Rainbow trout (Oncorhynchus mykiss) possess two somatostatin mRNAs that are differentially expressed.

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Moore, Craig A., Jeffrey D. Kittilson, Melissa M. Ehrman, and Mark A. Sheridan. Rainbow trout (Oncorhynchus mykiss) possess two somatostatin mRNAs that are differentially expressed. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R1553–R1561, 1999.—Previously, we isolated a 624-bp cDNA encoding for a 115-amino acid preprosomatostatin containing [Tyr7,Gly10]-somatostatin (SS)-14 at its COOH-terminus. The amino acid sequences of PPSS-II products obtained directly from islet extracts are known for coho salmon (33), eel (9), goldfish (40), sculpin and flounder (8), and tilapia (28). Evidence that teleost PPSSs derive from different mRNAs was first reported in angelfish (16–18); two cDNAs were obtained from pancreatic islets of this species, one encoding for PPSS-I and the other encoding for PPSS-II. Despite the existence of multiple cDNAs encoding for PPSSs in angelfish (16–18) and catfish (21, 24), definitive information regarding the potential differential expression of somatostatin genes has not been reported.

In this study, we used rainbow trout to characterize further the polygenic origin of SS in vertebrates and to evaluate the expression of SS gene products. Rainbow trout are particularly well-suited for this investigation because of the organization of their pancreas, in which the endocrine component (Brockmann body) is anatomically separate from the exocrine component and because the Brockmann body contains comparatively large amounts of SS peptide isoforms localized in discrete cell populations (29).

MATERIALS AND METHODS

Animals. Juvenile rainbow trout, Oncorhynchus mykiss, were obtained from the Garrison National Fish Hatchery near Riverdale, ND. Fish were maintained at North Dakota State University in well-aerated, dechlorinated municipal freshwater (14°C) under 12:12-h light-dark photoperiod and fed to satiety twice daily with SuperSweet Feeds (Glenco, MN) trout grower, except 24 h before experiments. In the nutritional state experiment, fish were either fed as usual or fasted for 2 wk before sample collection.

RNA extraction. Tissues were removed from rainbow trout of both sexes after the animals had been anesthetized with 0.01% (wt/vol) 3-amino benzoic acid ethyl ester (MS-222, Sigma) buffered with 0.2% (wt/vol) sodium bicarbonate. Tissue samples (~25 mg) were placed in 2-ml microfuge tubes and immediately frozen on dry ice. Total RNA was extracted by a modification of the RNAzol method (Cinna/Biotecx Laboratories, Friendswood, TX) described previously (25). Total RNA was quantified by spectrophotometry and diluted to 15 µg/µl. RNA samples were stored at −80°C until used.

Isolation and sequence analysis of PPSS cDNA. A two-phase rapid amplification of cDNA ends (RACE) PCR-based approach was used for the isolation and characterization of selected cDNA sequences as described previously (25). Briefly, in phase I (Fig. 1A), endogenous poly-A RNA was reverse transcribed from 15 µg of trout pancreatic total RNA with Superscript II reverse transcriptase (GIBCO BRL, Gaithersburg, MD) and a 37-nucleotide antisense adapter primer (GIBCO BRL). Five microliters of the reverse transcription reaction were used as a template for 3′-RACE PCR with a...
Fig. 1. Rapid amplification of cDNA ends (RACE) of preprosomatostatin-II′ (PPSS-II′) and PPSS-II′′ from endocrine pancreas of rainbow trout. A: diagrammatical outline of sequence strategy using 3′- (left) and 5′- (right)-RACE procedures. GSP, gene-specific primer; UAP, universal amplification primer. B: agarose gel electrophoresis of 3′- and 5′-RACE PCR fragments visualized by ethidium bromide staining and transillumination. Lane 1 is an HaeIII digest of 4× 174 DNA for size determination. Lane 2 contains 250-bp somatostatin (SS)-II′ and 243-bp SS-II′′ 3′-RACE PCR (phase I) products (observed as a single band). Lane 3 contains 561-bp SS-II′′ 5′-RACE PCR fragment. Lane 4 contains 544-bp SS-II′′ 5′-RACE-PCR product.

21-base somatostatin gene-specific primer (GSP-1; 5′-GGCT-GCAAGAATTCTTCTCTCG-3′) and the universal amplification primer (GIBCO BRL). After an initial denaturation cycle of 94°C for 5 min, 39 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 identified by agarose gel electrophoresis, as described above, of restriction enzyme digests (EcoRI; Promega, Madison, WI) of purified plasmid preparations (12). One to two micrograms of plasmid DNA was denatured and sequenced by the dideoxy chain-termination method (Sequenase Kit; US Biochemicals, Cleveland, OH) according to the manufacturer’s protocol. All sequences were confirmed by sequencing multiple colonies from at least three independent PCR reactions and with two or more different primers in both directions. In phase II (Fig. 1A), isolation of the 5′ cDNA sequence was accomplished by 5′-RACE PCR (GIBCO BRL). SS mRNA was exclusively reverse transcribed from pancreatic total RNA using a 20-base antisense oligonucleotide primer complementary to a region of the 3′ fragment isolated in phase I (GSP-2; 5′-GGTGGCGGTGTGACGTGATTG-3′). The resulting cDNA was purified twice over Glass Max spin columns (GIBCO BRL) to remove unincorporated dNTPs and primer and then “tailed” at the 3′ end with dCTP using terminal deoxynucleotidyl transferase (GIBCO BRL). Five microliters of the tailing reaction were used as a template for 5′-RACE PCR with GSP-2 and anchor primer (GIBCO BRL). Thirty-nine PCR cycles were performed as in 3′-RACE PCR, except Taq polymerase (Perkin-Elmer, Norwalk, CT) was pipetted beneath the layer of mineral oil after the initial 5-min denaturation cycle (26). The amplified product was identified by agarose gel electrophoresis, cloned, and sequenced as described above.

Slot-blot quantitation of mRNA. The amount of PPSS-II′ and PPSS-II′′ mRNA in pancreas and stomach was quantitated by slot-blot analysis (6), a technique similar to RNase protection assay in that it relies on reference to in vitro-synthesized RNA standards and has a sensitivity of ~10⁶ molecules but lends itself more readily to the analysis of numerous samples. RNA standards were made by first cloning full-length SS-II′ and SS-II′′ cDNAs in the sense orientation into the PCR 2000 cloning vector (Invitrogen). After linearization with EcoRI (Promega; for SS-II′ insert) or BamHI (Promega; for SS-II′′ inserts), in vitro RNA synthesis was performed using T7 RNA polymerase (40 units; Promega), according to the manufacturer’s protocol. Full-length cRNA was separated from unincorporated NTPs by ultracentrifugation (100,000 mol wt cutoff; Millipore, Bedford, MA) followed by ethanol precipitation (1/4 volume NaCl, 2× volume absolute ethanol) at −20°C overnight. After recovery of RNA by centrifugation (12,000 g for 20 min at 4°C), RNA was resuspended in 100 ml sterile water and quantitated by UV A₃₂₅⁺ spectrophotometry. The homogeneity of cRNA standard preparations was assessed by electrophoresis on a 6% polyacrylamide/8.0 M urea gel and verified by sequence analysis. Northern analysis (19) was performed to evaluate the number and size of transcripts as well as to verify that the specific oligonucleotide probes hybridized only with SS-II′ and SS-II′′ transcripts in the total RNA extracted from the Brockmann bodies of trout. Four hundred-fifty microfilter replicate dilutions of standards (serially diluted in sterile water containing yeast tRNA (10 µg/ml) and RNasin (80 U/ml; Promega)) and pancreatic total RNA samples (10 µg) were initially diluted with sterile water to a final volume of 50 µl, to which was added 20 µl of 37% formaldehyde and 30 µl of 20× saline sodium citrate (3 M NaCl, 0.3 M Na₂HPO₄·2H₂O, pH 7.0); after incubation at 65°C for 15 min, the RNA samples were immediately placed on ice and diluted further with 1,000 µl of ice-cold 10× saline sodium citrate) were slotted directly onto 0.2 µm Nytran membrane (Schleicher and Schuell) and hybridized, individually, with either SS-II′-specific, SS-II′′-specific, or SS-II′/SS-II′′-common (standards only; for normalization of RNA amount) radiolabeled oligonucleotide probes as described above. The resulting autoradiograms were quantified by scanning laser densitometry (Molecular Dynamics, Sunnyvale, CA). Statistical differences
were estimated by a two-tailed Student's t-test \( (n = 12; P < 0.05) \).

**RESULTS**

Rainbow trout possess two cDNAs encoding PPSSs that contain \([\text{Tyr}^7,\text{Gly}^{10}]\)-SS-14. An \( \sim 260 \)-bp fragment was amplified by 3'-RACE PCR from reverse-transcribed total RNA isolated from trout pancreas using an SS-specific upstream primer (GSP-1, Fig. 1). Sequence analysis of this 3' fragment (actual length = 243 bp) revealed six codons followed by a stop codon with 100% identity to the last six codons (+9 to +14) of a trout PPSS containing \([\text{Tyr}^7,\text{Gly}^{10}]\)-SS-14 recently identified and reported by our laboratory (25); the remainder of the fragment consisted of 3' untranslated region, including a polyadenylated tail at the most 3' end. Reverse transcription and 5'-RACE PCR with our previously designed GSP-2 primer resulted in the amplification of a 561-bp fragment identical in sequence to that which we reported previously (25). Reverse transcription and 5'-RACE PCR with a newly designed antisense primer unique to the new 3' fragment resulted in the amplification of a 544-bp fragment (Fig. 1B). Overlapping sequence of the 243-bp 3'-RACE and 544-bp 5'-RACE fragments identified a novel 600-bp cDNA encoding for a second PPSS containing \([\text{Tyr}^7,\text{Gly}^{10}]\)-SS-14, which we have designated PPSS-II'*, with a single putative initiation site 101 bases downstream from the 5' end and two putative polyadenylation signal sites. Exhaustive screening of 18–23 colonies from each of three independent 3'-RACE and 5'-RACE PCRs confirmed the existence of only two cDNAs, one encoding PPSS-II' and one identical to our previously reported sequence (25) that encodes for the precursor we now designate PPSS-II'*. A comparison between PPSS-II' and our previously reported sequence (25) is shown in Fig. 2. Although PPSS-II' is a 115-amino acid protein containing numerous putative recognition sites for posttranslational modification by converting enzymes, potentially yielding a 28-amino acid SS peptide with \([\text{Tyr}^7,\text{Gly}^{10}]\)-SS-14 at its COOH terminus, PPSS-II' is a 111-amino acid protein potentially processed to a 25-amino acid SS
peptide containing [Tyr7,Gly10]-SS-14 at its COOH terminus. SS-II\textsubscript{8} and SS-II\textsubscript{88} share 82.3% nucleotide and 80.5% amino acid identity. Despite the similarity of sequence between SS-II\textsubscript{8} and SS-II\textsubscript{88}, we took advantage of a 50-base region immediately upstream from the COOH termini of the SS coding regions to design three 20-base oligonucleotides that would specifically bind to SS-II\textsubscript{8} mRNA, SS-II\textsubscript{88} mRNA, or to both SS-II\textsubscript{8} and SS-II\textsubscript{88} mRNAs (the specificity of these probes was verified by hybridization to in vitro synthesized RNA; see Fig. 6A). Northern analysis using these probes revealed that there was a single transcript encoding PPSS-II\textsubscript{8} and a single transcript encoding PPSS-II\textsubscript{88} (Fig. 3).

Two PPSS-II mRNAs are differentially expressed in various tissues. RNA from various tissues was extracted and reverse transcribed. The resulting cDNAs encoding for PPSS-II\textsubscript{8} and PPSS-II\textsubscript{88} were coamplified by RS-PCR, electrophoresed on agarose, and subjected to Southern blot analysis using a full-length SS-II cDNA probe (which does not distinguish between SS-II\textsubscript{8} and SS-II\textsubscript{88}). With this approach, PPSS-II mRNA was detected in brain, esophagus, pyloric ceca, stomach, upper and lower intestine, and Brockmann bodies (Fig. 4). Duplicate samples pretreated with RNase demonstrated that amplified products were exclusively derived from RNA templates and not false positives derived from contaminating genomic DNA.

When slot-blot analysis of RS-PCR products was performed using gene-specific oligonucleotide probes that distinguish PPSS-II\textsubscript{8} and PPSS-II\textsubscript{88} mRNA, we detected the presence of PPSS-II\textsubscript{8} and PPSS-II\textsubscript{88} mRNA in esophagus, pyloric ceca, stomach, upper and lower intestine, and Brockmann bodies, although only PPSS-II\textsubscript{88} mRNA was present in brain (Fig. 5).

Abundance of PPSS-II mRNAs is different in various tissues. Hybridization of the gene-specific oligonucleotide probes to replicate slot-blot containing known quantities of in vitro-synthesized PPSS-II\textsubscript{8} and PPSS-II\textsubscript{88} cRNA standards, in the range of 6.5 x 10\textsuperscript{8} to 5.0 x 10\textsuperscript{9} molecules, and RNA extracted from selected tissues allowed for the accurate evaluation of the amounts of PPSS-II\textsubscript{8} and PPSS-II\textsubscript{88} mRNAs (Fig. 6). We used this approach to examine the expression of PPSS-II\textsubscript{8} and of PPSS-II\textsubscript{88} mRNAs in Brockmann bodies (endocrine pancreas) and stomachs removed from animals under
normal (fed to satiety twice per day except 24 h before sampling) physiological conditions. Under these conditions, pancreatic SS-II mRNA levels were nearly threefold higher than those of SS-II, estimated to be \(8.7 \times 10^8\) molecules/µg total RNA and \(3.2 \times 10^8\) molecules/µg total RNA, respectively (Fig. 7A). The concentrations of PPSS-II mRNAs were lower in stomach than in pancreas. In addition, the relative abundance PPSS-II mRNA species in the stomach was opposite that in the pancreas, such that the levels of PPSS-II mRNA were 10-fold higher than those of PPSS-II mRNA (Fig. 7B).

Abundance of PPSS-II mRNA is modulated by nutritional state. Nutritional state modulated the pattern of pancreatic PPSS-II mRNA expression. Fish that were fasted for 2 wk displayed levels of PPSS-II mRNA that were twofold higher than their continuously fed counterparts (Fig. 8). The levels of PPSS-II mRNA, however, were not affected by food deprivation.

**DISCUSSION**

In the present study, we characterized two cDNAs that encode PPSS containing [Tyr7,Gly10]-SS-14 at their COOH terminus (designated PPSS-II' and PPSS-II'') and demonstrated that the two PPSS-II mRNAs are differentially expressed. This is the first report of the coexistence of two different PPSS-IIs. The nucleotide identity between the two cDNAs is 82.3%; the position and extent of the differences suggests the existence of two nonallelic PPSS-II genes. The two PPSS-IIs in rainbow trout are in addition to a single PPSS-I encoding SS-14, which also presumably arise from a separate gene (19).

The deduced PPSS-II' and PPSS-II'' proteins in rainbow trout Brockmann bodies contain 115 and 111 amino acids, respectively, both slightly shorter than the precursors of anglerfish (16–18) and goldfish (20), the only other known PPSS-IIs containing [Tyr7,Gly10]-SS-14. Rainbow trout PPSS-II' shared 43.5% amino acid identity with anglerfish PPSS-II and 51.3% amino acid identity with goldfish PPSS-II. The amino acid identity...
between rainbow trout PPSS-II’ and anglerfish PPSS-II was 38.7%, whereas the identity between trout PPSS-II’ and goldfish PPSS-II was 41.4%. Amino acid identities between rainbow trout PPSS-IIs and precursors derived from gene 1 were lower, between 37.9 and 22.5%. Rainbow trout PPSS-IIs were least similar to the PPSS, giving rise to catfish SS-22. Although the evidence is limited, it appears that evolutionary selection has acted to conserve the biologically active COOH-terminal domain of PPSSs (Fig. 9).

A comparison of nucleotide and predicted amino acid sequences between SS-II’ and SS-II’’ mRNAs in endocrine pancreas (A) and stomach (B) of rainbow trout under normal physiological conditions. RNA extracted from tissues was subjected to slot-blot quantitation as shown in Fig. 6. Data are presented as means ± SE (n = 8); *significant difference compared with the PPSS-II’’ group within same tissue (P < 0.05).
Structural data also point to the emergence of additional SS genes. Lamprey, for example, possess variant forms of SS (three successively longer peptides extended at their NH₂ terminus) in addition to SS-14 (1). A variety of peptide sequence data as well as limited cDNA information suggests that teleost fish also possess multiple SS genes. A majority of the reports suggests the existence of two genes: one encoding [Tyr⁷,Gly¹⁰]-SS-14 and one encoding SS-14 (8). Recent reports suggest that some teleosts possess more than two SS genes. For example, the present study shows that rainbow trout possess two distinct cDNAs that give rise to two different PPSSs containing [Tyr⁷,Gly¹⁰]-SS-14 at their COOH termini as well as a third cDNA encoding SS-14 (19). In addition, a recent report also showed that goldfish possess three distinct cDNAs: one encoding SS-14, a second encoding [Glu¹,Tyr⁷,Gly¹⁰]-SS-14, and a third encoding [Pro²]-SS-14 (20). The presence of multiple SS genes also extends to the tetrapods.

Fig. 8. Effects of nutritional state on pancreatic levels of PPSS-IIIs. RNA extracted from tissues was subjected to slot-blot quantitation as shown in Fig. 6. Data are presented as means ± SE (n = 8); *significant difference compared with continuously fed group (P < 0.05).

Fig. 9. Comparison of amino acid sequences of PPSS-II' and PPSS-II' with those of PPSSs from 9 other species of vertebrates. Sequence identity was maximized by inserting gaps (dashed lines). Conserved amino acids are shaded. Amino acid sequences were deduced from cDNA sequences from H, human (35); M, monkey (39); B, bovine (37); R, rat (15); C, chicken (27); FR I and FR II, frog I and frog II (38); AF I, anglerfish I (18); AF II, anglerfish II (16–18); CF I, catfish I (13); CF II, catfish II (14); GF I-III, goldfish I-III (20); TRI, trout I (19), and TRII, trout II (25).
Frogs possess one cDNA that encodes for a PPSS containing [Pro\textsuperscript{2},Met\textsuperscript{13}]-SS-14 and a second cDNA that encodes a PPSS that contains SS-14 (38). In addition, mammalian cortistatin, a peptide sharing considerable identity to SS-14 that was isolated from the brain of rats (11) may be derived from an alternate SS gene form that emerged in early tetrapod evolution. Whether or not the various SS genes in vertebrates arose through several independent gene duplication events or through a single duplication event preDating or concomitant with the appearance of Agnatha, as suggested by Conlon et al. (10), is not known.

Because SS-II\textsuperscript{1} and SS-II\textsuperscript{11} of rainbow trout are more closely related to each other than either is to other SS-II or SS-I cDNAs, the duplication event leading to their emergence, probably the tetraploidization event that appears common to salmonids (30), likely occurred after the duplication, giving rise to the two teleost SS genes, an event estimated to have occurred some 160 million years ago (37). Tetraploidy may also help to explain the presence of multiple SSs in goldfish (20).

The two PPSS-II mRNAs of rainbow trout are differentially expressed. This conclusion is based on several observations. First, the pattern of PPSS-II mRNA and PPSS-II\textsuperscript{1} mRNA is tissue specific. For example, only PPSS-II\textsuperscript{1} mRNA was detected in the brain of rainbow trout, whereas both PPSS-II and PPSS-II\textsuperscript{11} mRNAs were detected in pancreas and various regions of the gut. Brain-specific expression of the mRNA encoding the alternate form of SS in frogs (denoted PSS2) (38) and cortistatin (11) also has been reported. Previous immunocytochemical studies support a similar distribution of [Tyr\textsuperscript{2},Gly\textsuperscript{10}]-SS-14-containing peptides in the intestine (4) and stomach (3) of rainbow trout. Second, the abundance of PPSS-II mRNAs was different with specific tissues. Within the Brockmann body of rainbow trout, the predominant message form was that encoding PPSS-II\textsuperscript{11}, whereas in the stomach the predominant form was that encoding PPSS-II\textsuperscript{1}. Finally, the pattern of PPSS-II expression within the endocrine pancreas of rainbow trout was modulated by nutritional state. Together, these results suggest that rainbow trout produce two forms of gene 2 SS peptides and that there exist mechanisms to independently regulate the expression of each.

The alternate forms of SS (containing [Tyr\textsuperscript{2},Gly\textsuperscript{10}]-SS-14) in rainbow trout are in addition to SS-14 (19). The functions of the various SS peptides remain to be fully elucidated; however, previous research has suggested that distinctive roles for the gene 1 and gene 2 forms exist. For example, peptides derived from gene 1 (e.g., SS-14, SS-28) were equipotent in their ability to inhibit the release of growth hormone from goldfish pituitary fragments in vitro, whereas peptides derived from alternate genes (e.g., sSS-25, catfish SS-22) had no effect on growth hormone release (23). Similarly, salmon SS-25 (from gene 2) inhibited insulin in rainbow trout, but SS-14 (from gene 1) did not (13).

In summary, the present report describes the characterization of two cDNAs encoding for PPSSs that contain [Tyr\textsuperscript{2},Gly\textsuperscript{10}]-SS-14, consistent with the existence of two nonallelic SS genes and that the mRNAs for the two PPSSs (PPSS-II\textsuperscript{1} and PPSS-II\textsuperscript{11}) are differentially expressed. These results support the notion of a polygenic origin of somatostatins and suggest the existence of mechanisms to control the differential expression of the multiple SS genes. The regulation of differential gene expression may underlie aspects of the multifunctional nature of the SS family of peptides. Future studies will be conducted to evaluate how differential expression of PPSS-II\textsuperscript{1} and PPSS-II\textsuperscript{11} is regulated.

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