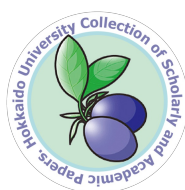




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Changes in the Levels of Gonadotropin Subunit mRNAs in the Pituitary of Pre-Spawning Chum Salmon

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ABSTRACT—We only have little information on expression of gonadotropin (GTH) subunit genes during spawning migration in salmonids. Changes in the levels of mRNAs for GTH subunits (GTH $\alpha 2$, I β and II β) were therefore analyzed in the pituitaries of chum salmon (*Oncorhynchus keta*) during the final stages of spawning migration to the Ishikari river. The fish were caught at Atsuta, a fisherman's village facing the Ishikari bay, and at Chitose, a tributary of the Ishikari river, in 1993 and 1994. The former is referred to as seawater (SW) fish, and the latter as freshwater (FW) fish. The levels of GTH subunit mRNAs in the pituitaries were determined by a quantitative dot blot analysis, using single-stranded sense DNA as the standard. The sense DNAs have the same nucleic acid sequences of mRNAs. The level of GTH $\alpha 2$ mRNA in the FW males was higher than that in the SW ones. Similar tendency was seen in the females. No significant changes were observed in the levels of GTH I β mRNA in both the males and females. Whereas, the level of GTH II β mRNA in the FW fish was higher than that in the SW fish regardless of sexes in 1994. Although not statistically significant in the males, similar tendency was seen in the 1993 fish. The present study thus showed that the level of GTH II β mRNA was increased concomitantly with that of GTH $\alpha 2$ mRNA during the final stages of spawning migration.

INTRODUCTION

Teleosts have two gonadotropins (GTH), referred to as GTH I and GTH II (Kawauchi *et al.*, 1989). GTH I is considered as vitellogenic GTH, whereas GTH II as maturational one. They are composed of common α and unique β subunits. Complete amino acid sequences of α subunit (Itoh *et al.*, 1990) and β subunit (Itoh *et al.*, 1988) of salmon GTHs were first determined in chum salmon, *Oncorhynchus keta*. Complete nucleotide sequences of cDNAs encoding the GTH subunits were determined in chum salmon (Kitahara *et al.*, 1988; Sekine *et al.*, 1989), masu salmon, *Oncorhynchus masou*, (Gen *et al.*, 1993; Kato *et al.*, 1993) and chinook salmon, *Oncorhynchus tshawytscha* (Trinh *et al.*, 1986; Suzuki *et al.*, 1995).

In female rainbow trout, *Oncorhynchus mykiss*, which are non-migratory, the plasma level of GTH I was increased during the early vitellogenesis and then decreased until ovulation when the plasma level of GTH I was rapidly increased again (Prat *et al.*, 1996). In male, the plasma level of GTH I

was maximal during mid- to late-testicular growth (Prat *et al.*, 1996). The level of GTH II remained very low until shortly before spermiation and ovulation when they began to rise (Prat *et al.*, 1996).

Unlike the situation observed in plasma, the levels of GTHs (mainly GTH II) in the pituitary of rainbow trout began to be increased about six months before spawning and attained the peak at the beginning of spawning (Sumpter and Scott, 1989). They remained high many months thereafter.

The changes in expression of genes encoding GTH subunits (α , I β and II β) were examined only in a few studies in rainbow trout during gonadal maturation (Naito *et al.*, 1991; Weil *et al.*, 1995). In female, the level of GTH I β mRNA was increased coincidentally with the onset of vitellogenesis, and that of GTH II β mRNA was increased from a later stage of vitellogenesis. The level of latter remained high throughout the final oocyte maturation and ovulation.

Although salmon pituitary glands contain two distinct α subunit proteins ($\alpha 1$ and $\alpha 2$ subunits), it is suggested that only $\alpha 2$ subunit is utilized as the composer of intact GTH II β . $\alpha 1$ subunit may be released in its free form or intracellularly degraded in GTH II-cells (Naito *et al.*, 1997). Hence, we quantified the amount of $\alpha 2$ mRNA encoding $\alpha 2$ subunit protein in

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the present study.

In coho salmon, *Oncorhynchus kisutch*, which are migratory and spawn only once in their life time, the plasma level of GTH II was maximal during mid- to late-vitellogenesis and then decreased (Swanson, 1991).

In female masu salmon, the pituitary content of GTH II β was increased prior to ovulation following elevation of the pituitary content of salmon gonadotropin-releasing hormone (sGnRH), while those of GTH I β showed clear seasonal increase in autumn (Amano *et al.*, 1992). In immature coho salmon, the pituitary content of GTH I was higher than that of GTH II (Swanson *et al.*, 1989).

In the migratory salmon, change in expression of genes encoding GTH subunits during the spawning migration has not been examined yet. Such information is required for understanding of neuroendocrine mechanisms of salmonid spawning migration and final maturation.

In the present study, we quantitatively analyzed changes in the levels of mRNAs for GTH subunits (GTH α 2, I β and II β) during the final stages of spawning migration in chum salmon. To quantitatively measure the levels of pituitary hormonal mRNAs, we adopted an assay method reported by Hiraoka *et al.* (1997) in which single-stranded sense DNA (ssDNA) was used as the standard, and developed it for the use in GTH study.

MATERIALS AND METHODS

Fish

Mature male and female chum salmon in the final stages of spawning migration were caught at Atsuta, a fishermen's village facing the Ishikari bay, and Chitose, a town through which a tributary of

the Ishikari river runs, on October 7, 1993 and October 13 and 15, 1994. Since the Ishikari is a prominent big river, and a vast number of juveniles are released only from the Hokkaido Salmon Hatchery in Chitose, the salmon caught at Atsuta and Chitose are considered to belong to the same genetic group. The fish caught at Atsuta are referred to as seawater (SW) fish, and those captured at Chitose as freshwater (FW) fish. The 1993 FW females were separated into two groups: those that had not yet ovulated and those that had. Only FW females that had ovulated were sampled in 1994. The number of fish in each group was six or seven. The body weight and fork length were measured. Afterward the fish were decapitated to remove the pituitary.

The maturity of the gonads, assessed by the gonadosomatic index (GSI, gonad weight/body weight $\times$ 100), in SW fish was almost the same in 1993 and 1994 (Table 1). The changes in GSI values in the SW and FW fish, an increase in the females and a decrease in the males, indicate that the final maturation of the gonads in the experimental fish might occur during migration from Atsuta to Chitose.

Tissue preparation

Immediately after decapitation, the pituitaries were taken out, frozen in liquid nitrogen and stored at -80°C . Total RNA was extracted from single pituitaries by the acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). The pituitary weights were about 30-70 mg and the amounts of total RNA extracted from single pituitaries were about 100-300 μg . To develop and validate the assay method, standard total RNA was extracted from pooled pituitaries of about 30 chum salmon captured at Atsuta in 1995 autumn.

Assay method

Single-stranded DNAs which have the same sequences of mRNAs encoding GTH α , I β and II β were synthesized by use of a polymerase chain reaction (PCR) method with a single common sense primer. Template DNA for PCR was prepared by the restriction enzyme digestion at one site of plasmid DNA containing cDNA insert for the salmon GTH subunit (Sekine *et al.*, 1989). The GTH α cDNA used in the present study encodes α 2 subunit (Kitahara *et al.*, 1988). Synthetic 20 mer oligonucleotide, 5'-GTTACTTCTGCTCTAAAGC-

Table 1. Maturity of gonads assessed by the gonadosomatic index (GSI) in the SW and FW fish in 1993 and 1994

1993					
	Male		Female		
	SW	FW	SW	FW (non-ovulated)	FW (ovulated)
n	6	6	6	7	7
FL(cm)	58.37 $\pm$ 2.04	71.00 $\pm$ 1.02*	59.13 $\pm$ 1.50	68.46 $\pm$ 1.99*	66.55 $\pm$ 0.96
BW(kg)	2.77 $\pm$ 0.16	3.67 $\pm$ 0.18*	2.85 $\pm$ 0.21	3.73 $\pm$ 0.40	3.37 $\pm$ 0.14
GW(g)	149.57 $\pm$ 13.16	127.71 $\pm$ 5.08	428.14 $\pm$ 34.77	751.14 $\pm$ 58.15*	ovulated
GSI	5.41 $\pm$ 0.34	3.51 $\pm$ 0.13*	15.04 $\pm$ 0.59	20.60 $\pm$ 1.17*	ovulated
1994					
	Male		Female		
	SW	FW	SW	FW (ovulated)	
n	6	6	6	7	
FL(cm)	62.00 $\pm$ 2.27	68.50 $\pm$ 1.67	64.02 $\pm$ 2.36	67.60 $\pm$ 1.94	
BW(kg)	2.58 $\pm$ 0.33	3.84 $\pm$ 0.34	3.04 $\pm$ 0.37	3.62 $\pm$ 0.32	
GW(g)	150.00 $\pm$ 19.15	138.33 $\pm$ 14.70	518.33 $\pm$ 69.30	778.57 $\pm$ 47.13	
GSI	5.82 $\pm$ 0.26	3.99 $\pm$ 0.19*	17.02 $\pm$ 0.91	22.02 $\pm$ 1.32	

Fork length (FL), body weight (BW) and gonad weight (GW).

Values are mean $\pm$ SEM.

*, $P < 0.01$; compared with SW fish.

3', was used as the PCR primer (Fig. 1A). The PCR mixture contained 1.6 pmol template DNA described above, PCR buffer (50 mM KCl, 1.5 mM MgCl₂ and 10 mM Tris-HCl, pH 8.3), 200 μ M dNTPs, 1.0 μ M primer, 1 unit *Taq* DNA polymerase (TaKaRa) and sterile distilled water to 50 μ l, overlaid with 30 μ l light mineral oil. The profile of amplification was: 30 cycles of incubation at 93°C for 1.5 min, at 53°C for 1.5 min and 72°C for 2 min after denaturation at 95°C for 4 min. Amplified DNA was subjected to low melting temperature agarose gel electrophoresis to separate the band that contained ssDNA. Afterward, ssDNA was purified by phenol extraction. We obtained ssDNAs for GTH α 2, I β and II β whose lengths were about 780, 790 and 840 bases, respectively (Fig. 1A).

Labeled cDNA probes which were specific to particular GTH subunit mRNAs were prepared by a primer extension method using Megaprime DNA labeling system (Amersham) and [α -³²P]dCTP (Amersham) with synthetic oligonucleotide primers. They are about 200 bases long and specific to corresponding standard DNAs (Fig. 1B). The sequences used as cDNA probes were carefully selected to avoid cross-hybridization. Although the α 1 mRNA has 72.3% sequence identity with the α 2 mRNA, the cDNA probe for α 2 mRNA corresponds to the region in the α 2 mRNA which lacks the sequence seen in the α 1 mRNA, and thus has low sequence identity with the α 1 mRNA. Furthermore, the cDNA probe made by primer extension method has particular size and sequence. Hence, the cDNA probe

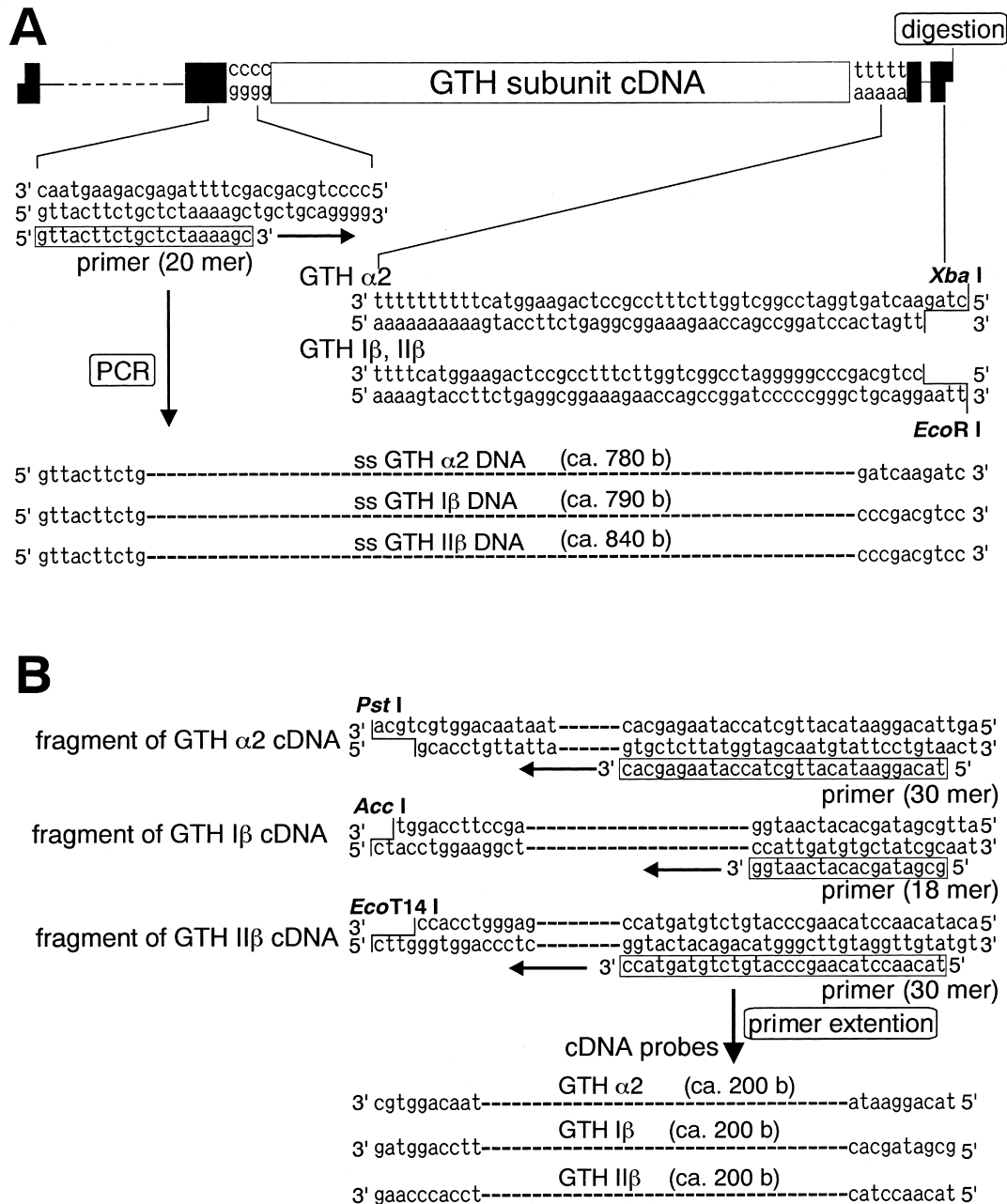


Fig. 1. Preparation of standards and cDNA probes. (A) A schematic representation of ssDNAs for GTH subunits with a common PCR primer. The plasmid DNAs were digested at one site by *Xba* I (GTH α 2) or *Eco*R I (GTH I β and II β) and used as a template for PCR. (B) A schematic representation of cDNA probes specific to the corresponding standards. The plasmid DNA was digested and its fragment was used as a template for PCR with a distinct primer to prepare specific cDNA probe.

for $\alpha 2$ mRNA used in the present study can not cross-hybridize with $\alpha 1$ mRNA.

Total RNA extracted from the pituitaries was blotted to HybondTM-N⁺ membranes (Amersham) using a MilliBlotTM-D (Millipore) in duplicate. As the standard to determine the amount of mRNA, serially diluted ssDNA (1-333 amol) were blotted to each membrane in triplicate. The amounts of total RNA blotted in single dot were 1/50 and 1/150 of total RNA obtained from single pituitaries for GTH $\alpha 2$; 1/500 and 1/1500 for GTH I β and 1/200 and 1/600 for GTH II β .

Hybridization with the labeled probe was performed in a solution containing $5 \times$ SSPE (0.05 M phosphate buffer, pH approx. 7.4, containing 0.76 M NaCl and 5 mM EDTA), $5 \times$ Denhardt's solution (0.1% solution of bovine serum albumin, Ficoll and polyvinylpyrrolidone), 100 μ g/ml denatured calf thymus DNA (Sigma) and $1.5-2 \times 10^6$ cpm of labeled cDNA probe/ml hybridization buffer at 65°C for 20 hr. The membranes were then washed with $2 \times$ SSPE/0.1% SDS at room temperature (RT) for 15 min, twice with $2 \times$ SSPE/0.1% SDS at 65°C for 30 min, and twice with $0.1 \times$ SSPE/0.1% SDS at 65°C for 30 min. The membranes were then exposed to a Fuji imaging plate (Fuji Photo Film Co., Ltd.) for 24-48 hr. Radioactivity expressed as the intensity of photostimulated luminescence (PSL) was analyzed by a Bioimaging analyzer (Fuji Photo Film Co., Ltd.), and the intensity of signals were estimated by subtraction of background.

Validation of assay method

Serially diluted standard DNA (1 amol-3.33 fmol) were hybridized with the corresponding probe to check the linear range of standard curve. At the same time, serially diluted pituitary total RNA (0.01-3.33 μ g) were also hybridized to check the linear range and parallelism of the curve for total RNA with the standard curve.

Northern blot analysis of the pituitary total RNA was performed to confirm whether the labeled probe was hybridized only with particular mRNA for GTH subunit. Serially diluted total RNA (1-10 or 0.3-3 μ g) was electrophoresed in a 1.0% agarose/formaldehyde gel and transferred to Hybond-N⁺ membranes (Amersham) according to the manufacturer's instruction. They were then hybridized with the corresponding cDNA probes labeled with [α -³²P]dCTP as was mentioned above.

Cross-hybridization of the cDNA probes was carefully checked in detail using ssDNAs for other GTH subunits, thyrotropin β (TSH β) and somatolactin (SL). Serially diluted standard (1-333 amol) and these ssDNAs (1-3.33 fmol) blotted to the same membrane were hybridized with the probe which is specific to the standard. The ssDNAs for TSH β and SL were synthesized by use of TSH β cDNA (Ito *et al.*, 1993) and SL cDNA (Takayama *et al.*, 1991) by the method similar to that used for preparation of GTH subunit ssDNAs.

Intra- and inter-assay variations (standard deviation/mean $\times 100$) were calculated to confirm reliability of measured values. Serially diluted standard DNAs (1-333 amol) and plasmid DNAs (1 ng) which contains GTH subunit cDNAs were blotted in triplicate to the membrane. A set of three membranes was prepared for each GTH subunit cDNA, and was hybridized with the labeled probe. Radioactivity of each dot was counted by use of Bioimaging analyzer, as is mentioned above. Intra-assay variations were calculated for every dose on each membrane, while inter-assay variation was obtained from the measured amounts of plasmid cDNA on all three filters. Further, intra-assay variations were checked when samples were analyzed. Range of intra-assay variations was 6.17 to 7.30, and that of inter-assay variations was 2.52 to 10.40.

Statistical analysis

For statistical analysis, Student's *t*-test was applied after Scheffé's F test for variance. The correlations between GTH subunit mRNA levels were analyzed using Pearson's correlation coefficient method.

RESULTS

Validation of assay method

The standard curves for estimation of the amounts of GTH subunit mRNAs were linear within the range of 1 amol to 1 fmol, although the value for GTH I β ssDNA at low concentration did not fit the line (Fig. 2). The curves of total RNA were parallel to the standard curves at low doses, became gradual and seemed to reach plateau, when the applied amounts were over 1 μ g. Since the amounts of standard ssDNA were at most 1 ng, 1/1000 of those of total RNA, we considered that the

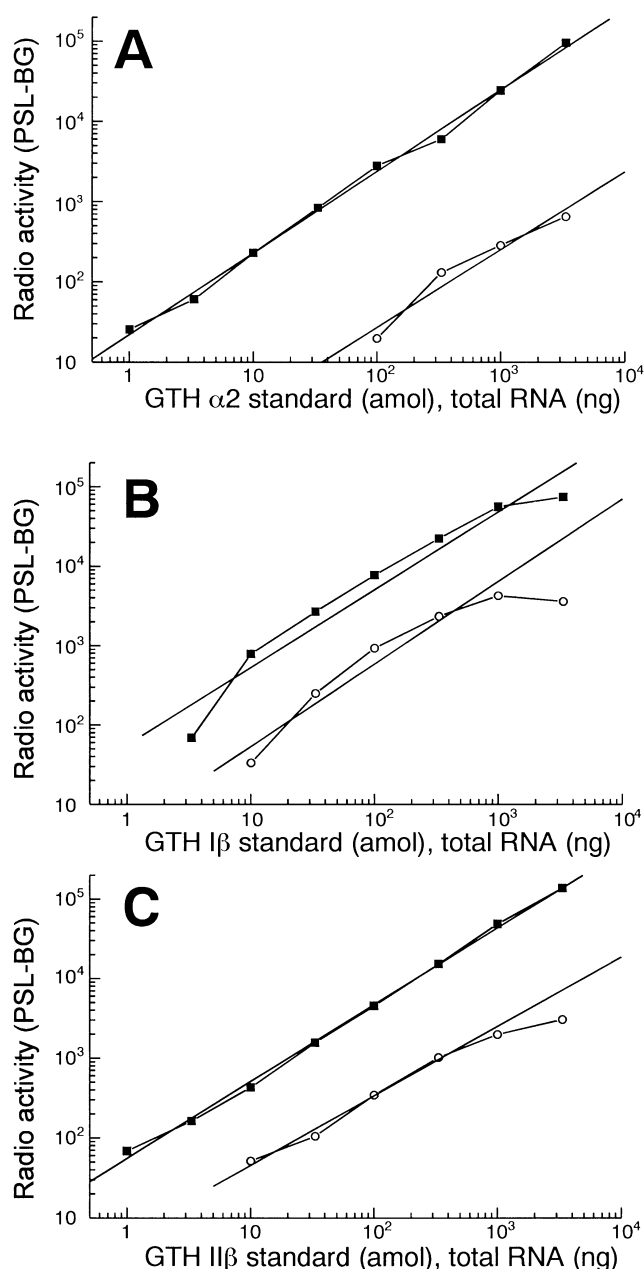


Fig. 2. Comparison of curves for serially diluted total RNA (—○—) and standard curves (—■—) for ssGTH $\alpha 2$ (A), ssGTH I β (B) and ssGTH II β (C).

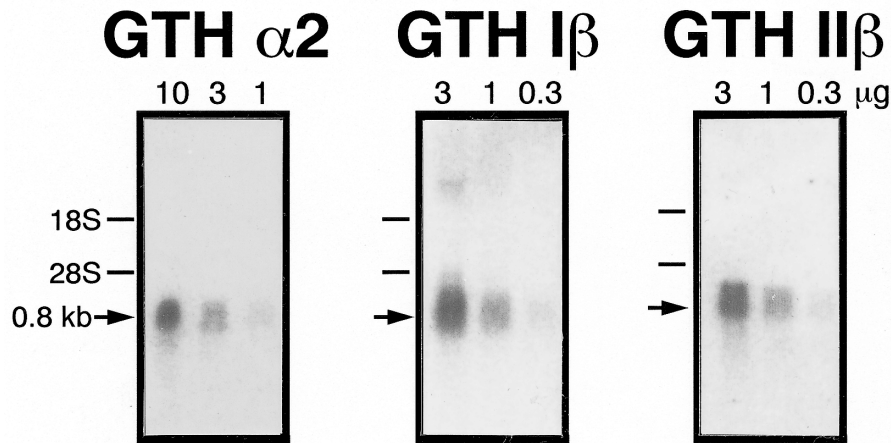


Fig. 3. Northern blot analysis of total RNA extracted from chum salmon pituitaries. After electrophoresis and blotting, RNA was hybridized with the GTH $\alpha 2$ cDNA probe, the GTH I β cDNA probe and the GTH II β cDNA probe.

Table 2. Cross-hybridization of cDNA probes with other ssDNAs than corresponding single-stranded cDNA

cDNA probe for	ssGTH $\alpha 2$	ssGTH I β	ssGTH II β	ssTSH β	ssSL
GTH $\alpha 2$	—	2.0%	2.6%	0.4%	0.7%
GTH I β	1.6%	—	2.0%	< 0.1%	< 0.1%
GTH II β	2.6%	2.8%	—	0.1%	0.2%

Radioactivity of unique hybridization was considered as 100%.

presence of too much RNA molecules on the limited area of membrane interfere base-pair matching of probe molecules to corresponding mRNA. Hence, the amounts of total RNA from the samples were carefully decided (see materials and methods section). The result of Northern blot analysis clearly shows that the labeled probes to GTH subunit mRNAs detected single species of RNA in total RNA in a dose-dependant manner (Fig. 3). The positions of the bands were just at the expected places corresponding to the sizes of mRNAs. Moreover, cross-hybridization to other ssDNAs of the present cDNA probes was sufficiently low, when overall homologies among GTH subunits were considered (Table 2). These results surely confirmed that the present cDNA probes, which were prepared by primer extension, are highly specific and can detect the amounts of GTH subunit mRNAs.

GTH subunit mRNAs

The levels of mRNAs encoding GTH $\alpha 2$, GTH I β and GTH II β were almost in the same order in the pituitary of pre-spawning chum salmon. Noticeable consistent differences were not observed between the males and females through 1993 to 1994. The amounts of particular hormonal mRNAs within the same group were not so deviated to be statistically analyzed.

The amount of GTH $\alpha 2$ mRNA in the pituitaries of FW males, which are ready to spawn, was 6.06 ± 0.46 fmol/pituitary, significantly higher than that of the SW fish (3.56 ± 0.16 fmol/pituitary) (Fig. 4). The GTH $\alpha 2$ mRNA level in the fe-

males showed similar tendency. In 1993 fish, GTH $\alpha 2$ mRNA was undetectable.

The levels of mRNA encoding GTH I β , a subunit of so-called vitellogenic GTH at least in salmonids, were similar in both the males and females (Fig. 5). Since the amount of 12 fmol/pituitary in 1993 fish was higher than that of 4 fmol/pituitary in 1994, we assume that the basal levels of GTH I β mRNA fluctuate year by year. Such year by year deviation may be derived of changes in global seawater temperature that has influences on sexual maturation. Actually, 1993 fish were sil-

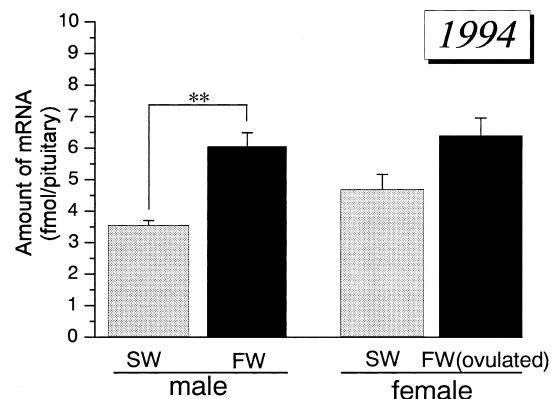


Fig. 4. Changes in the pituitary levels of GTH $\alpha 2$ mRNA between the SW (Atsuta) and FW (Chitose) fish in 1994. Vertical bars represent the mean $\pm$ SEM ($n = 6$). **, $P < 0.01$; by Student's t -test.

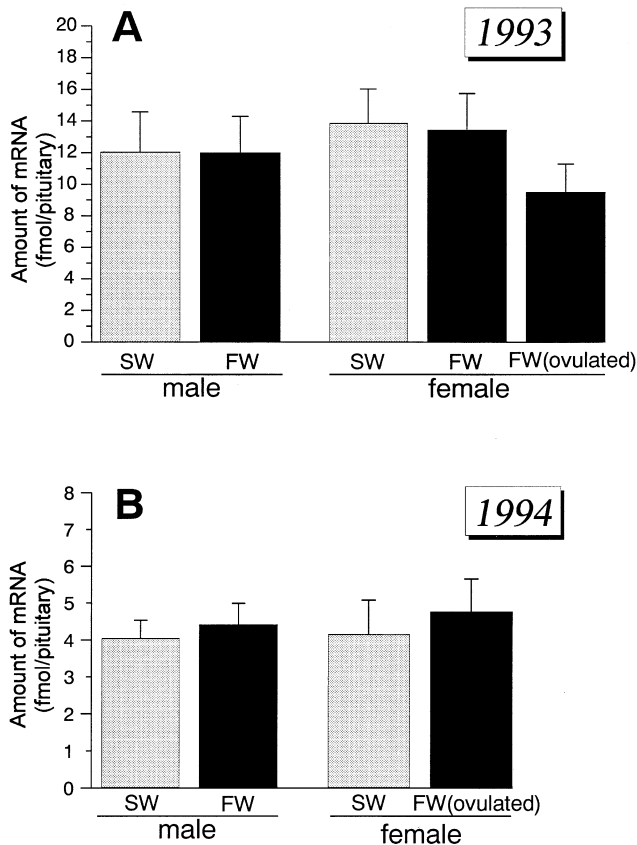


Fig. 5. Changes in the pituitary levels of GTH I β mRNA between the SW (Atsuta) and FW (Chitose) fish in both 1993 (A) and 1994 (B). Vertical bars represent the mean $\pm$ SEM ($n = 6$ or 7).

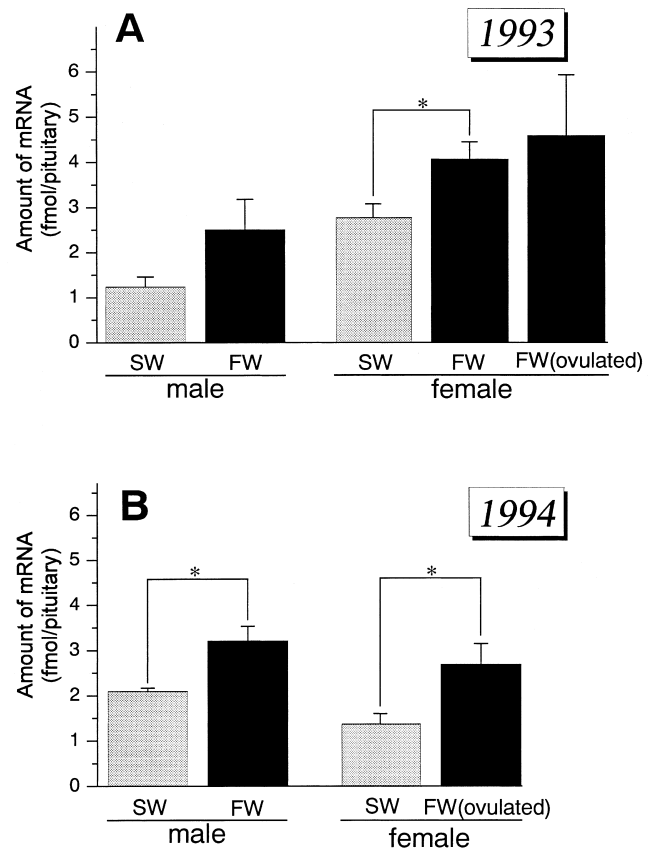


Fig. 6. Changes in the pituitary levels of GTH II β mRNA between the SW (Atsuta) and FW (Chitose) fish in both 1993 (A) and 1994 (B). Vertical bars represent SEM ($n = 6$ or 7). *, $P < 0.05$; **, $P < 0.01$; by Student's *t*-test.

ver, whereas 1994 fish showed weak nuptial coloring.

The levels of mRNA encoding GTH II β seemed to increase in both sexes during the final stages of spawning migration from seawater to fresh water, although some of the changes were statistically not significant (Fig. 6). In 1994, significant changes in the levels of GTH II β mRNA were found in both sexes: the levels of GTH II β mRNA in the FW fish (3.21 ± 0.32 fmol/pituitary in the males and 4.06 ± 0.39 fmol/pituitary in the females) were significantly higher than those in the SW fish (2.08 ± 0.08 fmol/pituitary in the males and 2.77 ± 0.30 fmol/pituitary in the females).

DISCUSSION

In the present study, we found that, in pre-spawning chum salmon, the levels of GTH $\alpha 2$ and II β mRNAs in the pituitary of FW fish were higher than those of the SW fish, whereas no significant differences were detected in the levels of GTH I β mRNA between the SW and FW fish. The changes in the level of GTH $\alpha 2$ mRNA correlated with that in GTH II β mRNA ($R^2 = 0.44$, $P < 0.05$). These results indicate that expression of GTH $\alpha 2$ and II β genes, but not I β gene, is stimulated during the final stages of spawning migration in chum salmon of the Ishikari stock.

As is described in the result section, the assay method developed in the present study is sufficiently reliable to determine the levels of GTH subunit mRNAs in the pituitary of chum salmon. The fact that the level of GTH $\alpha 2$ mRNA was undetectable in 1993 fish may be due to the destruction of the mRNA in the samples, because the level of SL mRNA was also very low in the same samples (Taniyama *et al.*, personal communication). Although the supposed destruction of certain species of mRNAs, GTH I β and II β mRNAs were detectable in 1993 samples. The changes of their amounts coincided with the changes seen in 1994 samples, and thus support the above hypothesis that expression of GTH II β gene is stimulated in pre-spawning chum salmon.

In female rainbow trout, *in situ* hybridization study showed that GTH subunit genes are synthesized separately in distinct cells and expressed in different manner (Naito *et al.*, 1991). GTH α and I β mRNA levels in GTH I-cells are increased coincidentally with the onset of vitellogenesis. Only the level of GTH α mRNA is decreased in GTH I-cells prior to the final oocyte maturation. Whereas, the levels of GTH α and II β mRNAs in GTH II-cells are increased from a later stage of vitellogenesis, and remain high throughout oocyte maturation and ovulation. Therefore, the changes in the levels of GTH I β and II β mRNAs observed in the present study should reflect

the changes in expression of corresponding GTH genes in distinct cells. The increase in the levels of GTH $\alpha 2$ mRNA observed in the present study may reflect the increase of GTH $\alpha 2$ gene expression in GTH II-cells.

It is generally accepted that gonadotropin-releasing hormone (GnRH) stimulates pituitary GTH synthesis in salmonids. In mammals, GnRH and its analog also stimulate release of luteinizing hormone and follicle-stimulating hormone *in vitro* and *in vivo* (Gautron *et al.*, 1992). In rainbow trout, GnRH analog released two GTHs from the *in vitro* pituitary in a different manner depending on maturity (Kawauchi *et al.*, 1989); GnRH analog stimulated mainly GTH I secretion in immature fish, and GTH II in mature animals. Recently, Kudo *et al.* (1996) reported that hybridization signals for salmon GnRH (sGnRH) mRNA in the preoptic area and telencephalon of chum salmon were stronger in the fish from spawning ground when compared to those from coastal sea. The sGnRH neurons in these areas are considered to be involved in the control of synthesis and release of pituitary GTH II in maturing salmonids (Amano *et al.*, 1997).

We conclude that expression of GTH $\alpha 2$ and II β subunit genes, but not I β gene, should be elevated by the increase of brain sGnRH during the upstream migration in chum salmon, since the levels of their transcripts, mRNAs, in the FW fish were higher than those in the SW fish. The elevation of GTH gene expression, which may selectively occur in GTH II-cells, accelerates final maturation during the final stages of spawning migration.

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