Molecular Cloning, Nucleotide Sequence and Tissue Distribution of Equine Testicular 3β -Hydroxysteroid Dehydrogenase/ $\Delta 5$ - $\Delta 4$ Isomerase Messenger Ribonucleic Acid

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Complementary DNA (cDNA) clones encoding equine testicular steroidogenic enzyme 3β -hydroxysteroid dehydrogenase/ $\Delta 5$ - $\Delta 4$ isomerase (3β -HSD) have been isolated from equine testicular cDNA library. Polymerase chain reaction (PCR) with 3β -HSD specific primers was performed with equine testicular cDNA, and PCR fragment was used as a probe for screening of equine testicular λ ZAP II phage cDNA library. Positive clones were excised into plasmid vector pBluescript and determined their nucleotide sequences. Total 1651 bp of the cDNA sequence was consisted with 112 bp of 5' flanking untranslated sequence, 1122 bp open reading frame encodes 373 amino acids, and putative poly-adenylation signal "AATAAA" at 385 bp downstream of the stop codon. The nucleotide and the deduced amino acid sequence of equine 3β -HSD were very similar to the known mammalian 3β -HSD sequences. From the deduced amino acid sequence, equine testicular 3β -HSD may be concluded to catalyze 3β -dehydrogenation and Δ -isomerization, but 3-ketoreduction as well as murine isoforms. As the clone was used in Northern blot analysis of equine tissues, the expression of equine 3β -HSD mRNA was prominent in the classical steroidogenic tissues including placenta.

Key words: *hydroxysteroid dehydrogenase, 3β -HSD, mRNA, steroidogenesis*

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We report here the first attempt to identify the mRNA for the equine testicular steroidogenic enzyme, 3β -hydroxysteroid dehydrogenase/ $\Delta 5$ - $\Delta 4$ isomerase (EC 1.1.1.145, EC 5.3.3.1; 3β -HSD). As the most common enzyme in various mammalian steroidogenic tissues, 3β -HSD catalyzes the conversion of $\Delta 5$ - 3β -hydroxypregnenone and $\Delta 5$ - 3β -hydroxyandrostene steroid precursors into $\Delta 4$ -3-ketosteroids progesterone, 17α -hydroxy-progesterone, $\Delta 4$ -androstenedione, and testosterone. In addition to these activities, 3β -HSD is also involved in the interconversion of 3β -hydroxy- and 3-keto- 5α -androstane steroids [1]. Consequently, 3β -HSD plays a critical function in the biosynthesis of all classes of hormonal steroids, and immunological or histochemical detection of 3β -HSD activity is commonly used in histological

studies as a cytochemical marker of the steroidogenic cells [2]. Furthermore, the expression of 3β -HSD has a broad distribution even in the non-steroidogenic tissues such as skin [1, 3-5]. In human and rodents, distinct isoforms of 3β -HSD mRNAs are identified from various tissues, e.g., adrenal gland, gonads, placenta, liver and skin [6-8]. For this reason, it is commonly accepted that 3β -HSD genes organize a multiple gene family [9]. While the most of members of 3β -HSD function as a NAD⁺-dependent 3β -hydroxysteroid dehydrogenase/ $\Delta 5$ - $\Delta 4$ isomerase, some isoforms of 3β -HSD are known to function as NADPH-dependent 3-ketosteroid reductase [10, 11].

In horses, however, there are a few studies on steroidogenesis approached by molecular basis [12, 13], except several immunohistochemical studies on the localization of equine steroidogenic enzymes include 3β -HSD [14], and on the activities of equine steroidogenic

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enzymes [15]. There remain a lot of characteristics in the equine pregnancy to investigate. Regardless of fetal gender, hypertrophic changes of equine fetal gonads are observed in mid- to late pregnancy [16]. In that period, fetal gonads produce high level of estrogen precursor steroids [17, 18]. Thus, it is considerable interest to investigate the pathway of estrogen production in the equine fetoplacental unit. The investigation of the localization of 3β -HSD gene expression in the equine fetoplacental unit should render to interpretation of the maternal or fetal cause of gestation failure.

Materials and Methods

mRNA Preparation and cDNA Synthesis

A 2-years-old Thoroughbred colt was anesthetized and castrated. Decapsulated testes were cut into 10 mm cubes, immediately frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Total RNA fraction was extracted from equine testis by guanidinium/CsCl ultracentrifugation [19]. Poly-adenylated RNA rich RNA fraction (poly(A)⁺ RNA) was obtained by affinity column chromatography with Oligo(dT)-Cellulose type 3 (Becton Dickinson Labware, Bedford), and double-stranded cDNAs were prepared with Superscript II Preamplification System (GIBCO BRL, Bethesda).

Polymerase Chain Reaction (PCR)

PCR amplification of the cDNA fragment was performed with GeneAmp PCR System 9600 (Perkin-Elmer, Urayasu) and TaqPlus DNA polymerase (Stratagene, San Diego) [20]. Nucleotide sequences of primers specific to human type II 3β -HSD [7] were as follows;

3β F2-EB 5'-GAATTCAATGTGAAAGGATCCC-AGCTCCTGTTAGAGG-3' (Forward)

3β R1-EB 5'-GAATTCGGATCCAGGGCCCTCA-AGGCCAGAATGTGGG -3' (Reverse)

The thermal cycle was 95°C for 4 min of pre-denature, and 30 cycles of 95°C for 1 min denature 55°C for 2 min annealing and 72°C for 3 min extension were followed by 72°C for 7 min extension in 100 μl of reaction mixture.

Cloning of the PCR Fragment

Molecular size of PCR-amplified fragments was determined by agarose gel electrophoresis (1% agarose in $1 \times \text{TAE}$), and purified with GENE CLEAN II kit (BIO 101, La Jolla). Purified PCR fragments were ligated into plasmid vector pCR2.1 (Original TA Cloning Kit; Invitrogen, Carlsbad). *Escherichia Coli* XL-1 Blue MRF⁺

cells (Stratagene, San Diego) were transformed with the ligated DNA. The transformants were selected by blue-white colony selection with IPTG and X-gal on the LB agar plates containing 50 $\mu\text{g}/\text{ml}$ ampicillin.

Construction of Equine Testicular cDNA Library

Five microgram of poly(A)⁺ RNA extracted from equine testis was dissolved in diethyl pyrocarbonate-treated water. First and second strand cDNA were synthesized by Amersham cDNA Synthesis System Plus (Amersham, Tokyo). Double stranded cDNA was ligated with *EcoRI*-*NotI*-*BamHI* adapter (Takara, Kyoto) and then with *EcoRI*-predigested lambda ZAP II phage DNA arms (Stratagene, San Diego) after 5'-phosphorylation (T4 polynucleotide kinase; Boehringer Mannheim, Tokyo). Ligated DNA was packaged into phage particle by Gigapack II Gold Packaging Extract (Stratagene, San Diego).

Screening of Lambda Phage Library

The equine testicular cDNA library was screened with Gene Images Non-Radioactive Labeling and Detection System (Amersham, Tokyo) using a plasmid clone for PCR fragment of equine testicular cDNA as a probe [21]. Positive clones were picked and subcloned as inserts of plasmid vector pBluescript [22].

Nucleotide Sequencing and Computer Analysis

Nucleotide sequence of the plasmid inserts were determined by Sanger's dideoxynucleotide chain termination method [23] with the A.L.F. automated sequencer (Pharmacia-Biotech, Tokyo), fluorescein labeled sequence primers (M4 and RV-M; Takara, Kyoto), and SequiTherm Long-Read Cycle Sequencing Kit (Epicentre Technologies, Madison). Obtained sequences were analyzed with the DNA analyzing software package DNASIS for Macintosh (Hitachi Software, Yokohama).

Northern Blot Analysis

Multiple tissue blot was performed with adrenal gland, testis, ovary, corpus luteum (125 days of pregnancy), placentas (125, 180, 270 days of pregnancy), fetal testis (155 days of pregnancy), fetal ovary (125 days of pregnancy), kidney, liver, and muscle. Twenty micrograms of total RNA from each tissue was separated by electrophoresis in 1.0% agarose 3-[N-morpholino]-propanesulfonic acid (MOPS)-formamide-formaldehyde gel [24]. Separated nucleic acids were transferred to positively charged nylon membrane (HyBond N⁺; Amersham), and hybridized with the same probe as used in screening of the lambda phage library labeled with [α - ^{32}P] dCTP.

Lane P: PCR product, M: *Eco*T14I-digested λ DNA as molecular size marker.

Results

An equine testicular cDNA library was constructed by testicular mRNA purification and reverse transcription *in vitro*. The 437 bp testicular cDNA fragment was successfully amplified with primers specific to β 3-HSD mRNA sequences (Fig. 1). The amplified fragment was excised, purified, and cloned into plasmid vector pCR2.1. The nucleotide sequence of the PCR clone and its deduced amino acid sequence, on the assumption that no frame shifts occurred, are shown in Fig. 2.

In the next step, lambda ZAP II phage cDNA library of equine testis was screened by this fragment as a probe. Out of 1×10^5 plaques, about 20 strong positive clones were detected. Several clones were sequenced until two clones including the identical complete coding sequence of this enzyme were identified. The 1651 bp nucleotide sequence of the cDNA clone consisted of 112 bp of the 5' flanking untranslated sequence, total 1122 bp of open reading frame encodes 373 amino acids, and putative poly-adenylation signal "AATAAA" at 385 bp downstream of the stop codon (Fig. 3). The deduced amino acid sequence is compared with those of bovine and human β -HSD to review the structure-function

primer 3βF2-EB→																									
9						18				27				36				45				54			
GAA	TTC	AAT	GTG	AAA	GGA	TCC	CAG	CTC	CTG	TTA	GAG	GCC	TGT	TCC	CAG	GCT	AGC								
Glu	Phe	Asn	Val	Lys	Gly	Ser	Gln	Leu	Leu	Leu	Glu	Ala	Cys	Ser	Gln	Ala	Ser								
63						72				81				90				99				108			
GTG	CCA	ATC	TTC	ATC	TAC	ACC	AGC	TCC	GTA	GCG	GTG	GCT	GGG	CCC	AAC	TCC	TAC								
Val	Pro	Ile	Phe	Ile	Tyr	Thr	Ser	Ser	Val	Ala	Val	Ala	Gly	Pro	Asn	Ser	Tyr								
117						126				135				144				153				162			
AGG	GAG	ATC	ATC	CAG	AAT	GGC	CAT	GAA	GAA	GCA	CAT	CTC	GAA	ACA	AAA	TGG	TCC								
Arg	Glu	Ile	Ile	Gln	Asn	Gly	His	Glu	Glu	Ala	His	Leu	Glu	Thr	Lys	Trp	Ser								
171						180				189				198				207				216			
TCT	CCA	TAC	CCA	TAC	AGC	AAA	AAG	CTT	GCT	GAG	AAG	GCT	GTG	CTG	GCA	GCT	AAT								
Ser	Pro	Tyr	Pro	Tyr	Ser	Lys	Lys	Leu	Ala	Glu	Lys	Ala	Val	Leu	Ala	Ala	Asn								
225						234				243				252				261				270			
GGG	TTG	CCT	CTT	AAA	AAT	GGT	GGC	ACC	TTA	TAC	ACT	TGT	GCC	TTA	AGG	CCC	ATG								
Gly	Leu	Pro	Leu	Lys	Asn	Gly	Gly	Thr	Leu	Tyr	Thr	Cys	Ala	Leu	Arg	Pro	Met								
279						288				297				306				315				324			
TTT	ATC	TAT	GGG	GAA	GGA	AGC	CCA	ACC	CTT	TAT	TAC	CTT	ATG	CAT	GAG	GGC	CTG								
Phe	Ile	Tyr	Gly	Glu	Gly	Ser	Pro	Thr	Leu	Tyr	Tyr	Leu	Met	His	Glu	Gly	Leu								
333						342				351				360				369				378			
AAC	AAC	AAT	GGC	ATC	CTG	ACA	CAC	AAC	TGC	AAG	TTC	TCC	AGA	GCC	AAC	CCA	GTC								
Asn	Asn	Asn	Gly	Ile	Leu	Thr	His	Asn	Cys	Lys	Phe	Ser	Arg	Ala	Asn	Pro	Val								
387						396				405				414				423				432			
TAT	GTT	GGC	AAC	ATA	GCC	TGG	GCC	CAC	ATT	CTG	GCC	TTG	AGG	GCC	CTG	GAT	CCG								
Tyr	Val	Gly	Asn	Ile	Ala	Trp	Ala	His	Ile	Leu	Ala	Leu	Arg	Ala	Leu	Asp	Pro								
← primer 3βR1-EB																									
LAT																									
Asn																									

Fig. 2. Nucleotide sequence of PCR product.
A pair of primers used for PCR were indicated with box shadow, and deduced amino acid sequence is shown below (*Italic*).

ctgaactacttgatctctcttttttagctctctccagggtca																		41
ctctctaataccaatctgcttgccctgctttttctgggtcctggcgagcatttctgttactcaggtttacc																		112
ATG	GCT	GGG	TGG	AGC	TGC	CTT	GTG	ACG	GGA	GCA	GGA	GGC	TTT	CTG	GGT	CAG	AGG	166
Met	Ala	Gly	Trp	Ser	Cys	Leu	Val	Thr	Gly	Ala	Gly	Gly	Phe	Leu	Gly	Gln	Arg	18
ATT	GTC	CGC	TTG	CTG	GTG	GAG	GAG	AAG	GAG	GTA	CAG	GAG	ATC	AGA	GCA	CTG	GAC	220
Ile	Val	Arg	Leu	Leu	Val	Glu	Glu	Lys	Glu	Val	Gln	Glu	Ile	Arg	Ala	Leu	Asp	36
AAA	GTC	TTC	AGA	CCA	GAA	TTG	CGG	GAA	GAG	TTT	TCC	AAA	CTC	CAG	AGC	AAG	GTC	274
Lys	Val	Phe	Arg	Pro	Glu	Leu	Arg	Glu	Glu	Phe	Ser	Lys	Leu	Gln	Ser	Lys	Val	54
AAG	CTG	ACC	GTG	CTG	GAG	GGA	GAC	ATT	CTG	GAT	GAG	CAG	TTC	CTG	AAG	AGA	GCT	328
Lys	Leu	Thr	Val	Leu	Glu	Gly	Asp	Ile	Leu	Asp	Glu	Gln	Phe	Leu	Lys	Arg	Ala	72
TGC	CAG	GGC	GCC	TCG	GCT	GTC	ATT	CAC	ACC	GCC	TCT	ATC	ATC	GAC	GTC	ACG	AAC	382
Cys	Gln	Gly	Ala	Ser	Ala	Val	Ile	His	Thr	Ala	Ser	Ile	Ile	Asp	Val	Thr	Asn	90
CTC	TTT	AAC	CCA	CAG	GTC	ACC	ATG	AAT	GTC	AAT	GTG	GAA	GGT	ACC	CAG	CTC	CTA	436
Leu	Phe	Asn	Pro	Gln	Val	Thr	Met	Asn	Val	Asn	Val	Glu	Gly	Thr	Gln	Leu	Leu	108
TTG	GAG	GCC	TGT	TCC	CAG	GCT	AGC	GTG	CCA	ATC	TTC	ATC	TAC	ACC	AGC	TCC	GTA	490
Leu	Glu	Ala	Cys	Ser	Gln	Ala	Ser	Val	Pro	Ile	Phe	Ile	Tyr	Thr	Ser	Ser	Val	126
GCG	GTG	GCT	GGG	CCC	AAC	TCC	TAC	AGG	GAG	ATC	ATC	CAG	AAT	GGC	CAT	GAA	GAA	544
Ala	Val	Ala	Gly	Pro	Asn	Ser	Tyr	Arg	Glu	Ile	Ile	Gln	Asn	Gly	His	Glu	Glu	144
GCA	CAT	CTC	GAA	ACA	AAA	TGG	TCC	TCT	CCA	TAC	CCA	TAC	AGC	AAA	AAG	CTT	GCT	598
Ala	His	Leu	Glu	Thr	Lys	Trp	Ser	Ser	Pro	Tyr	Pro	Tyr	Ser	Lys	Lys	Leu	Ala	162
GAG	AAG	GCT	GTG	CTG	GCA	GCT	AAT	GGG	TTG	CCT	CTT	AAA	AAT	GGT	GGC	ACC	TTA	652
Glu	Lys	Ala	Val	Leu	Ala	Ala	Asn	Gly	Leu	Pro	Leu	Lys	Asn	Gly	Gly	Thr	Leu	180
TAC	ACT	TGT	GCC	TTA	AGG	CCC	ATG	TTT	ATC	TAT	GGG	GAA	GGA	AGC	CCA	ACC	CTT	706
Tyr	Thr	Cys	Ala	Leu	Arg	Pro	Met	Phe	Ile	Tyr	Gly	Glu	Gly	Ser	Pro	Thr	Leu	198
TAT	TAC	CTT	ATG	CAT	GAG	GGC	CTG	AAC	AAC	AAT	GGC	ATC	CTG	ACA	CAC	AAC	TGC	760
Tyr	Tyr	Leu	Met	His	Glu	Gly	Leu	Asn	Asn	Asn	Gly	Ile	Leu	Thr	His	Asn	Cys	216
AAG	TTC	TCC	AGA	GCC	AAC	CCA	GTC	TAT	GTT	GGC	AAC	ATA	GCC	TGG	GCC	CAC	ATT	814
Lys	Phe	Ser	Arg	Ala	Asn	Pro	Val	Tyr	Val	Gly	Asn	Ile	Ala	Trp	Ala	His	Ile	234
ATG	GCC	TTG	AGG	GCC	CTG	AGG	GAC	CCC	AAG	AAG	GCC	CCA	AGC	ATC	CAA	GGA	CAG	868
Met	Ala	Leu	Arg	Ala	Leu	Arg	Asp	Pro	Lys	Lys	Ala	Pro	Ser	Ile	Gln	Gly	Gln	252
TTC	TAC	TAC	ATC	TCA	GAT	GAC	ACA	CCT	CCC	CAA	AGC	TAT	GAT	GAC	TTA	ACT	TAC	922
Phe	Tyr	Tyr	Ile	Ser	Asp	Asp	Thr	Pro	Pro	Gln	Ser	Tyr	Asp	Asp	Leu	Thr	Tyr	270
ACT	TTG	AGC	AAA	AAG	TGG	GGC	TTC	TGC	CTT	GAT	TCC	AGA	ATG	AGA	CTT	CCC	ATA	976
Thr	Leu	Ser	Lys	Lys	Trp	Gly	Phe	Cys	Leu	Asp	Ser	Arg	Met	Arg	Leu	Pro	Ile	288
TTT	CTG	AAG	TAC	TGG	CTT	GCC	TTC	CTG	CTG	GAA	ATA	GTG	AGC	TTC	CTG	CTC	AGT	1030
Phe	Leu	Lys	Tyr	Trp	Leu	Ala	Phe	Leu	Leu	Glu	Ile	Val	Ser	Phe	Leu	Leu	Ser	306
CCA	ATT	TAC	AAA	TAT	CGA	CCT	CCC	TTC	GAC	CGC	CAC	CTA	GTG	ACA	TGG	CAA	AAC	1084
Pro	Ile	Tyr	Lys	Tyr	Arg	Pro	Pro	Phe	Asp	Arg	His	Leu	Val	Thr	Trp	Gln	Asn	324
AGT	GTT	TTT	ACC	TTC	TCC	TAT	AAG	AAA	GCT	CAG	CGA	GAT	ATG	GGA	TAT	GAG	CCG	1138
Ser	Val	Phe	Thr	Phe	Ser	Tyr	Lys	Lys	Ala	Gln	Arg	Asp	Met	Gly	Tyr	Glu	Pro	342
CTC	TTC	AGC	TGG	GAG	GAA	GCC	AAG	AAG	AGA	ACC	ACT	GAG	TGG	ATC	GAC	GCC	CTG	1192
Leu	Phe	Ser	Trp	Glu	Glu	Ala	Lys	Lys	Arg	Thr	Thr	Glu	Trp	Ile	Asp	Ala	Leu	360
GTA	GAA	CCG	CAC	CAG	GAG	GCC	CTG	AAA	ACA	AAG	ACT	CTC	TGA	tctacaggtgacatg				1251
Val	Glu	Pro	His	Gln	Glu	Ala	Leu	Lys	Thr	Lys	Thr	Leu	***					373
gatgcggtatgttaggagatgtctgctagactctcccttctgtcttcaaacagcaaataccatgagcagca																		1322
gcccagatcctactgcctctctttcacaagatgccacggttatcgtcttctccttggccactagaaactttc																		1393
ccagtcactcgcgcagcagcaaatcttctgccctaccaccttccagaggacagacaagatgatttgctgca																		1464
gctgttgccacaaagtcttagttgctgattctgagctcttcaggcctcttttaacttagagttttgcctg																		1535
ttagttcccggttcccttggtaaatgcaaaagcatttccctatcttttaaaaattccattacttcagacagc																		1606
tcaatgaaagaataataaattggttttactgcctaactgcctaactctg																		1651

Fig. 3. Nucleotide sequence and deduced amino acid sequence for equine testicular 3β -HSD cDNA. The putative poly(A) addition signal sequence is underlined. Numbers on the right of the line indicate nucleotide and amino acid (*Italic*) position.

relationship (Fig. 4). Equine 3β -HSD has been found to have high similarity to those of cattle and other species in both nucleotide and amino acid sequence (Table 1).

To investigate the tissue distribution of 3β -HSD expression, Northern blot analysis was performed with cDNA clone as a probe. Equine 3β -HSD was expressed in the testis, adrenal gland, corpus luteum, and placentas,

but not detectable in liver, kidney, muscle, and fetal gonads (Fig. 5).

Discussion

Multiple isoforms of 3β -HSD cDNAs have been isolated and characterized in human, rat and mouse.

Equine	10	20	30	40	50	60
1)	MAGWSC	LVIG	ACGFLG	QRIV	RLIVEE	KEVQ
2)	-----	G-----	I-----	C-----	DLQ-----	--V-----
3)	-T-----	-----	I-----	--K-----	LK-----	--V-----
4)	M-----	-----	-----	-----	LK-----	--A-----
Equine	70	80	90	100	110	120
	GDILDE	QFLK	RACQGAS	AVI	HTASIID	VTN
Bovine	-----	C-----	G-----	T-----	V-----	--V-----
Human I	-----	P-----	--DV-----	VI-----	--C-----	FG-----
Human II	-----	P-----	--DV-----	V-----	--C-----	FG-----
Equine	130	140	150	160	170	180
	IYTSSV	AVAG	PNSYREI	IQN	GHEEAH	LETK
Bovine	-H-----	TIE-----	-----	D-----	-R-----	E-H-SA
Human I	-----	IE-----	-----	K-----	-----	EP-NT
Human II	-----	IE-----	-----	K-----	-----	EP-NT
Equine	190	200	210	220	230	240
	YTCALR	PMFI	YGEGSP	TLTY	LMHEGL	NNNG
Bovine	-----	Y-----	-----	F-SA	Y--GA-----	--NH-----
Human I	-----	Y-----	-----	RF-SA	SIN-A-----	--SSVG-----
Human II	-----	TY-----	-----	GPF-SA	SIN-A-----	--SSVG-----
Equine	250	260	270	280	290	300
	RDPKKAP	SIQ	GQFYI	ISDDT	PPQSYD	DLTY
Bovine	-----	V-N-----	-----	-----	-H-----	N-----
Human I	Q-----	R-----	-----	-----	-H-----	N-N-----
Human II	-----	VR-----	-----	-----	-H-----	N-N-----
Equine	310	320	330	340	350	360
	VSFLLS	PIYK	YRPPFD	RHLV	TWQNSV	FTFS
Bovine	-----	N-C-N-----	-----	LS-----	-----	L-----
Human I	-----	R-----	T-----	N-I-----	LS-----	-----
Human II	-----	S-----	Q-----	N-T-----	LS-----	-----
Equine	370					
	VEPHQE	ALKT	CTL			
Bovine	-KQ-K-T--	--IH				
Human I	-DR-K-T--	--S	--Q			
Human II	-DR-K-T--	--S	--Q			

Fig. 4. Comparison of the deduced amino acid sequence of equine 3 β -HSD with cattle and human. 1) Equine amino acid sequence predicted from nucleotide sequence (accession D89666) in this study. 2) Bovine (accession X17614 [34]), 3) human type I 3 β -HSD (accession X53321 [1]) and 4) human type II 3 β -HSD amino acid sequences (accession M67466 [7]) were modified from [4]. The box-shadowed amino acid residues are commented in the text.

Table 1. Sequence similarity in the equine 3 β -HSD with other mammalian species

Species	Sequence Similarity (%)	
	Amino Acid	Nucleotide
Cattle	79.8	79.7
Human type I	74.5	78.3
Human type II	74.5	77.9
Rat type I	69.6	67.9
Rat type II	68.8	68.2

Two types of rat 3 β -HSD mRNA sequences (accession M38178 and M38179 [35]) are isolated from gonads.

Fig. 5. Northern blot of equine 3 β -HSD. Each lane represents 20 μ g of total RNA. The numbers indicate the days of pregnancy (dpc), or the days post delivery (dpd). C.L.: Corpus Luteum.

These cDNAs are described by roman numerals in the order that they had been isolated, and the same number in the different species does not indicate that these types are orthologous. While human type I 3β -HSD, first isolated placental clone, is expressed in placenta, skin and mammary gland, human type II 3β -HSD is expressed in adrenal gland and gonads [1, 7]. Mouse type I 3β -HSD is expressed in adrenal gland, gonads, placenta, fetal and neonatal liver, but type II and type III 3β -HSD is expressed both in liver and kidney [25, 26]. These isoforms showing distinct tissue specificities are considered as a multiple gene family. From linkage analysis, two genes for human 3β -HSD isoforms are clustered in chromosome #1 [9]. Between these isoforms of 3β -HSD, there are distinct groups of proteins, that likely act as NADPH-dependent 3-ketosteroid-reductase rather than NAD^+ -dependent 3β -hydroxysteroid dehydrogenase/isomerase [10]. Rat type III, mouse type IV, V, and hamster type III 3β -HSD enzymes function as a 3-ketosteroid-reductase and are expressed in liver or kidney [10, 27].

In this study, the cDNA for equine testicular 3β -HSD mRNA was identified and determined its nucleotide sequence. The nucleotide sequence of equine testicular 3β -HSD mRNA showed high similarity both with type I and II 3β -HSD human mRNAs, and bovine one (Fig. 5). Furthermore, alignment study of predicted amino acid sequence revealed that equine testicular 3β -HSD contains common structures of other 3β -HSD enzymes, such as C-terminal 26 residue segment (aa 284-309) of transmembrane domain [28] and glycine-rich conserved sequence (Gly-X-X-Gly-X-X-Gly at aa 10-16) of NAD^+ -binding enzymes at the hydrophobic pocket [29]. Asp36 residue is conserved in most members of the 3β -HSD family, except in isoenzymes with 3-ketoreductase activity exhibiting a preference for NADPH over NADH [11]. Identification of two peptides, Gly251 to Lys275 and Asn176 to Arg186 in the active site of human type I 3β -HSD, by substrate protection against a site-directed affinity radioalkylator suggested that His262 and Cys183 are in close adjacency in the three-dimensional structure of the enzyme [30]. These regions are also conserved in the equine 3β -HSD, and one of two characteristic YXXXK sequences (Tyr155 to Lys159 and Tyr270 to Lys274) found in the active site of short-chain alcohol dehydrogenases, has been located in this region [31]. Thus, it is suggested that the equine testicular 3β -HSD, cloned in this study, may function both as 3β -hydroxysteroid dehydrogenase and isomerase, but 3-ketoreductase.

By Northern blot hybridization analysis, mRNAs

electrophoresed close to 18S ribosomal RNA carrying this sequence were found to be intensively expressed in the classical steroidogenic tissues such as adrenal gland, testis, corpus luteum, and placentas (Fig. 5). In contrast, no signs of expression were found in liver, muscle, kidney. It is not clear whether the equine 3β -HSD has multiple isoforms. Relatively higher expression of 3β -HSD mRNA in placenta compared with corpus luteum implicates the major contribution of placental tissue to the progesterone production in the mid- and late pregnancy of the mare. These results may agree with the immunohistochemical study [14], that demonstrates the equine 3β -HSD expression in trophoblasts. Han *et al.* [32] concluded that the fetal adrenal gland seems unlikely to be the major source of pregnenolone in the umbilical arterial plasma, in which pregnenolone is found to be high concentrations [33]. Though the source of umbilical pregnenolone is still unclarified, low abundance of 3β -HSD mRNA in the fetal gonads may not contradict with these findings. Our results also support the argument that the trophoblast, not fetal gonads, should be a major source of progesterone necessary for maintenance of pregnancy.

Acknowledgments

The nucleotide sequence data reported in this paper will appear with the accession number D89666 in the DDBJ/ EMBL/ NCBI nucleotide sequence databases.

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