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11β-Hydroxysteroid Dehydrogenase Complementary Deoxyribonucleic Acid in Rainbow Trout: Cloning, Sites of Expression, and Seasonal Changes in Gonads

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11β-Hydroxysteroid dehydrogenases (11β-HSDs) are important steroidogenic enzymes for catalyzing the interconversion of active glucocorticoid (cortisol and corticosterone) and inert 11-keto forms (cortisone and 11-dehydrocorticosterone) in mammals. In teleosts, 11β-HSD also plays a role in the production of the predominant androgen, 11-ketotestosterone, in male fish. In this study we cloned cDNAs encoding rainbow trout 11β-HSD (rt11β-HSD) from testes and head kidney. The predicted amino acid sequence, hydrophobicity analysis, and transient transfection assays with rt11β-HSD in HEK293 cells showed that rt11β-HSD is a homolog of mammalian 11β-HSD type 2. rt11β-HSD transcripts are present in steroidogenic tissues and in a number of other tissues. Strong *in situ* hybridization signals for rt11β-HSD transcripts were found in Leydig cells of testes, in thecal cells of the early vitellogenic ovarian follicles, and in thecal and granulosa cells of the mid-vitellogenic and postovulatory follicles. Weaker signals were also found in head kidney interrenal cells from juvenile rainbow trout. Seasonal changes in rt11β-HSD transcripts in testes showed a pattern similar to that of stress-induced serum cortisol levels, but not to serum androgen levels. High levels of rt11β-HSD transcripts were found in ovarian follicles from late vitellogenesis through ovulation. These results raise the possibility of a role for rt11β-HSD in the protection of developing gonads from the inhibitory effects of stress-induced cortisol. (*Endocrinology* 144: 2534–2545, 2003)

MEMBERS OF THE short-chain alcohol dehydrogenase (SCAD) superfamily (1), 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes act as dehydrogenases and 11-oxo-reductases. Two distinct forms of 11β-HSD have been identified and characterized in mammals. 11β-HSD type 1 is a low affinity, NADP(H)-dependent dehydrogenase/11-oxo-reductase that is principally found in glucocorticoid target tissues, such as liver, gonad, and kidney (2). Kotelevtsev *et al.* (3) demonstrated that 11β-HSD type 1 knockout mice were unable to convert inert 11-dehydrocorticosterone to corticosterone *in vivo*. These results suggest that 11β-HSD type 1 has a role in amplifying glucocorticoid action (3). 11β-HSD type 2 is a high affinity, NAD-dependent, 11β-dehydrogenase (4) localized mainly in classical mineralocorticoid target tissues such as kidney and colon, but also found in other tissues such as pancreas, placenta, and prostate (5). In mammals, 11β-HSD type 2 performs the key physiological role of metabolizing bioactive 11-hydroxyglucocorticoids (cortisol and corticosterone) to inert 11-keto forms (cortisone and 11-dehydrocorticosterone), and this protects the mineralocorticoid receptor (MR) from overstimulation by glucocorticoids (4, 6, 7). This unique system is necessary because the MR is nonselective and binds the glucocorticoids, cortisol and corticosterone, with equal affinity to the mineralocorticoid aldosterone.

Abbreviations: DIG, Digoxigenin; GR, glucocorticoid receptor; 11β-HSD, 11β-hydroxysteroid dehydrogenase; 11-KT, 11-ketotestosterone; MR, mineralocorticoid receptor; 11β-OHT, 11β-hydroxytestosterone; ORF, open reading frame; poly(A)$^+$, polyadenylated; RACE, rapid amplification of cDNA ends; rt, rainbow trout; SCAD, short-chain alcohol dehydrogenase; T, testosterone; TLC, thin layer chromatography; UTR, untranslated region.

11β-HSD has also been implicated in protecting gonadal tissue from the inhibitory actions of corticosteroids. A number of studies have demonstrated direct suppression of steroidogenesis by corticosteroids. ACTH treatment of male guinea pigs resulted in lower levels of plasma testosterone (T) accompanying the highly significant elevation in plasma cortisol levels (8). Excessive exposure to cortisol induced apoptosis of Leydig cells, which could lead to suppression of circulating T levels (9). Monder *et al.* (10) demonstrated that the inhibitory effect of corticosterone on T production by rat Leydig cells *in vitro* was enhanced by carbenoxolone, an inhibitor of 11β-HSD, suggesting that 11β-HSD alleviates glucocorticoid inhibition of steroidogenesis. Gao *et al.* (11) demonstrated predominant 11β-dehydrogenase activity of rat 11β-HSD in Leydig cells. However, Leckie *et al.* (12) showed by RT-PCR that rat Leydig cells in primary culture express the 11β-HSD type 1, but not the 11β-HSD type 2 gene, demonstrating that reductase activity predominated in these cells. The function of 11β-HSD type 1 in Leydig cells therefore still remains unclear.

11β-HSD type 1 and 2 genes have been found to be expressed in human ovarian granulosa cells (13). An *in vitro* study using granulosa cells of rats treated with hCG showed up-regulation of 11β-HSD type 1 and down-regulation of 11β-HSD type 2, suggesting that 11β-HSDs in granulosa cells regulate glucocorticoid metabolism. Tetsuka *et al.* (14) suggested that this mechanism protects immature follicles from excessive stimulation by glucocorticoids, whereas ovulatory follicles would be exposed to higher levels of glucocorticoids needed for completion of development or follicular rupture.

In male teleost fish, 11β-HSD is essential for biosynthesis of the potent androgen, 11-ketotestosterone (11-KT) in testes.

In rainbow trout (*Oncorhynchus mykiss*), 11-KT is synthesized as follows: T–*11β-hydroxylase*→11β-hydroxytestosterone (11β-OHT)–*11β-HSD*→11-KT (15, 16). 11-KT production by testes of salmonid species increases with the progression of spermatogenesis and declines after the onset of spermiation (17, 18). Recently, cloning and characterization studies of 11β-hydroxylase have revealed substantial changes in 11β-hydroxylase mRNA levels during the reproductive cycle of rainbow trout (19, 20), reflecting the changes in serum levels of 11-KT (20). A cDNA encoding Japanese eel testicular 11β-HSD has been cloned (21), but other information on regulation of the final step in 11-KT synthesis is not available.

The activation of glucocorticoid receptors (GR) and MR is regulated by cortisol/corticosterone and aldosterone, respectively. Therefore, the activation or inactivation of glucocorticoids by 11β-HSD is important for controlling the transcriptional activity of the GR and MR. In rainbow trout, cDNAs encoding separate receptors for glucocorticoids (22, 23) and mineralocorticoids (24) have recently been cloned and characterized. However, it is generally believed that most fish do not produce aldosterone (25–27). Cortisol is the predominant corticosteroid in fish and exhibits both mineralocorticoid and glucocorticoid actions (28). Whether 11β-HSD and the GR or MR are functionally linked in fish is an intriguing question.

To explore the question of the basic functions of 11β-HSD in teleosts and to understand the molecular basis for 11-KT production in fish further, we cloned cDNAs encoding 11β-HSD from rainbow trout testis and head kidney (containing cortisol-producing interrenal cells). We show the sites of expression of the 11β-HSD gene in rainbow trout, and we present data on seasonal changes in the expression of the 11β-HSD gene during gametogenesis.

Materials and Methods

Cloning and sequencing of 11β-HSD cDNA from rainbow trout testis and head kidney

Study animals. Testes and head kidney from 2-yr-old male rainbow trout (Wanaka Trout Hatchery, Wanaka, New Zealand) were used for the initial isolation of a partial 11β-HSD cDNA fragment. Testis and head kidney cDNA libraries for rapid amplification of cDNA ends (RACE) were constructed using two different individuals: 2-yr-old rainbow trout (Samegai Trout Hatchery, Shiga, Japan) for the testis library and 3-yr-old rainbow trout (steelhead strain; Inland Station, National Research Institute of Aquaculture, Tamaki, Japan) for the head kidney library. For sampling, fish were anesthetized with 300 mg/liter MS222 (3-aminobenzoic acid ethyl ester; Sigma-Aldrich Corp., St. Louis, MO) buffered in sodium bicarbonate. Testes and head kidney were isolated and frozen using liquid nitrogen and stored at −70 C until RNA extraction.

RNA extraction. Total RNA extraction in this study was carried out by the single step guanidinium isothiocyanate-phenol-chloroform extraction method (29) using TRIzol (Invitrogen, Carlsbad, CA). Polyadenylated [poly(A)$^+$] RNA isolation was conducted using Oligotex-dT30 (Takara Shuzo, Shiga, Japan) as described by the manufacturer.

Sequencing. All PCR products in this cloning study were separated on a 1% agarose gel and isolated using a QIAEX II gel extraction kit (QIAGEN, Hilden, Germany). Subsequently, the isolated cDNA fragments were inserted into pGEM-T Easy vector (Promega Corp., Madison, WI) and sequenced using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems, Foster City, CA). The sequence data were analyzed by SeqEd (PE Applied Biosystems) and DNASIS software (Hitachi Software Engineering Co., Tokyo, Japan). The homology search based on the GenBank database was performed by BLAST and ClustalW homology search (30).

PCR amplification of 11β-HSD cDNA. First strand cDNAs were synthesized from 1 μg testis and head kidney total RNA collected from two fish using oligo(deoxythymidine) primer (Invitrogen) and Superscript II RNase H$^-$ reverse transcriptase (Invitrogen). A partial 11β-HSD homolog cDNA was isolated using degenerate primers, 5′-ATH CAN GGN TGY GAY TCN GGN TTY GG-3′ and 5′-CCR AAR AAR TTN ACY TTC ATR CA-3′ (H = A + C + T, R = A + G, N = A + C + T + G, Y = C + T), designed from deduced amino acid sequences of eel 11β-HSD (21) and mammalian 11β-HSD type 2 of cow (31), sheep (4), mouse (32), rat (33), rabbit (34), and human (35). PCR amplifications were performed under the following conditions: 30 sec at 94 C for denaturing, 30 sec at 55 C for annealing, and 1 min at 72 C for extension, for 30 cycles. The final extension was at 72 C for 5 min. The 330-bp PCR products were amplified from both testis and head kidney cDNAs. These PCR products were sequenced and identified as 11β-HSD homologs.

Rainbow trout testis and steelhead trout head kidney cDNA libraries for the RACE method were synthesized using a Marathon cDNA amplification kit (CLONTECH Laboratories, Inc., Palo Alto, CA). Based on the sequence of the partial rainbow trout 11β-HSD (rt11β-HSD) homolog, two primers were designed to amplify full-length rt11β-HSD cDNAs. The 5′RACE-PCR primer was 5′-GCC CCA CAG ACC TCT GAG GCC CAG TTT G-3′, and the 3′RACE-PCR primer was 5′-CAG CGC GGC GTC TGG ACG CCC AGG GTT T-3′. RACE PCR amplifications of 5′ and 3′ regions were carried out using synthetic oligonucleotide primers and Adapter Primer 1 (CLONTECH Laboratories, Inc.) under the following conditions: 1 min at 94 C, 30 sec at 94 C, and 4 min at 72 C for 5 cycles; 30 sec at 94 C and 4 min at 70 C for 5 cycles; and 30 sec at 94 C and 4 min at 68 C for 25 cycles.

Based on the sequences of 5′- and 3′RACE PCR products, rt11βHSD forward (5′-ATG GAA GAC TAC TTA GAC TG-3′) and rt11βHSD reverse primers (5′-GAG CCC TTC AAT ATG AAC ACC AAC GC-3′) were designed to characterize the full-length open reading frame (ORF) of rt11β-HSD. PCR amplification was performed under the following conditions: 1 min at 94 C for denaturing, 1 min at 60 C for annealing, and 2 min at 72 C for extension for 30 cycles. The final extension was at 72 C for 5 min. Advantage 2 Polymerase Mix (CLONTECH Laboratories, Inc.) was used for this PCR reaction.

Enzymatic activity of rt11β-HSD expressed in HEK293 cells

Construction of the expression vector. The rt11β-HSD ORF was isolated by PCR using two primers (forward, ATG GAA GAC TAC TTA GAC TG; reverse, GAG CCC TTC AAT ATG AAC ACC AAC GC). The amplified rt11β-HSD cDNA was inserted into pGEM-T Easy vector (Promega Corp., Madison, WI) and subsequently inserted into the *Eco*RI site of expression vector, pcDNA 3.1$^+$ (Invitrogen).

Transient expression and enzymatic activity. To investigate the 11β-dehydrogenase activity of rt11β-HSD using cortisol as a substrate, thin layer chromatography (TLC) was used. HEK293 cells (1.0×10^6 cells/well) were plated in a six-well cell culture plate and incubated for 16–18 h in 2.0 ml DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen) at 37 C under an atmosphere of 5% carbon dioxide. Four micrograms of the expression vectors, pcDNA 3.1$^+$ containing rt11β-HSD cDNA (pcDNA11βHSD) or pcDNA 3.1$^+$ containing no insert DNA (pcDNA), were transfected into HEK293 cells (three replicates each) using Lipofectamine (Invitrogen) as described by the manufacturer. The cells were incubated for 16 h, then exposed to 40 pmol/well (2,000,000 cpm) tritiated cortisol. After 40 h of further incubation, media were collected. Steroids were extracted from 2.5 ml incubation medium using diethyl ether as previously described (36). Extracts were developed on a TLC plate (Whatman, Kent, UK) using a mixture of dichloromethane/diethyl ether (5:2, vol/vol). One milligram each of cortisol and cortisone was also subjected to TLC as mobility makers. The TLC plate was exposed to a Hyperfilm-3H (Amersham International, Amersham, UK) for 3 d. The band intensities were measured using NIH Image 1.62. The percent conversion was calculated using the following formula: band density of cortisone/(band density of cortisone + cortisol).

To investigate the 11-oxo-reductase activity of the expressed protein using cortisone as a substrate, a cortisol RIA was used. HEK293 cells

(4.0×10^4 cells/well) were plated in 8 wells of a 24-well cell culture plate and incubated under the same conditions as described above. The expression vector, pcDNA11βHSD or pcDNA (0.5 μg), was transfected into HEK293 cells (4 replicates each) using Lipofectamine (Invitrogen). The cells were incubated for 16 h, then exposed to 100 ng/ml cortisone. After 24 h of further incubation, media were collected, and steroids were extracted from 100 μl incubation medium using diethyl ether (36). As cross-reactivity of cortisone in the cortisol RIA is approximately 3% (37), cortisol and cortisone were separated by subjecting samples to chromatography on a TLC plate (Whatman). Authentic cortisol (15.6 ng/ml) was subjected to TLC as a reference. One milligram of cortisol was also subjected to TLC as a mobility maker. Subsequently, the area corresponding to cortisol was scraped off and eluted in acetone. Cortisol levels were then measured by RIA as previously described (37, 38). The percent conversion was normalized by the value of reference:

$$\text{Conversion of cortisol to cortisone (\%)} = \frac{\text{cortisol concentration} \times [15.6\ \text{(ng/ml)/concentration of reference}]}{100\ \text{(ng/ml cortisone)}}.$$

11β-Dehydrogenase activity using 11β-OHT as a substrate was examined using same procedures as those described above (reductase activity assay on cortisone), except that the cells were exposed to 100 ng/ml 11β-OHT. Tritiated 11β-OHT was not available, so 11-KT and 11β-OHT RIA were used for analysis. Steroids were extracted and chromatographed on a TLC plate (Whatman) to avoid cross-reactivity between 11-KT and 11β-OHT. One milligram each of 11-KT and 11β-OHT was also subjected to TLC as mobility makers. The areas corresponding to 11-KT and 11β-OHT were extracted, and 11-KT and 11β-OHT levels were measured by RIA as previously described (39–41). The percent conversion was calculated using the following formula: 11-KT concentration/(11-KT + 11β-OHT concentration). Differences in percent conversion between the mock transfection group and the rtβ-HSD cDNA transfection group were analyzed by unpaired *t* test.

Analysis of rt11β-HSD mRNA tissue distribution

Northern blot. Total RNAs from various tissues (brain, pituitary, gill, heart, head kidney, posterior kidney, liver, spleen, pyloric caeca, intestine, testis, ovary, muscle, blood, and skin) were extracted from 2-yr-old female and male fish (Wanaka Trout Hatchery). Poly(A)$^+$ RNA (250 ng for all tissues and 2 μg for brain) from the tissues obtained from the female plus testis poly(A)$^+$ RNA were subjected to Northern blot analysis as previously described (42). Poly(A)$^+$ RNA (25 ng) extracted from tissues of a male plus ovarian poly(A)$^+$ RNA were subjected to RT-PCR for confirmation of the Northern blot results.

In situ hybridization. Brain, head kidney, posterior kidney, spleen, pyloric caeca, intestine, testis, and ovary were collected from 1- and 2-yr-old male and female fish (Wanaka Trout Hatchery) and fixed in 4% paraformaldehyde in 0.1 M PBS. They were embedded in paraffin, sectioned (8 μm) and mounted on poly-*L*-lysine-coated slides (BDH Laboratory Supplies, Poole, UK). The 750-bp rt11β-HSD cDNA isolated by 3′RACE-PCR in pGEM-T Easy plasmids (Promega Corp.) was linearized using *Spe*I (Roche Molecular Biochemicals, Mannheim, Germany). Digoxigenin (DIG)-labeled sense and antisense cRNA probes were synthesized using a DIG RNA labeling kit (Roche Molecular Biochemicals). *In situ* hybridization analysis was performed as previously described (42).

Seasonal studies on serum steroids. Serum samples from 2-yr-old rainbow trout (Samegai Trout Hatchery) were collected monthly (5 male and 5–10 female fish at a time) from May to December 1996. Fish in the production pond were crowded with a seine net by hatchery staff and then transferred to a small net cage held in the hatchery pond 1–2 h before sampling. Fish were killed with an overdose of MS222 as described above. Blood was collected and allowed to clot, and serum was obtained by centrifugation and stored at −20 C until RIA. Serum T and 11-KT levels for males were measured as part of a separate study (20). Serum cortisol levels for both males and females were measured as previously described (37, 38). Cortisol data were log transformed, and differences between each sampling point for both males and females were analyzed by one-way ANOVA and Fisher's protected least significant difference test.

Seasonal studies on rt11β-HSD mRNA levels. Testes were also collected from the rainbow trout sampled from the Samegai hatchery in 1996, frozen using liquid nitrogen, and stored at −70 C until RNA extraction. For analysis of seasonal changes in rt11β-HSD mRNA levels in testes, 2 μg poly(A)$^+$ RNA were isolated from at least three fish each month and subjected to Northern blot analysis as previously described previously (42). Testes were fixed with Bouin's fixative to assess developmental stage and categorized into one of the following stages: early spermatogenesis (spermatogonia and spermatocytes present), midspermatogenesis (all sperm developmental stages present), late spermatogenesis (sperm become dominant in seminal lobule), and spermiating (inside of seminal lobule and testicular duct are filled with sperm). Due to the huge significant increase in gonadal size during the reproductive cycle (20), the amount of total RNA per gonad is different in each developmental stage. Thus, comparing gene expression levels on a unit of RNA basis does not reflect the changes in gene expression per gonad. To assess seasonal changes in rt11β-HSD mRNA levels per testis, we measured the rt11β-HSD mRNA levels as follows (20). We measured the total testis weight in each fish, the weight of frozen tissue used for RNA extraction, and the yield of poly(A)$^+$ RNA extracted from each frozen testis sample. We were then able to calculate the total amount of poly(A)$^+$ RNA contained within the testis. By using the band density obtained from Northern blots in the following formula, we were able to give an estimate of the relative amount of testicular rt11β-HSD mRNA per animal:

$$\text{Relative testicular 11}\beta\text{-HSD mRNA levels} = \text{Band density} \times \frac{\text{Total gonad weight}}{\text{Tissue weight used for RNA extraction}} \times \text{Poly(A)}^+ \text{ RNA weight from RNA extraction}.$$

Ovaries from 2-yr-old female rainbow trout (Wanaka Trout Hatchery) were collected monthly (three fish per sample) from March to September 2000. For RNA extraction, yolk was removed from the ovary samples, and the follicles were washed thoroughly with rainbow trout Ringer (39). The follicles were frozen using liquid nitrogen and stored at −70 C until RNA extraction, and 30 follicles/sample were separately frozen to measure the total RNA yield per follicle. Poly(A)$^+$ RNAs were isolated from each sample, and 100 ng were used for Northern blot analysis as described previously (42). Oocyte diameters were measured to determine developmental stage and were categorized as follows: early vitellogenesis (oocyte diameter, 1.0–2.5 mm), midvitellogenesis (2.5–4.0 mm), late vitellogenesis (>4.0 mm), and postovulation. The number of follicles per ovary was determined by dissection and did not appear to change significantly throughout the reproductive cycle. Therefore, the rt11β-HSD mRNA levels are presented as a ratio of the rt11β-HSD levels per follicle. To obtain the value, the following formula was used:

$$\text{Relative ovarian 11}\beta\text{-HSD mRNA levels} = \text{Band density} \times \frac{\text{Total RNA weight from RNA extraction}}{\text{Number of follicles used for RNA extraction}}.$$

As expression of genes that are generally recognized as housekeeping genes such as those encoding β-actin also change with reproductive stage, these genes cannot be reliably used for normalization (42). Therefore, concentrations of poly(A)$^+$ RNAs were carefully measured three times using Ribo Green RNA Quantitation Reagent (Molecular Probes, Inc., Eugene, OR) and a VersaFluor Fluorometer (Bio-Rad Laboratories, Inc., Hercules, CA). It was then possible to standardize Northern blots by loading equal amounts of mRNA per lane. Differences in rt11β-HSD mRNA levels between each sampling point for males and females were analyzed by one-way ANOVA and Fisher's protected least significant difference test.

Seasonal studies of the site of rt11β-HSD gene expression in gonads. Testes of 2-yr-old male rainbow trout (Wanaka Trout Hatchery) were collected monthly from January to September 2001. Ovaries of 2-yr-old female rainbow trout (Wanaka Trout Hatchery) were collected monthly from March to September 2000. Testis cross-sections (~3 mm) from three males and ovarian follicles from six females at each developmental stage

were fixed with 4% paraformaldehyde in 0.1 M PBS and subjected to *in situ* hybridization analysis as described previously (42).

Results

Cloning and sequencing of rt11β-HSD

To confirm the sequence, 13 plasmids containing 1.6 kb of the full-length ORF 11β-HSD were sequenced. Three different ORFs were found: 8 plasmids contained an 1194-bp ORF encoding a predicted protein consisting of 398 amino acids, 4 plasmids contained an 1191-bp ORF (397 amino acids), and 1 plasmid contained an 1188-bp open reading (396 amino acids). These differences were due to the different numbers of asparagine (AAC) repeats in the C-terminal region. Furthermore, 7 different 3′-untranslated regions (UTR), spanning approximately 150 bp, were found among the 13 plasmids. These differences were due to minor variations in the length of the thymine repeats and adenine repeats localized between 20–40 bp downstream from the stop codon. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession no. AB104415.

The deduced amino acid sequence shares 62.3% homology with Japanese eel (*Anguilla japonica*) testicular 11β-HSD (21), 42.2% homology with human 11β-HSD type 2 (35), 44.0% homology with rat 11β-HSD type 2 (33), 21.8% homology with human 11β-HSD type 1 (43), and 22.1% homology with rat 11β-HSD type 1 (2). As significant homology has been reported between mammalian 11β-HSD type 2 and 17β-HSD type 2 (5), we also compared the rt11β-HSD sequence with a range of 17β-HSDs. The highest homology we found was between rt11β-HSD and human 17β-HSD type 2 (44), which share 37.4% homology. Two regions commonly conserved among members of the SCAD superfamily (1) were compared (Fig. 1A): the A domain, containing the putative cofactor binding region of rt11β-HSD (5), shares 60.0% homology with those of mammalian 11β-HSD type 2 sequences, but 36.7% homology with mammalian 11β-HSD type 1 sequences; and the B domain containing the putative active site of rt11β-HSD (5) shares 50.0% homology with both mammalian 11β-HSD types 1 and 2. Hydrophobicity analysis (45) revealed that three successive hydrophobic peaks exist before the cofactor-binding domain in the rt11β-HSD homolog (Fig. 1B). The putative active site of the enzyme in the rt11β-HSD homolog exists within a hydrophobic segment (Fig. 1B). The overall pattern of hydrophobicity for the rt11β-HSD homolog is similar to that of mammalian 11β-HSD type 2.

Enzymatic activity of rt11β-HSD

Using HEK293 cells transfected with pcDNA11βHSD, the conversion of cortisol to cortisone was 75.9%, whereas 24.8% of cortisol was converted to cortisone in HEK293 cells transfected with pcDNA ($P = 0.0002$; Fig. 2A). The conversion of cortisone to cortisol was 14.4% in pcDNA-transfected cells, but there was no significant difference in the conversion with the pcDNA11βHSD-transfected cells (6.8%; $P = 0.1125$; Fig. 2B). HEK293 cells transfected with pcDNA11βHSD showed significantly higher conversion of 11β-OHT to 11-KT (77.6%) than pcDNA-transfected cells (1.6%; $P = 0.0001$; Fig. 2C).

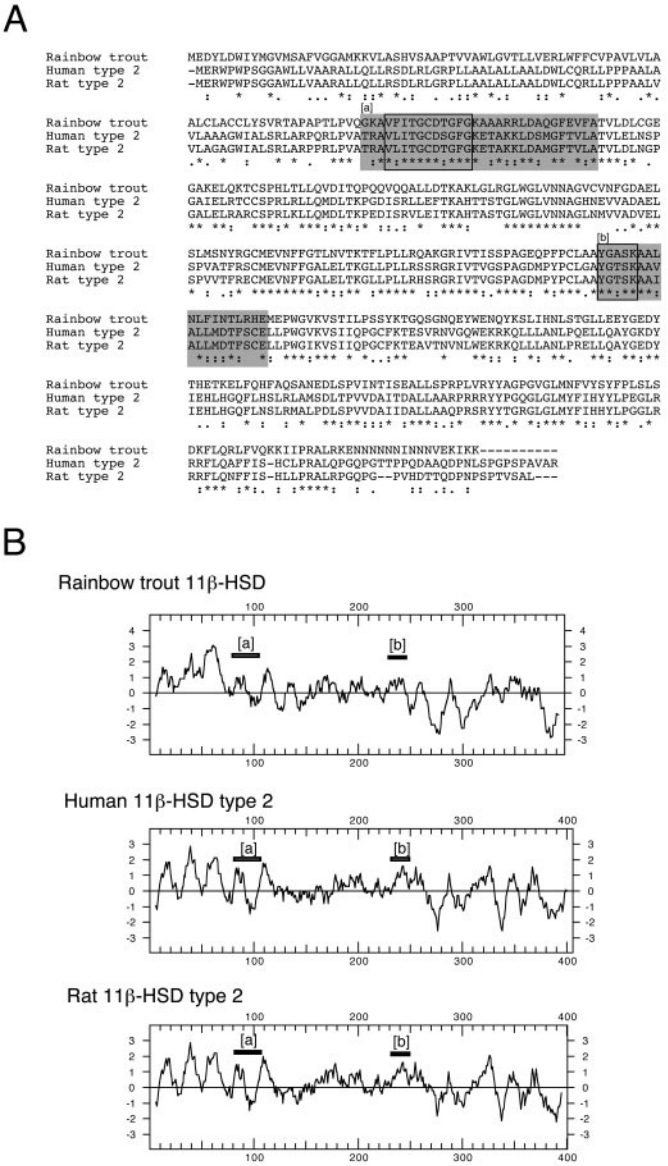

FIG. 1. A, Homology alignment of predicted amino acid sequences between rt11β-HSD and those in mammals. Amino acids identical to those in humans and rats are indicated by *asterisks*. A *colon* indicates conserved substitution, and a *period* indicates semiconserved substitution. Two regions, A and B, previously shown as conserved domains within the SCAD superfamily are enclosed by *gray boxes* in the homology alignment and indicated by *solid lines* in the hydrophobicity analysis. The *rectangle* in the A domain indicates the predicted cofactor-binding region, and the *rectangle* in the B domain indicates the active enzyme site. B, Hydrophobicity analysis between rt11β-HSD and those in mammals.

Transcript size and sites of expression of rt11β-HSD

Northern blot analysis using 250 ng poly(A)$^+$ RNAs obtained from the tissues of a female plus testis poly(A)$^+$ RNA revealed 3.5-kb rt11β-HSD transcripts in pituitary, gill, heart, pyloric caeca, intestine, head kidney, posterior kidney, liver, spleen, testis, ovary, muscle, and skin (Fig. 3). Two micrograms of poly(A)$^+$ RNA obtained from brain gave a weak signal. No signal was obtained from blood RNA. RT-PCR using cDNAs synthesized from the tissues of a male plus

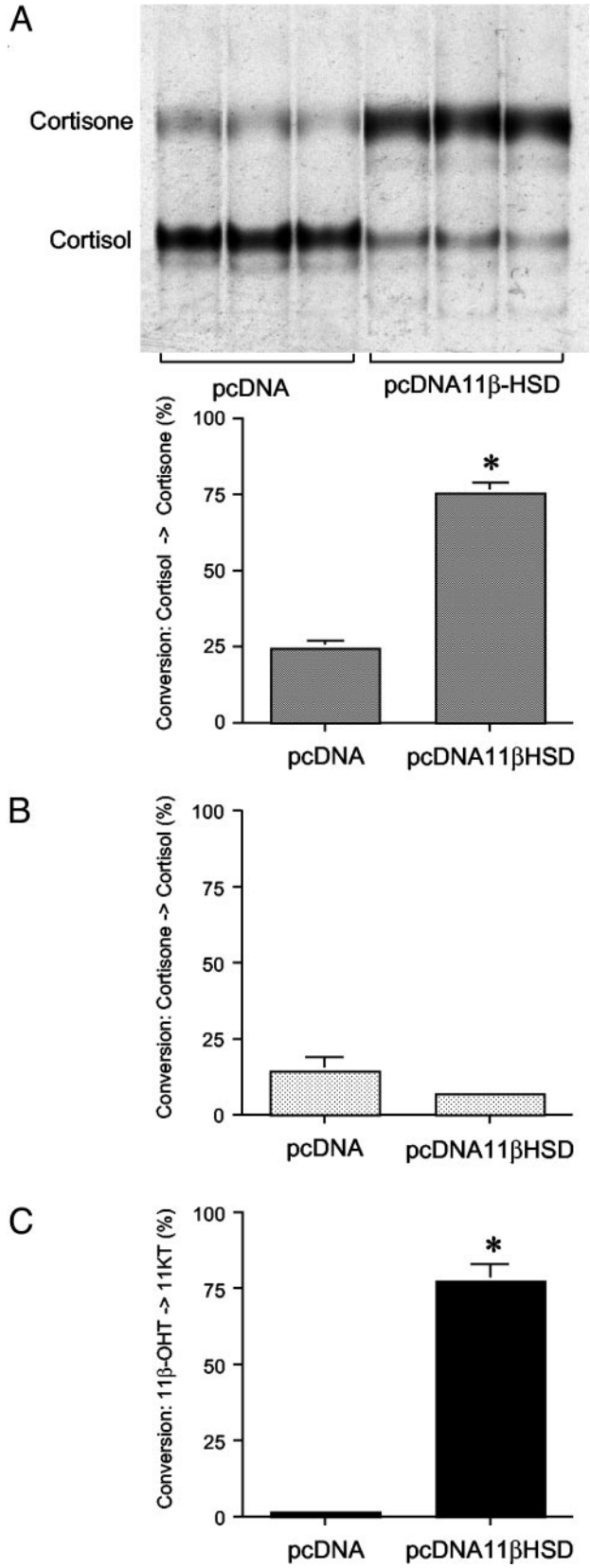

FIG. 2. Enzymatic activity of rt11β-HSD expressed in HEK293 cells. [pcDNA], HEK293 cells transfected with pcDNA 3.1$^+$ containing no rt11β-HSD cDNA. [pcDNA11βHSD], HEK293 cells transfected with pcDNA 3.1$^+$ containing rt11β-HSD cDNA. An *asterisk* indicates values significantly different from the mock transfection group. A, 11β-Dehydrogenase activity of rt11β-HSD on cortisol examined by TLC. *Top*, Autoradiogram of TLC plate. *Bottom*, Conversion of cortisol to cortisone. The values are the mean ± SE (n = 3; P = 0.0002). B, 11-Oxo-reductase activity on cortisone examined by RIA. The values are the mean ± SE (n = 4; P = 0.1125). C. 11β-Dehydrogenase activity on 11β-OHT examined by RIA. The values are the mean ± SE (n = 4; P = 0.0001).

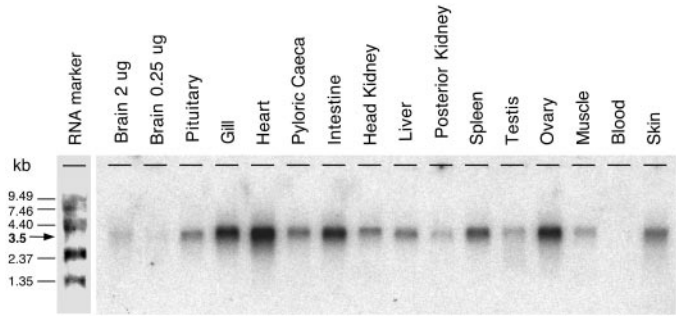

FIG. 3. Tissue distribution of rt11β-HSD mRNA. Poly(A)$^+$ RNA from various tissues of 2-yr-old female rainbow trout and testis poly(A)$^+$ RNA of 2-yr-old male rainbow trout were isolated. Poly(A)$^+$ RNA (250 ng for all tissues and 2 μg for brain) was subjected to Northern blot and hybridized with ^{32}P-labeled rt11β-HSD probe.

ovarian poly(A)$^+$ RNA gave the same results as those from Northern blot analysis (data not shown).

In situ hybridization revealed strong signals in Leydig cells and weaker signals in head kidney interrenal cells from juvenile rainbow trout (Fig. 4). In ovary sections, two different types of cell expressing rt11β-HSD were found. Strong signals were found in some thecal cells corresponding to steroid-producing cells identified in other studies (46) of the early vitellogenic ovarian follicle and in thecal and granulosa cells of the postovulatory follicles (Fig. 4). Although Northern blot showed rt11β-HSD mRNA in a wide range of tissues, *in situ* hybridization signals were not detectable in samples of brain, posterior kidney, spleen, pyloric caeca, or intestine (data not shown).

Seasonal changes in serum steroid levels

T and 11-KT levels in serum sampled from the Samegai Trout Hatchery in 1996 were presented in the report by Kusakabe *et al.* (20). Serum cortisol levels in rainbow trout after 1–2 h of confinement are shown in Fig. 5. Cortisol levels in males were not significantly different between sampling points (P = 0.1002). However, there was a trend for stress-induced cortisol levels to be stable at less than 100 ng/ml from early to midspermatogenesis (May to early August), except for one sample in June (238 ng/ml). Stress-induced cortisol levels increased until the beginning of the late maturation stage (early October). As most animals reached the late spermatogenesis (late October) and spermiating (early December) stages, cortisol levels appeared to decline.

Cortisol levels in females were significantly different between sampling points (P = 0.0051). In females, stress-induced cortisol levels were 3 times as high (213 ng/ml) as those in males in May (beginning of early vitellogenic stage). Cortisol levels declined during early vitellogenesis until early August (95 ng/ml). During the midvitellogenic stage

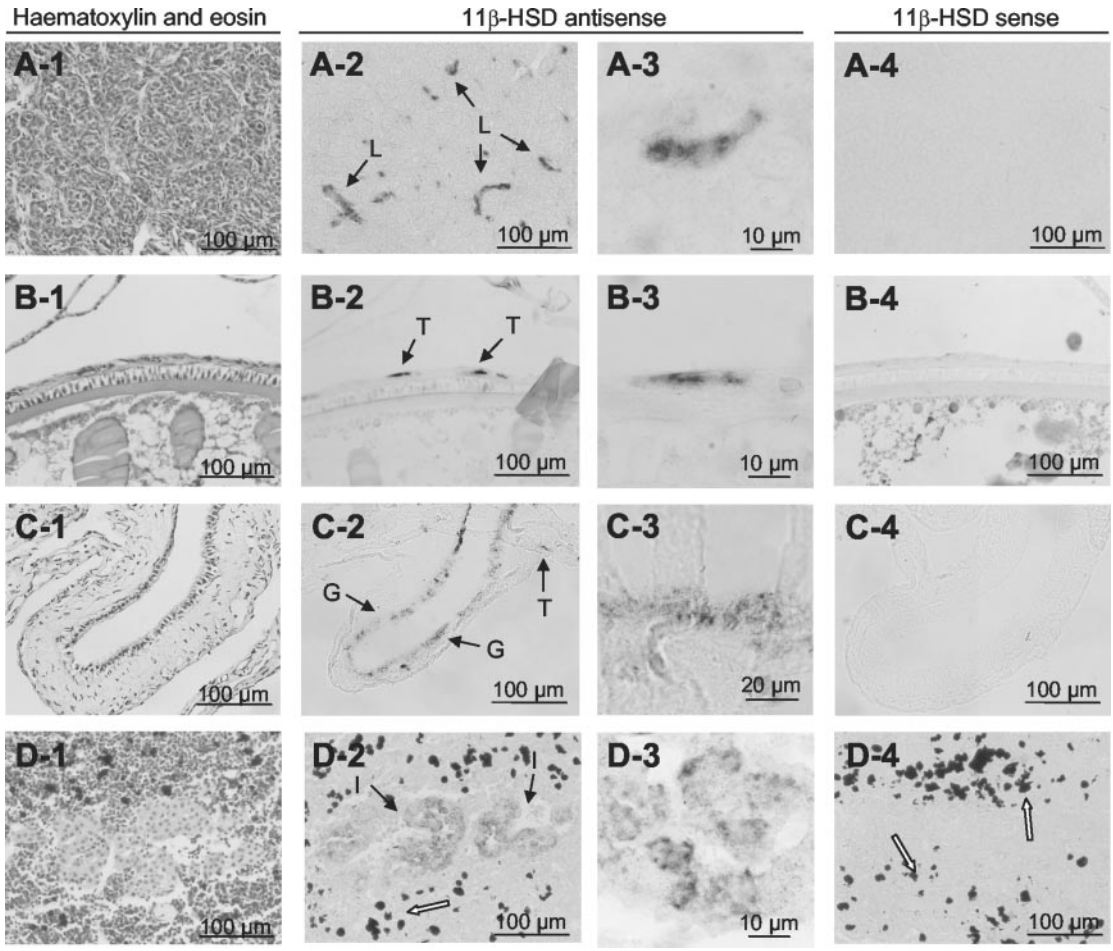

FIG. 4. *In situ* hybridization for rt11β-HSD mRNA with DIG-labeled rt11β-HSD cRNA probe. A-1–4, Testis of juvenile fish; B-1–4, ovarian follicle of early vitellogenic stage; C-1–4, ovarian follicle of postovulatory stage; D-1–4, head kidney of juvenile fish. Column 1, Hematoxylin/eosin-stained sections; columns 2 and 3, *in situ* hybridization sections, hybridized with antisense probe; column 4, *in situ* hybridization sections, hybridized with sense probe. Cells positive for DIG-labeled rt11β-HSD antisense probe are indicated by *solid arrows*. *Open arrows* indicate melanin granules. rt11β-HSD mRNA signals were detected in Leydig cells (L) of testis, thecal (T), and granulosa (G) cells of ovary and in head kidney interrenal cells (I).

(September to early October), cortisol levels were significantly elevated (208–290 ng/ml; $P < 0.05$), but levels tended to decrease thereafter and were significantly lower than peak values (290 ng/ml) by early December (125 ng/ml; $P < 0.05$).

Seasonal changes in rt11β-HSD gene expression in gonads

Northern blot. The results of the Northern blot analysis for seasonal changes in gonadal rt11β-HSD gene expression are shown in Fig. 6. Seasonal changes in testicular rt11β-HSD mRNA levels are presented as a ratio of the levels in May 1996 (Fig. 6A). There was no significant difference detected in total testicular rt11β-HSD mRNA levels between each sampling point ($P = 0.1953$) due to the large variation in samples from August to October. However, there was a trend that rt11β-HSD mRNA levels were relatively low during the early spermatogenesis (May to June), but appeared to increase during mid and late spermatogenesis (July to late October). As the testes reached the spermiating stage (early December), rt11β-HSD mRNA levels appeared to decline. Only two testis samples were available in June 1996, and the value in June was therefore presented as an average, but not included for the statistical analysis.

Seasonal changes in rt11β-HSD mRNA levels during the Southern fall-spring (Wanaka Trout Hatchery fish) are presented as a ratio of the levels in March 2000 (Fig. 6B). There was a significant difference between sampling points ($P = 0.004$). Ovarian rt11β-HSD mRNA levels were low between the early and midvitellogenic stages, but showed a trend for a gradual increase from March to June. As the ovaries reached the late vitellogenic stage (July), the levels of rt11β-HSD mRNA significantly increased ($P < 0.05$) and were maintained at that level through the postovulatory stage (September). As only two ovary samples were available in June 2000, the June mean is presented as an average, but was not included for statistical analysis.

In situ hybridization. Representative micrographs from *in situ* hybridization of testis and ovary with a rt11β-HSD cRNA probe are shown in Figs. 7 and 8. rt11β-HSD mRNA signals appeared to correlate well with the seasonal profile of rt11β-HSD mRNA levels measured by Northern blot analysis.

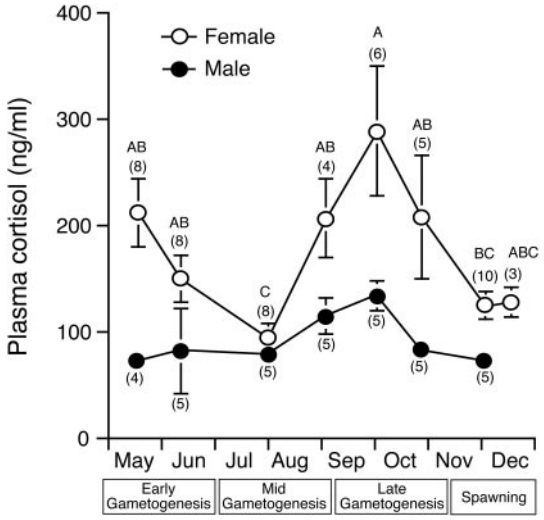

FIG. 5. Seasonal changes in the plasma stress-induced cortisol levels of mature female and male rainbow trout. Data are shown as the mean, and number of samples per data point are shown in *parentheses*. Bars indicate ±SE. Cortisol data were log transformed and analyzed by one-way ANOVA [male: $F(6,27) = 2.003$; $P = 0.1002$; female: $F(7,44) = 3.434$; $P = 0.0051$]. Values *not sharing a letter* are significantly different by Fisher's protected least significant difference test ($P < 0.05$; female, A–C).

In testes, Leydig cells during early spermatogenesis were often found as clusters of two or three cells, and the rt11β-HSD mRNA signal intensity appeared to be low (Fig. 7A). Leydig cells in mid- and late spermatogenesis testes (Fig. 7, B and C) were found in interstitial tissue surrounding the spermatogenic cysts. However, they were not distributed equally, but were often found beside the main sperm duct and/or at the periphery of testes. Hybridization signals were more intense in the mid to late spermatogenesis stages (Fig. 7, B and C). After spermiation, as the size of the gonad declined, Leydig cells appeared at higher density and often formed clusters, but the hybridization signal intensity declined (Fig. 7C).

In ovaries, no signal was detected during the very early vitellogenic stage in March (Fig. 8A). Hybridization signals were found in thecal cells of the early vitellogenic stage in April (Fig. 8B). rt11β-HSD mRNA signal was seen in thecal cells from early vitellogenic (April; Fig. 8B) until postovulatory stages (September; Fig. 8E). In the midvitellogenic stage in May, rt11β-HSD mRNA was found for the first time in granulosa cells, but the signals were weak (Fig. 8C). The signal intensity in granulosa cells noticeably increased in June (Fig. 8D). Although no results are available from the late vitellogenic stage (July and August) because of the poor adherence and integrity of the large, yolky, late vitellogenic sections during the *in situ* hybridization procedure, strong hybridization signals were detected in granulosa cells and in some scattered thecal cells of postovulatory follicles in September (Fig. 8E).

Discussion

We have cloned 11β-HSD cDNAs from rainbow trout. We found three different ORFs for rt11β-HSD due to the different lengths of the asparagine repeat at the C-terminal domain. The rt11β-HSD cDNAs, including the 3′UTR sequence, were found in seven different forms. As differences in the 3′UTR sequences are due to a few variations in the length of the thymine and adenine repeats near the stop codon, it is possible that the variation in the 3′UTR is due to PCR error. However, the three different ORFs are probably not due to PCR error, because the differences are always due to disappearance of either one or two AAC (asparagine) codons.

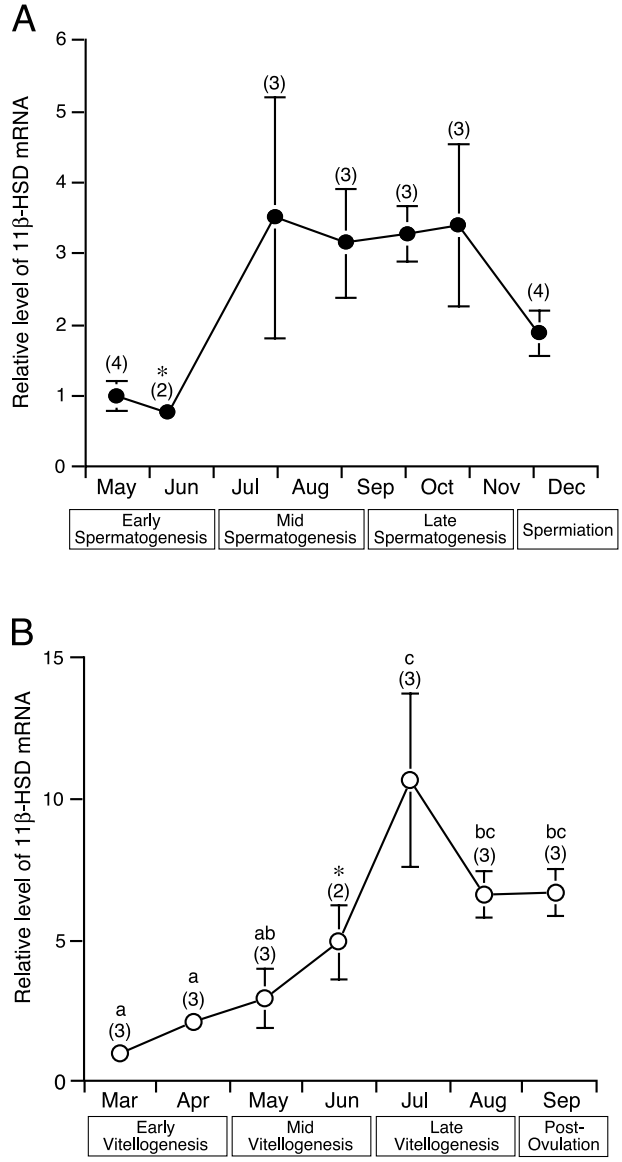

FIG. 6. Seasonal changes in rt11β-HSD mRNA levels in testes of adult males (A) and ovary of adult females (B) during gametogenesis. mRNA was visualized by Northern blot. rt11β-HSD mRNA levels are presented as a ratio of the levels in May 1996 for testes and in March 2000 for ovary. Data are shown as the mean, and the number of samples per data point is shown in *parentheses*. *Bars* indicate ±SE. One-way ANOVA was performed [male: $F(5,14) = 3.209$; $P = 0.1953$; female: $F(5,12) = 6.408$; $P = 0.004$]. Values *not sharing a letter* are significantly different by Fisher's protected least significant difference test ($P < 0.05$; female, a–c). An *asterisk* indicates that only two samples were available in June 1996 for testes and in June 2000 for ovary; therefore, the values for these sampling points are presented as an average and were not included for the statistical analysis.

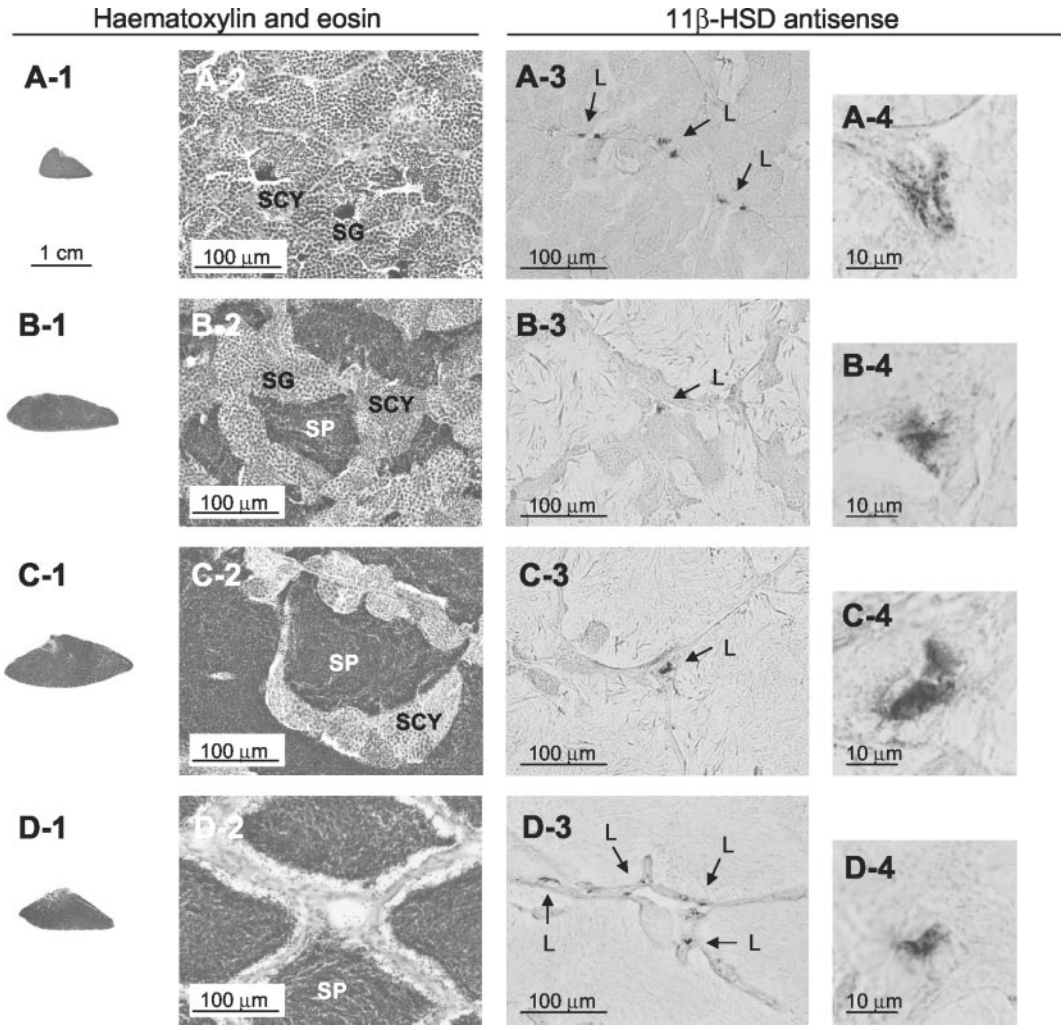

FIG. 7. Representative *in situ* hybridization analysis showing seasonal changes in rt11β-HSD mRNA in testes. A-1–4, Early spermatogenesis stage; B-1–4, midspermatogenesis stage; C-1–4, late spermatogenesis stage; D-1–4, spermiating testes. Column 1, Cross-sections of hematoxylin/eosin stain; column 2, higher magnification of hematoxylin/eosin stain; columns 3 and 4, *in situ* hybridization sections, hybridized with antisense probe. Cells positive for DIG-labeled rt11β-HSD antisense probe are indicated by *solid arrows*. SG, Spermatogonia; SCY, spermatocytes; SP, sperm. Signals were found in Leydig cells (L).

These sequences are not sufficiently different to suggest alternative splicing, but these isoforms may be formed by genome duplication (47). Whether there are functional differences between three different ORFs is not known. Northern blot analysis revealed a major 3.5-kb transcript. The rt11β-HSD cDNA clones isolated in this study were approximately 1.6 kbp and lacked the poly(A) tail. Difficulty in amplifying the full 3′UTR to the poly(A) tail by PCR is possibly due to the structure of the 3′UTR, such as the adenine and thymine repeat region. The adenine repeat would have interfered with synthesis of full-length 3′UTR cDNA using oligo(deoxythymidine) primer.

The predicted amino acid sequence of rt11β-HSD revealed higher homology to mammalian 11β-HSD type 2 (42–44%) than 11β-HSD type 1 (21–22%). The putative cofactor-binding region and the putative active site are more highly conserved compared with mammalian 11β-HSD type 2 rather than type 1. Furthermore, the predicted secondary structure analysis for rt11β-HSD and mammalian 11β-HSDs showed features similar to those of mammalian 11β-HSD type 2, such as a hydrophobic region before the putative cofactor-binding site. The results of transient transfection assays with rt11β-HSD in HEK293 cells showed significant 11β-dehydrogenase activity, but no significant 11-oxo-reductase activity. HEK293 cells transfected without the rt11β-HSD insert also showed moderate 11β-dehydrogenase activity, probably due to endogenous 11β-HSD activity of the cells, because the HEK293 cell line is derived from human kidney cells. Although there is some homology with mammalian 17β-HSD, rt11β-HSD does not show significant 17β-HSD activity in the same transient transfection assay (Kusakabe, M., and G. Young, unpublished data). These results strongly suggest that the rt11β-HSD encoded by the cDNAs cloned in this study is a homolog of mammalian 11β-HSD type 2, being predominantly an 11β-dehydrogenase.

In mammals the 11β-HSD type 1 gene is expressed in a number of tissues, including liver, lung, adipose tissue, vasculature, ovary, kidney, testis, and central nervous system,

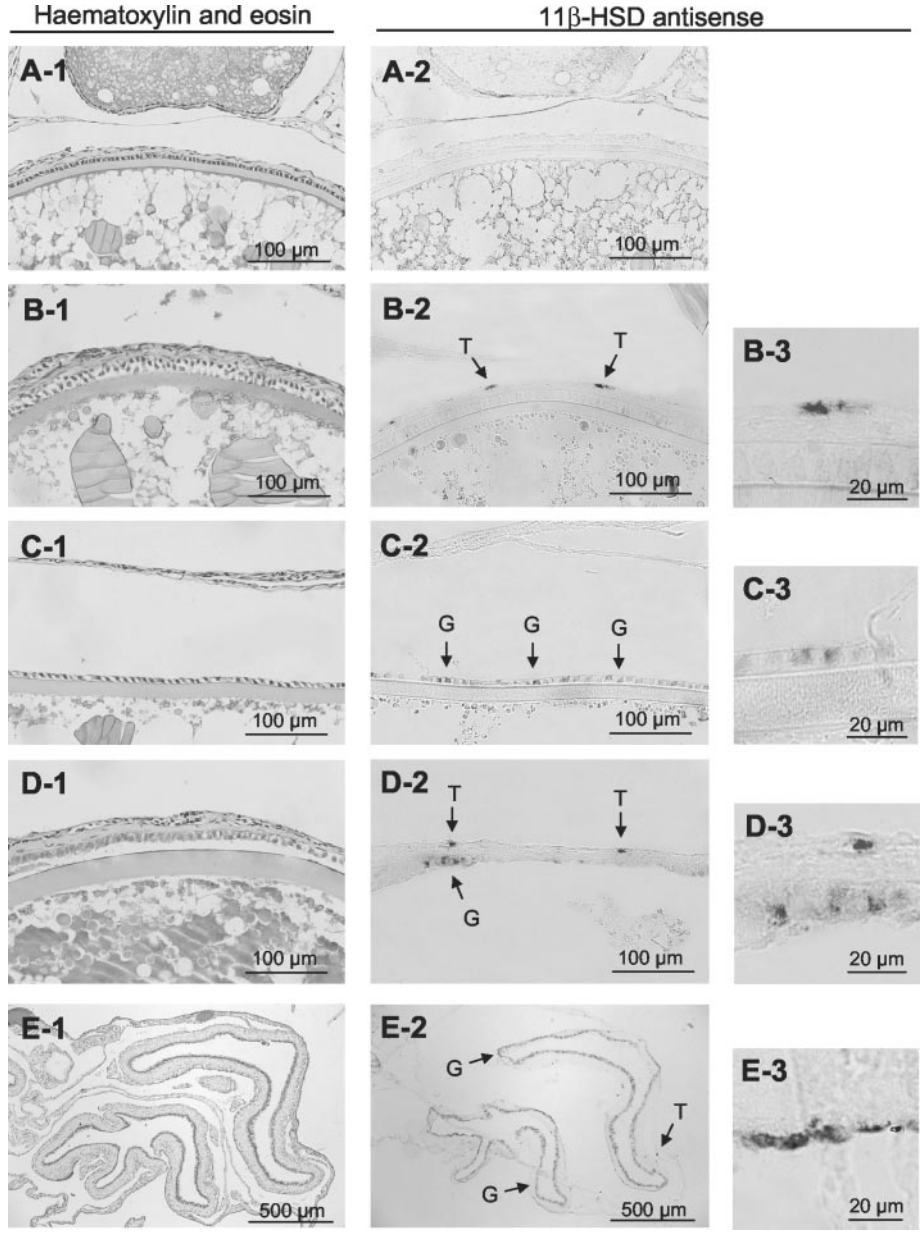

FIG. 8. Representative *in situ* hybridization analysis showing seasonal changes in rt11β-HSD mRNA in ovarian follicles. A-1–4, Early vitellogenic stage; B-1–4, early vitellogenic stage; C-1–4, midvitellogenic stage; D-1–4, late vitellogenic stage; E-1–4, postovulatory stage. Column 1, Hematoxylin/eosin stained sections; columns 2 and 3, *in situ* hybridization sections, hybridized with antisense probe. Cells positive for DIG-labeled rt11β-HSD antisense probe are indicated by *solid arrows*. Signals were found in thecal cells (T) and granulosa cells (G).

whereas 11β-HSD type 2 gene is highly expressed in classical aldosterone-selective target tissues, such as distal nephron, colon, sweat gland, and placenta (48). In this study, tissue distribution analysis of rt11β-HSD gene expression by Northern blot and RT-PCR analysis showed rt11β-HSD mRNA in brain, pituitary, gill, heart, pyloric caeca, intestine, head kidney, posterior kidney, liver, spleen, testis, ovary, muscle, and skin. Although the rt11β-HSD is a homolog of mammalian 11β-HSD type 2, the widespread tissue distribution of rt11β-HSD mRNA is similar to that of mammalian 11β-HSD type 1 genes. We also conducted *in situ* hybridization analysis to identify the cellular localization of rt11β-HSD mRNA in various tissues, and rt11β-HSD mRNA was only detected in known steroidogenic tissues: head kidney interrenal cells, Leydig cells of testis, granulosa cells, and some cells in the thecal layer of the ovarian follicle. Possibly cells in nonsteroidogenic tissues may equally, but weakly, express the rt11β-HSD gene at levels too low to detect by *in situ* hybridization. Dehydrogenase activity of 11β-HSD is essential for 11-KT synthesis from 11β-OHT in testes. However, the functions of rt11β-HSD in other tissues are unclear.

Suppressive effects of stress-induced cortisol on sex steroid production have been reported in a wide range of fish species (49–53). High circulating cortisol levels typically lead to inhibition of gonadal development (54). In salmonids, plasma androgen levels were also suppressed by acute stress in male (49) and female (55) rainbow trout. Pottinger *et al.* (56) reported that sexual maturity lowered the responsiveness to stress-related cortisol production in male rainbow trout and suggested that the reduction of cortisol production could be a physiological adaptation to protect the testes from the inhibitory effects of cortisol. Various lines of evidence suggest that mechanisms to protect developing gonads from corticosteroid-mediated stress may exist. Previous mamma-

lian studies have shown that high levels of plasma cortisol suppressed T secretion in testes (8, 57). Therefore, 11β-HSD type 1 in rat Leydig cells was proposed to have a role in the protection of testes from glucocorticoid-mediated inhibition of sex steroidogenesis (10, 11). However, this idea has been questioned by Leckie *et al.* (12), who demonstrated that 11β-HSD type 1 in rat Leydig cells is predominantly a reductase. Interestingly, unlike rat Leydig cells, rainbow trout Leydig cells express an 11β-HSD type 2 (11β-dehydrogenase) homolog. Positive signals for rt11β-HSD in some thecal cells and in granulosa cells of rainbow trout ovarian follicles were also found. As rainbow trout follicles do not express the 11β-hydroxylase gene (20) and therefore cannot produce 11β-hydroxyandrogens, the likely substrate for the ovarian enzyme is cortisol. Pickering and Christie (58) reported that the basal concentrations of plasma cortisol were markedly elevated toward the end of the reproductive cycle in male and female brown trout. Therefore, it is possible that rt11β-HSD in gonads may be part of a mechanism serving to protect gamete development from high circulating corticosteroid levels.

To explore this possibility further, we analyzed the relationship between stress-induced cortisol levels and gonadal rt11β-HSD mRNA. Unfortunately, it was not possible to obtain samples from unstressed fish because of the difficulties of rapidly catching and sampling fish in the production pond. Previous work has shown that chronic stress results in a peak in cortisol levels in rainbow trout 1–2 h after the application of a confinement stressor (56), which is the time frame used in this study. In this study stress-induced serum cortisol levels peaked during the late reproductive stages in both males and females, but levels in females were consistently higher than those in males. Differences in stress-induced cortisol production between males and females have been reported previously (59) and are most likely due to the differential effects of androgens and estrogens on the hypothalamic-pituitary-interrenal axis (60). Analysis of seasonal changes in rt11β-HSD gene expression in testes and ovarian follicles by Northern blot revealed an increase during late reproductive stages, although this was only statistically significant for females. In testes used for *in situ* hybridization analysis, the signal intensity in Leydig cells seemed to agree with the patterns seen by Northern blot. In females, the significant increase in rt11β-HSD gene expression (7- to 10-fold increase) during the late vitellogenesis and postovulatory stages revealed by Northern blots was supported by *in situ* hybridization results showing an abundance of rt11β-HSD transcripts in the granulosa cells of postovulatory follicles, although results for the late vitellogenic stage are not available. Interestingly, the changes in testicular rt11β-HSD mRNA levels tended to be more closely related to the pattern of the cortisol stress response than the seasonal changes in serum 11-KT levels, which increased with the progression of spermatogenesis, peaked in the late spermatogenesis stage, and declined after the onset of spermiation (20). In contrast, rainbow trout 11β-hydroxylase mRNA levels were correlated with the seasonal serum 11-KT levels, suggesting that 11-KT production is regulated in part by changes in the expression of the 11β-hydroxylase gene (20) rather than the rt11β-HSD gene. High levels of rt11β-HSD mRNA during the late reproductive stages in males and females may point to an involvement of rt11β-HSD in protecting gonads from high cortisol levels that might result from the heightened stress response compared with the early reproductive stages. Despite earlier work showing a direct suppressive action of cortisol on 17β-estradiol and T production by ovarian follicles of rainbow trout *in vitro* (61), Pankhurst *et al.* (62) found that cortisol was not directly associated with the inhibition of steroid production by teleost ovarian follicles. Nonetheless, rt11β-HSD in gonads and other tissues may be involved in reducing the impact of excessive circulating cortisol during the late reproductive stages.

The factors regulating rt11β-HSD gene expression are not known. LH induced 11β-HSD type 1 gene expression and suppressed 11β-HSD type 2 gene expression in human granulosa cells (13). These results were confirmed by an *in vitro* study using rat granulosa cells (14). Rat granulosa cells treated with hCG induced an increase in 11β-HSD type 1 mRNA and a decrease in 11β-HSD type 2 mRNA. However, Japanese eel injected with hCG showed a significant increase in testicular 11β-HSD mRNA (11β-HSD type 2 homolog). In female rainbow trout, Northern blot analysis revealed that ovarian rt11β-HSD mRNA levels peaked in the late vitellogenic and postovulatory stages, a time when plasma LH levels increased significantly (63, 64), suggesting that LH may also regulate 11β-HSD gene expression in rainbow trout.

In vitro studies using head kidney of juvenile coho salmon demonstrated that cortisone is not a detectable product of salmonid interrenal cells (65). However, after fish were acutely stressed by a 30-sec handling stressor, both plasma cortisol and cortisone levels were significantly elevated (65). These results suggested that cortisol secreted by interrenal cells is converted peripherally to cortisone. The widespread tissue distribution of rt11β-HSD may point to the enzyme having a physiological role in the conversion of cortisol to cortisone. In this study the *in situ* hybridization signal intensity for rt11β-HSD mRNA in interrenal cells of juvenile fish was faint compared with that in Leydig cells. The low rt11β-HSD mRNA levels in interrenal cells in juvenile fish could explain why head kidneys of juvenile coho salmon did not produce cortisone *in vitro*. The function of rt11β-HSD in interrenal cells is still not clear. The salmonid interrenal does contain a suite of steroidogenic enzymes capable of producing 11β-oxygenated androgens (66), and expression of the rt11β-HSD gene within these cells raises the question of a contribution of this tissue to 11-KT production. In addition, it is known that cortisol exerts direct ultra-short-loop negative feedback on cortisol production (67). Possibly rt11β-HSD could perform a role in regulating short-loop negative feedback in this tissue.

Mammalian 11β-HSD type 2 performs the vital role of converting glucocorticoids to their inactive 11-keto forms in mineralocorticoid target organs such as kidney and colon to allow selective access of aldosterone to MR. In teleosts, aldosterone is believed to be absent (25–27), and a single corticosteroid acts as both a mineralocorticoid and a glucocorticoid (28). Therefore, it had been assumed that only a single corticoid receptor existed in teleosts. However, a cDNA encoding an rtMR has recently been characterized, and cortisol

appears to be a major ligand for the rtMR (24). Although the details of physiological functions and tissue distribution of rtMR are unclear as yet, further investigation of the link between rt11β-HSD and rtMR may provide important information regarding the evolution of corticosteroid actions in vertebrates.

In summary, we have cloned cDNAs encoding rt11β-HSD, which is a homolog of mammalian 11β-HSD type 2. The rt11β-HSD gene appears to be expressed in a much wider range of tissues than mammalian 11β-HSDs. The analysis of seasonal changes in rt11β-HSD mRNA raises the possibility of roles for this enzyme in ameliorating cortisol-induced suppression of gonadal development and possible up-regulation by LH. The potential for rt11β-HSD to reduce the impact of stress-induced cortisol on reproduction is a question we are currently addressing, and the possibility of a role for rt11β-HSD in the function of the MR is of interest in the context of the evolution of corticosteroid-mediating systems.

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