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In Situ Hybridization of Putative Somatostatin mRNA Within Hypothalamus of the Rat Using Synthetic Oligonucleotide Probes

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The distribution of mRNA with high sequence homology to somatostatin mRNA within the periventricular hypothalamus of rat was assessed using in situ hybridization techniques with synthetic oligodeoxyribonucleotide probes, complementary to the 3′ coding region of rat somatostatin mRNA. The probes (22- and 24-mers) were 5′-end labeled using T4 polynucleotide kinase and γ-^{32}P-ATP. They were used either individually or after ligation with T4 DNA ligase to form a 46-mer. Serial tissue sections (< 10 μm) were taken from the level of the preoptic/anterior hypothalamus through the paraventricular hypothalamus. In situ hybridizations were conducted at room temperature in hybridization buffer. Neurons immunoreactive with antiserum raised against somatostatin were identified in alternate sections using standard immunocytochemical procedures. The anatomical location of the hybridization signal was determined by autoradiography. Our results show that the peri- and paraventricular hypothalamus is rich in transcripts putatively coding for somatostatin and that these transcripts are co-distributed with neurons immunoreactive with antisomatostatin immunoglobulin.

Key words: in situ hybridization, somatostatin, mRNA, immunocytochemistry, hypothalamus

Somatostatin is a tetradecapeptide, originally isolated from bovine hypothalamus as a modulator of growth hormone release [1]. Since then, neurons recognized by antisomatostatin immunoglobulin have been found not only within neurosecretory cells of the periventricular hypothalamus [2,3] but also in extrahypothalamic regions of the central nervous system [4,5]. These findings suggest that the function of somatostatin may not be limited to the neuroendocrine system.

Immunocytochemistry, however, is not a reliable means of identifying sites of somatostatin synthesis. A better approach is to identify somatostatin mRNA directly in brain tissue sections by in situ hybridization with labeled DNA probes [6,7,8]. The

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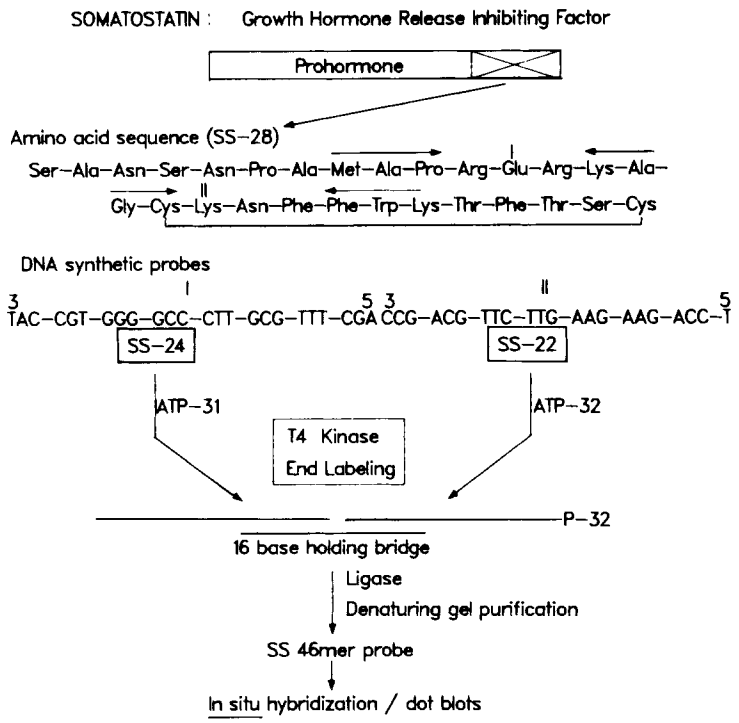

Fig. 1. Schematic illustration of the probe sequence and ligation reaction used to construct the 46-base oligonucleotide probe (SS = somatostatin).

recent determination of a cDNA sequence of preprosomatostatin from rat medullary thyroid carcinoma [9] has made it possible to synthesize specific oligonucleotide probes for use in such experiments.

In this study, the distribution of putative somatostatin mRNA within the periventricular portion of the preoptic area and paraventricular nucleus of the rat was examined using synthetic DNA probes complementary to the 3′ coding region of rat somatostatin mRNA (Fig. 1). The pattern of hybridization within these regions was compared to the distribution of neurons immunoreactive with antiserum which recognizes the C-terminal portion of somatostatin. Throughout this report, the use of the terms somatostatin mRNA and somatostatin immunoreactivity implies their putative nature.

MATERIALS AND METHODS

Preparation of Oligonucleotide Probes

Oligodeoxyribonucleotides (22- and 24-mers) were synthesized on a Model 380A DNA synthesizer (Applied Biosystems, Inc.), and purified by reverse phase HPLC on a μBondapak C_{18} column (Waters Associates) [10]. Oligonucleotides were 5'-end labeled using T4 polynucleotide kinase and γ-^{32}P-ATP [11]. The ^{32}P-labeled probes were either used individually, or after ligation, with the aid of a 16-mer holding-fragment and T4 DNA ligase to form a 46-mer, as shown in Figure 1. All probes had final activities of 1–5 $\times$ 10^8 cpm/μg.

In Situ Hybridization Histochemistry

Hybridization procedures were adopted from those published previously [6–8, 12–15]. Adult Sprague-Dawley rats were perfused transcardially with phosphate-buffered saline (PBS), followed by phosphate-buffered 10% formalin. The brains then were removed and placed in PBS containing 30% sucrose overnight at 4°C. Transverse 5–10 μm sections were cut from the hypothalamus on a cryostat and mounted on slides that had been dipped briefly in sterile diethylpyrocarbonate (50 μl/250 ml H_2O). The slide-mounted sections then were dipped briefly in the diethylpyrocarbonate solution, rinsed twice with sterile, distilled, deionized H_2O and incubated for 15 min at 37°C with proteinase K (1 μg/ml in 20 mM Tris-HCl, 2 mM $CaCl_2$, pH = 7.2). In some experiments, alternate sections were incubated at 37°C for 30 min with ribonuclease I (10 μg/ml in 50 mM Tris-HCl, 10 mM NaCl, and 10 mM EDTA, pH = 8.0). The slides were delipidated with absolute ethanol, rehydrated, and then rinsed in sterile, distilled, deionized H_2O. The washed slides then were placed in a hybridization buffer consisting of 0.3 M NaCl, 0.03 M sodium citrate, (pH = 7.0), 0.2% Ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 50% deionized formamide, 0.1% SDS, 0.1% denatured salmon sperm DNA and 0.1% denatured yeast RNA. Twenty μl of ^{32}P-labeled probe (1,000–50,000 cpm in hybridization buffer) was applied to each section for an overnight incubation at room temperature (23°C). The slides were rinsed repeatedly in 0.3 M NaCl, 0.03 M sodium citrate (pH = 7.0), air dried, and exposed to X-ray film (Kodak XAR-5 or SB) for three to seven days. After the X-ray films were processed, the tissue sections were stained with cresyl violet to determine the anatomical location of the hybridization signal.

Immunocytochemical Localization of Somatostatin Immunoreactivity

Alternate sections were mounted on slides and processed for immunocytochemistry with antisomatostatin (Immunonuclear) using the avidin-biotin method [16,17] and diaminobenzidine as a chromogen.

RESULTS

The hybridization signal obtained with the short probes (22- and 24-mers) was distinctly observable along the wall of the third ventricle in the preoptic area (Fig. 2A). More caudally, hybridization also was observed in the paraventricular nucleus. The distribution of somatostatin-immunoreactive neurons within the periventricular region (Fig. 2B) corresponds to the location of the hybridization signal.

Utilization of the 46-base probe permitted reproducible localization of somatostatin mRNA along the wall of the third ventricle from the level of the optic chiasm to that of the ventromedial nucleus. A substantial amount of hybridization was observed within the paraventricular nucleus (Fig. 3). The pattern of hybridization seen with the 46-mer (Fig. 4A) in the periventricular region was comparable to the distribution of somatostatin-immunoreactive perikarya in alternate sections (Fig. 4B). Hybridization with this probe was also observed in the neocortex, pyriform cortex, and the amygdaloid complex, regions previously shown to contain somatostatin-immunoreactive neurons [17]. On the other hand, hybridization was not observed in the cerebellar cortex and regions of the prosencephalon, where somatostatin immunoreactivity has not been identified. In addition, hybridization did not occur in tissue pretreated with ribonuclease I.

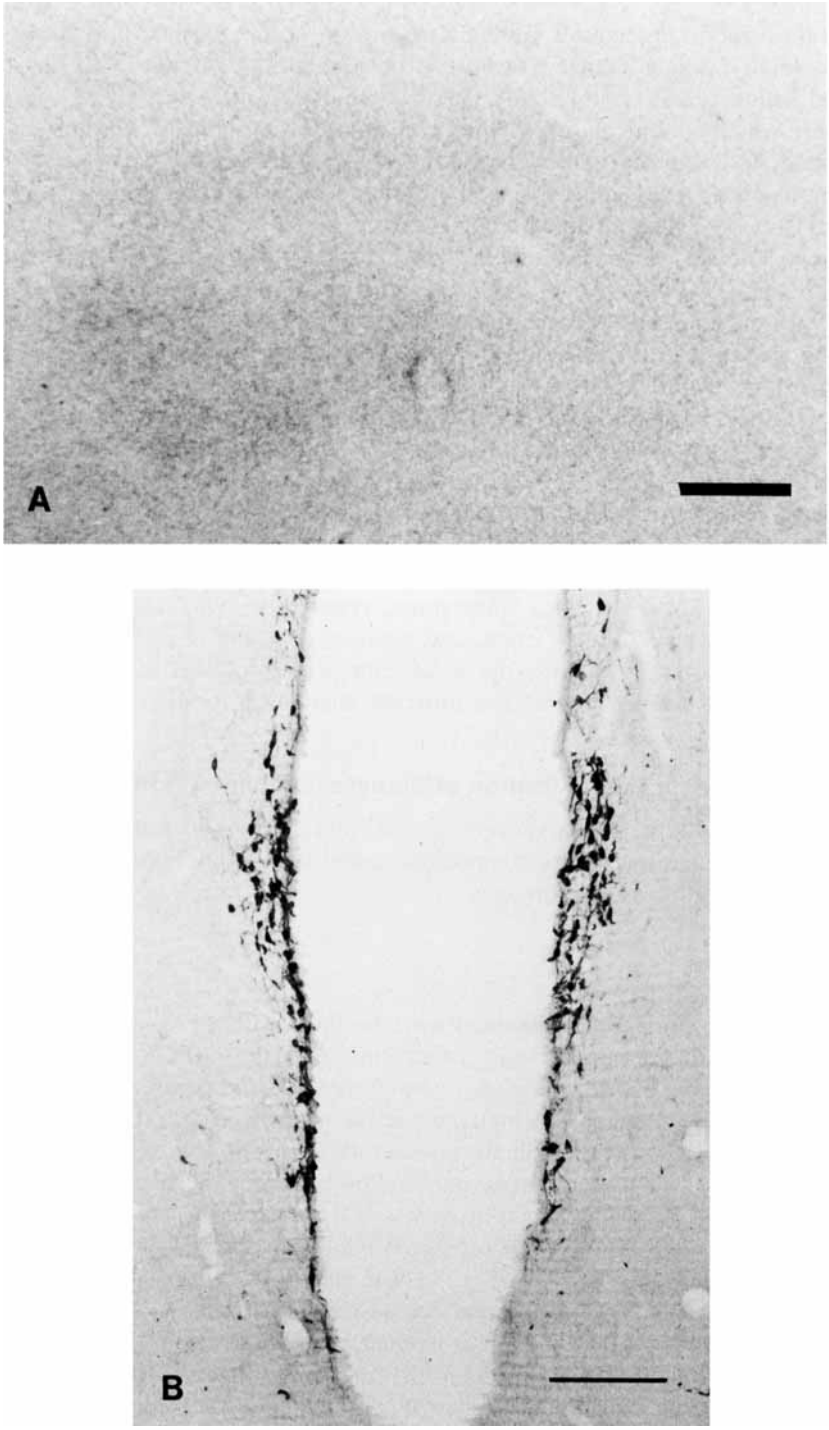

Fig. 2.

DISCUSSION

In this study, we used ^{32}P-labeled synthetic DNA probes to localize somatostatin mRNA within the hypothalamus of the rat. Both the short (22- and 24-mers) probes and the longer 46-mer gave a hybridization signal with the greatest intensity in the periventricular hypothalamus, an area that contains a high density of somatostatin-immunoreactive neurons. The absence of hybridization signal in tissue sections pretreated with ribonuclease I confirmed that the hybridization was specific for RNA. The inability to detect hybridization in tissue regions where somatostatin immunoreactivity was not observed suggests that the observed signal was specific for somatostatin mRNA.

The 46-nucleotide probe gave the best resolution of the three probes. It consistently hybridized to mRNA in the periventricular preoptic area as well as in the paraventricular nucleus. In contrast, the shorter probes produced less robust results, with hybridization often limited to the preoptic area. The difference in the signal-to-

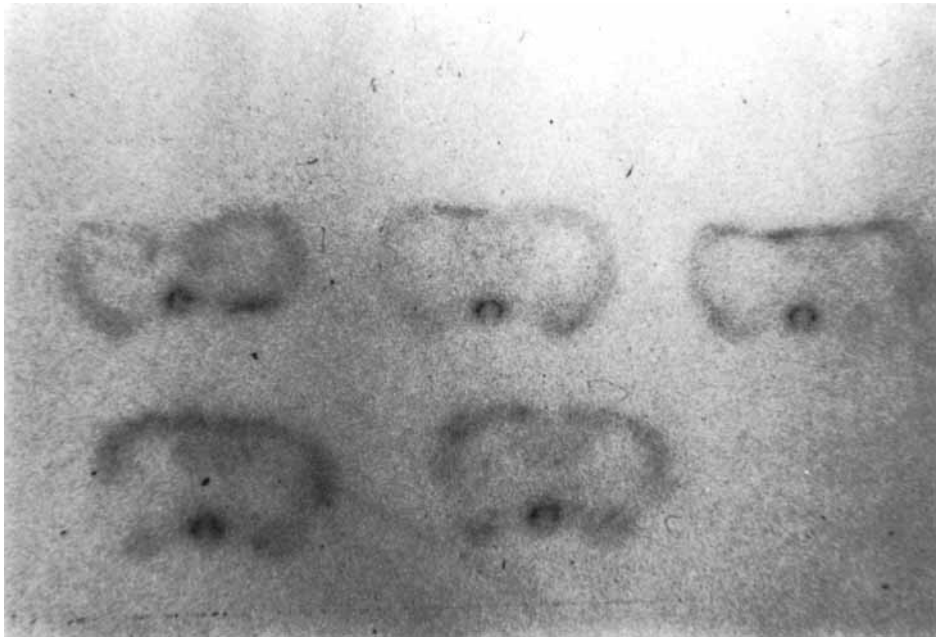

Fig. 3. In situ hybridization with a ^{32}P-labeled 46-base synthetic probe for somatostatin mRNA. Autoradiograph of serial 5 μm tissue sections (50,000 cpm/section), including the paraventricular nucleus. Note the hybridization signal in the periventricular portion of this nucleus.

Fig. 2. In situ hybridization with a 24-base synthetic probe for somatostatin mRNA. A) Autoradiograph of a tissue section (5 μm) incubated with ^{32}P-labeled 24-mer (2000 cpm/section). Note the hybridization signal along the wall of the third ventricle and in the neocortex, particularly the pyriform lobe. (Similar results have been obtained with the 22-mer) Bar = 2mm. B) Somatostatin-immunoreactive cells, surrounding the third ventricle of an adult rat, detected in an alternate 5 μm section by peroxidase-antiperoxidase immunocytochemistry. Bar = 200μm.

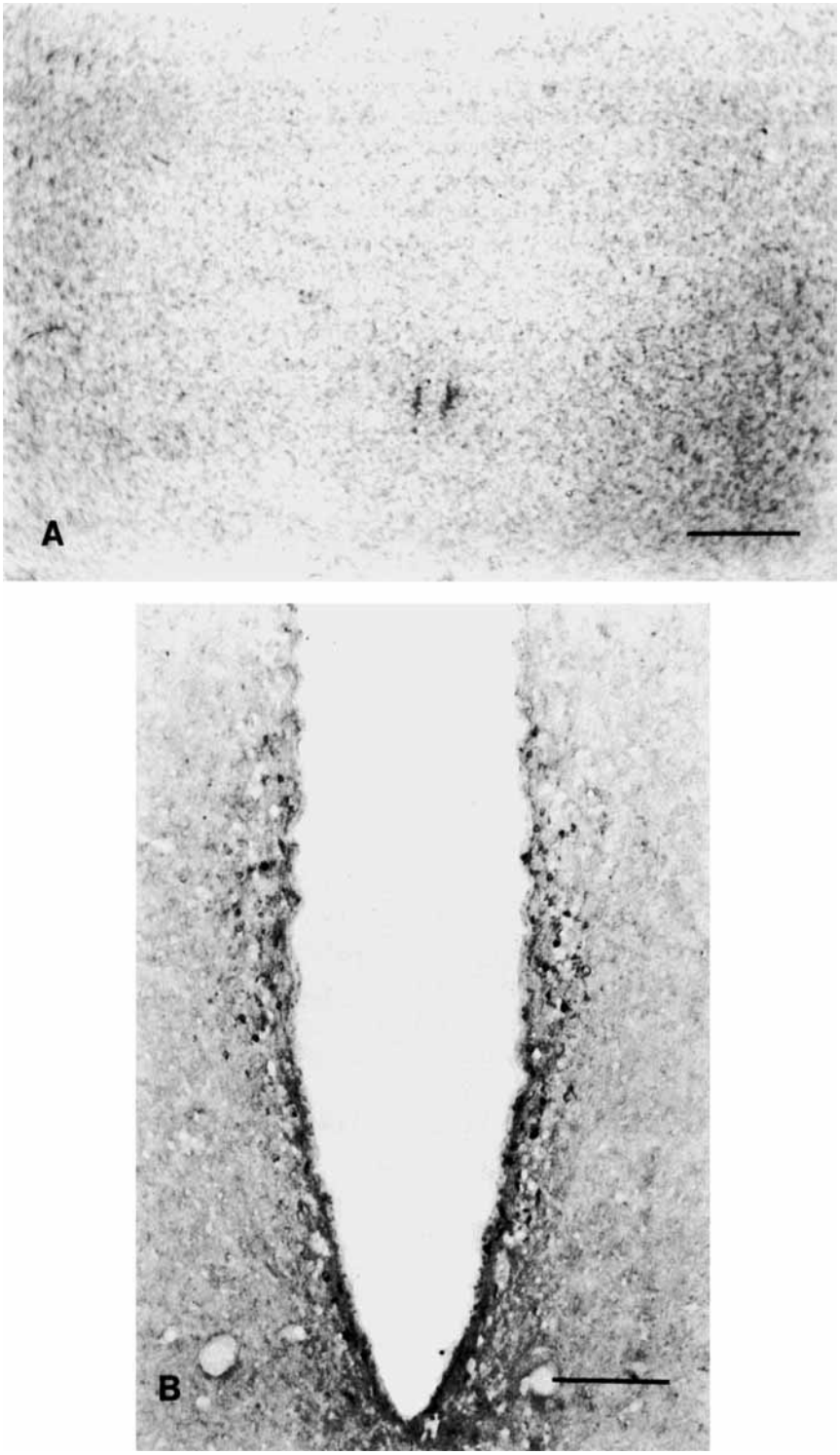

Fig. 4.

noise ratio observed between the short and long probes might reflect differences in mRNA levels between these regions.

Although the coexistence of somatostatin mRNA and somatostatin immunoreactivity within the hypothalamus, or even within individual hypothalamic neurons, implies a transcript-product relationship, it is not proof of such a relationship. Cells and neurons may actively take up peptides or contain untranslated mRNA. Other authors [6,7] have made similar implications concerning the sites of biosynthesis of proopiomelanocortin-related products within the pituitary.

Although these data indicate discrete anatomical sites for somatostatin biosynthesis, they provide no evidence as to the final active peptide synthesized within these regions, as somatostatin-14 and somatostatin-28 are derived from the same prohormone [18]. The presence of hybridization signal within the peri- and paraventricular hypothalamus nevertheless suggests that the transcripts necessary to code for these peptides is present within these hypothalamic regions.

In conclusion, the use of a rapid autoradiographic method to identify somatostatin mRNA in discrete brain regions has been demonstrated in this study. Similar achievements in the pituitary using ^{32}P-labeled cDNA probes have been described previously [8]. One important consequence of these techniques may be the ability to study the regulation of neuropeptide biosynthesis in discrete brain regions at the level of mRNA and, perhaps ultimately, the gene.

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Fig. 4. In situ hybridization with a 46-base oligonucleotide probe for somatostatin mRNA. A) Autoradiograph of a tissue section (7 μm) incubated with ^{32}P-labeled 46-mer (2000 cpm/section). This section includes the paraventricular nucleus. The hybridization signal is limited to the region surrounding the third ventricle. Bar = 2mm. B) Somatostatin-immunoreactive neurons along the wall of the third ventricle detected by the avidin-biotin method in an alternate 7 μm section. Bar = 200μm.

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