Changes in Transcript Levels of Gill Cortisol Receptor during Smoltification in Wild Masu Salmon, Oncorhynchus masou

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Changes in Transcript Levels of Gill Cortisol Receptor during Smoltification in Wild Masu Salmon, *Oncorhynchus masou*

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ABSTRACT—We developed a quantitative PCR assay to investigate transcript levels of gill cortisol receptor (CR) in masu salmon (*Oncorhynchus masou*). Using this system, we examined changes in transcript levels of gill CR during smoltification of wild masu salmon while tracking serum cortisol and growth hormone (GH) concentrations patterns. The masu salmon were parr in January and February, and thereafter smoltified, migrating from the river to the sea in May. Gill CR transcript levels were very low in January and February, but thereafter increased and reached a maximum in April (5 fold increase over levels observed in January). In May, when smolt enter the sea, the gill CR transcript levels decreased. Serum cortisol concentrations were also low from January to March and increased to the peak in April. These changes correlated well with changes in CR transcript levels from March to April. In contrast, serum GH concentration began to increase in January, peaked in March and decreased from March to May. These results elucidated patterns in transcript levels of gill CR during smoltification of wild masu salmon and suggested that gill CR transcription was positively regulated by cortisol until serum cortisol level reached a peak, and negatively done after the peak in masu salmon.

INTRODUCTION

When juvenile anadromous salmonids migrate from freshwater to a marine environment, a series of complex physiological changes, known as smoltification, occurs which is regulated by many endocrinological factors (Hoar, 1988; Sakamoto *et al*., 1995). Many of these changes occur in the gill. To enhance seawater adaptability in the gill, Na^+,K^+-ATPase activity increases as do the size and number of chloride cells, which facilitate ion exchange between the internal and external environment (McCormick, 1995). The ultrastructure of the chloride cell also changes dramatically (Mizuno *et al*., 2000). It has been shown that these changes are mainly regulated by the action of cortisol and growth hormone (GH) (McCormick, 1995). In general, the physiological effects of these hormones are thought to be mediated by their specific receptors. However, the action mechanism of the hormones in smoltification has not been examined at the molecular level with an attention to hormone receptors.

At present, the assumption that cortisol acts through the glucocorticoid receptor (GR) including cortisol receptor (CR), is widely accepted (Ducouret *et al*., 1995). Previously, the presence of CR in the gill has been demonstrated in salmonids by specific binding of radiolabeled glucocorticoid (Shrimpton *et al*., 1994, 1995). Therefore, it has been proposed that cortisol acts directly in the gill to stimulate the osmoregulatory response. GR cDNA has been isolated from rainbow trout (*Oncorhynchus mykiss*) liver and intestine (Ducouret *et al*., 1995) and from Japanese flounder (*Paralichthys olivaceus*) liver (Tokuda *et al*., 1999). A partial CR cDNA has also been reported for tilapia (*Oreochlomis niloticus*) liver (Tagawa *et al*., 1997). High levels of GR mRNA have been detected in the gill of rainbow trout (Ducouret *et al*., 1995). CR mRNA and protein have been localized to the gill chloride cell in chum salmon (Uchida *et al*., 1998). Besides, mineralocorticoid-like receptor, which is closely related to GR, cDNA has been recently isolated from rainbow trout testis (Colombe *et al*., 2000). Nonetheless, changes in CR transcript levels during smoltification have not been studied.

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In teleosts, hormonal regulation of CR transcription is not always revealed clearly. Only some studies have characterized gill CR changes in response to hormones: cortisol treatment (Maule and Schreck, 1991; Shrimpton and Randall, 1994), bovine GH treatment (Shrimpton *et al.*, 1995) or thyroid hormone treatment (Shrimpton and McCormick, 1998) in coho salmon (*Oncorhynchus kisutch*). Taken together, these studies suggest that CR number may be regulated in some way by cortisol, GH, and thyroid hormones. However, it is not clear whether these hormones regulate gill CR transcript levels in salmonids.

For these reasons, a sensitive quantitative assay for detection of masu salmon CR mRNA by competitive PCR was developed and changes in gill CR transcript levels were analyzed during smoltification of wild masu salmon (*Oncorhynchus masou*). At the same time, changes in serum cortisol and growth hormone levels were examined, and a relationship among CR transcript levels, serum cortisol, and GH concentrations was considered.

MATERIALS AND METHODS

Fish

Wild masu salmon were collected from the freshwater in the Kenichi River, Hokkaido, Japan. The samplings were performed monthly by electric shocker (Benkei Turbo, Frontier Electric, Japan) from January to April 1996 or by fishing from March to May 1996 at ten in the morning. The fish were rapidly transferred to buckets containing 200 mg/L tricaine methanesulphonate (MS-222), buffered with 400 mg/L sodium bicarbonate and were anaesthetized. This dosage of MS-222 has been shown to be sufficient to prevent the release of cortisol in captured fish (Barton *et al.*, 1985). There was not significant difference in the serum cortisol concentration between masu salmon caught by electric shocker and that by fishing in March and April 1996 (Mizuno *et al.* unpublished data). Therefore, it was thought that there was no effect of difference between the two capture methods in serum cortisol concentration of masu salmon in the present study. Average of body weight gradually increased from January (13.5±0.18 g) to May (24.2±0.22 g). Parr marks were evident without any body silvering in the parr (January-February) and were obscured in pre-smolt (March-April). In the full-smolt (May), parr marks were completely absent, while the outer extremities of dorsal and caudal fin lobes pigmentized black. After sacrificing the fish (N=5 per month) the first gill arches on either side were removed. The tissue was frozen and stored at –80°C for subsequent of CR mRNA and analysis of Na^+,K^+-ATPase activity analyses. At the same time gill samples were gathered, the caudal peduncle was severed, and blood samples were collected in plastic tubes. The blood was centrifuged, and the plasma was separated and collected. Plasma samples were frozen at –80°C for subsequent measurement of cortisol and GH concentrations.

Total RNA extraction and cDNA synthesis

Extraction of total RNA from frozen gill arches and cDNA synthesis were done according to the method of Nagae *et al.* (1997). Total gill RNA was extracted using ISOGEN (Wako Pure Chemical Industries) and contaminating genomic DNA was removed by deoxyribonuclease I (Wako Pure Chemical Industries) treatment. cDNA was synthesized from 1 μg total RNA with random hexadeoxynucleotide primers (Gibco BRL) using M-MLV reverse transcriptase (Gibco BRL) in a 20 μl reaction. Reverse transcriptions were performed for 60 min at 37°C, then inactivated for 5 min at 99°C

Partial masu salmon CR cDNA cloning and sequence determination

A partial CR cDNA was amplified by PCR from the gill cDNA. The forward and reverse primers used for competitive PCR were designed based on the nucleotide sequence of rainbow trout GR cDNA (Ducouret *et al.*, 1995).
The primer sequences are as follows.
forward: 5' ACGATGGAGCCGAACCCTCC 3'
reverse: 5' GGTTCCGGAACCCTGGTAGG 3'.
PCR conditions and procedures for cloning and sequence determination of cDNA were as previously described in Nagae *et al* (1996). The CR cDNA sequence was determined for 10 independent PCR products.

Preparation of Competitor Internal Standard

Competitor: An internal standard for competitive PCR was made by amplifying a partial β-actin cDNA using primers consisting of β-actin sequence flanked by the CR primer sequences to be used in the competitive PCR reaction. The primers for internal standard amplification, primers 1 and 2, included β-actin nucleotide sequence that is conserved in the common carp (Liu *et al.*, 1990), goose (Kolattukudy *et al.*, 1987) and rat (Nudel *et al.*, 1983) flanked by the competitive PCR primer sequences.
The primer sequences are as follows.
primer 1: 5' ACGATGGAGCCGAACCCTCCTGTACGCCTCTGGCCGTACC 3'
primer 2: 5' GGTTCCGGAACCCTGGTAGGGTGATGACCTGTCCGTCAGG 3'
Following amplification by PCR, the internal standard fragment was cloned into pBluescript II (Stratagene). Prior to use as an internal standard in competitive PCR reactions, the recombinant plasmid was digested by EcoRI and the insert fragment was purified.

Competitive PCR

Quantification of masu salmon CR mRNA was done according to the competitive PCR method of Sasaki and Enami (1996). Competitive PCR was performed for 35 cycles (94°C, 1 min; 62°C, 1 min; 72°C 1 min). The reaction mixture contained 5 μl of gill cDNA, various amounts of the competitor, 2.5 pM forward and reverse CR primers, 2.5 nM dNTPs and 0.25 units of Taq DNA Polymerase (Greiner) in a final volume of 50 μl. Subsequently, 5 μl samples were separated by electrophoresis through 2% agarose gels. Gels were stained with ethidium bromide and photographed on Polaroid 665 positive/negative film. Photographs were scanned by an image scanner and the intensity of each band on the negative film was quantified using the NIH-Image (Ver. 1.59) analysis system. The logarithm of the ratio of the densities of the CR and competitor bands (CR/competitor) multiplied by 1.46 (molecular weight correction factor), were plotted against the logarithm of the amount of added competitor expressed in moles. The amount of double-stranded CR cDNA was determined from the amount of competitor corresponding to zero on the vertical axis. This value was multiplied by 2 to yield the total amount of single-stranded CR cDNA present in the PCR reaction mixture and by 40 to give the total amount of single-stranded CR cDNA present in the cDNA synthesized from the gill total RNA (1μg). The amount of single-stranded CR cDNA thus obtained was directly used to express relative amount of CR mRNA in the original samples.

Accuracy test

Total RNA extracted from the gill in April was used to test for the assay variability. The between-assay coefficient of variation (CVb) was calculated from 5 assays done in triplicate. The within-assay coefficient of variation (CVw) was determined by measuring concentrations of products in ten parallel samples (5 μl each) from a single 50 μl competitive PCR reaction. The CVb and CVw were 4.8% and 5.9%, respectively.

Measurement of serum cortisol concentrations

Cortisol concentration was measured according to the radioimmunoassay method reported by Nagae *et al*. (1994).

Measurement of serum GH concentrations

GH concentration was determined using the chemiluminescent immunoassay reported by Fukada *et al.* (1997).

```
C CCA CCT GAC GAG AGG GCC TGC GCC TTG ATC CCC AAG TCT
  Pro Pro Asp Glu Arg Ala Cys Ala Leu Ile Pro Lys Ser

                50
ATG CCC CAG CTG GTA CCC ACC ATG CTA TCC CTG CTC AAA GCC
Met Pro Gln Leu Val Pro Thr Met Leu Ser Leu Leu Lys Ala

                    100
ATT GAG CCG GAA GCC ATT TAC TCT GGC TAC GAC AGC ACC ATT
Ile Glu Pro Glu Ala Ile Tyr Ser Gly Tyr Asp Ser Thy Ile

                          150
CCC GAC ACC TCC ACC CGC CTC AGC ACC ACC CTC AAC AGG CTG
Pro Asp Thy Ser Thy Arg Leu Ser Thy Thy Leu Asn Arg Leu

                               200
GGC GGA CAG CAG GTT GTT TCA GCC GTC AAA TGG GCC AAG TC
Gly Gly Gln Gln Val Val Ser Ala Val Lys Trp Ala Lys
```

Fig. 1. Nucleotide sequence deduced amino acid sequence of the partial cDNA encoding CR of masu salmon. Numbering refers to the nucleotide sequence.

```
                                                    50
ACGATGGAGCCGAACCCTCCTACGCCTCTGGCCGTACC ACC GGT ATC GTC ATG
                                        Thr Gly Ile Val Met

                                                   100
GAC TCC GGT GAC GGC GTG ACC CAC ACA GTA CCC ATC TAC GAG GGC TAC
Asp Ser Gly Asp Gly Val Thr His Thr Val Pro Ile Tyr Glu Gly Tyr

GCT CTG CCC CAC GCC ATC CTG CGT CTG GAT CTG GCC GGC CGC GAC CTC
Ala Leu Pro His Ala Ile Leu Arg Leu Asp Leu Ala Gly Arg Asp Leu

150
ACA GAC TAC CTG ATG AAG ATC CTG ACG GAG CGA GGC TAC AGC TTC ACC
Thr Asp Tyr Leu Met Lys Ile Leu Thr Glu Arg Gly Tyr Ser Phe Thr

 200
ACC ACG GCC GAG AGG GAA ATC GTA CGA GAC ATC AAG GAG AAG CTG TGC
Thr Thr Ala Glu Arg Glu Ile Val Arg Asp Ile Lys Glu Lys Leu Cys

    250
TAC GTG GCG CTG GAC TTT GAG CAG GAG ATG GGC ACC GCT GCC TCC TCT
Tyr Val Ala Leu Asp Phe Glu Gln Gln Met Gly Thr Ala Ala Ser Ser

        300
TCC TCT CTG GAG AAG AGC TAC GAG CTG CCTGACGGACAGGTCATCACCCTACCA
Ser Ser Leu Glu Lys Ser Tyr Glu Leu

 350
GGGTTCCGGAACC
```

Fig. 2. Nucleotide sequence of the competitor, which is a partial masu salmon β-actin cDNA coupled to the primer sequence for competitive PCR (underlined). The deduced amino acid sequence is shown for β-actin. Numbering refers to the nucleotide sequence.

Measurement of gill Na^+,K^+-ATPase activity

Gill Na^+,K^+-ATPase activity was analyzed according to Ura *et al.* (1997). The release of inorganic phosphate was measured according to the method of Goldenberg and Fermandoz (1966). Measurement of protein concentrations was performed according to Lowry *et al.* (1951) and enzyme activity was expressed as μmol Pi/ mg protein/hr.

Statistical analysis

All data are expressed as mean ± SEM. One-way analysis of variance and Student-Newman-Kuels multiple range tests were conducted on the data. Differences between groups were considered significant at $p < 0.05$.

RESULTS

Partial masu salmon CR cDNA cloning and sequence determination

All of the 10 PCR products analyzed had identical nucleotide sequences. The CR cDNA nucleotide sequence (207 bp) and amino acid sequence (68 amino acids) deduced from the nucleotide sequence are shown in Fig. 1. The salmon sequence is 98.5% identical to the published rainbow trout GR sequence (Ducouret *et al.*, 1995) and 39.7% identical to the rainbow trout mineralocorticoid-like receptor sequence (Colombe *et al.*, 2000) at amino acid levels.

Competitor sequence determination

Fig. 2 presents the nucleotide sequences of the competitor (360 bp) used for competitive PCR. The competitor was constructed by amplification of a partial masu salmon β-actin cDNA using primers composed of β-actin sequence flanked by the nucleotide sequence of the primer for competitive PCR. The nucleotide sequence of this β-actin cDNA displayed 85.0% homology with common carp (Liu *et al.*, 1990), 87.9% with goose (Kolattukudy *et al.*, 1987) and 82.2% with rat (Nudel *et al.*, 1983). In addition the deduced amino acid sequence of masu salmon β-actin was 95.8% identical to these 3 species (94 amino acids).

Quantitative competitive PCR system

To measure the content of CR cDNA prepared from CR mRNA in the gill, a sensitive quantification method using competitive PCR was established. The competitor, which served as an internal standard, was constructed so that it differed from the CR cDNA in size and in sequence except for the primer regions (Fig. 2). To circumvent the problem of variabil-

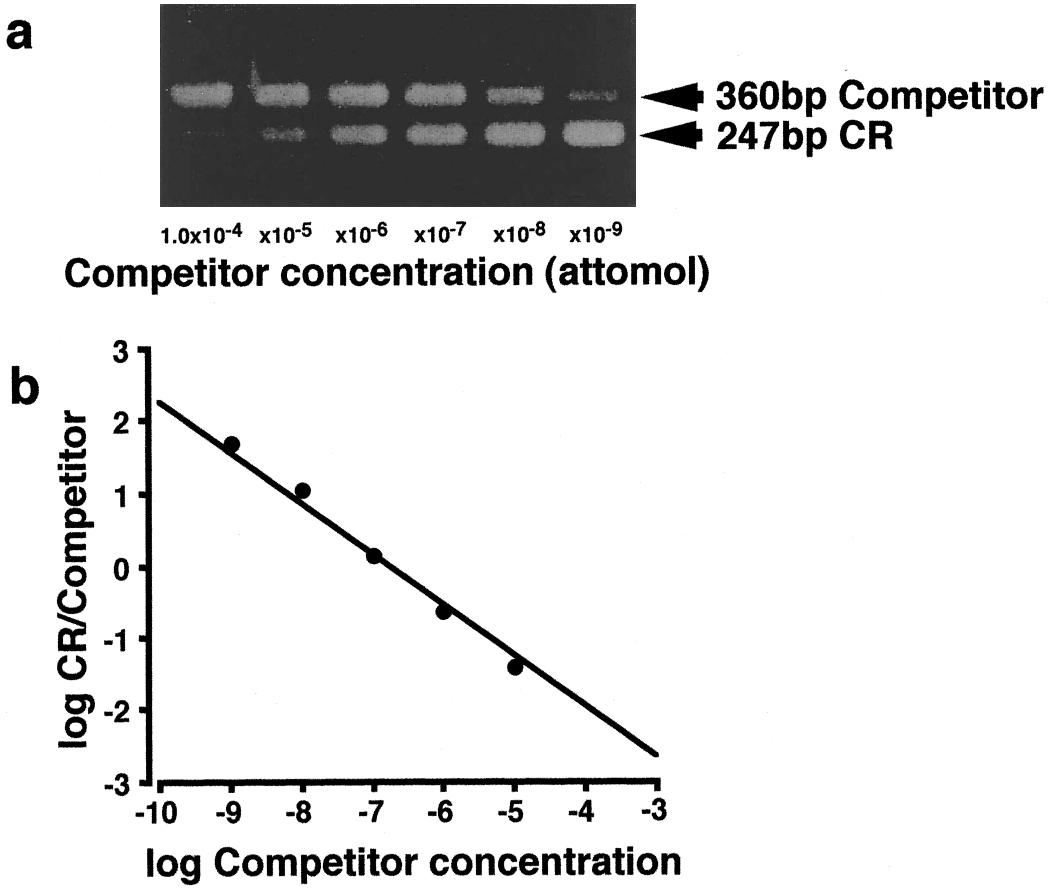

Fig. 3. Quantitative analysis of gill CR cDNA by competitive PCR. Halfquarter volumes (5 μl) of cDNA (40 μl) prepared from total RNA (2 μg) were amplified with a dilution series of internal standard (1.0×10^{-4}–1.0×10^{-9} attomol). (a) After competitive PCR, amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide. (b) The intensities of the bands corresponding to the CR cDNA and competitor were quantified, and the ratio of the log of CR/competitor was plotted.

ity in efficiency of amplification, cDNA (40 μL) prepared from 2 μg of total RNA collected in April was used in 5 μL aliquots as a template for competitive PCR reactions spiked with a dilution series of internal standard (1.0×10^{-4}–1.0×10^{-9} attomol). An electrophoretic analysis of the PCR products showed that the presence of the competitor (360 bp) in the same reaction mixture inhibited the amplification of CR cDNA (247 bp) competitively (Fig. 3a). As would be predicted for a competition reaction, a plot of the ratio of CR cDNA versus competitor was linear when plotted on a log-log scale (Fig. 3b).

Changes in transcript levels of gill CR during smoltification in wild masu salmon

Fig. 4 shows changes in transcript levels of gill CR during smoltification in wild masu salmon. Transcript levels of gill CR were expressed as the amount relative to 1.0×10^{-6} attomol of CR cDNA. The levels were very low in January and February. However, the level increased markedly during smoltification and reached its highest value in April (5-fold increase over levels in January and February). There was a significant difference in the levels between January and April ($P<0.05$). The level declined significantly from April to May ($P<0.05$).

Changes in serum cortisol and GH levels, and gill Na^+,K^+-ATPase activity during smoltification in wild masu salmon

Changes in serum cortisol levels during smoltification in wild masu salmon are shown in Fig. 5a. Serum cortisol concentrations remained low from January to March. In April, the concentration increased to a maximum ($P<0.05$). Fig. 5b shows the changes in serum GH levels observed during smoltification in wild masu salmon. Serum GH concentrations began to increase gradually from January and reached their maximum value in March. There was a significant difference in the concentration between January and March ($P<0.05$). The concentration decreased significantly from March to May ($P<0.05$).

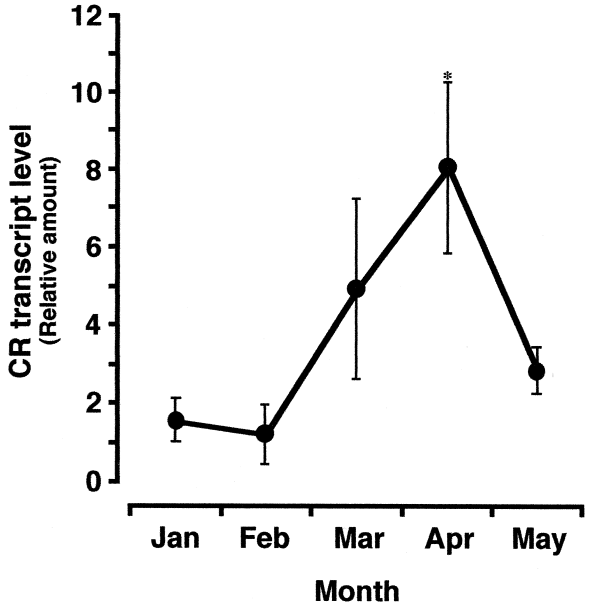

Fig. 4. Changes in transcript levels of gill CR during smoltification in masu salmon. Transcript levels of gill CR are expressed as the amount relative to 1.0×10^{-6} attomol of CR cDNA. Vertical bars represent the mean ± SEM (n=5). *: Significantly different from the value in January ($P<0.05$).

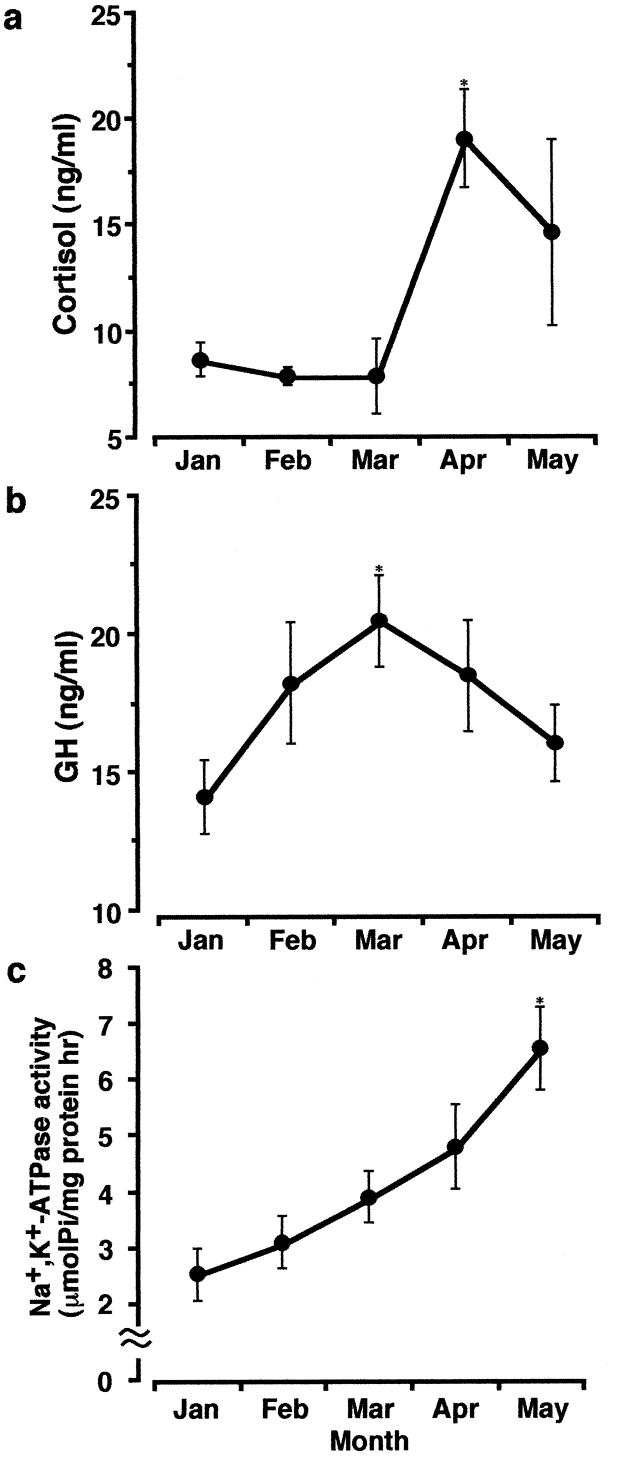

Fig. 5. Changes in serum concentration of cortisol (a) and growth hormone (GH) (b), and gill Na^+,K^+-ATPase activity (c) during smoltification in wild masu salmon. Vertical bars represent the mean ± SEM (n=5). *: Significantly different from the value in January ($P<0.05$).

Changes in gill Na^+,K^+-ATPase activity during smoltification in wild masu salmon are shown in Fig. 5c. Gill Na^+,K^+-ATPase activity increased substantially from January to May ($P<0.05$).

DISCUSSION

This is the first study to establish a sensitive quantitative system for gill CR mRNA and to accurately assess gill CR transcript levels. A partial masu salmon CR cDNA (207 bp) was amplified by non-competitive PCR. This cDNA exhibited high homology (98.5%) with rainbow trout GR and low one (39.7%) with rainbow trout mineralocorticoid-like receptor. Ten sequenced PCR products possessed the same nucleotide sequences. These results suggest that the competitive PCR reaction yields only one type of masu salmon CR product. To make an internal control for competitive PCR, a fragment of β-actin cDNA flanked by the competitive PCR primers was generated by PCR amplification of cDNA from the gill of masu salmon. A partial β-actin cDNA was used as an internal standard because the amplified CR cDNA has no adequate restriction enzyme sites for generating a deletion mutant. The isolated β-actin cDNA (284 bp) shared very high homology with common carp, goose and rat β-actins. The high homology suggests that the clone encoded part of masu salmon β-actin cDNA.

Competitive PCR methods require that a target gene and a competitor amplify with equal efficiencies and that only the target gene and competitor are amplified (Gilliland *et al.*, 1990; Siebert and Larrik, 1992). When competitive PCR was performed on cDNA prepared from the gill in April mixed with a dilution series of competitor, the plot of log competitor concentration versus log amplified PCR product ratio (CR/competitor) was linear. The linearity indicates that there is no difference between the efficiency in amplification of the target and of the competitor. These findings indicate that the developed quantitation system for CR cDNA using competitive PCR is highly accurate.

Analysis of transcript levels for gill CR indicated that the levels changed dramatically during smoltification in wild masu salmon. In the gill, CR transcripts and their translated products are mainly localized in the chloride cell of salmon (Uchida *et al.*, 1998). A previous report suggests that activation of filament chloride cells functions in development of seawater adaptability, whereas lamellar chloride cells assume osmoregulation in freshwater adaptation (Uchida *et al.*,1996). Moreover, Uchida *et al.* (1998) indicate that CR transcript levels of filament chloride cells in seawater are high as compared with that in freshwater. Therefore, it is suggested that changes in transcript levels for gill CR in the present study mainly originate in those for CR in the filament chloride cell.

Transcript levels for gill CR and serum cortisol concentrations increased synchronously from March to April. However, its transcript levels decreased and cortisol concentrations did not change from April to May. This relationship between transcript levels for gill CR and serum cortisol concentrations suggests that gill CR transcription is regulated positively until the peak in serum cortisol levels and done negatively after the peak. As for total GRs, it has been established in mammals that the GR population is composed of both free receptors in the cytoplasm and cortisol-bound receptors in the nucleus (Gorski and Gannon. 1976). From the model, it is speculated that smolting masu salmon must need to synthesize new unbound receptors in the gill to compensate for a decrease in free receptors caused until the peak of serum cortisol levels in April. For this reason, gill CR transcript levels may increase until the peak of serum cortisol levels in April. Moreover, it is found that acute and extensive increase in serum cortisol levels following transfer from freshwater to seawater or cortisol treatment induces decrease in gill CR number by negative regulation of cortisol in some salmonids (Weisbart *et al.*, 1987; Shrimpton *et al.*, 1994). The previous reports suggest that negative regulation of cortisol to gill CR in the present study is due to that serum cortisol concentrations attain to the level to start to negatively regulate gill CR in April. On the contrary, Shrimpton *et al.* (1994) report that during smoltification of juvenile coho salmon, a surge in serum cortisol levels corresponds to a reduction in gill CR number and affinity for cortisol as measured by specific binding of radiolabeled glucocorticoid to gill receptor preparations. The positive regulation of cortisol to gill CR is suggested in the present study, meanwhile it is not found in the previous study. Furthermore, it is regarded that the negative regulation of cortisol to gill CR starts in the peak in serum cortisol levels in the present study, whereas it is considered that the negative regulation starts before the peak in the previous study. These differences in regulational system of gill CR by cortisol may be due to difference in species between masu salmon and coho salmon, although the real cause is unclear in the present study.

Another notable finding was that gill CR transcript levels began to increase in March while serum cortisol levels remained constant. Therefore, it is likely that other endocrine factors regulate transcript levels of gill CR. Binding assay experiments using radiolabeled glucocorticoid demonstrated that the gill CR number increased after bovine GH treatment in coho salmon (Shrimpton *et al.*, 1995), suggesting that GH is also a regulator of gill CR. In the present study serum GH levels were observed to increase from February, reaching maximum levels in March, and decreasing thereafter until May. In addition, serum GH levels increased prior to an observed increase in serum cortisol levels in March. These results maybe suggest that GH is an upregulator of gill CR during smoltification. However, it is reported that insulin-like growth factors enhance corticosteroid receptor mRNA levels in cultured human adrenocortical cells (l'Allemand *et al.*, 1996). In salmonids, the action of GH is thought to be through its major influence on insulin-like growth factor I during smoltification (McCormick *et al.*, 1991; Madsen, 1993; Sakamoto *et al.*, 1993). Therefore, the consideration that the regulation of gill CR by GH action is probably mediated by the action of insulin-like growth factor I should be taken into account.

Moreover, the present study revealed that gill Na^+,K^+-ATPase activity increased gradually during smoltification, and

reached its highest value in May. A peak in gill Na^+,K^+-ATPase activity occurred in late April when gill CR transcript levels and serum cortisol concentrations reached their maximum. It is reported that Na^+,K^+-ATPase α-subunit is immunolocalized in gill chloride cell using polyclonal antibody against a synthetic oligopeptide corresponding to part of the α-subunit (Ura *et al*., 1996). Cortisol induces increase in transcript levels and synthesis of gill Na^+,K^+-ATPase α-subunit in masu salmon (Ura *et al*., unpublished data). In masu salmon, development of the rough endoplasmic reticulum and Golgi apparatus were observed in gill chloride cells in March and April, suggesting active synthesis of new proteins including Na^+,K^+-ATPase (Mizuno *et al*., 2000). These informations may suggest that cortisol acts directly in CR of the gill chloride cell, then it induces increase in synthesis of Na^+,K^+-ATPase α-subunit in the gill chloride cell during increase in serum cortisol concentrations. However, the real cause of that the peak in gill Na^+,K^+-ATPase activity drops behind the peaks in gill CR transcript levels and serum cortisol concentrations is unclear in the present study. To understand more relationship between cortisol and gill Na^+,K^+-ATPase, it is necessary to elucidate what role cortisol plays in mechanisms of Na^+,K^+-ATPase β-subunit expression and activation of Na^+,K^+-ATPase.

In conclusion, we have developed a quantitative system for sensitive and accurate measurement of CR transcript levels in masu salmon. Using this system, we have demonstrated that gill CR transcript levels change dramatically during smoltification in wild masu salmon. Transcript levels for gill CR and serum cortisol concentrations increased synchronously from March to April. However, its transcript levels decreased and cortisol concentrations did not change from April to May. These results suggest that gill CR transcription is positively regulated by cortisol until serum cortisol level reaches a peak, and negatively done after the peak. However, the hormonal regulation of gill CR transcription has not been demonstrated directly. For a better understanding of the hormonal regulation of CR transcript levels, it will be necessary to examine the effects of various hormones *in vivo* and *in vitro*.

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