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Biochemistry

Cell-free biosynthesis of somatostatin precursors: Evidence for multiple forms of preprosomatostatin*

(mRNA from islets of Langerhans/*in vitro* protein synthesis/formylmethionine-tRNA$_f$/two-dimensional gels)

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ABSTRACT **It has been demonstrated [Shields, D. (1980) J. Biol. Chem. 255, 11625–11628] that mRNA isolated from the islets of Langerhans codes for two preprosomatostatin molecules of apparent molecular weights 18,000 and 19,000, respectively. Here evidence is presented that *in vitro* translation of pancreatic islet mRNA in two different cell-free protein-synthesizing systems directs the synthesis of up to nine distinct forms of somatostatin-immunoreactive polypeptides. The multiplicity of the preprosomatostatin molecules was the result of initiation of translation from separate species of mRNA as demonstrated by amino-terminal labeling with *N*-formyl-[^{35}S]Met-tRNA$_f^{Met}$. Translation of islet mRNA isolated from different individual animals showed that all of the preprosomatostatin polypeptides were present amongst the cell-free products, which implies that the multiple forms were not due to genetic variation in the wild population. Based on their apparent molecular weights and distinctly different isoelectric points, the different preprosomatostatin molecules could be classified into two major families. These results suggest that the anglerfish preprosomatostatins are encoded by separate mRNA species and are consistent with the existence of a multigene family for somatostatin.**

Somatostatin is a 14-amino acid polypeptide hormone that was originally isolated from the hypothalamus (1) and has been found since in many tissues, including the endocrine pancreas (2), where it functions to inhibit the secretion of insulin and glucagon. It is now evident that somatostatin is synthesized as part of a larger precursor, prosomatostatin, that undergoes a series of proteolytic processing steps to yield the mature hormone (3–6). To investigate the primary events in the biosynthesis of somatostatin precursors, we have translated mRNA isolated from anglerfish (*Lophius americanus*) islets of Langerhans *in vitro* and demonstrated two distinct precursors to somatostatin, designated preprosomatostatins, of M_rs 18,000 and 19,000, respectively (7, 8). Peptide mapping experiments established that the mature somatostatin is located at the carboxyl terminus of each precursor. Similar results have been obtained by Goodman *et al.* (9, 10), who also showed that anglerfish intestinal mRNA codes for only a single M_r 16,000 preprosomatostatin; its relationship to the islet translation products is unknown at present.

By using recombinant DNA techniques, the complete amino acid sequence of two preprosomatostatin molecules, I and II, of 121 and 125 amino acids, respectively, has been determined from anglerfish islets (11). The mature hormone sequence in preprosomatostatin I was identical to that of the mammalian hypothalamus, whereas preprosomatostatin II differed at two residues: tyrosine-7 (replaced by phenylalanine) and glycine-10 (replaced by tyrosine). Furthermore, there was little homology in the predicted amino acid sequence of these two precursors, which showed significant sequence divergence in both the signal peptide and "proregions". Goodman *et al.* (12) also have analyzed a cDNA library from anglerfish islet mRNA and predicted the sequence of a single 119-amino acid preprosomatostatin that was almost identical to preprosomatostatin I except for a continuous stretch of 19 amino acids (residues 11 through 29). These results suggested that there could be several mRNA species coding for preprosomatostatin. To investigate this possibility, we have extended our earlier studies (7, 8) and here present evidence for at least seven to nine distinct forms of preprosomatostatin amongst the cell-free translation products of islet mRNA. Our data are consistent with the interpretation that the precursors to somatostatin are encoded by a multigene family.

MATERIALS

L. americanus pancreatic islets were purchased from S. Testaverde (Biofish Associates, Gloucester, MA). Somatostatin antiserum S203 (specific for residues 5–8) was a gift from W. Vale and RS-16 (specific for residues 8–14) was a gift from P. Davies. Synthetic somatostatin was a gift from R. Guillemin. L-[^{35}S]Methionine was from Amersham/Searle at the highest specific activity available. Calcium leucovorin was purchased from Lederle Laboratories (Pearl River, NY). *Escherichia coli* tRNA synthetase was a gift from U. Maitra. Carrier Ampholines were purchased from LKB.

METHODS

Most of the methods used for these experiments have been described, including mRNA isolation, cell-free protein synthesis (13, 14), and antibody precipitation (7).

Preparation of *N*-Formyl-[^{35}S]Met-tRNA$_f^{Met}$. *N*-Formyl-[^{35}S]Met-tRNA$_f^{Met}$ was prepared from rabbit liver tRNA exactly as described by Dubnoff and Maitra (15). The degree of formylation was assessed by translating bovine pituitary mRNA *in vitro*, in the absence and presence of microsomal membranes. In the presence of membranes, no radioactively labeled products were observed, whereas, in the absence of membranes, both preprolactin and pre-growth hormone were evident. These results are consistent with the removal of a signal peptide labeled only at its amino-terminal methionine residue and demonstrated that no radioactive methionine was incorporated into internal positions.

Gel Electrophoresis. NaDodSO$_4$ gel electrophoresis using a 15–20% gradient of polyacrylamide (13) was performed in the absence or presence of 7 M urea in the resolving gel. Isoelectric focusing and two-dimensional electrophoresis were done essentially by the method of O'Farrell (16).

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* Portions of these data were presented at the 21st Meeting of the American Society for Cell Biology, November 9–13, 1981, Anaheim, CA (24).

RESULTS

The islets of Langerhans from anglerfish are a rich source of mRNA coding for the precursors to the polypeptide hormones insulin, glucagon, and somatostatin. A small number of major translation products are synthesized in the wheat germ cell-free system (Fig. 1*A*, lane 1) and these correspond to (*i*) a M_r 11,000 preproinsulin (13), (*ii*) three (two of M_r 14,000 and one of M_r 16,000) preproglucagons (17), and (*iii*) M_rs 18,000 and 19,000 preprosomatostatins (7, 8). To improve the resolution of these translation products, urea was included in the $NaDodSO_4$ gel electrophoresis system (Fig. 1*A*). With increasing concentrations of urea (up to 7 M), the M_r 18,000 polypeptide was seen to migrate as a doublet consisting of a major and minor component (Fig. 1*A*, lanes 2 and 3, upward pointing arrows). Because this new polypeptide migrated very closely to the M_r 18,000 preprosomatostatin, it was a candidate for being a minor form of this precursor. To test this possibility, we treated the translation products with anti-somatostatin antiserum S203 (which has been shown to effect precipitation of both the M_r 18,000 and 19,000 preprosomatostatins), and the resulting immunoprecipitates were resolved on gels containing 7 M urea (Fig. 1*B*, lane 3, arrows). Four polypeptides were immunoprecipitated: the M_r 19,000 preprosomatostatin, the M_r 18,000 doublet, and a fourth polypeptide of apparent M_r 16,000. The presence of unlabeled somatostatin completely abolished antibody binding to these translation products (Fig. 1*B*, lane 4), suggesting that they all contained a somatostatin-like polypeptide sequence. Comparison of these immunoreactive polypeptides with those obtained with another, higher titer somatostatin antiserum (RS-16) showed (Fig. 1*B*, lane 5) similar results; in addition, the M_r 16,000 polypeptide was particularly evident. Most likely the M_r 16,000 preprosomatostatin was not previously observed (7, 8) because the S203 antiserum was unable to distinguish it above background, whereas RS-16 (a higher titer antiserum) significantly enhanced its precipitation relative to the M_r 18,000 precursor (compare lanes 3 and 5 in Fig. 1*B*). It is noteworthy from this analysis, that four separate forms of preprosomatostatin were detected.

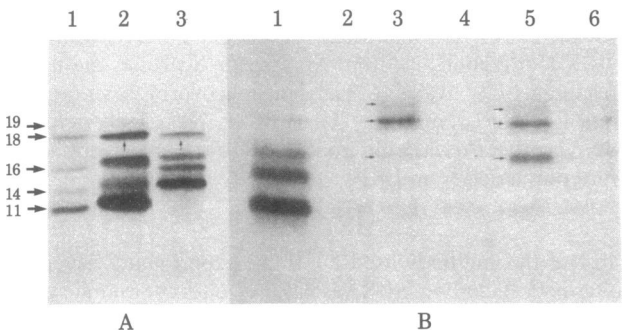

FIG. 1. *In vitro* biosynthesis of islet preprosomatostatins. Islet mRNA (1 A_{260} unit/ml) was translated in the wheat germ system containing 350 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine per ml, and the translation products were either prepared for electrophoresis directly (*A*) or were treated with anti-somatostatin antibodies (*B*). The products were then analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis with a gradient of 15–20% acrylamide. M_rs are shown $\times 10^{-3}$ with the autoradiographs of the dried gels. (*A*) Effect of urea on the resolution of islet mRNA translation products. Total translation products were applied to a gel lacking urea (lane 1) or to gels containing 4 M urea (lane 2) or 7 M urea (lane 3). The arrows (lanes 2 and 3) indicate the minor form of the M_r 18,000 preprosomatostatin. (*B*) Analysis of somatostatin-immunoreactive translation products by $NaDodSO_4$/polyacrylamide gels containing 7 M urea. Total translation products were treated with two antisera to somatostatin (S203 and RS-16) or with nonimmune serum. Lanes: 1, total translation products; 2, products treated with nonimmune serum; 3, immunoprecipitated products with S203 antiserum (arrows indicate the preprosomatostatins of M_rs 19,000, 18,000, and 16,000, respectively; 4, as lane 3 except that the immunoprecipitation reactions contained 10 μg of unlabeled somatostatin; 5, products precipitated by RS-16 antiserum (arrows as in lane 3); and 6, as lane 5 except that RS-16 antiserum was used in the presence of 10 μg of unlabeled somatostatin.

To characterize the M_r 16,000 somatostatin-immunoreactive polypeptide and further test the specificity of the immunoprecipitation reactions shown in Fig. 1, competition experiments were performed. This approach has been used to successfully identify the M_r 18,000 preprosomatostatin (7) and multiple forms of preproglucagon (17). Total radiolabeled translation products were incubated with RS-16 antiserum in the presence of increasing amounts of unlabeled somatostatin covering a millionfold concentration range, and the immunoprecipitates were resolved upon $NaDodSO_4$/polyacrylamide gels (Fig. 2). With increasing concentrations of unlabeled somatostatin, there was a diminution in antibody binding to all three major forms of the somatostatin-immunoreactive polypeptide (Fig. 2 *Upper*, arrows). In this experiment the minor form of the M_r 18,000 preprosomatostatin was not well resolved and was not analyzed as a separate component. Quantitative analysis of the major immunoprecipitated polypeptides by liquid scintillation assay after solubilization of the gel slices (Fig. 2 *Lower*) showed that

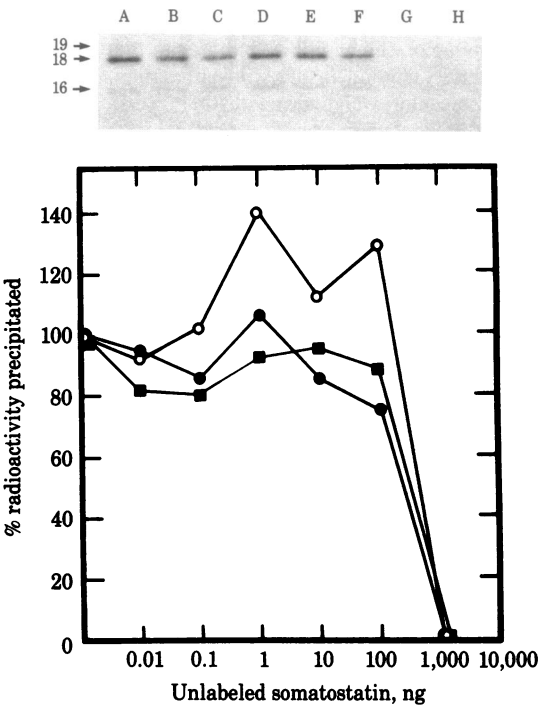

FIG. 2. Competition between the M_rs 19,000, 18,000, and 16,000 preprosomatostatins and unlabeled somatostatin for binding by anti-somatostatin antibodies. (*Upper*) Equal aliquots equivalent to 100,000 cpm of radioactivity of wheat germ translation products were treated with 2 μl of RS-16 antiserum as described (7) in the presence of no somatostatin (lane A), 0.01 ng of unlabeled somatostatin (lane B), 0.1 ng (lane C), 1 ng (lane D), 10 ng (lane E), 100 ng (lane F), 1 μg (lane G), and 10 μg (lane H). The immunoprecipitates were resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis. M_rs are shown $\times 10^{-3}$. (*Lower*) After location by autoradiography, the individual bands were excised from the dried gel and prepared for liquid scintillation assay (17). Competition curves for the M_r 19,000 preprosomatostatin (■), the M_r 18,000 preprosomatostatins (not separately resolved) (○), and the M_r 16,000 precursor (●) are shown. The data for 100% precipitation was the radioactive material precipitated by RS-16 antiserum in the absence of competitor: 1,000 cpm (M_r 19,000), 3,000 cpm (M_r 18,000), and 1,600 cpm (M_r 16,000).

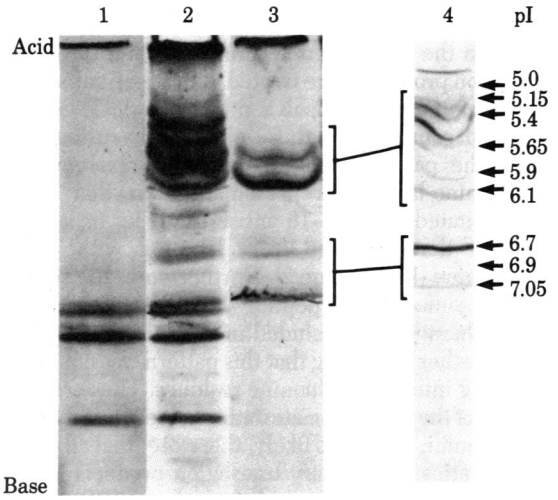

FIG. 3. Analysis of islet translation products by isoelectric focusing. Islet translation products synthesized in the wheat germ system were treated with either RS-16 antiserum (lanes 3 and 4) or anti-glucagon antiserum (lane 1) or were prepared directly for isoelectric focusing (lane 2). The samples in lanes 1, 2, and 3 were analyzed on an isoelectric focusing gel with Ampholines that produced a gradient from pH 4.0 to 8.0, and those in lane 4 were analyzed on a pH gradient from 4.9 to 8.5. The apparent pIs of the somatostatin immunoprecipitates (lane 4) are shown.

all of the somatostatin-immunoreactive polypeptides exhibited parallel competition curves, including slight enhancement of immunoprecipitation in the presence of 1–10 ng of unlabeled somatostatin. In addition, the amount of unlabeled somatostatin needed to cause 50% inhibition of antibody binding was almost identical for the three immunoprecipitates examined. On the basis of this data and our previous demonstration by peptide mapping that the M_r 18,000 and M_r 19,000 polypeptides were preprosomatostatins, we concluded that the M_r 16,000 immunoprecipitable polypeptide was also a precursor to somatostatin. Together with the data from Fig. 1, these results suggested that there could be four forms of preprosomatostatin.

In view of the cryptic nature of the islet somatostatin precursor polypeptides (Fig. 1), the putative multiplicity of the preprosomatostatins was analyzed by both isoelectric focusing (Fig. 3) and two-dimensional gel electrophoresis (Fig. 4). Translation products obtained from both the wheat germ and rabbit reticulocyte lysate cell-free systems were treated with RS-16

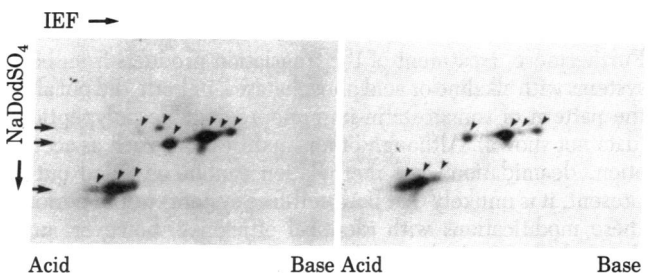

FIG. 4. Analysis of somatostatin-immunoreactive polypeptides by two-dimensional gel electrophoresis. Translation products synthesized in the wheat germ (*Left*) or the rabbit reticulocyte system (*Right*) were treated with RS-16 antiserum and analyzed by isoelectric focusing (IEF), followed by $NaDodSO_4$/polyacrylamide gel electrophoresis with gradients of 15–20% acrylamide. Arrows indicate the spots corresponding to somatostatin-immunoreactive polypeptides. Note the absence of spots corresponding to the M_r 19,000 preprosomatostatin (pI ≈ 5.9) in the reticulocyte system. The acidic end of the isoelectric focusing gel is at the left.

antiserum, and the immunoprecipitates were analyzed. The isoelectric focusing gel was able to resolve the immunoprecipitated preprosomatostatins into a number of distinct species (Fig. 3, lanes 3 and 4) which range in apparent isoelectric point from pH 5 to pH 7: four or five major and four minor preprosomatostatins were noted on each of two different isoelectric focusing gels. It should be noted that loading the samples from the basic end of the isoelectric focusing gel produced exactly the same pattern of preprosomatostatin molecules. For comparison, the immunoprecipitates obtained from treating the wheat germ translation products with glucagon antibodies were also analyzed (Fig. 3, lane 1); in this case, three major forms of preproglucagon were identified. Furthermore, these precursors had distinctly different isoelectric points (ranging from pH 6.8 to 8) from those of the preprosomatostatin molecules. These results are consistent with our previous work indicating at least two or three preproglucagon molecules (17).

To correlate the major preprosomatostatin polypeptides identified by isoelectric focusing with those identified using $NaDodSO_4$/gel electrophoresis, the immunoprecipitates were analyzed by two-dimensional gel electrophoresis (Fig. 4). It can be seen that the M_r 16,000 and M_r 18,000 preprosomatostatins represent several discrete polypeptides. Furthermore, the different preprosomatostatin molecules fall into two major "species": (*i*) those with an apparent M_r of 18,000, consisting of 3 or 4 forms with a pI range of 6–7; and (*ii*) those of M_r 16,000, of which three different isoelectric forms were detected with a pI range of 5–6. In addition the M_r 19,000 preprosomatostatins synthesized in the wheat germ system consisted of two isoelectric forms of pI 5.7–5.9. However, the latter peptides were absent from the translation products obtained from the reticulocyte lysate and were not detected by two-dimensional gel electrophoresis. The explanation for the apparent lack of translation of the M_r 19,000 preprosomatostatin mRNA in the reticulocyte cell-free system is unclear but may be related to inefficiency of ribosome binding or poor initiation under our *in vitro* conditions. It has been shown for example that the relative amounts of thyroid-stimulating hormone mRNA translation products are influenced significantly by the ionic conditions of the cell-free system (18); the possibility that a similar situation could exist for somatostatin mRNA is currently being investigated. Apart from this difference, the two-dimensional electrophoretic patterns of the preprosomatostatins synthesized in the wheat germ and reticulocyte lysate systems were identical. These results suggest that the different isoelectric forms of preprosomatostatin were not due to nonspecific modifications of the primary translation products by the cell-free systems.

The mRNA preparation routinely used in the foregoing experiments was obtained from pooled pancreatic islets obtained from many animals. It could be argued, therefore, that the multiplicity of preprosomatostatins reflects genetic variation or heterogeneity in the wild population of fish and represents translation products derived from different alleles that reside at one or two genetic loci. To test this possibility, the somatostatin-immunoreactive translation products obtained from islet mRNA isolated from individual animals (obtained from different geographical regions at different times of the year) were examined by isoelectric focusing (Fig. 5). It can be seen that the mRNA extracted from two individual animals directed the synthesis of about eight species of immunoprecipitable preprosomatostatins that were identical to those obtained from the pooled mRNA (compare lane 1 with lanes 2 and 3 in Fig. 5). Although this analysis is not exhaustive, isoelectric focusing can be used as a stringent test of genetic variation (19); hence, this result strongly suggested that multiple forms of preprosomatostatins were not the result of genetic variation in the wild

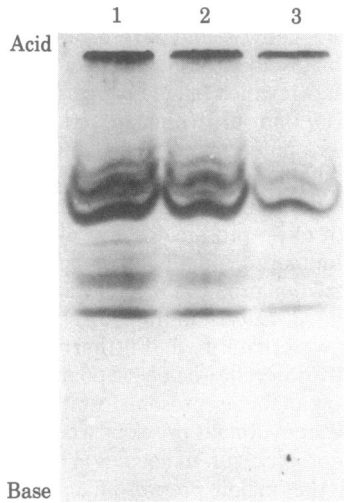

FIG. 5. Identification of preprosomatostatins synthesized from individual islet mRNA's. mRNA was isolated from the islets of two individual animals and translated in the wheat germ system. The cell-free products were treated with RS-16 antiserum (lanes 2 and 3), and the immunoprecipitates were compared to the immunoprecipitates obtained by using total mRNA from pooled islets (lane 1). The immunoprecipitated translation products were analyzed by isoelectric focusing under the conditions outlined in Fig. 3, lane 3.

population of fish but represented the translation products of multiple mRNA species present in the islets of each individual animal.

To determine if each preprosomatostatin resulted from translation of an individual species of mRNA, we used *N*-formyl-Met-tRNA$_f^{Met}$. In eukaryotic cells the initiator tRNA is not usually formylated; however, if *N*-formyl-methionine is incorporated by use of *N*-formyl-Met-tRNA$_f^{Met}$ in a cell-free system, the presence of the formyl group prevents the enzymatic cleavage of the amino-terminal initiator methionine (20). Because the mature somatostatin molecule is located at the carboxyl terminus of the preprosomatostatin molecules (7, 9, 11), if all of the preprosomatostatin molecules were radioactively labeled from incorporation of *N*-formyl-[^{35}S]Met-tRNA$_f^{Met}$, then it could be concluded that each different precursor resulted from initiation on separate mRNAs and was not formed by proteolytic cleavage

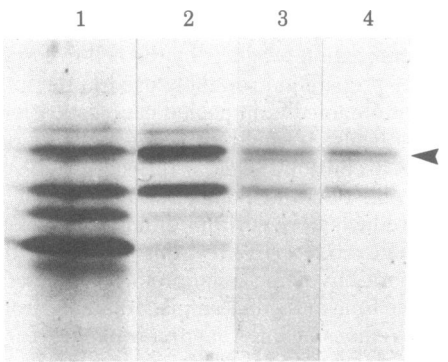

FIG. 6. Comparison of islet translation products synthesized with [^{35}S]methionine and *N*-formyl[^{35}S]methionine. Islet mRNA was translated in the wheat germ system containing either 300 μCi of [^{35}S]methionine (lane 1 and 2) or 90 μCi of *N*-formyl-[^{35}S]Met-tRNA$_f^{Met}$ (lanes 3 and 4) per ml. The incubation in lane 4 also contained 1 mM unlabeled methionine. The translation products in lanes 2, 3, and 4 were treated with RS-16 antiserum, and the immunoprecipitates were analyzed by NaDodSO$_4$ gel electrophoresis. The arrow indicates the position of the M_r 18,000 preprosomatostatins.

of a smaller number of larger molecules. *In vitro* translation was performed in the presence of *N*-formyl-[^{35}S]Met-tRNA$_f^{Met}$, and the translation products were treated with RS-16 antiserum; the immunoprecipitates were analyzed by NaDodSO$_4$ gel electrophoresis and compared to those obtained by using [^{35}S]methionine. The pattern resulting from the resolution of the [^{35}S]methionine-labeled preprosomatostatins that were immunoprecipitated with RS-16 antiserum (Fig. 6, lane 1) was identical to that obtained from labeling with *N*-formyl-[^{35}S]methionine (Fig. 6, lane 2). Furthermore, the same number of "formylmethionine peptides" were obtained when unlabeled methionine was included in the cell-free system (Fig. 6, lane 3), further indicating that this pattern was not generated from labeling internal methionine residues. These results suggest that all of the preprosomatostatins possess intact amino and carboxyl termini; it appears likely, therefore, that all of the preprosomatostatins are primary translation products of separate mRNA species and did not result from nonspecific proteolytic cleavage of a larger precursor.

DISCUSSION

Multiple Precursors to Somatostatin. It has been shown (7–9, 11) that anglerfish islet mRNA codes for two distinct forms of preprosomatostatin polypeptide. By using polyacrylamide gels of improved resolution and isoelectric focusing, we now report that the islet mRNA directs the synthesis of approximately seven to nine distinct preprosomatostatin molecules. Analysis of the translation products by two-dimensional gel electrophoresis suggested that the somatostatin immunoprecipitates fell into two major "families": one of M_r 18,000 that has four isoelectric forms (pI 6–7) and the other of M_r 16,000 that possesses three different preprosomatostatin molecules (pI 5–6). In addition, the M_r 19,000 preprosomatostatin appears to have two isoelectric forms. It might be argued that the different isoelectric forms of preprosomatostatin molecule represent nonspecific modifications of the primary translation products by components of the cell-free systems; however, this is unlikely for two reasons. First, the translation products were obtained from cell-free systems lacking microsomal membranes; consequently, none of the post-translation processing enzymes capable of core-glycosylation (for example) were present. Second, these forms are unlikely to be due to other modifications, such as phosphorylation by a protein kinase(s) present in the wheat germ system, because similar translation products were obtained with the rabbit reticulocyte lysate cell-free system, and it is unlikely that both cell-free systems would have protein kinases with identical affinities for heterologous substrates. Furthermore, treatment of the translation products from both systems with alkaline or acid phosphatases, or both, did not alter the pattern of somatostatin-immunoprecipitable polypeptides (data not shown). Although other modifications such as acetylation, deamidation, and methylation cannot be ruled out at present, it is unlikely that both cell-free systems would perform these modifications with identical efficiency; however, until detailed chemical characterization of each precursor is performed, these possibilities cannot be entirely excluded.

The finding of multiple polypeptide hormone precursors is not restricted to somatostatin. We have shown (17) that there are three preproglucagons and here showed (Fig. 3) that these represent three or four major and several minor isoelectric forms of glucagon precursor. It is also noteworthy that only one major and a minor isoelectric form of anglerfish preproinsulin was detected (data not shown)—results that correlate well with the finding of two insulin genes in several species (21). Furthermore, these observations suggest that the cell-free protein-

synthesizing systems are not randomly modifying the islet translation products to generate nonspecific isoelectric forms of these polypeptides.

Translation of islet mRNA in the wheat germ cell-free protein-synthesizing system in the presence of *N*-formyl-[^{35}S]-Met-tRNA$_f^{Met}$ showed that the same number of different forms of immunoreactive preprosomatostatin were radioactively labeled (Fig. 6). Because only the initiator methionine residue of each nascent polypeptide was labeled in this experiment, this result implies that there are distinct mRNA species coding for each preprosomatostatin. Furthermore, a structural feature of the preprosomatostatin molecule—i.e., that the mature somatostatin is located at the carboxyl terminus of each precursor (11)—allowed us to exclude the possibility that these precursors were generated by proteolysis in the cell-free system. Because all of the somatostatin precursors were radioactively labeled with *N*-formyl[^{35}S]methionine and because they were also immunoprecipitable with anti-somatostatin antisera (which could only recognize the carboxyl terminus of the antigens synthesized *in vitro*), it can be concluded that each precursor had intact amino and carboxyl termini.

Translation of islet mRNA isolated from different individual animals (Fig. 5) showed that the same spectrum of somatostatin-immunoreactive products were present in each animal as in the mixed population. This result implies that the different preprosomatostatins did not arise as a result of the heterogeneous distribution of several closely related mRNA species within the wild population. It strongly suggests that the islets of each animal within this species contains qualitatively the identical set of mRNAs coding for somatostatin precursors. Taken together, these results indicate that each different preprosomatostatin molecule is the primary translation product of a distinct species of mRNA.

A Multigene Family Coding for Multiple Preprosomatostatins. Although we do not know the physiological significance for different forms of preprosomatostatin at present, it is clear that each precursor is the result of translation of an active mRNA species and, hence, each preprosomatostatin represents the products of an actively transcribed gene. In recent years it has become apparent that several proteins are encoded by a small family of genes, each gene coding for a polypeptide that is related to the other members of the family yet clearly distinct (22). The data presented here together with those obtained from cloned islet cDNA (11) are consistent with the interpretation that the preprosomatostatins in anglerfish islets are coded for by a multigene family. Definitive proof of this interpretation however must await examination of the genomic DNA coding for these polypeptides. In this context one question of considerable interest would be to determine if the different preprosomatostatin mRNAs are each transcribed from a distinct gene or whether more than one mRNA is derived from a single gene by differential splicing events as has been found to be the case for IgM (23).

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