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Distribution of 3α-Hydroxysteroid Dehydrogenase in Rat Brain and Molecular Cloning of Multiple cDNAs Encoding Structurally Related Proteins in Humans

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3α-Hydroxysteroid dehydrogenase in the brain is responsible for production of neuroactive tetrahydrosteroids that interact with the major inhibitory gamma-aminobutyric acid receptor complexes. Distribution of 3α-hydroxysteroid dehydrogenase in different regions of the brain in rats was evaluated by activity assay and by Western immunoblotting using a monoclonal antibody against liver 3α-hydroxysteroid dehydrogenase as the probe. The olfactory bulb was found to contain the highest level of 3α-hydroxysteroid dehydrogenase activity, while moderate levels of the enzyme activity were found in other regions such as cerebellum, cerebral cortex, hypothalamus and pituitary. Some activity was found in the rest of the brain such as amygdala, brain stem, caudate putamen, cingulate cortex, hippocampus, midbrain, and thalamus. The protein levels of 3α-hydroxysteroid dehydrogenase in different regions of the brain as detected by Western immunoblotting are comparable to those of the enzyme activity. We used the rat cDNA as the probe to screen a human liver λ gt11 cDNA library. A total of four different cDNAs were identified and sequenced. One of the cDNAs is identical to that of the human chlordecone reductase cDNA except that our clone contains a much longer 5′-coding sequence than previously reported. The other three cDNAs display high degrees of sequence homology to those of both rat 3α-hydroxysteroid dehydrogenase and human chlordecone reductase. We are currently investigating the functional relationship between the enzymes encoded by these human cDNAs and 3α-hydroxysteroid dehydrogenase.

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INTRODUCTION

3α-Hydroxysteroid dehydrogenase (3α-HSD) was first identified by its activity in converting dihydrocortisone to 3α-tetrahydrocortisone. It was subsequently found to reduce dihydrotestosterone and dihydroprogesterone to their respective tetrahydro metabolites. In addition to its role in the metabolism of steroid hormones, 3α-HSD has been shown to metabolize a wide range of other endogenous substrates as well as xenobiotics. Stress, as defined by Selye [1], develops in three stages: an initial alarm reaction, characterized by an immediate sympathoadrenomedullary discharge, a subsequent "stage of resistance" characterized by the activation of the hypothalamic–pituitary–adrenocortical axis (HPA), and, finally, by exhaustion. Two hypothalamic peptides which appear to play an important role in stress-related HPA activation are the corticotropin-release factor (CRF) and vasopressin (AVP) [2]. The secretion of both CRF and AVP to the portal system of the anterior pituitary during stress prompts an increase in the release of adrenocorticotropin (ACTH) into circulation [3, 4]. The elevated ACTH levels stimulate the release of glucocorticoids and progestins from the adrenal gland [5]. As these steroids reach the brain, they are quickly converted to tetrahydrosteroids by sequential reactions involving two brain enzymes: steroid 5α-reductase and 3α-hydroxysteroid dehydrogenase. The production of the tetrahydrosteroid metabolites is rapid and robust, but transient, and may last only an hour or so [6]. Recent studies have demonstrated that the steroid hormone metabolites, both tetrahydrodeoxycorticosterone

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(THDOC) and tetrahydroprogesterone (THP), serve as agonist-ligands for the major inhibitory $GABA_A$ receptor complex [7, 8]. Interaction between these tetrahydrosteroids and the $GABA_A$ receptor complex induces an anxiolytic response that may protect the neurons from overstimulation and therefore preserve the homeostasis of the CNS [9].

Because the neuroactive tetrahydro-steroid metabolites induce anxiolytic, analgesic, anaesthetic, and anticonflict behavior in animals and humans, it has been postulated that the lack of these steroid metabolites may cause adverse effects in brain functions [10]. As 3α-hydroxysteroid dehydrogenase is the key brain enzyme involved in the production of neuroactive tetrahydro-steroid hormones, its distribution in brain may have significant implications with regard to the physiology and pathology of brain functions. In order to characterize the enzyme we have previously made several monoclonal antibodies and used these monoclonal antibodies as probes to isolate a full-length cDNA encoding the rat liver 3α-hydroxysteroid dehydrogenase and several human cDNAs encoding enzymes structurally related to the rat 3α-HSD [11–14]. In this communication we studied the distribution of 3α-hydroxysteroid dehydrogenase by activity assays and by Western immunoblotting. We also present results regarding the isolation of several cDNAs encoding structurally related proteins in humans.

MATERIALS AND METHODS

Preparation of cytosolic proteins

Rats (200–250 g) were sacrificed by carbon dioxide euthanasia. Brains were removed and homogenized in a solution containing 0.25 M sucrose and 10 mM Tris–HCl, pH 7.2. The homogenate was centrifuged at 10,000 g for 10 min. Supernatant containing cytosols and microsomes was recovered and subjected to a second centrifugation at 100,000 g for 30 min. The supernatant containing cytosolic proteins was recovered for protein assay as described by Lowry [15].

3α-hydroxysteroid dehydrogenase activity assays

3α-HSD activities were measured by a radioactive procedure [16]. The reaction mixture contained 300 μg of brain cytosolic protein, 20 nmol of dihydrotestosterone, 20 nCi of 14C-5α-dihydrotestosterone in 900 μl of 100 mM sodium phosphate buffer, pH 7.2. The reaction mixture was preincubated at 37°C for 3 min prior to the addition of 100 μl of 10 mM NADPH to start the reaction. The reaction was allowed to continue for 10 min and was stopped by the addition of 5 ml methylene chloride. Dihydrotestosterone and androsterone were extracted into methylene chloride using three successive 5 ml aliquot of the solvent. The solvent was dried under a stream of nitrogen. The dry residue was dissolved in 100 μl of methylene chloride and applied to a TLC plate. The plate was developed using

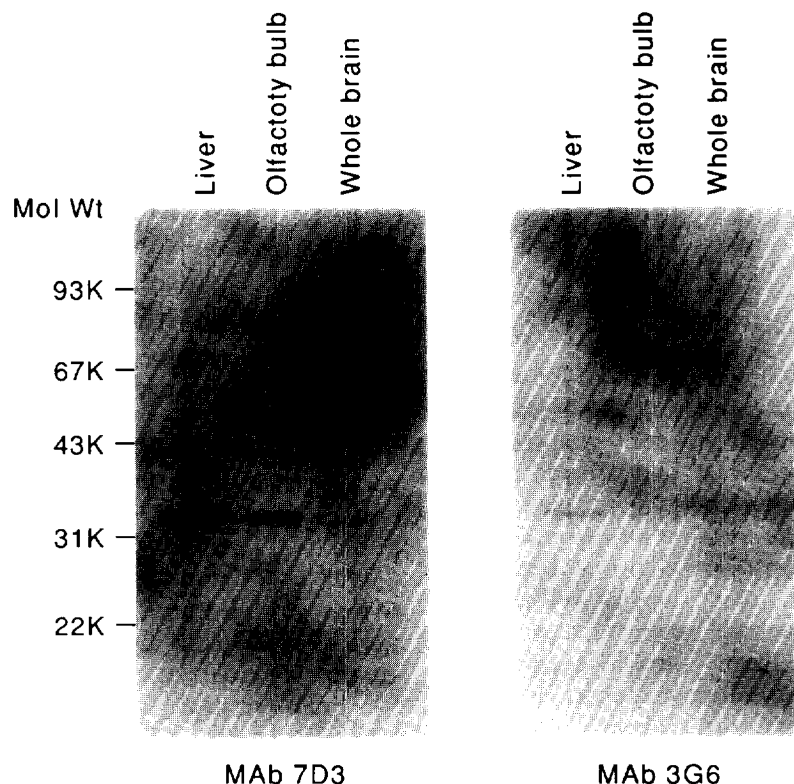

Fig. 1. Immunological cross-reactivity between the liver and the brain 3α-HSD. Two μg of the liver cytosols were used for the immunoblot, whereas 300 μg of the olfactory bulb and the whole brain cytosols were used. The positions of the protein size markers are shown on the left side of the blots.

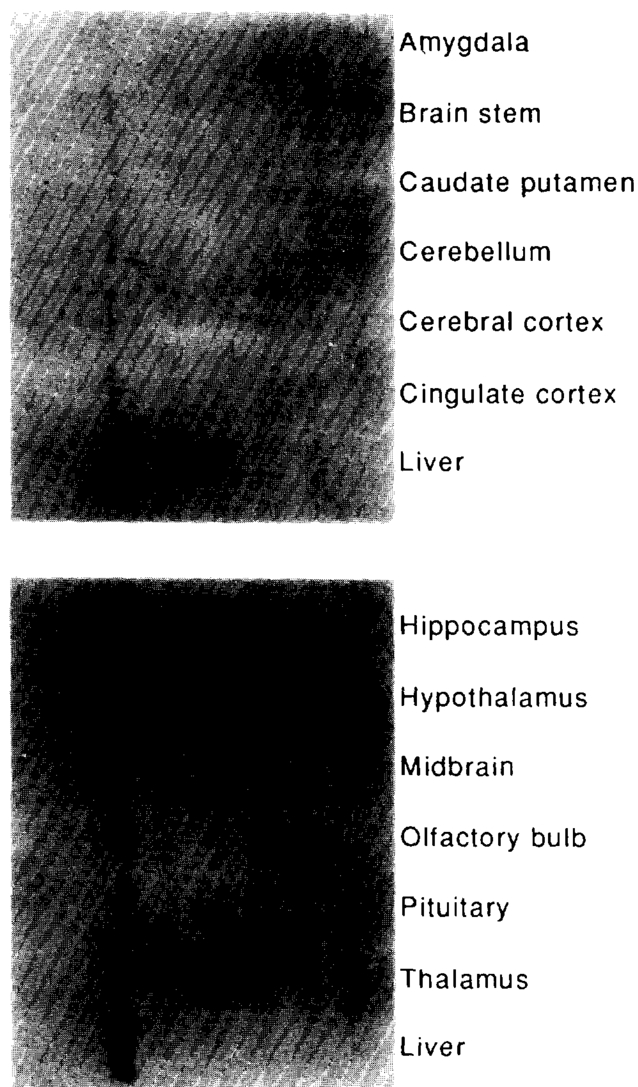

Fig. 2. Western immunoblotting of 3α-HSD in different regions of the brain using monoclonal antibody MAb 7D3. Each lane contains 300 μg of the cytosolic proteins. The positions of the protein size markers are shown on the left side of the blot.

a mixed solvent system of chloroform:ethyl acetate:ethanol/4:1:0.6. Radioactive spots were localized by autoradiography.

Immunoblotting

Proteins were subjected to electrophoresis in a 8% polyacrylamide–0.1% SDS gel and then electrotransferred to a nitrocellulose filter [17]. The filter was sequentially treated with 3% BSA in PBS, hybridoma supernatant and goat anti-mouse IgG-horseradish peroxidase conjugate. The proteins which interacted with monoclonal antibody were visualized by incubating the filter with a solution containing 4-chloronaphthol and hydrogen peroxide.

Library screening using monoclonal antibody as the probe

A human liver cDNA library in the expression vector λ gt11 was screened using monoclonal antibody 3G6 prepared against 3α-HSD [18]. Fusion proteins adsorbed to nitrocellulose filters were detected by sequential incubation with primary monoclonal antibodies, goat anti-mouse IgG-peroxidase, and 4-chloronaphthol plus hydrogen peroxide.

Positive plaques were replated at lower titers and rescreened with monoclonal antibodies until all plaques reacted positively. Phage were isolated by the plate lysate method [18], and DNA was extracted after treatment with phenol/chloroform.

Library screening with cDNA probes under low stringency washing conditions

The library was plated out and lifts were taken on nylon membranes. Phage DNA attached to the membranes was denatured, neutralized, and cross-linked to the membrane by UV light. Plaque hybridization was performed at 55°C in a solution containing 6 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.5), 0.1% SDS, and 5 × Denhardt's solution. Washing was performed at 55°C in solution containing 2 × SSC and 0.1% SDS. Positive plaques were isolated and rescreened after dilution.

Subcloning and sequence analysis

Phage DNA was digested with Eco RI, subjected to electrophoresis in a low-melting agarose gel and isolated by binding to glass beads. Recovered DNA fragments were subcloned into the Eco RI site of pUC19. Chain termination sequencing was performed on denatured supercoiled plasmid DNA using T7 DNA polymerase [19].

RESULTS

Immuno-cross reactivity between the rat liver and brain enzyme

Figure 1 shows the Western immunoblotting of the 3α-HSD in liver as well as in brain using two monoclonal antibodies, MAbs 7D3 and 3G6, made against the liver enzyme. These two monoclonal antibodies have been shown to interact with the liver 3α-HSD at different epitopes [12]. Both MAbs 7D3 and 3G6 recognized a single protein in the liver, the olfactory bulb and the brain. The molecular size of the protein in these different tissues appears to be the same. The intensities of the protein bands detected by MAb 3G6 are much lighter than those detected by MAb 7D3, which is likely due to a lower binding affinity of MAb 3G6.

Distribution of 3α-HSD in different regions of the brain

The regional expression of the 3α-HSD in the brain was first analyzed by Western immunoblotting using MAb 7D3 as the probe. As shown in Fig. 2, expression of 3α-HSD protein was detectable in every part of the brain that was analyzed. Nonetheless, the levels of

3α-HSD protein as reflected by the intensities of the protein bands on the blots vary in different areas of the brain. We have performed at least five blots which showed reproducible band patterns. For example, the protein band in the olfactory bulb was the strongest. Other regions, such as cerebellum, cerebral cortex, hypothalamus, and pituitary, contained less but moderate amounts of the 3α-HSD protein. Much less 3α-HSD was found in amygdala, brain stem, caudate putamen, cingulate cortex, hippocampus, midbrain and thalamus.

A similar pattern of 3α-HSD activities was also seen in various regions of the brain. The brain tissues from four animals were used for the activity assays. As shown in Fig. 3, the olfactory bulb also contains the highest level of 3α-HSD activity. Moderate levels of 3α-HSD activity were also found in cerebellum, cerebral cortex, hypothalamus, and pituitary. This suggests that the differences in activities of 3α-HSD in various regions of the brain are due to different levels of the 3α-HSD enzyme.

Isolation of multiple human cDNA clones

As reported previously, a monoclonal antibody (MAb 3G6) raised against rat liver 3α-HSD also cross-reacted with a human protein of molecular weight similar to that of the rat enzyme [12]. Therefore, in our initial screening of the human cDNA library we used this monoclonal antibody as the probe. From approx. 1 million phages we isolated 50 positive clones. Sequencing and comparison of the cDNA inserts in these positive clones indicated that we had isolated four distinct cDNAs. Subsequent use of these cDNAs as probes to re-screen the cDNA library produced several longer clones that contained three full-length and one near full-length cDNAs.

Sequence comparison reveals a high degree of similarity (approx. 70%) between the human cDNAs and the rat 3α-HSD. Search of the Genebank revealed that one of the human cDNAs (HAKRa) isolated in this study had the same DNA sequence as human chlordecone reductase [20] except the HAKRa contains a much longer 5′-coding sequence than that of human chlordecone reductase. The start codons that were assigned are located within the potential eucaryotic translation initiation consensus sequences [21]. The same start codon was also shown to be used by several other rat enzymes belonging to the aldo-keto reductase superfamily [13]. Because these human cDNAs resemble both 3α-HSD and human chlordecone reductase, and both of them belong to the rat aldo-keto reductase superfamily, we tentatively named them human aldo-keto reductase (HAKR). In contrast to the 3α-HSD cDNA, the human cDNAs contain much shorter 3′-non-coding sequences. In two of the cDNAs, HAKRa and HAKRb, a polyadenylation signal (AATAAA) was found, and in HAKRd a polyadenylation tail was located.

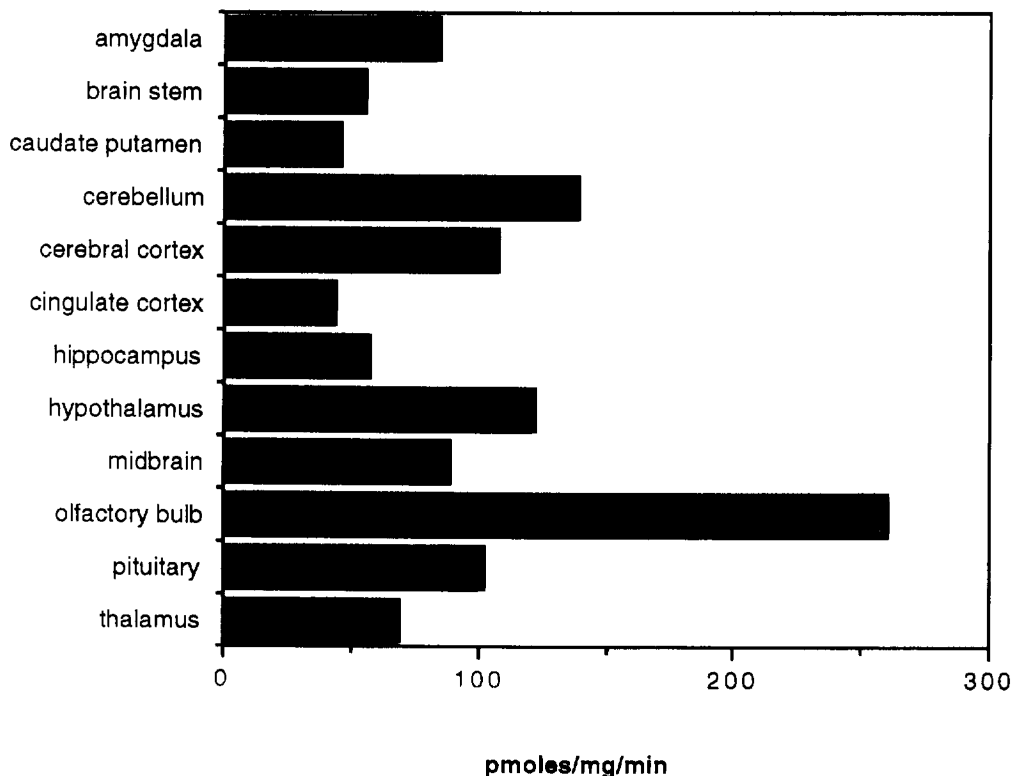

Fig. 3. 3α-HSD activities in various regions of the rat brain. Assay of 3α-HSD activity is described in the Materials and Methods.

```
         1                                                                   65
3α-HSD   MDSISLRDALNDGNFIPVLGFGTTVPEKVAKDEFIKATKIAIDNGFFHFDSAYLYELEEDVGQSI
HAKRa    M PKYQ VE     H M        YA PE PRNRAVEV  L  EA  R I       NN  Q  LA
HAKRb    M PKHKCVK     H M        YA PE PRSKALEV  L  EA  R I    H  NN  Q  LA
HAKRc      AKYQCVK     H M        YA AE PKSKALEA  L  EA  R I    H  NN  Q  LA
HAKRd    M SKYQCVK     H M        YA AE PKSKALEAV L  EA  H I    HV NN  Q  LA

         66                                                                 130
3α-HSD   RSKIEGKSGKREDIFYTSKLWSTFHRPELVRPCLEKTLEKHQQHYVDLYIIHFPMALQPGDIFFP
HAKRa        ADG V               C  FQ QM Q A   SS K L LD      LL        K  ETPL
HAKRb        ADG VE                 HR EL R A   NS K A LD      L  S   S K  EELS
HAKRc        ADG V               CNSHR ELDR A   RS KNL LD      LI F VSVK  EEVI
HAKRd        ADG V                NSHR EL   A ERS KNL LD      LI F VSVK  EEVI

         131                                                                195
3α-HSD   RDEHGKLLFETVDICDTWEAMEKCKDAGLASSIGGSNFNCRRLERILNKPGLKYKPVCNQVECHL
HAKRa    K  N  VI D   LSA   V          K   V      Q  M                     P
HAKRb    T  N  VI DI  L T              K   V    R Q  I                     P
HAKRc    K  N  IL D   L A     V     E  K   V    RST  M        QV           P
HAKRd    K  N  IL D   L A              K   V    H L  M   E      E          P

         196                                                                260
3α-HSD   YLNQSKMLDYCKSKDIILVSYCTLGSSRGKTWVDQKSPVLLDDPVLCAIAKKYKQTPALVALRYQ
HAKRa          L  F      V  AHSA  TQ H L    PN      E      L   H R    I
HAKRb     F R  L  F      V  A SA   Q D R    PN      E      L   H R    I
HAKRc     F  R L  F      V  A SA   L EEP    PN      E      L   H R    I
HAKRd     F  R L  F      V  A SA   H EEP    PN      E      L   H R    I

         261                                                             322
3α-HSD   LQSGVEPLIRSFKPKRIKEPTQVFEFQLASEDMKALDGLNRNFRYNNAKSFHDHPNHPFTDE.
HAKRa      R  VV AK YNEQ  R NI        T       V        Y  VVMDFLM   DY  S  Y.
HAKRb      R  VV AK YNEQ  RQNV        TA      AI   D  LH F SD  AS   Y YS  Y.
HAKRc      R  VV AK YNEQ  RQNV        T   E   AI      V  LTLDI AGP  Y IS  Y.
HAKRd      R  VV AK YNEQ  RQNV        T   E   AI      V  LTLDI AGP  Y IS  Y.
```

Fig. 4. Comparison of deduced amino acid sequences of 3α-HSD, HARKa, HARKb, HARKc, and HARKd. Only differences from the 3α-HSD sequence are displayed. Boldface one-letter codes denote the beginning of the sequences derived from the cDNA sequences. The amino acid residues are numbered in boldface letters in the amino terminus to craboxyl terminus direction.

Figure 4 shows the alignment of the deduced sequences of the rat 3α-HSD and the human proteins. The 3α-HSD cDNA encodes a protein of 322 amino acids, whereas the open reading frames of the human cDNAs encode proteins of 323 amino acids. A high degree of sequence homology (about 65%) was found between the rat 3α-HSD and each of the human proteins. A much greater degree of homology (>85%) was found among the human proteins. Of the four human proteins, HAKRc and HAKRd showed the highest degree of homology (95%).

DISCUSSION

Over the last 50 years since Selye demonstrated that 3α-tetrahydrosteroids induced anxiolytic, analgesic and anticonvulsive effects in animals [10], the mechanism by which these neuroactive steroids interact with neurons at molecular levels has been well delineated. As demonstrated by competitive ligand binding, these 3α-tetrahydrosteroids interact with the major inhibitory $GABA_A$ receptor complex near the binding site for barbiturates [22]. Upon binding to the $GABA_A$ receptor, tetrahydrosteroids induce a prolonged opening of the chloride channel leading to the transport of chloride ion and hyperpolarization of the neurons. As a result of the modulating function of 3α-tetrahydrosteroids on $GABA_A$ receptor complex, it has been speculated that some psychiatric disorders, such as depression, premenstrual syndrome, and anxiety, may be due to abnormality in the metabolism of neuroactive steroid metabolites [17, 18]. Since 3α-hydroxysteroid dehydrogenase is one of the key enzymes responsible for the production of the neuroactive tetrahydrosteroids, its expression and regulation may have direct implication to various functions of the tetrahydrosteroids in the brain. It is rather interesting that olfactory bulb contains a much higher concentration of the 3α-HSD than any other area of the rat brain. Since $GABA_A$ receptors in the olfactory bulb is involved in the odor processing, it is possible that the neuroactive steroid metabolites may play a role in the odor coding pathway [23].

Deprivation of steroid hormones by adrenalectomy did not affect the levels of 3α-HSD in the brain; neither did administration of various steroid hormones affect

the enzyme activity in the brain. No sex difference was found in the brain 3α-HSD activity. In contrast, the liver 3α-HSD has been shown to exhibit sexual dimorphism. These results suggest that the regulation of brain enzyme, which is steroid hormone-independent, is different from that of the liver enzyme.

Several groups of researchers have suggested the existence of multiple 3α-HSD in the liver [24, 25]. Penning has suggested that the brain enzyme is much less active than the liver enzyme [26]. However, in this study we found that the liver and the brain enzyme are immunologically identical. Using monoclonal antibodies made against the liver enzyme to screen a λ gt11 cDNA library derived from the rat brain we have isolated a cDNA clone that showed identical sequence to that of the liver enzyme (data not shown). Our studies, therefore, suggest the enzymes expressed in the liver and the brain are identical.

Multiple human 3α-HSD have been purified from the cytosolic fraction of human liver. Since a monoclonal antibody previously raised against rat 3α-HSD in our laboratory recognizes a human protein with a molecular weight similar to the rat enzyme, we used it as the probe to isolate cDNAs in a human liver library. As shown in this study, human liver expresses multiple forms of enzymes structurally related to rat 3α-HSD. Our recent studies suggest that two of the proteins encoded by the cDNAs we isolated have 3α-HSD activity.

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