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Analysis of Salmon Calcitonin I in the Ultimobranchial Gland and Gill Filaments during Development of Rainbow Trout, *Oncorhynchus mykiss*, by *in situ* Hybridization and Immunohistochemical Staining

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ABSTRACT—We have cloned two distinct cDNAs encoding salmon-type calcitonin (sCT)-I cDNAs from the ultimobranchial gland of rainbow trout, *Oncorhynchus mykiss*. Both cDNAs were predicted to encode nearly identical sCT-I precursors which consisted of an N-terminal peptide of 80 amino acid residues, a putative cleavage site Lys-Arg, sCT-I, a cleavage and amidation sequence Gly-Lys-Lys-Arg, and a C-terminal peptide of 18 amino acids. Development of sCT-I-expressing cells was then examined by employing conventional histochemical staining, *in situ* hybridization with a specific cRNA probe, and further immunohistochemistry. The primordium of the ultimobranchial gland was first identified, as two cell masses, in the region between the alimentary canal and sinus venosus behind the heart 17 days postfertilization (dpf; 14°C). However, expression of sCT-I mRNA could not be detected in this gland until one day later, and appeared at 18 dpf. sCT-I immunoreactivity was first observed at 19 dpf (two days before hatching), and the ultimobranchial gland began to assume a follicular structure at 20 dpf (one days before hatching). As ontogeny proceeded, the sCT-I-immunoreactive cells increased in both number and stainability. The sCT-I mRNA was also expressed on the developing gill filaments, but immunoreactive sCT-I was not detected in these sites. These results provide basic data for further research on the organogenesis of the trout ultimobranchial gland.

Key words: salmon-type calcitonin, rainbow trout, ultimobranchial gland, gills, development

INTRODUCTION

Calcitonin (CT) is a vertebrate peptide hormone first identified as a hypocalcemic substance in mammals (Copp *et al.,* 1962). Mammalian CT, abundantly produced from the thyroidal C-cells, is now considered to have a primary role in skeletal conservation (Zaidi *et al.,* 2002). However, recent studies have suggested that CT is synthesized in a variety of extrathyroidal tissues (Beker *et al.,* 1979; Sexton *et al.,* 1999), exerting additional functions as a local factor: e.g. regulation of the sperm mobility and capacitation (Gnessi *et al.,* 1984; Fraser *et al.,* 2001), and of the embryonic implantation and development (Ding *et al.,* 1994; Wang *et al.,* 1998). As for fish, accumulating evidence also indicates that CT may be synthesized in various tissues including the ultimobranchial gland, the major production site of fish CT (Martial *et al.,* 1994; Sasayama 1999; Clark *et al.,* 2002). Although its physiological significance is controversial, it is suggested that serum fish CT might protect scales from excessive resorption in the goldfish (*Carassius auratus*) and nibbler fish (*Girella punctata*) (Suzuki *et al.,* 2000a), and be involved in the reproductive physiology of rainbow trout (*Oncorhynchus mykiss*) (Bjornsson *et al.,* 1986). Okuda *et al.,* (1999) further proposed that CT, produced locally in the small intestine, might suppress acute absorption of calcium and nutrition in the goldfish. The mRNA encoding a possible calcitonin receptor is also detected in a number of tissues of flounder (Suzuki *et al.,* 2000b), implying diverse functions of fish CT in the adult. To our knowledge, however, there is no report describing the early development and differentiation of CT-producing cells with a survey of the whole fish body. Identification of CT-producing cells during fish ontogeny is important for the understanding of their physiological roles in development. In the present study, we have cloned the mRNA encoding salmon-type CT-I (sCT-I) from the rainbow trout, and established a whole mount *in situ* hybridization

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system for trout embryos and alevins. Temporal and spatial expression of the sCT-I mRNA during the early development was then investigated using this technique. Production of sCT-I peptide was further examined by immunohistochemical staining.

MATERIALS AND METHODS

Animals and sampling

Two-year-old and 1.5-year-old rainbow trout, *Oncorhynchus mykiss*, of both sexes were collected at Fuji Trout Hatchery, Shizuoka, Japan, in March and May. After being carefully dissected out, ultimobranchial glands were immediately frozen in liquid nitrogen, or fixed in 4% paraformaldehyde (PFA)/0.01M phosphate-buffered saline (PBS) (pH 7.4) at 4°C. In September, eggs and sperm of rainbow trout were transported from Fuji Trout Hatchery to Shizuoka University, where artificial fertilization was carried out. Embryos and alevins were reared in an aquarium with recirculating fresh water at 14°C. The eyed stage was reached 13 days after fertilization, and the trout started hatching 21 days after fertilization. Nearly every day, from 7 days to 24 days after fertilization, embryos or alevins were collected, and some of them were fixed in Bouin's solution without acetic acid at 4°C overnight for histochemical analysis. Others were fixed in 4% PFA /PBS (pH 7.4) at 4°C overnight for *in situ* hybridization. Both the embryos and alevins of the chum salmon were reared at 10°C in the Otsuchi Marine Research Center of the Ocean Research Institue, University of Tokyo, and fixed nearly every week, from 13 days to 56 days after fertilization, for histological analysis.

Construction of cDNA library and DNA cloning

Total RNA was extracted from 120 ultimobranchial glands of the trout using TRIZOL reagent (Invitrogen, Tokyo, Japan), and poly(A)$^+$ RNA was prepared with oligo-dT coated latex beads (Oligotex-dT30 super; Takara, Kyoto, Japan), according to the manufacturer's instructions. cDNA was prepared with a ZAP Express cDNA synthesis kit (Stratagene, La Jolla, CA, USA), and then a cDNA library was constructed using a ZAP Express cDNA Gigapack III Gold cloning kit (Stratagene). The resultant cDNA library contained 7.0×10^6 recombinant rhambda phages, and was amplified to 7.6×10^8 plaque-forming units (pfu) /ml.

Preparation of cDNA probe

Poly (A)$^+$ RNA (0.5 μg) from the ultimobranchial gland was reverse-transcribed in 20 μl buffer containing 80 pmol of oligo-dT19 primer, 1 mM dNTP, 20 units RNase inhibitor (Toyobo, Osaka, Japan), and 10 units Rous-associated virus 2 (RAV-2) reverse transcriptase (Takara), as described previously (Suzuki *et al*., 1992). Polymerase chain reaction (PCR) was subsequently carried out with the sCT primers (5'-CAAGCGTTGCTCCAACCTCA, 20-mer; and 5'-CGTTTCTTGCCAGGCGTGCC, 20-mer) (Fig. 1a), using a Program Temp Control System, PC-701 (Astec, Fukuoka, Japan). The amplification profile for 30 cycles was dissociation at 94°C for 1.5 min, annealing at 55°C for 1.5 min, and extension at 72°C for 2.5 min. The final cycle included polymerization for 8 min. Amplification products were separated by electrophoresis, and a major band was subcloned into pGEM-3Z vector (Promega, Madison, WI, USA). Sequencing reactions were conducted with a thermo sequenase cycle sequencing kit (USB, Cleveland, OH, USA), and nucleotide sequences were analysed using a Li-Cor automated DNA sequencer model 4200L-2G (Li-Cor, Lincoln, NE, USA). A DNA probe was synthesized from a cDNA fragment encoding sCT-I, using a digoxigenin (DIG)-high prime kit (Roche Diagnostics GmbH, Mannhaim, Germany).

DNA cloning and sequence analysis

Approximately 18,000 recombinants from the amplified cDNA library were screened by plaque hybridization. Hybridization with the above cDNA probe and post-hybridization washing were performed, according to the manufacturer's instructions. Hybridization signals were detected with 25mM CSPD, a 1, 2-dioxetane chemiluminescent enzyme substrate (Tropix, Bedford, MA, USA), on Hyperfilm-ECL film (Amersham Biosciences, Piscataway, NJ, USA) after incubation with alkaline phosphatase-conjugated anti-DIG antibody (Roche). The pBK-CMV phagemid vectors with inserts were excised *in vivo* from the ZAP express vectors of positive recombinants, using the ExAssist helper phage (Stratagene). The nucleotide sequences of these DNAs were analysed using a Li-Cor automated DNA sequencer.

In situ hybridization histochemistry

Antisense and sense RNA probes of 113 bases (Fig. 1a) were prepared by *in vitro* transcription from the CT-encoding region of rainbow trout sCT-I cDNA, using a DIG RNA labelling kit (Roche). The procedure of whole-mount *in situ* hybridization for rainbow trout embryos and alevins was established based on several protocols for mammalian (Wilkinson, 1992) and zebrafish embryos (http://www.shigen.nig.ac.jp:6070/zf_info/zfbook/chapt9 /9.9.html). Briefly, fixed embryos and alevins were digested with 10 μg/ml proteinase K for 10 min (15 dpf and 18 dpf embryos) or 20 min (21 dpf and 24 dpf alevins), refixed in 0.2% glutaraldehyde/4% PFA for 20 min, and then incubated at 65°C overnight with the hybridization buffer containing 1 μg/ml DIG-labelled cRNA. The composition of the buffer was 50% formamide, 5x SSC, 50 μg/ml yeast tRNA, 40 μg/ml heparin, and 1% SDS. After hybridization, the specimens were washed twice in 50% formamide/5x SSC/1% SDS at 65°C for 30 min each time, then treated twice with 20 μg/ml RNase A at 37°C for 30 min each time, and further washed twice in 50% formamide/2×SSC at 65°C for 30 min each time. For signal detection, they were incubated with anti-DIG antibody coupled to alkaline phosphatase (Roche) at a 1:2,000 dilution at 4°C overnight. The antibody had been preadsorbed with homogenization powders from rainbow trout alevins. The specimens were then stained with NBT (nitroblue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolylphosphate) (Roche). Digital images were recorded with a Leica MZ95 stereomicroscope (Leica, Vienna, Austria) equipped with a Polaroid PDMC/OL CCD camera system (Polaroid, Cambridge, MT, USA). Some specimens were embedded in paraplast wax, and 6 μm sections were cut for observation at the cellular level. As for the hybridized gills, 20 μm sections were prepared using Technovit 7100 resin (Kulzer and Co., GmbH, Wehrheim, Germany). *In situ* hybridization for tissue sections was carried out, as previously described (Suzuki *et al*. 1997).

Western blot analysis

Homogenate proteins were prepared from the ultimobranchial gland, as described previously (Suzuki *et al.,* 1999). The proteins and sCT peptide (Bachem, Bubendorf, Switzerland) were denatured at 100°C for 3 min in a modified buffer (Takata *et al.,* 1990), and electrophoresed on a 16.5% T/3% C SDS-polyacrylamide gel in Tris/tricine buffer, according to the methods by Schagger and von Jagow (1987). Electroblotting and signal detection was carried out as described previously (Suzuki *et al.,* 1997). Blotted polyvinylidene fluoride membranes (Immobilon, Millipore, Tokyo Japan) were incubated with rabbit anti-sCT-I antibody, a gift from Prof. K. Wakabayashi, Gunma University, Japan, at a 1:5,000 dilution.

Immunohistochemistry

The peroxidase-antiperoxidase method was applied for the detection of CT. Serial sections (6 μm) were pretreated with 0.3% H_2O_2 for 30 min, and washed in deionized water and PBS. They were then incubated overnight with the anti-sCT-I antibody at a

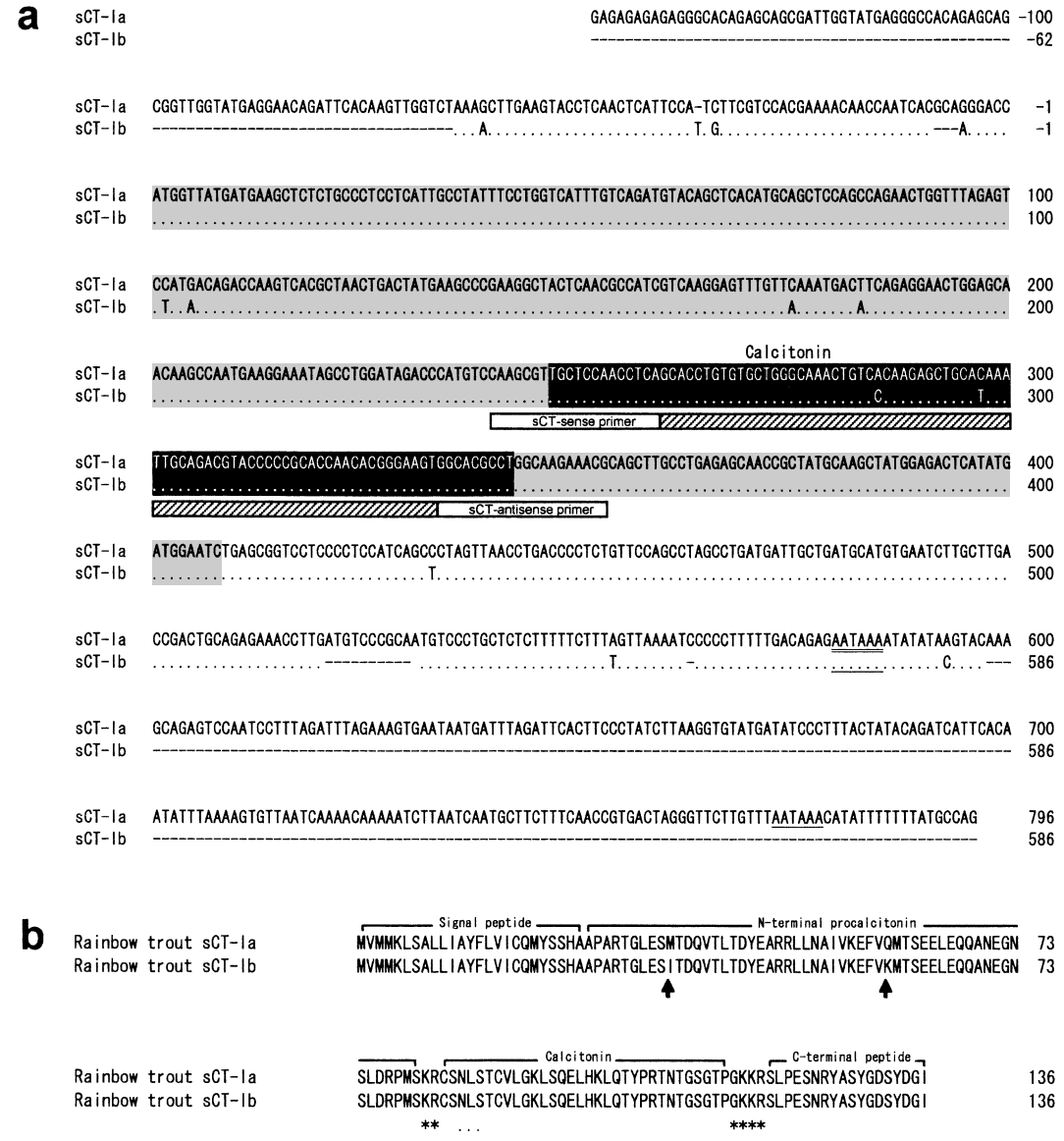

Fig. 1. Nucleotide sequences of sCT-Ia and -Ib cDNAs from the rainbow trout (a), and their deduced precursors (b). (a): The coding region for sCT precursors is indicated by gray boxes and a black box that specifies the sCT-encoding sequences. The positions of the sCT primers, utilized for initial DNA cloning, are shown under the sequences. The cRNA probes, used for *in situ* hybridization histochemistry, were prepared from the internal portion of sCT-Ia cDNA, which is indicated by a bar encompassing the sCT primer regions. Gaps marked by hyphens (–) are inserted to optimize homology. Dots represent the nucleotides identical with those of sCT-Ia cDNA. Nucleotides are numbered from 5' to 3', beginning with the first base in the coding region. The AATAAA polyadenylation signals are underlined, and a potential polyadenylation signal is double underlined. (b): The sCT-Ia and –Ib precursors are identical except for two amino acid residues (arrows). Asterisks denote potential signals for proteolytic processing, and full points indicate a sequence for potential N-linked glycosylation.

1:8,000 dilution. After washing in PBS, the sections were incubated with the bridge antiserum of goat anti-rabbit IgG antibody (Dako, Kyoto, Japan) at a 1:200 dilution for 2 h. Peroxidase-conjugated swine anti-rabbit IgG (Dako) was then applied, as the third antibody, at a 1:200 dilution for 1.5 h, and visualized with diaminobenzidine solution (15 mg/100 ml 0.05 M Tris/HCl buffer, pH7.6) including H_2O_2 (16.7 μl/100 ml). Immunohistochemical controls, in which the primary antibody was omitted, did not show positive staining, confirming the specificity. Other sets of serial sections were stained with hematoxylin and eosin. Digital images were recorded with an Olympus BX60 microscope equipped with a Polaroid PDMC Ie CCD camera system (Polaroid).

RESULTS

Molecular cloning of sCT cDNA

There was a distinct cDNA band of approximately 100 bp observed after gel electrophoresis of RT-PCR products, which had been amplified from the ultimobranchial gland mRNA using the sCT primers. After isolation and subcloning of the cDNA fragment, the nucleotide sequences coding for sCT-I were determined by sequence analysis. The sCT-I-encoding fragment was then used as a probe to screen 18,000 recombinants from the rainbow trout cDNA library.

About 1,080 positive CT clones were detected, and sequence analysis revealed the complete nucleotide sequences of two forms of sCT-I (sCT-Ia and -Ib) cDNAs (Fig. 1). In total, 22 positive clones were sequenced, and sCT-Ia and -Ib cDNAs were found in four clones and three clones, respectively.

Rainbow trout sCT cDNA and precursor

The rainbow trout sCT-Ia (Accession number: AB094135) and -Ib cDNAs (Accession number: AB094136) consisted of 944 and 648 bases, respectively, excluding the poly(A) tail (Fig. 1a). Both the cDNAs were predicted to encode an N-terminal peptide of 80 amino acid residues, a putative cleavage site Lys-Arg, sCT-I, a cleavage and amidation sequence Gly-Lys-Lys-Arg, and a C-terminal peptide of 18 amino acids, and the precursors shared a 99% amino acid identity (Fig. 1b). The N-terminal peptide may be cleaved into a signal peptide of 25 amino acids and an N-terminal procalcitonin, by analogy with other teleost N-terminal peptides (Suzuki *et al.,* 1998) and human preprocalcitonin (Russwurm *et al.,* 1999). The rainbow trout sCT-I conserved a sequence for potential N-linked glycosylation (Asn88-Leu89-Ser90), although the biological significance of CT glycosylation has yet to be determined (Fig. 2).

Histological analysis of the ultimobranchial gland

Development of the ultimobranchial gland was first examined by traditional histochemical methods with hema-

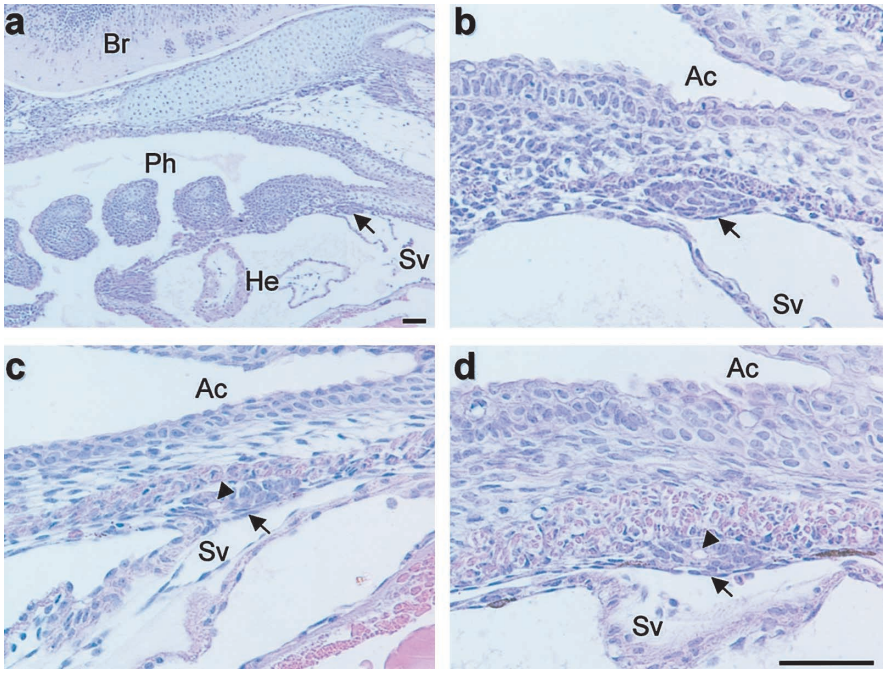

Fig. 2. Embryonic ultimobranchial gland in the parasagittal sections of rainbow trout embryos at 17 dpf (a, b) and 20 dpf (c), and of the alevin at 24 dpf (d), stained with hematoxylin and eosin. The ultimobranchial gland (arrows) is seen just caudal to the pharynx (Ph), characterized by the branchial arches and pouches, (a) and is positioned between the alimentary canal (Ac) and sinus venosus (Sv) (b, c, d). Folliculogenesis (arrow heads) has begun in this gland at 20 dpf (c). Presumptive ultimobranchial glands could not be localized at earlier stages. Br, brain; He, heart. Anterior is left. The scale bar = 50 μm.

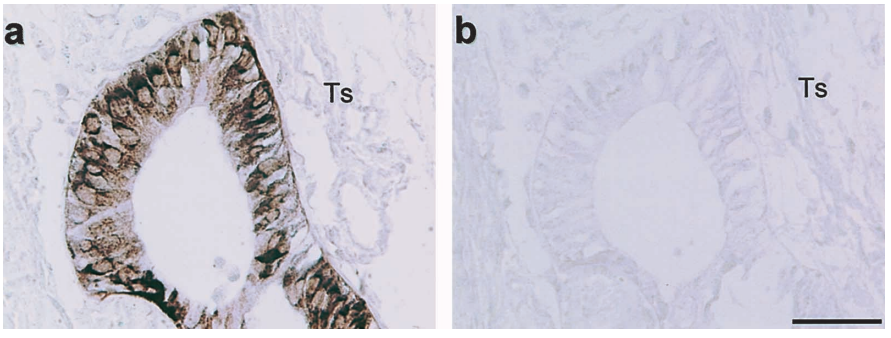

Fig. 3. *In situ* hybridization histochemistry for sCT-I mRNA in wax sections (6 μm) of the ultimobranchial gland of adult trout. (a): Cells in the ultimobranchial gland show an intense hybridization signal with the DIG-labelled antisense cRNA probe. (b): Control section to (a). No signal is seen by hybridization with the sense probe. Ts, transverse septum. The scale bar = 50 μm.

toxylin and eosin. Distinct early rudiments of the ultimobranchial gland were observed 17 days postfertilization (dpf), as two disk-like cell masses posterior to the pharynx (Fig. 2a, b). They were located bilaterally close to the sagittal plane, facing the sinus venosus just beneath the alimentary canal. Both the location and overall structure of the ultimobranchial gland appeared to be relatively unchanged from 17 dpf to 24 dpf (3 days posthatch) (Fig. 2), but follicle-like structures became visible from 20 dpf (one day before hatching) (Fig. 2c, d).

The sCT-I gene expression was then investigated by whole mount *in situ* hybridization histochemistry with a DIG-labelled antisense RNA probe (Fig. 1a). Specificity of the probe was confirmed by detecting a strong signal throughout the ultimobranchial gland of the adult trout (Fig. 3a). This probe is considered to detect both sCT-Ia and –Ib mRNAs because the DNA region, from which the RNA probe was synthesized, has a 98% nucleotide identity between the sCT-Ia and -Ib cDNAs. The whole mount *in situ* hybridization detected a pair of weak signals under the alimentary canal,

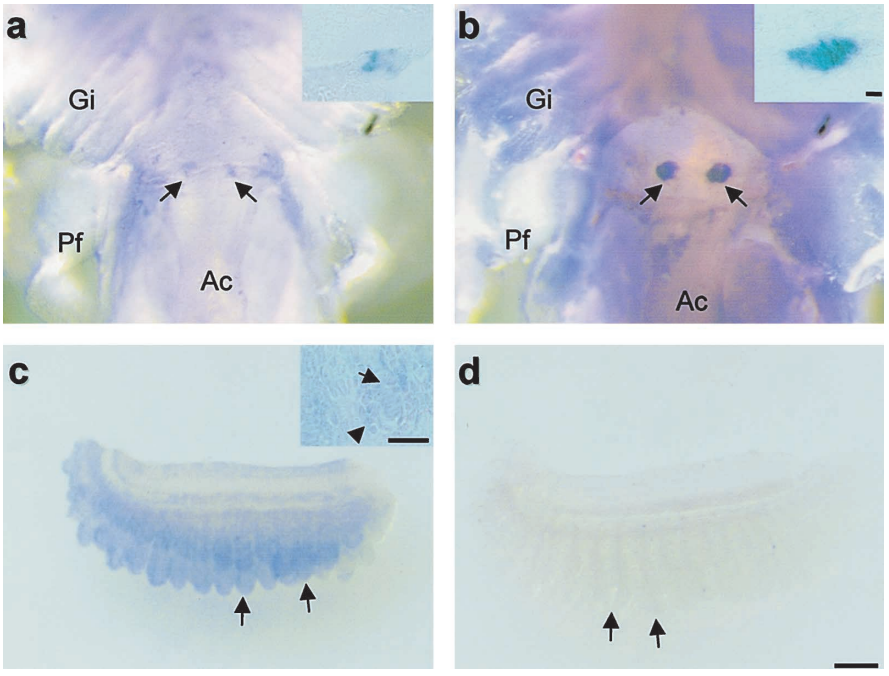

Fig. 4. Expression of sCT-I mRNA in the developing ultimobranchial gland (a, b) and gill filaments (c) of rainbow trout, by whole mount *in situ* hybridization. (a): Ventral view of the hybridized 18 dpf embryo shows the earliest signal in a pair of the ultimobranchial glands (arrows) sited on the alimentary canal (Ac), and a parasagittal section of this embryo (inset) reveals that the signal originates in only some cells of the ultimobranchial gland. (b): The hybridization signal from the ultimobranchial gland (arrows) intensifies in the 24 dpf alevin, and a parasagittal section (inset) shows the signal coming from all the cells of this gland. Gi, gills; Pf, pectoral fin. Anterior is up. (c): The gill, dissected out of the hybridized 24 dpf alevin, shows distinctive signals over the elongating gill filaments (arrows). A parasagittal section (inset) of the distal part of gill filaments shows the signal to occur in all the cells of each gill filament, including the epithelial cells (arrowhead) and central cord cells (arrow). (d): Control gill to (c). The gill from the 24 dpf alevin hybridized with the sense probe exhibits no distinct signal. The scale bar to whole mounts = 100 μm; the bar to insets of (a) and (b) = 10 μm; the bar to the inset of (c) = 50 μm.

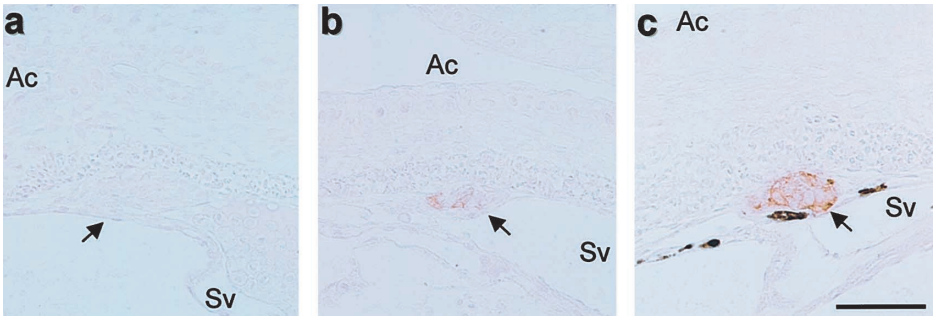

Fig. 6. Production of sCT-I peptide in the developing ultimobranchial gland of rainbow trout, by immunohistochemical staining of wax sections (6 μm). No sCT-I immuno-labelling is seen in the ultimobranchial gland (arrow) of the 18 dpf embryo (a), but immunoreactive sCT-I appears in some cells of this organ (arrow) at 19 dpf (b). The number of immunoreactive cells and the intensity of the immuno-labelling have increased in the ultimobranchial gland (arrow) of the 24 dpf alevin (c). Ac, alimentary canal; Sv, sinus venosus. Anterior is left. The scale bar = 50 μm.

just caudal to the gills, in 18 dpf embryos (Fig. 4a). Paraffin sectioning of the 18 dpf whole mounts revealed that the hybridization signals for sCT-I mRNA were scattered within the ultimobranchial gland and distributed only in some population of the cells (Fig. 4a, inset). Three in five embryos at 18 dpf showed sCT-I-positive signals in this organ, suggesting a developmental variation in the onset of sCT-I gene expression in the ultimobranchial gland. Thereafter, both the number of cells showing the hybridization signal and the signal intensity in this organ increased progressively with age (Fig. 4b).

To localize sCT-I peptide, a polyclonal antibody was applied for immunohistochemistry. Specificity of the antibody was assessed by Western blot analysis. As shown in Fig. 5 (lane 1), the anti-sCT antibody recognized sCT-I peptide as a prominent band of 3.5 kDa. This band was also detected intensely in the homogenate of trout ultimobranchial gland (Fig. 5, lane 2), indicating that this antibody detects sCT-I, but not its precursor. Immunohistochemical staining of wax sections with this antibody exhibited no labels until 19 dpf (Fig. 6a), at which time sCT-I immunoreactivity was observed in some cells of the ultimobranchical gland (Fig. 6b). As ontogeny proceeded, the sCT-I-positive cells increased in both number and stainability (Fig. 6c), which agrees with the expression pattern of the sCT-I mRNA (Fig. 4a, b).

As for tissues other than the ultimobranchial gland, hybridization signals for sCT-I mRNA were observed in the developing gill filaments. As the gill filaments just emerged from the pharyngeal arches, distinctive signals were not detected in these sites. However, the signals became evident by 24 dpf (3 days posthatching), when the gill filaments elongated (Fig. 4c). Sectioning of the hybridized 24 dpf alevins displayed that the signal for sCT-I mRNA was distributed over the epithelial cells of each gill filament and in the distal part of the central cell cord running through the filament. (Fig. 6a, inset). However, no immunoreactive sCT-I was detectable in these cells (data not shown).

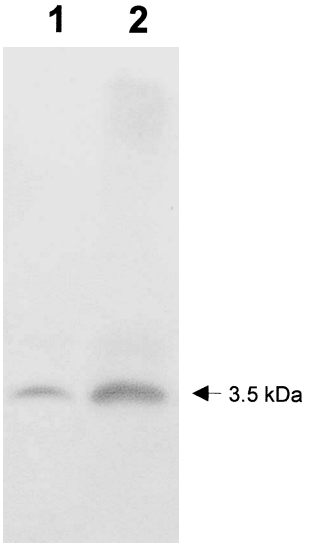

Fig. 5. Western blot analysis with a rabbit anti-sCT-I antibody for sCT-I peptide (2 μg: lane 1) and the proteins prepared from the ultimobranchial gland of adult rainbow trout (10 μg: lane 2). After separated on 16.5% T/3% C SDS-PAGE, a prominent band of 3.5 kDa was immuno-detected in both lanes, confirming the specificity of this antibody and the production of sCT-I peptide in the ultimobranchial gland.

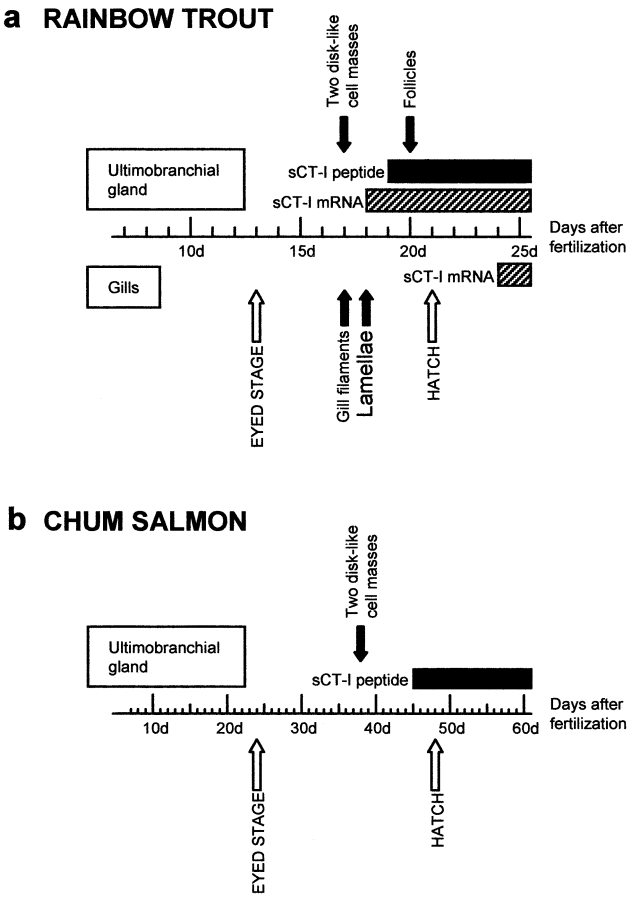

Fig. 7. Diagram showing the differentiation events concerning the sCT-I-producing cells during development of the rainbow trout (a) and chum salmon (b). The immunohistochemical data for chum salmon sCT-I (b) are cited from Sasayama (1989).

Histological analysis of chum salmon embryos and alevins with hematoxylin and eosin stainings also showed that a pair of primitive ultimobranchial glands occurred at 38 dpf (10°C) and developed in a similar manner to the rainbow trout (data not shown). All the results concerning the rainbow trout and chum salmon are summarized in Fig. 7.

DISCUSSION

In the present study, two distinct cDNAs encoding sCT-I were cloned from the ultimobranchial gland of rainbow trout. The cloned sCT-Ia cDNA was 296 bases longer than sCT-Ib cDNA, but shorter transcripts for sCT-Ia precursor might be generated by use of a potential polyadenylation signal at positions 580–585 in the 3' non-coding region (Fig. 1a), as in the pink salmon that produces two sCT-I mRNA

species by alternative polyadenylation (Martial *et al.,* 1994). The initiation site for translation was predicted to be ATG at positions 1 to 3 in each cDNA (Fig. 1) because the sequence ACCATGG at positions -3 to 4 was identical with the consensus sequence for initiation sites in other eukaryotic genes (Kozak, 1981).

The present study surveyed both the expression of sCT-I mRNA and the synthesis of sCT-I peptide over the whole bodies of rainbow trout embryos and alevins, by whole mount *in situ* hybridization and immunohistochemistry. Hybridization signals for sCT-I mRNA were observed in a pair of the ultimobranchial glands and in the growing gill filaments. In mammals, it is well known that the ultimobranchial body develops from the ventral portion of the last pharyngeal pouches (Moore and Persaud, 1993; Manley and Capecchi, 1995), and it is therefore speculated that fish ultimobranchial gland may be derived in a similar manner (Copp, 1969). However, the present histological analysis could not localize the site from which the anlage of this gland is formed in early trout embryos.

In the trout ultimobranchial gland, sCT-I mRNA was first detected in 18 dpf embryos, and immunoreactive sCT-I was visible from 19 dpf. It is well documented that CT is synthesized and secreted by the ultimobranchial gland or thyroid C-cells after proteolytic processing of a larger precursor molecule (Sexton *et al.,* 1999; Pondel, 2000). Accordingly, the present molecular analysis has clarified the amino acid sequence of rainbow trout CT precursor in which the sCT-I portion is flanked by two putative cleavage sites: Lys-Arg and Gly-Lys-Lys-Arg, like other CT precursors (Clark *et al.,* 2002). In mammals, it is suggested by immunohistochemical staining that either prohormone convertase 2 (PC2) together with PC1 or PC2 alone are involved in the cleavage of procalcitonin in the C-cells (Tomita, 2000; Kurabuchi and Tanaka, 2002). Although neither PC1 nor PC2 have yet been cloned in the salmonids, homologous convertases seem to occur in the trout ultimobranchial gland just before or at 19 dpf, contributing to the production of sCT-I in this site.

The present conventional histochemical staining showed that in chum salmon embryos, the primitive ultimobranchial gland began to be formed at 38 dpf (10°C), and developed in a similar fashion to the rainbow trout. It is reported that immunoreactive sCT-I occurs in the ultimobranchial gland of chum salmon from 45 dpf (three days before hatching) onwards (Sasayama *et al.,* 1989). Although the developmental stages of rainbow trout have not been fully compared with those of chum salmon (Vernier, 1969), ontogeny of the ultimobranchial gland basically seems similar for these two species. It is possible that the developmental characteristics of the ultimobranchial gland, observed in the rainbow trout, might be also seen in all salmonid fish.

In teleosts, active sites of calcium deposition are considered to be the skeletal bone and scales (Sasayama, 1999), and it is reported that sCT stimulates the growth of these tissues in a cichlid fish (Wendelaar Bonga and Lammers, 1982). CT also suppresses osteoclastic activities in the scales of other fishes (Suzuki *et al.,* 2000a). In the rainbow trout, however, neither hard bones nor scales appeared to be formed during the present observation period, suggesting that the commencement of sCT production is not directly involved in the formation of hard tissues. On the other hand, recent reports have indicated that CT may have various roles in embryonic/foetal development (Pondel, 2000). For example, an *in vitro* study demonstrated that CT can increase avian embryonic cartilage growth primarily by promoting cartilage maturation through accelerating chondrocyte hypertrophy and matrix formation (Burch, 1984). In addition, CT receptor (CTR) mRNAs are expressed in most tissues of adult mammals (Kuestner *et al.,* 1994; Sexton *et al.,* 1999), and an embryological study utilizing transgenic mice with a 4.9-kb human CTR promoter/beta-galactosidase (LacZ) construct exhibits that CTR gene is expressed in various tissues such as the brain, spinal cord, muscle, and skin, during ontogeny (Jagger *et al.,* 2000). Similarly, a possible CTR mRNA is shown to exist in a number of tissues from a teleost (Suzuki *et al.,* 2000b). Although there is no direct evidence, it is possible that sCTR might occur in various tissues during ontogeny of rainbow trout, and that sCT, secreted from the ultimobranchial gland, might subserve a role in these tissues.

The present study showed hybridization signals for sCT-I mRNA in the gill filaments at 24 dpf, but the immunohistochemical staining failed to detect clear labeling for sCT-I peptide in these sites. One possible explanation for this discrepancy could be that sCT-I is secreted in very small amounts and/or via a rapid vesicular transport pathway. In murines, ectopic CT production is reported in uterine epithelial cells during the early stages of pregnancy (Ding *et al.,* 1994). Furthermore, blastocyst differentiation can be accelerated through receptor-mediated Ca signaling by CT treatment *in vitro*, suggesting a local function of the ectopic CT (Wang *et al.,* 1998). As mentioned above, in mice various tissues express CTR mRNA during ontogeny (Jagger *et al.,* 2000), and it is also shown using a HEK293 cell line stably expressing CTR1a isoform that CT can induce activation of the mitogen-activated protein kinase (MAPK) pathway (Chen *et al.,* 1998), which could mediate multiple cellular functions such as differentiation, transformation, and proliferation (Tanoue and Nishida, 2003). Because the specific binding of CT is detected in trout gills (Ariot-Bonnemaines *et al.,* 1983), sCT-I, produced in the developing gill filaments, might have some autocrine or paracrine actions.

Our failure to detect sCT-I immunoreactivity in the trout gills could be also interpreted as evidence that procalcitonin (proCT) is produced in these sites, because the anti-sCT antibody used in this study does not seem to recognize the pro-sCT-I molecule, judging from the results of the Western blot analysis (Fig. 5). In the last decade, numerous clinical studies have shown that blood concentrations of proCT are increased in systemic inflammation and sepsis (Russwurm *et al.,* 1999; Whicher *et al.,* 2001). Unexpectedly, proCT levels were elevated even in septic, thyroidectomized patients

(Assicot *et al.,* 1993), suggesting that the majority of proCT are secreted from cells other than thyroid C-cells. Whereas blood mononuclear cells and the liver are candidate sites of origin of proCT in mammals (Russwurm *et al.,* 2001; Whicher *et al.,* 2001), rainbow trout pro-sCT-I might be secreted from the gills. However, the physiological role of proCT is not yet known in fish.

Our *in situ* hybridization analysis revealed that the ultimobranchial gland of rainbow trout begins to express sCT-I mRNA at 18 dpf, and its expression levels increased drastically as ontogeny proceeded. Nothing is, however, known about the molecular mechanisms for initiating and intensifying sCT gene expression in this site. Our elucidation of the sCT-I cDNAs has now made it possible to identify the whole sCT-I genes of the trout and determine whether homologous enhancer elements occur in the 5'-flanking DNA of these sCT genes. In mammals, luciferase reporter assay with HeLa and 44-2C cell lines revealed that an 18-bp element (5'-GGCAGCTGTGCAAATCCT), located approximately 1 kbp upstream of the transcriptional initiation site of rat CT gene, has a strong enhancer activity (Tverberg and Russo, 1993). A nearly identical sequence to this element is also present in the human CT/CGRP enhancer region (Braod *et at.,* 1989; Peleg *et al.,* 1990). These distal enhancers contain potential binding elements for helix-loop-helix (HLH) protein and octamer-binding protein designated OB2. It is suggested (Lanigan and Russo, 1997) that the major HLH complex can be a heterodimer of the ubiquitous upstream stimulatory factor-1 and -2. Although OB2 protein has not yet been cloned, mobility shift competition assays indicate the existence of this factor in the CT/CGRP-producing CA77 and 44-2C cells, but not in non-C-cell lines such as HeLa, GH3, and Rat-1, suggesting that OB2 protein is a cell-specific activator. Therefore, OB2-like protein might occur in the ultimobranchial gland of rainbow trout by 18 dpf, enhancing sCT gene expression.

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