Biochemical and Biophysical Research Communications **276,** 258–263 (2000)
doi:10.1006/bbrc.2000.3410, available online at http://www.idealibrary.com on IDEAL®

Cloning, Functional Characterization, and Expression of Thyrotropin Receptors in the Thyroid of Amago Salmon (*Oncorhynchus rhodurus*)

Yuichi Oba,* Toshiaki Hirai,†,‡ Yasutoshi Yoshiura,*
Tohru Kobayashi,* and Yoshitaka Nagahama*,[1]

**Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan; and*
†Department of Biosciences, and ‡Biotechnology Research Center, Teikyo University of Science and Technology, Uenohara, Yamanashi 409-0193, Japan

Received August 2, 2000

Two thyrotropin receptor cDNAs (sTSH-Ra and sTSH-Rb) were cloned from thyroid tissue of the amago salmon, *Oncorhynchus rhodurus*. sTSH-Ra and sTSH-Rb showed the highest degrees of sequence homology to mammalian TSH receptors. Functional characterization in COS-7 cells transiently transfected with sTSH-Ra or sTSH-Rb showed the largest increase in cAMP when exposed to bovine TSH. RT-PCR analysis demonstrated that sTSH-Ra and sTSH-Rb were expressed in the basibranchial region, but not in the ovary, testis, liver, kidney or brain. *In situ* hybridization revealed that sTSH-Ra and sTSH-Rb were exclusively expressed in thyroid follicular epithelial cells of amago salmon undergoing smoltification. These results indicated that the cloned cDNAs encode functional TSH receptor proteins. This is the first report of isolation of TSH receptor molecules from nonmammalian vertebrates.

In teleosts, as in other vertebrates, thyrotropin (TSH) is a key regulator of thyroid function. The hypophysectomy-induced decrease in thyroid function and its reversal by TSH or pituitary substitution have well been documented (1–3). Thyrotropic activity can be stimulated *in vitro* by TSH or pituitary extracts (3, 4). It has also been reported that the pituitary–thyroid axis plays important roles in salmonid smoltification (5) and flounder metamorphosis (6, 7), comparable to its well-known developmental role in amphibian metamorphosis (8).

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under Accession Nos. AB030954 and AB030955.

[1] To whom correspondence should be addressed. Fax: +81-564-55-7556. E-mail: nagahama@nibb.ac.jp.

TSH is a heterodimer, similar to the gonadotropins (FSH and LH in tetrapods; GTH I and GTH II in teleosts), composed of common α subunit and hormone-specific β subunit (9). The TSH β subunit has been cloned in mammals (10), chicken (10), *Xenopus* (11) and also in some teleosts (12–14). The actions of TSH are mediated by its interaction with the TSH receptor on the plasma membrane of thyroid cells. Structurally, TSH, FSH and LH receptors belong to the glycoprotein hormone receptor family (GPHR). The cloning and characterization of the TSH receptors have been reported in some mammals (15). However, TSH receptors have not been identified to date in other vertebrates, despite the importance of TSH actions on thyroid glands in nonmammalian vertebrates.

Here, we describe the isolation and characterization of two amago salmon TSH receptor cDNAs containing the entire TSH receptor coding regions. The abilities of the recombinant sTSH-Ra and sTSH-Rb to generate cAMP were tested by challenging transfected COS-7 cells with various purified glycoprotein hormones. We also examined the tissue distribution and cellular localization of these two clones.

MATERIALS AND METHODS

Experimental animals, preparations, and pituitary hormones. Experimental animals and purified pituitary hormones were described previously (16, 17). Thyroid tissues were removed together with the basibranchial region after sacrificing the fish. For *in situ* hybridization, thyroid tissues were immediately fixed for 24 h at 4°C in 4% paraformaldehyde that was freshly prepared in 0.1 M phosphate buffer, pH 7.2, then equilibrated with 0.1 M phosphate buffer. After dehydration, they were embedded into paraffin.

Isolation of full-length cDNAs and sequencing. Thyroid follicles of most teleosts are typically found scattered in the basibranchial region around the ventral aorta and its branches to the first three gill arches (18–20). Poly(A)$^+$-enriched RNA was extracted from the basibranchial region of two female amago salmon in the pre-

0006-291X/00 $35.00

TABLE 1

Deoxyoligonucleotide Primers

Primer	DNA sequence
1a	5′-GGTCCAGAACCGCCGACATGGCATAGA-3′
1b	5′-GGCACAGGAGTTGAGAGGGTAGAAGAG-3′
2a	5′-GCCAGGATGTTGAGGATAAGGACTGAG-3′
2b	5′-GGGTTGTGCACAGCGCAGTAGATCTTC-3′
3a	5′-CCCGGGCAGAGTACTACAACCACGCCA-3′
3b	5′-CTCCTCCTCATCGCCTCAGTGGACCTT-3′
4a	5′-CTGACGGTGATCACGCTAGAGCGGTGG-3′
4b	5′-GTGTTCGCCAGCGAGCTGTCGGTTTAT-3′
5a	5′-ACTGGCACCATCACAGTACTC-3′
5b	5′-CCAAACCCATCATGGTCCTC-3′
6a	5′-GCCGTGGGCTTCAGGTGAGAAGGT-3′
6b	5′-GAGAAGGAAGCCGTCAGAATCTC-3′
7	5′-AAGAATTCAAAGAGATGACTGAAAACGAC-3′
8a	5′-ATTTGAATTCATGTGTCCAGGCTACGGTTC-3′
8b	5′-TGGGATCCCCCGGCTGAGTTAGGGCTCACG-3′
9a	5′-CACAGCTCTTCAGAAGGCAGACGGTC-3′
9b	5′-GAAGGCAAGCAGTCAGTTCGAAGGGTA-3′

vitellogenic stage (average GSI = 0.6). The degenerate primers were designed using highly conserved regions of seven-transmembrane domains in reported GPHRs (16). The reverse transcription and PCR conditions were the same as those described previously (16). Two different PCR products of approximately 0.7 kb were amplified.

5′-RACE and 3′-RACE were carried out using antisense primers (5′-RACE; Primer 1a or 1b) and sense primers (3′-RACE; Primer 3a or 3b) with Adapter Primer 1 (Clontech) (Table 1). Second nested amplifications were conducted using diluted aliquots of the first reaction as template with the antisense primer (5′-RACE; Primer 2a or 2b) and the sense primer (3′-RACE; Primer 4a or 4b) and Adapter Primer 2. Products of approximately 2.0 kb (5′-RACE) and 2.0 kb or 1.5 kb (3′-RACE) were amplified. PCR products were gel-purified and T-A cloned into the pGEM-T Easy vector.

cDNAs containing the full-length open reading frames (ORF) were generated by PCR using the 3′-end (Primer 5a or 5b) and 5′-end (Primer 6a or 6b) primer sets with high fidelity Pyrobest DNA polymerase (Takara). The resultant clones were named pB-sTSH-Ra and pB-TSH-RB.

RT-PCR for tissue distribution analysis. Total RNA was prepared from the ovary, liver, kidney and brain of a female amago salmon in the late-vitellogenic stage (gonadosomatic index, GSI = 18), and from the testis of a male amago salmon in the spermiation stage (GSI = 5.6) by a method described previously (16). Poly(A)$^+$-enriched RNA from the basibranchial region was utilized as described above. Poly(A)$^+$-enriched RNA for ovaries was prepared from four individual females in the pre-vitellogenic stage (average GSI = 0.6). The prepared RNAs were reverse-transcribed. The primers for specific amplification corresponded to the transmembrane domain (Primer 4a or 4b) or to the 3′-noncoding region (Primers 5a or 5b). The primer sets for sGTH-R and sGTH-RI and the PCR conditions were the same as described previously (16, 17).

Functional expression studies. To confirm that the cloned sTSH-Ra and sTSH-Rb cDNA encoded functional receptors, the entire coding regions were subcloned into the expression vector pSG5 (Amersham Pharmacia Biotech). For functional expression studies, ORFs for sTSH-Ra and sTSH-Rb were subcloned into the expression vector pSG5 (Amersham Pharmacia Biotech) as described previously (16). Briefly, each ORF was amplified by PCR with pB-sTSH-Ra and pB-sTSH-Rb as templates, and Primers 7a or 7b (sense) and Primers 8a or 8b (antisense). Restriction enzyme sites were introduced into the 5′-ends of these primers to allow insertion into pSG5. All nucleotide sequences of the constructs were confirmed by sequencing. Transient transfection and intracellular cAMP assays were conducted as described previously (16).

In situ hybridization. The RNA probe was produced using a DIG RNA labeling kit and DIG RNA labeling mix (Boehringer Mannheim) according to the manufacturer's protocol. As sTSH-Ra and sTSH-Rb were indistinguishable on dot blot analysis (data not shown), *in situ* hybridization studies of sTSH-Ra and sTSH-Rb were performed using the probe for sTSH-Ra. The fragment between nucleotide 68–1162 in sTSH-Ra was subcloned into pBluescript II SK. For antisense and sense probe syntheses, this clone was transcribed *in vitro* with T3 and T7 RNA polymerases, respectively, after linearization by digestion with restriction enzymes. *In situ* hybridization was performed according to a protocol developed by Young *et al.* (unpublished), based on the method of Braissant and Wahli (21). Briefly, the paraffin-embedded tissue was cut into sections 7 μm thick. The sections of the basibranchial region were deparaffinized, then treated with 10 μg/ml of proteinase K (Boehringer Mannheim). Hybridization was performed at 50°C for 22 h with 1 μg/ml of DIG-labeled RNA probe containing 200 μg/ml of tRNA (Boehringer Mannheim). After hybridization, sections were treated with 1 μg/ml of RNase A (Boehringer Mannheim), then incubated for 2 h with alkaline phosphatase-conjugated anti-DIG antibody and blocking reagent (Boehringer Mannheim). Staining was conducted with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim) as chromogen. After staining, sections were fixed in 4% paraformaldehyde and mounted.

RESULTS AND DISCUSSION

Isolation of cDNAs encoding sTSH-Ra and sTSH-R. Two GPHR-like fragments (designated as sTSH-Ra and sTSH-Rb) were isolated from the 0.7-kb RT-PCR product, when the basibranchial region was used as a template. The RACE technique was utilized to clone the full-length cDNAs.

Sequence analyses were performed as described previously (16). The nucleotide sequences of 3758 and 2776 bp clones revealed ORFs of 814 and 793 amino acids with putative signal peptides (28 and 26 amino acids), extracellular domain (435 and 411 amino acids), transmembrane domain (consistent 264 amino acids), and intracellular domain (87 and 92 amino acids), respectively. The extracellular domains for both sTSH-Ra and sTSH-Rb contained 3 identical potential sites for N-glycosylation. Intracellular domains contained 1 (sTSH-Ra) and 3 (sTSH-Rb) potential sites for phosphorylation by protein kinase C (Fig. 1).

Our preliminary analyses of the genomic organization of sTSH-Ra and sTSH-Rb showed the presence of at least one intron in their transmembrane domains (arrowheads in Fig. 1). The transmembrane domains of amago salmon gonadotropin receptor genes (sGTH-RI and sGTH-R) also contain one or two introns at the same site as found in sTSH-Ra and sTSH-Rb (16, 17). In contrast, tetrapod GPHRs (FSH, LH and TSH receptors) have been reported to be free of introns in their transmembrane domains; the introns were found only in their extracellular domains.

One of the sequence differences between mammalian TSH and GTH receptors is the presence of an extra

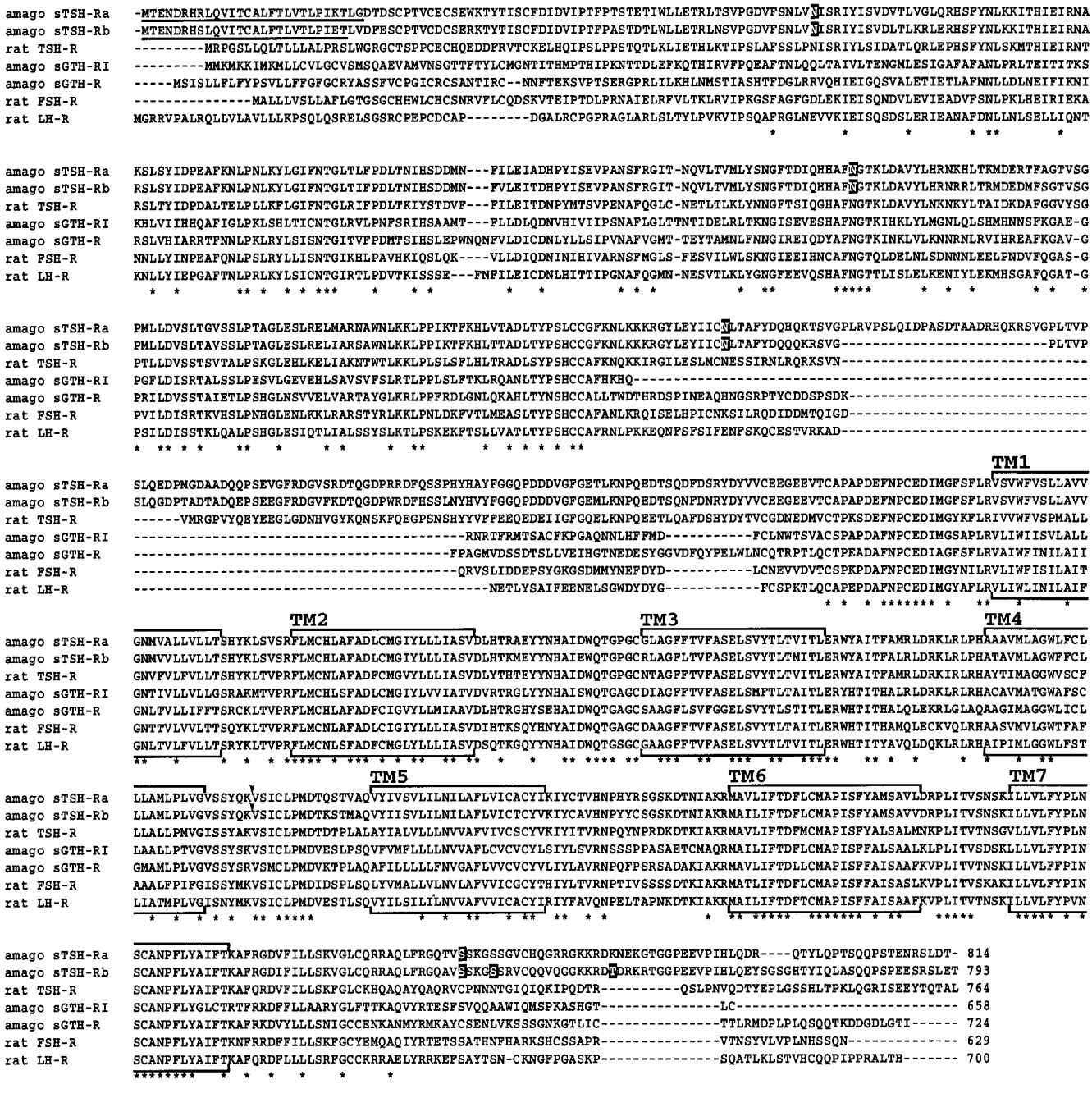

FIG. 1. Aligned amino acid sequences of amago salmon sTSH-Ra and sTSH-Rb with amago salmon GTH receptors (sGTH-RI and sGTH-R), rat FSH and LH receptors (FSH-R and rat LH-R). The numbers on the right refer to the amino acid position. The putative signal peptides are underlined. Potential N-glycosylation sites are indicated by bold italic ***N.*** Potential sites for phosphorylation by protein kinase C are indicated by bold italic ***T*** or ***S.*** Arrowheads indicate the intron site found in the seven-transmembrane domain. Gaps (−) were introduced to optimize the sequence alignment. Asterisks indicate residues conserved in all GPHR members. TM1-7, transmembrane domains 1–7.

amino acid insertion in TSH receptors close to the junction between the extracellular domain and the first transmembrane segment (15, 22). Interestingly, a similar insertion was also observed in both sTSH-Ra and sTSH-Rb (Fig. 1), but not in amago salmon GTH receptors.

Sequence comparison with other GPHRs. The sequence distances for sTSH-Ra to sTSH-Rb and various GPHRs were calculated (Fig. 2A). The overall homology percentages of sTSH-Ra were 87% (sTSH-Rb), 57% (rat TSH receptors, M34842), 44% (amago sGTH-RI, AB030012), 42% (amago sGTH-R, AB030005), 47% (rat

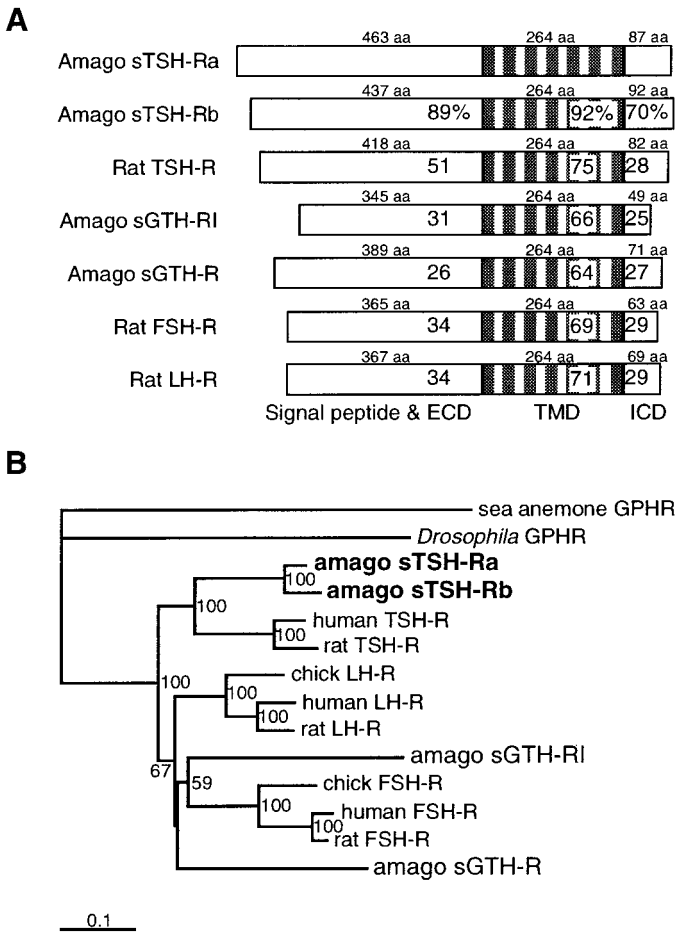

FIG. 2. (A) Domain structure and homology percentages of sTSH-Ra with sTSH-Rb, amago sGTH-RI, amago sGTH-R, and rat GPHRs. The numbers above each box refer to the size of amino acids for each domain. The figures within each box indicate the amino acid percentage homology of domain relative to the sTSH-Ra. (B) Phylogenetic tree of the 14 GPHRs products amino acid sequences of transmembrane domain constructed by the neighbor-joining method. The numbers indicate bootstrap values from 100 replicates. Only bootstrap values of 50% or more are shown. Horizontal lines indicate genetic distances. Sea anemone putative receptor (Z28332); *Drosophila* putative receptor (U47005); human FSH (M65085), LH (S57793), and TSH (M32215) receptors; chicken FSH (U51097), and LH (U31987) receptors.

FSH receptors, L02842), and 48% (rat LH receptors, M26199).

For phylogenetic analysis, only seven-transmembrane domain regions were compared as described previously (16, 17). The analysis revealed that sTSH-Ra and sTSH-Rb were grouped with mammalian TSH receptors with a high bootstrap value of 100% (Fig. 2B). We previously cloned two different GTH receptor cDNAs (probably encoding FSF and LH receptors, respectively) from amago salmon ovarian follicles. The overall sequence homology between these two GTH receptors was 44%. In contrast, sTSH-Ra and sTSH-Rb showed a high degree of homology, 86.1% at the nucleotide level and 87% at the amino acid level, within their ORFs. Southern blotting analysis showed that sTSH-Ra and sTSH-Rb are both single copy genes (data not shown). It is possible that sTSH-Ra and sTSH-Rb evolved due to the tetraploidy within salmonids (23, 24).

Transient expression of the sTSH-Ra and sTSH-Rb cDNAs. Most of the effects of TSH are mediated by cAMP (25). To monitor the functional responses of various ligand effects, we measured the intracellular cAMP accumulation in transfected cells. Although TSH has recently been purified from salmonids, no such preparations have been available for use in physiological experiments. In this connection, it is notable that salmon and bovine TSHs were reported to be equipotent in an *in vivo* TSH bioassay using intact juvenile coho salmon (26, 27). We therefore used bovine TSH in place of homologous TSH. Salmon and bovine gonadotropins were also used. Cyclic AMP measurement of transiently transfected cells showed that sTSH-Ra and sTSH-Rb functionally respond to bovine TSH. The cells transfected with pSG5 vector alone did not respond to any hormone treatment at 5 μg/ml (data not shown). It is of interest that both sTSH-Ra- and sTSH-Rb-transfected cells also responded to bovine FSH and LH, but not to salmon GTH I or GTH II (Fig. 3). These results were in agreement with earlier findings that salmon GTH I and GTH II have no thyrotropic activities in an *in vivo* bioassay using salmon parr (28) and that tetrapod gonadotropins have thyro-

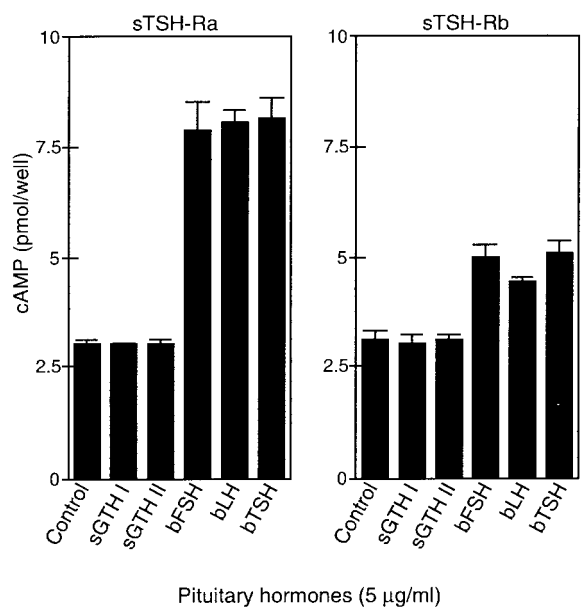

FIG. 3. Cyclic AMP accumulation in COS-7 cells transiently transfected with sTSH-Ra and sTSH-Rb cDNAs. After stimulation with 5 μg/ml of salmon sGTH I, sGTH II, bovine bTSH, bFSH, or bLH for 30 min, intracellular cAMP concentrations were measured by EIA.

tropic activity against teleost thyroid glands (29–33). The cAMP production in sTSH-Rb was less effective than sTSH-Ra, but the potential tendency of the hormones was the same as that of sTSH-Ra (Fig. 3), due probably to the differences in the nature of the receptors or their expression levels in COS-7 cells.

Tissue distribution and cellular expression of sTSH-Ra and sTSH-Rb. Tissue distributions of sTSH-Ra and sTSH-Rb were examined by RT-PCR using gene-specific primers. To distinguish from genomic amplification, at least one intron was included in these genomic regions. Both sTSH-Ra and sTSH-Rb were expressed only in the basibranchial region (Fig. 4). No amplification was detected in the ovary (Fig. 4), testis, liver, kidney or brain (data not shown). Interestingly, sGTH-RI and sGTH-R were also expressed in the basibranchial region (Fig. 4). A LH/CG receptor cDNA has been cloned from human thyroid glands (34). The significance of GTH receptors in thyroid tissue should be investigated further.

The thyroid structure of smolts collected in September appeared to be relatively active, consisting of numerous, small follicles (Figs. 5A and 5B). In precociously mature female parr collected in October, thyroid follicles appeared inactive as evidenced by low numbers of epithelial cells (Figs. 5C and 5D). *In situ* hybridization using sTSH-Ra as a probe produced a strong signal in thyroid follicular epithelial cells in smolts (Fig. 5A). No signals were observed in muscle, blood vessels or cartilage. The same results were obtained when sTSH-Rb was employed as a probe (data not shown).

Under hatchery conditions, some large amago salmon become precociously mature as 1-year-old fish, easily recognized by their dark color, which usually die after spawning with 2-year-old fish. Of the remaining hatchery-reared parr, variable numbers undergo smoltification in autumn and winter. The best hypoosmoregulatory performance coincided with the peak of smoltification assessed by external appearance (35). In this study, abundant TSH receptor mRNA transcripts were found in thyroid epithelial cells of smolts. These results were consistent with our earlier findings that in amago salmon the levels of thyroxine are high in smolts, but are consistently low in precociously mature male parr (35). Taken together, these results provide strong evidence that TSH, acting through it receptor, plays an important role in smoltification in salmonids.

In conclusion, TSH receptor cDNAs were cloned from the amago salmon thyroid. This indicated that TSH and its receptor system had already been established before the divergence of tetrapods and teleosts in the early phylogeny of vertebrates. The availability of salmon TSH receptor cDNAs will facilitate investigation of the actions of TSH in fish thyroid tissue.

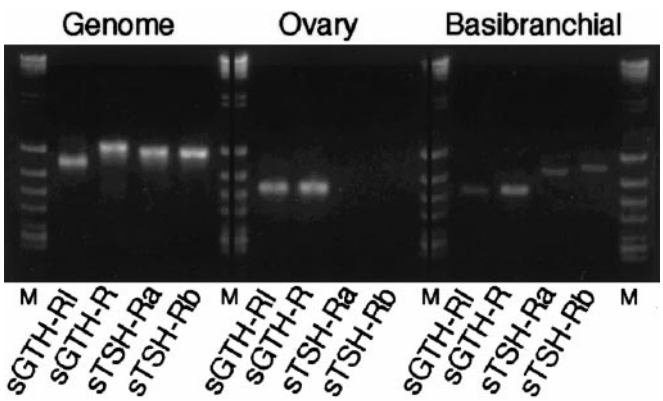

FIG. 4. Determination of the expression of sTSH-Ra and sTSH-Rb, and also of sGTH-RI and sGTH-R transcripts in the ovary and basibranchial region by RT-PCR using specific primer sets. The left panel shows amplification when genomic DNA was used as a PCR template. Expression in the ovary is shown in the middle panel. Expression in the basibranchial region is shown in the right panel. The lanes labeled M show the molecular size marker.

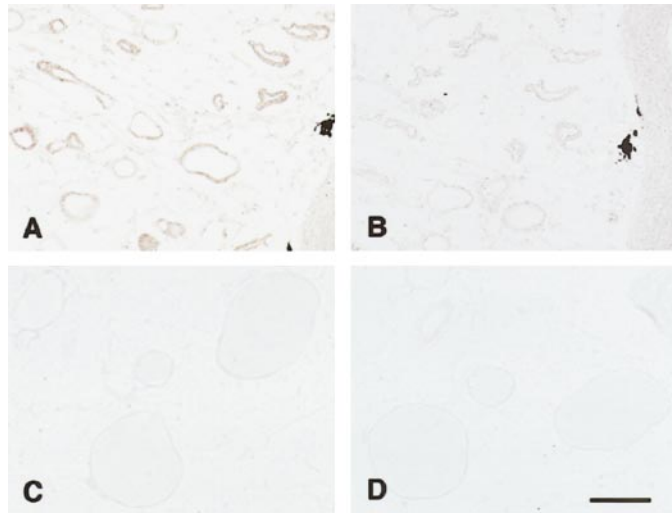

FIG. 5. Localization of sTSH-Ra and sTSH-Rb mRNAs in the basibranchial region of sexually immature female smolts collected in September (A and B) and precociously mature female parr collected in October (C and D). A and C show hybridization to antisense probes. B and D show hybridization to sense probes as negative controls.

ACKNOWLEDGMENTS

We thank Dr. H. Kawauchi, Kitasato University, Japan for providing purified salmon GTH I and GTH II. We are also grateful to Drs. K. Okuzawa and H. Kagawa, National Research Institute of Aquaculture, for providing the amago salmon. This work was supported in part by Grants-in-Aid for Research for the Future (JSPS-RFTF 96L00401) from the Japan Society for the Promotion of Science, for Scientific Research (072831049 and 10440247) and for Advanced Scientific Research on Bioscience/Biotechnology Areas from the Japanese Ministry of Education, Science, Sports, and Culture, Japan, and by a Grant-in-Aid (Bio Design Program) from the Ministry of Agriculture, Forestry and Fisheries, Japan.

REFERENCES

1. Eales, J. G. (1979) *in* Hormones and Evolution (Barrington, E. J. W., Ed.), Vol. I, pp. 341–436, Academic Press, London.
2. Leatherland, J. F. (1982) *Environ. Biol. Fishes* **7,** 83–110.
3. Swanson, P., Grau, E. G., Helms, L. M., and Dickhoff, W. W. (1988) *J. Exp. Zool.* **245,** 194–199.
4. Grau, E. G., Helms, L. M., Shimoda, S. K., Ford, C.-A., LeGrand, J., and Yamauchi, K. (1986) *Gen. Comp. Endocrinol.* **61,** 100–108.
5. Swanson, P., and Dickhoff, W. W. (1987) *Gen. Comp. Endocrinol.* **68,** 473–485.
6. Miwa, S., and Inui, Y. (1987) *Cell Tissue Res.* **249,** 117–123.
7. Inui, Y., Tagawa, M., Miwa, S., and Hirano, T. (1989) *Gen. Comp. Endocrinol.* **74,** 406–410.
8. Rosenkilde, P., and Ussing, A. P. (1996) *Int. J. Dev. Biol.* **40,** 665–673.
9. Pierce, J. G., and Parsons, T. F. (1981) *Annu. Rev. Biochem.* **50,** 127–139.
10. Li, M. D., and Ford, J. J. (1998) *J. Endocrinol.* **156,** 529–542.
11. Gregory, C. C., and Porter, T. E. (1997) *Gen. Comp. Endocrinol.* **107,** 182–190.
12. Ito, M., Koide, Y., Takamatsu, N., Kawauchi, H., and Shiba, T. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6052–6055.
13. Salmon, C., Malchelidon, J., Fontaine, Y.-A., Huet, J. C., and Quérat, B. (1993) *C.R. Acad. Sci. Ser. III* **316,** 749–753.
14. Yoshiura, Y., Sohn, Y. C., Munakata, A., Kobayashi, M., and Aida, K. (1999) *Fish Physiol. Biochem.* **21,** 201–210.
15. Kosugi, S., and Mori, T. (1995) *Endocrinol. J.* **42,** 587–606.
16. Oba, Y., Hirai, T., Yoshiura, Y., Yoshikuni, M., Kawauchi, H., and Nagahama, Y. (1999) *Biochem. Biophys. Res. Commun.* **263,** 584–590.
17. Oba, Y., Hirai, T., Yoshiura, Y., Yoshikuni, M., Kawauchi, H., and Nagahama, Y. (1999) *Biochem. Biophys. Res. Commun.* **265,** 366–371.
18. Gudernatsch, J. F. (1911) *J. Morphol.* **21,** 709–782.
19. Hoar, W. S. (1939) *J. Morphol.* **65,** 257–295.
20. Gorbman, A. (1969) *in* Fish Physiology (Hoar, W. S., and Randall, D. J., Eds.), Vol. 2, pp. 241–274, Academic Press, New York.
21. Braissant, O., and Wahli, W. (1998) *Biochemica* **1,** 10–16.
22. Parmentier, M., Libert, F., Maenhaut, C., Lefort, A., Gérard, C., Perret, J., Van Sande, J., Dumont, J. E., and Vassart, G. (1989) *Science* **246,** 1620–1622.
23. Lim, S. T., Kay, R. M., and Bailey, G. S. (1975) *J. Biol. Chem.* **250,** 1790–1800.
24. Allendorf, F. W., and Thorgaard, G. H. (1984) *in* Evolutionary Genetics of Fishes (Turner, B. J., Ed.), pp. 1–53, Plenum, New York.
25. Dumont, J. E., Vassart, G., and Refetoff, S. (1989) *in* The Metabolic Basis of Inherited Diseases (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., Eds.), pp. 1843–1880, McGraw–Hill, New York.
26. Swanson, P., Dickhoff, W. W., and Gorbman, A. (1987) *Gen. Comp. Endocrinol.* **65,** 269–287.
27. Moriyama, S., Swanson, P., Larsen, D. A., Miwa, S., Kawauchi, H., and Dickhoff, W. W. (1997) *Gen. Comp. Endocrinol.* **108,** 457–471.
28. Swanson, P., Suzuki, K., Kawauchi, H., and Dickhoff, W. W. (1991) *Biol. Reprod.* **44,** 29–38.
29. Fontaine, Y.-A. (1969) *Acta Endocr. Suppl.* **136,** 1–154.
30. Grau, E. G., and Stetson, M. H. (1977) *Am. Zool.* **17,** 857.
31. Grau, E. G., and Stetson, M. H. (1979) *Gen. Comp. Endocrinol.* **39,** 1–8.
32. Milne, R. S., and Leatherland, J. F. (1980) *Comp. Biochem. Physiol.* **66A,** 679–686.
33. MacKenzie, D. S. (1982) *Comp. Biochem. Physiol.* **72A,** 477–482.
34. Frazier, A. L., Robbins, L. S., Stork, P. J., Sprengel, R., Segaloff, D. L., and Cone, R. D. (1990) *Mol. Endocrinol.* **4,** 1264–1276.
35. Nagahama, Y., Adachi, S., Tashiro, F., and Grau, E. G. (1982) *Aquaculture* **28,** 81–90.