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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 α2, Iβ and IIβ) 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 α2 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 Iβ mRNA in both the males and females. Whereas, the level of GTH IIβ 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 IIβ mRNA was increased concomitantly with that of GTH α2 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 tschawytscha* (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, Iβ and IIβ) 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 Iβ mRNA was increased coincidentally with the onset of vitellogenesis, and that of GTH IIβ 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 (α1 and α2 subunits), it is suggested that only α2 subunit is utilized as the composer of intact GTH IIβ. α1 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 α2 mRNA encoding α2 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 IIβ was increased prior to ovulation following elevation of the pituitary content of salmon gonadotropin-releasing hormone (sGnRH), while those of GTH Iβ 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 α2, Iβ and IIβ) 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 × 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, Iβ and IIβ 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 α2 subunit (Kitahara *et al*., 1988). Synthetic 20 mer oligonucleotide, 5'-GTTACTTCTGCTCTAAAAGC-

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 ± 2.04	71.00 ± 1.02*	59.13 ± 1.50	68.46 ± 1.99*	66.55 ± 0.96
BW(kg)	2.77 ± 0.16	3.67 ± 0.18*	2.85 ± 0.21	3.73 ± 0.40	3.37 ± 0.14
GW(g)	149.57 ± 13.16	127.71 ± 5.08	428.14 ± 34.77	751.14 ± 58.15*	ovulated
GSI	5.41 ± 0.34	3.51 ± 0.13*	15.04 ± 0.59	20.60 ± 1.17*	ovulated

	1994			
	Male		Female	
	SW	FW	SW	FW (ovulated)
n	6	6	6	7
FL(cm)	62.00 ± 2.27	68.50 ± 1.67	64.02 ± 2.36	67.60 ± 1.94
BW(kg)	2.58 ± 0.33	3.84 ± 0.34	3.04 ± 0.37	3.62 ± 0.32
GW(g)	150.00 ± 19.15	138.33 ± 14.70	518.33 ± 69.30	778.57 ± 47.13
GSI	5.82 ± 0.26	3.99 ± 0.19*	17.02 ± 0.91	22.02 ± 1.32

Fork length (FL), body weight (BW) and gonad weight (GW).
Values are mean ± 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 $MgCl_2$ and 10 mM Tris-HCl, pH 8.3), 200 μM dNTPs, 1.0 μM primer, 1 unit *Taq* DNA polymerase (TaKaRa) and sterile distilled water to 50 μl, overlaid with 30 μl 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 α2, Iβ and IIβ 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-^{32}P]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 α1 mRNA has 72.3% sequence identity with the α2 mRNA, the cDNA probe for α2 mRNA corresponds to the region in the α2 mRNA which lacks the sequence seen in the α1 mRNA, and thus has low sequence identity with the α1 mRNA. Furthermore, the cDNA probe made by primer extension method has particular size and sequence. Hence, the cDNA probe

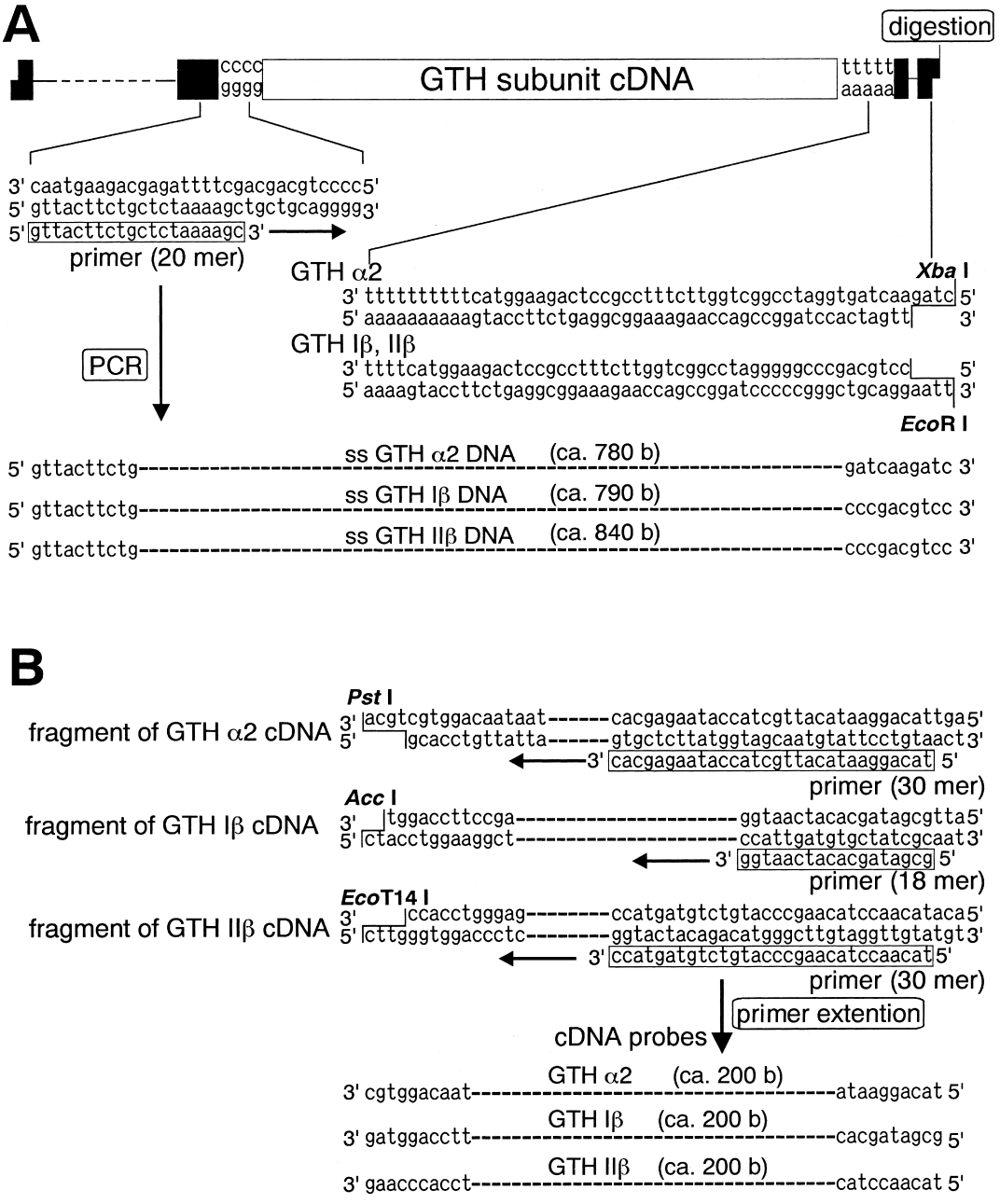

Fig. 1. Preparation of standards and cDNA probes. (**A**) A schematic representation of ssDNAs for GTH subunits with a common PCR primer. The plasmid DNAs were digested at one site by *Xba* I (GTH α2) or *Eco*R I (GTH Iβ and IIβ) and used as a template for PCR. (**B**) A schematic representation of cDNA 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 α2 mRNA used in the present study can not cross-hybridize with α1 mRNA.

Total RNA extracted from the pituitaries was blotted to Hybond™-N⁺ membranes (Amersham) using a MilliBlot™-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 α2; 1/500 and 1/1500 for GTH Iβ and 1/200 and 1/600 for GTH IIβ.

Hybridization with the labeled probe was performed in a solution containing 5 × SSPE (0.05 M phosphate buffer, pH approx. 7.4, containing 0.76 M NaCl and 5 mM EDTA), 5 × Denhardt's solution (0.1% solution of bovine serum albumin, Ficoll and polyvinylpyrrolidone), 100 μg/ml denatured calf thymus DNA (Sigma) and 1.5-2 × 10^6 cpm of labeled cDNA probe/ml hybridization buffer at 65°C for 20 hr. The membranes were then washed with 2 × SSPE/0.1% SDS at room temperature (RT) for 15 min, twice with 2 × SSPE/0.1% SDS at 65°C for 30 min, and twice with 0.1 × 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 μg) 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 μg) 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-^{32}P]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 × 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 Iβ 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 μg. Since the amounts of standard ssDNA were at most 1 ng, 1/1000 of those of total RNA, we considered that the

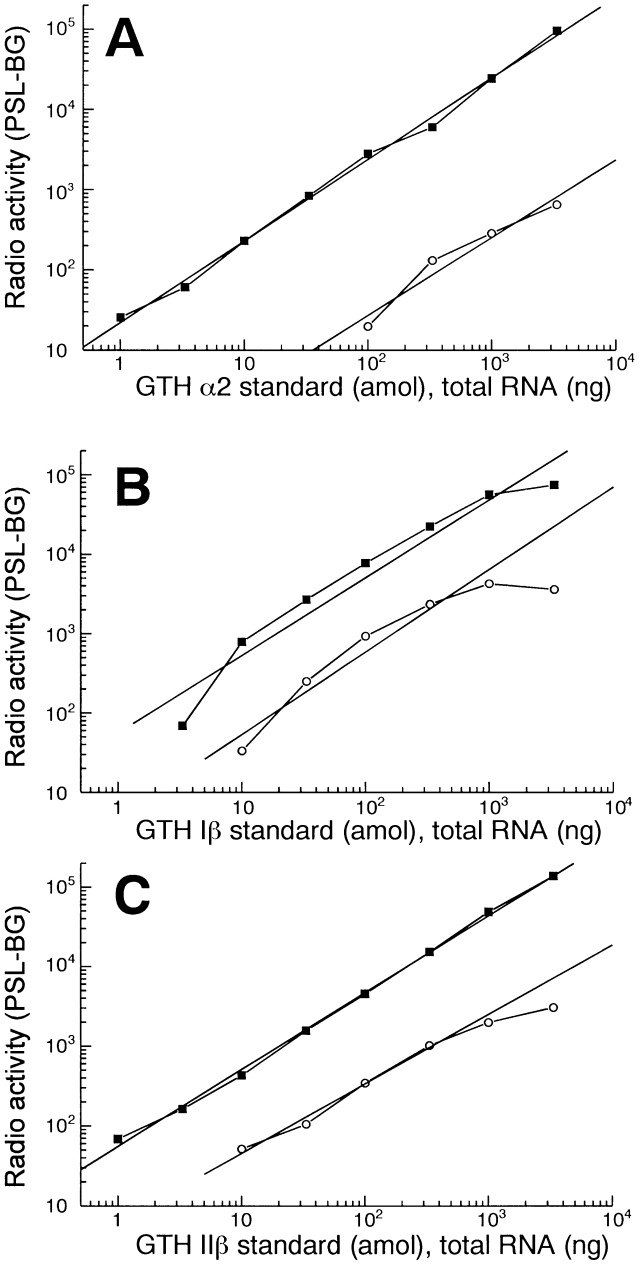

Fig. 2. Comparison of curves for serially diluted total RNA (–○–) and standard curves (–■–) for ssGTH α2 (**A**), ssGTH Iβ (**B**) and ssGTH IIβ (**C**).

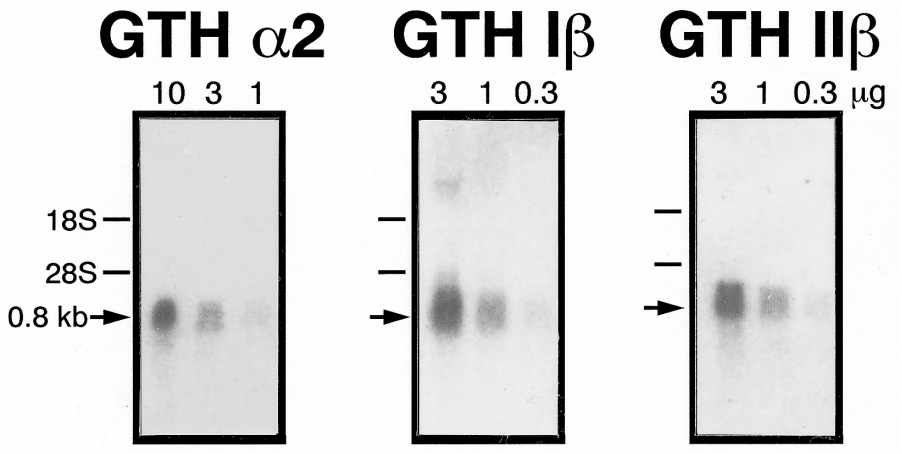

Fig. 3. Northern blot analysis of total RNA extracted from chum salmon pituitaries. After electrophoresis and blotting, RNA was hybridized with the GTH α2 cDNA probe, the GTH Iβ cDNA probe and the GTH IIβ cDNA probe.

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

cDNA probe for	ssGTH α2	ssGTH Iβ	ssGTH IIβ	ssTSH β	ssSL
GTH α2	——	2.0%	2.6%	0.4%	0.7%
GTH Iβ	1.6%	——	2.0%	< 0.1%	< 0.1%
GTH IIβ	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 α2, GTH Iβ and GTH IIβ 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 α2 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 α2 mRNA level in the females showed similar tendency. In 1993 fish, GTH α2 mRNA was undetectable.

The levels of mRNA encoding GTH Iβ, 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 Iβ 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-

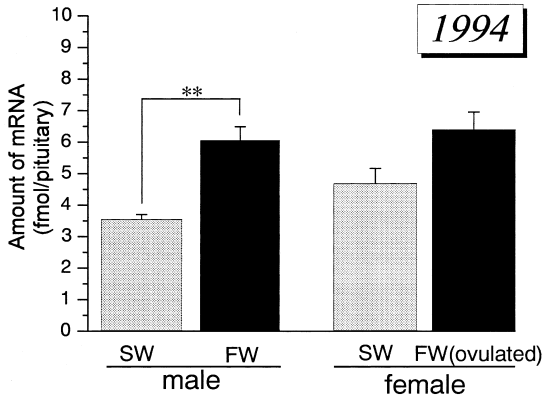

Fig. 4. Changes in the pituitary levels of GTH α2 mRNA between the SW (Atsuta) and FW (Chitose) fish in 1994. Vertical bars represent the mean ± SEM (n = 6). **, $P < 0.01$; by Student's *t*-test.

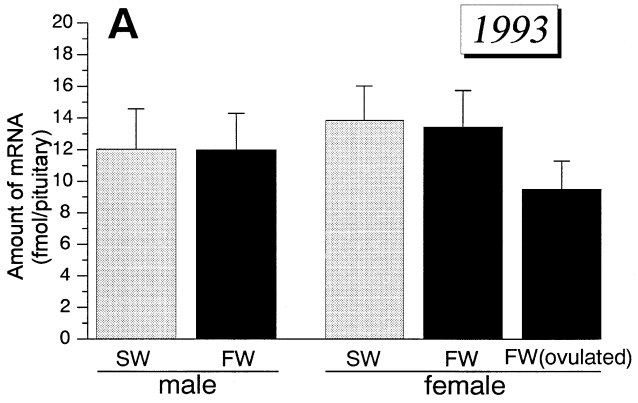

Fig. 5. Changes in the pituitary levels of GTH Iβ mRNA between the SW (Atsuta) and FW (Chitose) fish in both 1993 (**A**) and 1994 (**B**). Vertical bars represent the mean ± SEM (n = 6 or 7).

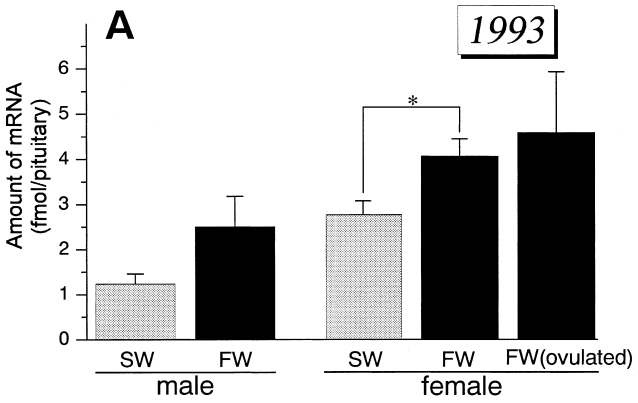

Fig. 6. Changes in the pituitary levels of GTH IIβ mRNA between the SW (Atsuta) and FW (Chitose) fish in both 1993 (**A**) and 1994 (**B**). Vertical bars represent SEM (n = 6 or 7). ∗, $P < 0.05$; ∗∗, $P < 0.01$; by Student's *t*-test.

ver, whereas 1994 fish showed weak nuptial coloring.

The levels of mRNA encoding GTH IIβ 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 IIβ mRNA were found in both sexes: the levels of GTH IIβ 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 α2 and IIβ 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 Iβ mRNA between the SW and FW fish. The changes in the level of GTH α2 mRNA correlated with that in GTH IIβ mRNA (R^2 = 0.44, $P < 0.05$). These results indicate that expression of GTH α2 and IIβ genes, but not Iβ 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 α2 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 Iβ and IIβ 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 IIβ gene is stimulated in pre-spawning chum salmon.

In female rainbow tout, *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 Iβ 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 IIβ 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 Iβ and IIβ 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 α2 mRNA observed in the present study may reflect the increase of GTH α2 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 α2 and IIβ subunit genes, but not Iβ 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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REFERENCES

Amano M, Aida K, Okumoto N, Hasegawa Y (1992) Changes in salmon GnRH and chicken GnRH-II contents in the brain and pituitary, and GTH contents in the pituitary in female masu salmon, *Oncorhynchus masou*, from hatching through ovulation. Zool Sci 9: 375–386

Amano M, Urano A, Aida K (1997) Distribution and function of gonadotropin-releasing hormone (GnRH) in the teleost brain. Zool Sci 14: 1–11

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159

Gautron JP, Leblanc P, Bluet-Pajot MT, Pattou E, L'Heritier A, Mounier F, Ponce G, Audinot V, Rasolonjanahary R, Kordon C (1992) A second endogenous molecular form of mammalian hypothalamic luteinizing hormone-releasing hormone (LHRH), (hydroxyproline^9) LHRH, releases luteinizing hormone and follicle-stimulating hormone *in vitro* and *in vivo*. Mol Cell Endocrinol 85: 99–107

Gen K, Maruyama O, Kato T, Tomizawa K, Wakabayashi K, Kato Y (1993) Molecular cloning of cDNAs encoding two types of gonadotropin α subunit from the masu salmon, *Oncorhynchus masou*: construction of specific oligonucleotides for the α1 and α2 subunits. J Mol Endocrinol 11: 265–273

Hiraoka S, Ando H, Ban M, Ueda H, Urano A (1997) Changes in expression of neurohypophysial hormone genes during spawning migration in chum salmon, *Oncorhynchus keta*. J Mol Endocrinol 18: 49–55

Ito M, Koide Y, Takamatsu N, Kawauchi H, Shiba T (1993) cDNA cloning of the beta subunit of teleost thyrotropin. Proc Natl Acad Sci USA 90: 6052–6055

Itoh H, Suzuki K, Kawauchi H (1988) The complete amino acid sequences of β-subunits of two distinct chum salmon GTHs. Gen Comp Endocrinol 71: 438–451

Itoh H, Suzuki K, Kawauchi H (1990) The complete amino acid sequences of alpha subunits of chum salmon gonadotropins. Gen Comp Endocrinol 78: 56–65

Kato Y, Gen K, Maruyama O, Tomizawa K, Kato T (1993) Molecular cloning of cDNAs encoding two gonadotrophin β subunits (GTH-Iβ and -IIβ) from the masu salmon, *Oncorhynchus masou*. J Mol Endocrinol 11: 275–282

Kawauchi H, Suzuki K, Itoh H, Swanson P, Naito N, Nagahama Y, Nozaki M, Nakai Y, Itoh S (1989) The duality of teleost gonadotropins. Fish Physiol Biochem 7: 29–38

Kitahara N, Nishizawa T, Gatanaga T, Okazaki H, Andoh T, Soma G (1988) Primary structure of two mRNAs encoding putative salmon alpha-subunits of pituitary glycoprotein hormone. Comp Biochem Physiol 91: 551–556

Kudo H, Hyodo S, Ueda H, Hiroi O, Aida K, Urano A, Yamauchi K (1996) Cytophysiology of gonadotropin-releasing hormone neurons in chum salmon (*Oncorhynchus keta*) forebrain before and after upstream migration. Cell Tissue Res 284: 261–267

Naito N, Hyodo S, Okumoto N, Urano A, Nakai Y (1991) Differential production and regulation of gonadotropins (GTH I and GTH II) in the pituitary gland of rainbow trout, *Oncorhynchus mykiss*, during ovarian development. Cell Tissue Res 266: 457–467

Naito N, Koide Y, Kawauchi H, Nakai Y (1997) Distinct alpha (α)-subunits of salmon glycoprotein hormones: production sites in the pituitary with sexual maturation. Fish Physiol Biochem 17: 39–44

Prat F, Sumpter JP, Tyler CR (1996) Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). Biol Reprod 44: 29–38

Sekine S, Saito A, Itoh H, Kawauchi H, Itoh S (1989) Molecular cloning and sequence analysis of chum salmon gonadotropin cDNAs. Proc Natl Acad Sci USA 86: 8645–8649

Sumpter JP, Scott AP (1989) Seasonal variations in plasma and pituitary levels of gonadotrophin in males and females of two strains of rainbow trout (*Salmo gairdneri*). Gen Comp Endocrinol 75: 376–388

Suzuki K, Liu D, Hew CL (1995) A gene encoding chinook salmon (*Oncorhynchus tschawytscha*) gonadotropin alpha subunit: gene structure and promoter analysis in primary pituitary cells. Mol Marine Biol Biotechnol 4: 10–19

Swanson P (1991) Salmon gonadotropins: reconciling old and new ideas. Reproductive Physiology of Fish 1991. Sheffield: FishSymp; 1991: 2–7

Swanson P, Bernard M, Nozaki M, Suzuki K, Kawauchi H, Dickhoff WW (1989) Gonadotropins I and II in juvenile coho salmon. Fish Physiol Biochem 7: 169–176

Takayama Y, Ono M, Rand-Weaver M, Kawauchi H (1991) Greater conservation of somatolactin, a presumed pituitary hormone of the growth hormone/prolactin family, than of growth hormone in teleost fish. Gen Comp Endocrinol 83: 366–374

Trinh KY, Wang NC, Hew CL, Crim LW (1986) Molecular cloning and

sequencing of salmon gonadotropin beta subunit. Eur J Biochem 159: 619–624

Weil C, Bougoussa-Houadec M, Gallais C, Itoh S, Sekine S, Valotaire Y (1995) Preliminary evidence suggesting variations of GtH 1 and GtH 2 mRNA levels at different stages of gonadal development in rainbow trout, *Oncorhynchus mykiss*. Gen Comp Endocrinol 100: 327–333

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