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IDENTIFICATION OF A PUTATIVE HYPOTHALAMIC mRNA CODING FOR SOMATOSTATIN AND OF ITS PRODUCT IN CELL-FREE TRANSLATION

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SUMMARY

Poly(A)-containing RNA from rodent hypothalamic tissue has been used to direct the synthesis of polypeptides in cell-free systems derived from wheat germ extract and rabbit reticulocyte lysate in the presence of [^{35}S]-L-cysteine. Immunoprecipitation of translation products with antiserum to somatostatin followed by sodium dodecylsulfate gel electrophoresis demonstrated the existence of a 15,000 dalton polypeptide species which was displaceable by synthetic somatostatin. In addition, hybridization of fractionated hypothalamic poly(A)-RNA, blotted against DBM-paper, with a probe containing a synthetic gene for somatostatin resulted in specific hybridization of a 550 nucleotide RNA species to the probe. These results suggest that the primary translation product for hypothalamic somatostatin is a 15,000 dalton polypeptide species.

INTRODUCTION

Somatostatin is a tetradecapeptide originally isolated from ovine hypothalamus as a hypophysiotropin which inhibited the release of growth hormone by the pituitary(1). Subsequent studies have suggested that this oligopeptide is widely distributed in the central and peripheral nervous systems and in other sites in the body, where it has been shown to exhibit a variety of inhibitory actions such as inhibition of the secretion of growth hormone and thyrotropin by the pituitary, glucagon and insulin by the pancreas, and various gastrointestinal hormones(2). Knowledge about the biosynthesis of somatostatin is necessary in order to understand the regulatory processes involving this peptide. To date, there is limited information on this subject; in vitro pulse-chase biosynthetic studies in

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fish pancreatic islets(3,4), and limited molecular weight characterization of constitutive rodent hypothalamic peptides recognized by anti-somatostatin antisera(5,6,7) all suggest that somatostatin is synthesized as part of a higher molecular weight precursor. However, no cell-free studies on the biosynthesis of a somatostatin precursor have been reported. In this communication, we report the demonstration of a polypeptide of molecular weight 15,000 immunoprecipitable by anti-somatostatin antisera whose synthesis is directed by rat hypothalamic poly(A)-RNA in wheat germ and rabbit reticulocyte cell-free systems and the correspondence of the molecular weight of this polypeptide species to the size (550 nucleotides) of a hypothalamic poly(A)-RNA species which specifically hybridizes with a plasmid bearing a synthetic gene for somatostatin.

MATERIALS

Oligo (dT)-cellulose (T_3) was purchased from Collaborative Research; guanidine-HCl was obtained from EM Laboratories; wheat germ was a gift from General Mills. Protein A-Sepharose CL 4B was purchased from Pharmacia. PMSF was obtained from Sigma. [^{35}S]-L-cysteine at 618 Ci/mmole was from New England Nuclear, as was the rabbit reticulocyte lysate (Leucine Translation Kit KO857 AWlot), α[^{32}P]ATP (3000 Ci/mmole), Protosol and Omnifluor. NBPC was purchased from Pierce Chemical Company. Synthetic somatostatin was from Peninsula.

METHODS

RNA Isolation

Total cytoplasmic RNA was prepared from rat or mouse hypothalami by guanidine-HCl extraction, and poly(A)-RNA by oligo (dT)-cellulose chromatography of the total RNA as previously described(8). The poly(A)-RNA so obtained was dissolved in sterile water, aliquoted and stored at -20°. Each poly(A)-RNA preparation was titrated for translation activity and only those preparations exhibiting at least 10 fold stimulation of [^{3}H]-L-leucine incorporation into TCA‡ precipitable radioactivity over endogenous levels were used for immunoprecipitation experiments.

Cell-Free Translation and Characterization of Translation Products

Translation of rodent hypothalamic poly(A)-RNA was carried out in a rabbit reticulocyte lysate system (New England Nuclear) as described by Pelham and Jackson(9). [^{35}S]-L-cysteine was used at 10μCi per 25μl of total incubation mixture. Aliquots of the total translation products were analyzed for TCA precipitable [^{35}S] radioactivity following urea treatment(10) and by

‡Abbreviations: ABM, azobenzyloxy; DBM, diazobenzyloxy; PMSF, phenylmethyl sulfonyl fluoride; TCA, trichloroacetic acid; SDS, sodium dodecylsulfate; IgG, immunoglobulin G; NBPC, 1-[(m-Nitrobenzyloxy) methyl] pyridinium chloride

SDS-polyacrylamide slab gel electrophoresis(11). The total translation product was then incubated with the IgG fraction (Protein A-sepharose purified (12)) of anti-somatostatin antisera in the presence and absence of synthetic somatostatin at a concentration known to displace maximally ^{125}I-somatostatin at the antibody concentration used. The antibody concentrations employed were higher than those used for radioimmunoassay (bound/total ^{125}I-somatostatin = 75%). We tested A-804, kindly provided by Dr. A. Arimura, rabbit anti-somatostatin kindly provided by Dr. J. B. Martin and S-39 and S-203 kindly provided by Dr. W. Vale. Of these, only S-203 gave rise to a significant increase in incorporation of radioactivity relative to non-immune IgG and to displaceability by cold somatostatin (results not shown). The immunoprecipitates were purified by protein A-sepharose column chromatography(8), the acid eluate (antigen) from the column was lyophilized and redissolved in Laemli buffer(11) and analyzed by SDS-polyacrylamide slab gel electrophoresis in 15% gels(11), using the following molecular weight standards: albumin (69K), catalase (60K), ovalbumin (46K), carbonic anhydrase (29.5K), chymotrypsin (25K), lysozyme (14K), ribonuclease (13K) and cytochrome C (12.3K) Gels were visualized by fluorography as described by Laskey and Mills(13).

Hybridization Analysis

The RNA fractions obtained from the high salt eluate of the oligo dT column (non-poly(A) species) and from the low salt eluate (poly(A)-enriched species) both from hypothalamus and from extra-hypothalamic brain were subjected to 1.5% agarose-acrylamide slab gel electrophoresis following glyoxal denaturation(14). The separated RNA species were then blotted onto DBM-paper according to the procedure of Alwine et al(15). The DBM-paper was prepared starting with commercial NBPC and Whatman 540 paper as described by the authors(15) and was stored in the form of ABM paper at 4^{0} and activated by dithionite just before use. Each batch of paper was tested before use by measuring the direct binding of ^{32}P-labeled PBR322 DNA(16)(1cm^{2} of paper exhibited 70% maximum binding in the range of 1-25µg DNA). As hybridization probe we used the plasmid containing the nucleotide sequence coding for somatostatin prepared synthetically(17). The purified pSom-I plasmid was nick-translated to a final specific activity of 5 x 10^{8} cpm/µg. After transfer of the RNA to the DBM-paper, the paper was incubated for 24 hours with hybridization buffer(15)(50% formamide, 0.75 M NaCl, 75 mM trisodium citrate, 25-50 mM sodium phosphate, pH 6.5, 0.2% SDS)(18), containing 1%(w/v) glycine. Hybridization was then performed with the ^{32}P-labeled pSom-I previously sonicated and denatured (100^{0}, 5 min), in 5 ml of hybridization buffer at 42^{0} in sealed plastic bags for 48 hours. The paper was then subjected to further washing in a buffer composed of 50% formamide, 0.75 M NaCl, 75 mM trisodium citrate, 25-50 mM sodium phosphate, pH 6.5, 0.2% SDS, blotted dry and exposed to x-ray film with a Dupont intensifying screen.

RESULTS

As shown in Fig. 1, translation of rodent hypothalamic poly(A)-RNA in the rabbit reticulocyte and wheat germ systems in the presence of [^{35}S]-L-cysteine gave rise to the synthesis of a spectrum of labeled proteins over a wide molecular weight range, attesting to the activity of the RNA preparation and the ability of the cell-free system to reflect the polypeptides encoded by the hypothalamic poly(A)-RNA. Also shown in Fig. 1 is the appearance on the

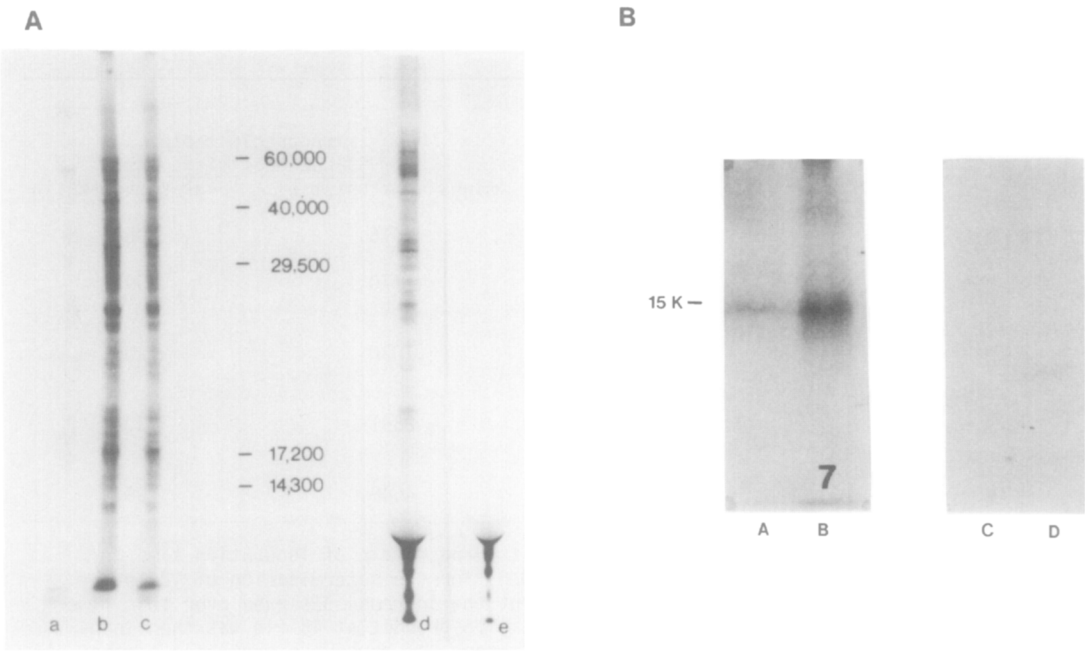

Figure 1: Fluorographs of SDS-polyacrylamide slab gel analysis of cell-free translations (12.5% acrylamide). A: spectrum of total translation products using [^{35}S]-L-cysteine as precursor amino acid (a-c in wheat germ system); a: proteins formed in the absence of exogenous RNA; b: proteins formed using 5µg of rat hypothalamic mRNA/50µl incubation mixture; c: proteins formed using 5µg/50µl incubation of mouse hypothalamic mRNA (d,e in rabbit reticulocyte lysate system); d: proteins formed using 5µg rat hypothalamic mRNA/50µl incubation volume; e: proteins formed in the absence of exogenous RNA. Molecular weight standards do not apply to d and e. The leading edge of the gel in d and e reflects the high concentration of globin proteins in the reticulocyte lysate system; B: electrophoresis of purified immunoprecipate derived from anti-somatostatin IgG addition to post-ribosomal supernatant following translation of rat hypothalamic mRNA in the wheat germ system in the presence of [^{35}S]-L-cysteine; A: product obtained from translation of 20µg rat hypothalamic mRNA; B: product obtained from translation of 40µg rat hypothalamic mRNA (in each case pooled products from translations of 5µg mRNA/50µl incubation mixture); C: same as A, except that non-immune IgG at same dilution was used in place of anti-somatostatin IgG; D: same as A, except that 2µg of purified globin mRNA was used in place of rat hypothalamic mRNA.

slab gel of a cysteine labeled polypeptide species of 15,000 molecular weight when the translation products were subjected to immunoprecipitation with the Vale S203 anti-somatostatin IgG fraction. The appearance of this band on the gel was both RNA and IgG specific (Fig. 1). The immunoprecipitation of cysteine radioactivity from the translation products was also sensitive to the presence of unlabeled synthetic somatostatin (Table I), when hypothalamic poly(A)-RNA was used to direct the synthesis of polypeptides in the reticu-

Table I. CELL-FREE TRANSLATION OF HYPOTHALAMIC POLY(A)-RNA IN THE RABBIT RETICULOCYTE LYSATE SYSTEM*

Addition	Increase in TCA Precipitable [^{35}S] Radioactivity, cpm	Immunoprecipitable [^{35}S] Radioactivity, cpm	
		minus somatostatin	plus somatostatin
no mRNA	1,092	916	950
globin mRNA	46,922	1170	993
whole rat brain Poly(A)-RNA	67,840	1030	1200
rat hypothalamic Poly(A)-RNA	70,978	2591	1307
mouse hypothalamic Poly(A)-RNA	241,105	4548	1504

*Duplicate aliquots of 2µl each were taken at 0 and 45 min of incubation time for each sample and treated with urea (see METHODS) for the determination of TCA precipitable cpm. The values presented represent the increase observed over this time interval. The remainder of the total translation product at 45 min was then incubated with the antibody in the presence and absence of exogenous synthetic somatostatin and the immunoprecipitate harvested and counted as specified in METHODS.

losyte lysate system. Neither globin mRNA nor extrahypothalamic rat brain poly(A)-RNA gave rise to the incorporation of significant radioactivity into the immunoprecipitate relative to the control with no added RNA in the reticulocyte system, even though these exogenous RNAs stimulated the incorporation of radioactivity into a TCA precipitable form (Table I). However, both rat and mouse hypothalamic poly(A)-RNA presented a stimulation of incorporation of radioactivity into the immunoprecipate, relative to the control with no exogenous RNA, which could be displaced by the prior addition of cold somatostatin (50% and 66% displacement for rat and mouse RNA, respectively).

Analytical gel electrophoresis of 25µg of glyoxal-denatured RNA in 1.5% agarose was performed on RNA obtained from both rat and mouse hypothalamus, as well as extrahypothalamic brain. After blotting of the RNA gel onto DBM-paper and hybridization with a plasmid DNA bearing the synthetic somatostatin gene(17), a signal visible after two weeks exposure was observed only in those lanes where poly(A)-RNA from hypothalamus was present (Fig. 2A) and not in the high salt eluate of the oligo dT column from hypothalamic or any

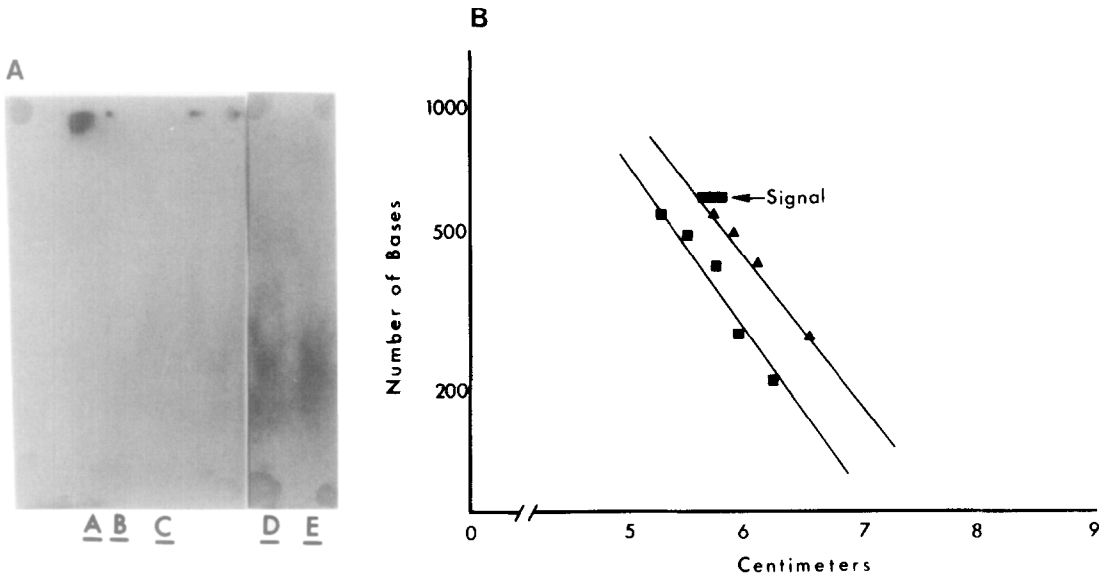

Figure 2: A: hybridization of ^{32}P-labeled pSomI DNA with glyoxal-denatured hypothalamic RNA fractionated on 1.5% agarose acrylamide gel and blotted onto DBM-paper
A: high-salt eluate of oligo-dT chromatography of extra-hypothalamic brain RNA (rat)
B: low-salt eluate of oligo-dT chromatography of extra-hypothalamic RNA (rat)
C: high-salt eluate of oligo-dT chromatography of hypothalamic RNA (rat)
D: low-salt eluate of oligo-dT chromatography of hypothalamic RNA (rat)
E: low-salt eluate of oligo-dT chromatography of hypothalamic RNA (mouse)
B. distance migrated by Hae III digested fragments of PBR322 in presence (■) and absence (▲) of glyoxal treatment. The area corresponding to the hybridization signal of ^{32}P-labeled pSomI DNA with hypothalamic poly(A)-RNA bound to DBM-paper is denoted by ▬

of the RNA forms of extra-hypothalamic brain. The molecular weight of the RNA which hybridized corresponded to a species of approximately 550 nucleotides (Fig. 2B).

DISCUSSION

In these studies, we have attempted to provide evidence for the existence and molecular weight of a precursor form of hypothalamic somatostatin using the approach of cell-free translation and hybridization analysis of hypothalamic poly(A)-RNA.

In cell-free translation studies, we found a 15,000 molecular weight polypeptide species which is labeled with [^{35}S]-L-cysteine and specifically

immunoprecipitated with an anti-somatostatin antibody. Studies attempting to define a precursor in fish pancreas, utilizing a pulse-chase paradigm, have specified a molecular weight of ca 12-13,000(3,4). Radioimmunoassay of fractionated extracts of rat hypothalamus also suggest the existence of a 12,000 molecular weight form of the peptide(6), but a similar analysis of mouse hypothalamic extracts proposes a molecular weight of 15,000(7). In these latter studies, however, the column chromatographic methods used, in which broad peaks were observed, may not be exact enough for this specification. Our results from hybridization analysis corroborate the molecular weight determined by translation studies: using a synthetic probe for hybridization of nucleic acids, an mRNA species of approximately 550 nucleotides was found, and suggests the existence of a short (ca 100 bases) non-coding region in the somatostatin mRNA.

The net burden of the evidence regarding the molecular weights of larger forms of somatostatin would seem to suggest that the single polypeptide chain forms found in in vivo biosynthetic experiments and extracts of tissue are smaller than that observed in our cell-free studies, and that a terminal extension which is removed co-translationally probably exists.

We must emphasize that in both approaches we have used probes which do not correspond to an exact matching between reagent and product; i.e. the antibody was raised against mature somatostatin (MW 1400), where the level of cross reactivity with the precursor is unknown, and the probe which was synthesized in terms of the genetic code in bacteria presents a theoretical match of 65% minimum and not exceeding 75% with rodent hypothalamic mRNA. These two factors make difficult a calculation of the level of somatostatin mRNA present in hypothalamus. Our results do suggest, however, that, on the basis of hybridization analysis, the hypothalamus is a site of synthesis of somatostatin, and that the concentrations of "extra-hypothalamic somatostatin" mRNA in the CNS are less than in the hypothalamus.

While further information, derivable by studies utilizing recombinant DNA techniques, is necessary to consider the structure of the hypothalamic

somatostatin precursor, it is clear that "mature" somatostatin represents only about 10% of the precursor molecule. It will be of interest to see if the somatostatin precursor contains other biologically active peptides, as observed with the opiocortin system(19).

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