

Isolation and characterization of a cDNA encoding mouse 3 α -hydroxysteroid dehydrogenase An androgen-inactivating enzyme selectively expressed in female tissues

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Abstract

3 α -Hydroxysteroid dehydrogenase catalyzes the transformation of 3-ketosteroids into 3 α -hydroxysteroids, thus playing an important role in androgen and progesterone metabolism. So far, mouse cDNA and gene encoding 3 α -HSD has not been reported. In this report, we describe the isolation of a mouse 3 α -HSD cDNA and the characterization of its substrate specificity and tissue distribution. Sequence analysis indicates that m3 α -HSD shares 87% amino acid identity with rat 3 α -HSD. Cells stably transfected with this enzyme catalyze the transformation of dihydrotestosterone (DHT), 5 α -androstenedione (5 α -dione) and dihydroprogesterone (DHP) into 5 α -androstane-3 α ,17 β -diol (3 α -diol), androsterone (ADT) and 5 α -pregnan-3 α -ol-20-one (allopregnanolone), respectively. Quantification of mRNA expression levels of this enzyme was determined in male and female mouse sex-specific tissues using quantitative Realtime PCR. We show that this enzyme is mainly expressed in female-specific tissues while being almost absent from male-specific tissues. In the liver, the same expression level is seen in both male and female, while there is 6-fold higher expression level in female pituitary than in male. These results strongly suggest that m3 α -HSD could play an important role in the female mouse physiology similar to that of type 1 5 α -reductase with which it works in tandem. This role could be related to the inactivation of excess of androgen and progesterone that are more severely regulated than in man.

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1. Introduction

The enzyme 3 α -HSD catalyzes the transformation of the most potent natural androgen DHT into one less active form, 3 α -diol, as well as the transformation of 5 α -pregnane-3,20-one into allopregnanolone, a naturally-occurring neuroactive steroid [1]. These enzymes are also involved in the metabolism of various 3-keto steroids including progestins, glucocorticoids and bile acid precursors [2,3]. 3 α -HSDs are members of the aldo-keto reductase (AKR) family. One special feature of this protein family is that despite sharing a particularly high degree of homology, the members of sub-

families exert different enzymatic activities. For example, human type 5 17 β -HSD, 20 α -HSD and types 1 and 3 3 α -HSD all belong to the AKR1c subfamily of AKRs: these enzymes catalyze dramatically different activities while sharing approximately 86% identity. Moreover, human 20 α -HSD and type 3 3 α -HSD share 98% identity although they possess different enzymatic activity pattern and function [4]. Consequently, the activity exerted by members of this family cannot be identified, or predicted by sequence analysis but must be determined by the activity of the transfected genes in cultured cells [5,6]. Members of this family also show high variability concerning the lability of the enzymatic activity upon homogenization of the cells.

In human, two types of 3 α -HSDs have been isolated, chronologically named type 1 [7] and type 3 3 α -HSD [4,8].

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3 α -HSD activity has been found in various mammalian tissues including the liver [9], prostate [10], brain [11] and epididymis [12]. The human type 1 3 α -HSD is specifically expressed in the liver while type 3 3 α -HSD is more widely expressed in various peripheral tissues including the skin, prostate, adrenals, brain and liver [6]. Three-dimensional structure of rat [13] and human 3 α -HSD have been previously described. While the apoenzyme of rat 3 α -HSD has been crystallized, the human enzyme could not be obtained free of its cofactor. This is most probably due to a much higher affinity of human 3 α -HSD for the cofactor binding than rat enzyme [14]. The substrates of the 3 α -HSD enzymes are mainly 5 α -reduced or 5 β -reduced steroids. 3 α -HSD is thus exerting its activity downstream 5 α -reductase. This observation is in good agreement with the study using type 1 5 α -reductase knock out mice [15].

In this report, we describe the isolation and characterization of the first mouse 3 α -HSD. The availability of the cDNA allowed us to study the tissue distribution and the substrate specificity of the mouse enzyme. Our data show that, in contrast to the general belief that 3 α -HSD is an androgen-inactivating enzyme and thus expected to be highly expressed in androgen-targeted tissues, mouse 3 α -HSD is mainly expressed in female-specific tissues, following the expression profile of type 1 5 α -reductase, which is now known as the female isoform of 5 α -reductase [16].

2. Materials and methods

2.1. Tissue collection and RNA preparation

C57BL6 mice at 12–15 weeks of age were obtained from Charles River Inc. (Saint-Constant, Que., Canada). The mice were housed individually in vinyl cages. The photoperiod was 12 h of light and 12 h of darkness (lights on at 07:15 h). Certified rodent food (Lab Rodent Diet) and tap water were provided ad libitum. The experiment was conducted in an animal facility approved by the Canadian Council on Animal Care (CCAC) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The study was performed in accordance with the CCAC Guide for Care and Use of Experimental Animals. The collected organs were rapidly trimmed, snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was isolated by Trizol (Invitrogen, Burlington, Ont.). Twenty micrograms of total RNA was converted to cDNA by incubation at 42°C for 1 h with 400 U SuperScript II reverse transcriptase (Invitrogen), using oligo-d(T)₂₄ as primer in a reaction buffer containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT and 0.5 mM dNTPs.

2.2. Construction of mouse 3 α -HSD expression vector

The DNA fragment of a coding region of mouse 3 α -HSD was amplified from a mouse liver cDNA, pre-

pared as described above, using PCR and the oligoprimers pair (5'-GGG-GTC-GAC-GCT-ATG-AAT-TCT-GTA-TCC-CAC-GTG-3' and 5'-GGG-TCT-AGA-GCA-AAG-GCC-ACC-ATG-TTA-ATA-TTCA-3') derived from the Riken DNA sequence NM_134072. The resulting cDNA fragments were subcloned into a pCMVneo expression vector (pCMVneo-m3 α -HSD) which was subsequently used to produce stably transfected HEK-293 cells, as described below. Plasmid DNA was prepared using the Qiagen Mega Kit (Qiagen, Chatsworth, CA, USA). Sequence of the pCMVneo-m3 α -HSD was determined using an ABI 3730/XL automatic sequencer, to verify the identity of the amplified sequence and orientation of the promoter.

2.3. Stable expression in HEK-293 cells

HEK-293 cells were cultured in 6-well falcon flasks to approximately 3×10^5 cells/well in Minimum Essential Medium (MEM) (Gibco, Grand Island, NY) supplemented with 10% (v/v) FCS (Hyclone, Logan, UT) at 37°C under a 95% air–5% CO₂ humidified atmosphere. Five micrograms of pCMVneo-m3 α -HSD was transfected using Exgen 500 reagent (MBI Fermentas, Amherst, NY) as described [17]. After a 6 h incubation at 37°C , the transfection medium was removed and 2 ml of MEM were added. Cells were further cultured for 48 h and then transferred into 10 cm Petri dishes and cultured in MEM containing 700 $\mu\text{g/ml}$ of G-418 in order to inhibit the growth of non-transfected cells. The medium containing G-418 was changed every two days until resistant colonies were observed.

2.4. Assay of enzymatic activity

The measurement of the enzymatic activities was performed in intact cells as previously described [18]. Briefly, 0.1 μM of the [¹⁴C]-labeled steroid (Dupont Inc., Mississauga, Ont., Canada) was added to freshly changed culture medium in a 6-well culture plate. After incubation, the steroids were extracted with 2 ml of ether. The organic phases were pooled and evaporated to dryness. The steroids were then solubilized in 50 μl of dichloromethane, applied to Silica gel 60 thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany), before separation by migration in the toluene–acetone (4:1) solvent system. Substrates and metabolites were identified by comparison with reference steroids and revealed by autoradiography and quantified using the Phosphorimager System (Molecular Dynamics, Sunnyvale, CA). For determination of lability, cells were broken by the application of three freeze-thaw cycles. The same number of cells was incubated in a final volume of 1 ml containing 50 mM sodium phosphate (pH 7.4), 20% glycerol, 1 mM EDTA, 1 mM NADPH, and 0.1 μM of [¹⁴C]-labeled steroid. Incubation, extraction, separation and measurement of the metabolites were carried out as described above.

2.5. Quantitative Realtime PCR (Q-RT-PCR)

cDNA corresponding to 30 pg of the initial total RNA was used to perform fluorescent-based Realtime PCR quantification using the LightCycler Realtime PCR apparatus (Hoffman-La Roche Inc. Nutley, NJ). Reagents were obtained from the same company and were used as described by the manufacturer. The conditions for the PCR reactions were: denaturation at 94 °C for 15 s, annealing at 50 °C for 10 s and elongation at 72 °C for 35 s. Oligoprimers (5'-gcaaaggccaccatgttaattca-3', 5'-ggatgcttgacagaaattta-3') allowed the amplification of approximately 200 bp of the mouse 3 α -HSD sequence. The data were normalized using the mRNA expression levels of a housekeeping gene, namely ATP5o (subunit O of ATPase) as internal standard. Atp5o has been shown to be a gene that has stable expression levels from embryonic life through adulthood in various tissues [19]. 3 α -HSD mRNA expression levels are expressed as

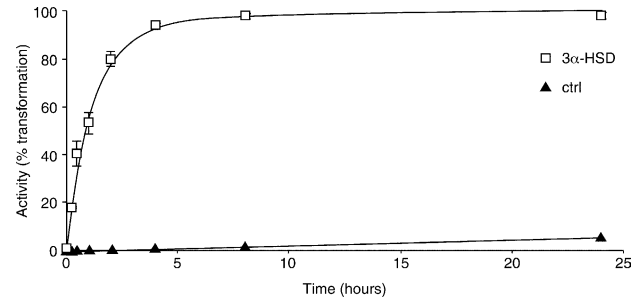


Fig. 1. 3 α -HSD activity of HEK-293 cells stably transfected with pCMVneo-m3 α -HSD. Time course of m3 α -HSD activity using DHT as substrate. Experiments were performed in intact cells using 0.1 μ M [14 C]-DHT. Error bars indicate mean \pm S.E.M. of triplicate. Incubation, extraction, separation and quantification of steroids were performed as described in Section 2.

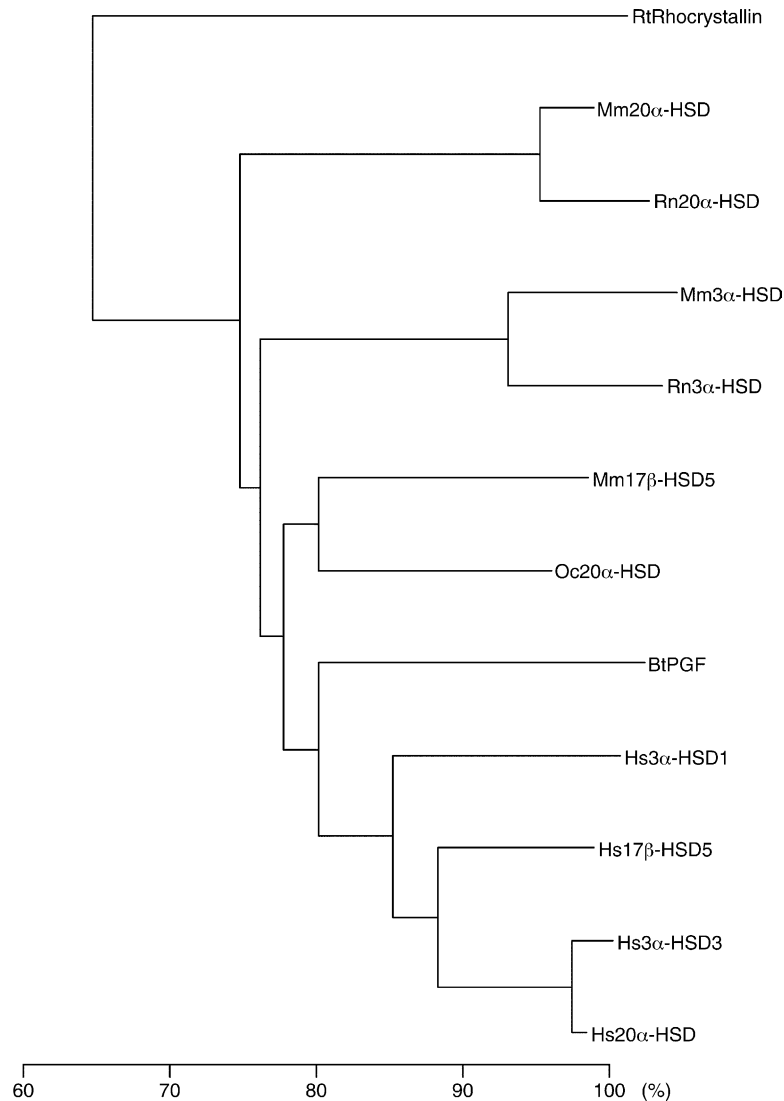


Fig. 2. Dendrogram representing a cluster analysis of steroid metabolizing members of the AKR family.

number of copies per μg total RNA using a standard curve of Cp versus logarithm of the quantity. The standard curve is established using known cDNA amounts of 0, 10^2 , 10^3 , 10^4 , 10^5 and 10^6 copies of cDNA and a LightCycler 3.5 program provided by the manufacturer (Roche Inc., Nutley, NJ).

3. Results

3.1. Characterization of the mouse 3α -HSD gene

The mouse genome project has made available the sequence of a cluster containing eight members of the aldo-keto reductase family located on the chromosome 13 [20]. This cluster includes the mouse type 5 17β -HSD gene, which was already located on chromosome 13 in region A2 [21] and a gene corresponding to a unknown transcript (GenBank 9030611N15Rik) encoding a protein product named as AKR1c14. This gene spans 33 kb on chromosome 13, contains 9 exons separated by 8 introns, is transcribed into a 2.5 kb mRNA species and encodes a protein of 323 amino acids. We have isolated a cDNA fragment containing the coding region of this gene and produced stably transfected HEK-293 cells, which we have deposited in GenBank database under the accession number AY730283. The enzyme overexpressed in HEK-293 cells exhibits a strong 3α -HSD activity (Fig. 1). We named this gene mouse 3α -HSD (m 3α -HSD). Interestingly, the m 3α -HSD gene is highly expressed in the liver and the mRNA size is 2.5 kb-long, similar to that of human type 1 and rat 3α -HSD. It is noteworthy that many members of this family display a mRNA size of 1.3 kb such as human and mouse type 5 17β -HSD, human and mouse 20α -HSD. It thus seems a 2.5 kb is selective for type 1 3α -HSD. We thus assumed that this enzyme was the mouse ortholog for human type 1 3α -HSD. The subsequent cluster analysis based on amino acid identity revealed some suspected association between different members in this protein subfamily (Fig. 2); mouse with rat 3α -HSD, just like 20α -HSD, seemed to be closely related, while all the human AKR1c members were nearly positioned on this scale. It is exciting to remind that this type of analysis gives important clues about the evolutionary path of these enzymes, and about their divergence period from other members of the family [22].

3.2. Substrate specificity of mouse 3α -HSD

Using the 3α -HSD stably expressed in HEK-293 cells, we have further characterized the substrate specificity of this enzyme in cultured cells by comparison with non-transfected cells. As illustrated in Fig. 3, mouse 3α -HSD catalyzes mainly the transformation of DHT into 3α -diol, 5α -dione into ADT and 5α -pregnane-3,20-dione into 5α -pregnane- 3α -ol-20-one. The absence of transformation of preg and prog into 20α -OHPreg and 20α -OHProg, respectively, indicates that

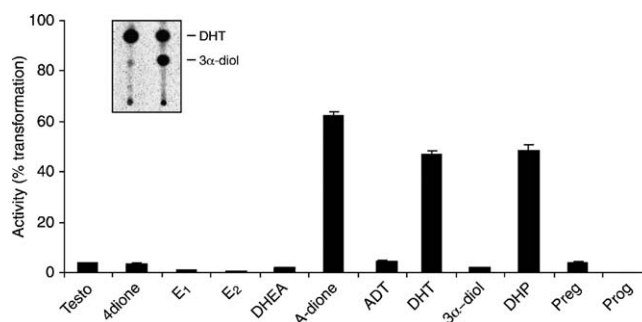


Fig. 3. Substrate specificity of m 3α -HSD. The experiments were conducted in intact transfected cells in culture using $0.1 \mu\text{M}$ of the indicated [^{14}C]- and [^3H]-labeled steroid. Separation of the metabolites was done by TLC; example shown is 3α -HSD enzymatic reaction with substrate DHT (2nd lane), versus control in non-transfected cells (1st lane). Name of the substrates are indicated in the x-axis. Testo represents a conversion of testosterone to androstenedione (4-dione) and vice-versa; E1, conversion of estrone to estradiol (E2) and vice-versa; DHEA, conversion of dehydroepiandrosterone to androstenediol; A-dione, conversion of androstenedione to androsterone (ADT) and vice-versa; DHT, conversion of dihydrotestosterone to 3α -diol and vice-versa; DHP, conversion of dihydroprogesterone to allo-pregnanolone; preg and prog, conversion of pregnenolone and progesterone to 20α -OHPreg and 20α -OHProg.

the 20α -hydroxy activity of this enzyme is not significant compared to its 3α -HSD activity.

3.3. Lability of mouse 3α -HSD

An interesting particularity of aldo-keto reductase enzymes is that some of the members of this family are labile upon homogenization of the HEK-293 cells stably expressing the enzyme whereas other members remain stable after homogenization. As shown in Fig. 4, m 3α -HSD is moderately labile, losing its activity after homogenization similar to that of human type 3 3α -HSD, in contrast to mouse type 5 17β -HSD [5] and human type 1 3α -HSD [6] that are stable after homogenization of the cells.

3.4. Tissue distribution of mouse 3α -HSD

The recently developed method of mRNA quantification by Realtime RT-PCR (Q-RT-PCR) has greatly improved mRNA quantification compared to classical PCR. The Q-RT-PCR permits to quantify the initial amount of mRNA using a standard curve of purified cDNA combined with the kinetics of DNA production [23,24] instead of quantifying end products which suffer from a high variability associated with the amplification process. Using this technique, we were able to establish the tissue distribution of the m 3α -HSD mRNA in both male and female tissues. The results indicate that m 3α -HSD mRNA is selectively expressed in female-specific tissues, namely the ovary, uterus, uterine horn, uterus and mammary gland and moderately in the vagina (Fig. 5C). On the other hand, the expression of the enzyme is negligible in androgenic-sensitive tissues (Fig. 5B). However, there is no significant difference in the expression levels of the

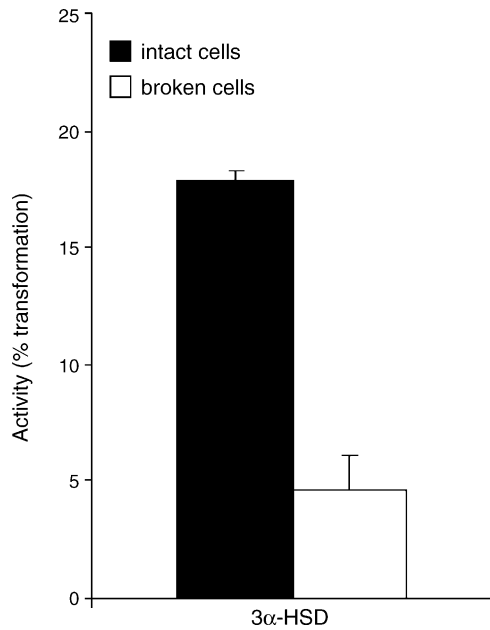


Fig. 4. Activity of m3α-HSD in intact and broken cells. The enzymatic activities of expressed cDNA encoding m3α-HSD in intact and broken cells were compared using 0.1 μM [¹⁴C]-DHT as substrate. The error bar indicates mean ± S.E.M. of triplicates.

enzyme in the male and female liver, lung, stomach, colon and heart, whereas there are much higher levels in the female (1.6 million copies/μg total RNA) versus male (0.25 million copies/μg total RNA) hypophysis (Fig. 5A).

4. Discussion

The present report describes the first structure of the m3α-HSD transcript, its tissue distribution, and the enzymatic characteristics of the encoded protein. It is noteworthy that m3α-HSD possesses a gene structure and mRNA size similar to that of human and rat 3α-HSD. Indeed, they show longer gene (30 kb) and mRNA (2.5 kb) size than many other members of the aldo-keto reductase family that possess a gene length of approximately 15 kb and a mRNA size around 1.3 kb. Another typical but confusing particularity of this gene family is that some members are labile upon homogenization of the cells whereas other members remain stable. This should be related to their protein structure since all other experimental conditions are the same except the cDNA sequence (Table 1).

Data from crystallographic structure studies of human [14] and rat [13] type 3 3α-HSD have suggested that the human enzyme binds the co-factor much stronger than rat 3α-HSD

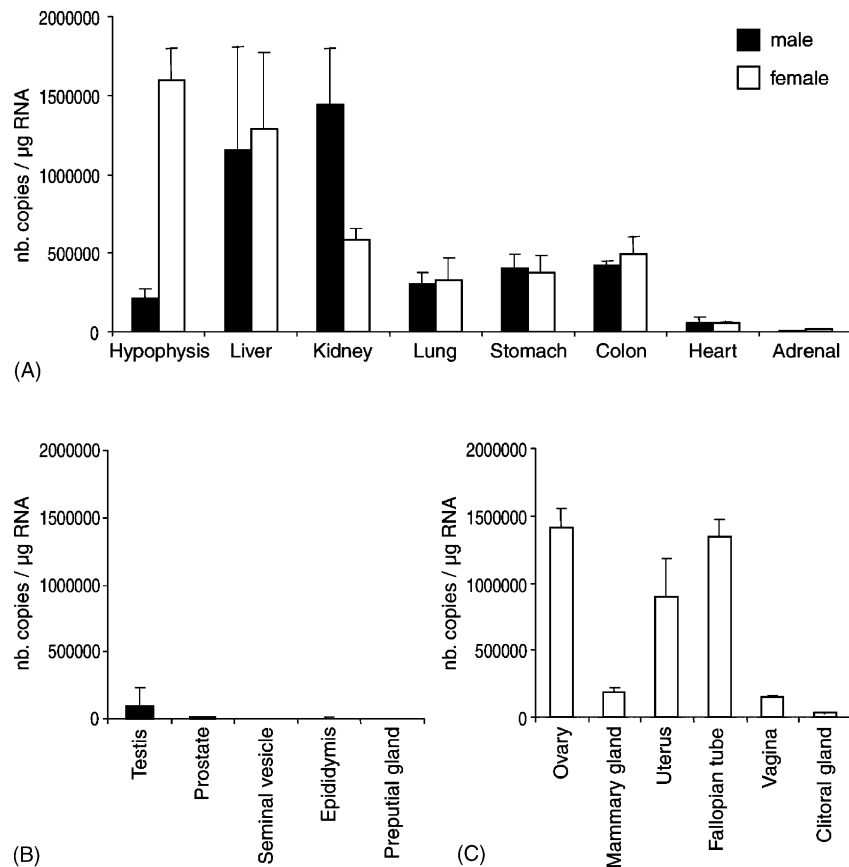


Fig. 5. Expression of m3α-HSD. The mRNA was quantified in non-sexual specific tissues (A); in male (B); and in female (C) sex-specific tissues. The expression level is indicated as the number of copies per μg of RNA. The error bar indicates mean ± S.E.M. of duplicates.

m3αHSD	MNSVSPRVLLNDGHFIPALGFGTVPDKVPKDELIKATKLAIDTGFRRHDSAYLQIEEEVQGAIRSKIEDGTVKREDIFYTSKLNSTFHRPELVRSCLKFTLKNQALDY	110
m20αHSD	---KIQKIE---S---V---YATEEHL-KKSMES---V---C-I-CSH---N---I---L---S---PS---NS-RFLN---	
m17βHSD	-D-KQQT-R-S---I---YA-QE---SKATE---A---I---SM-N-K---L---A---V-C---V---QS---QL---	
r3αHSD	-D-I-L-A---N---V---E-A---V---N---N---EV---T---ST---	
r20αHSD	---KIQKME---S---V---YATEENLRKKSMES---V---I-CSH---N---I---V---S---PS---NS-RFLN---	
rb20αHSD	-DPKFQ--A-S---V---YA-EE---SKAME---A---I---F-KN-K---L---A---C---PS---DS---L---	
h3αHSD1	-DPKYQ-E---M-V---YA-PE---RNRAVEV-L-EA---I---NN-Q-L---A-S---C-FQ-QM-QPA-SS-KL---	
h17βHSD5	-D-KHQC-K---M-V---YA-PE-RSKALEV-L-EA---I---H-NN-Q-L---A-S---PA-NS-K---	
h3αHSD3	-D-KYQC-K---M-V---YA-AE---SKALE-V-L-EA-H-I---HV-NN-Q-L---A-S---NS-PA-RS-L---	
h20αHSD	-D-KYQC-K---M-V---YA-AE---SKALE-L-L-EA-I-H-NN-Q-L---A-S---CNS-PA-RS-L---	
m3αHSD	VDLYI IHPPMALQPGDKLFRDEHGKLLAEAVLDCDTWEAMEKCKDAGLAKSIGVSNFNFRQLETILNKPGLKYKPVPCNQVECHLYLNQSQMLDYCKSKDILLVSYCTLG	220
m20αHSD	---L---VS-K---NE-L-K---N-IFDT---R---M---KL-A---MN-V---A-GA---	
m17βHSD5	---L---MK---ENYL-K-N---IYD---I---R---K-K---P---GKL-F-R---V---A-SA---	
r3αHSD	---L---IF---F-T-I---C-R---K---	
r20αHSD	---L---VS-K---E-L-Q---N-ILDT---R---K---HR---KL-A---MN-V---A-GA---	
rb20αHSD	---T---K---VEIL-T---AIFDT---I-A---R---M---P---GKL-EF---G-V---A-SA---	
h3αHSD1	---LL---K---ETPL-K-N---VIFDI---SA---V---C---M---P---KL-F---V---A-HSA---	
h17βHSD5	---L---S---S-K---EE-S-T---N---VIFDI---T---R---M---P-F-R-KL-F---V---A-SA---	
h3αHSD3	---L---VSVK---EEVI-K-N---I-FDT---A---H-L-M---P-F-RKL-F---V---A-SA---	
h20αHSD	---L---VSVK---EEVI-K-N---I-FDT---A---V---R---M---P-F-RKL-F---V---A-SA---	
m3αHSD	SSRDKIWDQKSPVLLDDPVLCCAMANKYKQTPALIAIRYQLQRGIVVLTFRSFKKRIKEFMKVFEFQLASEDMKVLGDLHRNLRNTASYFDDHFNHPFTDEY	323
m20αHSD	TQ-Y-YCINEDT---K-R---L-D---A-AK-N-E-R-N-Q-D---D-I---D---FP-DM-KA---F-F---	
m17βHSD5	-H-E-Q---S---N---GS-K-NR---L---V---AK-S---N-Q---T---D-NK-I---IGS-S-K---DF-W---	
r3αHSD	---T---I-K---V-L---V-P-I---NA---LTQ---A---N-F---N-K---	
r20αHSD	TQ-Y-YCINEDT---I-T-K---R---L---E---T-VK-N-E-R-NLQ-D---D-EI-N-D---FP-NM-KA---F-F---	
rb20αHSD	-H-EFE---SA---E-LIG-L-K-HQ---L---AK-T---NIQ---P---I-S-N-F-V---DFAIG---Y-S---	
h3αHSD1	TQ-H-L---PN---E---L-K-H---L---V---AK-YN-Q---R-NIQ---T---N-Y---VVMDFLM---DY-S---	
h17βHSD5	-Q---R---PN---E---L-K-H-R---L---V---AK-YN-Q---RQNVQ---TA---AI---D---H-FNSDS-AS---Y-S---	
h3αHSD3	-H-EFP---PN---E---L-K-H-R---L---V---AK-YN-Q---RQNVQ---T-E-AI---N-V-L-LDI-AGF---Y-S---	
h20αHSD	-H-EFP---PN---E---L-K-H-R---L---V---AK-YN-Q---RQNVQ---T-E-AI---N-V-L-LDI-AGF---Y-S---	

Fig. 6. Alignment of different hydroxysteroid dehydrogenases from the AKR family. Amino acid sequence of m3α-HSD was aligned with amino acid sequences of mouse (m), rat (r), rabbit (rb) and human (h) members of the AKR1c subfamily. Amino acid sequences are presented in the conventional single-letter code and numbered on the right. Dashes and dots represent identical and missing amino acid residues, respectively.

Table 1

Comparison of lability characteristic in mouse and human 3α-HSD, 20α-HSD and 17β-HSD5

	Mouse	Human
3α-HSD1	+	–
3α-HSD3	n.i.	+
20α-HSD	–	+
17β-HSD5	–	+

(+) and (–) indicate positive and negative lability characteristic, respectively. n.i. represents not identified.

due to two substitutions in the co-factor binding site: Asn²⁸⁰ (human) instead of Leu²⁸⁰ (rat) and Lys²⁷⁰ (human) in place of Arg²⁷⁰ (rat). Leu²⁸⁰ and Arg²⁷⁰ do not form any hydrogen bond, while Asn²⁸⁰ and Lys²⁷⁰ form two hydrogen bonds with the adenine part of NADP as well as a salt bridge with the 2'-phosphate of NADP, respectively. The corresponding amino acid in the mouse Arg²⁷⁰ and Phe²⁸⁰ strongly suggest that m3α-HSD will have the enzymatic characteristics similar to rat enzyme with a weaker affinity for the cofactor. Further, more, the two important amino acids in the steroid binding site at position 24 and 129 are identical in the mouse and rat sequences, but are different from that of all other members of the aldo-keto reductase family (Fig. 6).

The mRNA tissue distribution of m3α-HSD displays an unexpected pattern. Indeed, 3α-HSD is well recognized to be involved in the inactivation of DHT, the most potent natural androgen; it is thus expected that this enzyme is expressed in androgen-sensitive tissues. The expression profile we obtained shows that m3α-HSD is not expressed appreciably in male-specific tissues, but is highly expressed in female-

specific tissues such as the ovary, mammary gland, uterus, fallopian tube and vagina. This expression pattern is similar to that of type 1 5α-reductase which 3α-HSD acts downstream to inactivate progesterone and testosterone. Indeed, studies using type 1 5α-reductase knockout mice show that this particular enzyme seems to play a major role in female-specific tissues [25]. Since 5α-reductase is precisely responsible for the formation of DHT and DHP, we suggest that m3α-HSD and type 1 5α-reductase work in tandem to regulate the levels of DHT and DHP in female-specific tissues.

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