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Molecular cloning, cDNA structure and predicted amino acid sequence of bovine 3β -hydroxy-5-ene steroid dehydrogenase/ Δ^5 - Δ^4 isomerase

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We have used our recently characterized human 3β -hydroxy-5-ene steroid dehydrogenase/ Δ^5 - Δ^4 -isomerase (3β -HSD) cDNA as probe to isolate cDNAs encoding bovine 3β -HSD from a bovine ovary λ gtll cDNA library. Nucleotide sequence analysis of two overlapping cDNA clones of 1362 bp and 1536 bp in length predicts a protein of 372 amino acids with a calculated molecular mass of 42093 (excluding the first Met). The deduced amino acid sequence of bovine 3β -HSD displays 79% homology with human 3β -HSD while the nucleotide sequence of the coding region shares 82% interspecies similarity. Hybridization of cloned cDNAs to bovine ovary poly(A)⁺ RNA shows the presence of an approximately 1.7 kb mRNA species.

Steroidogenesis; 3β -Hydroxy-5-ene-steroid dehydrogenase/ Δ^5 - Δ^4 -ene isomerase; DNA, complementary; (Bovine ovary)

1. INTRODUCTION

The enzyme complex 3β -hydroxy-5-ene-steroid dehydrogenase (EC 1.1.1.145) and steroid $\Delta^5 - \Delta^4$ -eneisomerase (EC 5.3.3.1), hereafter called 3β -HSD, catalyzes the oxidative conversion of Δ^5 -ene-3 β hydroxy steroid precursors into Δ^4 -3-ketosteroids. The 3β -HSD enzymatic system plays a crucial role in the biosynthesis of all classes of hormonal steroids, namely progesterone, mineralocorticoids, glucocorticoids, androgens and estrogens. The 3*β*-HSD enzyme complex is thus present in the adrenals, testes, ovaries and placenta as well as in many peripheral tissues, including the prostate, breast, liver and skin [1-6]. This enzymatic system is found in both microsomes and mitochondria and it shows a strict requirement for NAD⁺ as co-factor [5,7,8]. The two activities of 3β -HSD seem to reside within a single protein in human placenta [5,9,10], ovine adrenals [7], rat adrenals [4], rat testes [11], bovine ovaries [12] and bovine adrenals [13].

Congenital deficiency of 3β -HSD activity causes a severe depletion of steroid formation by the adrenals and gonads and is frequently lethal in early life [14,15]. The classical form of this disease includes the association of severe salt-losing adrenal insufficiency and am-

biguity of external genitalia in both sexes. However, clinical variants of 3β -HSD deficiency present various degrees of salt-loss [14,16]. Moreover, the late-onset form of 3β -HSD includes other misdesignated causes of virilization [17].

In order to obtain information about the evolution of the structural domains of 3β -HSD and to make available bovine 3β -HSD cDNA which would permit detailed studies of the control of 3β -HSD gene expression and its enzymatic activity in this species, we have isolated and characterized the full length cDNA structure of bovine ovary 3β -HSD using a human 3β -HSD cDNA [10].

2. MATERIALS AND METHODS

2.1. Construction and screening of the bovine ovary $\lambda gt11$ cDNA library

Total RNA was isolated from bovine ovary by homogenizing tissue in guanidinium isothiocyanate followed by centrifugation through a cushion of 5.7 M CsCl as previously described [18,19]. Poly(A)⁺ RNA was purified by two successive cycles of chromatography through an oligo(dT)-cellulose column. A bovine ovary cDNA library was constructed in λ gt11 vector using the oligo-dT-primed method by Clontech Laboratories (Palo Alto, CA, USA). The amplified cDNA library was screened with human 3β -HSD cDNA hp 3β -HSD63 [10] as probe. The cDNA was labeled with [α -³²P]dCTP (Amersham) using the random primer method [20]. Prehybridization was performed for 3 h at 37°C in 30% formamide, 5 × SSPE (1 × SSPE being 0.18 M NaCl, 10 mM NaH₂PO₄, pH 7.4, 1 mM EDTA), 0.1% sodium dodecyl sulfate (SDS), 0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone and 200 µg/ml denatured salmon testis DNA. Thereafter, 1 × 10⁶ cpm [³²P]hp3 β -HSD63 cDNA probe/ml was added to pre-

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hybridization buffer containing 4% dextran sulfate. After 20 h of hybridization at 37°C, the filters were washed twice (20 min each) in $2 \times SSC/0.1\%$ SDS at 25°C, once for 20 min in $2 \times SSC/0.1\%$ SDS at 40°C and 1 h in $1 \times SSC/0.1\%$ SDS at 40°C. Nitrocellulose filters were then exposed overnight at -80°C. In order to obtain clones containing the 5' coding region, [³²P]-labeled 3 β -HSD cDNA fragment corresponding to the first 121 nucleotides of the 5' end of human 3 β -HSD cDNA hp3 β -HSD63 [10] was used as probe. Prehybridization and hybridization buffer were as described above except that 20% formamide was used instead of 30%.

2.2. RNA blot analysis

Northern blot analysis was carried out under highly stringent conditions as previously described [21]. In brief, $5 \mu g$ of poly(A)⁺ RNA isolated from bovine ovary were electrophoresed on 1.2% agarose/2.2 M formaldehyde gel and immobilized on a nylon membrane (Hybond N, Amersham). Hybridization with *Eco*RI inserts of bo3 β -HSD113 or bo3 β -HSD123 cDNA clones was performed at 42°C for 16 h. The fragments were labeled with [α -³²P]dCTP to a specific activity of 1 × 10⁹ dpm/ μg using a random primer method. The probes (2 × 10⁶ cpm/ml) were added to hybridization buffer [21]. The filters were washed for 15 min in 2 × SSC/0.1% SDS at 25°C and 65°C (two times each). The autoradiographs were obtained after exposing the filters to Kodak X-OMAT AR films for 1 h at -80°C.

2.3. DNA sequencing

Complementary DNA clones were sequenced by the dideoxy chain termination method using T7 DNA polymerase [22] (Sequenase kit, United States Biochemical Corp., Cleveland, OH). Fragments were subcloned in the Bluescript SK vector (Stratagene, San Diego, CA) and synthetic oligonucleotides, as well as T7 or T3 vector primers, were used as sequencing primers as shown in fig.1.

3. RESULTS AND DISCUSSION

A bovine ovary $\lambda gt11$ cDNA library was screened with human cDNA clone hp3 β -HSD63 [10] as probe. Out of 5×10^5 recombinants, 90 positive clones were isolated and purified. Among the 90 clones obtained, two clones, bo3\beta-HSD113 and bo3\beta-HSD123, which hybridized with ³²P-labeled cDNA fragment corresponding to the first 121 nucleotides of hp3\beta-HSD63 clone, were characterized by a combination of restriction endonuclease mapping and DNA sequence analysis according to the strategy described in fig.1. The bo3 β -HSD113 (1362 bp) and bo3*β*-HSD123 (1536 bp) clones have identical sequences in their overlapping regions. The merged nucleotide sequence of the two cDNAs as well as the predicted amino acid sequence are illustrated in fig.2. The nucleotide sequence of the expected coding region shares 82% homology with that of human 3β -HSD [10] while the interspecies sequence similarity of the 3' non-coding region is 65%. The sequence GCCAT-GG containing the initiating codon ATG corresponds to a consensus sequence for initiation by eukaryotic ribosomes [23]. In addition, a consensus polyadenylation signal AATAAA [24] starts 29 nucleotides upstream of the poly(A) tail.

The deduced bovine 3β -HSD protein consists of 372 amino acids, excluding the first Met, with a calculated molecular mass of 42 093 (fig.2) which is in close agreement with that of 42 126 of the human 3β -HSD enzyme. As shown in fig.3, the bovine 3β -HSD amino acid sequence shows 79% homology with that of human 3β -HSD [10]. Accordingly, both 3β -HSD proteins have very similar hydropathy profiles (fig.4). As observed for human 3β -HSD (fig.3), a consensus sequence corresponding to a potential *N*-glycosylation site (Asn-X-Thr) is located at amino acids 268–270 (excluding the first Met) in bovine 3β -HSD.

In order to examine the characteristics of 3β -HSD expression in bovine ovary, ³²P-labeled *Eco*RI fragments of clones bo3 β -HSD113 (fig.5, lane 1) or bo3 β -HSD123 (fig.5, lane 2) were separately hybridized to 5 μ g poly(A)⁺ RNA. Following 1 h exposure at -80° C, a



Fig.1. Restriction map of bovine ovary cDNA clones $bo3\beta$ -HSD113 and $bo3\beta$ -HSD123 encoding 3β -HSD and sequence analysis strategy. The protein coding region is represented by the black box and the 5'- and 3'-noncoding regions by the solid lines. The arrows beneath the schematic cDNA indicate the direction and extent of sequencing using synthetic oligonucleotide primers (\rightarrow) or T3 or T7 vector primers ($\circ \rightarrow$). A scale in base pairs (bp) is shown below.

			т	I'TCA'	TCTG	SCTTO	GCCA	CAATO	-80 CTGA	ccgci	ATCG	FCCT	CTGG	CCCA.	AACT	GCCCG	CCCA	GTGC	-40 TTTC	FGGT	гссто	GTG	AGCGI	TTC	I'CAG'	rgere	CAGAT	TTG	-1 SCC
ATG Met	GCA Ala	GGG Gly	TGG Trp	AGC Ser	TGC Cys	CTC Leu	GTG Val	ACC Thr	30 GGA Gly 10	GGA Gly	GGA Gly	GGC Gly	TTT Phe	CTG Leu	GGC Gly	CAG Gln	AGG Arg	ATC Ile	60 ATC Ile 20	TGC Cys	CTG Leu	TTG Leu	GTG Val	GAG Glu	GAG Glu	AAG Lys	GAT Asp	CTG Leu	90 CAG G1n 30
GAA Glu	ATC Ile	CGG Arg	GTG Val	CTA Leu	GAC Asp	AAA Lys	GTC Val	TTC Phe	120 AGA Arg 40	CCA Pro	GAA Glu	GTT Val	CGG Arg	GAG Glu	GAA Glu	TTT Phe	TCT Ser	AAG Lys	150 CTC Leu 50	CAG Gln	A GC Ser	AAG Lys	ATC Ile	AAG Lys	CTG Leu	ACC Thr	CTG Leu	CTG Leu	180 GAA Glu 60
GGA Gly	GAC Asp	ATT Ile	CTG Leu	GAT Asp	GAG Glu	CAG Gln	TGC Cys	CTG Leu	210 AAG Lys 70	GGG Gly	GCC Ala	TGC Cys	CAG Gln	GGC Gly	ACC Thr	TC A Ser	GTG Val	GTC Val	240 ATC Ile 80	CAC His	ACC Thr	GCC Ala	TCT Ser	GTC Val	ATT Ile	GAC Asp	GTC Val	AGG Arg	270 AAT Asn 90
GCT Ala	GTC Val	CCG Pro	CGA Arg	GAG Glu	ACC Thr	ATC Ile	ATG Met	AAC Asn	300 GTC Val 100	AAT Asn	GTG Val	AAA Lys	GGT Gly	ACC Thr	C A G Gln	CTG Leu	CTG Leu	TTG Leu	330 GAG Glu 110	GCC Ala	ŤGT Cys	GTC Val	CAG Gln	GCC Ala	AGC Ser	GTA Val	CCG Pro	GTC Val	360 TTT Phe 120
ATC Ile	CAC His	ACC Thr	AGC Ser	ACC Thr	ATA Ile	GAA Glu	GTG Val	GCT Ala	390 GGG Gly 130	CCC Pro	AAC Asn	TCC Ser	TAC Tyr	AGG Arg	GAG Glu	ATC Ile	ATC Ile	C AA Gln	420 GAC Asp 140	GGC Gly	CGT Arg	G AA Glu	GAA Glu	GAG Glu	CAT His	CAT His	GAA Glu	TCG Ser	450 GCA Ala 150
ТСС Тгр	TCC Ser	TCT Ser	CCA Pro	TAC Tyr	CCA Pro	TAC Tyr	AGC Ser	AAG Lys	480 AAG Lys 160	CTT Leu	GCC Ala	GAG Glu	AAG Lys	GCT Ala	GTG Val	CTG Leu	GGA Gly	GCT Ala	510 AAT Asn 170	GGG Gly	TGG Trp	GCT Ala	CTG Leu	AAA Lys	AAT Asn	GGT Gly	GGC Gly	ACC Thr	540 TTG Leu 180
ТАС Туг	ACT Thr	TGT Cys	GCC Ala	CTG Leu	AGG Arg	CCC Pro	ATG Met	TAC Tyr	570 ATC 11e 190	TAC Tyr	GGG G1y	GAG Glu	GGG Gly	AGC Ser	CCA Pro	TTC Phe	CTT Leu	TCT Ser	600 GCC Ala 200	TAC Tyr	ATG Met	CAC His	GGA Gly	GCC Ala	TTG Leu	AAT Asn	AAC Asn	AAC Asn	630 GGC Gly 210
ATC Ile	CTG Leu	ACC Thr	AAT Asn	CAC His	TGC Cys	AAG Lys	TTC Phe	ŤCA Ser	660 AGA Arg 220	GTC Val	AAC Asn	CCA Pro	GTC Val	TAT Tyr	GTT Val	GGC Gly	AAT Asn	GTG Val	690 GCC Ala 230	TGG Trp	GCC Ala	CAC His	ATT Ile	CTG Leu	GCC Ala	TTG Leu	AGG Arg	GCC Ala	720 CTG Leu 240
AGG Arg	GAC Asp	CCC Pro	AAA Lys	AAG Lys	GTC Val	CCA Pro	AAC Asn	ATC Ile	750 CAA Gln 250	GGA Gly	CAG Gln	TTC Phe	TAC Tyr	TAC Tyr	ATC Ile	TCA Ser	GAC Asp	GAC Asp	780 ACG Thr 260	CCA Pro	CAC His	CAA Gln	AGC Ser	TAC Tyr	GAT Азр	GAC Asp	CTC Leu	AAT Asn	810 TAC Tyr 270
ACT Thr	TTG Leu	A GC Ser	AAA Lys	GAA Glu	ТGG Тгр	GGC Gly	TTC Phe	TGC Cys	840 CTG Leu 280	GAT Asp	TCC Ser	CGG Arg	ATG Met	AGC Ser	CTT Leu	CCT Pro	ATT Ile	TCT Ser	870 CTG Leu 290	CAG Gln	TAC Tyr	TGG Trp	CTT Leu	GCC Ala	TTC Phe	CTG Leu	CTG Leu	GAA Glu	900 ATA Ile 300
GTG Val	AGC Ser	TTC Phe	CTG Leu	CTC Leu	AGT Ser	CCA Pro	ATT Ile	TAC Tyr	930 AAA Lys 310	TAT Tyr	AAC Asn	CCT Pro	TGC Cys	TTC Phe	AAC Asn	CGC Arg	CAC His	CTA Leu	960 GTG Val 320	ACT Thr	CTT Leu	TCC Ser	AAC Asn	AGC Ser	GTG Val	TTC Phe	ACC Thr	TTC Phe	990 TCC Ser 330
ТАТ Tyr	AAG Lys	AAA Lys	GCT Ala	CAG Gln	CGA Arg	GAT Asp	CTG Leu	GGG Gly	1020 TAT Tyr 340	GAG Glu	CCC Pro	CTC Leu	TAC Tyr	ACT Thr	TGG Trp	GAG Glu	GAA Glu	GCC Ala	1050 AAG Lys 350	CAG Gln	AAA Lys	ACC Thr	AAG Lys	GAG Glu	TGG Trp	ATT Ile	GGC Gly	TCC Ser	1080 CTG Leu 360
GTG Val	AAA Lys	C A G Gln	CAC His	AAA Lys	G A G Glu	ACC Thr	CTG Leu	AAA Lys	1110 ACA Thr 370	AAG Lys	ATT Ile	CAC His	TGA End	ссте	GGA	GTGAG	CAATO	GATG	1145 GGAA	CGTG	GACA	FTAG 1	TAG	CAGA	IGTC:	TATCO	GAGGO	CTC	1185 CCCT
тса	GGCT	гсат.	ACAG.	алас	таас	ACCG	ACAC	AAGC	1224 CTGG	STTC	гссто	SCCT	CCCT	FTTA	GAAG	ATGCI	CACO	TTA	1264 СТАТО	CTGC	TTC	cgccł	ACCAC	GAAAG	TGT	этсто	STCAP	TGG	1304 CCCA
AGT	GGGA	GCTT	гстт	ссст	ACCC	GCCT	CCAG	GGGA	1343 CAGA	CAAG	GTGA	FTTG	CTGC	AGCTO	SCTG	GCACO	CAAA	ATCT	1383 CAGTO	GCA	GATTO	CTGAC	STTAT	TTTGC	GCT	PCTTO	GTAAC	TTC	1423 GAGT
1462 1502 1542 TTTGCCTCTTAGTCCCACTTTCTTTGTTAAATGTGGAAGCATTTCTTTTAAAAGTTCATATTCCTTCATGTAGCTC <u>AATAAA</u> AATGATCAACATTTTCATGACTCAAAAAAAAAA											1542 AAAA																		

Fig.2. Nucleotide sequence of bovine 3β -HSD cDNA and deduced amino acid sequence. The full length cDNA structure was determined from the two overlapping bovine ovary λ gt11 clones bo3 β -HSD113 which included nucleotides – 104 to + 1248 and bo3 β -HSD123 which included nucleotides + 7 to + 1542. The single open reading frame beginning at the ATG codon is shown below the nucleotide sequence which is numbered in the 5' to 3' direction. Nucleotides are numbered above the sequence while amino acids (including the first Met) are numbered below the sequence. Nucleotides 5' of the ATG codon are given negative numbers. The putative polyadenylation signal AATAAA is underlined.

strong hybridization signal corresponding to 1.7 kb transcripts was obtained with both probes. When electrophoresed in parallel, the bovine ovary mRNA species migrates at the same position as human placental 3β -HSD mRNA [10].

Direct sequencing of the N-terminal amino acids of purified human placental 3β -HSD has shown that the first amino acid is a threonine [10] while the first two N- terminal amino acids of ovine adrenal 3β -HSD are alanine and glycine [7]. The deduced bovine 3β -HSD protein sequence thus shows the same two NH₂-terminal amino acids as reported for the ovine adrenal enzyme. Interestingly, the predicted NH₂-terminal amino acid sequence of bovine ovary 3β -HSD (fig.2) is in perfect agreement with the sequence NH₂-Ala-Gly-X-Ser-Cys-Leu-Val-Thr-Gly-Gly-X-X-Phe-Leu-Gly-Gln

HUMAN 3 β -HSD -TARKE-KA-GL	46
BOVINE 3 β -HSD MAGWSCLVTGGGGFLGQRIICLLVEEKDLQEIRVLDKVFRPEVREE	46
N-TVPFRDVICIFGVTHS	104
FSKLQSKIKLTLLEGDILDEQCLKGACQGTSVVIHTASVIDVRNAVPRETIMNVNVKG	104
N-HPL-NT-PAH	162
TQLLLEACVQASVPVFIHTSTIEVAGPNSYREIIQDGREEEHHESAWSSPYPYSKKLA	162
RNSSVGT	220
EKAVLGANGWALKNGGTLYTCALRPMYIYGEGSPFLSAYMHGALNNNGILTNHCKFSR	220
NF-L	278
VNPVYVGNVAWAHILALRALRDPKKVPNIQGQFYYISDDTPHQSYDDLNYTLSKEWGF	278
RW-F-LMIGRT-R-PI	336 336
A-KSVVDRS-TQ 373 DLGYEPLYTWEEAKQKTKEWIGSLVKQHKETLKTKIH 373	

Fig.3. Comparison of the deduced amino acid sequences of human and bovine 3\mathcal{\beta}-HSD. The complete bovine 3\mathcal{\beta}-HSD amino acid sequence is illustrated using the single letter code. Identical amino acids of human 3\mathcal{\beta}-HSD are represented by dashes (-) while different amino acids are identified.

very recently determined for the protein purified from bovine adrenal gland microsomes by Tamaoki's group [25]. Despite evidence that 3β -HSD isolated from human placental microsomes and mitochondria has the same immunological size as well as substrate- and inhibitor-specificity criteria [5,9], the possibility of more than one 3β -HSD exists.

Characterization of bovine 3β -HSD cDNA indicates that this enzyme is very well conserved throughout the course of evolution. The availability of the full-length bovine 3β -HSD cDNA will allow one to investigate in detail the tissue distribution of 3β -HSD expression and activity. In addition, study of the regulation of bovine



Fig.4. Comparison of the hydropathy profiles of human and bovine 3β -HSD proteins. The hydropathicity analysis of human and bovine 3β -HSD deduced amino acid sequences was performed according to the algorithm of Kyte and Doolittle [26] with a window of 9 amino acids. Positive and negative values on the y-axis indicate the degree of hydrophobicity and hydrophilicity, respectively.

 3β -HSD gene expression should provide much information on the regulation and role in different tissues of this crucial steroidogenic enzyme.



Fig.5. RNA blot analysis of bovine ovary 3β -HSD. 5 μ g of poly(A)⁺ RNA purified from bovine ovary were hybridized under stringent conditions with ³²P-labeled cDNA probes bo3 β -HSD113 (lane 1) or bo3 β -HSD123 (lane 2). Elements of the BRL 0.24-9.5 kb RNA ladder were used as molecular size markers.

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