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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 λgtll 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 Δ^5-Δ^4-ene-isomerase (EC 5.3.3.1), hereafter called 3β-HSD, catalyzes the oxidative conversion of Δ^5-ene-3β-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 3β-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 ambiguity 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 λgt11 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 λgt11 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 hp3β-HSD63 [10] as probe. The cDNA was labeled with [α-^{32}P]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 NaH_2PO_4, 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, 1×10^6 cpm [^{32}P]hp3β-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 × SSC/0.1% SDS at 25°C, once for 20 min in 2 × SSC/0.1% SDS at 40°C and 1 h in 1 × 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, [^{32}P]-labeled 3β-HSD cDNA fragment corresponding to the first 121 nucleotides of the 5′ end of human 3β-HSD cDNA hp3β-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 μg 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 bo3β-HSD113 or bo3β-HSD123 cDNA clones was performed at 42°C for 16 h. The fragments were labeled with [α-^{32}P]dCTP to a specific activity of 1×10^9 dpm/μg using a random primer method. The probes (2×10^6 cpm/ml) were added to hybridization buffer [21]. The filters were washed for 15 min in 2 × SSC/0.1% SDS at 25°C followed by 15 min washes in 0.1 × 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 λgt11 cDNA library was screened with human cDNA clone hp3β-HSD63 [10] as probe. Out of 5×10^5 recombinants, 90 positive clones were isolated and purified. Among the 90 clones obtained, two clones, bo3β-HSD113 and bo3β-HSD123, which hybridized with ^{32}P-labeled cDNA fragment corresponding to the first 121 nucleotides of hp3β-HSD63 clone, were characterized by a combination of restriction endonuclease mapping and DNA sequence analysis according to the strategy described in fig.1. The bo3β-HSD113 (1362 bp) and bo3β-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 GCCATGG 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, ^{32}P-labeled *Eco*RI fragments of clones bo3β-HSD113 (fig.5, lane 1) or bo3β-HSD123 (fig.5, lane 2) were separately hybridized to 5 μg poly(A)$^+$ RNA. Following 1 h exposure at −80°C, a

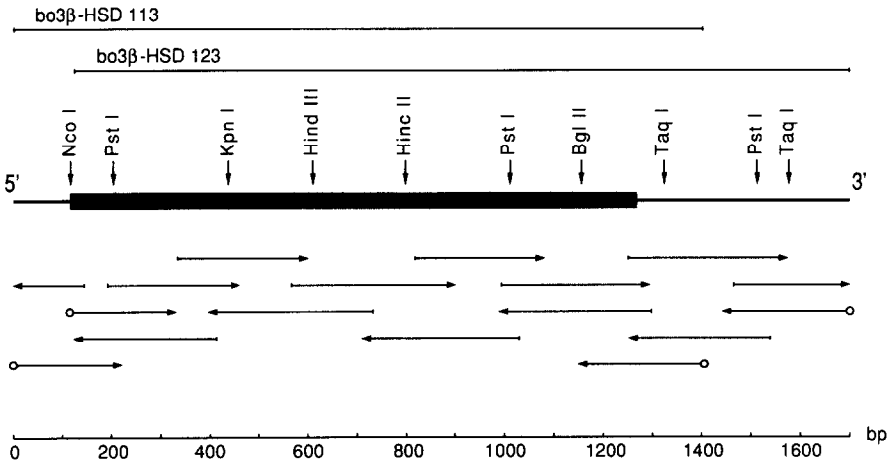

Fig.1. Restriction map of bovine ovary cDNA clones bo3β-HSD113 and bo3β-HSD123 encoding 3β-HSD and sequence analysis strategy. The protein coding region is represented by the black box and the 5′- and 3′-noncoding regions by the solid lines. The arrows beneath the schematic cDNA indicate the direction and extent of sequencing using synthetic oligonucleotide primers (→) or T3 or T7 vector primers (○→). A scale in base pairs (bp) is shown below.

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                                           -80                                         -40                                        -1
                   TTTCATCTGGCTTGCCACAATCTGACCGCATCGTCCTCTGGCCCAAACTGCCCCCCAGTGCTTTCTGGTTCCTGGTGAGCGTTTCTCAGTGCTCAGATTTGGCC

                                       30                                      60                                      90
ATG GCA GGG TGG AGC TGC CTC GTG ACC GGA GGA GGA GGC TTT CTG GGC CAG AGG ATC ATC TGC CTG TTG GTG GAG GAG AAG GAT CTG CAG
Met Ala Gly Trp Ser Cys Leu Val Thr Gly Gly Gly Gly Phe Leu Gly Gln Arg Ile Ile Cys Leu Leu Val Glu Glu Lys Asp Leu Gln
                                    10                                      20                                      30

                                       120                                     150                                     180
GAA ATC CGG GTG CTA GAC AAA GTC TTC AGA CCA GAA GTT CGG GAG GAA TTT TCT AAG CTC CAG AGC AAG ATC AAG CTG ACC CTG CTG GAA
Glu Ile Arg Val Leu Asp Lys Val Phe Arg Pro Glu Val Arg Glu Glu Phe Ser Lys Leu Gln Ser Lys Ile Lys Leu Thr Leu Leu Glu
                                    40                                      50                                      60

                                       210                                     240                                     270
GGA GAC ATT CTG GAT GAG CAG TGC CTG AAG GGG GCC TGC CAG GGC ACC TCA GTG GTC ATC CAC ACC GCC TCT GTC ATT GAC GTC AGG AAT
Gly Asp Ile Leu Asp Glu Gln Cys Leu Lys Gly Ala Cys Gln Gly Thr Ser Val Val Ile His Thr Ala Ser Val Ile Asp Val Arg Asn
                                    70                                      80                                      90

                                       300                                     330                                     360
GCT GTC CCG CGA GAG ACC ATC ATG AAC GTC AAT GTG AAA GGT ACC CAG CTG CTG TTG GAG GCC TGT GTC CAG GCC AGC GTA CCG GTC TTT
Ala Val Pro Arg Glu Thr Ile Met Asn Val Asn Val Lys Gly Thr Gln Leu Leu Leu Glu Ala Cys Val Gln Ala Ser Val Pro Val Phe
                                    100                                     110                                     120

                                       390                                     420                                     450
ATC CAC ACC AGC ACC ATA GAA GTG GCT GGG CCC AAC TCC TAC AGG GAG ATC ATC CAA GAC GGC CGT GAA GAA GAG CAT CAT GAA TCG GCA
Ile His Thr Ser Thr Ile Glu Val Ala Gly Pro Asn Ser Tyr Arg Glu Ile Ile Gln Asp Gly Arg Glu Glu Glu His His Glu Ser Ala
                                    130                                     140                                     150

                                       480                                     510                                     540
TGG TCC TCT CCA TAC CCA TAC AGC AAG AAG CTT GCC GAG AAG GCT GTG CTG GGA GCT AAT GGG TGG GCT CTG AAA AAT GGT GGC ACC TTG
Trp Ser Ser Pro Tyr Pro Tyr Ser Lys Lys Leu Ala Glu Lys Ala Val Leu Gly Ala Asn Gly Trp Ala Leu Lys Asn Gly Gly Thr Leu
                                    160                                     170                                     180

                                       570                                     600                                     630
TAC ACT TGT GCC CTG AGG CCC ATG TAC ATC TAC GGG GAG GGG AGC CCA TTC CTT TCT GCC TAC ATG CAC GGA GCC TTG AAT AAC AAC GGC
Tyr Thr Cys Ala Leu Arg Pro Met Tyr Ile Tyr Gly Glu Gly Ser Pro Phe Leu Ser Ala Tyr Met His Gly Ala Leu Asn Asn Asn Gly
                                    190                                     200                                     210

                                       660                                     690                                     720
ATC CTG ACC AAT CAC TGC AAG TTC TCA AGA GTC AAC CCA GTC TAT GTT GGC AAT GTG GCC TGG GCC CAC ATT CTG GCC TTG AGG GCC CTG
Ile Leu Thr Asn His Cys Lys Phe Ser Arg Val Asn Pro Val Tyr Val Gly Asn Val Ala Trp Ala His Ile Leu Ala Leu Arg Ala Leu
                                    220                                     230                                     240

                                       750                                     780                                     810
AGG GAC CCC AAA AAG GTC CCA AAC ATC CAA GGA CAG TTC TAC TAC ATC TCA GAC GAC ACG CCA CAC CAA AGC TAC GAT GAC CTC AAT TAC
Arg Asp Pro Lys Lys Val Pro Asn Ile Gln Gly Gln Phe Tyr Tyr Ile Ser Asp Asp Thr Pro His Gln Ser Tyr Asp Asp Leu Asn Tyr
                                    250                                     260                                     270

                                       840                                     870                                     900
ACT TTG AGC AAA GAA TGG GGC TTC TGC CTG GAT TCC CGG ATG AGC CTT CCT ATT TCT CTG CAG TAC TGG CTT GCC TTC CTG CTG GAA ATA
Thr Leu Ser Lys Glu Trp Gly Phe Cys Leu Asp Ser Arg Met Ser Leu Pro Ile Ser Leu Gln Tyr Trp Leu Ala Phe Leu Leu Glu Ile
                                    280                                     290                                     300

                                       930                                     960                                     990
GTG AGC TTC CTG CTC AGT CCA ATT TAC AAA TAT AAC CCT TGC TTC AAC CGC CAC CTA GTG ACT CTT TCC AAC AGC GTG TTC ACC TTC TCC
Val Ser Phe Leu Leu Ser Pro Ile Tyr Lys Tyr Asn Pro Cys Phe Asn Arg His Leu Val Thr Leu Ser Asn Ser Val Phe Thr Phe Ser
                                    310                                     320                                     330

                                      1020                                    1050                                    1080
TAT AAG AAA GCT CAG CGA GAT CTG GGG TAT GAG CCC CTC TAC ACT TGG GAG GAA GCC AAG CAG AAA ACC AAG GAG TGG ATT GGC TCC CTG
Tyr Lys Lys Ala Gln Arg Asp Leu Gly Tyr Glu Pro Leu Tyr Thr Trp Glu Glu Ala Lys Gln Lys Thr Lys Glu Trp Ile Gly Ser Leu
                                    340                                     350                                     360

                                      1110                                    1145                                    1185
GTG AAA CAG CAC AAA GAG ACC CTG AAA ACA AAG ATT CAC TGA CCTGGGAGTGACAATGATGGGAACGTGGACATTAGTTAGCAGATGTCTATCGAGGGCTCCCCT
Val Lys Gln His Lys Glu Thr Leu Lys Thr Lys Ile His End
                                    370

                                      1224                                    1264                                    1304
TGAGGCTTCATACAGAAAGTAACACGGACACAAGCCTGGGTTCTCCTGCCTCCCTTTTAGAAGATGCTCACCTTACTATCTGCCTTCCGCCACCAGAAACTGTGTCTGTCAATGGCCCA

                                      1343                                    1383                                    1423
AGTGGGAGCTTTCTTCCCTACCCGCCTCCAGGGGACAGACAAGGTGATTTGCTGCAGCTGCTGGCACCAAAATCTCAGTGGCAGATTCTGAGTTATTTGGGCTTCTTGTAACTTCGAGT

                                      1462                                    1502                                    1542
TTTGCCTCTTAGTCCCACTTTCTTTGTTAAATGTGGAAGCATTTCTTTTAAAAGTTCATATTCCTTCATGTAGCTCAATAAAAATGATCAACATTTTCATGACTCAAAAAAAAAAAAAA
```

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 λgt11 clones bo3β-HSD113 which included nucleotides −104 to +1248 and bo3β-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 NH_2-terminal amino acids as reported for the ovine adrenal enzyme. Interestingly, the predicted NH_2-terminal amino acid sequence of bovine ovary 3β-HSD (fig.2) is in perfect agreement with the sequence NH_2-Ala-Gly-X-Ser-Cys-Leu-Val-Thr-Gly-Gly-X-X-Phe-Leu-Gly-Gln

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HUMAN  3β-HSD -T--------A---------R---K--E-K-------A-G--L---  46
BOVINE 3β-HSD MAGWSCLVTGGGGFLGQRIICLLVEEKDLQEIRVLDKVFRPEVREE  46

-----N-T---V--------PF--R---DV--I----CI---FGVTH--S--------  104
FSKLQSKIKLTLLEGDILDEQCLKGACQGTSVVIHTASVIDVRNAVPRETIMNVNVKG  104

-----------------Y--S---------K----N-H---PL-NT-PA---H-----  162
TQLLLEACVQASVPVFIHTSTIEVAGPNSYREIIQDGREEEHHESAWSSPYPYSKKLA  162

-----A----N----------------------R----SINE--------SSVG---T  220
EKAVLGANGWALKNGGTLYTCALRPMYIYGEGSPFLSAYMHGALNNNGILTNHCKFSR  220

--------------------Q----A-S-R----------------N--------F-L  278
VNPVYVGNVAWAHILALRALRDPKKVPNIQGQFYYISDDTPHQSYDDLNYTLSKEWGF  278

R----W-F-L--M--IG----------R---T-R-P----I-----------------  336
CLDSRMSLPISLQYWLAFLLEIVSFLLSPIYKYNPCFNRHLVTLSNSVFTFSYKKAQR  336

--A-K---S---------V--V----DR------S-TQ  373
DLGYEPLYTWEEAKQKTKEWIGSLVKQHKETLKTKIH  373
```

Fig.3. Comparison of the deduced amino acid sequences of human and bovine 3β-HSD. The complete bovine 3β-HSD amino acid sequence is illustrated using the single letter code. Identical amino acids of human 3β-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 3β-HSD gene expression should provide much information on the regulation and role in different tissues of this crucial steroidogenic enzyme.

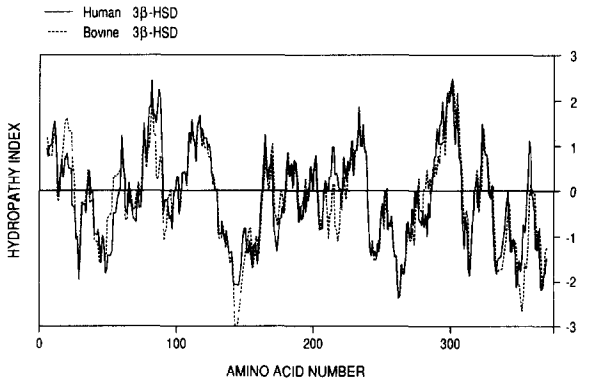

Fig.4. Comparison of the hydropathy profiles of human and bovine 3β-HSD proteins. The hydropathicity analysis of human and bovine 3β-HSD deduced amino acid sequences was performed according to the algorithm of Kyte and Doolittle [26] with a window of 9 amino acids. Positive and negative values on the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

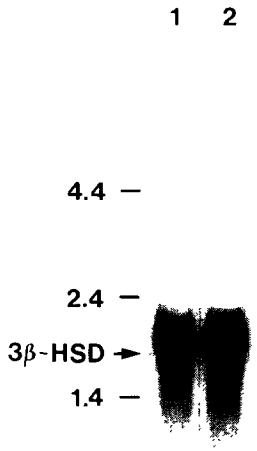

Fig.5. RNA blot analysis of bovine ovary 3β-HSD. 5 μg of poly(A)$^+$ RNA purified from bovine ovary were hybridized under stringent conditions with ^{32}P-labeled cDNA probes bo3β-HSD113 (lane 1) or bo3β-HSD123 (lane 2). Elements of the BRL 0.24-9.5 kb RNA ladder were used as molecular size markers.

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