Cloning and expression of chicken 20-hydroxysteroid dehydrogenase

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Abstract

The ligand specificity and activation of steroid receptors depend considerably on the enzymatic activities involved in local pre-receptor synthesis and the metabolism of the steroids. Several enzymes in particular, steroid dehydrogenases have been shown to participate in this process. Here we report the isolation of 20-hydroxysteroid dehydrogenase (ch20HSD) cDNA from chicken intestine and the distribution of ch20HSD mRNA and 20-reductase activity in various avian tissues. Using a reverse transcription PCR and comparison with the known sequences of mammalian 20βHSDs, we have isolated a new ch20HSD cDNA. This cDNA predicted 276 amino acid residues that shared about 75% homology with mammalian 20βHSD. Sequences specific to the short-chain dehydrogenase/reductase superfamily (SDR) were found, the *Gly-X-X-X-Gly-X-Gly* cofactor-binding motif (residues 11–17) and the catalytic activity motif *Tyr-X-X-X-Lys* (residues 193–197). The cDNA coding for ch20HSD was expressed in *Escherichia coli* by placing it under isopropylthiogalactoside (IPTG) inducible control. Both the IPTG cells of *E. coli* and the isolated recombinant protein reduced progesterone to 20-dihydroprogesterone, corticosterone to 20-dihydrocorticosterone and 5α-dihydrotestosterone to its 3-ol derivative. The 20-reductase and 3-reductase activities of ch20HSD catalyzed both 3α/β- and 20α/20β-epimers. The mRNA transcripts of ch20HSD were found in the kidney, colon, and testes; weaker expression was also found in the heart, ovaries, oviduct, brain, liver, and ileum. 20-Reductase activity has been proven in tissue slices of kidney, colon, ileum, liver, oviduct, testis, and ovary; whereas the activity was nearly absent in the heart and brain. A similar distribution of 20-reductase activity was found in tissue homogenates measured under V_{max} conditions. These results suggest that chicken 20HSD is the latest member of the SDR superfamily to be found, is expressed in many avian tissues and whose precise role remains to be determined.

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Introduction

Hydroxysteroid dehydrogenases (HSDs) are enzymes that play an important role in the biosynthesis and inactivation of steroid hormones. Due to their oxidase and reductase activities, they catalyze the interconversion of biologically active hormones and their less active or inactive derivatives and thus regulate the local concentration of steroid ligands and the transcription of corresponding genes (Penning 1997). HSDs catalyze the stereospecific and reversible conversion of hydroxyl and carbonyl groups at the C_3-, C_{11}-, C_{17}-, and C_{20}-positions and belong to two distinct protein superfamilies, short-chain dehydrogenases (SDR) and aldo-keto reductases (AKR; Jörnvall *et al.* 1995, Penning 1997). One of these enzymes is 20β-hydroxysteroid dehydrogenase (20βHSD) that was originally found in fish ovaries (Nagahama & Adachi 1985) and in pig neonatal testis (Nakajin *et al.* 1988*a*, Ohno *et al.* 1992). In addition to being present in pig testicular tissue where it may regulate hormone concentration during development (Ohno *et al.* 1992), later studies revealed that this enzyme is expressed in many other porcine tissues, including the kidney, liver, heart, lung, and brain (Kobayashi *et al.* 1996) but the role of 20βHSD is still ill-defined. Tanaka *et al.* (1992) showed that porcine 20βHSD is a cytosolic enzyme that belongs to the SDR superfamily.

We have reported that 20HSD activity in chicken intestine is co-localized with 11β-hydroxysteroid dehydrogenase (11βHSD) activity and demonstrated that 20HSD plays a role in modulating the potency of glucocorticoids to stimulate intestinal Na^+ transport (Vylitová *et al.* 1998, Mazancová *et al.* 2005). It has been suggested that the reduction of corticosterone by 20HSD might be an alternative system to the oxidation of this steroid via 11βHSD and thus 20HSD might facilitate aldosterone binding to mineralocorticoid receptors. In agreement with this, 20-dihydrocorticosterone does not possess any affinity for avian aldosterone receptors (DiBattista *et al.* 1989) and neither 20-dihydrocorticostetone nor 11-dehydro-20-dihydrocorticosterone is able to induce Na^+ transport in chicken intestine (Mazancová *et al.* 2005). In addition, avian 20HSD might not only be involved in corticosterone/cortisol, but also in progesterone metabolism, similar to 20HSDs in mammals

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(Penning 1997, Quinkler *et al.* 1999). The presence of both 20HSD and 11βHSD also implies that the functional coupling of 11β-oxidation followed by 20-reduction might amplify the effect of steroid dehydrogenases (Mercer & Krozowski 1992).

In this study, we report the isolation of cDNA encoding 20HSD from chicken intestine, its overexpression in *Escherichia coli*, and the distribution of 20HSD mRNA and enzyme activities in various tissues.

Materials and methods

Animals

Experiments were performed on Brown Leghorn chickens obtained from the hatchery of the Institute of Molecular Genetics (Czech Acad. Sci., Prague). They were raised under controlled conditions (12 h light:12 h darkness period), fed a commercial poultry diet, and had free access to water. The chickens were killed by decapitation and various tissues used for RNA extraction were quickly removed. To identify the expression of steroid dehydrogenase activity in oviduct, some chickens were treated with estrogens because this organ is extremely sensitive to female hormones that induce the differentiation and proliferation of oviduct cells (Dougherty & Sanders 2005). In this experiment, the females received a daily s.c. injection of 2 mg diethylstilbesterol (DES) per kg in polypropylene glycol for 7 days starting from day 22. The animal protocol was approved by the Institutional Animal Care Committee.

20HSD cloning strategy

Based on a comparison of the high sequence homology among the known sequences of 20βHSD (EC 1·1·1·184) in other vertebrates (human, pig, mouse, rat, rabbit, some teleosts), the predicted mRNA sequence of this enzyme in chickens was constructed using the program CLUSTAL W. Briefly, the known sequences were used to search chicken EST (expressed sequence tags) in the free internet National Center for Biotechnology Information (NCBI) database for cDNA fragments with the highest similarity. The fragments found were used to further search the database for other overlapping fragments and thus elongate the sequence. The generated sequence of more than 1200 bp was searched for as an open reading frame by the Basic Local Alignment Search Tool. Based on the sequence of putative ch20HSD, the open reading frame (ORF)-specific forward (5′→3′ CGCTAGGGAGTGCGGGAAGGT) and reverse primers (5′→3′ GCCACTTGCAAGGGTCCACAGA) were designed by the program Lasergene (DNASTAR, Madison, WI, USA) and used in a PCR to obtain the ch20HSD amplicon. The PCR mixture contained 2·5 U platinum tag polymerase, 20 pmol each of the sense and antisense primers, 200 μM dNTPs, 50 mM KCl, 15 mM Tris–HCl, 0·5 mM $MgCl_2$, and 1 μl chicken cDNA. Chicken cDNA was prepared from RNA isolated from the ileum by Trisol (Invitrogene) and reverse transcripted using anchorage oligo(dT) primers (Sigma) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogene) according to the manufacturer's instructions. Ileal cDNA was used because we have recently demonstrated 20HSD activity in this tissue (Mazancová *et al.* 2005).

All PCR products were separated on 1·5% agarose gel and isolated using a GenElute Gel extraction kit (Sigma). Subsequently, the isolated cDNA fragment was inserted into pGEM Easy Vector System (JM 109 competent cells; Promega), the plasmid DNA was purified with a QIAprep Spin Miniprep kit (Qiagen) and sequenced by the ABI PRISM 3100 DNA sequencer in Academy of Sciences Sequencing Facility.

Production and purification of ch20HSD recombinant protein

To produce the ch20HSD recombinant protein, two specific primers were designed close to the ORF region of ch20HSD. The protein was cloned and expressed using the Champion pET SUMO Protein Expression System (Invitrogene) according to the manufacturer's instructions. Briefly, the purified PCR product from chicken ileal cDNA was inserted into the bacterial expression vector pET SUMO and transformed to One Shot Match 1-T1 Chemically Competent *E. coli*. We extracted the plasmid from cells possessing kanamycine resistance and after confirming the proper sequence orientation by nucleotide sequence analysis, the plasmid was transformed to One Shot BL21 (DE3) Chemically Competent *E. coli*. The same cells transformed with the pET SUMO/CAT plasmid were used as a positive control. This plasmid allows expression of N-terminally tagged chloramphenicol acetyl transferase (CAT).

The soluble ch20HSD recombinant protein was prepared in cells cultivated in bacterial growth medium (LB medium) containing 50 μg/ml kanamycine. After the cells reached a cell density of 0·5, isopropyl-1-thio-β-D-galactopyrasonide (1 mM; IPTG) was added to induce protein synthesis and the cells were incubated for another 6 h at 37 °C. The cells were then harvested, and lysed by sonication, freezing, and thawing. The purified protein was prepared according to the manufacturer's instruction using a Ni-CTA HC RESIN (Sigma). Pure protein was eluted by 250 mM imidazole, 300 mM NaCl, and 50 mM sodium phosphate (pH 8·0). Fractions containing purified ch20HSD were pooled and the purity and molecular weight of the protein was verified by 12% SDS-PAGE, fixed, and stained using the standard Coomassie blue protocol.

Assays of enzymatic activities

Determination of the activity of recombinant ch20HSD was performed in a cell suspension of intact *E. coli* and that of native ch20HSD in tissue slices and homogenates using a steroid assay. The suspension of *E. coli* or the purified recombinant protein of ch20HSD was utilized to determine the enzyme activity, its stereospecificity, and substrate specificity. The enzyme assay was conducted in incubation buffer (100 mM KCl; 50 mM Tris–HCl (pH 8·5)) containing 0·8 mM NADPH and [1,2,6,7-^{3}H] corticosterone, [1,2,6,7-^{3}H]progesterone, 5α-dihydro[1,2,4,6,7-^{3}H]testosterone, or [1,2,6,7-^{3}H]aldosterone (65 nM). The reaction was stopped by cooling and the steroids extracted from the incubation medium using Sep-Pak cartridges. The SUMO/CAT protein was used as a negative control and showed no steroid activity. The incubation times were selected to ensure that the velocities of the individual reactions would be linear. To identify the stereospecificity of ch20HSD, progesterone instead of corticosterone was used because α-epimer of 20-dihydrocorticosterone was not available in the market. The conversion of androstanolone (5α-dihydrotestosterone) was studied because some 20HSDs also show 3α/3β-hydroxysteroid dehydrogenase activity.

The distribution of ch20HSD activity in chickens was determined in tissue slices and homogenates of the kidney, liver, brain, heart, ileum, testes, ovaries, and oviduct using an enzyme assay carried out as previously described (Mazancová *et al.* 2005) with some modifications. Briefly, tissue slices (<1 mm thick; 100 mg) were incubated in Dulbecco's Modified Eagle's Medium (DMEM) containing [^{3}H]corticosterone (17 nM) in the presence of 95% O_2/5% CO_2 at 37 °C. Tissue homogenates were prepared by homogenization (1:9, w/v) in an ice-cold buffer containing 200 mM sucrose and 10 mM Tris–HCl (pH 8·5) with a Polytron homogenizer. The homogenates were centrifuged at 1000 ***g*** for 10 min and the supernatant was assayed for protein concentration using the Coomassie blue method. The conversion of corticosterone was assayed in tubes containing the buffer (100 mM KCl, 50 mM Tris–HCl (pH 8·5)), cosubstrate NADPH or NADH (0·4 mM), homogenate (0·05–1 mg protein depending on the tissue), and [^{3}H]corticosterone (25 nM). To remove the oxidized cosubstrate produced by 20HSD from the reaction mixture, the enzyme activity was determined in the presence of 2U glucose-6-phosphate dehydrogenase purified from baker's yeast (Sigma) and 1 mM glucose-6-phosphate (Agarwal *et al.* 1990). The reactions were stopped by cooling and the steroids extracted using Sep-Pak cartridges. All assays were carried out in triplicate.

Analysis of steroids

The analysis of the steroids was performed by HPLC using an Agilent 1100 system (Agilent, Palo Alto, CA, USA) consisting of a degasser, a binary pump, an autosampler, a thermostated column compartment, a multiwavelength detector, and a radioactivity detector (Radiomatic 150TR; Canberra Packard, Meriden, CT, USA) with a flow cell (flow rate of scintillation cocktail/mobile phase was 3:1). The reduction product of corticosterone (4-pregnen-11β,21-diol-3,20-dione) was detected according to a previously described procedure (Vylitová *et al.* 1998, Mazancová *et al.* 2005). Although we were unable to identify whether 20α-dihydrocorticosterone (4-pregnen-11β,20α,21-triol-3-one) and 20β-dihydrocorticosterone (4-pregnen-11β,20β,21-triol-3-one) co-elute, we have seen in all runs only two peaks of radioactivity that co-chromatographed with corticosterone and 20β-dihydrocorticosterone.

Progesterone (4-pregnen-3,20-dione) derivatives 20α- and 20β-dihydroprogesterone (4-pregnen-20α-ol-3-one; 4-pregnen-20β-ol-3-one) were separated on the Zorbax Eclipse XDB-C18 column (150×4·6 mm I.D., 5 μm, Rockland Technologies (Agilent)). A 20 μl sample was injected and elution was achieved by a linear gradient between mobile phases A (water) and B (acetonitrile: tetrahydrofuran:methanol 50:20:30, v/v/v). Gradient started from 42% B to 60% B at 20 min with flow rate 1 ml/min, then the column was eluted with 100% B for 5 min at elution flow 1 ml/min. Equilibration before the next run was achieved by 10 min washing with buffer A. Column temperature was held at 30 °C. Detection was made at 254 nm (standards) or by radioactivity detector (samples).

Analysis of androstanolone (5α-androstan-17β-ol-3-one) derivatives (5α-androstan-3α,17β-diol; 5α-androstan-3β,17β-diol) was carried out on the Zorbax Eclipse XDB-C18 column (150×4·6 mm I.D., 5 μm, Rockland Technologies (Hewlett-Packard)). A 20 μl sample was injected and elution was achieved by a linear gradient between mobile phases A (water) and B (methanol). Gradient started from 42% B to 62% B at 5 min and the following gradient was from 62% B to 100% B at next 15 min with flow rate 1 ml/min, then the column was eluted with 100% B for 5 min at elution flow 1 ml/min. Equilibration before the next run was achieved by 10 min washing with buffer A. Column temperature was held at 30 °C. Detection was made by mass spectrometry (standards) or radioactivity detector (samples). Mass spectrometric instrument was MSD-Trap XCT Ultra (Agilent). There was used atmospheric pressure ionization–electrospray ionization at positive mode. Operating conditions were determined as follows: drying gas (N_2), 12 l/min; drying gas temperature, 350 °C; nebulizator pressure, 55 psi; and capillary voltage, 3500 V; ions were observed at mass range m/z 100–400. Selected steroids were monitored by extracted ion chromatogram of previously selected ions (i.e. 275, 291, and 257 m/z).

Analysis of ch20HSD mRNA expression levels

The tissue distribution of ch20HSD mRNA was studied by real-time RT-PCR. The total RNA (1·3–1·8 μg) from various tissues was reversed transcribed using oligo(dT) primers. The cDNA products were quantified for ch20HSD and β-actin with a LightCycler-Fast Start DNA Master SYBR Green I kit (Roche) and a LightCycler instrument. The primers used for ch20HSD were: forward AGGGCTGCATCCACTCTTCC and reverse TTTGGCCAACCTTCTTTCTC; and for β-actin: forward TGATATTGCTGCGCTCGTTGTTGA and reverse CATGGCTGGGGTGTTGAAGGTCTC. PCR was performed in a total volume of 10 μl containing 1 μl of tenfold diluted cDNA; 4 mM $MgCl_2$; 0·5 μM of each primer and the PCR mix (1×). The LightCycler was programmed as follows: pre-incubation and denaturation of the template cDNA for 10 min at 95 °C, followed by 45 cycles of amplification: 95 °C for 15 s, followed by 56 °C for 10 s (ch20HSD) or 64 °C for 10 s (β-actin), and then 72 °C for 17 s (ch20HSD) or 16 s (β-actin). For quantification, we prepared standard curves for both pairs of primers from serial dilutions of chicken cDNA. The results were calculated as the relative expression of ch20HSD mRNA to β-actin mRNA. Samples for real-time PCR experiments were measured on two occasions for each sample.

Results

Chicken ch20HSD cDNA sequence and the deduced primary structure

The cloning strategy to identify a putative ch20HSD was based on a procedure that searched the free internet chicken EST database for the cDNA fragments with the highest similarity to the conserved domains of known vertebrate 20βHSDs. The fragments found were then used to further search the database for other overlapping fragments and thus elongate the sequence. Figure 1 shows a comparison of the deduced amino acid sequence of the ch20HSD with other steroid dehydrogenases. Similar to other members of SDR family (Jörnvall *et al.* 1995), the deduced ch20HSD protein contains the well-conserved putative cosubstrate-binding domain (AAs 10–34) with the well-conserved motif Gly-X-X-X-Gly-X-Gly. Similarly, the triad Tyr, Lys, and Ser can also be found in ch20HSD, including the highly conserved Tyr-X-X-X-Lys segment assigned to the catalytic center. The deduced ch20HSD amino acid sequence exhibits 72–78% homologies with human, pig, rat, mouse, and rabbit 20βHSD, and a little less with teleosts, zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), and ayu fish (*Plecoglossus altivelis*; Table 1). This process resulted in the generation of a 930 bp sequence. The sequence was verified *in vitro* by cloning in *E. coli* and then published in the GenBank nucleotide sequence database with the accession number NM001030795. The ORF encodes a protein of 276 amino acid residues with a calculated molecular mass of 30·25 kDa.

Recombinant expression of ch20HSD in *E. coli*

To confirm that the cDNA encodes active 20HSD, we cloned the ORF of putative 20HSD into the pET SUMO plasmid. As shown in Fig. 2, the transfection of *E. coli* with the plasmid construct resulted in conversion of corticosterone to 20-dihydrocorticosterone, but *E. coli* transfected with the plasmid without ch20HSD did not show any 20-reductase activity. Subsequent overexpression resulted in a SUMO/ch20HSD fusion protein. This protein was purified by chromatography and the purity of the recombinant protein was determined by SDS-PAGE (Fig. 3). The molecular mass of the recombinant protein shown in Fig. 3 corresponds with the sum of the molecular mass of the SUMO protein (11 kDa) and that predicted for ch20HSD (30·25 kDa).

The HPLC profile of progesterone incubated with the recombinant protein exhibited two metabolites, 20α- and 20β-dihydrotestosterone (Fig. 4), but catalyzed the production of β-epimer more efficiently (27·9 ± 2·5 pmol/h per mg protein; $n=6$) than the α-epimer (11·5 ± 1·0 pmol/h per mg protein; $n=6$). The ability of ch20HSD to reduce progesterone (39·4 ± 3·5 pmol/h per mg protein; $n=6$) was very similar to that of reduction of corticosterone to 20-dihydrocorticosterone (38·9 ± 0·4 pmol/h per mg protein; $n=7$). Since there have been several reports on bifunctional hydroxysteroid dehydrogenases having 20HSD and 3HSD activities (Ohno *et al.* 1991, Penning 2003), we further investigated the catalytic activity of ch20HSD for the reduction of the oxo group at position C_3. When androstanolone was incubated with ch20HSD in the presence of NADPH, two peaks corresponding to the reduced products of androstanolone, 5α-androstan-3α,17β-diol and 5α-androstan-3β,17β-diol, were identified (Fig. 5) and this reduction at C_3 was more efficient (α-epimer, 83·3 ± 2·5 pmol/h per mg protein, $n=6$; β-epimer, 104·8 ± 11·9 pmol/h per mg protein, $n=6$) than the reduction at C_{20} (see above). When aldosterone was incubated with ch20HSD in the presence of NADPH, no metabolites were identified.

As shown in Fig. 6, ch20HSD efficiently catalyzed the reduction of substrate using NADPH as cosubstrate but no conversion was found with NADH.

Tissue distribution of ch20HSD mRNA and activity

Using specific primers for ch20HSD, we determined the expression levels of ch20HSD mRNA in various tissues. As can be seen in Fig. 7, RT-PCR revealed the highest level of expression in the kidney, colon, and

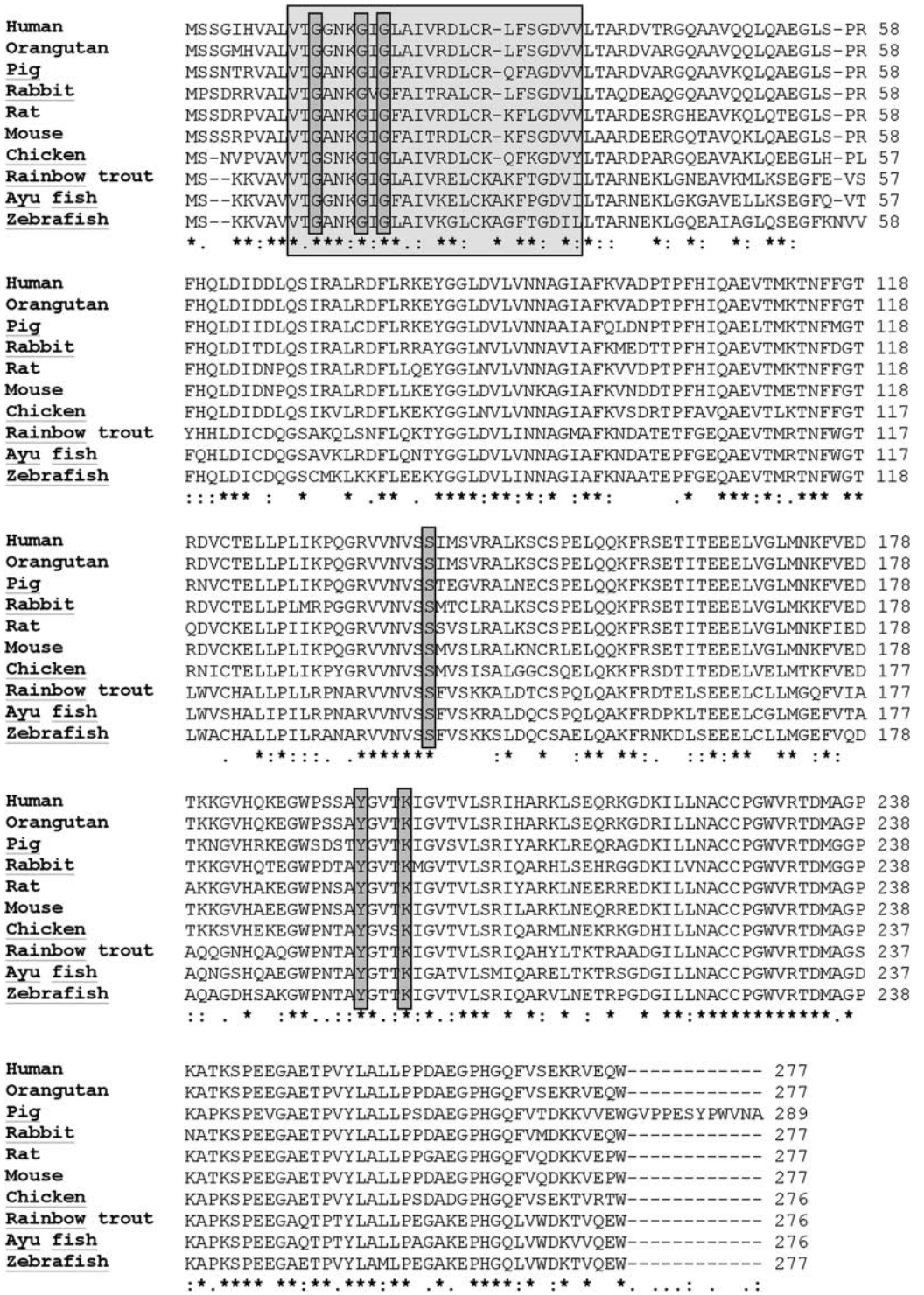

Figure 1 Protein sequence alignment of chicken 20HSD with other known 20HSDs. Putative cosubstrate-binding site AAs 10–34, motif (*Gly-X-X-X-Gly-X-Gly*), substrate-binding site (*Ser*), and catalytic site (*Tyr-X-X-X-Lys*) are boxed. Amino acid residues are numbered on the right. (*) All amino acid residues in this column are identical in all sequences in the alignment; (:) conserved substitutions have been observed; (.) semi-conservative substitutions were observed (according to UniProKB/Swiss-Prot).

Table 1 Protein sequence homology of putative chicken 20HSD sequence with known sequences of other vertebrates expressed in percentage of identical amino acid residues

	Zebrafish	Ayu fish	Rainbow trout	Chicken	Mouse	Rat	Rabbit	Pig	Orangutan
Human	61	61	60	78	86	85	83	84	98
Orangutan	61	61	60	78	86	85	83	84	×
Pig	58	59	58	73	79	80	78	×	84
Rabbit	60	60	58	72	80	80	×	78	83
Rat	62	63	61	75	89	×	80	80	85
Mouse	61	61	60	75	×	89	80	79	86
Chicken	64	60	60	×	75	75	72	73	78
Rainbow trout	81	84	×	60	60	61	58	58	60
Ayu fish	79	×	84	60	61	63	60	59	61

testes, whereas lower expression was detected in other tissues. The single band of a 400 bp amplified fragment was detected in all tissues (not shown) using electrophoretic analysis of the reaction product. The expression of ch20HSD was further compared with the 20-reductase activity of intact tissue slices and tissue homogenates. The incubation of tissue slices with [^{3}H]corticosterone revealed 20-reductase activity in all investigated tissues with the exception of the brain and heart (Fig. 8). A similar pattern was also observed in homogenates. As illustrated in Table 2, the tissue homogenates efficiently reduced corticosterone at C_{20} in the presence of NADPH but not of NADH.

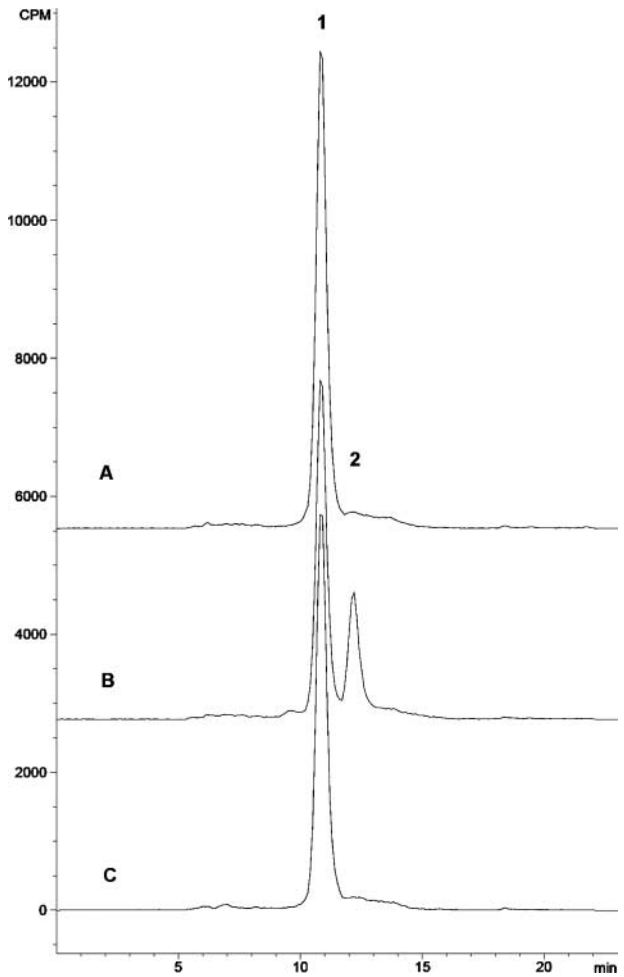

Figure 2 Conversion of corticosterone in (A) non-transfected and (B) transfected *E. coli* with plasmids pET SUMO/20HSD and (C) pET SUMO/CAT. 1, Corticosterone and 2, 20-dihydrocorticosterone.

Discussion

We have succeeded in cloning chicken 20HSD. The cloned ch20HSD cDNA predicted a protein of 276 amino acid residues that was 13 amino acid residues shorter than the pig protein (Tanaka *et al.* 1992). Using ch20HSD cDNA, we found that the transfection of *E. coli* with this cDNA leads to the conversion of corticosterone to 20-dihydrocorticosterone and progesterone to 20-dihydroprogesterone with higher 20βHSD than 20αHSD activity. Similarly, β-epimers of 20-dihydrocorticosterone and 20-dihydroprogesterone were demonstrated as the main products of 20HSD in the duck intestine (DiBattista *et al.* 1989). In addition, the reduction catalyzed by ch20HSD was cosubstrate-specific, required NADPH instead of NADH and apart

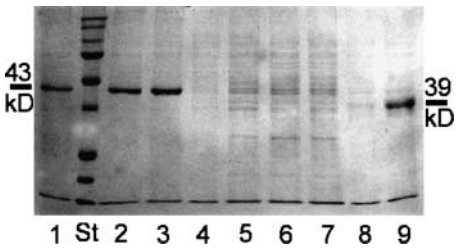

Figure 3 Overexpression and purification of chicken 20HSD. St, molecular mass marker; lanes 1–3, lysate from cells transfected with pET SUMO/ch20HSD plasmid 3, 5, and 7 h after induction with IPTG; lanes 4–7, lysate from cells without plasmid 1, 3, 5, and 7 h after induction with IPTG; and lanes 8 and 9, lysate from cells transfected with pET SUMO/CAT plasmid 1 and 5 h after induction with IPTG.

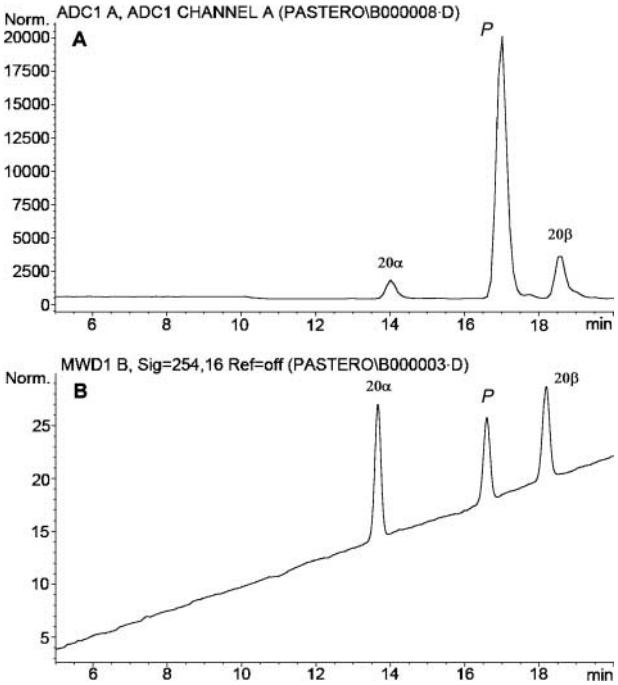

Figure 4 Conversion of progesterone by chicken 20HSD. (A) Sample (radioactivity detection); (B) standards (u.v. detection). 20α, 4-Pregnen-20α-ol-3-one; P, 4-pregnen-3,20-dione; 20β, 4-pregnen-20β-ol-3-one.

from 20HSD activity also possessed 3α- and 3βHSD activity. Finally, the tissues that expressed ch20HSD mRNA also had 20-reductase activity. The differences in 20-ketosteroid activity and ch20HSD mRNA expression indicate that either mRNA expression does not correlate with active enzyme or that the protein cloned in our experiments is not the only 20HSD operating in some chicken tissues. Comparison of Figs 7 and 8 suggests that ch20HSD plays a smaller role in reduction of 20-ketosteroids in liver, oviduct, ovary, and especially in brain and heart and that additional 20-ketosteroid reductase(s) have to operate in liver and oviduct. This is consistent with previously reported 20HSD activities of mammalian hydroxysteroid dehydrogenases, such as 17βHSD and 3αHSD (Penning 2003) and discrete tissue distribution of enzymes that possess 3-, 17-, and 20-ketosteroid reductase activities in varying ratios (Penning *et al.* 2000). We cannot also exclude the role of other carbonyl reductases (Nishinaka *et al.* 1992, Maser 1995).

The predicted amino acid sequence of ch20HSD revealed high homology to the mammalian 20βHSD reaching more than 70% on average. Similar to other steroid dehydrogenases, ch20HSD displays polyfunctional enzyme activity, 3- and 20HSD activity, but in comparison to some other enzymes, it shows an unusual absence of stereochemical specificity as both 3α/3β- and 20α/20β-epimers were identified. In contrast, 20HSD in pigs operates as 3α/3β,20βHSD (Ohno *et al.* 1991) and in *Streptomyces hydrogenans* only as 3α,20βHSD (Edwards & Orr 1978), whereas cyprinid fishes coexpress both 20α- and 20βHSD activities although it is not known whether these activities reflect one single or two different enzymes (Thibaut & Porte 2004). However, the results in the present study point to a possibility that ch20HSD has similar widespread distribution as 20βHSD in fishes and neonatal pigs (Kobayashi *et al.* 1996, Guan *et al.* 1999).

The physiological role of 20βHSDs is not completely clear. These enzymes show high similarity to carbonyl reductases that have been implicated not only in elimination of reactive carbonyl compounds, but also in reduction of biologically active compounds, such as prostaglandins and steroid hormones (Maser 1995). The role of 20βHSD is well documented in oocyte maturation in lower vertebrates, in which 17α,20β-dihydroprogesterone has been proven to be an important maturation-inducing factor (Nagahama 1997).

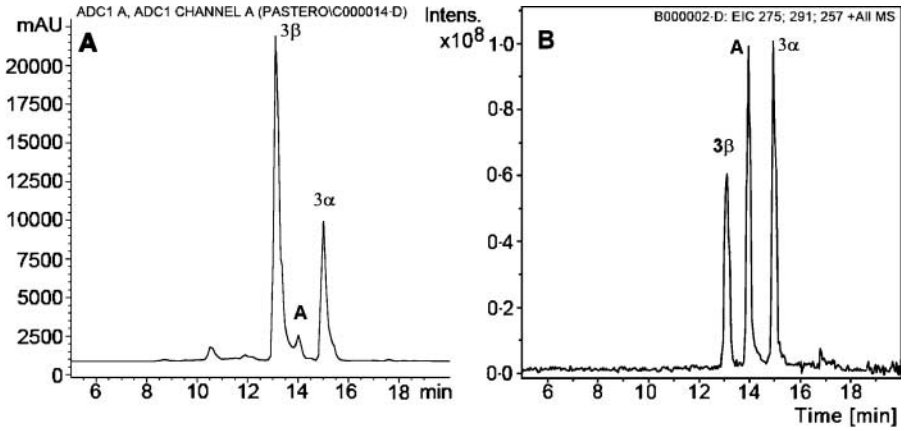

Figure 5 Conversion of androstanolone by chicken 20HSD. (A) Sample (radioactivity detection); (B) standards (MS detection). 3β, 5α-androstan-3β,17β-diol; A, 5α-androstan-17β-ol-3-one; 3α, 5α-androstan-3α,17β-diol.

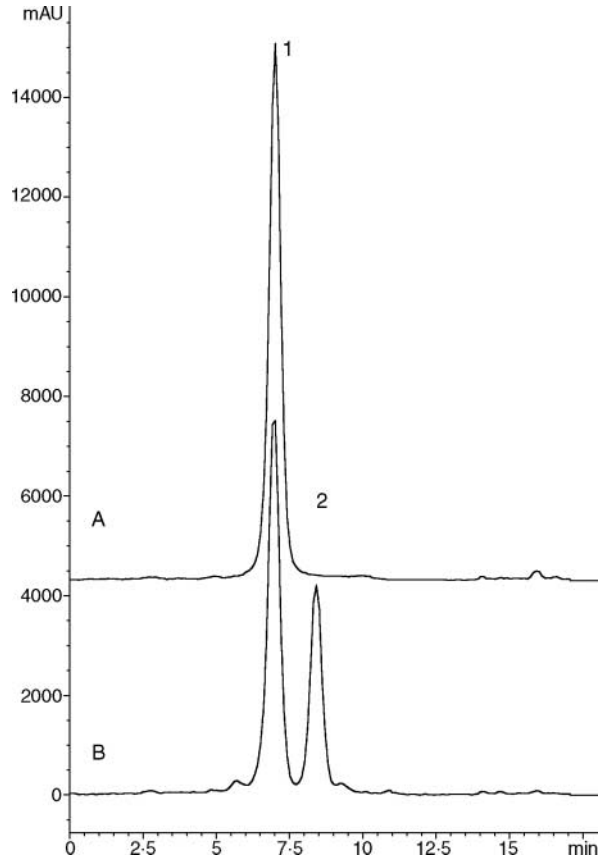

Figure 6 HPLC chromatogram of corticosterone metabolites from enzymatic assay of ch20HSD purified protein overexpressed in *E. coli*. Metabolism of corticosterone in the presence of (A) NADH and (B) NADPH. 1, corticosterone and 2, 20-dihydrocorticosterone.

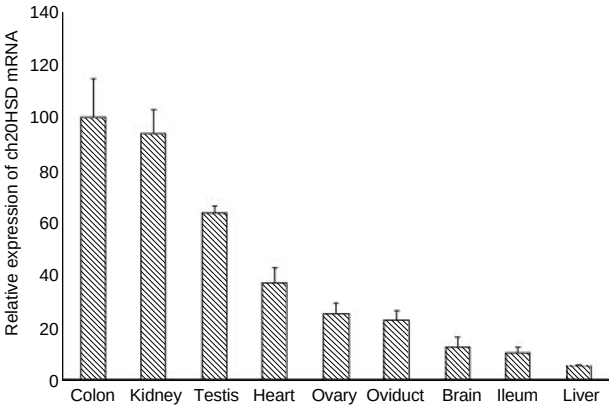

Figure 7 Tissue distribution of 20HSD in chicken. Relative levels of expression of ch20HSD. Colon was normalized to 100% and compared with other tissues. ΔCT values (expressed as mean ΔCT=CT of the 20HSD gene – CT of the β-actin) were: colon, 3·2±0·2; kidney, 2·7±0·2; testis 3·3±0·1; heart 3·7±0·2; ovary, 4·9±0·4; oviduct, 4·7±0·1; brain, 5·6±0·3; ileum, 6·1±0·3; and liver, 6·5±0·2. The ch20HSD mRNA abundances in oviduct were measured in chicks treated with DES (for further details, see Materials and methods). Values are means±S.E.M. of seven animals.

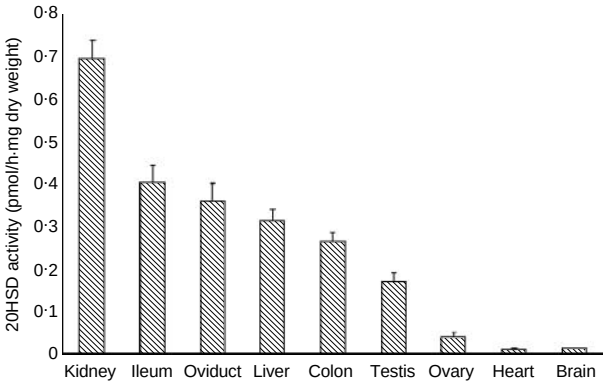

Figure 8 Reduction of corticosterone to 20-dihydrocorticosterone and 11-dehydro-20-dihydrocorticosterone in tissue slices. The 20-reductase activities in oviduct were measured in chicks treated with DES (for further details, see Materials and methods). Values are means±S.E.M. of 9–12 animals.

This steroid is synthesized from 17α-hydroxyprogesterone by 20βHSD in the follicular layers of the ovaries. Similarly, 20βHSD seems to be also involved in the regulation of spermatogenesis in fishes (Sakai *et al.* 1989). In mammals, 20-ketosteroid reductases are generally considered to play a role in progesterone activity. First, they protect against the occupancy of progesterone receptors by inappropriate ligands, because 20-reduction considerably decreases progestin potency. Second, they are able to prevent the conversion of C_{21} to C_{19} steroids, because the conversion of progesterone to 20-dihydroprogesterone prevents 17,20-lyase from producing the precursors to C_{19} sex hormones. Several enzymes responsible for this reaction were found and it was shown that they have predominantly 20α-stereospecificity (Penning 1997, Bumke-Vogt *et al.* 2002). 20β-Stereospecificity was identified in testes of neonatal pigs (Nakajin *et al.* 1988*a*), where it seems to regulate steroid hormone concentration in the testes during development. However, later studies revealed a

Table 2 Cosubstrate dependence of activity of 20HSD in tissue homogenates

	NIL	+NADPH	+NADH
Tissue			
Kidney	0	143±10	2±1
Liver	0	29±5	3±1
Colon	0	22±4	0
Ileum	0	19±3	0
Oviduct	0	8±2	0
Heart	0	0·1±0·0	0
Brain	0	0·1±0·1	0·3±0·2

Values are means±S.E.M. of six animals. Data are given in picomole of corticosterone reduced to 20-dihydrocorticosterone plus 11-dehydro-20-dihydrocorticosterone per hour and milligram of protein. The 20-reductase activity in oviduct homogenates was measured in chicks treated with DES (for further details, see Materials and methods).

widespread distribution of 20βHSD in neonatal pigs, which suggest a broader role for this enzyme in steroid hormone metabolism than merely in the synthesis of androgens in testicular cells (Kobayashi *et al.* 1996). The substrate specificity of pig 20βHSD is not identical to ch20HSD. Although pig 20βHSD catalyzes the reduction of progesterone, it does not reduce 11-oxo/11-hydroxy-C_{21} steroids, such as cortisol, corticosterone, and cortisone (Nakajin *et al.* 1988*a*).

Presently, it is not known whether ch20HSD exhibits some of the physiological roles described herein. However, recent experiments in our laboratory have shown its significance in mineralocorticoid target tissues (Mazancová *et al.* 2005). In these tissues, such as kidney and intestine, ch20HSD together with 11βHSD might play a role in preventing corticosterone from binding to the mineralocorticoid receptors. Previous studies showed that avian mineralocorticoid receptors bind aldosterone and corticosterone with nearly equal affinities (Rafestin-Oblin *et al.* 1989, Sandor *et al.* 1989) and reduction of corticosterone to 20-dihydrocorticosterone might be an alternative system to 11β-oxidation to facilitate aldosterone binding. 20-Dihydrocorticosterone has much lower affinity to mineralocorticoid receptor than corticosterone (DiBattista *et al.* 1989) and in contrast with corticosterone, it is not able to induce electrogenic Na^+ transport usually regulated by aldosterone (Mazancová *et al.* 2005). In addition, the functional coupling of 11β-oxidation followed by reduction of the ketone group at position C_{20} via cosubstrates might amplify the effect of steroid dehydrogenases (Mercer & Krozowski 1992). Similarly, the expression of ch20HSD in reproductive organs might modulate steroid synthesis via cytochrome $P450_{C17}$ because 20β-hydroxy-C_{21} steroids have been shown to inhibit $P450_{C17}$ (Nakajin *et al.* 1988*b*) and this cytochrome is expressed in both avian gonadal and non-gonadal tissues (Boswell *et al.* 1995, Schlinger *et al.* 1999).

In summary, it is well known that steroid hormone receptors demonstrate considerable promiscuity in binding various steroid ligands. Therefore, the reduction of a potent steroid hormone into less potent or inactive derivatives might be an alternative system to the well-known 11βHSD pre-receptor modulation of steroid activity or might modulate the concentration of C_{19} hormones in gonadal and extragonadal tissues. The advantage of 20HSD compared with 11βHSD is that this enzyme might modulate not only 11β-hydroxy/11-keto-C_{21}-steroid hormones but also progesterone and sex hormones. The detailed role of this enzyme in various tissues remains to be determined, but the availability of chicken 20HSD cDNA offers an opportunity for further studies.

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References

Agarwal AK, Tusle-Luna M-T, Monder C & White PC 1990 Expression of 11β-hydroxysteroid dehydrogenase using recombinant vaccinia virus. *Molecular Endocrinology* **4** 1827–1832.

Boswell T, Hall MR & Goldsmith AR 1995 Testosterone is secreted extragonadally by European quail maintained on short days. *Physiological Zoology* **68** 967–984.

Bumke-Vogt C, Bähr V, Diederich S, Herrmann SM, Anagnostopoulos I, Oelkers W & Quinkler M 2002 Expression of the progesterone receptor and progesterone-metabolising enzymes in the female and male human kidney. *Journal of Endocrinology* **175** 349–364.

Dougherty DC & Sanders MM 2005 Estrogen action: revitalization of the chick oviduct model. *Trends in Endocrinology and Metabolism* **16** 414–419.

DiBattista JA, Mehdi AZ & Sandor T 1989 Steroid C-20 oxidoreductase activity of duck intestinal mucosa: the interrelations of the enzymatic activity with steroid binding. *General and Comparative Endocrinology* **74** 136–147.

Edwards CA & Orr JC 1978 Comparison of the 3α-and 20β-hydroxysteroid dehydrogenase activities of the cortisone reductase of *Streptomyces hydrogenans*. *Biochemistry* **17** 4370–4376.

Guan G, Tanaka M, Todo T, Young G, Yoshikuni M & Nagahama Y 1999 Cloning and expression of two carbonyl reductase-like 20β-hydroxysteroid dehydrogenase cDNAs in ovarian follicles of rainbow trout (*Oncorhynchus mykiss*). *Biochemical and Biophysical Research Communications* **255** 123–128.

Jörnvall H, Persson B, Krook M, Atrian S, Gonzalez-Duarte R, Jeffery J & Ghosh D 1995 Short-chain dehydrogenases/reductases (SDR). *Biochemistry* **34** 6003–6013.

Kobayashi K, Ohno S, Shinoda M, Toyoshima S & Nakajin S 1996 Immunochemical distribution and immunohistochemical localization of 20β-hydroxysteroid dehydrogenase in neonatal pig tissues. *Journal of Steroid Biochemistry and Molecular Biology* **59** 485–493.

Maser E 1995 Xenobiotic carbonyl reduction and physiological steroid oxidoreduction. The pluripotency of several hydroxysteroid dehydrogenases. *Biochemical Pharmacology* **49** 421–440.

Mazancová K, Kučka M, Mikšík I & Pácha J 2005 Glucocorticoid metabolism and Na^+ transport in chicken intestine. *Journal of Experimental Zoology* **303A** 113–122.

Mercer WR & Krozowski ZS 1992 Localization of an 11β-hydroxysteroid dehydrogenase activity to the distal nephron. Evidence for the existence of two species of dehydrogenase in the rat kidney. *Endocrinology* **130** 540–543.

Nagahama Y 1997 17α/20β-Dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: mechanisms of synthesis and action. *Steroids* **62** 190–196.

Nagahama Y & Adachi S 1985 Identification of maturation-inducing steroid in a teleost, the amago salmon (*Oncorhynchus rhodurus*). *Developmental Biology* **109** 428–435.

Nakajin S, Ohno S & Shinoda M 1988*a* 20β-hydroxysteroid dehydrogenase of neonatal pig testis: purification and some properties. *Journal of Biochemistry* **104** 565–569.

Nakajin S, Takahashi K & Shinoda M 1988*b* Inhibitory effects and spectral changes on pig testicular cytochrome P-450 (17α-hydroxylase/lyase) by 20β-hydroxy-C_{21}-steroids. *Yakugaku Zasshi* **108** 1188–1195.

Nishinaka T, Kinoshita Y, Terada N, Terada T, Mizoguchi T & Nishihara T 1992 Characterization of multiple forms of carbonyl reductase from chicken liver. *Enzyme* **46** 221–228.

Ohno S, Nakajin S & Shinoda M 1991 20β-Hydroxysteroid dehydrogenase of neonatal pig testis: 3α/β-hydroxysteroid dehydrogenase activities catalyzed by highly purified enzyme. *Journal of Steroid Biochemistry and Molecular Biology* **38** 787–794.

Ohno S, Nakajin S & Shinoda M 1992 Ontogeny of testicular steroid dehydrogenase enzymes in pig (3α/β-, 20α- and 20β-). Evidence for two forms of 3α/β hydroxysteroid dehydrogenase. *Journal of Steroid Biochemistry and Molecular Biology* **42** 17–21.

Penning TM 1997 Molecular endocrinology of hydroxysteroid dehydrogenases. *Endocrine Reviews* **18** 281–305.

Penning TM 2003 Hydroxysteroid dehydrogenases and pre-receptor regulation of steroid hormone action. *Human Reproduction Update* **9** 193–205.

Penning TM, Burczynski ME, Jez JM, Hung CF, Lin HK, Ma H, Moore M, Palackal N & Ratnam K 2000 Human 3α-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. *Biochemical Journal* **351** 67–77.

Quinkler M, Johanssen S, Grossmann C, Bähr V, Müller M, Oelkers W & Diederich S 1999 Progesterone metabolism in the human kidney and inhibition of 11β-hydroxysteroid dehydrogenase type 2 by progesterone and its metabolites. *Journal of Clinical Endocrinology and Metabolism* **84** 4165–4171.

Rafestin-Oblin ME, Couette B, Radanyi C, Lombes M & Baulieu EE 1989 Mineralocorticoid receptor of the chick intestine. Oligomeric structure and transformation. *Journal of Biological Chemistry* **264** 9304–9309.

Sakai N, Ueda H, Suzuki N & Nagahama Y 1989 Steroid production by amago salmon (*Oncorhynchus rhodurus*) testes at different development stages. *General and Comparative Endocrinology* **75** 231–240.

Sandor T, Skadhauge E, DiBattista JA & Mehdi AZ 1989 Interrelationships of the intestinal glucocorticoid and mineralocorticoid receptor systems with salt homeostasis. In *Progress in Avian Osmoregulation*, pp 305–332. Eds MR Hughes & A Chadwick. Leeds: Leeds Philosophical and Literary Society.

Schlinger BA, Lane NI, Grisham W & Thompson L 1999 Androgen synthesis in a songbird: a study of cyp 17 (17α-hydroxylase/C17,20-lyase) activity in the zebra finch. *General and Comparative Endocrinology* **113** 46–58.

Tanaka M, Ohno S, Adachi S, Nakajin S, Shinoda M & Nagahama Y 1992 Pig testicular 20β-hydroxysteroid dehydrogenase exhibits carbonyl reductase-like structure and activity. *Journal of Biological Chemistry* **267** 13451–13455.

Thibaut R & Porte C 2004 Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish. *Journal of Steroid Biochemistry and Molecular Biology* **92** 485–494.

Vylitová M, Mikšík I & Pácha J 1998 Metabolism of corticosterone in mammalian and avian intestine. *General and Comparative Endocrinology* **109** 315–324.

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