Cloning of mouse 17β-hydroxysteroid dehydrogenase type 2, and analysing expression of the mRNAs for types 1, 2, 3, 4 and 5 in mouse embryos and adult tissues

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17β-Hydroxysteroid dehydrogenases (17HSDs) are responsible for the conversion of low-activity sex steroids to more potent forms, and vice versa. 17HSD activity is essential for the biosynthesis of sex steroids in the gonads, and it is also one of the key factors regulating the availability of active ligands for sex-steroid receptors in various extragonadal tissues. In this study, we have characterized mouse 17HSD type 2 cDNA, and analysed the relative expression of 17HSD types 1, 2, 3, 4 and 5 mRNAs in mouse embryos and adult male and female tissues. The cDNA characterized has a open reading frame of 1146 bp, and encodes a protein of 381 amino acids with a predicted molecular mass of 41837 kDa. Northern-blot analysis of adult mouse tissues revealed that, of the different 17HSDs, the type 2 enzyme is most abundantly expressed. High expression of the enzyme, which oxidizes both testosterone and oestradiol, in several large organs of both sexes indicates that it is the isoform having the most substantial role in the metabolism of sex steroids. Interestingly, four of the five 17HSD enzymes were also detected by Northern blots of whole mouse embryos, and each of the enzymes showed a unique pattern of expression. The oestradiol-synthesizing type 1 enzyme predominates in early days of development embryonic day 7, but after that the oxidative type 2 enzyme becomes the predominant form of all 17HSDs. The data therefore suggest that there is transient oestradiol production in the early days of embryonic development, after which inactivation of sex steroids predominates in the fetus and placenta.

INTRODUCTION

The physiological activities of oestrogens and androgens are regulated by redox reactions at position C-17. The enzymes mediating such redox reactions, 17β-hydroxysteroid dehydrogenases (17HSDs), are responsible for the conversion of low-activity sex steroids, such as 3-hydroxy-1,3,5(10)-oestratrien-17-one (oestrone), 4-androstene-3,17-dione (androstenedione) and 5α-androstane-3,17-dione (5α-androstanedione), to their more potent forms, 1,3,5(10)-oestratriene-3,17β-diol (oestradiol), 4-androsten-17β-ol-3-one (testosterone) and 5α-androstan-17β-ol-3-one (5α-dihydrotestosterone), and vice versa. 17HSD activity is essential for the biosynthesis of sex steroids in the gonads, but it is also present in various extragonadal tissues of several species, including certain steroid-hormone target tissues ([1,2] and refs. therein). In addition, high 17HSD activity is present in the liver [2,3], being predominantly responsible for inactivation of sex steroids from the blood circulation. The presence of 17HSD activity is, therefore, one of the key factors regulating the availability of highly active ligands for sex-steroid receptors at target cells. The primary structures of five distinct mammalian 17HSD enzymes have been reported [4–8], suggesting that a multitude of enzymes acting at C-17 are involved in the control of steroid-hormone concentrations available for receptor binding.

Current data indicate that 17HSD type 1 and type 3 enzymes, predominantly catalysing the reductive reaction [4,6,9,10], are principally involved in gonadal oestradiol and testosterone biosynthesis respectively ([2] and refs. therein; [6,11,12]). In contrast, 17HSD type 2 and type 4 enzymes preferentially catalyse the opposite reaction, thereby inactivating the sex steroids [5,7,10,13]. A mouse 17HSD type 5 enzyme has also been recently characterized [8].

All the 17HSD enzymes catalyse the 17β-oxidation/reduction of sex steroids, but increasing evidence suggests that, in addition to 17HSD activity, other functions might be associated with some of the enzymes. For example, (1) 17HSD type 2 has been shown to be highly expressed in the liver and small intestine [10,14], and it has significant similarities to 11β-hydroxysteroid dehydrogenase type 2, β-hydroxybutyrate dehydrogenase and retinol dehydrogenase [15]. (2) In addition to being involved in the inactivation of oestradiol to oestrone, 17HSD type 4 has been shown to catalyse hydratase and dehydrogenase reactions involved in the D-β-oxidation of fatty acids [16–18]. (3) 17HSD type 5 belongs to a family of aldoketoreductases and shows great similarity to several other enzymes involved in the metabolism of steroids (e.g. 3α-hydroxysteroid dehydrogenase and 20α-hydroxysteroid dehydrogenase), prostaglandins, alcohols and xenobiotics [8]. Therefore detailed analysis of tissue distribution, developmental expression and substrate specificities of the 17HSD enzymes forms the background for understanding the metabolic pathways and biological processes in which the enzymes play key roles.

Recently, we reported the primary structure of mouse 17HSD type 1 [19], and, in the present study, we describe the cloning and characterization of mouse 17HSD type 2 cDNA. In addition, the

Abbreviations used: androstenedione, 4-androstene-3,17-dione; 5α-androstanedione, 5α-androstane-3,17-dione; 5α-dihydrotestosterone, 5α-androstan-17β-ol-3-one; oestradiol, 1,3,5(10)-oestratriene-3,17β-diol; oestrone, 3-hydroxy-1,3,5(10)-oestratrien-17-one; testosterone, 4-androsten-17β-ol-3-one; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 17HSD, 17β-hydroxysteroid dehydrogenase; poly(A)$^+$ RNA, polyadenylated RNA; RT-PCR, reverse transcriptase-PCR; E, embryonic.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Y09517.

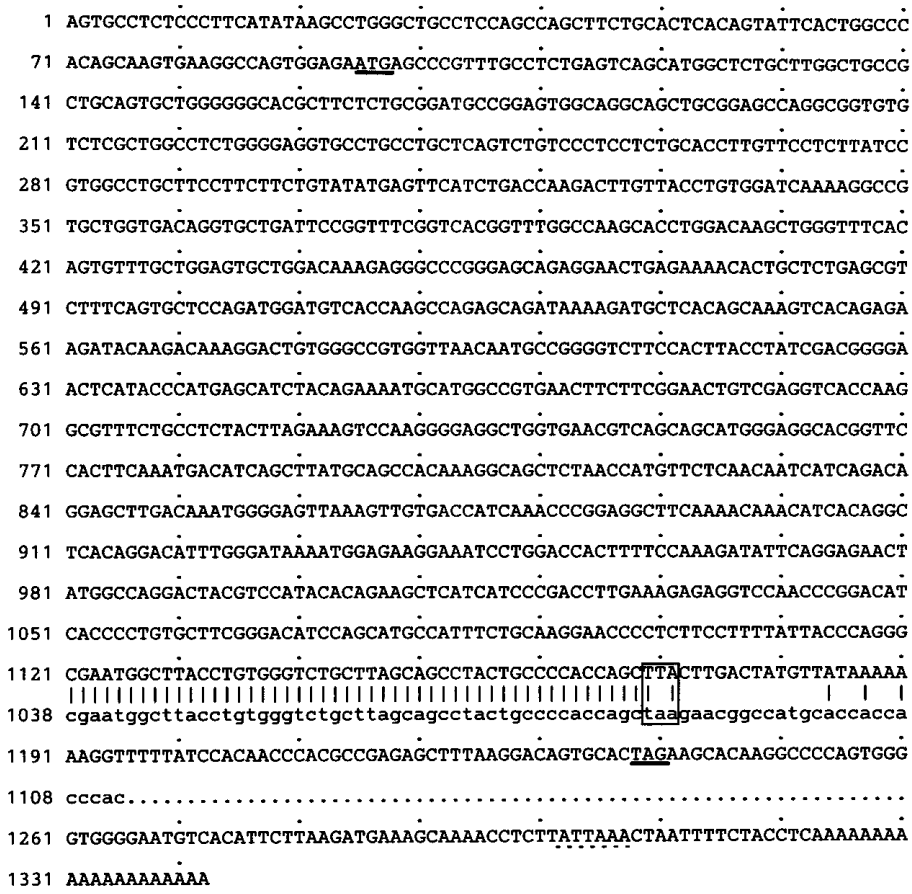

Figure 1 Nucleotide sequence of mouse 17HSD type 2 cDNA

The mouse 17HSD type 2 cDNA characterized in the present study is indicated by capital letters, and the sequence obtained by Stoffel and Weiss [25a] is shown in lower-case letters. Translation initiation and stop codons are underlined. The mutated stop codon (TAA) of the truncated cDNA is shown by a box, and a putative poly(A)$^+$ adenylation signal is marked by a dashed underline.

cDNAs for mouse 17HSD types 4 and 5, and specific PCR primers for mouse 17HSD type 3, have been recently characterized [8,20,21]. Using these tools we have now compared the levels of mRNA expression of 17HSD types 1, 2, 3, 4 and 5 in the mouse embryo and several tissues of adult male and female mice.

EXPERIMENTAL

Materials

[2,4,6,7-^{3}H]Oestradiol (75 Ci/mmol), [2,4,6,7-^{3}H]oestrone (99 Ci/mmol), [1,2,6,7-^{3}H]testosterone (100 Ci/mmol), [1,2,6,7-^{3}H]androstenedione, [^{32}P]dCTP (3000 Ci/mmol) and [^{35}S]dATP ($>$ 1000 Ci/mmol) were purchased from Amersham International (Amersham, Bucks, U.K.). Unlabelled oestradiol and oestrone were obtained from Steraloids (Wilton, NH, U.S.A.), and cell-culture media and supplements were obtained from Gibco (Grand Island, NY, U.S.A.). Reagents not mentioned in the text were purchased either from Sigma Chemical (St. Louis, MO, U.S.A.) or Merck (Darmstadt, Germany) and were of the highest purity grade available.

Construction of liver cDNA library and cloning of mouse 17HSD type 2

Mouse liver poly(A)$^+$ RNA (5 μg) was prepared similarly to the way described for Northern-blot analysis (see below). An oligo(dT) and random-primed cDNA library was constructed from the mRNA using a cDNA synthesis kit, following the instructions of the manufacturer (Stratagene, La Jolla, CA, U.S.A.). The synthesized cDNAs with *Eco*RI and *Xho*I overhangs were subcloned into ZAP Express™ vector (Stratagene), and the constructs were packaged into phage particles using Gigapack II Packaging extract (Stratagene). An unamplified library was screened in *Escherichia coli* XL-1 Blue MRF ' cells (Stratagene) using a [^{32}P]-labelled rat 17HSD type 2 fragment as a probe [22]. From multiple positive clones obtained, six positive clones were purified using a QIAGEN™ Lambda purification kit (Qiagen, Hilden, Germany), and after this they were cloned in Bluescript (KS)$^+$-plasmid (Stratagene). Plasmids were amplified in *E. coli* JM109 cells and thereafter were purified using a QIAGEN™ plasmid purification kit (Qiagen). The mouse 17HSD type 2 nucleotide sequence was determined from three clones using an automatic DNA sequencer (ABI Prism 377 DNA Sequencer; Perkin–Elmer, Foster City, CA, U.S.A.). The nucleotide sequences of both strands at the 3′-ends of the cDNA were also determined at least twice by the dideoxy termination method described by Sanger et al. [23], using a ^{T7}sequencing kit (Pharmacia Biotech, Uppsala, Sweden). Sequence analyses were carried out by using a GCG sequence analysis software package (Genetics Computer Group, Madison, WI, U.S.A.) and Geneskipper software (version 1.2; EMBL, Heidelberg,

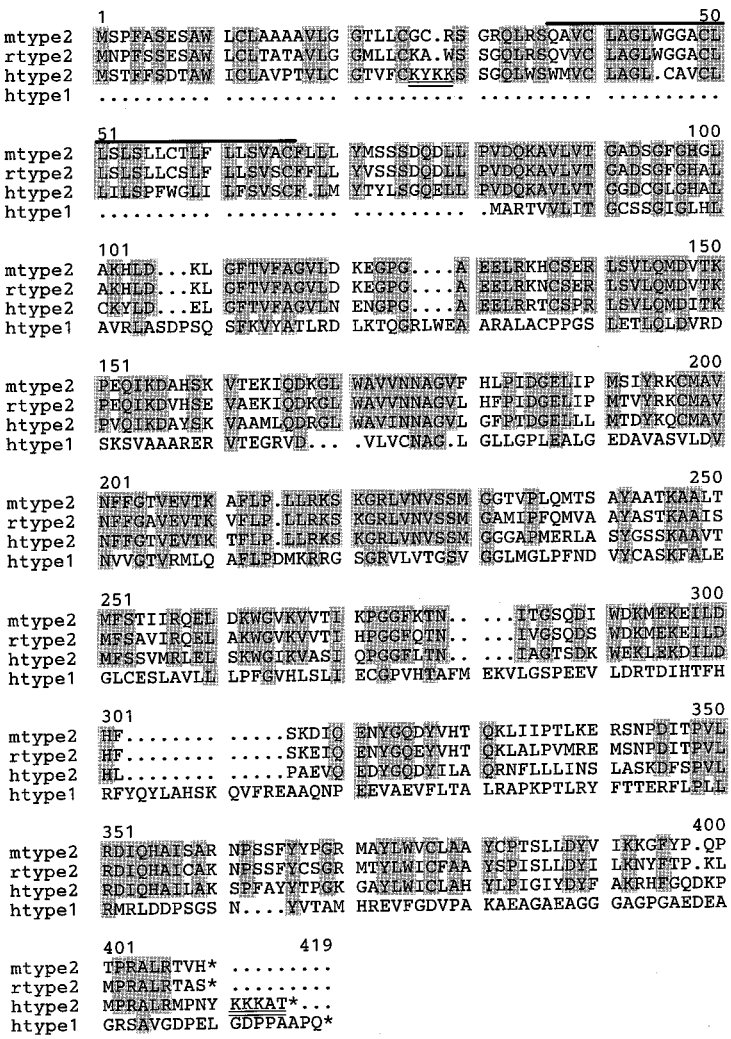

Figure 2 Comparison of amino acid sequences of mouse, rat and human 17HSD type 2 and human type 1 enzymes

The putative transmembrane region of the type 2 enzymes is indicated by a line above the sequence. The two lysine-rich amino acid motifs present only in human type 2 enzyme are indicated by double underlining. The conserved amino acids in all type 2 enzymes are indicated by shaded boxes, as are the conserved amino acids between type 2 enzymes and human type 1 enzyme.

Germany). Activity of the cloned enzyme was measured in transiently transfected 293-cells using a pcDNA3-mouse 17HSD type 2 expression plasmid as previously described [10,19].

Preparation of mouse 17HSD type 3 probe

Total RNA from mouse testes was similarly prepared as described for Northern-blot analysis (see below). Using mouse testis RNA as a template, reverse transcriptase-PCR (RT-PCR) was carried out with the primers previously described for mouse 17HSD type 3 [21]. The RT-PCR product was then inserted in pCRII vector (Invitrogen, San Diego, CA, U.S.A.), amplified and purified, and the sequence of the cDNA was confirmed using an automatic DNA sequencer (ABI Prism 377 DNA Sequencer). The 367 bp cDNA obtained corresponds to nucleotides 365–731 of human type 3 cDNA, and the identity between the cDNAs is 82%.

Northern-blot analysis of the 17HSDs

Total RNA and poly(A)$^+$ RNA were extracted from multiple adult female and male Balb/c mouse tissues using standard methods [24,25]. Briefly, male and female mouse tissues were homogenized in 4 M guanidine thiocyanate with 25 mM sodium acetate in an Ultra-Turrax homogenizer (IKA Labortechnik, Staufen, Germany). The homogenates were centrifuged (21 h, 179000 ***g***) through a cushion of 5.7 M CsCl, followed by ethanol precipitation. For each sample, poly(A)$^+$ RNA isolated by oligo(dT) cellulose (Boehringer Mannheim, Mannheim, Germany) chromatography was resolved by 1% gel electrophoresis and transferred to Hybond N nylon membrane (Amersham). Furthermore, a membrane containing 2 μg of poly(A)$^+$ RNA (Clontech, Palo Alto, CA, U.S.A.) from whole mouse embryos at different embryonic (E) stages (7, 11, 15 and 17 days) was used.

The membranes were prehybridized for 2 h at 42 °C in 5 × SSPE [1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate (pH 7.4) and 0.1 mM EDTA] containing 50% (v/v) formamide, 0.1% (w/v) BSA, 0.1% (w/v) Ficoll, 0.1% (w/v) poly(vinylpyrrolidone), 0.5% (w/v) SDS and 20 mg of salmon sperm DNA/ml. The membranes were then hybridized overnight at 42 °C using the following mouse 17HSD cDNA fragments as probes: 17HSD type 1: nucleotides 1–1059 [19], 17HSD type 2:

nucleotides 584–1320 (present study), 17HSD type 3: corresponding to nucleotides 365–731 in human enzyme [6], 17HSD type 4: nucleotides 11–1874 [20] and 17HSD type 5: nucleotides 1–1197 [8]. After hybridization and washing, the membranes were exposed to Kodak XAR films (Kodak, Rochester, NY, U.S.A.) for 1 and 4 days. The amount of mRNA applied to the gel was controlled by hybridizing the membranes with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin cDNA probes. Densitometric scanning of films derived from multiple exposure times was then performed with a densitometer (Molecular Dynamics 300 A Computing Densitometer; Molecular Dynamics, Sunnyvale, CA, U.S.A.), and the intensities of the mRNA signals of different 17HSD enzymes were analysed relative to GADPH or β-actin.

RESULTS

The cDNA coding for the mouse 17HSD type 2 enzyme was cloned from a Balb/c mouse liver library. The sequence, which was determined from three different clones, is shown in Figure 1. The sequence is 1342 bp in length and has an open reading frame of 1146 bp. The cDNA encodes a protein of 381 amino acids with a predicted molecular mass of 41837 kDa. The putative polyadenylation signal (ATTAA) was located at position 1302 in the 3′-non-coding region of the cDNA (Figure 1). A putative nucleotide sequence of the mouse 17HSD type 2 was submitted to nucleotide-sequence databases at the time that the present work was carried out [25a]. Between nucleotides 84 and 1169 of the present cDNA, the two sequences are identical, with the exception of a change in nucleotides 201 (A → G, Gln^{47} → Arg) and 203 (G → C, Ala^{48} → Pro). However, in the cDNA characterized in the present report the stop codon is located at nucleotide 1238, whereas the cDNA sequence reported by Stoffel and Weiss [25a] has a premature stop codon (TAA) at position 1170 (Figure 1). Furthermore, the 3′ region of the mouse 17HSD type 2 cDNA sequenced in the present work is closer to that recently found for the rat enzyme, suggesting that the previously reported mouse 17HSD type 2 cDNA sequence does not encode a full-length enzyme.

The amino acid sequence of the mouse 17HSD type 2 obtained in the present work shares 89 % similarity and 82 % identity with that obtained from rat cDNA. The similarity between human and mouse enzymes is 76 %, and the identity is 61 % (Figure 2). The hydrophilic lysine-rich motif (KKKAT) present in the C-

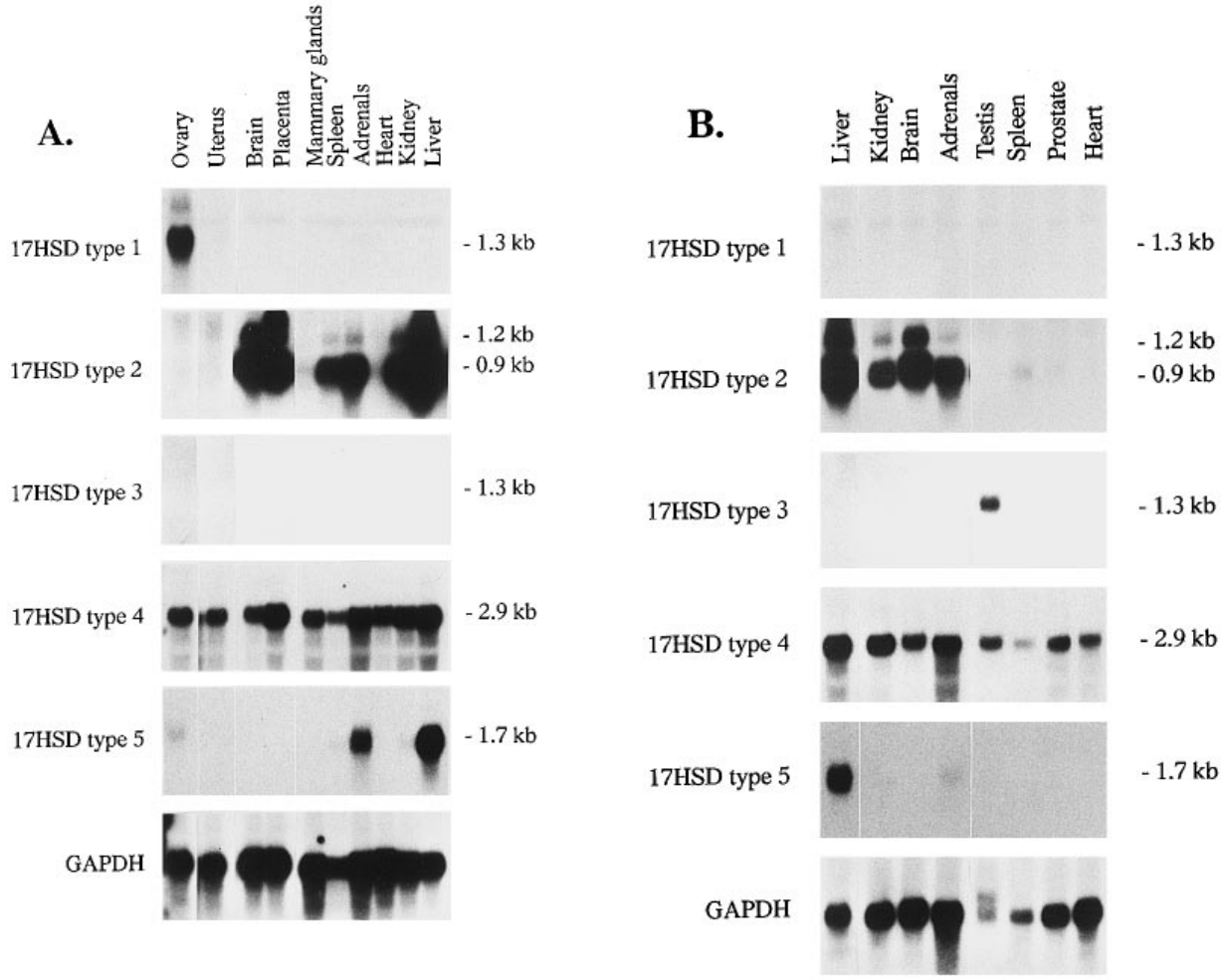

Figure 3 Northern-blot analysis of 17HSD types 1–5 in adult female (A) and male (B) tissues

Poly(A)$^+$ RNA (10 μg) from the indicated tissues was used to analyse mRNA expression of 17HSDs by Northern blot. The amount of mRNA applied was controlled by hybridizing the membranes with GAPDH cDNA.

Table 1 Relative expression of mRNAs of 17HSD types 1–5 in tissues of adult male (M) and female (F) mice

Intensities of the hybridization signals for 17HSD mRNAs obtained by Northern-blot analysis were quantified by densitometric scanning and were normalized with signals obtained for GAPDH.

		17HSD mRNAs				
Tissue	Gender	Type 1	Type 2	Type 3	Type 4	Type 5
Liver	M	−	+ + + + +	−	+ +	+
	F	−	+ + + + +	−	+ +	+ +
Kidney	M	−	+ +	−	+	+
	F	−	+ +	−	+	+
Heart	M	−	−	−	+	−
	F	−	−	−	+	−
Adrenal	M	−	+ +	−	+	+
	F	+	+ +	−	+	+
Spleen	M	−	+	−	+	−
	F	−	+ +	−	+	+
Brain	M	−	+ +	−	+	−
	F	−	+ +	−	+	−
Testis		−	−	+ +	+ +	+
Prostate		−	+	−	+	−
Ovary		+ +	−	−	+	−
Placenta		−	+ + +	−	+	−
Breast		−	+	−	+	−
Uterus		+	+	−	+	−

terminus of the human enzyme is not present in mouse or rat 17HSD type 2, and the same is true of the lysine cluster (KYKK) present proximal to the putative transmembrane region of the human enzyme. There is, however, a short conserved amino acid sequence (PRALR) in the C-terminus of all three enzymes. Furthermore, eight highly conserved amino acids of short-chain dehydrogenase/reductase family members are also found in the mouse type 2 enzyme. Similarly to the human and rat enzymes, mouse 17HSD type 2 also contains a highly hydrophobic region of 30 non-polar amino acids (Gln^{36}–Cys^{65}), a putative transmembrane region. The enzymic activity of mouse 17HSD type 2 was then determined in embryonic kidney 293 cells expressing the enzyme under the cytomegalovirus promoter. The enzyme predominantly catalyses the oxidative reaction inactivating the 17β-hydroxy forms of the sex steroids (testosterone and oestradiol) into their less potent 17-oxo forms (results not shown).

The tissue distribution of mouse 17HSD types 1–5 was next determined by Northern-blot analysis (Figure 3), using various female and male tissues, and the intensities of the 17HSD mRNA signals were normalized to GAPDH (Table 1). Similarly to the results previously shown [19], the 1.3 kb mRNA for mouse 17HSD type 1 is predominantly expressed in the ovary, but very low levels of type 1 mRNA were also found in the uterus and adrenals of female mice. The two 17HSD type 2 mRNAs were highly expressed in placenta, liver, kidney and, surprisingly, also in the brain in both sexes. Lower levels of mouse type 2 mRNAs were also detected in the adrenals and spleen of both sexes, and in uterus, mammary gland and prostate. The relative expression of the 0.9 kb and 1.2 kb mRNAs was constant at 10:1 in the tissues analysed, except in liver, brain and placenta, where the ratio was 2:1. Identically to the results previously found for the human enzyme [6], mouse 17HSD type 3 mRNA (1.3 kb) was significantly expressed only in the testes. 17HSD type 4 mRNA (2.9 kb) was moderately and constitutively expressed in every male and female tissue analysed. The only exceptions were the testes, liver and kidney of both sexes, where the expression of the enzyme was significantly higher than in other tissues analysed. 17HSD type 5 mRNA (1.7 kb) was found to be expressed only in the testis, and in liver, kidney and adrenals of both male and female animals. However, a faint signal of an mRNA longer than that observed in other tissues was also detected in the ovary.

We further analysed the expression of 17HSD enzymes in mouse embryos at different stages of embryonic development. Using Northern-blot analysis of whole mouse embryos, all the 17HSD enzymes, except type 3, were detected, and the enzymes showed differential expression patterns during fetal life (Figure 4). 17HSD type 1 mRNA was expressed only on fetal day E7, after which the signal was undetectable. 17HSD type 2 was slightly expressed at day E7, after which it gradually increased, and the greatest expression was observed at day E17. The strong increase in 17HSD type 2 expression detected between days E11 and E15 is consistent with the development of the tissues found predominantly to express the enzyme in adult animals (Table 1). 17HSD type 4 was constitutively expressed throughout fetal life,

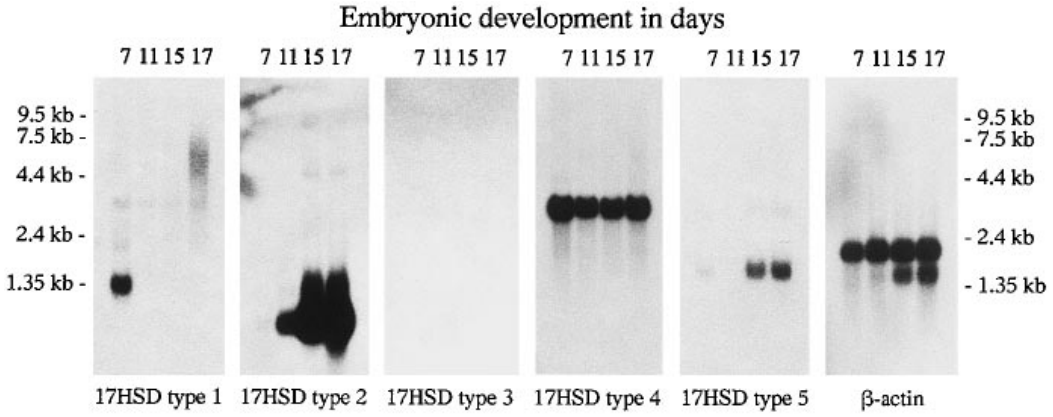

Figure 4 Northern-blot analysis of 17HSD types 1–5 in mouse embryos

Membranes containing 2 μg of poly(A)$^+$ RNA from different developmental stages (days 7, 11, 15 and 17) of whole mouse embryos were hybridized with cDNA probes from different 17HSD enzymes. In order to control the amount of mRNA applied, the membrane was also hybridized with β-actin cDNA.

which is in line with the results obtained from the tissues of adult animals. For 17HSD type 5 a faint signal was detected at day E7; thereafter the expression vanished and reappeared again on day E15, remaining constant to day E17.

DISCUSSION

Both the human and rodent type 2 enzymes contain a putative transmembrane region of 30 apolar amino acids at the N-terminus, proximal to the cofactor-binding area ([5,22] and the present study), and the enzymes are suggested to be associated with endoplasmic reticulum. Interestingly, only the human enzyme contains a putative anchor sequence (KYKK), whereas in the mouse and rat enzymes there are no charged amino acids at the N-terminal region proximal to the cofactor-binding area. Another difference between the human and rodent enzymes is the presence of three additional lysine residues in the extreme C-terminus of the human enzyme that do not exist in rodent type 2 enzymes. At the C-terminus of all characterized 17HSD type 2 enzymes there is, however, a short conserved charged motif containing two arginines (PRALR). Both lysine- and arginine-containing motifs have been shown to play a role as retrieval signals for ER-resident membrane proteins [26], and hence this structural motif may also have significance in the determination of the topology and localization of 17HSD type 2 enzymes.

Northern-blot analyses of 17HSD type 1–5 mRNAs were carried out in order to analyse the relative expression of the enzymes in various adult mouse tissues. The data indicated that the steroidogenic 17HSD type 1 and 3 enzymes were specific for females and males respectively. As regards expression of 17HSD types 2, 4 and 5, no difference between males and females was found. The data, furthermore, show that of the 17HSD enzymes, type 2 is the most abundantly expressed. The enzyme has a predominant oxidative activity, and the very high expression of the enzyme in several large organs such as liver, small intestine, kidney and placenta ([5,10,22] and the present study) indicates that 17HSD type 2 has the most substantial role in the inactivation of sex steroids, of all the enzymes characterized. The enzyme is able to inactivate oestradiol, testosterone and 5α-dihydrotestosterone, but the precise substrate specificity and cell-specific expression of the type 2 enzyme remain to be characterized. The data on adult mouse tissues also revealed that mouse 17HSD types 2, 4 and 5 are expressed in the liver more abundantly than in other tissues. This is in line with the strong steroid-metabolizing capacity of the liver.

It is evident that in both humans and rodents 17HSD type 4 has the widest tissue distribution among all the 17HSD enzymes ([13,20] and the present study), and the enzyme was detected in every tissue analysed. The full-length 17HSD type 4, containing three domains, is a peroxisomal protein [27], and the data indicating that expression of the type 4 enzyme is slightly higher in tissues with a greater number of peroxisomes (liver, testis and kidney) further indicate a constitutive role for the enzyme in peroxisomes. In addition to 17HSD activity the enzyme has been shown to catalyse, for example, D-3-hydroxyacyl-CoA dehydrogenase and 2-enoyl-CoA hydratase activities involved in β-oxidation of fatty acids [16–18]. Furthermore, the acyl-CoA dehydrogenase activity of the enzyme has been shown to be much higher than the oestradiol oxidation associated with rat 17HSD type 4 [17,18], and hence the physiological role of this widely expressed enzyme remains to be characterized in more detail. In the ovary 17HSD type 1 is the principal form, but, using mouse type 5 cDNA as a probe, a hybridization signal, slightly greater than that in other tissues, was also found in the ovary. This is most likely a result of the great similarity between several members of the aldoketoreductases, from which e.g. 20α-hydroxysteroid dehydrogenase has been shown to be highly expressed in rabbit and rat ovaries at least [28,29].

Previous activity measurements have shown the existence of 17HSD activity in both human and rodent fetuses [30,31]. However, in these previous studies the type of enzyme expressed could not be determined. The present results showing extensive expression of several forms of 17HSD in the embryos throughout fetal life suggest an important role for these enzymes in the control of the extent of steroid hormone action in the feto-placental unit. In mice, implantation of the embryo takes place at day E4–E5. Interestingly, our data suggest that activation of oestrone to oestradiol catalysed by the type 1 enzyme is the predominant 17HSD activity at day E7, which is the time immediately after blastocyst development. This is in line with previous data showing the presence of P450arom in porcine blastocysts [32], and with data showing that, in rabbits, oestradiol production peaks one day after implantation [33]. Previous data have shown that oestradiol production by the fetus is transient, which is in line with our results indicating that no significant amounts of 17HSD type 1 are expressed at any of the later stages of pregnancy. These results are in line with several reports suggesting that oestrogens play a crucial role in early embryogenesis and/or embryo–uterus interaction. In contrast, at the later stages the oxidative 17HSD type 2 enzyme predominates, leading to inactivation of sex steroids both in the fetus and placenta, thereby protecting the fetus from excessive sex-hormone action. However, biosynthesis and activation of testosterone in the fetus is essential for the normal development of male reproductive tract, and, although 17HSD type 3 was not detectable by Northern-blot analysis in whole mouse embryos, it is evident that the enzyme is expressed in the testis during fetal life [21]. Furthermore, the critical role of the enzyme in fetal testosterone biosynthesis is indicated by findings showing that inborn errors in 17HSD type 3 lead to genetic males with female external genitalia [6].

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