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Cloning and Expression of Two Carbonyl Reductase-like 20β-Hydroxysteroid Dehydrogenase cDNAs in Ovarian Follicles of Rainbow Trout (*Oncorhynchus mykiss*)

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In salmonid fish, 20β-hydroxysteroid dehydrogenase (20β-HSD) is a key enzyme involved in the production of oocyte maturation-inducing hormone (MIH), 17α,20β-dihydroxy-4-pregnen-3-one. Here we report the isolation of two cDNAs which encode proteins with high homology to carbonyl reductase-like 20β-HSD (CR/20β-HSD) from rainbow trout (*Oncorhynchus mykiss*) ovarian follicles. Genomic DNA analysis showed that the two CR/20β-HSD cDNAs are derived from two different genes. Northern blot and RT PCR analysis demonstrated that trout CR/20β-HSDs are broadly expressed in various tissues. Enzymatic characterization using recombinant CR/20β-HSD proteins produced in *E. coli* showed that the product of one of the two cDNAs had both 20β-HSD and CR activity, but the other had neither activity. Although the functional significance of the two genes remains unresolved, these results clearly demonstrate the presence of two distinct CR/20β-HSD transcripts in the trout ovary.

Meiotic maturation of the oocyte (oocyte maturation) is a crucial step in the development of a fertilizable gamete. In teleosts, oocyte maturation is regulated by an oocyte follicle-derived steroidal mediator, maturation-inducing hormone (MIH), under the control of gonadotropin (GTH). 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) has been identified as the natural MIH in salmonid fish (1), and a two cell-type model has been proposed for the production of 17α,20β-DP by salmonid ovarian follicles (2). In this model, GTH stimulates the thecal layer to produce 17α-hydroxyprogesterone (17α-HP), and also acts on the granulosa cell layer to rapidly enhance 20β-hydroxysteroid dehydrogenase (20β-HSD) activity, which converts 17α-HP to 17α,20β-DP. It has been suggested that GTH causes the *de novo* synthesis of 20β-HSD by a mechanism dependent on RNA synthesis (3), via an adenylate cyclase-cAMP pathway (4). Thus, 20β-HSD is the key enzyme involved in the production of MIH. However, the molecular events underlying the GTH-regulated 20β-HSD synthesis have not yet been elucidated because the cDNA(s) encoding salmonid 20β-HSD has not been cloned.

We have reported the cloning of 20β-HSD cDNA from neonatal pig testes (5). Interestingly, pig 20β-HSD shows high similarity to carbonyl reductase (CR) from human (6) and rat (7, 8), and pig 20β-HSD has a very strong carbonyl reductase activity (9), suggesting that pig 20β-HSD is identical to pig CR. We named it as CR/20β-HSD. In the present study, we cloned CR/20β-HSD cDNAs from rainbow trout ovarian follicles and analyzed the enzymatic activity of the proteins they encode.

MATERIALS AND METHODS

cDNA and genomic cloning. A CR/20β-HSD cDNA fragment from ayu (*Plecoglossus altivelis*) ovary (10) was used as a probe to screen a λgt 10 ovarian follicle cDNA library prepared from rainbow trout (*Oncorhynchus mykiss*) ovary containing fully-grown follicles just prior to maturation. A genomic λDASH rainbow trout library (gift from Dr. Thomas Chen, University of Connecticut) was screened using a rainbow trout CR/20β-HSD cDNA clone (clone B, see below) obtained from the rainbow trout cDNA library as a probe. Inserts of both cDNA and genomic clones were further subcloned into pBluescript SK⁻ vectors and sequenced with an ABI PRISM 377 DNA Sequencer. All the sequences were confirmed by bi-directional determination and deposited into Genbank (accession number AF 100930-33).

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5′ RACE. The 5′ end sequence of one of the rainbow trout CR/20β-HSD cDNAs (clone A) was determined by 5′ Rapid Amplification of cDNA End system (Gibco BRL). The nested gene-specific primer (primer G3A, 5′ CATTCACCACTCTGGCATTTGG 3′) was designed to anneal to 511nt-532nt of the rainbow trout CR/20β-HSD cDNA A. Three 5′ RACE products of different sizes were cloned into TA-cloning vector, pGEM T-easy (Gibco BRL), and subsequently characterized.

In vitro expression of rainbow trout CR/20β-HSD cDNA A and B in E. coli. Two primers were designed to introduce an *Nde*I site at the 5′ end and a *Bam*HI site at the 3′ end of the open reading frame (ORF) of the two rainbow trout CR/20β-HSD cDNAs (clone A and B). The PCR amplified products were inserted into a bacterial expression vector (pET21b$^+$) at the *Nde*I and *Bam*HI sites, and introduced into *E. coli* BL21 strain. The expression plasmids were verified by nucleotide sequence analysis. Fifty-ml cultures were harvested and 1-ml homogenized lysates in Tris-HCl buffer were recovered. After 10,000 rpm centrifugation, the supernatants were collected and used to measure 20β-HSD activity by the conversion of 17α-HP into 17α, 20β-DP. After incubation at 28°C with [^{3}H] 17α-HP (1.48-2.22 TBq/mmol; NEN), products were separated on a high performance TLC plate (Merck) in a benzene/acetone (4:1) solvent system, which was then exposed to Hyperfilm (Amersham). A spot with the same mobility as cold standard 17α,20β-DP was extracted with ethanol, and further identified by recrystallization according to Axelrod (11).

Carbonyl reductase activity was determined using the method described by Wermuth (6). Clear lysates were harvested from 200-ml cultures of BL21 transformed with expression constructs, as described above. The lysates were purified by fast protein liquid chromatography (FPLC) through DEAE Sepharose fast flow (Pharmacia). The absorbed proteins were eluted using a linear gradient of 3 to 100 mM potassium phosphate buffer with 0.1 mM EDTA and 0.1 mM DTT (pH 7.4). Carbonyl reductase activity was measured by the decrease in absorbance at 340 nm in the presence of 0.08 mM NADPH at 25°C.

Northern analysis. Total RNAs were prepared from adipose tissue, brain, gill, head kidney, posterior kidney, liver, heart, muscle, testis and ovary of rainbow trout in vitellogenic stage using ISOGEN (Nippon Gene), and poly (A)+ RNAs were purified using Oligotex-DT30 (Takara). The poly(A)+RNAs (2.5 μg) were separated on a formaldehyde-agarose gel, and transferred onto a nylon membrane (Hybond-N+, Amersham). The membrane was hybridized with the 5′ end of the cDNA A fragment, labeled with [^{32}P]-dCTP by PCR amplification using: primer AS (5′ CAACTCTGGTTTAAATAATG-TAATTAGCTGTGCTGGTGA 3′) and primer G3A (5′ CATTCAC-CACTCTGGCATTTGG 3′). After stripping hybridized probe from the membrane, a control rehybridization was performed with a rainbow trout β-actin cDNA probe. The data were quantified by a BAS 2000 Imager (Fuji).

Reverse-transcription and polymerase chain reaction (RT-PCR). One microgram of total RNA extracted from each tissue was reverse-transcribed to first-strand cDNA using a cDNA Synthesis Kit (Takara). Forward primer specific for CR/20β-HSD cDNA A was: 5′-TCACCAGCACAGCTAATTACATTATTTAAACCAGAGTTG (35-73) and the specific primer for CR/20β-HSD cDNA B was 5′-CTACAACCACAGTGCGGT (22-39). The reverse primer was the same for both CR/20β-HSD cDNAs A and B (primer G3A 5′-CATTCACCACTCTGGCATTTGG). PCR reaction was performed at 94°C for 1 min, 62°C for 1 min and 72°C for 1 min for 30 cycles using a Perkin-Elmer 480 thermal cycler.

RESULTS

Isolation of two CR/20β-HSD genes (clone A and clone B) from rainbow trout ovarian follicles. Thirteen positive clones were recovered from screening of two million λgt10 phage plaques. Two cDNAs whose sequences exhibited high homology to ayu (84%) and pig (61%) CR/20β-HSDs were obtained and termed rainbow trout CR/20β-HSD cDNA type A and B (Fig. 1). Type B cDNA contains an open reading frame of 828 nucleotides, with 114 nucleotides in the 5′ noncoding region, and 271 nucleotides in the 3′ noncoding region including one polyadenylation site. The predicted molecular weight of the deduced protein encoded by the ORF is 30.1 kDa, and the isoelectric point (pI) is 7.58. Type A contains an incomplete ORF missing the 5′-end with 324 nucleotides in the 3′ noncoding region including a poly(A) signal. Since the 5′-end of type A was truncated, 5′ RACE was carried out to fill the 5′ end of the clone A. Like type B, type A cDNA also contains an ORF of 828 nucleotides and encodes a 276 amino acids product with a pI of 7.89. Type A and type B shared high homology of 99% at the nucleotide level and 98.7% at the amino acid level within their ORFs. The homology of the 5′ noncoding regions is 77%, while that of the 3′ noncoding region is 91%. Comparison of the amino acid sequences of rainbow trout CR/20β-HSDs with other mammalian carbonyl reductases, members of the short-chain dehydrogenase/reductase (SDR) family, reveals that the Rossmann fold, GlyXXXGlyXGly, which is the co-factor binding site, is well conserved among these enzymes, as is the TyrXXXLys motif which is critical to the catalytic activity (12).

Using type B cDNA fragment as a probe, we screened the λDASH genomic library of rainbow trout. Four positive clones were isolated from one million phage plaques. Two clones, termed CR/20β-HSD genes I and II, showed different restriction patterns and were further sequenced. The sequence of the cDNA deduced from CR/20β-HSD gene I matched perfectly with CR/20β-HSD cDNA type A. cDNA sequence deduced from CR/20β-HSD gene II matched with CR/20β-HSD cDNA type B except for two nucleotides. Both rainbow trout CR/20β-HSD genes consist of four exons. The structural organization of the genes is very similar, with the introns interrupting the genes at the same locations. However, some of the introns are of different sizes. The sequences of genes I and II share 53% identity in the 5′ upstream regions up to −700 bp from the initiation site. There is a putative TATA box in the upstream region of both rainbow trout CR/20β-HSD I and II, but sequence homology decreases upstream from the TATA box.

Enzymatic characterization of recombinant CR/20β-HSD proteins. Both ORFs of cDNA clones A and B were inserted into the expression vector pET21b+ to yield pET21b+rcA and pET21b+rcB. Protein expressed from pET21b+rcA and pET21b+rcB were confirmed to be 30 kDa by SDS/PAGE electrophoresis, and the N-terminal amino acid sequences were confirmed by an amino acid analyzer (ABI 494). Products from pET21b+rcA, pET21b+rcB, or pET21b+ only, were

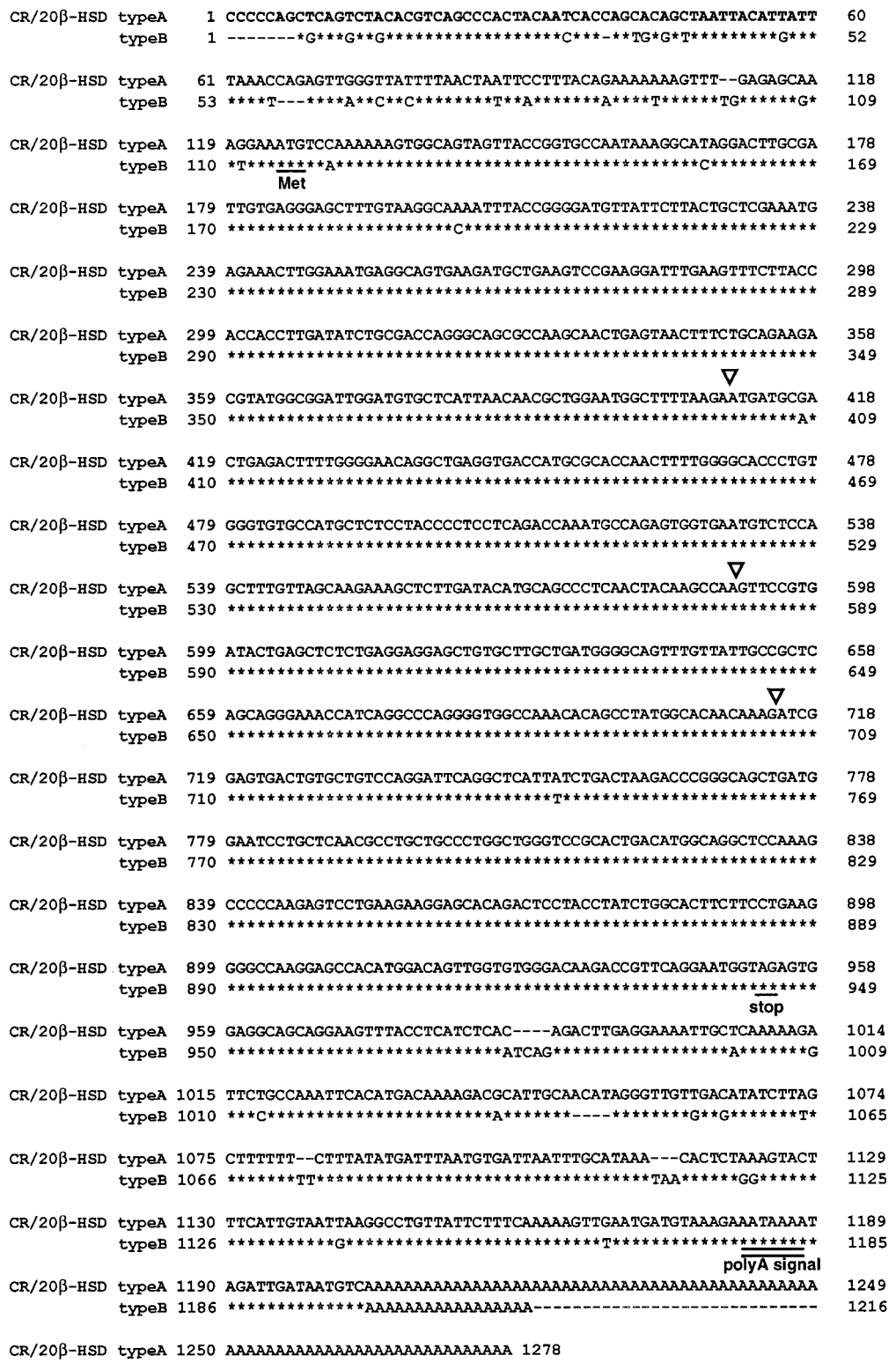

FIG. 1. Nucleotide sequences of rainbow trout CR/20β-HSD cDNA type A and B. Triangles indicate the places where the introns interrupt the gene. The polyadenylation signal is indicated by a double solid line. Met under the third line is the first methionine of the deduced ORF.

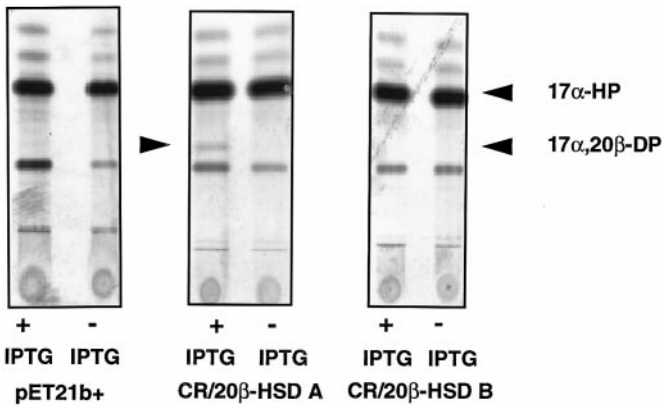

FIG. 2. 20β-HSD activity of recombinant CR/20β-HSD A and B proteins. Arrow heads at the right side indicate the location of authentic 17α,20β-DP and 17α-HP. The arrow head in the middle indicates the presence of a band corresponding to [3H]17α,20β-DP found only when CR/20β-HSD cDNA A was expressed. See text for further details.

incubated with [^{3}H] 17α-HP in the presence of NADPH and resulting steroids were separated by TLC (Fig. 2). A band which comigrated with authentic 17α,20β-DP was obtained from incubation of pET21b+rcA lysates with [3H] 17α-HP. This band was extracted and recrystallized. The extraction ratio from three crystallizations in different solvent systems was the same as that for cold 17α,20β-DP (before crystallization, 2910.6 cpm/mg; Ist, 2309.6 cpm/mg; 2nd, 2229.8 cpm/mg; 3rd, 2155.1 cpm/mg), indicating that CR/20β-HSD A cDNA encodes a protein with 20β-HSD activity. Lysates from pET21b+rcB or pET21b+ did not convert 17α-HP to 17α,20β-DP.

Recombinant enzyme from pET21b+rcA also catalyzed the reduction of a number of characteristic substrates of carbonyl reductase (Table 1). Like human carbonyl reductase and pig 20β-HSD, it efficiently catalyzed the reduction of quinones (4-nitrobenzaldehyde), or menadione, whereas prostaglandins and steroids including 17α-HP were reduced at lower rates. The CR/20β-HSD A recombinant enzyme also reduced both 5α- and 5β-dihydrotestosterone. Recombinant protein derived from pET21b+rc B did not recognize any of these substrates.

Expression of CR/20β-HSDs in various tissues of rainbow trout. Since the nucleotide sequences of CR/20β-HSD cDNAs A and B are very similar, their mRNAs are difficult to distinguish from each other by Northern hybridization. We used CR/20β-HSD cDNA A as the probe to investigate the tissue distribution of CR/20β-HSD mRNAs in rainbow trout. The result of Northern blot analysis is shown in Fig. 3a. A single band of 1.34 kb was detected in most of tissues, of greatest abundance in liver and gill, followed by brain, ovary and testis, adipose tissue, head kidney and posterior kidney. A very weak hybridization signal was obtained using muscle RNA. Result of RT-PCR employing primers specific for CR/20β-HSD cDNAs A and B was consistent with that of Northern blot, showing that CR/20β-HSD cDNA A and CR/20β-HSD cDNA B were expressed in most tissues (Fig. 3b), but only the expression of CR/20β-HSD A could be detected in liver. No signal could be detected in heart either by Northern analysis or by RT PCR, suggesting CR/20β-HSD cDNAs are not expressed in this tissue or that the level is below the limits of detectability.

DISCUSSION

In the present study, two closely related CR/20β-HSD cDNAs (A and B) and two DNA fragments (termed gene I and II) were isolated from rainbow trout. CR/20β-HSD A cDNA perfectly matched the gene I genomic sequence, indicating that gene I encodes CR/20β-HSD A. On the other hand, the sequence differed by two nucleotides between CR/20β-HSD B cDNA and gene II. This discrepancy may reflect gene polymorphism, or alternatively, gene II may encode another CR/20β-HSD different from CR/20β-HSD B. Both genes I and II consist of four exons, with the same exon-intron interruption, but the introns are of different sizes. The human carbonyl reductase gene contains three exons (13), and both human and trout CR/20β-HSD A exhibit similar enzymatic properties, suggesting that the early gene duplication and extensive divergence of CR/20β-HSD genes may have occurred early in the vertebrate lineage if they evolved from a common ancestor.

In vitro expression in *E. coli* and analysis of the enzymatic properties of the recombinant protein demonstrated that CR/20β-HSD A posseses both 20β-HSD activity as well as other broad carbonyl reductase activity, whereas the CR/20β-HSD B is devoid of activity. Rainbow trout CR/20β-HSD A and B differed by only 3 amino acid residues. Two of these residues are located

TABLE 1

Comparison of Substrate Specificity of Rainbow Trout CR/20β-HSD Type A with Pig 20β-HSD and Human Carbonyl Reductase

Substrate	Conc. mM	Relative velocity (%)		
		Pig	Human	Rainbow trout
4-Nitrobenzaldehyde	0.5	100	100	100
Menadione	0.25	62	350	44
PGE2	1	0	10	1.2
5α-Dihydrotestosterone	0.05	90	11	23
5β-Dihydrotestosterone	0.05	29	12	30
Progesterone	0.02	10	ND[a]	0
17α-Hydroxyprogesterone	0.02	10	ND[a]	1.2

[a] ND, not detectable.

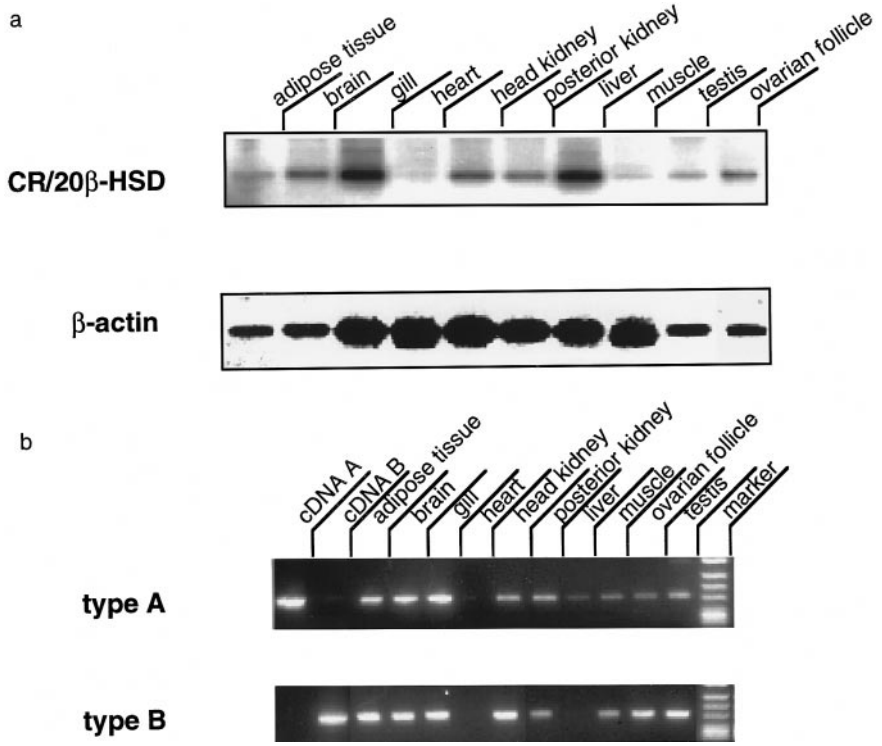

FIG. 3. Tissue distribution of rainbow trout CR/20β-HSD transcripts. (a) results of Northern blot of mRNAs hybridized with of CR/20β-HSD cDNA A probe (upper panel) or with β-actin cDNA probe (lower panel). (b) results of RT-PCR using specific primers for CR/20β-HSD cDNA A (upper panel) and cDNA B (lower panel).

in (I15T) or near (K27Q) the cofactor NADPH binding site. These substitutions may cause a conformational change in the binding pocket and interfere with binding of enzyme and co-factor or substrates. It is of interest to determine whether CR/20β-HSD type B could interfere with the function of CR/20β-HSD type A by competing for its substrates.

The physiological role of carbonyl reductase was thought to be in the NADPH-dependent reduction of a variety of endogenous and foreign carbonyl compounds. However, evidence for its involvement in steroid metabolism is increasing. It has been suggested that two classes of carbonyl reductase exist (7), one with high activity and steroid specificity and the other with low activity and broad specificity. Pig 20β-HSD and rat ovarian carbonyl reductase seem to belong to the former class. Recombinant rainbow trout CR/20β-HSD A has an enzymatic profile similar to that of pig 20β-HSD, suggesting that it belongs to the steroid specific carbonyl reductase class. In salmonid fish, 20β-HSD activity is required for MIH production. Activity in fully-grown follicles was elevated by salmon gonadotropin, forskolin and human chorionic gonadotropin *in vitro* (2). Two carbonyl reductases have been reported to exist in rat ovary, but only one is inducible by pregnant mare's serum gonadotropin (PMSG) (8).

Northern hybridization and RT-PCR analysis demonstrated that CR/20β-HSD genes are expressed in a variety of tissues. The significance of such widespread distribution is unclear. However, several studies have reported 20α-HSD and 20β-HSD activity in non-gonadal tissue of several teleosts, including salmonid steroidogenic interrenal tissue (14, 15), and salmonid gill, which has been proposed as a site of release of 17α,20β-DP into the environment where it performs a pheromonal function (16, 17). Thus, our finding of abundant CR/20β-HSD transcripts in gill (and liver) are consistent with these *in vitro* findings. However, rainbow trout tissues such as heart and muscle do not convert 17α-HP to 17α,20β-DP (17), which is consistent with the very low or absent levels of CR/20β-HSD transcript. It is not known whether extra-gonadal tissues are a quantitatively important sourse of 17α,20β-DP, or if the CR/20β-HSD in extra-gonadal tissues is under the control of reproductive hormones.

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REFERENCES

1. Nagahama, Y., and Adachi, S. (1985) *Dev. Biol.* **109,** 428–435.
2. Young, G., Adachi, S., and Nagahama, Y. (1986) *Dev. Biol.* **118,** 1–8.
3. Nagahama, Y., Kagawa, H., and Young, G. (1985) *Develop. Growth Differ.* **27,** 213–221.
4. Kanamori, A., and Nagahama, Y. (1988) *Gen. Comp. Endocrinol.* **72,** 39–53.
5. Tanaka, M., Ohno, S., Adachi, A., Nakajin, S., Shinoda, M., and Nagahama, Y. (1992) *J. Biol. Chem.* **267,** 13451–13455.
6. Wermuth, B., Bohren, K., Heinemann, G., Wartburg, J., and Gabbay, K. (1988) *J. Biol. Chem.* **263,** 16185–16188.
7. Wermuth, B., Mader-Heinemann, G., and Ernst, E. (1995) *Eur. J. Biochem.* **228,** 473–479.
8. Aoki, H., Okada, T., Mizutani, T., Numata, Y., Minegishi, T., and Miyamoto, K. (1997) *Biochem. Biophys. Res. Commun.* **230,** 518–523.
9. Nakajin, S., Tamura, F., Takase, N., and Toyoshima, S. (1997) *Biol. Pharm. Bull.* **20,** 1215–1218.
10. Tanaka, M., Nakajin, S., Kobayashi, D., Fukada, S., and Nagahama, Y. (1996) Abstracts, 3rd International symposium on fish endocrinology, p.73.
11. Axelrod, L. R., Mattijssen, C., Goldzieher, J. W., and Pulliam, J. E. (1965) *Acta Endocrinol.* **99,** 7–77.
12. Jornvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J., and Ghosh, D. (1995) *Biochemistry* **34,** 6003–6013.
13. Forrest, G. L., Akman, S., Doroshow, J., Rivera, H., and Kaplan, W. D. (1991) *Mol. Pharmacol.* **40,** 502–507.
14. Sangalang, G. B., and Freeman, H. C. (1988) *Gen. Comp. Endocrinol.* **69,** 406–415.
15. Barry, T. P., Riebe, J. D., Parrish, J. J., and Malison, J. A. (1997) *Gen. Comp. Endocrinol.* **107,** 172–181.
16. Kime, D. L., and Ebrahimi, M. (1997) *Fish Physiol. Biochem.* **17,** 117–121.
17. Ebrahimi, M., Scott, A. P., and Kime, D. E. (1996) *Gen. Comp. Endocrinol.* **104,** 296–303.