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Expression and Processing of the [Pro^2,Met^{13}]Somatostatin-14 Precursor in the Intermediate Lobe of the Frog Pituitary

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The biosynthesis of various hypothalamic neuropeptides has been previously reported in anterior pituitary cells but not in intermediate lobe cells. We have recently demonstrated the occurrence of two somatostatin isoforms in the frog brain, namely somatostatin-14 (SS1) and [Pro^2,Met^{13}]somatostatin-14 (SS2). In the present study, we demonstrate that the gene encoding the SS2 precursor (PSS2) is actively expressed in the intermediate lobe of the frog pituitary. High concentrations of PSS2 mRNA have been detected by Northern blot analysis and *in situ* hybridization in the frog pars intermedia but not in the pars distalis or pars nervosa. The distribution of PSS1- and PSS2-derived peptides has been investigated by immunohistochemistry using two antisera directed against SS1 and the sequence 54–66 of PSS2 (PSS2$_{54-66}$), respectively. The SS1 antiserum stained only a network of fibers in the neural lobe and a few nerve processes in the intermediate lobe. In contrast, the PSS2$_{54-66}$ antiserum produced intense labeling of melanotrope cells in the pars intermedia. Biochemical characterization of the immunoreactive materials present in pituitary extracts was performed by combining high-performance liquid chromatography analysis and RIA detection. The SS1 RIA revealed the existence of two major immunoreactive peaks that exhibited the same retention times as synthetic SS1 and SS2. The PSS2$_{54-66}$ RIA detected a single peak that likely corresponds to the N-flanking peptide of SS2 (PSS2$_{1-66}$). The present study reveals that melanotrope cells of the frog pituitary selectively express the PSS2 gene and fully process PSS2 to generate the mature somatostatin variant SS2. Taken together, these data provide the first evidence that the gene encoding a hypophysiotropic neuropeptide is intensely expressed in the intermediate lobe of the pituitary. (*Endocrinology* 143: 3472–3481, 2002)

THERE IS NOW evidence that various hypothalamic neuropeptides are synthesized within the anterior pituitary (see Ref. 1 for review). In particular, the occurrence of vasoactive intestinal polypeptide (2, 3), GnRH (4), somatostatin (5), TRH (4, 6), as well as their corresponding mRNAs (7–11) has been demonstrated in the rat anterior pituitary. In contrast, the biosynthesis of hypothalamic neuropeptides in the intermediate lobe of the pituitary has received little attention (12, 13).

The primary structure of somatostatin-14 (SS1) has been remarkably well conserved during vertebrate evolution and it has long been thought that only one form of somatostatin existed in tetrapods (see Refs. 14 and 15 for reviews). However, the characterization of two isoforms of somatostatin in the brain of the frog *Rana ridibunda, i.e.* SS1 and the variant [Pro^2,Met^{13}]somatostatin-14 (SS2), has shown that this view was not correct (16). Molecular cloning of the cDNAs encoding prepro-SS1 (PSS1) and prepro-SS2 (PSS2) in the brain of fish (17–19), amphibians (20), and mammals (21–23) has revealed that two somatostatin genes are actually expressed in the central nervous system of all vertebrates. While the C-terminal tetradecapeptides of PSS1 and PSS2 exhibit strong structural similarity (*e.g.* only two amino acid substitutions between SS1 and SS2 in frog), the sequences of the N-terminal flanking peptides strongly diverge. Notably, in the frog *R. ridibunda*, PSS2 by contrast to PSS1 does not contain a processing site within the N-flanking domain and thus cannot generate an extended form, which would be homologous to somatostatin-28 (Fig. 1).

In a previous report, we have shown that the mRNAs encoding PSS1 and PSS2 are differentially distributed in frog tissues. Specifically, the PSS1 gene is widely expressed in the CNS and in peripheral organs including the pancreas and gastrointestinal tract while the expression of the PSS2 gene seemed to be restricted to the brain (20). In the present study, we demonstrate that, in frog, the PSS2 gene but not the PSS1 gene, is intensely expressed in melanotrope cells, and we show that PSS2 is fully processed to generate the SS2 peptide in the intermediate lobe of the pituitary.

Materials and Methods

Animals

Adult male frogs (*R. ridibunda*, 30–40 g body weight) were obtained from a commercial source (Couétard, Saint-Hilaire de Riez, France). The animals were kept in glass tanks supplied with tap water in a temperature-controlled room (8 ± 0.5 C) under an established photoperiod (lights on, 0600–1800 h) for at least 1 wk before the experiments. Animal manipulations were performed according to the recommendations of

Abbreviations: CST, Cortistatin; NIL, neurointermediate lobe; PACAP, pituitary adenylate cyclase-stimulating polypeptide; PC, prohormone convertase; PCST, pro-cortistatin; POMC, proopiomelanocortin; PSS1, prepro-SS1; PSS2, prepro-SS2; SS1, somatostatin-14; SS2, [Pro^2,Met^{13}]somatostatin-14; SSC, standard saline citrate.

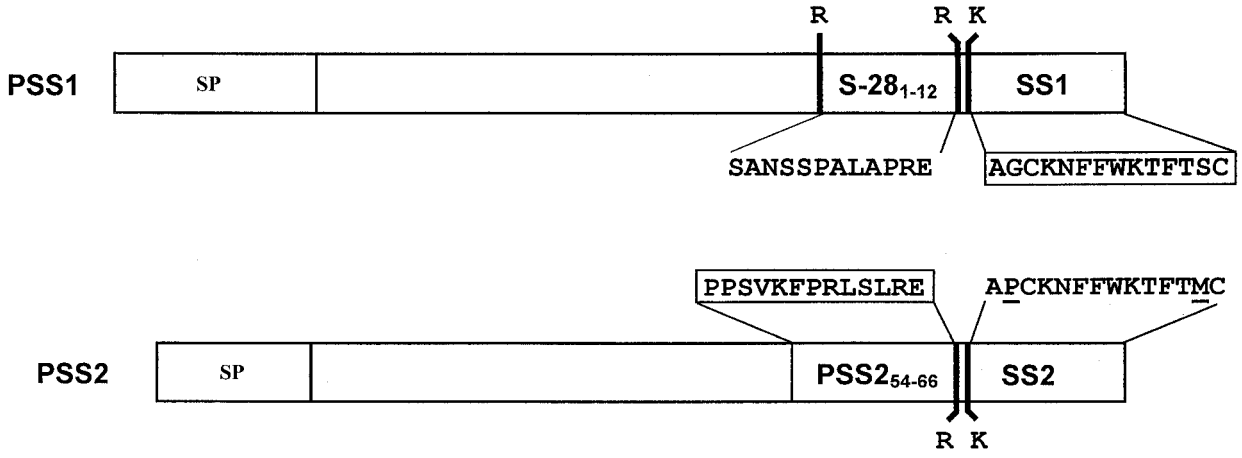

FIG. 1. Schematic representation of the structure of frog PSS1 and PSS2. *Vertical bars* indicate basic amino acid cleavage sites. The sequences of the two somatostatin variants (SS1 and SS2) are shown. The sequences of SS1 and PSS2$_{54-66}$ that were used for raising antibodies for immunohistochemistry and RIA are *boxed*. SP, Signal peptide.

the French ethical committee and under the supervision of authorized investigators.

Northern blot analysis

Total RNAs from whole brain, whole pituitary, distal lobe, and neurointermediate lobe were prepared by the acid guanidinium thiocyanate-phenol-chloroform method (24) using the Tri reagent (Sigma, Saint-Quentin-Fallavier, France). Equivalent amounts of RNA (20 μg) were analyzed as previously described (20) and hybridized under high stringency conditions with ^{32}P-labeled random primed PSS1 and PSS2 cDNA probes.

In situ hybridization

Frogs were anesthetized by immersion in a solution of 0.1% triaminobenzoic acid ethyl ester (MS 222, Sigma) and perfused transcardially with 0.1 M PBS (pH 7.4) and then with 4% paraformaldehyde in PBS. Whole pituitaries were quickly removed and postfixed in the same fixative solution for 4 h. The tissues were immersed in 0.1 M PBS containing 15% sucrose for 12 h and transferred into a 30% sucrose solution for 12 h. The pituitaries were embedded in O.C.T. Tissue Teck (Reichert-Jung, Nussloch, Germany) and frozen on dry ice. Frontal sections (12-μm thick) were cut in a cryomicrotome (Frigocut 2800 E, Reichert-Jung) and mounted on 0.5% gelatin/0.05% chrome alum/0.01% poly-L-lysine-coated slices. Tissue sections were processed for *in situ* hybridization as previously described (20). Briefly, sections were incubated in 0.1 M triethanolamine (pH 8.0) for 5 min, rinsed in 2× standard saline citrate (SSC; 0.3 M NaCl/0.03 M sodium citrate, pH 7.0), and covered with prehybridization buffer (50% formamide; 0.6 M NaCl; 10 mM Tris-HCl, pH 7.5), containing 0.02% Ficoll, 0.02% polyvinylpyrollidone, 0.1% BSA, 1 mM EDTA (pH 8.0), supplemented with 550 μg/ml denatured salmon sperm DNA and 50 μg/ml yeast tRNA. All sections were hybridized with a [^{35}S]uridine-5′-triphosphate-labeled PSS1 or PSS2 riboprobe synthesized as previously described (20). Hybridization was performed overnight at 60 C in the same buffer (except for salmon sperm, whose concentration was 60 μg/ml) supplemented with 10 mM dithiothreitol, 10% dextran sulfate, and 10^7 cpm/ml heat-denatured riboprobes. The sections were washed in 2× SSC at 60 C and treated with ribonuclease A (50 μg/ml) for 60 min at 37 C. Five final high stringency washes were performed in 0.1× SSC, 14 mM β-mercaptoethanol, 0.05% sodium pyrophosphate at 60 C. Pituitary slices were dehydrated in graded ethanols with salts and exposed onto Hyperfilm-βmax (Amersham Pharmacia Biotech, Les Ulis, France) for 2 wk. Tissue sections were subsequently dipped into Kodak (Rochester, NY) NTB-2 liquid emulsion, exposed for 4 wk, and developed. The slices were stained with hematoxylin and eosin for identification of anatomical structures.

Peptide synthesis and production of antibodies

Synthetic frog SS2, PPSVKFPRLSLRE (PSS2$_{54-66}$) (Fig. 1) and its *N*-tyrosylated analog ([Tyr^0] PSS2$_{54-66}$) were synthesized by solid phase methodology as previously described (25). Synthetic PSS2$_{54-66}$ (5 mg) was conjugated to 100 mg bovine thyroglobulin using glutaraldehyde as a coupling agent. After a 2-h incubation in 0.1 M PBS (pH 7.5) at 4 C, the conjugate was dialyzed. The coupling efficiency was 75%. Rabbits were injected intradermally with the synthetic peptide-thyroglobulin conjugate (equivalent of 200 μg synthetic peptide/animal per injection) emulsified with Freund's adjuvant. The titer of the antisera was determined by RIA as described below. The same antiserum (no. 49075) was used for immunohistochemical labeling and RIA quantification. The SS1 antiserum (no. SAB3638) was kindly provided by Dr. C. Rougeot (Institut Pasteur, Paris, France) (26). The specificity of this antibody has been previously characterized. This antiserum exhibits 60% cross-reactivity in RIA with the frog SS2 variant.

Immunohistochemistry

Frogs were anesthetized and perfused transcardially with 100 ml of 0.1 M PBS as indicated above. The perfusion was continued with 100 ml of McLean's fixative as previously described (27). The whole brains with the attached pituitaries were quickly dissected and postfixed overnight at 4 C with the same fixative solution. The tissues were immersed in 0.1 M PBS containing 15% sucrose for 12 h and transferred into 30% sucrose for 12 h. The tissues were embedded in O.C.T. Tissue Teck and frozen on dry ice. Frontal and parasagittal sections (10-μm thick) were cut in a cryomicrotome and mounted on glass slides coated with 0.5% gelatin and 5% chrome alum. Slices were rehydrated and processed for indirect immunofluorescence as previously described (28). Briefly, the sections were preincubated with normal goat serum (1:30) for 45 min to reduce nonspecific staining, and incubated in a moist chamber for 12 h at 4 C with either the SS1 or the PSS2$_{54-66}$ antiserum both diluted 1:400 in 0.1 M PBS containing 0.3% Triton X-100 and 1% BSA. The sections were rinsed three times, incubated for 90 min at room temperature with fluorescein isothiocyanate-conjugated goat antirabbit γ-globulins (Nordic Immunology, Tilburg, The Netherlands), diluted 1:100 in PBS. The slices were finally mounted in PBS-glycerol (1:1, vol/vol). The preparations were examined under a Leitz Orthophan microscope equipped with a Vario-Orthomat photographic system. Selected slices were also analyzed with a confocal laser scanning microscope (Leica Corp., Heidelberg, Germany) equipped with a diaplan optical system and an argon/krypton ion laser (excitation wavelengths: 488, 568, and 610 nm). To study the specificity of the immunoreaction, the following controls were performed: 1) substitution of the primary antisera with PBS; 2) incubation with nonimmune rabbit serum instead of the primary antisera; 3) preincubation of the SS1 antiserum with synthetic SS1 or SS2 (10^{-6} M each)

and preincubation of the $PSS2_{54-66}$ antiserum (diluted 1:400) with synthetic $PSS2_{54-66}$ (10^{-6} M).

Tissue extraction and peptide characterization

Frog pituitaries were boiled in acetic acid for 10 min to ensure inactivation of proteolytic enzymes (29). The tissues were homogenized by sonication, and the suspension was centrifuged (13,000 × *g*; 4 C; 30 min). Peptide material contained in the supernatant was prepurified on Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA). Bound material was eluted with acetonitrile-water-trifluoroacetic acid (60:39.96:0.04, vol/vol/vol) and evaporated in a Speed-Vac concentrator (Savant, Hicksville, NY). The dry extract was dissolved in water-trifluoroacetic acid (99.9:0.1, vol/vol) and chromatographed on a (250 × 4.6-mm) Vydac 218TP54 C_{18} reversed-phase HPLC column at a flow rate of 1 ml/min. The concentration of acetonitrile in the eluting solvent was raised from 21–42% over 60 min for characterization of SS1 and SS2 and from 3.5–68.5% over 60 min for characterization of $PSS2_{54-66}$. One-minute fractions were collected and dried in a Speed-Vac concentrator. Synthetic SS1 and SS2 used as reference peptides were chromatographed using the same gradient as for pituitary extracts.

RIA procedures

The concentrations of SS1- and $PSS2_{54-66}$-like immunoreactivity in crude tissue extracts or HPLC fractions were measured by RIA using antisera nos. SAB3638 and 49075, respectively. Synthetic [Tyr^0] SS1 and [Tyr^0] $PSS2_{54-66}$ (1 μg each) were iodinated by the chloramine-T method and separated from free iodine on Sep-Pak cartridges. The radioiodinated peptides were eluted with a gradient of acetonitrile (0–60%) and kept at –20 C in glycerol (1:1, vol/vol). The RIAs were performed in 0.06 M Na_2HPO_4 containing 0.01 M disodium EDTA, 0.5% NaN_3 and 0.1% Triton X-100 (30). The final dilutions of the antisera against SS1 and $PSS2_{54-66}$ were 1:18,000 and 1:5,400, respectively, and the total amount of tracer was 8,000 cpm/tube. The incubation was carried out for 48 h at 4 C. Separation of the antibody-bound fraction was performed by adding 100 μl of bovine γ-globulins (1%) and 2 ml of polyethylene glycol 8,000 (20%). The tubes were maintained 20 min at room temperature and centrifuged (5,000 × *g*; 4 C; 25 min). The supernatants were removed and the precipitates containing the bound fraction were counted in a γ counter (LKB Wallac, Inc., Rockville, MD).

Background color adaptation experiments

Ninety frogs were adapted on white (40 animals) or black background (50 animals) under constant illumination for 3 wk at 8 C. The neurointermediate lobes of the pituitary from four and five groups of ten white- or black-adapted animals were dissected and immediately processed for RNA extraction. RNA samples were analyzed by Northern blot using PSS2 or proopiomelanocortin (POMC) cDNAs probes. mRNA signals were quantified and corrected for RNA loading variations by scanning the ethidium-bromide-stained 18S ribosomal RNA of each sample using the Densylab 2.0.5 software (Bioprobe Systems, Montreuil, France). Statistical analysis was performed using the Student's *t* test.

Perifusion experiments

The perifusion technique used to determine the effect of test substances on α-MSH release has been previously described in detail (31). For each experiment, four neurointermediate lobes (NILs) were mixed with preswollen Bio-Gel P-2 beads and transferred into a plastic column (0.9 cm inner diameter). The tissues were perifused with Ringer's solution at constant flow rate (0.3 ml/min) and temperature (24 C). The effluent perifusate was collected as 2.5-min fractions during infusion of the secretagogues and 5-min fractions during stabilization periods. The collected samples were immediately chilled at 4 C, and the concentration of α-MSH was measured in each fraction on the same day as the perifusion experiment as previously described (32). The perifusion profiles were calculated and expressed as percentages of the basal secretory level. The basal level of α-MSH was calculated as the mean of six consecutive fractions (30 min) collected just before the infusion of each secretagogue.

Results

Distribution of frog PSS1 and PSS2 mRNAs

The tissue distribution of the PSS1 and PSS2 mRNAs was investigated by Northern blot analysis. Using the PSS1 cDNA probe, hybridization signals corresponding to mRNA species of approximately 750 nucleotides were observed in the brain, but not in the pituitary (Fig. 2A). Using the PSS2 cDNA probe, a weak hybridization signal (~550 nucleotides) was detected in the brain, and an intense signal was seen in the neurointermediate lobe of the pituitary. No PSS2 hybridization signal was observed in the distal lobe of the pituitary (Fig. 2B).

By means of *in situ* hybridization histochemistry, we did not detect PSS1 mRNA in the distal lobe, the intermediate lobe and the neural lobe of the pituitary (Fig. 3, A and D). In contrast, a dense accumulation of PSS2 mRNA was seen in the intermediate lobe (Fig. 3B). No hybridization signal was observed with the sense PSS2 riboprobe (Fig. 3C). Cyto-autoradiographic labeling confirmed that the three lobes of the pituitary were devoid of PSS1-expressing cells (Fig. 3E), whereas the pars intermedia appeared densely loaded with PSS2 mRNA (Fig. 3F).

Peptide concentration in frog pituitary extracts

The detection limit of the $PSS2_{54-66}$ RIA was 40 pg/tube. The IC_{50} value of the assay was 270 pg. Intra- and interassay coefficients of variation were 7.5% and 10%, respectively. The antibody did not exhibit any cross-reactivity with SS1, frog SS2, α-MSH, rat octadecaneuropeptide, human pituitary adenylate cyclase-activating polypeptide (PACAP), mouse joining peptide, or human secretoneurin. Serial dilutions (1:2) of frog pituitary extracts produced displacement curves that were parallel to the standard curve obtained with synthetic $PSS2_{54-66}$ (Fig. 4). The total content of $PSS2_{54-66}$ in Sep-Pak-prepurified extracts was 1.2 ng per pituitary gland.

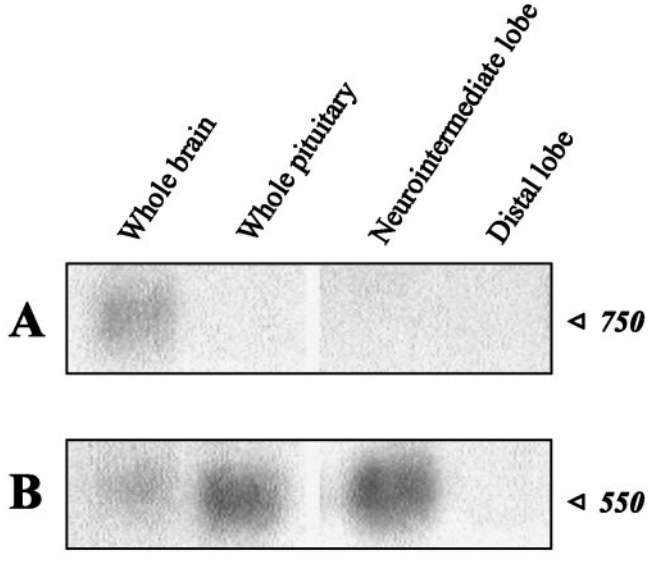

FIG. 2. Northern blot analysis of total frog RNA hybridized with the random-primed PSS1 (A) and PSS2 (B) cDNA probes. Twenty micrograms of RNA from each tissue sample were electrophoresed on a formaldehyde-agarose gel. Molecular weights were determined by using RNA markers.

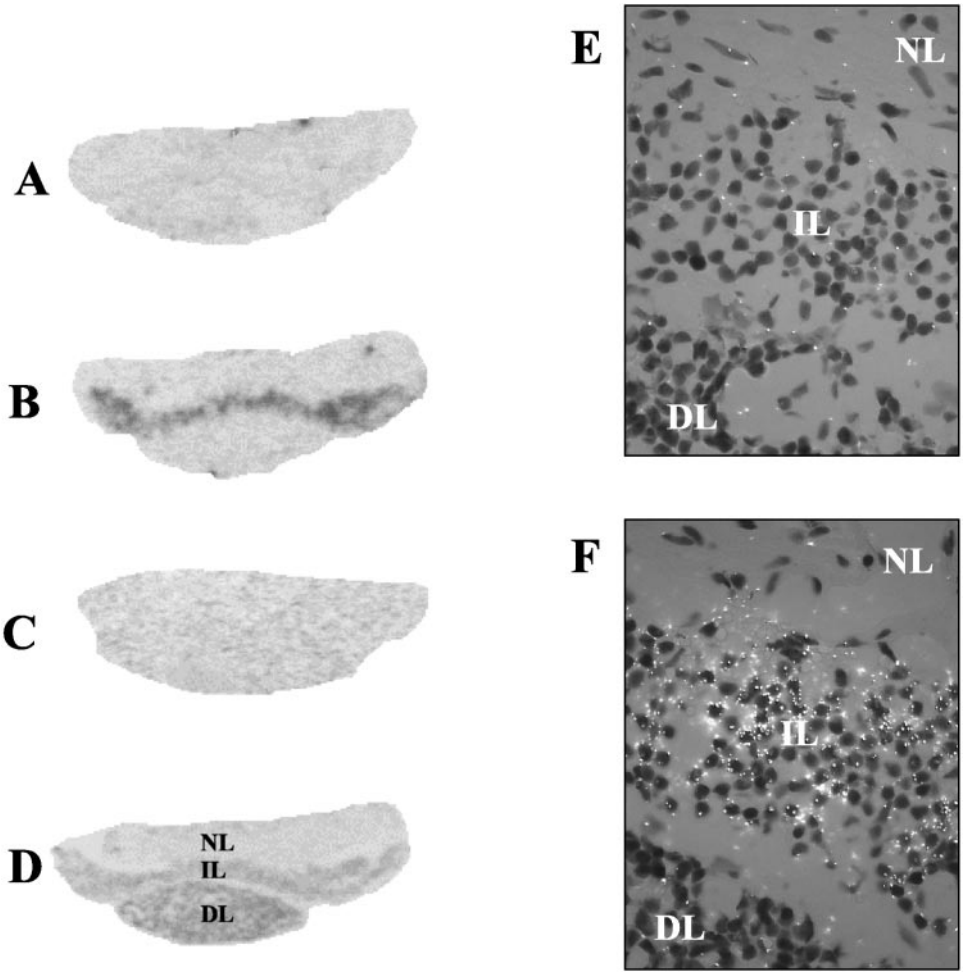

FIG. 3. Autoradiographic localization of PSS1 and PSS2 mRNA in the frog pituitary by *in situ* hybridization. A–C, Pituitary sections were hybridized with the antisense PSS1 (A) or PSS2 (B) cRNA probes, or sense PSS2 cRNA probe (C). The anatomical subdivisions of the pituitary were visualized after hematoxylin-eosin staining (D). E and F, Combined darkfield/brightfield microphotographs showing the cellular distribution of PSS1 (E) and PSS2 (F) mRNAs. DL, Distal lobe; IL, intermediate lobe; NL, neural lobe.

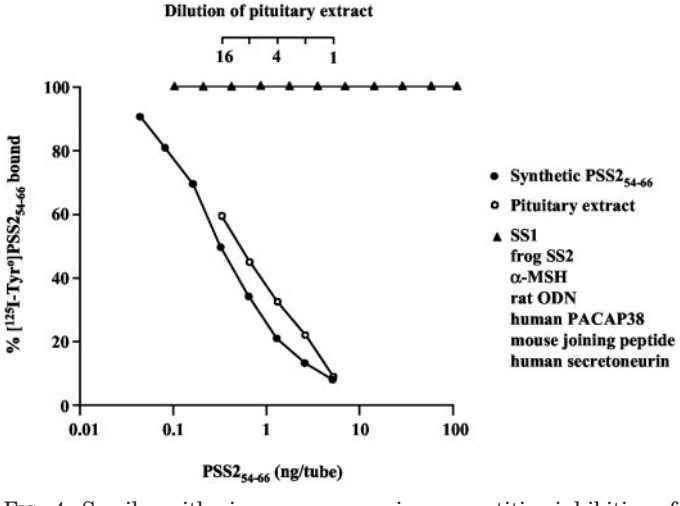

FIG. 4. Semilogarithmic curves comparing competitive inhibition of antibody-bound [I^{125}]-labeled [Tyr^0]PSS2$_{54-66}$ by synthetic PSS2$_{54-66}$ and by serial dilutions of whole pituitary extracts. ODN, Octadecaneuropeptide.

Distribution of SS1 and PSS2$_{54-66}$ in the frog pituitary

A dense network of SS1-immunoreactive fibers was observed in the neural lobe of the pituitary (Fig. 5A). A few SS1-positive fibers were also detected coursing between melanotrope cells (Fig. 5B). The PSS2$_{54-66}$ antiserum produced intense immunolabeling of melanotrope cells in the intermediate lobe of the pituitary (Fig. 5C). At a higher magnification, the PSS2$_{54-66}$-immunoreactive material exhibited a granular aspect and appeared confined to the cytoplasm of melanotrope cells (Fig. 5D). In contrast, no PSS2$_{54-66}$-like immunoreactivity was detected in the neural lobe (Fig. 5C). The distal lobe of the pituitary was totally devoid of SS1- (data not shown) and PSS2$_{54-66}$-immmunoreactive material (Fig. 5C).

Preincubation of the primary antiserum against SS1 with the homologous peptide (10^{-6} M) resulted in complete loss of the immunostaining (Fig. 5E). In contrast, preincubation of the SS1 antiserum with SS2 (10^{-6} M) did not affect the immunolabeling in the neural lobe of the pituitary (Fig. 5F). Preincubation of the antiserum against PSS2$_{54-66}$ with synthetic PSS2$_{54-66}$ (10^{-6} M) totally abolished the immunoreaction in the intermediate lobe of the pituitary (data not shown).

Reversed-phase HPLC analysis

The PSS1- and PSS2-derived peptides present in the prepurified pituitary extracts were characterized by combining reversed-phase HPLC analysis and RIA detection. The SS1 RIA revealed the existence of two major peaks exhibiting a

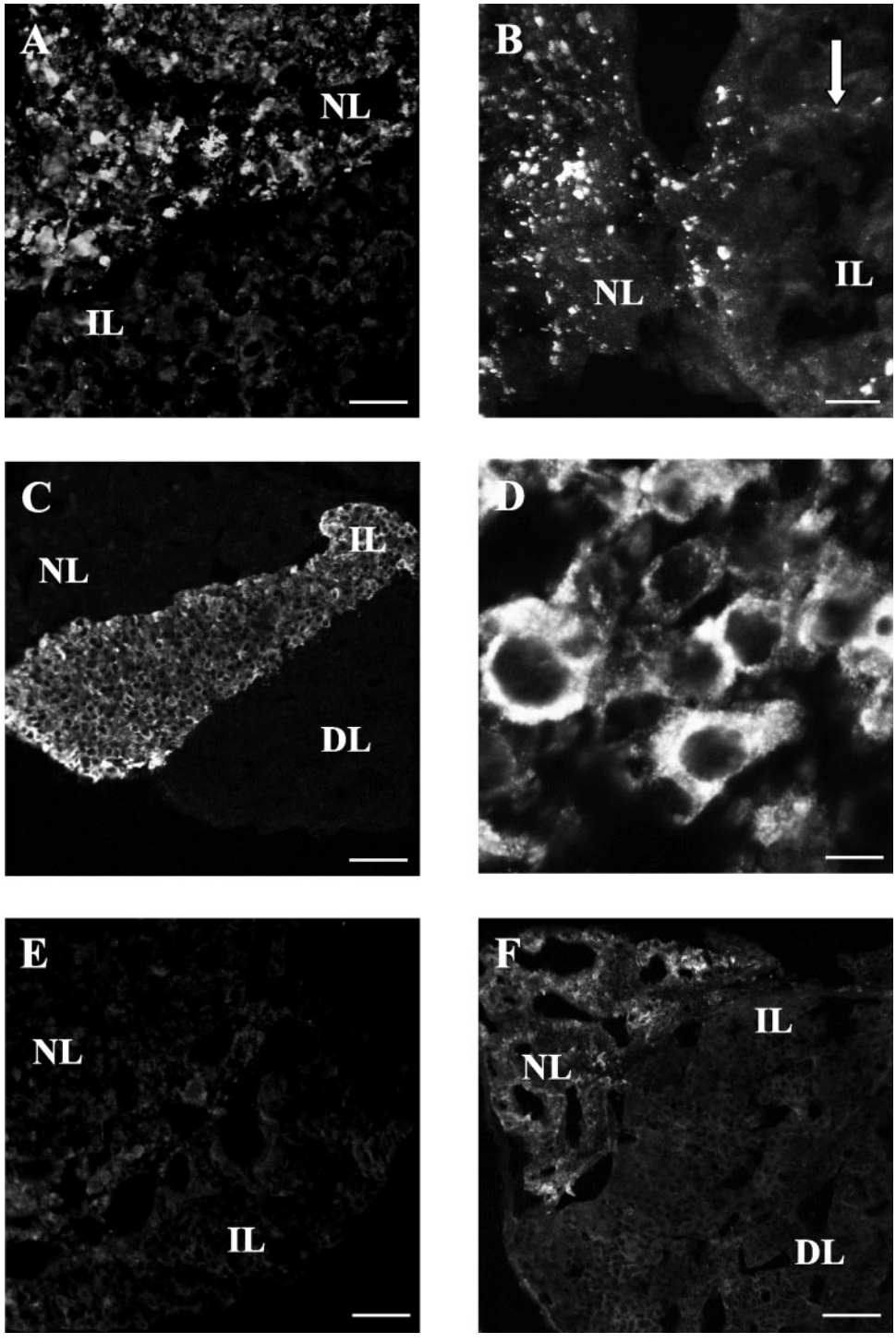

FIG. 5. Immunofluorescence photomicrographs of sagittal sections of the frog pituitary showing the distribution of SS1- and PSS2$_{54-66}$-immunoreactive structures. A and B, Sections incubated with the SS1 antiserum. The neural lobe (NL) exhibited numerous immunoreactive fibers, whereas the melanotrope cells of the intermediate lobe (IL) were virtually devoid of immunoreactive material (A). At a higher magnification, a few somatostatin-positive fibers were detected in the intermediate lobe (IL) at the boundary of the neural lobe (*arrow*) (B). C and D, Sections incubated with the PSS2$_{54-66}$ antiserum. Almost all melanotrope cells of the IL were strongly immunostained, whereas the NL and the DL were totally devoid of immunoreactivity (C). Confocal laser scanning microscope analysis of melanotrope cells showed that the immunoreactive material exhibited a granular aspect and was restricted to the cytoplasm (D). E and F, Specificity controls. When the SS1 antiserum was preabsorbed with synthetic SS1 (10^{-6} M), no labeling was observed in the NL and IL (E). When the SS1 antiserum was preabsorbed with SS2 (10^{-6} M), immunostaining was still present in the NL (F). *Scale bars*, A and E, 20 μm; B and D, 5 μm; C and F, 50 μm.

retention time of 25 min and 31 min. These two immunoreactive peptides coeluted with synthetic SS1 and SS2, respectively (Fig. 6A). The PSS2$_{54-66}$ RIA detected a predominant immunoreactive peak that eluted at 28 min (Fig. 6B).

Effect of background adaptation on PSS2 and POMC gene expression

The expression of PSS2 and POMC mRNA in the intermediate lobe of the pituitary was compared in white- and black-adapted frogs by Northern blot analysis. No significant difference in PSS2 mRNA level was detected between the two groups of animals ($P > 0.05$; Fig. 7A). In contrast, dark background adaptation provoked a significant increase ($P < 0.01$) in POMC mRNA level (Fig. 7B).

Effect of SS2 on α-MSH release

As previously reported (33), exposure of perifused frog neurointermediate lobes to TRH (10^{-8} M) induced a marked

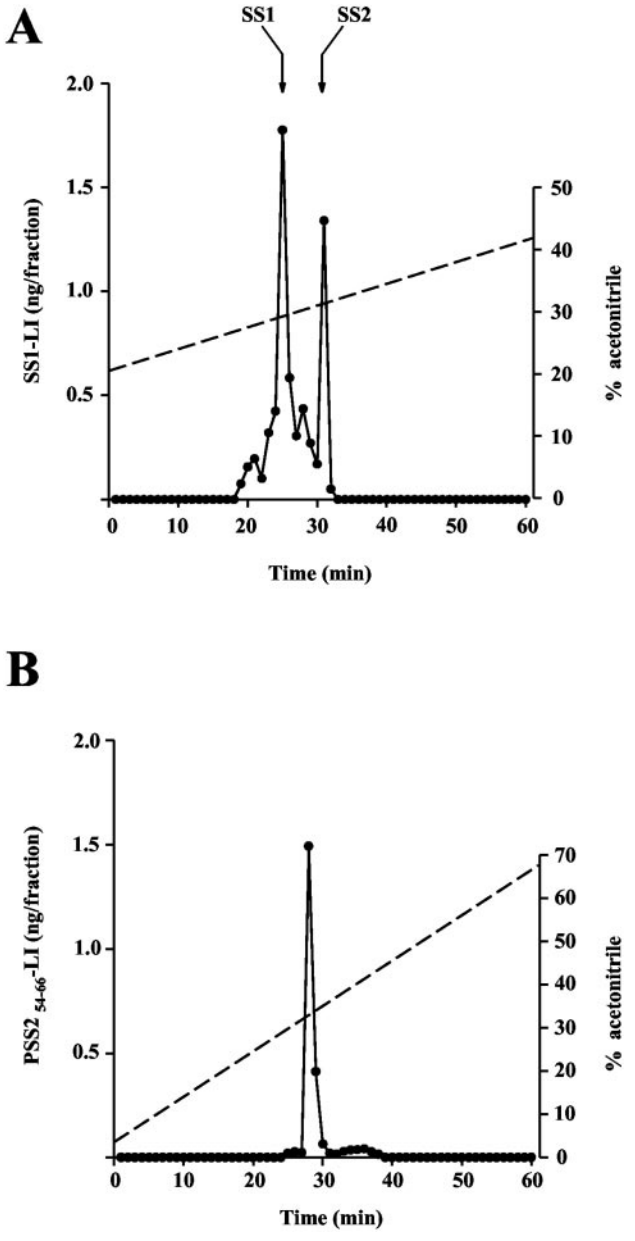

FIG. 6. Reversed-phase HPLC analysis of SS1- (A) and PSS2$_{54-66}$ -like immunoreactivity (B) in the frog pituitary. Tissue extracts were pre-purified on Sep-Pak C_{18} cartridges and chromatographed on a reversed-phase C_{18} HPLC column. Fractions (1 ml each) were collected, dried, and assayed for SS1 and PSS2$_{54-66}$ content. The *arrows* indicate the elution position of the synthetic standards. The *dashed lines* show the concentration of acetonitrile in the eluting solvent.

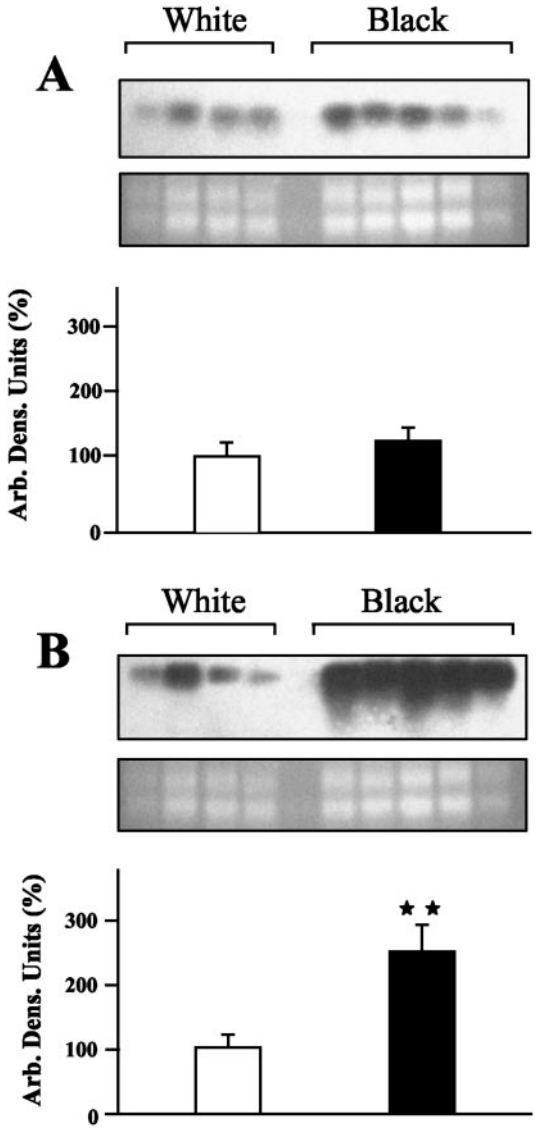

FIG. 7. Effect of background adaptation on PSS2 and POMC mRNA levels in the neurointermediate lobe of the frog pituitary. Total RNA from 4 or 5 pools of 10 neurointermediate lobes from white- or black-adapted animals was hybridized with the ^{32}P-labeled PSS2 (A) or POMC (B) probe, and the blots were autoradiographed. The autoradiograms with the corresponding ethidium bromide-stained ribosomal RNA are shown. The histograms represent the mean densitometry of the autoradiograms from the 4 or 5 groups of animals adapted either to *white* or *black* background. Values are mean arbitrary densitometric units (± SEM) expressed as percentages of white-adapted animals. Ribosomal 18S RNA was quantified to correct for RNA loading variations.

stimulation of α-MSH release. In contrast, synthetic SS2 (10^{-6} M) did not modify α-MSH secretion (Fig. 8A). During infusion of SS2, the stimulatory effect of TRH was not affected (Fig. 8B).

Discussion

Previous reports have shown that, in frog, the concentration of somatostatin-immunoreactive material is in the same range in the neurointermediate lobe as in the hypothalamus (34, 35). However, immunohistochemical studies have only revealed the presence of somatostatin-positive fibers in the frog pars nervosa (36) that could not account for the large amount of immunoreactive peptide found in neurointermediate lobe extracts. The recent discovery of the existence of two somatostatin isoforms in the European green frog, *i.e.* somatostatin-14 (SS1) and [Pro^2,Met^{13}]somatostatin-14 (SS2; Refs. 16 and 20), led us to re-examine this issue. Here, we demonstrate that biosynthesis of SS2, but not SS1, occurs specifically in melanotrope cells of the frog pituitary.

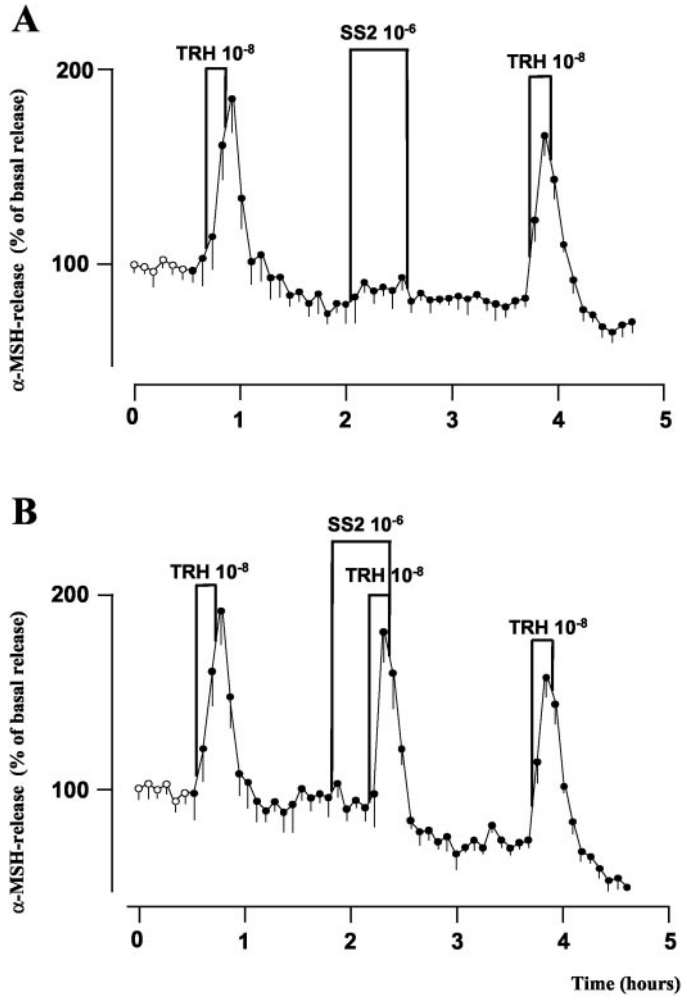

FIG. 8. Effect of frog SS2 on α-MSH secretion from perifused frog neurointermediate lobes. A, Synthetic frog SS2 (10^{-6} M) was infused between two pulses of TRH (10^{-8} M; 10 min) to verify that NILs were responding correctly. B, A pulse of TRH was administered during infusion of SS2. The profiles represent the mean (± SEM) secretion pattern of four independent perifusion experiments. The spontaneous level of α-MSH release (100% basal level) was calculated as the mean α-MSH concentration in six consecutive fractions preceding the first pulse of TRH (O-O; 30 min). The mean basal level of α-MSH release was 117 ± 24 pg/ml per NIL.

Expression of the PSS2 gene in the frog pars intermedia

Using Northern blot analysis, PSS1 mRNA was found neither in the distal lobe nor in the neurointermediate lobe, and PSS2 mRNA was not detected in the distal lobe of the pituitary. In contrast, intense expression of the PSS2 gene was observed in the neurointermediate lobe. *In situ* hybridization histochemistry confirmed that the PSS2 transcripts were not detected in the distal lobe and revealed that PSS2 mRNA is exclusively expressed in the intermediate lobe of the pituitary. Although the existence of an SS2 ortholog, called cortistatin (CST), has now been documented in mammals (21–23) and fish (17–19), transcription of the gene encoding this somatostatin variant in the pituitary has never been reported before. The present study thus provides the first evidence for selective and discrete expression of the PSS2 gene in the intermediate lobe of the pituitary.

Cellular localization of PSS2 in the frog pars intermedia

The pars intermedia of the frog pituitary is composed of two distinct populations of cells: the endocrine melanotrope cells that secrete POMC-derived peptides and glial-like cells called folliculo-stellate cells (37). Recent studies conducted in mammals indicate that the somatostatin-14 precursor gene is expressed in folliculo-stellate cells (13), whereas another hypothalamic hypophysiotropic neurohormone, PACAP, is expressed in melanotrope cells (38). It was thus important to determine which cell type expresses the PSS2 gene in the frog pars intermedia. To solve this question, we have raised antibodies against a tridecapeptide derived from PSS2, *i.e.* $PSS2_{54-66}$. This peptide sequence was chosen because $PSS2_{54-66}$ exhibits a very low degree of similarity to frog somatostatin-28_{1-12} (Fig. 1) so that the antibodies should not cross-react with any PSS1-derived peptides. A sensitive RIA was developed, and it was found that the antibodies did not show any cross-reactivity with various peptides present in melanotrope cells such as α-MSH, octadecaneuropeptide, PACAP, joining peptide, and secretoneurin. The fact that hypophysial extracts produced displacement curves that were parallel to the standard curve revealed that peptides immunologically related to PSS2 are actually present in the pituitary.

The $PSS2_{54-66}$ antibodies were then used to determine the cellular localization of the immunoreactive material in the frog pituitary. Intense labeling was observed throughout the intermediate lobe, whereas the distal lobe and the neural lobe were totally devoid of immunoreactivity. Confocal laser scanning microscope analysis revealed that $PSS2_{54-66}$-like immunoreactivity was restricted to the cytoplasmic domain of melanotrope cells. In contrast, the SS1-like immunoreactivity was contained in nerve fibers located in the neural lobe and in a few terminals innervating the intermediate lobe. Consistent with this latter observation, somatostatin-14 positive fibers have previously been visualized in the pars intermedia of fish (39–41) and amphibians (36). In rat, somatostatin-14-like immunoreactivity has been visualized in folliculo-stellate cells of the pars intermedia (13).

Processing of PSS2 in the frog pars intermedia

The mammalian ortholog of PSS2, pro-cortistatin (PCST), possesses two dibasic sites in its C-terminal region (21). Transfection of the PCST cDNA in AtT-20 cells has recently shown that these two cleavage sites are used so that processing of PCST can generate both CST-14 and its elongated form CST-29 (42). Unlike mammalian PCST, frog PSS2 only possesses one dibasic site located upstream the SS2 sequence (20), and the mature peptide SS2 has been isolated from the frog brain (16). To investigate whether PSS2 expressed in pars intermedia cells is actually processed to generate mature SS2, the immunoreactive peptides were characterized by combining HPLC analysis and RIA quantification. Using a RIA for SS1 that cross-reacts with SS2, two immunoreactive peptides, which exhibited the same retention as synthetic SS1 and frog SS2, were resolved. The presence of SS1 in neurointermediate lobe extracts is consistent with the occurrence of numerous SS1-immunoreactive terminals in the pars nervosa. Using the $PSS2_{54-66}$ RIA, a single peak that likely cor-

responds to PSS2$_{1-66}$ was resolved. The presence of both SS2 and PSS2$_{54-66}$-like immunoreactivity in neurointermediate lobe extracts indicates that the Arg-Lys processing site located upstream SS1 is efficiently cleaved by prohormone convertases (PCs). Because PC1 and PC2 are actively expressed in the frog pars intermedia (43–47), either one or the other enzyme may be responsible for the processing of PSS2 in melanotrope cells.

Physiological implications

In amphibians, melanotrope cells play a pivotal role in skin color adaptation (37). When animals are placed on a dark background, the transcription of the POMC gene is strongly activated (48) and the expression of several other genes, including chromogranins (49, 50), PC2 (51), and 7B2 (52) is also stimulated. Conversely, the present study has shown that the level of PSS2 mRNA in the frog pars intermedia is not affected during background color adaptation, indicating that the expression of POMC and PSS2 are not coordinately regulated.

The functional significance of SS2 produced by melanotrope cells is totally unknown. To explore this issue, we have investigated the possible role of SS2 on melanotrope cell activity. We found that SS2 had no effect on spontaneous or TRH-induced α-MSH secretion by perifused frog neurointermediate lobes, indicating that the peptide does not exert paracrine or autocrine activities. Consistent with this observation, we have previously found that SS1 does not affect α-MSH release in *R. ridibunda* (35). Therefore, it appears that neither SS1 released by nerve terminals in the vicinity of melanotrope cells nor SS2 produced by melanotrope cells themselves are involved in the control of the α-MSH secretion.

The pars intermedia of the pituitary is poorly vascularized, and it is generally accepted that the peptide hormones secreted by melanotrope cells must diffuse to the adenohypophysis before being released into the systemic circulation. Thus, after reaching the distal lobe, SS2 may control the secretion of anterior pituitary hormones. In agreement with this hypothesis, we have recently shown that SS2, like SS1, is able to compete for [^{125}I-Tyr^0,D-Trp^8]S-14 binding in the distal lobe of the frog pituitary, and that both SS1 and SS2 inhibit cAMP formation and GH release from cultured frog adenohypophysial cells (53). These data, together with the occurrence of a dense accumulation of somatotrope cells in the frog distal lobe at the boundary of the intermediate lobe (54), strongly suggest that SS2 released by melanotrope cells, in addition to SS1 released by hypothalamic nerve fibers in the median eminence, may contribute to the regulation of GH secretion (Fig. 9).

In conclusion, the present data have shown that the PSS2 gene, but not the PSS1 gene, is actively expressed in the frog pars intermedia. Immunohistochemical labeling combined with confocal laser scanning microscope analysis revealed that PSS2-like immunoreactivity is located in the cytoplasm of melanotrope cells. Biochemical characterization indicated that the immunoreactive peptide is indistinguishable from synthetic frog SS2. Collectively, these data suggest that frog melanotrope cells secrete the somatostatin variant [Pro^2,Met^{13}]somatostatin-14 that may contribute, together with hypothalamic SS1, in the control of GH secretion. Thus, the present study demonstrates for the first time that the gene encoding a hypophysiotropic neuropeptide is intensively expressed in the intermediate lobe of the pituitary and that the corresponding precursor is fully processed to generate the mature neuropeptide.

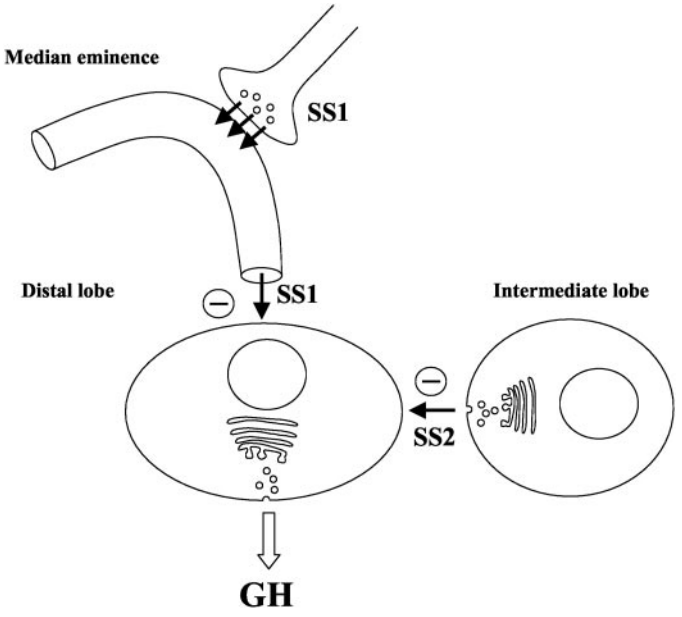

FIG. 9. Schematic representation summarizing the possible contributions of SS1 (from hypothalamic origin) and SS2 (released by melanotrope cells) in the control of GH secretion. Numerous somatotrope cells are located in the frog distal lobe close to the intermediate lobe (54). Both SS1 and SS2 compete for [^{125}I-Tyr^0,D-Trp^8]S-14 binding on frog pituitary slices (53). Both SS1 and SS2 inhibit cAMP formation and GH secretion from frog adenohypophysial cells (53). Because the intermediate lobe of the pituitary is very poorly vascularized, secretory products released by melanotrope cells diffuse toward the distal lobe before being released into the circulation. Thus, SS2 produced by melanotrope cells may contribute to the regulation of GH release by somatotrope cells.

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