Changes in the levels of mRNAs for GH/prolactin/somatolactin family and Pit-1/GHF-1 in the pituitaries of pre-spawning chum salmon

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ABSTRACT

Changes in the levels of pituitary mRNAs encoding GH, prolactin (PRL) and somatolactin (SL) were determined in pre-spawning chum salmon (*Oncorhynchus keta*) caught at a few key points along their homing pathway in 1994 and 1995. Furthermore, we analyzed relationships between expression of pituitary-specific POU homeodomain transcription factor (Pit-1/GHF-1) and GH/PRL/SL family genes. In 1994, seawater (SW) fish and matured fresh-water (FW) fish were sequentially captured at two points along their homing pathway, the coast and the hatchery. In addition to these two points, maturing FW fish were captured at the intermediate of the two points in 1995. The levels of hormonal mRNAs were determined by a quantitative dot blot analysis using single-stranded sense DNA as the standard. Relative levels of Pit-1/GHF-1 mRNAs were estimated by Northern blot analysis. In 1994, the levels of GH/PRL/SL family mRNAs except

for PRL mRNA in the male FW fish were 1.8–4 times higher than those in the SW fish. In 1995, the level of PRL mRNA was somewhat sharply elevated in the maturing FW fish soon after entry into the FW environment, while that of SL mRNA was gradually increased during upstream migration from the coast to the hatchery. The levels of 3 kb Pit-1/GHF-1 mRNA in the FW fish were higher than those in the SW fish in both 1994 and 1995. The present results indicate that expression of genes for the GH/PRL/SL family and Pit-1/GHF-1 is coincidentally enhanced in homing chum salmon. Moreover, the present study suggests that expression of the SL gene is elevated with sexual maturation, whereas that of PRL gene is elevated with osmotic change during the final stages of spawning migration.

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INTRODUCTION

Growth hormone (GH), prolactin (PRL) and somatolactin (SL) belong to the same hormone family, presumably derived from a common ancestral molecule (Ono *et al.* 1990, Rand-Weaver *et al.* 1991, Takayama *et al.* 1991). In homing salmonids, plasma levels of GH/PRL/SL family hormones in river fish are significantly higher than those in ocean fish during the final maturation in

chum salmon (*Oncorhynchus keta*) (Kakizawa *et al.* 1995). Furthermore, previous reports suggested that gonadotropin-releasing hormone stimulates secretion of GH (Peter *et al.* 1986) and SL (Kakizawa *et al.* 1997). These studies suggest that release of GH and SL would be accelerated with sexual maturation. A question arising here is whether synthesis, as well as release, of GH/PRL/SL family hormones is elevated in salmonid pituitaries in this period. However, changes in expression of

TABLE 1. Changes in body weight (BW), fork length (FL), GSI and plasma levels of Na⁺ and Cl⁻ in pre-spawning chum salmon in 1994 and 1995. Values are means ± S.E.M.

	Fish	Sex	n	BW (kg)	FL (cm)	GSI (%)	Na ⁺ (mM)	Cl ⁻ (meq/l)
Year 1994	SW	♂	6	2.6 ± 0.3	62.0 ± 2.3	5.8 ± 0.3	229.6 ± 1.8	163.1 ± 1.7
		♀	6	3.0 ± 0.4	64.0 ± 2.4	17.0 ± 0.9	227.2 ± 2.0	166.8 ± 1.8
	Matured FW	♂	6	3.8 ± 0.3	68.5 ± 1.7	4.0 ± 0.2**	171.5 ± 2.2**	131.0 ± 1.2**
		♀	7	3.6 ± 0.3	67.6 ± 1.9	22.0 ± 1.3**	171.2 ± 2.4**	135.9 ± 1.8**
1995	SW	♂	10	3.3 ± 0.3	65.2 ± 1.5	5.4 ± 0.5	212.1 ± 1.4	163.7 ± 2.5
		♀	10	3.3 ± 0.3	64.1 ± 1.3	15.5 ± 0.6	213.0 ± 1.6	151.3 ± 5.3
	Maturing FW	♂	11	3.6 ± 0.2	67.0 ± 1.1	5.4 ± 0.2	159.4 ± 5.2**	116.4 ± 4.1**
		♀	13	3.0 ± 0.2	62.4 ± 1.0	15.9 ± 0.6	161.1 ± 6.4**	116.5 ± 2.6**
	Matured FW	♂	9	3.2 ± 0.2	67.6 ± 1.1	4.5 ± 0.3 ⁺	163.2 ± 1.9**	129.1 ± 1.6**
		♀	10	3.8 ± 0.2	67.0 ± 0.9	19.0 ± 0.8 ⁺⁺	130.7 ± 4.6**	93.3 ± 5.7**

***P* < 0.01 by Student's *t*-test compared with SW fish. ⁺*P* < 0.05; ⁺⁺*P* < 0.01 compared with maturing FW fish.

GH/PRL/SL family genes have not been reported in homing salmonids. Changes in the levels of pituitary mRNAs encoding each of GH, PRL and SL were therefore determined in chum salmon during the final stages of sexual maturation by a dot blot analysis following Hiraoka *et al.* (1996, 1997) and Kitahashi *et al.* (1998).

We first found increases in the levels of mRNAs encoding GH, PRL and SL in the pituitaries of homing chum salmon in seawater (SW) and fresh-water (FW) fish which were captured at Atsuta and Chitose, respectively, in 1994. Homing chum salmon experience two major physiological changes, FW adaptation and sexual maturation, in the final stages of migration. Therefore, to clarify which factor is important for the enhancement of expression of these hormonal genes, we obtained maturing FW fish at the intermediate of the two points, Ebetsu, in the next experiment in 1995, in addition to the two points mentioned above, and analyzed the changes in the levels of GH, PRL and SL mRNAs in these fish.

We further examined changes in the levels of mRNA encoding Pit-1/GHF-1, hereafter referred to as Pit-1, which is a pituitary-specific POU homeodomain transcription factor involved in activation of GH and PRL promoters (Bodner *et al.* 1988, Ingraham *et al.* 1988). The presence of Pit-1 in the pituitaries of salmonids was first demonstrated by cloning studies (Elsholtz *et al.* 1992, Ono & Takayama 1992, Yamada *et al.* 1993). Two species of Pit-1 mRNAs, which are about 2 and 3 kb in size, were isolated in salmonids. Pit-1 protein may enhance SL gene expression *in vitro* (Xiong *et al.* 1993) and *in vivo* in rainbow trout (Ono *et al.* 1994). The levels of Pit-1 mRNAs were thus semiquantitatively estimated by Northern blot

analysis. Because of the unavailability of appropriate Pit-1 cDNAs as standards for assays, we had to employ a relative assay method.

MATERIALS AND METHODS

Fish

Pre-spawning chum salmon (*Oncorhynchus keta*) of the Ishikari stock were caught at a few key points along their homing pathway to the Chitose Salmon Hatchery in Hokkaido, Japan, in 1994 and 1995. In 1994, SW fish were captured at Atsuta in Ishikari bay, 20 km away from the mouth of the Ishikari river, and matured FW fish were captured at the Chitose Salmon Hatchery in October. In 1995, in addition to the two points mentioned above, maturing FW fish were captured at the intermediate of the two points, Ebetsu, in October. Since the Ishikari river is a prominent big river, and a vast number of juveniles are released only from the Chitose Salmon Hatchery of the National Salmon Resources Center, the salmon caught at Atsuta, Ebetsu and Chitose are considered to belong to the same genetic group. Hereafter, the fish caught at Atsuta, Ebetsu and Chitose are referred to as SW fish, maturing FW fish and matured FW fish respectively.

Fish were weighed and body length measured (Table 1), and blood was collected from the caudal vasculature. Blood samples kept in ice were later centrifuged at 2000 *g* for 15 min to obtain plasma. To confirm the magnitudes of FW adaptation, the levels of plasma Na⁺ and Cl⁻ were determined by ion analyzer (AVL 984-S, Graz, Austria) and chloridometer (Buchler Instruments, Fort Lee, NJ, USA) respectively. The values for ion

concentrations (Table 1) clearly showed that the fish of both sexes in Ebetsu were well adapted to an FW environment.

The maturity of the gonads was assessed by the gonadosomatic index (GSI, gonad weight/body weight $\times$ 100). In both 1994 and 1995, the gonads of male and female fish in Chitose were fully matured, easily spermiated by a press of abdomen or ovulated, when they were sampled. The matured FW fish also showed well-developed nuptial color. Increase in GSI values in the FW female fish in Ebetsu and Chitose, when compared with the SW fish, and also a decrease in those in the male fish, indicated that the final maturation of the gonads might occur during migration from Ebetsu to Chitose. It is noteworthy that the SW fish captured in 1994 showed considerable nuptial color, while the fish in 1995 were still silver, as had been seen in regular years.

Preparation of total RNA

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 guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi 1987) for the tissues collected in 1994, and by the guanidinium thiocyanate/hot phenol method (Chirgwin *et al.* 1979) for the samples of 1995. The pituitary weight was 30–70 mg, and the amounts of total RNA extracted from single pituitaries were within the range 100–300 μg . For preliminary experiments and validation of the assay method, standard total RNA was extracted from pooled pituitaries of chum salmon captured in 1995.

Preparation of single-stranded standard DNA

The levels of pituitary hormonal mRNAs were quantitatively measured by a dot blot analysis adopting the principles of assay methods reported by Hiraoka *et al.* (1996, 1997) and Kitahashi *et al.* (1998). Single-stranded sense DNAs (ssDNA) were used as the standards. They were prepared by a modified PCR method with a single sense primer. Template DNAs for PCR were prepared by restriction enzyme digestion at one site of plasmid DNA containing a cDNA insert for chum salmon GH/PRL/SL family. Amplified DNAs were applied to low melting point agarose gel electrophoresis and purified by phenol extraction.

GH ssDNA Plasmid DNA containing GH-I cDNA insert (a gift from Kyowa Hakko Co. Ltd, Tokyo, Japan; Sekine *et al.* 1985) was digested by

restriction enzyme SacI at one site, and was used as the template for PCR. Synthetic oligonucleotide corresponding to the chum salmon GH-I cDNA (89–108) was used as the primer for PCR. Amplification was performed for 30 cycles at 94°C for 1 min 20 s, 64°C for 2 min and 72°C for 3 min after one cycle at 94°C for 5 min, 64°C for 2 min and 72°C for 3 min. Although the sequence of salmon GH-II was reported by Sekine *et al.* (1989), we could not develop an assay system to measure the amounts of each of GH-I and -II mRNAs, because of their high homology.

PRL ssDNA Plasmid DNA containing PRL-I cDNA insert (a gift from Kyowa Hakko Co. Ltd; Kuwana *et al.* 1988) was digested by restriction enzyme EcoRI at one site, and was used as the template for PCR. Synthetic oligonucleotide, 5'-GTTACTTCTGCTCTAAAAGC-3', corresponding to the sequence of the vector, was used as the primer for PCR. Amplification was performed for 35 cycles at 94°C for 1 min 20 s, 55°C for 2 min and 72°C for 2 min after one cycle at 93°C for 5 min, 55°C for 2 min and 72°C for 2 min.

SL ssDNA Plasmid DNA containing SL cDNA insert (a gift from Dr M Ono, Rikkyo University; for the sequence see Ono *et al.* 1990) was digested by restriction enzyme SphI at one site, and was used as the template for PCR. Synthetic oligonucleotide corresponding to the chum salmon SL cDNA (2–21) was used as the primer for PCR. Amplification was performed for 30 cycles at 93°C for 1 min 20 s, 52°C for 2 min and 72°C for 3 min after one cycle at 93°C for 5 min, 52°C for 2 min and 72°C for 3 min.

Preparation of cDNA probes

Since cDNA probes for GH mRNA seriously cross-reacted with PRL and SL mRNAs in a preliminary experiment, we designed the sequences of synthetic oligonucleotides to be highly specific to both GH-I and -II mRNAs. A mixture of three synthetic oligonucleotide probes for the chum salmon GH cDNA complementary to the sequences at 400–419, 441–470 and 499–528 was labeled with a 3'-end labeling kit (Amersham International plc, Amersham, Bucks, UK) using $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ according to the manufacturer's instructions.

Labeled cDNA probes which were specific to PRL and SL mRNA were prepared by a primer extension method using Megaprime DNA labeling systems and $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ (Amersham). Synthetic oligonucleotides corresponding to chum salmon PRL-I cDNA (215–232) and SL cDNA (521–550)

were used as the primer in labeling reaction. Labeled probes for PRL and SL were about 350 and 550 bases long respectively. The sequences used as the probes were carefully selected to avoid cross-hybridization.

Dot blot analysis

Total RNA extracted from the pituitaries was mounted in duplicate on a nylon membrane, Hybond-N⁺ (Amersham) using Milliblot-D (Millipore, Nihon Millipore Ltd, Tokyo, Japan). As the standard to determine the amount of mRNA, serially diluted ssDNA (0.33–1000 pg) was mounted on each membrane in triplicate. The amount of total RNA mounted in a single dot was 1 µg and 333 ng obtained from single pituitaries.

A procedure of hybridization for the assay of GH mRNA with the mixture of oligonucleotide probes was performed at 42 °C overnight in a solution containing 5 × Denhardt's solution, 5 × SSPE (0.05 M phosphate buffer, pH 7.4, containing 0.76 M NaCl and 5 mM EDTA), 0.5% SDS, 100 µg/ml denatured calf thymus DNA (Sigma) and labeled probe (0.5×10^6 c.p.m./ml). Afterwards, the membranes were washed with 2 × SSPE/0.1% SDS at room temperature (RT) for 15 min, twice with 2 × SSPE/0.1% SDS at 50 °C for 30 min, and twice with 1 × SSPE/0.1% SDS at 50 °C for 30 min.

For the assay of PRL and SL mRNAs, hybridization was performed at 65 °C overnight in a solution containing 5 × Denhardt's solution, 5 × SSPE (0.05 M phosphate buffer, pH 7.4, containing 0.76 M NaCl and 5 mM EDTA), 0.5% SDS, 100 µg/ml denatured calf thymus DNA (Sigma) and labeled probe (2×10^6 c.p.m./ml). The membranes were washed with 2 × SSPE/0.1% SDS at 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.

To determine the intensity of specific hybridization signals, the membranes were exposed to a Fuji imaging plate (Fuji Photo Film, Tokyo, Japan) for 20 h. Radioactivity expressed as the intensity of photostimulated luminescence was analyzed by a Bioimaging analyzer (Fuji Photo Film), and the intensity of signals was estimated by subtraction of background.

Validation of assay method

Serially diluted standard DNA (0.1–1000 pg) was hybridized with the corresponding probe to check the linear range of the standard curve. At the same time, serially diluted pituitary total RNA (1–1000 ng) was also hybridized to check the linear

range and parallelism of the curve with the standard curve.

Northern blot analysis was used to confirm whether the labeled probe was hybridized only with the particular mRNA for GH, PRL or SL in the pituitary total RNA. Aliquots of pooled total RNA (1 µg) extracted for preliminary experiments were separately electrophoresed in a 1.0% agarose/formaldehyde gel and transferred to Hybond-N⁺ membrane according to the manufacturer's instructions. Then, the membranes were hybridized with the probes as above.

Cross-hybridization of the probes was carefully checked in detail using ssDNAs for other GH/PRL/SL family, thyroid-stimulating hormone β subunit and gonadotropin subunits. Serially diluted standard ssDNAs for GH, PRL or SL DNA (0.33 pg–1 ng) and the ssDNAs mentioned above (0.33 and 1.00 ng) were blotted to the same membrane, and were hybridized with the probe specific to the standard.

Intra- and inter-assay variations (standard deviation/mean × 100) in the present assay system were calculated to confirm reliability of measured values. Serially diluted standard DNAs (0.1–1000 pg) and total RNA were blotted in triplicate to the membrane. A set of these membranes was prepared for each hormonal mRNA, and hybridized with the labeled probe. Radioactivity of each dot was counted by use of the Bioimaging analyzer as above. Intra-assay variations were calculated for every dose on each filter, while inter-assay variation was obtained from the measured amounts of total RNA on all three filters. Furthermore, intra-assay variations were checked when samples were analyzed. Ranges of intra-assay variations were 0.2–7.6% for GH mRNA, 5.3–16.7% for PRL mRNA and 2.7–14.7% for SL mRNA. Inter-assay variation was 14.0% for GH mRNA, 12.3% for PRL mRNA and 8.0% for SL mRNA.

Northern blot analysis of Pit-1 mRNA

Northern blot analysis was used to analyze the changes in the levels of 2 and 3 kb Pit-1 mRNAs separately. Total RNA (3 µg) extracted from single pituitaries was used in each sample. A synthetic oligonucleotide probe for the chum salmon Pit-1 cDNA complementary to the sequence at 912–941 was labeled with a 3'-end labeling kit (Amersham) using [α -³²P]dATP according to the manufacturer's instructions. After prehybridization in a hybridization buffer (see below for composition) without labeled probe, hybridization with the labeled probe was performed at 42 °C overnight in a solution containing 50% formamide, 5 × Denhardt's

solution, $5 \times$ SSPE, 0.5% SDS, 100 μ g/ml denatured calf thymus DNA (Sigma, Tokyo, Japan) and 0.4×10^6 c.p.m./ml labeled probe. The membranes were washed with $2 \times$ SSPE/0.1% SDS at RT for 15 min, twice with $2 \times$ SSPE/0.1% SDS at 50 °C for 30 min and twice with $1 \times$ SSPE/0.1% SDS at 50 °C for 30 min. Radioactivity of each species of Pit-1 mRNA was counted by use of the Bioimaging analyzer as above.

Statistical analysis

For statistical analysis, Student's *t*-test was applied after an *F* test for variance.

RESULTS

Validation of assay method

The standard curves with GH, PRL and SL ssDNAs were linear within the ranges of 10 pg to 1 ng for ssGH DNA, 1 pg to 1 ng for ssPRL DNA and 3.3 pg to 1 ng for ssSL DNA (Fig. 1). Considerable portions of curves obtained with serially diluted total RNA were parallel to these standard curves. Northern blot analysis clearly showed that the labeled probes to GH, PRL and SL mRNAs detected single species of RNA in total RNA (Fig. 2).

Cross-hybridization to other ssDNAs of the present cDNA probes was sufficiently low. Cross-reactivity of GH probe was 0.11% to ssPRL DNA and 0.18% to ssSL DNA; that of PRL probe was 0.04% to ssGH DNA and 0.04% to ssSL DNA; and SL probe was 0.09% to ssGH DNA and 0.03% to ssPRL DNA. These values showed that specificities of the present assay system were satisfactory to determine the amount of a particular hormonal mRNA in the chum salmon pituitaries.

Changes in the levels of GH, PRL and SL mRNAs

In fish sampled in 1994, the levels of GH, PRL and SL mRNAs in matured male FW fish were significantly higher than those in the SW fish except for PRL mRNA (Fig. 3), although the level of PRL mRNA in the FW males tended to be higher than that in the SW males. The level of GH mRNA in FW fish was about 1.8-fold higher than that in SW fish in both males and females. The pituitaries of FW females contained about 4-fold the level of PRL mRNA when compared with the SW females. The levels of SL mRNA in the FW fish were about 2- and 3.4-fold those in the SW males and females respectively.

FIGURE 1. The standard curve prepared with ssDNAs as the standard (●) and intensity of hybridization signals for serially diluted total RNA (○). Serially diluted total RNAs were parallel to the standard DNAs within the ranges of 10 pg to 1 ng in GH, 1 pg to 1 ng in PRL, and 3.3 pg to 1 ng in SL. Values are mean $\pm$ S.E.M.

In the fish of 1995, changes in the level of GH mRNA were inconsistent with those in 1994, whereas the patterns of significant changes in the levels of PRL and SL mRNAs were similar to those found in the fish of 1994 (Fig. 4, upper panel). In the preliminary experiment in 1993, no significant changes in the levels of GH mRNA were observed (data not shown). The salmon in this experiment showed a silver color. The discrepancy in the

FIGURE 2. Northern hybridization analysis to confirm the specificity of each probe. Total RNA of pituitaries (1 µg) on different filters was hybridized with each probe for GH (lane 1), PRL (lane 2) and SL (lane 3).

changes in the levels of GH mRNA between 1994 and 1995 may be caused by a difference in sexual maturity observed in the fish captured at Atsuta.

The levels of PRL mRNA in both the maturing and matured FW fish were significantly higher than those in the SW fish in 1995 (Fig. 4, middle panel). No significant differences in the levels of PRL mRNA were seen between maturing and matured animals. These results indicated that expression of PRL gene was elevated soon after entry into an FW environment from hyperosmotic coastal sea. Two-fold increases in the levels of PRL mRNA in the females were more conspicuous than 1.5-fold increases in the males. Such sexual difference in the magnitudes of stimulated PRL gene expression was coincidentally observed in both 1994 and 1995.

In contrast to the rather sharp earlier increases in the levels of PRL mRNA, the pituitary levels of SL mRNA in the fish of both sexes of 1995 were gradually increased during upstream migration from the Atsuta coast to the Chitose Salmon Hatchery, although the profiles of increases slightly differed (Fig. 4, lower panel). The similar increases in the levels of SL mRNA in both sexes in 1994 and 1995 were apparently the most consistent among changes in expression of GH/PRL/SL family genes during the final stages of spawning migration.

Changes in the level of Pit-1 mRNAs

Northern blot analysis of chum salmon pituitary RNA clearly showed the presence of two distinct bands for Pit-1 mRNAs, sizes of which were about 2 and 3 kb in all fish examined (Fig. 5). The intensity of hybridization signals for 3 kb bands of Pit-1 mRNA was significantly higher in the FW fish of both sexes than in the SW fish in both 1994 and 1995 (Fig. 6). The intensity of signals for 3 kb Pit-1 mRNA in the males gradually increased and

FIGURE 3. Changes in the levels of GH, PRL and SL mRNAs in SW and matured FW fish in 1994. Values are mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$ by Student's *t*-test.

attained the highest level at Chitose, whereas the intensity of signals in the FW females rather sharply increased during the last phase of upstream migration between Ebetsu and the Chitose Salmon Hatchery. Although significant increases in the intensity of signals for 2 kb Pit-1 mRNA were observed in the FW females of 1994 and also in maturing FW males, no consistent changes were seen through 1994 and 1995.

FIGURE 4. Changes in the levels of GH, PRL and SL mRNAs in SW Atsuta, maturing FW Ebetsu and matured FW Chitose fish in 1995. Values are mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$ by Student's *t*-test.

As is shown in Table 1, the changes in the GSI values in the FW fish in Ebetsu and Chitose, when compared with the SW fish, indicated that the final maturation of the gonads apparently occurred during upstream migration from Ebetsu to Chitose. Thus, the gradual increases in the levels of SL and 3 kb Pit-1 mRNAs should be initiated in association with the progress of final maturation of gonads in pre-spawning chum salmon.

FIGURE 5. Northern blot analysis of chum salmon pituitary mRNA in SW and matured FW fish. Note the presence of two distinct bands for Pit-1 mRNAs, sizes of which were about 2 and 3 kb. Total RNA of pituitaries (3 μ g) was hybridized with the radiolabeled Pit-1 probe.

DISCUSSION

In the present study, we determined the changes in the pituitary levels of GH/PRL/SL and Pit-1 mRNAs during the final stages of spawning migration in chum salmon, and first found the significant elevation in the levels of PRL and SL mRNAs in the consecutive two years. A similar change was also seen in the level of GH mRNA in 1994, although it was not clear in 1995. Coincidentally with these increases in the levels of GH/PRL/SL mRNAs, the pituitary levels of Pit-1 mRNAs, particularly that of 3 kb species, were elevated in homing salmon. The present findings in the homing chum salmon agree well with the previous reports that the plasma levels of GH, PRL and SL in river fish were higher than those in ocean fish (Kakizawa *et al.* 1995). Since the levels of mRNAs generally reflect activity of gene expression, we consider that, in pre-spawning chum salmon, both gene expression and secretory activity

FIGURE 6. Changes in the levels of 2 and 3 kb Pit-1 mRNAs: upper panel, SW Atsuta and matured FW fish in 1994, and lower panel, SW Atsuta, maturing FW Ebetsu and matured FW Chitose fish in 1995. Values are mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$ by Student's *t*-test.

are stimulated in pituitary endocrine cells producing GH/PRL/SL family hormones.

A serious concern for the above results is cross-reactivity of the probes with mismatching hormonal mRNAs, since all of three GH/PRL/SL mRNAs showed almost coincident increases in their amounts, and they are considered to belong to the same family (see Ono *et al.* 1990). Therefore we carefully verified the validity of the assay method, as described in the Results sections. The sufficiently low cross-reactivity of the probes certified the validity of the assay data. The similar ranges of the quantities of GH/PRL/SL mRNAs, within the order between 1 and 10 fmol in individual pituitaries, verified that the magnitude of hybridization signals was not over-estimated by the presence of family mRNAs.

The large difference in the absolute values between the 1994 and 1995 fish (Figs 3 and 4) is another serious concern. The 1993 fish used in the preliminary experiment and the 1995 fish had similar amounts of GH mRNA, and also showed similar insignificant changes in the pituitary levels of GH mRNA (Taniyama S, Kitahashi T, Ando H, Ban M, Ueda H & Urano A, unpublished observations). Considering body color, both 1993 and 1995 fish were in a similar phase of sexual maturation, whereas 1994 fish, which showed considerable nuptial color, were in a more advanced stage. Furthermore, our SW to FW transfer

experiment using pre-spawning chum salmon showed that significant changes in the expression of GH/PRL/SL family genes could occur within 1–2 days (Taniyama S, Ueda H, Ando H, Urano A & Yamauchi K, unpublished observations). Based on these facts, we consider that the large differences between 1994 and 1995 fish can be accounted for by a difference in sexual maturity. The differences may not be due to degradation of total RNA, because the amounts of GH and PRL mRNAs were higher in 1994 than in 1995, in contrast to higher amounts of SL mRNA in 1995.

A noticeable result was that the patterns of changes in the amounts of hormonal mRNAs were different between PRL and SL mRNAs. In 1995, the level of PRL mRNA was somewhat sharply elevated in the maturing FW fish, while the pituitary levels of SL mRNA in the fish of 1995 were gradually increased during upstream migration from the Atsuta coast to the Chitose Salmon Hatchery. The changes in the levels of these mRNAs in 1994 were not contradictory to the changes in 1995 mentioned above. A similar difference between the changes in PRL and SL mRNA levels was also seen in pre-spawning chum salmon of the Otsuchi stock, which were homing to the Otsuchi river in the Sanriku coast (Taniyama S, Ueda H, Ando H, Urano A & Yamauchi K, unpublished observations). Although evidence for the involvement of GH in the regulation of sexual maturation, including increases in the plasma GH levels during sexual maturation, has been sufficiently accumulated in salmonids (see Björnsson 1997), consistent changes in the levels of GH mRNA were not observed in the present study.

The difference between the profiles of the pituitary levels of PRL and SL mRNAs may reflect the physiological roles of the two hormones. Many previous studies indicated that PRL is an important hormone for FW adaptation (see Hirano & Mayer-Gostan 1978), whereas SL may have some physiological functions in reproduction (Rand-Weaver *et al.* 1992, Rand-Weaver & Swanson 1993, Kakizawa *et al.* 1995). In 1995, the levels of PRL mRNA were elevated in the maturing FW fish captured at Ebetsu, and the elevated levels were maintained in the fully matured fish. Considering the location of Ebetsu, we concluded that the expression of PRL gene was stimulated soon after entry into an FW environment, although at present we do not have any lines of evidence for elevation of PRL gene expression by hypo-osmotic stimulation. The marked or greater increases in the levels of PRL mRNA in the females in the present study agree well with the previous findings that the plasma level of PRL in homing females was higher

than that in males (Hirano *et al.* 1985, 1990, Kakizawa *et al.* 1995, Ogasawara *et al.* 1996). Expression of PRL gene in pre-spawning chum salmon thus may be elevated by an osmotic change during the upstream migration. Since the pituitary levels of SL mRNA in the fish of 1995 were gradually increased during upstream migration from the Atsuta coast to the Chitose Salmon Hatchery, and the changes in the GSI values indicate that the final maturation of the gonads in the experimental fish occurred during migration from Ebetsu to Chitose, we considered that expression of the SL gene was enhanced not by the osmotic change but with the progress of sexual maturation.

In agreement with the changes in the levels of GH/PRL/SL family mRNAs, the levels of Pit-1 mRNAs were elevated during the last phases of homing migration in chum salmon in both 1994 and 1995. Such coincidence can be viewed as good evidence that Pit-1 is crucial for the expression of GH/PRL/SL family genes during the final stages of sexual maturation in chum salmon. Supporting evidence for the above idea is that Pit-1 was involved in the gene expression of SL in rainbow trout (Ono *et al.* 1994), and that fish Pit-1 functionally binds to the promoter region of SL gene (Yamada *et al.* 1993). The profiles of the changes in the levels of Pit-1 mRNAs in the consecutive two years, like the changes in the levels of SL mRNA, appear to show that the levels of Pit-1 mRNAs were increased in association with sexual maturation. Since an immunocytochemical study showed the strongest Pit-1 immunoreactivity in SL cells among GH/PRL/SL cells in the pituitary of ovulated female chum salmon (Salam MA, Ando H, Ban M, Ueda H & Urano A, unpublished observation), we presumed that Pit-1 was involved in stimulation of SL gene expression during the final stages of sexual maturation.

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