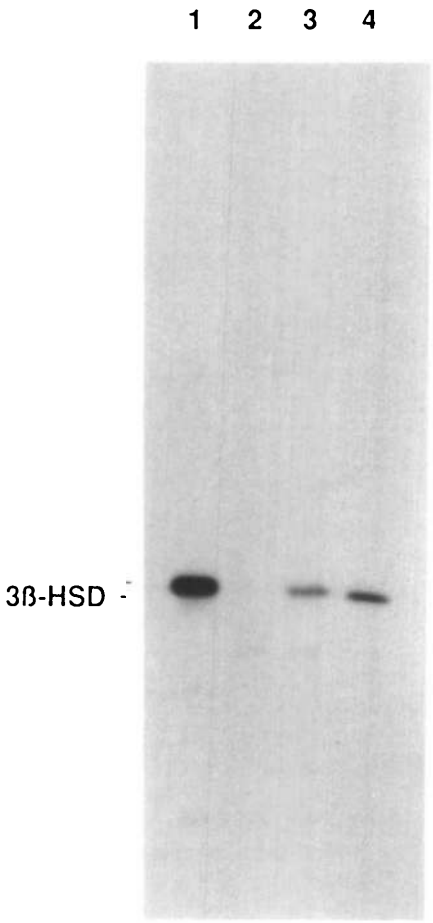

Fig. 3. Immunoblot Analysis of Purified Human Placental 3βHSD (Lane 1) and Expressed 3βHSD Protein Encoded by Human Type I (Lane 3) and Type II (Lane 4) 3βHSD cDNA

Immunoblot analysis was performed using antiserum raised in rabbits against purified h3βHSD, as previously described (1–3, 12). Purified hp3βHSD protein and homogenates from HeLa cells transfected with control pCMV vector (lane 2), pCMV-type I h3βHSD (lane 3), or pCMV-type II h3βHSD (lane 4) were separated on 5–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose, treated with rabbit anti-3βHSD serum, and exposed to ^{125}I-labeled goat antirabbit immunoglobulin G. Autoradiography was performed at −80 C for 2 h.

for the three substrates tested. The same situation is found for the type I protein. In fact, analysis of the kinetic properties of both expressed 3βHSD proteins reveals that the relative enzymatic activity (V_{max}/K_m) of type I is 5.9-, 4.5-, and 2.8-fold higher than that of the type II 3βHSD protein using PREG, DHEA, and DHT as substrate, respectively.

Tissue-Specific Expression of Human Type I and Type II 3βHSD mRNA Species

To determine the tissue-specific expression of human type I and type II 3βHSD genes and the relative abundance of both types of 3βHSD mRNA populations, we performed a ribonuclease protection assay which offers

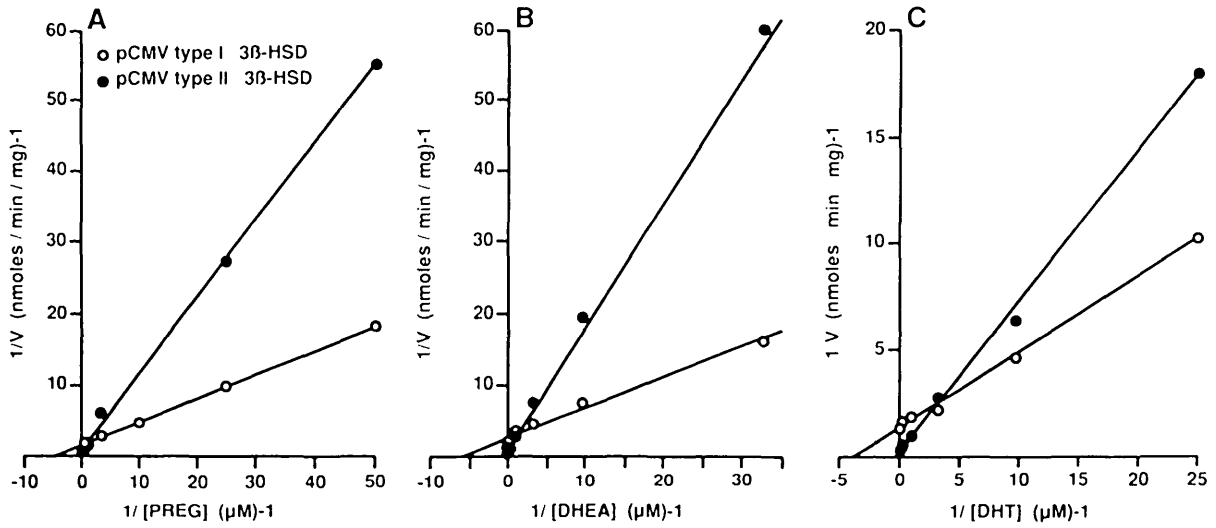

Fig. 4. Lineweaver-Burk Plot Analysis of the Kinetic Properties of the Expressed Human Type I and Type II 3βHSD Proteins Using the Indicated Concentrations of PREG (A), DHEA (B), or DHT (C)

Incubations were performed for 30 min at 37 C, as described in *Materials and Methods*, using 2.1 and 4.8 mg protein from homogenates of cells transfected with pCMV-type I h3βHSD and pCMV-type II h3βHSD plasmids, respectively.

the opportunity to discriminate accurately a few base-pair mismatches occurring after annealing of type I or type II cRNA probes to the expected 3βHSD type I or type II mRNA species. Somewhat surprisingly, using specific cRNA probes, it can be seen in Fig. 5A that the type II 3βHSD mRNA population is the almost exclusive species detectable in the human adrenal gland, testis, and ovary, as revealed by the presence of the expected full-length (220 nucleotides) protected fragment using the specific type II cRNA probe as well as by the occurence of the expected small fragments (~98 and 64 nucleotides) when the type I cRNA probe was used. It was possible, however, to detect the presence of type I 3βHSD protected mRNA fragments after a longer time of exposure of testicular and ovarian mRNA. However, even with overexposed autoradiographs, it was not possible to detect human type I 3βHSD mRNA in total RNA from either human adrenal or human type II 3βHSD mRNA in human placenta (data not shown). It can also be seen in Fig. 5, B and C, that the human type I 3βHSD mRNA population corresponds to the sole detectable species in human placenta and skin under the experimental conditions used. In addition, ribonuclease protection assay analysis of mammary gland RNA showed that the type I 3βHSD mRNA is the predominant species, while some type II 3βHSD mRNA population could be detected on the original autoradiograph (Fig. 5A). Note that this study was performed using total RNA from placenta, adrenal, ovary, and skin, while poly(A)$^+$ RNA from testis and mammary gland tissue was required to detect hybridization signal.

DISCUSSION

The present study describes the molecular cloning and characterization of the structure and expression of cDNA clones isolated from a human adrenal library which encode a functional new type of 3βHSD protein (type II) with 371 amino acids and a molecular mass of 41,921. We also demontrate by transient expression of human type I and type II 3βHSD cDNAs in HeLa cells that type II possesses a significantly lower affinity for PREG, DHEA, and DHT than type I. The present data show, in addition, that the type II 3βHSD transcripts correspond to the almost exclusive mRNA species detected by RNase protection in the adrenal, ovary, and testis, in contrast to the type I 3βHSD mRNA population, which is the almost exclusive mRNA species revealed in placenta as well as skin and is the predominant species in mammary gland tissue. The existence of two 3βHSD mRNA species offers many possibilities for the tissue-specific regulation of the activity of these isoenzymes.

The present elucidation of the structure of human type II 3βHSD, which represents the almost exclusive isoenzyme expressed in the adrenals and gonads, should provide the necessary tools for studies on the characterization of the molecular basis of classical as well as nonclassical 3βHSD deficiencies (Refs. 29–45 and references therein). Such studies should also provide useful information on the heterogeneity of the related disease, while offering the opportunity of an earlier diagnosis and a possible improvement of the therapeutical approaches. The present finding of expression of the same 3βHSD isoenzyme in human adrenals and gonads is in agreement with clinical observations of the impairement of steroidogenesis in these tissues in patients suffering from classical as well as nonclassical 3βHSD deficiencies (30, 34, 35, 37–39, 44). However, the present demonstration that the type II 3βHSD protein possesses equivalent affinity for C-21 (PREG) and C-19 (DHEA and DHT) steroids, as also

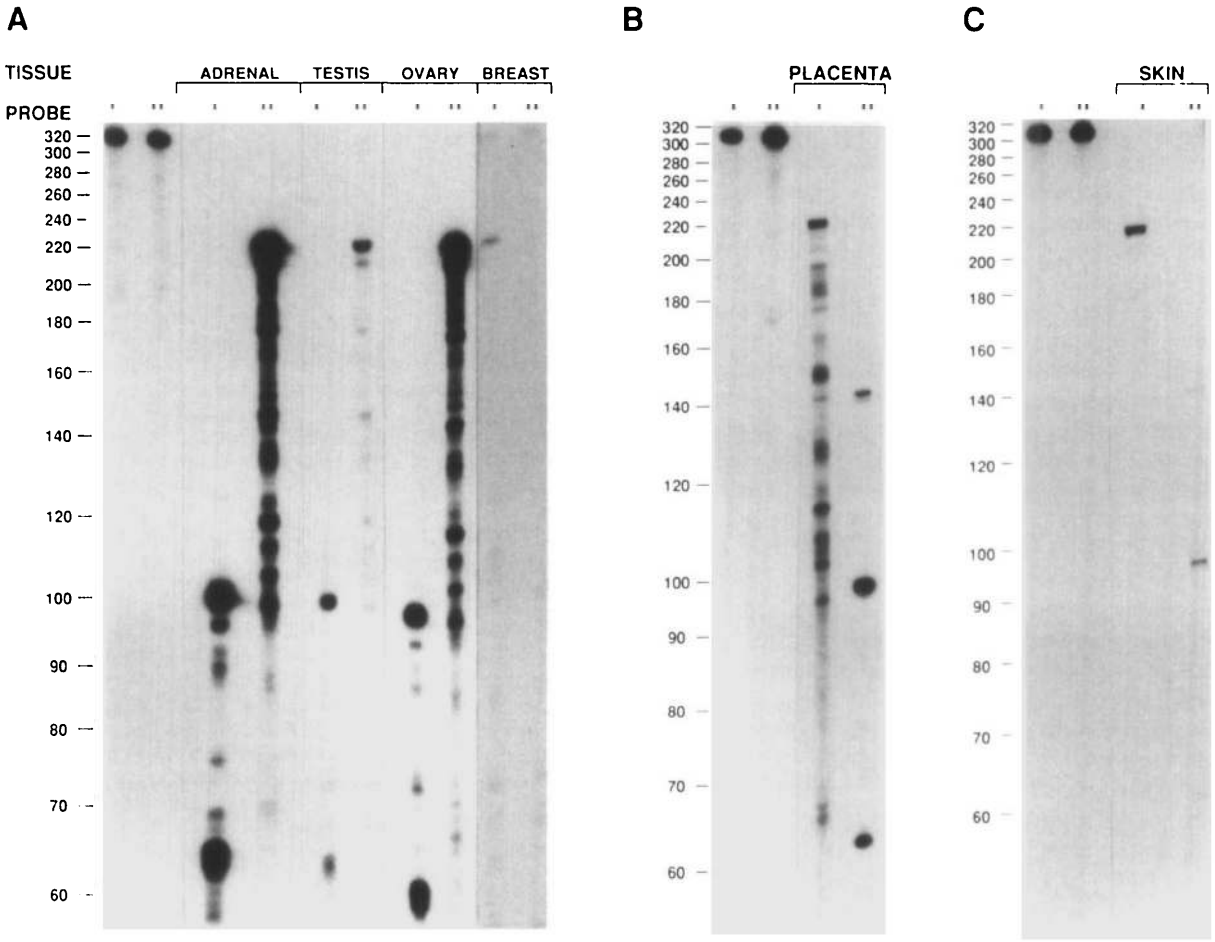

Fig. 5. Ribonuclease Protection Analysis of the Distribution of Human Types I and II 3βHSD mRNAs in Classical Steroidogenic as Well as Peripheral Tissues in the Human

Samples of total RNA from the human adrenal (3 mg), ovary (10 mg), placenta (20 mg), and skin (from the breast region; 20 mg) or poly(A)$^+$ from the testis (10 mg) or breast (normal mammary gland; 10 mg) were hybridized to the type I or type II cRNA probes for 14 h at 37 C and digested with ribonuclease-A and -T1. The protected fragments were resolved on 6% denaturing polyacrylamide-7 M urea sequencing gels, as described in *Materials and Methods*. With either probe (315 nucleotides), the longest protected fragment (220 nucleotides), which included nucleotides 688–909 for human type I 3βHSD and 685–904 for human type II 3βHSD, corresponds to the homologous RNA species protected by the cRNA probe. Protected fragments of 98 and 64 nucleotides correspond to predicted well recognized mismatches for RNase-A (71). Lanes corresponding to the adrenal and placenta were exposed to x-ray film for 14 h, those from testis, breast, and ovary were exposed for 7 days, and the skin sample was exposed for 9 days. Lanes corresponding to adrenal, ovary, and placenta are overexposed in order to further demonstrate the type I- or II-specific mRNA expression in these tissues.

observed for the type I 3βHSD protein, argues against the existence of 3βHSD isoenzymes having a different substrate specificity, as previously suggested to explain the clinical observations in a 3βHSD-deficient patient (33).

The tissue-specific gene expression of the two human types of 3βHSD, especially the specific or predominant expression of type I 3βHSD in the mammary gland and skin, is in agreement with the evidence for intact peripheral (extraadrenal and extragonadal) 3βHSD activity in patients with classical congenital adrenal 3βHSD deficiency as well as in those with the late-onset form of the disease (29–31, 34–39, 44, 45).

The present original data demonstrating type I 3βHSD gene expression in human skin, the largest organ in the body, are in agreement with the detection of 3βHSD activity in epidermal keratinocytes in culture obtained from human breast skin as well as from foreskin of newborn infants and from leg skin. Moreover, 3βHSD/isomerase activity has been detected in sebaceous glands in skin from various anatomical regions (13–17, 19). Furthermore, conversion of DHEA into Δ^4-steroids has been reported in human axillary apocrine glands (17) as well as in plucked terminal hair follicles (51). The physiological importance of 3βHSD gene expression in skin is supported by the observation that DHEA can stimulate sebaceous gland secretion in humans (52), as a result of its conversion into the potent

androgens testosterone and DHT (17), thus indicating the presence of 17β-hydroxysteroid dehydrogenase and 5α-reductase activity in human skin.

The formation of active sex steroids from the inactive adrenal precursors DHEA, DHEA sulfate, and/or Δ^4-androstenedione locally in the same cells where synthesis took place without release in the extracellular space has been recently described as intracrine activity (27, 53). Intracrine activity corresponds to an economical system that requires minimal amounts of hormones in order to exert optimal function in peripheral target tissues, in contrast to the classical endocrine system which requires dilution of hormones in the general circulation before action in distant tissues. While the ovaries and testes are the exclusive sources of androgens and estrogens in the lower mammals, the situation is completely different in higher primates, especially the human, where a large proportion of the active sex steroids is synthezized locally from precursor steroids secreted in large amounts by the adrenals, thus giving autonomy to the target cells and the possibility of adjusting steroid formation as well as metabolism to local requirements (27). The major importance of steroid biosynthesis by peripheral tissues is clearly indicated by the widespread distribution of expression of key steroidogenic enzymes, namely 3βHSD, 17βHSD, 5α-reductase, and/or aromatase, in human peripheral normal and/or cancerous tissues, including, in addition to skin, the prostate, breast, endometrium, adipose tissue, lung, and liver (Refs. 12, 20–24, 27, and 54–60 and references therein).

The novel 3βHSD isoenzyme has been arbitrarily designated type II by reference to our previously characterized human type I 3βHSD from a placental library (1, 12), without any relationship to rat type I and type II, which are both expressed in adrenals and gonads (2). However, computer analysis of the human type II 3βHSD protein, using the method of Klein *et al.* (48), predicts the presence of two membrane-spanning domains between residues 75–91 and residues 287–303, as previously reported in the human type I, macaque, bovine, and rat type I enzymes (2, 8). Moreover, such analysis indicates that introduction of residues 83, 85, 87, and 89 (excluding the first Met) from rat type II into the rat type I enzyme prevents the formation of the predicted membrane-associated α-helical segment between residues 75–91. In fact, we have recently demonstrated by site-directed mutagenesis that the much lower 3βHSD activity of rat type II compared to that of rat type I can be explained by the lack of the membrane-spanning domain 75–91 in rat type II (61). On the basis of these observations, it could be speculated that human type II 3βHSD protein has evolved from a common ancestor to that of the rat type I 3βHSD.

The finding that the human type II 3βHSD differs from the 3βHSD protein deduced from macaque ovarian cDNA by only 13 residues, while 23 differents residues distinguish the former from the human type I 3βHSD protein, could suggest that the human type II evolved from the same duplicated ancestor gene as that of the deduced macaque 3βHSD protein, while the human type I may have evolved from another member of this gene family which has diverged after a duplication that took place earlier in evolution.

MATERIALS AND METHODS

Isolation and Subcloning of cDNA Clones

A λgt22A directionally cloned cDNA library was generated from poly(A)$^+$ RNA isolated as previously described (2) from six human female adrenals using a Superscript Lambda System (Bethesda Research Laboratories, Gaithersburg, MD). Approximately 4×10^5 recombinant phages were screened with 1×10^6 cpm/ml ^{32}P-labeled (62) human placental 3βHSD cDNA, as previously described (2, 8). Nitrocellulose filters (Amersham Corp., Arlington Heights, IL) were then autoradiographed for 5 h at −80 C. Positive clones were obtained at a frequency of 75/10^4 plaques. The second screening was performed with a ^{32}P-labeled *Eco*RI-*Kpn*I cDNA fragment corresponding to the first 318 5′-nucleotides of clone human placental 3βHSD63 in order to optimize the chances of obtaining full-length cDNA clones. The hybridization conditions were the same as those used in the first screening, except that the formamide concentration was reduced from 30% to 20%. The second screening, performed with 400 positive plaques from the first screening, yielded 100 positive plaques which were used to isolate the corresponding phage DNA (63), then subjected to restriction endonuclease digestion and hybridization analyses. The cDNA inserts were excised by *Sal*I and *Not*I double digestion, purified, and subcloned into the Bluescript KS II+ (BSKS) vector (Stratagene, La Jolla, CA).

Sequence Determination and Computer Analyses

Oligonucleotides synthesized with a Biosearch DNA synthesizer, T7 or T3 vector primers, and T7 DNA polymerase (64) were used to sequence both strands of double stranded plasmid DNA using the dideoxy chain-termination method (65) with a T7 sequencing kit (Pharmacia LKB Biotechnologies, Piscataway, NJ). DNA sequences were analyzed with Microgenie (Beckman, Palo Alto, CA) and PC/GENE (IntelliGenetics, Inc, Mountain View, CA) softwares. Putative membrane-spanning protein segments were determined by the programs SOAP (48), RAOARGOS (49), and HELIXEM (50), with PC/GENE software.

Transient Expression of Human Type I and Type II 3βHSD cDNAs

We used the previously described pCMV-3βHSD construction (12), hereafter named pCMV-type I h3βHSD for the human type I 3βHSD cDNA. The full-length cDNA insert corresponding to the human type II (3βHSD; ha3βHSD34) clone was double digested with *Eco*RI/*Not*I, ligated to the unique *Eco*RI site of the pCMV vector (kindly provided by Dr. Michael B. Mathews, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY), and finally blunt-end ligated down-stream from the CMV promoter to produce the recombinant plasmid pCMV-type II h3βHSD. These constructions were sequenced in both orientations, amplified, and subsequently purified by two cesium chloride/ethidium bromide equilibrium centrifugations. HeLa human cervical carcinoma cells (American Type Culture Collection, Rockville, MD) were obtained at their 94–106th passage and grown in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 15 mM HEPES, 100 IU penicillin/ml, and 100 mg streptomycin sulfate/ml. HeLa cells were then plated at an initial density of 10^4 cells/cm^2 in T-75

flasks, and the medium was changed 24 h later, immediately before transfection. The pCMV type I h3βHSD and pCMV type II h3βHSD plasmids were introduced into HeLa cells by the calcium phosphate precipitation procedure, as previously described (66). Mock transfections were carried out with the pCMV alone, while transfection efficiency was monitored by cotransfection of the pCMV-type I or II h3βHSD plasmids with the pXGH5 plasmid (67). The pXGH5 constitutively expresses GH, which is secreted into the culture medium. The media were collected 72 h after transfection and stored at −20 C until assayed for GH release by RIA, while cells were harvested by scraping with a rubber policeman and resuspended at a concentration of 10×10^6 cells/ml buffer containing 50 mM potassium phosphate (pH 7.4), 20% glycerol, and 1 mM EDTA. Cell homogenates were then submitted to three freeze/thaw cycles. To determine 3βHSD activity, cells were incubated for 30 min at 37 C in the presence of ^{3}H-labeled steroid substrates in 50 mM Tris buffer (pH 7.5) containing 1 mM of the appropriate cofactor, namely NAD^+ for DHEA and PREG and NADH for DHT. Kinetic parameters were measured by adding increasing concentrations of DHEA (Steraloids, Wilton, NH), PREG (Steraloids), and DHT (Steraloids) in the presence of 2 nM [1,2,6,7-^{3}H]DHEA (89.8 Ci/mmol; New England Nuclear, Boston, MA), 1 nM [1,2,4,5,6,7,16,17-^{3}H]DHT (185 Ci/mmol; New England Nuclear), and 3 nM [7-^{3}H]PREG (22.6 Ci/mmol; Amersham), respectively. The enzymatic reaction was stopped by chilling the incubation mixture in an ice-water slurry and adding 4 vol ether-acetone (9:1, vol/vol). The organic phase was then evaporated and separated on TLC plates, using a 4:1 mixture of benzene and acetone. Substrates and formed steroids were identified by co-migration on each TLC plate of the nonlabeled steroid, and the area was cut and transferred to scintillation vials containing 0.1 ml ethanol to which 10 ml scintillation fluid were added for measurement of radioactivity in a scintillation spectrometer. Tissue protein content was measured by the method of Bradford (68), using BSA as standard. K_m and V_{max}values were calcualted by the Lineweaver-Burk method.

Immunoblot Analysis

HeLa cell proteins were size-separated on a 5–15% polyacrylamide gel (1.5 mm thick) and transferred to nitrocellulose filters, as previously described (2). Purified human placental 3βHSD (1, 3) was used as a positive control. The blots were treated with wash solution [5% fat-free milk (Carnation) and 0.1% Nonidet P-40 in PBS; 3 × 30 min] and incubated with a 1:2000 dilution of rabbit polyclonal antibodies raised against purified human placental 3βHSD for 18 h at 4 C. The blots were washed three times (30 min/wash) and incubated for an additional 14 h at 4 C in a 1:1000 dilution of ^{125}I-labeled goat antirabbit immunoglobulin G. After three washes in the same solution, autoradiography was performed at −80 C with intensifying screens and XAR-5 films (Eastman Kodak, Rochester, NY).

Messenger RNA Purification

Term placental tissue as well as normal ovaries, mammary gland, and breast skin were removed from otherwise healthy adult individuals during routine surgical procedures. Adrenal tissue was obtained from one normal individual. Testicular tissue was obtained after being removed for the treatment of prostate cancer. Total RNA was extracted in 4 M guanidinium isothiocyanate, 50 mM Tris-HCl (pH 7.5), 10 mM Na_2EDTA (pH 8.0), and 5% β-mercaptoethanol and collected by CsCl density ultracentrifugation in a Beckman SW40 or Ti-45, as previously described (2). The RNA was phenol, phenol-chloroform, and chloroform (twice) extracted; precipitated with ethanol; resuspended in sterile water; and quantitated by absorbance at 260 nM. Poly(A)$^+$ RNA was purified by two cycles of affinity chromatography on oligo(dT)-cellulose columns, as previously described (63).

Ribonuclease Protection Assay

Subclones used to produce cRNA probes were generated by PCR technology (69). The synthetic oligonucleotides used were from two highly conserved regions of human type I 3βHSD and human type II 3βHSD and have the following sequences: 5′-GGGGCGGCCGCGCCTGGGCCCACATTCTGG-3 and 5′GGGGGATCCAGCTCACTATTTCCAGCAG-3′ (underlined sequences are introduced *Not*I and *Bam*HI restriction sites). The amplified region corresponds to nucleotides 688–907 for the human type I 3βHSD and to nucleotides 685–904 for the human type II 3βHSD. Polymerase chain reaction was performed with plasmid vectors containing human type I or II 3βHSD cDNA, and the 220-bp fragment was subcloned in Blue Script KS vector and sequenced in both orientations. The recombinant plasmids were linearized with *Not*I and labeled with [α-^{32}P]UTP (800 Ci/mmol; Amersham), using T3 RNA polymerase of the Transprobe T kit (Pharmacia LKB Biotechnology) to generate type I- and II-specific cRNA probes of 315 nucleotides. After verifying the quality of the probes on a 5% polyacrylamide gel, 3–20 mg human total or poly(A)$^+$ RNA were immediately hybridized with 8×10^4 cpm type I or the type II cRNA probe. Briefly, RNA samples were resuspended in 30 ml hybridization buffer containing 40 mM 1,4-piperazinediethanesulfonic acid (pH 6.4), 0.4 M NaCl, 1 mM EDTA, 80% deionized formamide, and the type I or type II cRNA probe, treated at 85 C for 5 min, and then hybridized at 37 C for 14 h. Thereafter, RNA samples were diluted 10-fold with 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 5 mM EDTA in the presence of 40 mg/ml RNase-A and 250 U/ml RNase-T1 and then incubated at 37 C for 1 h. Samples were treated with a final concentration of 0.2 mg/ml proteinase-K and 0.5% sodium dodecyl sulfate for 15 min at 37 C, extracted, and then purified as previously described (70). RNase-resistant hybrids were analyzed onto a denaturating polyacrylamide urea sequencing gel. Autoradiography was performed at −80 C with two intensifying screens.

Acknowledgments

This work was supported by the Medical Research Council (MRC) of Canada, le Fonds de la Recherche en Santé du Québec (FRSQ), and le Fonds pour la Formation de Chercheurs et l'Aide à la Recherche and Endorecherche (to F.L., J.S., and V.L.T.). The new nucleotide sequence reported in this paper has been submitted to the GeneBank/EMBL Data Bank with accession number M67466.

Received May 10, 1991. Revision received June 4, 1991. Accepted June 4, 1991.

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* Supported by studentships from the FRSQ.
† Recipient of a MRC postdoctoral fellowship.
‡ Recipient of a Scholarship from the MRC of Canada.

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