

# Differential expression of two somatostatin receptor subtype 1 mRNAs in rainbow trout (*Oncorhynchus mykiss*)

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## Abstract

Somatostatins (SSs) play important roles in the growth, development and metabolism of vertebrates. In this study, cDNAs for two unique somatostatin receptor variants were cloned and sequenced from rainbow trout. The two cDNAs, one consisting of 1755 bp and the other of 1743 bp, share 63.6% identity in nucleotide sequence and 94.1% identity in deduced amino acid sequence and presumably arose through gene duplication. Each cDNA encodes for a putative 371-amino acid somatostatin receptor (one designated sst1A and the other sst1B) containing seven transmembrane domains. Rainbow trout sst1A and sst1B have 64.4 and 65.5% similarity respectively with human sst1 and only 43–60% similarity with other subtypes.

Trout sst1 mRNAs are differentially expressed, both in terms of distribution among tissues as well as in terms of abundance within selected tissues. Both sst1A and sst1B mRNAs were present in brain, stomach, liver, pancreas, upper and lower intestine, pyloric cecum, kidney and muscle, whereas only sst1B mRNA was present in the esophagus. sst1A mRNA was more abundant than sst1B in the optic tectum, whereas sst1B mRNA was more abundant than sst1A in liver. sst1A and sst1B mRNAs were equally abundant in pancreas. These findings contribute to the understanding of the evolution of the SS signaling system and provide insight into the mechanisms that regulate the expression of SS receptors.

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## Introduction

Somatostatins (SSs) are a relatively diverse family of peptide hormones that regulate a wide range of physiological processes in vertebrates, including the modulation of growth, development and metabolism (Sheridan *et al.* 2000). The multifunctional nature of this peptide hormone family arises from an elaborate, multifaceted signaling system that consists of numerous forms of SS molecules that are capable of binding to a variety of receptors. The molecular heterogeneity of the SS family arises from tissue-specific variations in biosynthesis from larger precursor molecules as well as from the existence of multiple SS genes. The different forms of SS seen in mammals such as SS-25 or SS-28 are

N-terminal extensions of SS-14 and result from differential processing of the same precursor, preprosomatostatin I (PPSS I) (Conlon 1989). Several non-mammalian species, including teleost fish, possess multiple PPSSs, each capable of being processed to an SS isoform (Conlon *et al.* 1997, Sheridan *et al.* 2000, Lin & Peter 2001). Rainbow trout, for example, possess a PPSS I that contains the highly conserved SS-14 at its C-terminus (Kittilson *et al.* 1999) as well as two different PPSS IIs, each containing [Tyr<sup>7</sup>,Gly<sup>10</sup>]-SS-14 at their C-termini (Moore *et al.* 1999).

Somatostatins exert their actions through binding to specific plasma membrane receptors. Since 1992, five somatostatin receptor (sst) subtypes, sst1–sst5, have been identified by molecular cloning



of their cDNAs or genes in mammals (Hoyer *et al.* 1995).

The ssts are seven transmembrane domain (TMD) guanine nucleotide binding (G)-protein-coupled receptors; the receptors display subtype-specific selective binding to ligands (Reisine & Bell 1995, Patel & Srikant 1997, Meyerhof 1998). Although SS binding in non-mammalian species has been known for some time (Pesek & Sheridan 1996), details regarding the structural characterization of ssts is just emerging (Lin & Peter 2001). In this study, we used rainbow trout to further understand the polygenic origins of ssts and to provide insight into mechanisms which may serve to regulate their expression.

## Materials and methods

### Experimental animals

Juvenile rainbow trout of both sexes were obtained from Dakota Trout Ranch near Carrington, ND, USA and were transported to North Dakota State University. They were maintained in well-aerated, 800 l circular tanks supplied with recirculated (10% make-up volume per day) dechlorinated municipal water at 14 °C under a 12 h light:12 h darkness photoperiod. Fish were fed to satiety twice daily with PMI AquaMax Grower (Brentwood, MO, USA), except for 24–36 h before experiments. Animals were acclimated to laboratory conditions for at least 4 weeks prior to experimentation.

### RNA extraction

The fish were anaesthetized in 0.05% 2-phenoxyethanol. Selected tissues (e.g. brain, Brockmann body, etc.) were removed, placed in 2 ml microcentrifuge tubes, and immediately frozen on dry-ice. Total RNA was extracted using TRI Reagent (Molecular Research Center, Inc.,

Cincinnati, OH, USA) as specified by the manufacturer. Each RNA pellet was redissolved in 75 µl RNase-free deionized water and quantified by u.v. A<sub>260</sub> spectrophotometry. RNA samples were then stored at –90 °C until further use. Brain and Brockmann bodies (endocrine pancreas) were selected as source material for sequence analysis because of the central importance of these organs to the function of animals and because they are known target organs of SS in fish and mammals (Sheridan *et al.* 2000)

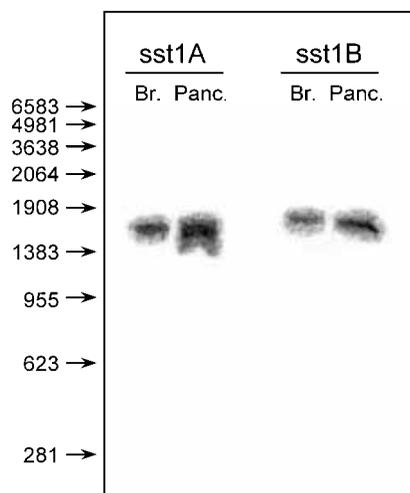
### Oligonucleotide primers and probes

Gene-specific oligonucleotide primers and probes were custom synthesized by Sigma-Genosys (The Woodlands, TX, USA) or supplied with 3'- and 5'-RACE kits (Gibco/BRL, Gaithersburg, MD, USA). Primers were used for reverse transcription (RT) and PCR without further purification. Oligonucleotides were 5'-end labeled with  $\gamma$ -[<sup>32</sup>P]ATP (Amersham) using T4-polynucleotide kinase (Promega) (Sambrook *et al.* 1989). The full-length  $\gamma$ -actin probe (human fibroblast; Gunning *et al.* 1983) was labeled with [<sup>32</sup>P]CTP by random priming (cf. Promega). All radiolabeled probes were purified over Elutip-D columns (Schleicher and Schuell, Keene, NH, USA) as detailed by the supplier.

### Isolation and sequence of somatostatin receptor cDNAs

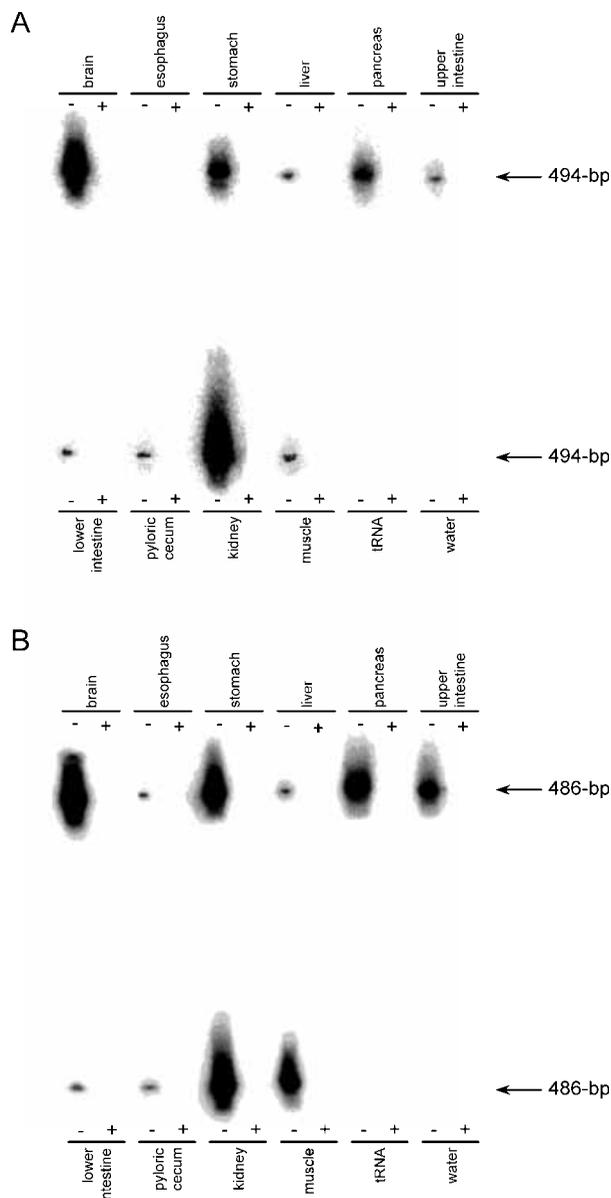
A three-phase approach was adopted for the isolation of selected cDNAs using RT-PCR and rapid amplification of cDNA ends (RACE)-PCR. In phase I, endogenous poly(A)<sup>+</sup> RNA was reverse transcribed from 1 µg trout pancreatic and brain total RNA with Superscript II reverse transcriptase and a 37-nucleotide antisense adapter primer

**Figure 1** Comparison of the cDNA nucleotide and deduced amino acid sequences of rainbow trout sst1A and sst1B. Nucleotides are numbered from 5' to 3' and the amino acid residues (denoted using single-letter abbreviations) are numbered with the start codon in the open reading frame. A gap (indicated by asterisk) has been inserted just prior to the start codon for maximum alignment of the 5' untranslated region. The complete amino acid sequence for sst1A is shown. Amino acid residues of the corresponding sst1B that differ from those of sst1A are shown in boldface type following sst1A residues with a slash. The stop sequence is denoted by '###'. Putative polyadenylation signals are overlined for sst1A and underlined for sst1B. Potential N-linked glycosylation sites are denoted by a □ around the amino acid. A potential site for a palmitoyl anchor is denoted by a ○ around the amino acid. Amino acids that potentially are associated with a disulfide bond are surrounded by a rectangle with rounded corners. Potential phosphorylation sites are denoted with a oval around the amino acid.



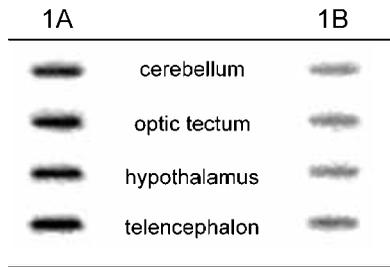
**Figure 2** Northern blot analysis of poly(A)<sup>+</sup> RNA reveals single transcripts for rainbow trout sst1A and sst1B. Approximately 500 ng of poly(A)<sup>+</sup> RNA per lane was size fractionated, electroblotted to a nylon membrane, and then hybridized with gene-specific radiolabeled oligonucleotide probes. Blots were visualized with a Cyclone phosphor imaging system on a type "ST" (supersensitive) screen. BR, brain; Panc., pancreas.

containing 17 thymine bases at its 3' end (Gibco/BRL technical bulletin). Five microliters of the RT reaction were used as template for PCR with a 20-base forward SS receptor gene-specific primer (GSP-1; 5'-GACCGITACSTGGCYGTG GT-3') and a 23-base reverse SS receptor gene-specific primer (GSP-2; 5'-AGGATRGGG TTGGCRCAGCTGTT-3'). Degenerate GSP-1 and GSP-2 primers were designed from previously known mammalian SS receptor sequences in transmembrane domain regions that contained highly conserved nucleotide sequences. Following an initial denaturation cycle of 94 °C for 5 min, 35 PCR cycles were performed, each consisting of 1 min denaturation (94 °C), 1 min annealing (42 °C) and 1 min extension (72 °C). In the last cycle, the extension time was increased to 10 min to ensure complete extension. The resulting PCR product was visualized by electrophoresis on an agarose gel containing 1% (w/v) OmniPur agarose (EM Science, Gibbstown, NJ, USA) and 1% (w/v) NuSieve GTG agarose (BioWhittaker Molecular Applications, Rockland, ME, USA) in 1 × Tris-borate-EDTA (TBE) buffer followed by ethidium bromide staining and u.v. transillumination. Amplified fragments were directly cloned into the pGEM-T Easy Vector (Promega). Positive



**Figure 3** Expression of sst1A (A) and sst1B (B) mRNAs in various tissues of rainbow trout. Total RNA was treated with (+) or without (-) RNase and then subjected to RNA template-specific (RS)-PCR. Resulting products were analyzed by Southern blot hybridization using gene-specific <sup>32</sup>P-labeled oligonucleotide probes. The size of the RS-PCR products matched the size (494 and 486 bp for sst1A and sst1B respectively) predicted from cDNA sequences and primer locations.

colonies were identified by agarose gel electrophoresis of restriction enzyme digests (EcoRI; Promega) of purified plasmid preparations (Ahn *et al.* 2000). Plasmid DNA (75 fmol) was sequenced

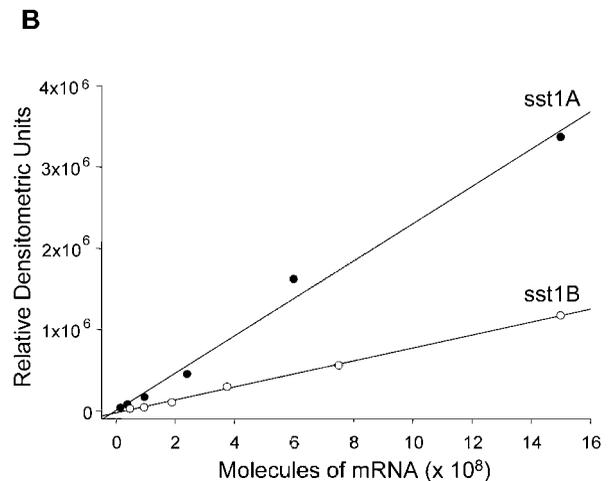
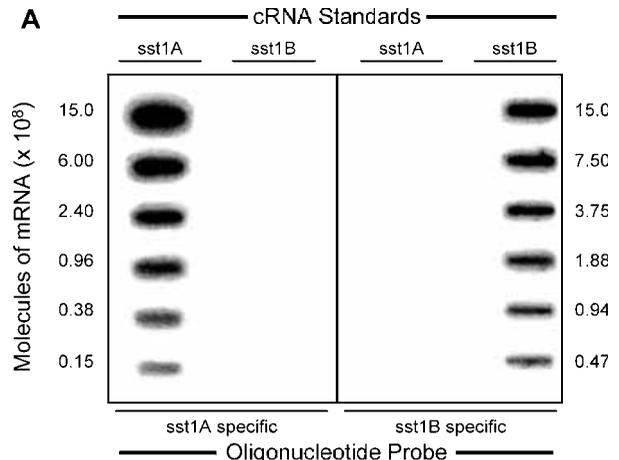


**Figure 4** Northern slot-blot analysis of total RNA isolated from various regions of the brain. Approximately 10  $\mu$ g of total RNA were slotted in duplicate directly onto nylon membrane, hybridized with radiolabeled sst1A- or sst1B-specific oligonucleotide probes and visualized with a Cyclone phosphor imaging system.

with the CEQ 2000 sequencer using the Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's protocol.

In phase II, isolation of the 3' cDNA sequence was accomplished by 3'-RACE PCR (Gibco/BRL technical bulletin). Endogenous poly(A)<sup>+</sup> RNA was again reverse transcribed as described previously; 5  $\mu$ l of the RT reaction was used as template for 3'-RACE PCR with a 23-base sst gene-specific primer (GSP-3; 5'-TGCCCTTCTACATCGTCCAGCTG-3') and the universal amplification primer (Gibco/BRL). PCR was performed as in phase I. The amplified product was identified, cloned and sequenced as described previously.

In phase III, isolation of the 5' cDNA sequence was accomplished by 5'-RACE PCR (Gibco/BRL technical bulletin). Somatostatin receptor mRNA was exclusively reverse transcribed from pancreatic and brain total RNA using GSP-2. The RT reaction conditions were the same as those described previously. The resulting cDNA was purified twice over a Glass Max spin column (Gibco/BRL) to remove unincorporated dNTPs and primer and eluted with 50  $\mu$ l sterile water. One-fifth of the purified cDNA was tailed at the 3' end with dCTP using the enzyme terminal deoxynucleotidyl transferase (Gibco/BRL); 5  $\mu$ l of the tailing reaction mixture was used as template for 5'-RACE PCR with sst GSP-4 (5'-GCGGTAGCGGGCAGCCTTGA-3') and anchor primer (Gibco/BRL). Thirty-five PCR cycles were performed as described in phase I, except that the reaction mixture was heated to 94  $^{\circ}$ C for 4.5 min prior to the addition of Taq DNA polymerase (Qiagen, Chatsworth, CA, USA). The amplified

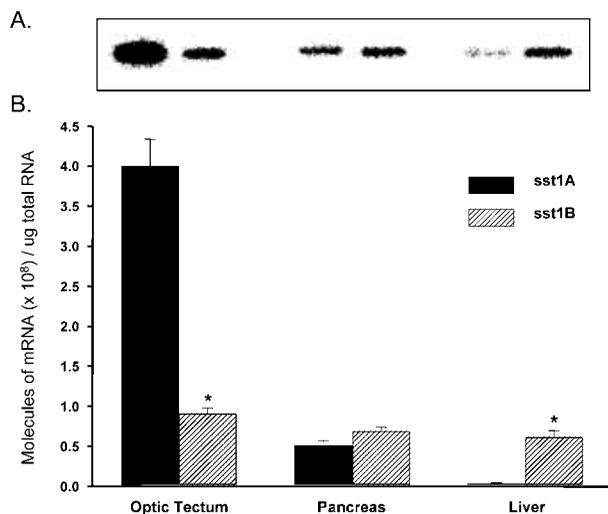


**Figure 5** Quantitative slot-blot analysis for sst1A and sst1B mRNAs. Sample total RNA (*c.* 10  $\mu$ g) and serial dilutions of *in vitro*-synthesized cRNA standards for sst1A and sst1B (A) were slotted in duplicate directly onto nylon membrane, hybridized with radiolabeled sst1A- or sst1B-specific oligonucleotide probes and visualized with a Cyclone phosphor imaging system. Sample RNA was quantitated after correction for background by relation to standards plotted as a function of the number of sst1A or sst1B molecules per microgram of total RNA (B).

product was visualized by agarose gel electrophoresis, cloned and sequenced as described previously.

### Analysis of RNA and DNA

Northern blot analysis (Moore *et al.* 1999) was performed to evaluate the number and size of transcripts as well as to verify that the gene-specific



**Figure 6** Relative abundance of sst1A and sst1B mRNAs in the optic tectum, pancreas and liver of rainbow trout. Sample total RNA was subjected to slot-blot quantitation as shown in Fig. 5. Data are presented as (A) representative slot blots and (B) means  $\pm$  S.E.M. ( $n=8$ ); \* Significant difference compared with the sst1A group within the same tissue ( $P<0.05$ ).

oligonucleotide radiolabeled probes (sst1A(as): 5'-GACAAAAACCGCCACCTCACACAC-3'; or sst1B(as): 5'-CACGAAGACCGCCACAATGCACAA-3') hybridized only with the respective sst1A and sst1B transcripts in poly(A)<sup>+</sup> RNA extracted from the brain and pancreas of rainbow trout.

RNA template-specific PCR (RS-PCR) was used to qualitatively evaluate the expression of sst1A and sst1B mRNAs among various tissues because of its high specificity (amplification of false positives derived from contaminating genomic DNA is excluded) and high sensitivity (Shuldiner *et al.* 1991). A d<sub>26</sub>t<sub>30</sub> primer (5'-CATGTACCTTGATCAACCGTCTCGTGCCAGAGGATRGGGTTGGCRCAGCTGTTRGC-3'), containing 26 bases at its 3' end complementary to both sst1A and sst1B (d<sub>26</sub>) and 30 bases of non-specific tagging sequence at its 5' end (t<sub>30</sub>), was used to co-reverse transcribe sst1A and sst1B mRNA in total RNA (*c.* 15  $\mu$ g) isolated from tissues. Five microliters of the RT reaction were incubated with or without RNase-A and subjected to PCR as previously described (Moore *et al.* 1999) using a gene-specific upstream primer (usst1A: 5'-CCCGCTACCGCCGACCGACTGT-3'; or usst1B: 5'-ACCGCCGTCACACCGTAGCAAAG-3') and a downstream t<sub>30</sub> primer (identical to the t<sub>30</sub> region of the d<sub>26</sub>t<sub>30</sub>

primer: 5'-CATGTACCTTGATCAACCGTCTCGTGCCAG-3'). Following an initial denaturation cycle of 94 °C for 5 min, 39 PCR cycles were performed; each cycle consisted of 1 min denaturation (94 °C), 1 min annealing (65 °C for sst1A and 62 °C for sst1B) and 1 min extension (72 °C), except in the last cycle when the extension time was increased to 10 min. The resulting RS-PCR products were subjected to Southern blot analysis using sst1A(as) or sst1B(as) gene-specific radiolabeled probes (Moore *et al.* 1999).

The amount of sst1A and sst1B mRNA in brain (optic tectum), pancreas and liver was measured using a quantitative slot-blot technique (Moore *et al.* 1999) in which *in vitro*-synthesized cRNA standards were blotted onto nylon membrane along with sample total RNA (10  $\mu$ g). The membranes were hybridized with radiolabeled sst1A(as) or sst1B(as) oligonucleotide probes, washed and visualized with the Packard Cyclone Imaging System. Sample blots were then stripped and rehybridized with a radiolabeled full-length  $\gamma$ -actin probe. Sample RNA abundance was calculated after correction for background and after  $\gamma$ -actin normalization.

## Data analyses

Nucleotide and deduced amino acid sequences were aligned and analyzed with the GeneTool and PepTool sequence analysis programs (BioTools Inc., Edmonton, Alberta, Canada). ClustalX (default parameters) was used in conjunction with the neighbor-joining method (Saitou & Nei 1987) to generate the phylogenetic tree; the tree was visualized with TreeView (Page 1996). Quantitative data are expressed as means  $\pm$  S.E.M. A two-tailed Student's *t*-test was used to estimate differences ( $P<0.05$ ) between treatment groups (Sigma Stat, SPSS Inc., Chicago, IL, USA).

## Results

### Characterization of two somatostatin receptor subtype 1 cDNAs

Two distinct cDNA fragments, each approximately 500 bp in size, were amplified by RT-PCR from total RNA isolated from the brain and Brockmann body (endocrine pancreas) of rainbow trout using two sst-specific primers (GSP-1 and GSP-2). These cDNAs (denoted fragment A and fragment B) had

**sst1 Nucleotide Percent Identity**

Tsst1A	63.6*	62.4	58.7	59.2	70.4	66.2
94.1	Tsst1B	62.7	60.2	58.4	62.1	59.6
62.4	62.9	Gsst1A	92.8	68.4	70.8	66.4
62.1	62.4	98.6	Gsst1B	65.3	71.3	66.3
64.4	65.5	77.7	77.1	Hsst1	88.1	83.2
63.9	65.0	77.1	76.6	98.7	Msst1	96.1
62.5	63.6	75.2	74.7	94.6	95.6	Rsst1

**sst1 Amino Acid Percent Identity**

**Figure 7** Amino acid (lower left) and cDNA nucleotide (upper right) sequence identities of vertebrate sst1 subtypes. Percentage identities were calculated on sequences arranged for maximum alignment using the GeneTool 1.0 analysis program (BioTools, Inc.; Edmonton, Alberta, Canada). Sequences are obtained from: goldfish sst1A (Gsst1A) and sst1B (Gsst1B) (Lin *et al.* 1999a); human sst1 (Hsst1) and mouse sst1 (Msst1) (Yamada *et al.* 1992); rat sst1 (Rsst1) (Meyerhof *et al.* 1991); rainbow trout sst1A (Tsst1A) and rainbow trout sst1B (Tsst1B) (this study). \*When the extreme 5' portion of sst1A and the extreme 3' portion of sst1B is removed, nucleotide sequence identity between trout sst1A and sst1B is 88.1%.

nucleotide sequences similar to the coding region from TMD3 to TMD7 of mammalian ssts and were very similar in nucleotide sequence to each other. Further investigation using 3'-RACE PCR with GSP-3 (designed from the RT-PCR fragment obtained in phase I) revealed two distinct products, one *c.* 500 bp and the other *c.* 850 bp. The 500 bp product had an overlapping sequence with fragment A. The 850 bp product had an overlapping sequence with fragment B. Reverse transcription using GSP-2 and 5'-RACE PCR with GSP-4 resulted in the amplification of two distinct fragments: one that was *c.* 950 bp and had an overlapping sequence with fragment A and another that was *c.* 475 bp and had an overlapping sequence with fragment B. Overlapping sequences of the 950 bp 5'-RACE product, fragment A, and the 500 bp 3'-RACE product revealed a novel 1755 bp cDNA encoding a subtype 1 sst (designated sst1A; Fig. 1). This sequence contained a single initiation site 500 bases from the most 5' end, and two potential putative polyadenylation sites:

one 75 bases from the most 3' end and the other 20 bases from the most 3' end. Overlapping sequences of the 475 bp 5'-RACE product, fragment B, and the 850 bp 3'-RACE product revealed a second distinct 1743 bp cDNA which we designated sst1B (Fig. 1). This sequence contained a single initiation site 25 bases from the most 5' end, and also contained two potential putative polyadenylation sites: one 465 bases from the most 3' end and the other 18 bases from the most 3' end.

The sst1A and sst1B cDNAs each contain a single open reading frame of 1113 bp encoding a 371-amino acid receptor protein with seven hydrophobic TMDs, a feature found in the G-protein-coupled receptor family. They also contain the DRY motif at the border of TMD3 and the second intracellular loop, a sequence highly conserved in ssts as well as in other seven TMD receptors. The YANS.ANP.LY motif was also found in TMD7, a signature sequence in mammalian ssts (Fig. 1). The designation of these ssts as subtype 1 was based on similarity to other



known sst sequences. Rainbow trout sst1A and sst1B share 63.6% nucleotide identity and 94.1% amino acid identity.

Despite the similarity of sequence between rainbow trout sst1A and sst1B, we took advantage of two regions of the nucleotide sequence within the 500 bp RT-PCR fragments to design oligonucleotide probes that would distinguish between the two transcripts. Southern blot analysis of positive size-fractionated sst1 PCR products from phase I RT-PCR showed specific binding of these radiolabeled oligonucleotide probes to the RT-PCR fragments; the sst1A-specific probe only hybridized to sst1A cDNA (fragment A), the sst1B-specific probe only hybridized to sst1B cDNA (fragment B) and neither probe hybridized to a partial rainbow trout sst2 cDNA (data not shown). Northern blot analysis of size-fractionated poly(A)<sup>+</sup> RNA from brain and pancreas revealed that there appears to be a single transcript encoding sst1A and a single transcript encoding sst1B (Fig. 2).

### Differential expression of sst1 mRNAs among tissues

RNA from various tissues was extracted and reverse transcribed. The resulting cDNAs encoding sst1A and sst1B were amplified by RS-PCR, electrophoresed and subjected to Southern blot analysis using the sst1A(as) or sst1B(as) radiolabeled nucleotide probes. Using this method, rainbow trout sst1A was found in brain (whole), stomach, liver, pancreas, upper and lower intestine, pyloric cecum, kidney and muscle (Fig. 3A). In addition to the tissues in which sst1A mRNA was found, sst1B mRNA was also found in the esophagus (Fig. 3B). Duplicate samples pretreated with RNase demonstrated that amplified products were exclusively obtained from RNA templates and not false positives originating from contaminating genomic DNA. The sizes of the amplified cDNAs for sst1A and sst1B from RS-PCR were 494 and 486 bp respectively, and matched the sizes predicted from primer locations on the cDNA sequences.

Northern slot-blot analysis showed that sst1A and sst1B mRNA were uniformly distributed among the major regions of the trout brain (Fig. 4).

### Differential abundance of sst1 mRNAs within tissues

Hybridization of gene-specific oligonucleotide probes to replicate slot-blots containing known quantities of *in vitro*-synthesized sst1A and sst1B cRNA standards (in the range  $1.5 \times 10^7$ – $1.5 \times 10^9$  molecules) and RNA extracted from selected tissues allowed for accurate evaluation of the amounts of sst1A and sst1B mRNAs (Fig. 5; note the lack of cross-hybridization). This approach was used to determine the abundance of sst1A and sst1B mRNAs in the optic tectum, pancreas and liver (tissues that play central roles in the coordination of growth, development and metabolism) removed from rainbow trout under normal physiological conditions (Fig. 6). Under these conditions, levels of sst1A mRNA were significantly higher (16-fold) than those of sst1B in the optic tectum. In contrast, sst1B mRNA levels were significantly higher (4-fold) than those of sst1A in the liver. No difference between the levels of sst1A mRNA and sst1B mRNA were detected in the pancreas.

### Discussion

In this study, we characterized two cDNAs that encode for distinct type 1 SS receptors from the endocrine brain and endocrine pancreas of rainbow trout, designated sst1A and sst1B. The nucleotide identity between the cDNAs is 63.6% (88.1% when the 5' and 3' untranslated regions are excluded); the position and extent of the differences suggests the existence of two non-allelic sst 1 genes. We also demonstrate for the first time to our knowledge that the mRNAs encoding sst isoforms are differentially expressed, both in terms of distribution among tissues as well as in terms of abundance within tissues.

**Figure 8** Comparison of amino acid sequences of rainbow trout sst1A and sst1B with those of: goldfish sst1A and sst1B (Gsst1A and Gsst1B) (Lin *et al.* 1999a); human sst1 (Hsst1) and mouse sst1 (Msst1) (Yamada *et al.* 1992); rat sst1 (Rsst1) (Meyerhof *et al.* 1991). Sequence identity was maximized by inserting gaps (dashed lines). Conserved amino acid residues are indicated by asterisks. The putative TMDs are overlined. Notable structural motifs are boxed.

To date, molecular cloning has revealed the existence of five subtypes of somatostatin receptor (sst1–sst5) in several mammalian (Hoyer *et al.* 1995, Reisine & Bell 1995, Patel & Srikant 1997, Meyerhof 1998) and non-mammalian species (Lin *et al.* 1999a, 2000, 2002, Zupanc *et al.* 1999, Bossis & Porter 2001, Lin & Peter 2003). Somatostatin receptor subtype 1 has been found in human, mouse (Yamada *et al.* 1992) and rat (Meyerhof *et al.* 1991) as well as two isoforms in goldfish (Lin *et al.* 1999a). The characterization of trout ssts contributes further to our understanding of the evolution of the sst family and provides new insight into structure–function relationships of the SS signaling systems (cf. Sheridan *et al.* 2000). When compared with nucleotide sequences from other cloned sst1 subtypes, rainbow trout sst1A is most homologous to that of mouse sst1 with 70.4% similarity (Fig. 7; Yamada *et al.* 1992). Rainbow trout sst1B on the other hand is most similar to goldfish sst1A, sharing 62.7% nucleotide identity (Fig. 7; Lin *et al.* 1999a). Even lower is the degree to which rainbow trout sst1A and sst1B share similarities with other sst subtypes, i.e. sst2–sst5 (data not shown). As expected, the largest number of differences between the nucleotide sequences occurs in the untranslated regions of the cDNAs.

The deduced sst1A and sst1B proteins produced in rainbow trout islet cells and brain tissue share 94.1% amino acid identity and both contain 371 amino acids (Figs 7 and 8), slightly longer than the 367-amino-acid proteins in goldfish sst1A and sst1B (Lin *et al.* 1999a), but shorter than the 391-amino-acid proteins in human and mouse sst1 (Yamada *et al.* 1992) and rat sst1 (Meyerhof *et al.* 1991). The trout sst1A and sst1B deduced amino sequences are most similar to human sst1, sharing 64.4 and 65.5% identity respectively (Figs 7 and 8). Rainbow trout sst1A and sst1B share only 43–60% amino acid identity with mammalian sst2–sst5 (data not shown).

Structurally, the deduced amino acid sequences of rainbow trout sst1A and sst1B share many similarities with the other cloned vertebrate sst1s. The extracellular N-terminus and the intracellular C-terminus of trout sst1A and sst1B account for the greatest number of differences when compared with other vertebrate sst1s, whereas the TMDs and the intra- and extracellular loops are highly conserved. Several conserved sequence patterns are found in the trout sst1s which are also found in

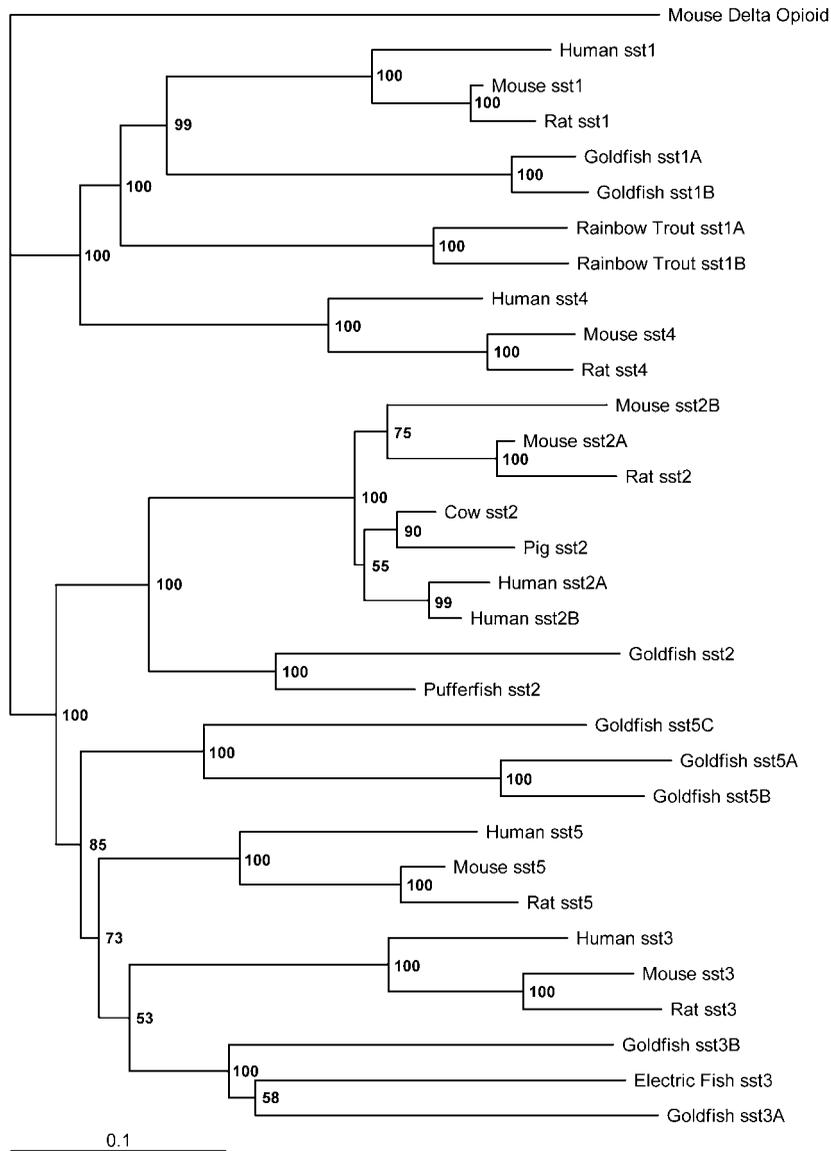
other sst1s as well as other sst subtypes. Many members of the DRY-containing family of G-protein-coupled receptors also contain these conserved motifs. These amino acid motifs may play a role in conferring the appropriate tertiary structure necessary for functional activity and include the motifs GN..V, NLA.AD, S...L...S.DRY, W..S.....P, F..P, F..CW.P and NS..NP..Y in  $\alpha$ -helical TMDs 1–7 respectively (Reisine & Bell 1995). The search for structural motifs that are specific for the sst family has revealed a putative phosphorylation site R..SE in the third intracellular loop and the motif L.YANS.ANP.LY.F.S in TMD7, both of which are found in the trout sst1s (Fig. 8).

Believed to be glycoproteins (Rens-Domiano & Reisine 1991, 1992), ssts contain consensus sequences in the N-terminal domain of the receptors with the sequence Asn-X-Ser/Thr (N-X-S/T), where X is any amino acid except proline. Trout sst1A and sst1B contained two potential Asn-linked glycosylation sites at amino acid positions Asn<sup>7</sup> and Asn<sup>22</sup>. The nature and extent to which receptors are glycosylated may play a role in high-affinity ligand binding (Rens-Domiano & Reisine 1992). Both Asn-linked glycosylation sites were either identical or similar in sequence to the corresponding site in human, rat, mouse and goldfish sst1s (Fig. 8; Meyerhof *et al.* 1991, Li *et al.* 1992, Yamada *et al.* 1992, Lin *et al.* 1999a).

Another structural feature found in the rainbow trout sst1s is two conserved cysteine residues (Fig. 8). These are found at positions Cys<sup>110</sup> and Cys<sup>188</sup> in the first and second putative extracellular loops respectively, and are thought to be associated with the formation of a disulfide bridge that would stabilize the tertiary structure of the receptor as seen in other G-protein-coupled receptors (Strader *et al.* 1994).

Three potential amino acids were found which could be cAMP-dependent protein kinase phosphorylation sites at positions Thr<sup>152</sup>, Ser<sup>243</sup> and Thr<sup>248</sup> for both sst1A and sst1B. In addition, potential protein kinase C phosphorylation sites were found at position Thr<sup>365</sup> for sst1A and position Thr<sup>365</sup> for sst1B (Fig. 8; Kemp & Pearson 1990).

An additional highly conserved amino acid residue at position Cys<sup>317</sup>, which is found in the C-terminus, has been shown to be palmitoylated in  $\beta_2$ - and  $\alpha_2$ -adrenergic receptors as well as the



**Figure 9** Phylogenetic tree based on the alignment of amino acid sequences of known somatostatin receptors in vertebrates. The branch 'lengths' represent amino acid substitutions per site from a common ancestor and are proportional to the estimated elapsed time since the divergence occurred. The bold numbers at each branch point represent the bootstrap values, scaled to a maximum of 100%. The following sequences were obtained from GenBank: mouse opioid receptor, AF062753; human sst1, M81829; human sst2A and human sst2B, AF18174, human sst3, M96738; human sst4, D16826; human sst5, D16827; mouse sst1, M81831; mouse sst2A, M81832; mouse sst2B, X68951; mouse sst3, M91000; mouse sst4, U26176; mouse sst5, U82697; rat sst1, M97656; rat sst2, M93273; rat sst3, X63574; rat sst4, U04738; rat sst5, L04535; pig sst2, D21338; cow sst2, L06613; goldfish sst1A, AF097726; goldfish sst1B, AF097727; goldfish sst2, AF139597; goldfish sst3, AF311307; goldfish sst5, AF252879; pufferfish sst 2, AF329947. The electric fish (*Apteronotus*) sst3 sequence is from Zupanc *et al.* (1999).

rhodopsin family of receptors and could be used to anchor the C-terminal end of the receptor to the plasma membrane (Fig. 8; O'Dowd *et al.* 1989, Papac *et al.* 1992, Kennedy & Limbird 1993).

Other important features of the sst1 receptors are conserved amino acids that may play a role in the binding of the ligand SS-14. Kaupmann *et al.* (1995) proposed a model based upon site-directed mutagenesis studies of different ssts. These findings show that the core residues of SS-14, Phe<sup>6</sup>-Phe-Trp-Lys-Thr-Phe<sup>11</sup>, interact with a ligand-binding pocket located in TMDs 3–7, which is lined with hydrophobic and charged amino acids. In trout sst1s, the ligand-binding pocket is lined by Phe<sup>265</sup> and Gln<sup>269</sup> in TMD6 and by Thr<sup>283</sup> in TMD7 (Fig. 8). This pocket may provide a hydrophobic environment for interaction with the Phe<sup>6</sup>-Phe<sup>11</sup> region of SS-14; this interaction may be enhanced by an electrostatic interaction between Lys<sup>9</sup> of the ligand and Asp<sup>117</sup> of TMD3 (Sheridan *et al.* 2000).

The phylogenetic relationships of the cloned trout sst1s with other known members of the sst family is shown in Fig. 9. The pattern that emerges supports a multigenic origin of ssts that arose from a series of gene duplication events throughout the course of vertebrate evolution. Two major clades can be identified: an sst1/sst4 group and an sst2/sst3/sst5 group. Subtype 1 ssts appear to have diverged early from sst4s, similar to the divergence of sst2s from sst5/sst3s. The branching of the sst1 clade is consistent with the phylogeny of vertebrates, with rainbow trout representing one of the oldest species so far examined. The emergence of sst1 isoforms in fish, both rainbow trout and goldfish (Lin *et al.* 1999a), probably results from a recent duplication event such as the tetraploidization of these species (Ohno 1970). The existence of sst isoforms in other tetraploid or non-tetraploid species is not known. The significance of different sst isoforms is not clear, but they may play a role in selective binding of SSs that circulate in fish (e.g. SS-14, [Tyr<sup>-7</sup>,Gly<sup>10</sup>]-SS-14, [Tyr<sup>7</sup>,Gly<sup>10</sup>]-SS-25) or in activating specific biological responses.

The two sst1 mRNAs are differentially expressed. This is supported by several observations. First, the expression pattern of sst1A and sst1B is tissue specific. While sst1A and sst1B mRNAs are distributed widely in the brain, pancreas, kidney and selected regions of the gut, only sst1B mRNA was detected in the esophagus; sst1 has also been found broadly distributed in mammals (Sheridan

*et al.* 2000). Lin *et al.* (1999a) also reported a pattern of tissue-specific expression for sst1 isoforms in goldfish. Secondly, the abundance of the rainbow trout sst1 mRNAs was different within specific tissues. Within the optic tectum, the predominant form was that encoding for sst1A, whereas in the liver, sst1B mRNA was the predominant form. There appeared to be no difference in the abundance of sst1A and sst1B mRNAs in the pancreas. These results suggest the production of two forms of sst1 in trout and that there may be independent mechanisms which regulate the expression of each variant. The factors which serve to regulate sst expression remain to be investigated, but likely candidates include factors from systems that are known to be affected by SS (e.g. growth hormone and insulin).

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