Molecular cloning of cDNA for rat ovarian 20α-hydroxysteroid dehydrogenase (HSD1)

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20α-Hydroxysteroid dehydrogenase (20α-HSD, EC 1.1.1.149) catalyses the conversion of progesterone into 20α-dihydroprogesterone (20α-OHP). Previously, we purified the enzyme (37 kDa) from rat ovary and determined its N-terminal amino acid sequence. In the present study we succeeded in cloning a full-length 20α-HSD cDNA. mRNA was extracted from immature rat ovaries after successive treatment with equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG). A cDNA library was constructed in λZAP. For screening, a 576 bp probe was amplified by the PCR using mixed primers based on the N-terminal sequence of 20α-HSD, and labelled with [^{32}P]dCTP. Eight positive clones were isolated from 1.2×10^4 recombinants. Analysis of the nucleotide sequence revealed that one clone of 1.2 kbp cDNA (pHSD12-07) contained a polyadenylation site and an open reading frame encoding 323 amino acids with the N-terminal sequence of 20α-HSD. The fusion protein of pHSD12-07 produced by *Escherichia coli* reacted with a specific polyclonal antibody generated against rat ovarian 20α-HSD. In addition, the *in vitro* transcription–translation product produced by *Xenopus* oocytes showed 20α-HSD activity and Northern-blotting analysis revealed that the ovaries from normal adult rats contained a 1.2 kb mRNA. Thus we succeeded in isolating a clone encoding the full length of rat ovarian 20α-HSD. The sequence showed high similarity with those of rat liver 3α-hydroxysteroid dehydrogenase (3α-HSD), bovine lung prostaglandin F synthase (PGFS), human liver chlordecone reductase (CDR), frog lens ρ-crystallin and aldose reductases, indicating that 20α-HSD belongs to the aldo–keto reductase family.

INTRODUCTION

20α-Hydroxysteroid dehydrogenase (20α-HSD, EC 1.1.1.149) is an NADPH-dependent oxidoreductase which catalyses the conversion of progesterone into 20α-dihydroprogesterone (20α-OHP), a biologically inactive steroid. The biological functions of 20α-HSD have been studied intensively in the rat because the enzyme plays a pivotal role in murine reproduction [1–5]. Immunohistochemical studies have revealed that the enzyme is located in the cytoplasm of ovarian cells, including luteal cells and granulosa cells. Since the activity of 20α-HSD in the corpus luteum is suppressed by prolactin during the luteal phase and increased at end of pseudopregnancy and pregnancy [6,7], the activity seems to be under the regulation of pituitary hormones. However, the mechanism of induction of the enzyme is still obscure.

20α-HSD activity has been reported in various steroid-producing tissues such as ovary, testis, adrenal, uterus and placenta [1,8–11]. Since progesterone is the precursor of biologically active steroids such as androgen, oestrogen and corticoids, 20α-HSD may be involved in the regulation of biosynthesis of these hormones. 20α-HSD activity was also found in non-steroid-producing normal cells (lymphocytes, haemopoietic cells, erythrocytes and certain micro-organisms) [12–16] and in spontaneous neoplasms in animals [17], and is known to be induced by cytokines in the lymphocytes, including interleukin-3 and granulocyte macrophage-colony-stimulating factor [13]. Thus further knowledge about the enzyme would contribute to various fields including haematology and immunology.

The importance of 20α-HSD in reproduction has prompted interest in the mechanism of regulation of the enzyme's activity at the molecular level. Previously we purified 20α-HSD from rat ovarian cytosol and found that it consisted of two types of isoenzymes (HSD1 and HSD2) [18]. Recently we determined the partial amino acid sequence of HSD1. We also reported that the ovarian 20α-HSD mRNA was abundant in immature rats treated with equine chorionic gonadotropin/human chorionic gonadotropin (eCG/hCG) using a *Xenopus* oocyte translation system [19]. In the present paper we describe the molecular cloning of the cDNA of rat ovarian 20α-HSD (HSD1).

MATERIALS AND METHODS

Reagents

pfu DNA polymerase, λZAP, and pBluescript were purchased from Stratagene (La Jolla, CA, U.S.A.). Oligotex dT30, ligase, T4 polynucleotide kinase and pUC119 were from Takara Shuzo (Kyoto, Japan). Restriction endonucleases, avian-myeloblastosis-virus (AMV) reverse transcriptase, random primers and blocking reagent were from Boehringer Mannheim Yamanouchi (Tokyo, Japan). Agarose, an AutoRead sequencing kit and a TransProbe T kit were from Pharmacia LKB (Uppsala, Sweden). Progesterone, 20α-OHP, NADP, and NADPH were fwrom Sigma (St. Louis, MO, U.S.A.). [^{32}P]dCTP (3000 Ci/mmol)

Abbreviations used: 20α-HSD, 20α-hydroxysteroid dehydrogenase; 20α-OHP, 20α-dihydroprogesterone; eCG, equine chorionic gonadotropin; hCG, human chorionic gonadotropin; 3α-HSD, 3α-hydroxysteroid dehydrogenase; PGFS, prostaglandin F synthase; CDR, chlordecone reductase; IPTG, isopropyl β-D-thiogalactopyranoside; AMV, avian myeloblastosis virus; 1 × SSC, 0.15 M NaCl/0.015 M sodium citrate.

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The nucleotide sequence data reported in this paper have been submitted to the EMBL Nucleotide Sequence Database under the accession number D14424.

(a)

	3	4	5	6	7	8	9
20α-HSD	Ser	Lys	Ile	Gln	Lys	Met	Glu
	TCN	AAR	ATY	CAR	AAR	ATG	GAR
	AGY		ATA				
Forward primers	5'ACC	AAA	ATA	CAA	AAA	ATG	GA 3'
	TGT	G	C	G	G		
			T				
3α-HSD FGFS	188						194
	Cys	Asn	Gln	Val	Glu	Cys	His
	Cys	Asn	Gln	Val	Glu	Cys	His
	TGY	AAY	CAR	GTN	GAR	TGY	CAY
Reverse primers	3'ACA	TTA	GTC	CAN	CTC	ACA	GT 5'
	G	G	T		T	G	

(b)

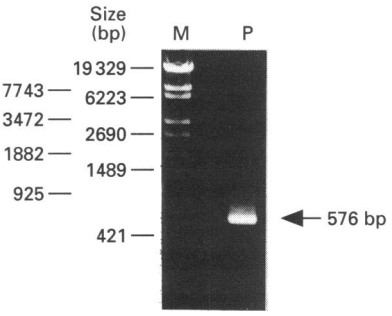

Figure 1 Design of PCR primers for screening of rat ovarian 20α-HSD

(**a**) Forward mixed primers were designed on basis of the N-terminal amino acid sequence of 20α-HSD (HSD1) (Ser^3 to Glu^9) [23]. The reverse mixed primers were conserved oligonucleotide sequences between the cDNAs of 3α-HSD and bovine lung [26–29]. Numbers above the amino acids indicate the positions in the amino acid sequences of 3α-HSD and PGFS. (**b**) PCR amplification using the primers. The PCR product was analysed by 1.0%-agarose electrophoresis, and a DNA fragment (576 bp) with the N-terminal sequence of 20α-HSD (HSD1) was amplified. M, marker; P; PCR product.

was from New England Nuclear (Boston, MA, U.S.A.). eCG and hCG were from Teikoku Zoki (Tokyo, Japan). Other reagents were from Wako Pure Chemicals (Osaka, Japan).

Animal treatments and tissue preparation

Immature and adult female rats (Wistar–Imamichi strain) were purchased from Imamichi Institute for Animal Reproduction (Ibaraki, Japan) and maintained under controlled lighting conditions of 14 h light and 10 h dark (light on at 05.00 h). Ovaries for the cDNA library were collected from the immature rats 18 days after hCG injection (25 i.u./0.1 ml of saline per rat, intravenously) following eCG injection [50 i.u./0.1 ml of saline per rat (26 days old), subcutaneously] [19]. For Northern-blotting analysis ovaries were also collected from normal adult rats (10 weeks old). The ovaries were stored at −80 °C until RNA extraction.

Synthesis of ovarian 20α-HSD cDNA

RNA was extracted by the CsCl ultracentrifugation method as reported previously [20]. Poly(A) RNA was selected with Oligotex dT30 by the method described previously [21]. A yield of 1–3 mg of total RNA or 50–200 μg of poly(A) RNA was obtained from 1 g of tissue. The poly(A) RNA was used for the synthesis of first-strand cDNA for PCR by AMV reverse transcriptase or double-strand cDNA for construction of the cDNA library in λZAP.

Amplification of the DNA probe by PCR for 20α-HSD screening

The probe used for screening of the cDNA library was amplified by PCR [22]. Mixed primers were designed based on the N-terminal amino acid sequence of 20α-HSD (HSD1) [23]. The forward primer, 5′-(A)(T)/(C)(G)/(C)(T) AA/(A)(G) AT/(A)(C)(T) CA/(A)(G) AA/(A)(G) ATG GA-3′, and the reverse primer, 3′-AC/(A)(G) TT/(A)(G) GT/(C)(T) CAN CT/(C)(T) AC/(A)(G) GT-5′, were synthesized with a DNA Cyclone Plus (Milligen Bioresearch, MA, U.S.A.) apparatus. PCR was carried out in a Quick Thermo (Nippon Genetics, Tokyo, Japan) instrument for 30 cycles of 91 °C for 1 min, 45 °C for 1 min and 72 °C for 2 min. The final volume was 100 μl, containing the cDNA, 20 mM Tris/HCl, pH 8.8, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 mM dNTP, each primer at 1.0 mM and 2.5 units of *pfu* DNA polymerase. The PCR produced was cloned in pUC119, which was transformed in *Escherichia coli* strain XL1-blue. After sequencing, the specific DNA fragment of 20α-HSD (hsd23-7) was obtained and labelled with [^{32}P] dCTP by random primers.

Construction of cDNA library

A cDNA library was constructed in λZAP, and 1.2×10^6 recombinants were obtained. Approx. 1.2×10^4 plaques of the library were plated and transferred to nylon filters (Hybond-N; Amersham, Tokyo, Japan). They were hybridized with the [^{32}P]dCTP-labelled probe (hsd23-7) for 15 h at 45 °C in 5 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.02% SDS, 0.1% dodecanoylsarcosine and 1% blocking reagent. After washing with 1 × SSC/0.1% SDS at 25 °C for 5 min and with 0.2 × SSC/0.1% SDS for 10 min at 60 °C, the filters were dried and exposed to X-ray film for 15 h at −80 °C. The phagemids from eight selected plaques were transformed into *E. coli* XL1-blue, and single-stranded DNAs were prepared as described elsewhere [24].

DNA sequencing and sequence analysis

DNA sequencing was performed by the dideoxy chain-termination method using an AutoRead sequencing kit and A.L.F. DNA sequencer (Pharmacia LKB, Uppsala, Sweden). The sequences were obtained for both strands and with all restriction sites overlapped. Sequence analysis was performed with MacMollyTetra computer software (Soft Gene, Berlin, Germany).

Production of the 20α-HSD–β-galactosidase fusion protein and analysis of Western blotting

Cultures of *E. coli* containing the phagemids (pHSD12-07) with or without 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) were grown for 3 h at 37 °C. The cultures were pelleted and resuspended in SDS/PAGE loading buffer. Proteins of *E. coli* were separated by SDS/10%-PAGE and transferred to poly(vinylidene difluoride) membrane (Millipore, Bedford, MA, U.S.A.). Western-blotting analysis was performed using a specific polyclonal antibody against rat ovarian 20α-HSD produced by

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                                                    Met Asn Ser Lys Ile Gln Lys Met
       GAC TCT TCT AGG GAA GAG CAG CAT CTG AGA ATG AAT TCC AAA ATT CAG AAG ATG
     -30                                            1
Glu Leu Asn Asp Gly His Ser Ile Pro Val Leu Gly Phe Gly Thr Tyr Ala Thr Glu Glu
GAG TTA AAC GAT GGT CAC TCC ATC CCT GTA CTG GGC TTT GGC ACC TAT GCA ACC GAA GAG
  30                                            60
Asn Leu Arg Lys Lys Ser Met Glu Ser Thr Lys Ile Ala Ile Asp Val Gly Phe Arg His
AAT CTC AGA AAA AAG TCT ATG GAG TCC ACG AAA ATA GCT ATA GAT GTT GGG TTC CGC CAT
  90                                            120
Ile Asp Cys Ser His Leu Tyr Gln Asn Glu Glu Glu Ile Gly Gln Ala Ile Val Ser Lys
ATT GAT TGT TCT CAC TTG TAC CAG AAT GAA GAA GAG ATA GGT CAG GCC ATT GTA AGC AAG
 150                                            180
Ile Glu Asp Gly Thr Val Lys Arg Glu Asp Ile Phe Tyr Thr Ser Lys Leu Trp Ser Thr
ATT GAA GAT GGC ACT GTG AAA AGG GAA GAT ATA TTC TAT ACT TCA AAG CTT TGG TCA ACT
 210                                            240
Ser His Arg Pro Glu Leu Val Arg Pro Ser Leu Glu Asn Ser Leu Arg Lys Leu Asn Leu
TCC CAT CGT CCA GAG TTG GTC AGA CCC AGC TTG GAA AAT TCA CTG AGG AAA CTT AAT TTG
 270                                            300
Asp Tyr Val Asp Leu Tyr Leu Ile His Phe Pro Val Ser Leu Lys Pro Gly Asp Glu Leu
GAC TAT GTA GAC CTC TAT CTC ATT CAT TTC CCG GTA TCT CTG AAG CCA GGG GAT GAG CTT
 330                                            360
Leu Pro Gln Asp Glu His Gly Asn Leu Ile Leu Asp Thr Val Asp Leu Cys Asp Thr Trp
TTA CCT CAA GAT GAG CAT GGA AAC TTA ATA CTT GAC ACA GTG GAT CTC TGC GAC ACC TGG
 390                                            420
Glu Ala Met Glu Lys Cys Lys Asp Ala Gly Leu Ala Lys Ser Ile Gly Val Ser Asn Phe
GAG GCC ATG GAG AAG TGT AAG GAT GCA GGA TTG GCC AAG TCC ATC GGG GTG TCC AAC TTT
 450                                            480
Asn Arg Arg Gln Leu Glu Lys Ile Leu Asn Lys Pro Gly Leu Lys His Arg Pro Val Cys
AAC CGC AGG CAG CTG GAG AAG ATC CTG AAT AAG CCG GGG CTC AAG CAC AGG CCT GTG TGC
 510                                            540
Asn Gln Val Glu Cys His Leu Tyr Leu Asn Gln Ser Lys Leu Leu Ala Tyr Cys Lys Met
AAC CAG GTA GAA TGC CAT CTT TAT CTC AAC CAG AGC AAG CTG CTC GCT TAC TGC AAG ATG
 570                                            600
Asn Asp Ile Val Leu Val Ala Tyr Gly Ala Leu Gly Thr Gln Arg Tyr Lys Tyr Cys Ile
AAT GAC ATC GTT CTG GTT GCC TAT GGT GCC CTG GGA ACT CAG AGA TAC AAA TAC TGT ATT
 630                                            660
Asn Glu Asp Thr Pro Val Leu Leu Asp Asp Pro Ile Leu Cys Thr Met Ala Lys Lys Tyr
AAT GAA GAT ACC CCA GTT CTC TTG GAT GAT CCC ATT CTT TGT ACC ATG GCA AAG AAG TAC
 690                                            720
Lys Arg Thr Pro Ala Leu Ile Ala Leu Arg Tyr Gln Leu Glu Arg Gly Ile Val Thr Leu
AAG CGG ACT CCA GCC CTG ATT GCC CTT CGC TAC CAG CTG GAG CGT GGG ATT GTG ACC CTA
 750                                            780
Val Lys Ser Phe Asn Glu Glu Arg Ile Arg Glu Asn Leu Gln Val Phe Asp Phe Gln Leu
GTC AAG AGT TTC AAT GAG GAG AGA ATC AGA GAG AAC CTG CAG GTC TTT GAT TTC CAG TTG
 810                                            840
Ala Ser Asp Asp Met Glu Ile Leu Asp Asn Leu Asp Arg Asn Leu Arg Tyr Phe Pro Ala
GCT TCA GAT GAC ATG GAA ATT TTA GAT AAC CTG GAC AGA AAT CTT CGG TAC TTT CCT GCT
 870                                            900
Asn Met Phe Lys Ala His Pro Asn Phe Pro Phe Ser Asp Glu Tyr TER
AAT ATG TTT AAG GCT CAC CCT AAC TTT CCA TTC TCT GAT GAA TAC TAA GAT GGC AGC CCT
 930                                            960         969
AGC CAT GAG TTC TGC TCG AAG CTC CTT TGT GTG ATG CTG GAC TCT CAG AGG CCA ATA ATA
 990                                           1020
CAA CAC ACT GAC TCC AAT CCA TAC TGC TTA GCA ACT CAC CCC CCA GTT TGA GCT GTG TCT
1050                                           1080
GTA CAT CGG GGA GCA AAT TCA CTA AAT TTT CCT GCT TTT CTG TAA ATA AAT AAA AAT ATT
1110                                           1140
TTG CTT CAG CCT C.....
1170
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Figure 2 Sequences of nucleotides and deduced amino acids of rat ovarian 20α-HSD cDNA

The deduced amino acid sequence (pHSD12-07) is given above the nucleotide sequence of rat ovarian 20α-HSD. Nucleotides and amino acids are numbered from the ATG initiation codon. 20α-HSD contains 969 nucleotides encoding 323 amino acids. The shaded boxed amino acid sequence is identical with a 19-amino-acid sequence of 20α-HSD (HSD1). The boxed nucleotide sequence is the polyadenylation signal (AATAAA). The underlined nucleotides correspond to the forward and reverse primers.

immunizing a mouse with purified 20α-HSD (HSD1) [18] by the method reported previously [19].

In vitro transcription of the rat 20α-HSD mRNA and translation in *Xenopus* oocytes

Capped mRNA was synthesized by *in vitro* transcription of pHSD12-07 cleaved by *Kpn*I using T3 RNA polymerase (Trans-Probe T kit). The mRNA was translated using a *Xenopus laevis* oocyte system according to the method reported previously [25]. Oocytes, 100 in all, were injected with the mRNA (1 μg/μl; 20 nl/oocyte) and then incubated at 20 °C for 36 h in modified Barths' saline. mRNA extracted from immature rat ovary was injected as a positive control. The oocytes were homogenized with a Teflon/glass homogenizer in 2 ml of 5 mM Tris/HCl buffer, pH 7.6, containing 0.1 M NaCl, 10% glycerol, 1 mM phenylmethanesulphonyl fluoride and 5 μg/ml leupeptin. After centrifugation at 10000 *g* for 60 min at 4 °C, 20α-HSD activity in

the supernatant was measured by *in vitro* conversion of progesterone into 20α-OHP and expressed as μg of 20α-OHP/ml of cytosol according to the method described previously [19].

Northern-blotting analysis

The total RNA was resuspended in electrophoresis buffer [20 mM Mops (pH 7.0)/5 mM sodium acetate and 1 mM EDTA], containing 50% formamide and 6.5% formaldehyde and heated at 65 °C for 15 min. The RNA (40 μg) was electrophoresed on 1.2% agarose/formamide gel and then transferred to a nylon membrane. The membrane was hybridized with [^{32}P]dCTP-labelled 20α-HSD cDNA, which was a 1.2 kb fragment of pHSD12-07 cleaved by *Eco*RI and *Pst*I, in 5 × SSC containing 50% formamide, 0.02% SDS, 0.1% dodecanoylsarcosine, and 5% blocking reagent. After washing with 1 × SSC/0.1% SDS at 25 °C for 5 min and 0.2 × SSC/0.1% SDS at 60 °C for 1 min, the membrane was exposed to X-ray film at −80 °C for 15 h.

RESULTS

Amplification of the cDNA probe for screening of the rat ovarian cDNA library

The N-terminal amino acid sequence (3–21) of rat ovarian 20α-HSD was as follows: N-Ser^{3}-Lys^{4}-Ser^{5}-Leu^{6}-Arg^{7}-Met^{8}-Glu^{9}-Leu^{10}-Asn^{11}-Asp^{12}-Gly^{13}-His^{14}-Ser^{15}-Ile^{16}-Pro^{17}-Val^{18}-Leu^{19}-Gly^{20}-Phe^{21}-C [23]. Since a search of the SWISS-PROT protein database revealed that the sequence (Leu^{10}–Phe^{21}) was very similar to those of rat liver 3α-HSD (with Asn and Phe only instead of His^{14} and Ser^{15}) [26–28] and bovine lung PGFS (with Phe only instead of Ser^{15}) [29], we designed mixed primers for PCR in order to screen to the rat ovarian cDNA library (Figure 1a). The forward primer corresponded to the sequence Ser^{3}–Glu^{9}, which was specific to rat ovarian 20α-HSD. The reverse primer, selected from 3α-HSD (Cys^{188}–His^{194}), was a region common with that of PGFS (Cys^{188}–His^{194}).

Using these primers and the first-strand cDNA transcripted from the immature rat ovarian mRNA, a 576 bp band was amplified by PCR (Figure 1b). The nucleotide sequence of the PCR product (hsd 23-7) was found to encode a peptide of 192 amino acids, including 19 amino acids from the N-terminal sequence of rat ovarian 20α-HSD (HSD1). Since hsd23-7 showed high similarity to, but was not exactly identical with, the corresponding regions of 3α-HSD (73.6%) and PGFS (75.3%), we assumed that the cDNA encoded part of the amino acid sequence of 20α-HSD. Although Ser^{3} in 20α-HSD was changed to Cys (TGC) in the amplified cDNA fragment, this site was thought to be mismatched by PCR because this was the 5′ end of the forward primer. Then we screened the λZAP-cDNA library of immature rat ovaries to isolate the full length of 20α-HSD using ^{32}P-labelled hsd23-7.

Isolation of the 20α-HSD cDNA clone

More than 100 positive signals were detected in 1.0 × 10^{4} recombinants. After the second screening, eight clones were selected and their nucleotide sequences were determined. Four clones encoded a common long nucleotide sequence. As shown in Figure 2, the nucleotide sequence of one of the clones (pHSD12-07) contained an open reading frame of 969 bp encoding a 323-amino-acid sequence. The non-coding region at the 5′ end was 30 bp long. A polyadenylation signal and a poly(A) tail were contained in the 408 bp 3′ untranslated region. The deduced amino acid sequence completely matched the N-terminal 19-amino-acid sequence of 20α-HSD determined previously [23].

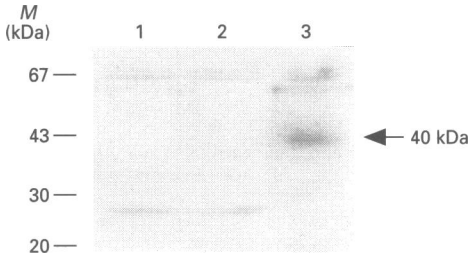

Figure 3 Expression of the 20α-HSD–β-galactosidase fusion protein by *E. coli*

The lysate of the transformant obtained with 20α-HSD cDNA (pHSD12-07) was analysed by Western blotting. Lane 1, *E. coli* lysate without vector (pBluescript) or 20α-HSD clone (pHSD12-07); lane 2, vector; lane 3, transformant with 20α-HSD clone. Note the specific protein with molecular mass (*M*) of 40 kDa in lane 3.

Table 1 20α-HSD activity in *Xenopus* oocytes microinjected with mRNA transfected *in vitro*

Synthesized mRNA was transcribed *in vitro* from pHSD12-07 cleaved by *Kpn*I using T3 RNA polymerase. Ovarian mRNA extracted from immature rats treated with eCG/hCG was used as a positive control. *Xenopus* oocytes microinjected with the mRNA preparation were incubated in modified Barths' saline at 20 °C for 36 h. Enzymic activity of 20α-HSD in the oocyte cytosol is expressed as the amount of 20α-OHP converted from progesterone in the presence of NADPH. Results are means ± S.E.M. for five determinations. Abbreviation: ND, not detected.

mRNA preparation	20α-HSD activity (20α-OHP ng/μg of mRNA)
None	ND
Ovarian mRNA	202.3 ± 11.9
Synthesized mRNA	320.8 ± 27.8

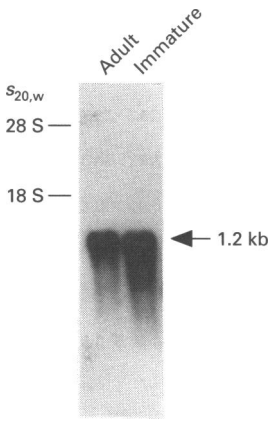

Figure 4 Northern-blotting analysis of rat ovarian 20α-HSD mRNA

A 40 μg sample of total RNAs from normal adult rat (lane 1) or immature rat treated with eCG/hCG (lane 2) was subjected to 1.2%-agarose/formamide-gel electrophoresis and transferred to a nylon membrane. It was then hybridized with a ^{32}P-labelled rat ovarian 20α-HSD cDNA. The mobilities of 28 S and 18 S are indicated on the left, and arrow shows 1.2 kb 20α-HSD mRNA.

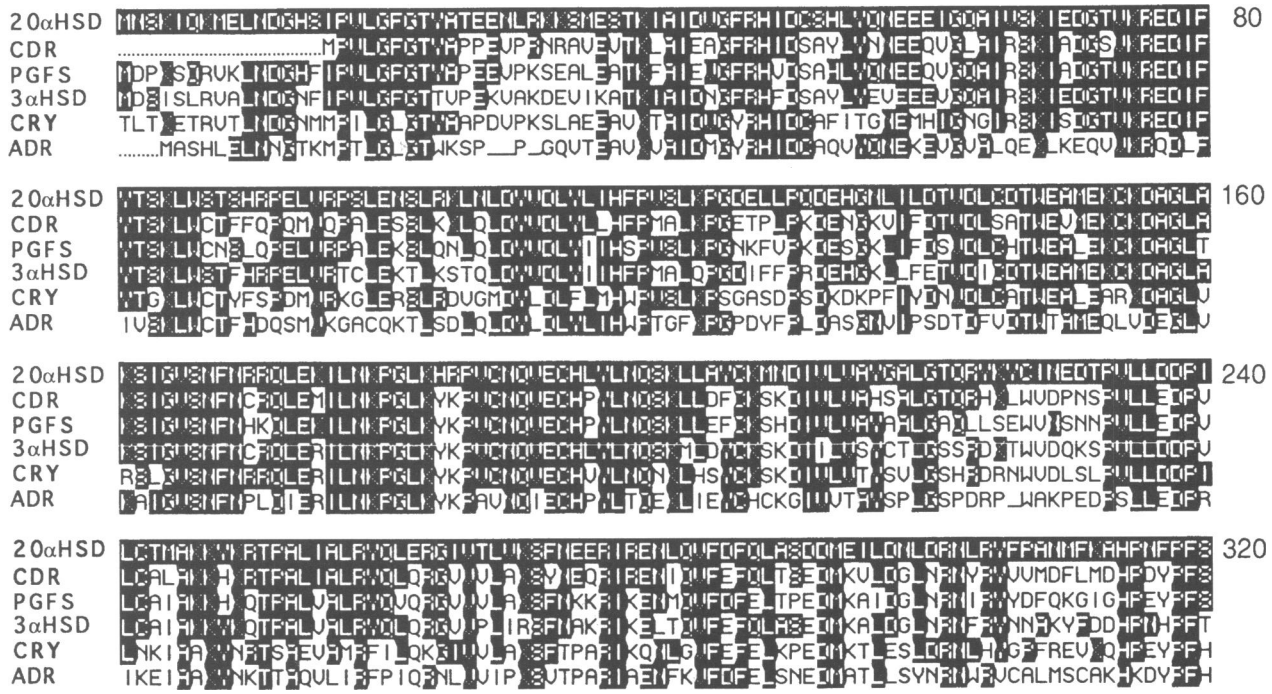

Figure 5 Comparison of deduced amino acid sequence of 20α-HSD with the aldo–keto reductase family

20αHSD, rat ovary (the present work); PGFS, bovine lung, [29]; 3αHSD, [26–28]; CDR, [30]; CRY, [31]; ADR, rat aldose reductase [32]. Black-boxed amino acids are those in CDR, PGFS, 3α-HSD, CRY and ADR corresponding to the 20α-HSD sequence.

Table 2 Structural similarity among 20α-HSD and representative members of the aldo–keto reductase family

The similarity of amino acid and nucleotide sequences of 20α-HSD with members of the aldo–keto reductase family is shown. Values above the diagonal indicate nucleotide sequence similarity. Values under the diagonal indicate coding amino-acid-sequence similarity. The names over each column represent the sequence with the corresponding row names. 20αHSD, rat ovary 20α-HSD (the present work); PGFS, bovine lung PGFS [29]; 3αHSD [26–28]; CDR, human liver CDR [30]; CRY, bullfrog lens ρ-crystallin [31]; ADR rat aldose reductase [32]. The nucleotide sequence of bullfrog lens ρ-crystallin has not been published.

	20αHSD	3αHSD	PGFS	CDR	CRY	ADR
20αHSD		71.7	65.6	72.9	–	48.2
3αHSD	65.6		71.0	69.2	–	44.8
PGFS	66.1	66.0		74.5	–	35.3
CDR	65.3	65.1	70.2		–	41.6
CRY	40.9	31.5	37.6	39.7		–
ADR	38.1	36.3	40.7	44.0	38.1	

Expression of a 20α-HSD–β-galactosidase fusion protein in *E. coli* and 20α-HSD activity in the *Xenopus* oocyte translation system

20α-HSD–β-galactosidase fusion protein of pHSD12-07 was detected by Western-blotting analysis (Figure 3). The molecular mass of 20α-HSD evaluated by SDS/PAGE was 33 kDa [18] and that of the fusion protein was calculated to be 40 kDa. As shown in lane 3, a 40 kDa specific protein was produced in *E. coli* containing pHSD12-07, indicating that the clone (pHSD12-07) represented rat ovarian 20α-HSD (HSD1).

The mRNA transcribed from pHSD12-07 *in vitro* was subjected to translation in *Xenopus* oocytes (Table 1). 20α-HSD activity was detected in the product of the mRNA as well as that of ovarian mRNA from eCG/hCG-treated immature rat, but was not detected in control oocytes. Thus the transcription product from the clone (pHSD12-07) had 20α-HSD activity. The lower activity of the synthesized 20α-HSD mRNA was probably due to its shorter half-life, because its poly(A) tail would be expected to be shorter than that of ovarian mRNA. Another reason may be related to the percentage of mRNA molecules carrying caps. The efficiency of capped RNA generation is estimated to be 30–90 % according to the manufacturer's instructions (TransProbe T kit).

Northern-blotting analysis of immature-rat and normal-adult-rat ovarian mRNAs

Rat corpus luteum is the tissue richest in 20α-HSD. Ovaries in normal adult rats consist of multiple sets of corpora lutea, whereas those in immature rats treated successively with eCG and hCG possess a single generation of corpora lutea. Using pHSD12-07 as a cDNA probe, a band of approx. 1.2 kb was detected in ovarian total RNAs from immature rat and normal adult rat (Figure 4). Thus the size of the mRNA matched that of the cDNA (pHSD12-07), and was similar between the tissue preparations. Although the washing conditions for the Northern blotting were low stringency (0.2 × SSC/0.1 % SDS at 60 °C for 1 min), the cDNA probe did not cross-hybridize with other mRNAs, including 1.4 or 2.4 kb 3α-HSD mRNA [28]. A similar result was obtained when analysis was done under high-stringency conditions (0.2 × SSC/0.1 % SDS at 55 °C for 30 min; results not shown).

Analysis of 20α-HSD sequence

A search of GenBank/EMBL and the SWISS-PROT databases revealed several proteins that were similar to 20α-HSD. The proteins with similar sequences included rat liver 3α-HSD [26–28], bovine lung prostaglandin F synthase (PGFS) [29], human liver chlordecone reductase (CDR) [20], frog lens ρ-crystallin [31] and mammalian aldose reductase [32–35] (Figure 5). Similarities of the nucleotide and deduced amino acid sequences among these proteins are shown in Table 2. Similarity among 20α-HSD, 3α-HSD, PGFS, and CDR was more than 65% in both amino acid and nucleotide sequences.

DISCUSSION

We succeeded in isolating a 1.2 kb cDNA encoding rat ovarian 20α-HSD (HSD1) from a cDNA library in λZAP using a PCR-amplified probe based on the N-terminal sequence of 20α-HSD. The cDNA contained a polyadenylation signal and an open reading frame encoding 323 amino acids corresponding to the N-terminal sequence of 20α-HSD (HSD1) we had determined previously [23]. Using the cDNA (pHSD12-07), (1) the fusion protein with β-galactosidase produced by *E. coli* reacted with a specific antibody against rat ovarian 20α-HSD, (2) the *in vitro* transcription–translation product produced by *Xenopus* oocytes showed 20α-HSD activity, and (3) the ovarian mRNA from immature rats treated with eCG/hCG and that from normal adult rats were found to be of similar size (1.2 kb) by Northern-blotting analysis. From these results we concluded that the clone (pHSD12-07) encodes the full length of rat ovarian 20α-HSD.

Analysis of the secondary structure of rat ovarian 20α-HSD by Chou–Fasman folding revealed that the enzyme contains repeated α-helix and β-sheet structures. There is a βαβαβ structure between Met^{1} and Cys^{51}, and this motif contains Gly^{20}, Gly^{22} and Gly^{45}, which are common amino acids in this motif in other NADPH-dependent enzymes [36]. Taken together with previous reports that the βαβαβ structure located at the N-terminal in many other oxidoreductases is responsible for binding to NADPH [36], rat ovarian 20α-HSD seems to possess the cofactor-binding site in this domain.

Sequence analysis revealed that the 20α-HSD is similar to other cytosolic proteins of the aldo–keto reductase family [37]. The amino acid sequence showed high similarity with rat liver 3α-HSD, which has been recognized to be multi-functional, since the enzyme metabolizes steroids, prostaglandins, bile acids and xenobiotics [38–41]. On the basis of the molecular size and amino-acid-sequence similarity the members of this family are divided into two groups: proteins of about 250 amino acids (short-chain-alcohol dehydrogenase-type) and 300 amino acids (long-chain-alcohol dehydrogenase-type) [42]. We found previously that purified 20α-HSD from rat ovary had an estimated molecular mass of 33 kDa by SDS/PAGE [18] and 37 kDa by time-of-flight mass-spectrometric analysis [23]. The cDNA cloned in the present study indicated that rat ovarian 20α-HSD consists of 323 amino acids with a calculated molecular mass of 37304 Da. Thus we confirmed our previous result [23] and clarified that rat ovarian 20α-HSD seems to be a member of the long-chain-alcohol dehydrogenase-type aldo–keto reductase family.

To date, several 20α-HSDs have been purified from rat ovary [18], bovine testis [8], porcine adrenal [10], porcine testis [43], fetal bovine erythrocytes [14], fetal ovine erythrocytes [44], human placenta (17β-HSD) [45], *Streptomyces hydrogenas* [15] and *Clostridium scindens* [46]. Of these, the human placental and porcine adrenal 20α-HSDs have different substrate specificity from the rat ovarian enzyme. However, the 20α-HSDs from fetal bovine erythrocytes, fetal ovine erythrocytes and *Streptomyces hydrogenas* preferably metabolize progesterone and have molecular masses of 50–55 kDa, and thus seem to belong to the long-chain-alcohol dehydrogenase type. The 20 α-HSDs from bovine and porcine testis and porcine adrenal are single polypeptides of 34–40 kDa and seem to be structurally similar to the rat ovarian 20α-HSDs, although molecular cloning of these enzymes has not been reported. Recently, a cDNA encoding rabbit ovarian 20α-HSD has been isolated [47]. The similarity of the amino acid sequences of the rat and rabbit 20α-HSDs was 70%.

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