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Occurrence of two somatostatin variants in the frog brain: Characterization of the cDNAs, distribution of the mRNAs, and receptor-binding affinities of the peptides

(neuropeptide precursors/nucleotide sequence/amphibians/evolution)

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ABSTRACT **In tetrapods, only one gene encoding a somatostatin precursor has been identified so far. The present study reports the characterization of the cDNA clones that encode two distinct somatostatin precursors in the brain of the frog *Rana ridibunda*. The cDNAs were isolated by using degenerate oligonucleotides based on the sequence of the central region of somatostatin to screen a frog brain cDNA library. One of the cDNAs encodes a 115-amino acid protein (prepro-somatostatin-14; PSS1) that exhibits a high degree of structural similarity with the mammalian somatostatin precursor. The other cDNA encodes a 103-amino acid protein (prepro-[Pro^2, Met^{13}]somatostatin-14; PSS2) that contains the sequence of the somatostatin analog (peptide SS2) at its C terminus, but does not exhibit appreciable sequence similarity with PSS1 in the remaining region. *In situ* hybridization studies indicate differential expression of the PSS1 and PSS2 genes in the septum, the lateral part of the pallium, the amygdaloid complex, the posterior nuclei of the thalamus, the ventral hypothalamic nucleus, the torus semicircularis and the optic tectum. The somatostatin variant SS2 was significantly more potent (4–6 fold) than somatostatin itself in displacing [^{125}I-Tyr^0, D-Trp^8] somatostatin-14 from its specific binding sites. The present study indicates that the two somatostatin variants could exert different functions in the frog brain and pituitary. These data also suggest that distinct genes encoding somatostatin variants may be expressed in the brain of other tetrapods.**

Somatostatin is a cyclic tetradecapeptide initially isolated from the ovine hypothalamus on the basis of its ability to inhibit the secretion of growth hormone (1). Subsequent studies have shown that somatostatin-14 (SS1) is widely distributed in the central nervous system and in peripheral tissues such as pancreas, intestine, and stomach, where it acts both as a neurotransmitter/neuromodulator and a hormone (2).

The primary structure of SS1 has been strongly conserved during evolution. In particular, the sequence of somatostatin is identical in mammals (1), birds (3), reptiles (4, 5), amphibians (6), teleosts (see ref. 7 for review), holosteans (8), elasmobranchs (9), and agnathans (10–12). The cDNA encoding preprosomatostatin from various mammalian species, including human (13), monkey (14), ox (15), and rat (16), has been characterized. Processing of preprosomatostatin generates two biologically active peptides—i.e., somatostatin-28 and SS1. A second form of prepro-somatostatin cDNA has been cloned from the pancreas of the anglerfish (17), trout (18), and catfish (19). Concurrently, a few molecular variants of somatostatin have been identified in the pancreas of the lamprey (11, 20, 21) and Pacific ratfish (22), and in the pituitary of the sturgeon (23).

It has long been thought that a single gene encoding prepro-somatostatin was present in the genome of tetrapods (24). This hypothesis was invalidated when two molecular forms of somatostatin were identified in the central nervous system of an amphibian, the frog *Rana ridibunda* (25). In this species, both peptide SS1 and a variant ([Pro^2, Met^{13}]somatostatin-14; peptide SS2) are found in the brain. However, the structure of the precursor generating peptide SS2 and the physiological significance of this somatostatin variant are totally unknown. We report here the characterization of the cDNAs encoding the precursors for peptides SS1 and SS2, the distribution of the corresponding mRNAs and the receptor-binding affinities of the mature peptides.

MATERIALS AND METHODS

Animals. Adult male frogs (*R. ridibunda*) weighing 30–40 g were obtained from a commercial source (Couétard, Saint-Hilaire de Riez, France). The animals were maintained in controlled conditions of temperature (8 ± 0.5°C) and illumination (12-hr light/12-hr dark) with free access to running water for at least 1 week before death. Animal manipulations were performed according to the recommendations of the French Ethical Committee and under the supervision of authorized investigators.

Peptides. The somatostatin analog [Tyr^0, D-Trp^8] SS1 was a gift from D. H. Coy (Tulane University, New Orleans). Synthetic SS1 was provided by J. Chanteclair (Sanofi, Paris). SS2 was synthesized by the solid phase method as previously described (26). [Tyr^0, D-Trp^8]SS1 was radioiodinated by means of the lactoperoxidase technique as described (27), and the monoiodinated radioligand was purified by reversed-phase HPLC on a Zorbac C-18 column (25 × 0.4 cm; Merck) using a gradient of acetonitrile in triethylamine-phosphate buffer (0.25 M; pH 3). The specific radioactivity of the tracer was ≈2000 Ci/mmol (1 Ci = 37 GBq).

Isolation of Frog Prepro-SS1 and -SS2 (PSS1 and PSS2) cDNAs. An amplified frog brain cDNA library constructed in λgt10 (28) was screened with a degenerate (64-fold) 23-mer oligonucleotide [5′-GT(CT)TTCCA(AG)AA(AG)AA-

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Abbreviations: SS1, somatostatin-14; SS2, [Pro^2, Met^{13}]SS1; PSS1, prepro-SS1; PSS2, prepro-SS2.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U68136 (PSS1) and U68137 (PSS2)].

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(AG)TT(CT)TT(AG)CA)-3′] (Genosys) designed from the amino acid sequence CKNFFWKT common to frog SS1 and SS2. The oligonucleotides were 3′-end labeled with [α-^{32}P]dCTP using terminal transferase (Promega). Filters (Hybond; Amersham) were prehybridized at 40°C for 4 hr in 5× SSPE (0.9 M NaCl/0.05 M sodium phosphate buffer, pH 7.7/0.005 M EDTA), 0.1% SDS, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA, 50 μg/ml tRNA, and 50 μg/ml salmon sperm DNA. Hybridization was performed in 5× SSPE/1% SDS, overnight at 40°C. Filters were then washed twice at room temperature in 5× SSPE/0.1% SDS for 15 min. The membranes were exposed onto Kodak X-OMAT film for 48 hr at −70°C with high-speed intensifying screens.

Nine positive clones were obtained and their inserts were amplified using PCR. The resulting PCR products were then subcloned into pGEM-T vector (Promega) and sequenced by the dideoxynucleotide chain-termination procedure (29). Seven clones contained the full-length nucleotide sequence of the PSS1 gene transcript. The two other positive clones contained part of the sequence of the PSS2 gene transcript. Another library was constructed in λZap (Stratagene) using poly(A$^+$) RNA isolated from frog brain and screened under high stringency conditions (30) with the PSS2 cDNA probe. Numerous positive clones were identified. pBluescript SK-phagemids were isolated from the bacteriophage clones by *in vivo* excision, according to the manufacturer's instructions. Sequence data were analyzed with the MULTALIN sequence software (31).

Northern Blot Analysis. Total RNA from frog brain, spinal cord, stomach, intestine, pancreas, liver, kidney, heart, lung, and testis was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (32). Twenty micrograms of total RNA from each tissue was separated on a formaldehyde-agarose denaturing gel and transferred onto a nylon support (Amersham). The membrane was hybridized under high stringency conditions with the frog PSS1 and PSS2 cDNA probes.

***In Situ* Hybridization.** A 312-bp DNA fragment of the PSS1 cDNA (position 1–312) and a 148-bp fragment of the PSS2 cDNA (position 111–258) were PCR amplified and subcloned into the pGEM-T vector. Full-length ^{35}S-labeled sense and antisense single-stranded PSS1 and PSS2 RNA probes were synthesized by using a Promega riboprobe kit.

Frogs were anesthetized and perfused transcardially with 4% paraformaldehyde. The brains were postfixed for 3 hr at 4°C in the same solution, transferred into 0.1 M phosphate buffer containing 15% saccharose for 12 hr, and frozen in isopentane at −30°C. Coronal sections (10-μm thick) were cut on a cryomicrotome (2800 Frigocut, Leica) and collected on 0.5% gelatin/0.05% chrome alum/0.01% polylysine-coated slices. Sections were incubated in 0.1 M triethanolamine (pH 8) for 5 min, rinsed in 2× standard saline citrate (SSC; 0.3 M NaCl/0.03 M sodium citrate, pH 7), and covered with prehybridization buffer (50% formamide/0.6 M NaCl/0.01 mM Tris·HCl/0.02% Ficoll/0.02% polyvinylpyrrolidone/0.1% BSA/0.001 M EDTA/550 μg/ml salmon sperm DNA/50 μg/ml yeast tRNA). Hybridization was performed overnight at 60°C in the same buffer (except for salmon sperm that was at 60 μg/ml) supplemented with 0.01 mM dithiothreitol, 10% dextran sulfate, and 10^7 cpm/ml heat-denatured (15 min at 65°C) antisense PSS1 or PSS2 RNA probe, as described (33). Briefly, brain slices were washed in 2× SSC at 60°C, and treated with RNase A (50 μg/ml) for 60 min at 37°C. Five final high-stringency washes were performed in 0.1× SSC, 14 mM 2-mercaptoethanol, and 0.05% sodium pyrophosphate at 60°C. The slices were dehydrated and exposed onto Hyperfilm-βmax (Amersham) for 2 weeks. Control sections were hybridized with the ^{35}S-labeled sense PSS1 or PSS2 RNA probe.

Receptor Autoradiography. Unfixed frog brains were placed in an embedding medium (O.C.T. Tissue Teck, Reichert-Jung, Nussloch, Germany) and immediately frozen. The tissues were sectioned coronally at 20 μm on a cryomicrotome. Brain sections taken from the telencephalon and diencephalon regions were thaw-mounted on gelatin-coated slides, dried overnight under vacuum, and kept at −80°C until incubation with the radioligand. Tissue sections were preincubated for 30 min at 10°C with 0.05 M Tris buffer (pH 7.4) containing 0.005 M $MgCl_2$, 0.032 M sucrose, 0.5% BSA, 5 μg/ml bacitracin, and 10^{-6} M GTP. The sections were rinsed in the same buffer without GTP and incubated with 30 pM of [^{125}I-Tyr^0, D-Trp^8]SS1 in the absence or presence of various concentrations of SS1 and SS2 at 10°C, as described (34). The sections were rinsed in ice-cold buffer, dried under a cold air-stream, and apposed onto Hyperfilm-[^{3}H] (Amersham) for 4 weeks. Autoradiograms were quantified by means of a computer-assisted image analysis system (Bio 500; Biocom, Paris). The kinetics parameters of the competition curves were calculated using the SIGMAPLOT software (Jandel, San Rafael, CA).

RESULTS

Characterization of the cDNAs Encoding PSS1 and PSS2. The PSS1 clones encompassed a length of 557 nt comprising a 345-nt coding region flanked by 45 nt at the 5′ end and 164 nt at the 3′ end (Fig. 1*a*). Two polyadenylylation signal motifs (AATAAA) are present at positions 506–511 and 534–539, followed by the poly(A) tail. The open reading frame encodes for a 115-amino acid protein including a putative 24-amino acid signal peptide (35). The primary structure of frog PSS1 contains a monobasic processing site (Arg) potentially generating somatostatin-28, and a dibasic processing site (Arg–Lys) potentially generating SS1. The sequence of frog somatostatin-28 shows only two substitutions (Ser for Asn^5, and Leu for Met^8) compared with human and rat somatostatin-28.

The nucleotide sequence of the cloned PSS2 cDNA and the deduced amino acid sequence are shown in Fig. 1*b*. Sequence analysis of the 469-nt cDNA revealed a single potential initiation codon 27 nt downstream from the 5′ end. The initiation site TCACAATGC represents a reasonable Kozak consensus sequence (36). The 127-nt 3′-untranslated sequence contains a variant consensus polyadenylylation motif (ATTAAA) 22 nt upstream from the poly(A) tail. The open reading frame encodes for a 103-amino acid protein that possesses a putative signal sequence of 21 amino acids (35). Frog PSS2 exhibits a dibasic Arg–Lys site potentially yielding SS2. Otherwise, the N-terminal flanking polypeptide of PSS2 does not exhibit appreciable sequence similarity with frog PSS1 or mammalian prepro-somatostatin.

Distribution of PSS1 and PSS2 mRNAs. The presence of PSS1 and PSS2 mRNAs in various tissues was investigated by Northern blot analysis. Using the PSS1 cDNA probe, a major band (≅750 nt) was detected in the brain and stomach and, to a lesser extent, in the pancreas, intestine, and spinal cord (Fig. 2*a*). The PSS1 probe also revealed the occurrence of a minor band (≅600 nt) in the same tissues. In contrast, the PSS2 cDNA probe showed the existence of a single band (≅550 nt) in the brain extract only, with no signal apparent in the other tissues examined (Fig. 2*b*).

The distribution of PSS1 and PSS2 mRNAs was studied in the frog brain by *in situ* hybridization (Fig. 3). The PSS1 gene was widely expressed in various brain regions. In the telencephalon, prominent expression was evident in the pallium, the septum, and the amygdaloid complex (Fig. 3*a*). In the diencephalon, a strong hybridization signal was observed in the posterior thalamic nucleus, the anterior preoptic area, and the ventral hypothalamic nucleus (Fig. 3*b*). In the mesencephalon, PSS1 mRNA expression was detected in the nucleus of the medial longitudinal fasciculus, the interpeduncular nucleus, the anterior tegmental nuclei, the torus semicircularis, and the periventricular layer of the optic tectum (Fig. 3*c*). The distribution of PSS2 mRNA exhibited only partial overlapping with

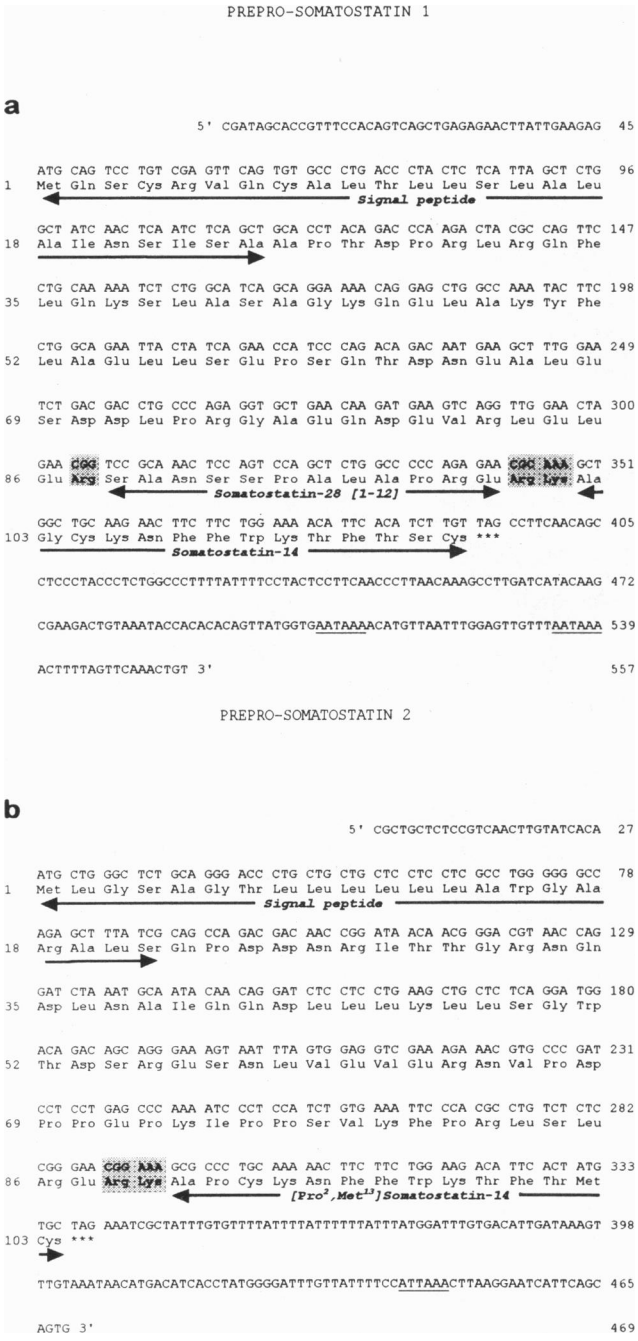

FIG. 1. Nucleotide and deduced amino acid sequences of *R. ridibunda* PSS1 (*a*) and PSS2 (*b*). Numbers on right correspond to the last nucleotide of each line. Amino acid residues are numbered on the left from the putative starting methionine [1]. Shaded amino acids indicate potential cleavage sites. Polyadenylylation signals are underlined.

that of PSS1 mRNA. Specifically, a high density of PSS2 mRNA was found in the median and dorsal aspects of the pallium, in the median area of the amygdaloid complex, the anterior preoptic area (Fig. 3*d*), the tegmental, and the interpeduncular nuclei (Fig. 3*f*). A moderate density of PSS2 was also observed in the ventral hypothalamic nucleus, the posterior thalamic nucleus, and the nucleus of the medial longitudinal fasciculus (Fig. 3*e*). Several regions containing PSS1 mRNA were virtually devoid of PSS2 mRNA, such as the septum, the lateral part of the pallium (Fig. 3*d*), the torus semi circularis, and the optic tectum (Fig. 3*f*).

Binding Affinity of Frog SS1 and SS2. The ability of SS1 and SS2 to compete for [^{125}I-Tyr^0, D-Trp^8]SS1 binding sites in the

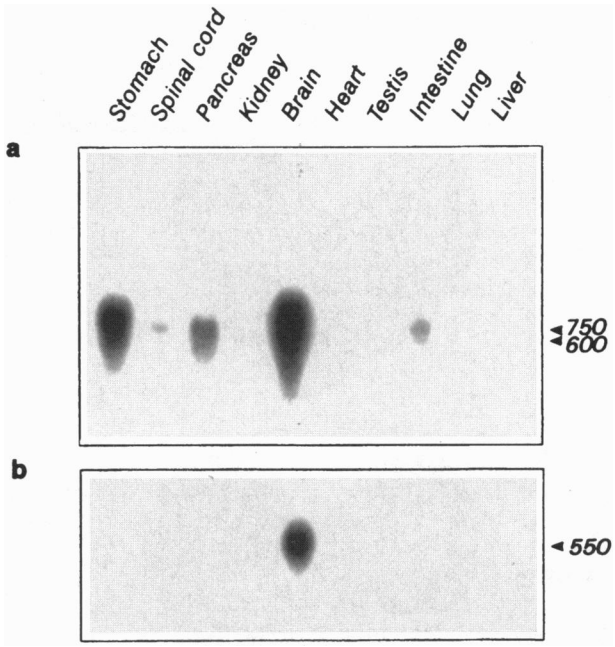

FIG. 2. Northern blot analysis of total frog RNA hybridized with the random-primed PSS1 (*a*) and PSS2 (*b*) cDNA probes. Twenty micrograms of RNA from each tissue sample was electrophoresed on a formaldehyde-agarose gel. Molecular weights were determined by using RNA markers.

frog pallium mediale (telencephalon) and stratum griseum superficiale (mesencephalon) was studied by autoradiography. In both brain regions, SS2 was significantly more potent than SS1 in displacing the radioligand from its binding sites (Fig. 4). The IC_{50} of SS1 and SS2 were 24.0 ± 1.4 and 5.6 ± 0.7 nM in the pallium mediale ($P < 0.001$) and 8.3 ± 1.6 and 1.4 ± 0.07 nM in the stratum griseum superficiale ($P < 0.05$), respectively. Frog urotensin II, which exhibits sequence similarities with SS1 (39), did not significantly inhibit radioligand binding in the frog brain (Fig. 4).

DISCUSSION

The present studies have characterized the cDNAs encoding two distinct somatostatin precursors in the brain of the frog *R. ridibunda*. The amino acid sequence of PSS1 exhibits 75% and 85% identity with human (13) and chicken (data from K. Nata; GenBank accession no. X60191) prepro-somatostatins, respectively. Specifically, the sequence of PSS1-derived somatostatin-28 only differs by two substitutions compared with the human sequence and one substitution with the chicken sequence (Fig. 5). The N-terminal pentadecapeptide of the prosegment is also highly conserved with only one substitution (Ser → Thr at position 3) as compared with the mammalian sequence. In contrast, the structure of PSS2 shows wide divergences with those of PSS1 and mammalian prepro-somatostatins (13–16). In particular, the N-terminal flanking peptide of SS2 exhibits only a few sequence similarities with frog or mammalian somatostatin-28[1–12], and the upstream Arg monobasic cleavage site present in all other somatostatin precursors (see ref. 24 for a review) is lacking, indicating that PSS2 probably is not processed to yield a somatostatin-28-like molecule (Fig. 5). In addition, the structure of the N-terminal domain of pro-SS2 diverges completely from those of pro-SS1 and mammalian pro-somatostatins (16). These observations indicate that a strong evolutionary pressure has acted to conserve the sequence of the biologically active domain of PSS2, while the prosegment of PSS2 has very few similarities with other prepro-somatostatins, including PSS1.

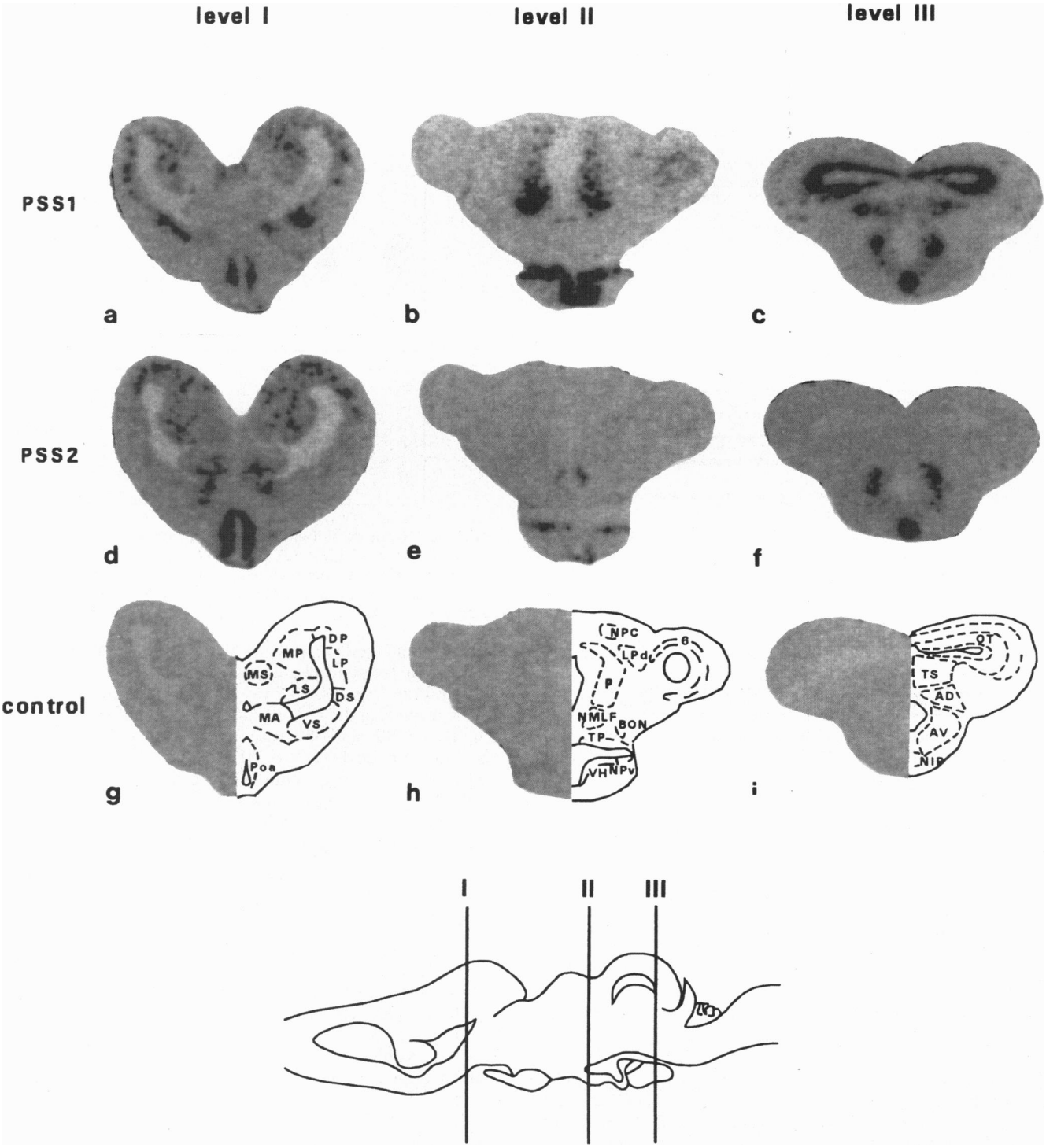

FIG. 3. X-ray autoradiographs showing the distribution of PSS1 (*a–c*) and PSS2 (*d–f*) mRNAs in frog telencephalon (level I), diencephalon (level II), and mesencephalon (level III). Coronal brain sections were hybridized with the antisense PSS1 or PSS2 cRNA probes (*a–f*), or with the sense PSS1 (*g* and *h*) or PSS2 (*i*) cRNAs, and exposed onto x-ray films for 20 days. The anatomical structures are designated on the right hemisections (*g–i*) according to refs. 37 and 38. The scheme of the parasagittal section indicates the coronal planes chosen for the illustrations. AD, anterodorsal tegmental nucleus; AV, anteroventral tegmental nucleus; BON, basic optic nucleus; DP, dorsal pallium; DS, dorsal septum; LP, lateral hypothalamic nucleus; Lpd, lateral thalamic nucleus, posterodorsal division; LS, lateral septum; MA, medial amygdala; MP, medial pallium; MS, medial septum; NIP, nucleus interpeduncularis; NMLF, nucleus of the medial longitudinal fasciculus; NPv, periventricular nucleus; NPC, nucleus of the posterior commissure; OT, optic tectum; P, posterior thalamic nucleus; Poa, anterior preoptic area; TP, posterior tuberculum; TS, torus semicircularis; VH, ventral hypothalamic nucleus; VS, ventral septum; 6, tectal lamina six.

The occurrence of two somatostatin precursors has been reported in the pancreas of anglerfish (17) and catfish (19, 40). Statistical analysis of the cDNA sequences indicates that the two somatostatin genes present in teleosts results from a single duplication event that occurred ≈160 million years ago in a common ancestor of this lineage (15). The present data reveal that duplication of the somatostatin gene has probably also occurred in the tetrapod lineage that diverged from the fish lineage some 400 million years ago. While frog PSS1 exhibits a high degree of similarity with fish prepro-somatostatin I (17,

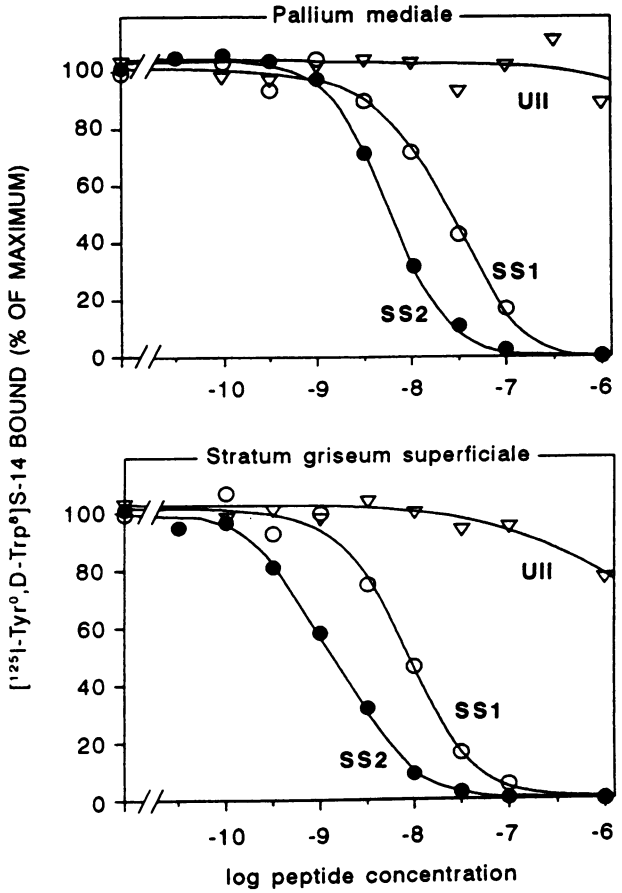

FIG. 4. Displacement of [^{125}I-Tyr^0, D-Trp^8]SS1 binding by SS1, SS2, and urotensin II (UII) on frog brain slices as determined by quantitative autoradiography in the pallium mediale and in the stratum griseum superficiale.

40), several lines of evidence indicate that frog PSS2 and teleost prepro-somatostatin II are not homologous. (*i*) No sequence similarities were found between the prosegments of frog PSS2 and anglerfish prepro-somatostatin II (17), even though the alignment of the prosegment of PSS2 with other somatostatin precursors including PSS1 revealed the existence of 6 identical amino acids in the N-terminal flanking region of SS1 (Fig. 5). (*ii*) The prepro-somatostatin II gene of teleosts is only expressed in pancreatic islets (see ref. 18 for a review) while the mRNA encoding frog PSS2 was detected only in the brain. The wide divergence between the structures of PSS1 and PSS2 suggests that the duplication event has occurred at an early stage of evolution of terrestrial vertebrates, possibly before the divergence of amphibians and mammals. If this hypothesis is true, the PSS2 gene may not be restricted to amphibians but could also be present in other tetrapods.

In situ hybridization studies showed that the PSS1 and PSS2 genes are differentially expressed in the brain. A high density of PSS1 and PSS2 mRNA was detected in the medial and dorsal aspects of the pallium, the anterior preoptic area and the tegmental and interpeduncular nuclei. Other brain regions including the septum, the lateral part of the pallium, the torus semicircularis and the optic tectum contained only PSS1 mRNA. In contrast, expression of the PSS2 gene was predominant in a few regions including the medial area of the amygdaloid complex and the ventral part of the anterior preoptic area. These observations suggest that SS1 and SS2 could exert distinct functions in the frog brain and pituitary.

A good correlation was generally observed between the distribution of somatostatin receptors in the brain (41) and the distribution of PSS1 and PSS2 mRNA (this study). In particular, high concentrations of binding sites and PSS1/PSS2 mRNAs are present in the medial pallium and the anterior preoptic area. In contrast, a few regions expressing exclusively the PSS1 gene, such as the septum and the torus semicircularis are virtually devoid of somatostatin receptors (41), suggesting that the cell bodies expressing the PSS1 gene may send projections in other regions that possess somatostatin receptors.

To compare the biological potencies of SS1 and SS2, competition studies were performed using a nonselective radioligand which, in mammals, exhibits high affinity for all subtypes of somatostatin receptors (42). In the pallium mediale and the stratum griseum superficiale, two regions of the frog brain that contain a high density of somatostatin receptors (42), SS2 appeared respectively 4.3 and 5.9 times more potent than SS1 in displacing [^{125}I-Tyr^0, D-Trp^8]SS1 binding. Similarly, in the rat occipital cortex, SS2 exhibited a higher affinity than SS1 for [^{125}I-Tyr^0, D-Trp^8]SS1 binding sites (unpublished results). These data indicate that the SS2 variant is potentially more active than SS1 in both amphibians and mammals.

In conclusion, the present report has described the characterization of the cDNAs encoding two distinct somatostatin

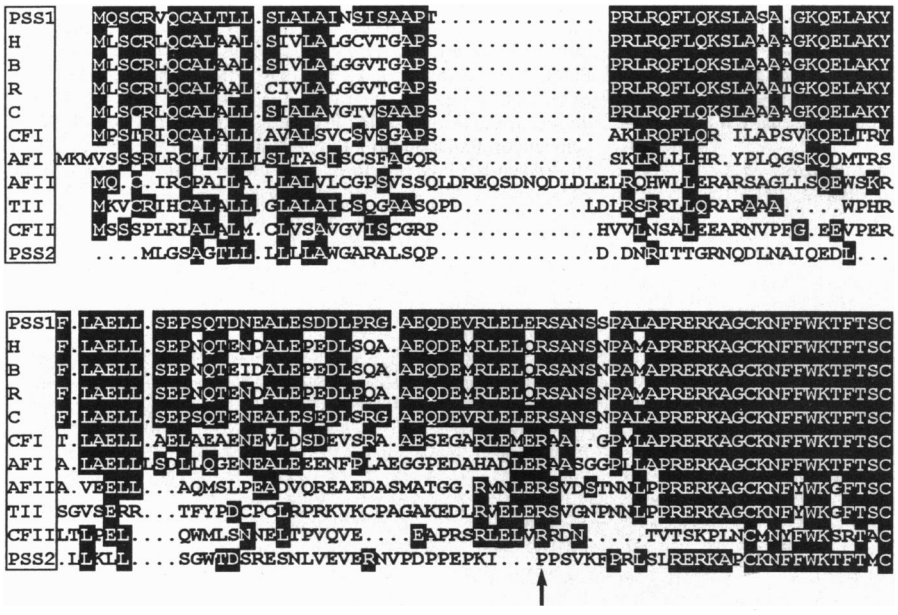

FIG. 5. Comparison of the amino acid sequences of PSS1 and PSS2 with those of prepro-somatostatins from seven other vertebrate species. Similarity between the sequences was maximized by inserting gaps (dots). Conserved amino acids are shaded. The arrow points to the single Arg residue lacking in PSS2. The amino acid sequences deduced from the cDNA sequences were taken from the following references: H, human (13); B, (15); R, rat (16); C, chicken (data from K. Nata; GenBank accession no. X60191); CFI, catfish I (40); AFI, anglerfish I (17); AFII, anglerfish II (17); TII, trout II (18); and CFII, catfish II (19).

precursors in the brain of a tetrapod. Further studies will be conducted to determine whether a second somatostatin gene encoding a PSS2-related precursor is present in reptiles, birds and/or mammals.

Note Added in Proof. While this manuscript was submitted, the characterization of a second somatostatin precursor cDNA has been reported in mammals (43). This cDNA encodes a precursor that contains at its C terminus the sequence of a tetradecapeptide, called cortistatin, that exhibits 11 amino acid identity with somatostatin 14. The fact that cortistatin posseses a proline at position 2, and is expressed exclusively in the brain, suggests that it could be the mammalian counterpart of frog SS2.

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