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CELL-FREE TRANSLATION AND PARTIAL CHARACTERIZATION OF PROENKEPHALIN MESSENGER RNA FROM BOVINE STRIATUM

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SUMMARY: The biosynthesis of the primary precursor of the enkephalins (proenkephalin) was investigated by cell-free translation of bovine striatum polyadenylated RNA. ^{35}S-Labeled proteins encoded by striatum mRNA were digested with trypsin and carboxypeptidase B, and released [^{35}S]Met-enkephalin was identified by immunoprecipitation and high-performance liquid chromatography. The apparent molecular weight of putative proenkephalin synthesized in cell-free systems was found to be M_r 31,000 ± 1000. Digestion of cell-free translated ^{3}H-labeled proteins of M_r 31,000 ± 3000 yielded both [^{3}H]Met-enkephalin and [^{3}H]Leu-enkephalin. Putative proenkephalin mRNA of striatum was found to be 1450 ± 200 nucleotides in length.

The opioid pentapeptides methionine-enkephalin (Tyr-Gly-Gly-Phe-Met) and leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu) (1) are synthesized in the form of a precursor protein (proenkephalin) which contains multiple enkephalin sequences flanked by basic amino acid residues (2-8). The size of the largest putative enkephalin precursor extracted from bovine adrenal medulla has been reported to be approximately M_r 50,000 by gel filtration (4). In contrast, the size of the most abundant putative enkephalin precursor extracted from guinea pig striatum is reported to be approximately M_r 90,000 by SDS-polyacrylamide gel electrophoresis (8). This discrepancy in size suggests that different enkephalin gene products may be synthesized in the two tissues examined.

Recently we have employed cell-free translation of messenger RNA from bovine adrenal medulla to study proenkephalin biosynthesis (9,10). We have presented evidence that the primary proenkephalin gene product of adrenal medulla has an apparent molecular weight of 31,000 ± 1000, and that the major proenkephalin mRNA of adrenal medulla has a chain length of 1450 ± 150 nucleotides (10). In the present study we have determined the sizes of the primary proenkephalin gene product and proenkephalin mRNA of bovine striatum by cell-free translation in order to determine whether markedly different proenkephalin genes are preferentially expressed in the brain and adrenal medulla.

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MATERIALS AND METHODS

Materials. Sources of materials have been described previously (10) or are indicated below. The RB-4 anti-Met-enkephalin serum, which binds Met-enkephalin, Leu-enkephalin, and Met-enkephalin sulfoxide with high affinity, has been described (10).

Preparation of mRNA. Striata were removed from bovine brains and frozen within 20 min of slaughter. Total RNA was extracted by the method of Chirgwin, et.al. (11). Poly(A)-RNA was isolated by adsorption to poly(U)-Sepharose 4B (Pharmacia) and eluted with 70% formamide. Per gram of tissue, 0.4 mg total RNA and 8 µg poly(A)-RNA were typically obtained.

Cell-free translation of mRNA. Reaction mixtures with the rabbit reticulocyte lysate contained 25 mM Hepes buffer pH 7.4, 1.2 mM $MgCl_2$, 0.17 mM EDTA, 35 mM KOAc (in addition to reticulocyte K^+ ions), 1.2 mM dithiothreitol, 8 µM hemin, 0.33 mM $CaCl_2$, 0.67 mM EGTA, 23 mM NaCl, 10 mM creatine phosphate, 37 µg/ml creatine phosphokinase (Calbiochem), 19 unlabeled amino acids (minus methionine) at 25 µM each, 0.8-1.0 mCi/ml [^{35}S]methionine (1100 Ci/mmole, New England Nuclear), 0.33 volume of micrococcal-nuclease treated (12) rabbit reticulocyte lysate (Bethesda Research Labs), 667 units/ml human placental ribonuclease inhibitor (JEM Research), 40 µg/ml rabbit liver tRNA, and poly(A)-RNA as indicated in figure legends. Reaction volumes are indicated in figure legends. Incubations were for 60 min at 37° C. The wheat germ system was employed as described (10). Protein synthesis was assayed by [^{35}S]methionine incorporation into hot 5% trichloroacetic acid-insoluble material.

Proteolysis. Reaction mixtures were diluted to 400 µl with 0.05 M Tris-HCl pH 7.5, 0.001 M $CaCl_2$, and heated for 2 min at 100° C. Samples were incubated with trypsin (EC 3.4.21.4, treated with L-(1-tosylamide-2-phenyl-ethyl chloromethyl ketone, Millipore) at 50 µg/ml for 5-6 hr at 37° C., then heated for 4 min at 100° C. They were then incubated with carboxypeptidase B (EC 3.4.12.3, treated with diisopropyl fluorophosphate, Sigma) at 2 µg/ml for 30 min at 37°C., then heated for 4 min at 100° C. Samples were centrifuged for 2 min at 9400 x g and supernatant fractions used for immunoprecipitation.

Immunoprecipitation. Mixtures (400 µl) contained cell-free translation products treated with proteases, 10 mM sodium phosphate pH 7.4, 75 mM NaCl, 0.5 mM EDTA, 0.5 mM methionine, 0.25 mg/ml bovine serum albumin, 0.5% Triton X-100, 0.8 unit/ml aprotinin (Sigma), 25 µg/ml bacitracin, 8 µl/ml RB-4 anti-Met-enkephalin serum. After incubation for 16-24 hr at 4° C., 6 mg Protein A-Sepharose CL-4B beads (Pharmacia) were added and the samples were agitated for 2-4 hr at 4°C. The beads were centrifuged at 3000 x g for 20 min through 3 ml of 1 M sucrose in Buffer A (10 mM sodium phosphate pH 7.4, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mg/ml bovine serum albumin, 1 mM methionine). The beads were washed thrice by resuspension and recentrifugation in 2 ml Buffer A, then heated for 4 min at 100° C. in 1 ml 17 µM Met-enkephalin, and finally centrifuged at 1000 x g for 10 min. The supernatant fractions were then lyophilized. The recovery of Met-enkephalin during proteolysis and immunoprecipitation was approximately 60% (10).

High-performance liquid chromatography (HPLC). Samples in 16 mM H_3PO_4 were applied to a 4 mm x 30 mm Waters µBondapak C_{18} column equilibrated with 16 mM H_3PO_4. Elution proceeded at 2 ml/min with linear gradients, as indicated in figures, of solvent B (90% acetonitrile, 10% 160 mM H_3PO_4). Absorbance at 210 nm was monitored, and fractions of 1 ml (0.5 min) were collected and counted for radioactivity.

Gel electrophoresis of proteins. Proteins of translation mixtures (not heated at 100° C.) were precipitated with 5% trichloroacetic acid/1 mM methionine and separated by SDS-polyacrylamide gel electrophoresis (13) in 1.5 mm thick gels polymerized from 10% acrylamide and 0.27% methylene bis-acrylamide. Slicing of the gel, elution of proteins, digestion with trypsin and carboxypeptidase B, and immunoprecipitation were performed as described (10).

Gel electrophoresis of RNA. Poly(A)-RNA was electrophoresed in slab gels containing 0.8% low-melting agarose and 5 mM methylmercuric hydroxide (14) as described (10). Gels were sliced and RNA was extracted (15) and precipitated with ethanol in the presence of 6 μg deacylated rabbit liver tRNA as carrier.

RESULTS

Polyadenylated RNA from bovine striatum stimulated amino acid incorporation into protein 30- to 60-fold in the wheat germ and 9- to 20-fold in the rabbit reticulocyte cell-free translation systems. Half-maximal and maximal stimulations were achieved with approximately 8 and 24 μg poly(A)-RNA, respectively, per ml of reaction mixture with the reticulocyte system. Maximal incorporation was approximately 65 pmol [^{35}S]methionine per ml of reaction mixture (results not shown). Synthesis of Met-enkephalin-containing protein, determined as described below, was optimal when translation mixtures contained mRNA at rate-limiting rather than saturating concentrations with respect to total [^{35}S]methionine incorporation.

To quantitate proenkephalin biosynthesis in the absence of antibodies that recognize intact proenkephalin, ^{35}S-labeled proteins were digested with trypsin and carboxypeptidase B to liberate enkephalin residues from precursors (4,10). The resultant [^{35}S]Met-enkephalin was immunoprecipitated with anti-Met-enkephalin serum and further purified by reverse-phase HPLC. The HPLC elution profiles of immunoprecipitated material are shown in Fig. 1. No [^{35}S]Met-enkephalin was obtained from translation mixtures lacking exogenous mRNA (panel A). From translation mixtures containing 1 μg striatum mRNA, 0.43 fmol [^{35}S]Met-enkephalin was recovered and identified by the criteria of retention time and immunocompetition by unlabeled Met-enkephalin (panel B). A small amount (0.04 fmol) of [^{35}S]Met-enkephalin sulfoxide was also identified by the same criteria. These results demonstrate that protein containing the Met-enkephalin sequence is synthesized by cell-free translation of striatum mRNA. Approximately 0.017% of the [^{35}S]methionine incorporated into protein was recovered in [^{35}S]Met-enkephalin sequences.

To determine the molecular weight of the protein(s) containing the Met-enkephalin sequence, ^{35}S-labeled proteins translated in the rabbit reticulocyte system were fractionated according to size by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2, most of the Met-enkephalin-containing protein migrated as a single band of M_r 31,000 $\pm$ 1000. Trace amounts also migrated at M_r 42,000 in this experiment and two similar experiments. Translation of striatum mRNA in the wheat germ system resulted in the appearance of Met-enkephalin-containing proteins of M_r 31,000, 26,000, 21,000, and <13,000 (not shown); those smaller than M_r 31,000 probably result from proteolysis or premature chain termination. We conclude that the major putative proenkephalin gene product of striatum is a protein of M_r approximately 31,000.

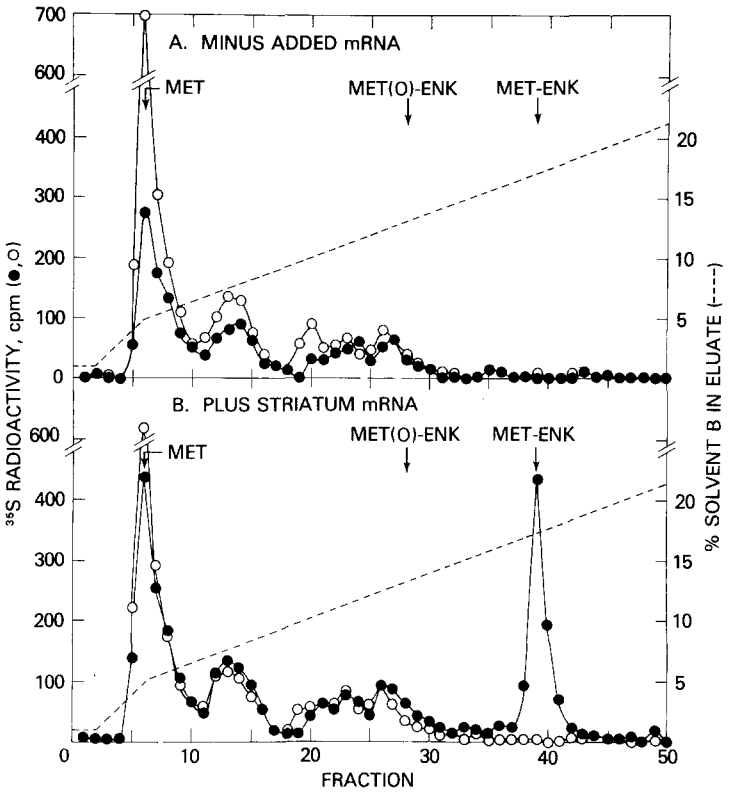

Fig. 1. HPLC analysis of ^{35}S-labeled striatum peptides synthesized in the rabbit reticulocyte system, then digested and immunoprecipitated as described in Materials and Methods. The profiles represent radioactivity immunoprecipitated in the absence (●) or presence (○) of 50 μM unlabeled Met-enkephalin. Arrows indicate elution of standard methionine (MET), Met-enkephalin sulfoxide (MET(O)ENK) and Met-enkephalin (MET-ENK). Panel A: No added mRNA; 0.7 x 10^6 cpm [^{35}S]methionine incorporated into protein. Panel B: Striatum mRNA (1 μg) added to 60 μl reaction mixture; 6.1 x 10^6 cpm incorporated. Each profile represents material from 50% of a translation reaction; data were corrected for 32% loss due to volume sacrificed during the entire procedure.

To provide evidence for the presence of both Met- and Leu-enkephalin sequences in the M_r 31,000 protein, cell-free translated proteins labeled with [^3H]tyrosine, [^3H]phenylalanine, and [^3H]leucine were analyzed by SDS-polyacrylamide gel electrophoresis. Proteins with molecular weights of 28,000 through 34,000 were digested with trypsin/carboxypeptidase B, immunoprecipitated, and analyzed by HPLC. As shown in Fig. 3, peptides resembling [^3H]Met-enkephalin and [^3H]Leu-enkephalin were recovered and identified by retention time and immunocompetition. An additional heterogeneous peak (fractions 32-36) of immunocompeted radioactivity, which would include [^3H]Arg-Tyr-Gly-Gly-Phe-Met, also was noted. The molar ratio of recovered [^3H]Met-enkephalin (plus sulfoxide) to recovered [^3H]Leu-enkephalin was estimated to be 4.7, assuming that the relative specific radioactivities of the three labeled amino acids were not altered by the reticulocyte lysate. These results

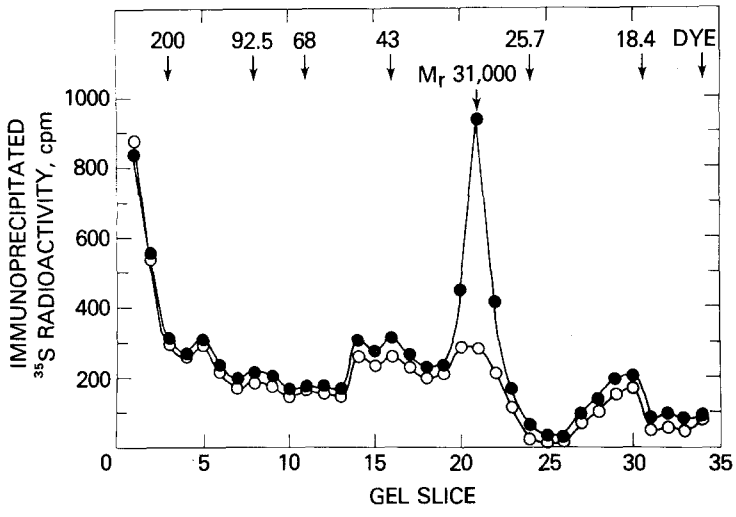

Fig. 2. SDS-polyacrylamide gel electrophoresis of Met-enkephalin-containing proteins synthesized by cell-free translation of striatum mRNA. [^{35}S]Methionine-labeled proteins (24 x 10^6 cpm) were synthesized in the reticulocyte system (240 µl) containing 4 µg striatum mRNA, then electrophoresed, eluted from gel slices, digested with trypsin and carboxypeptidase B, and immunoprecipitated in the absence (●) or presence (○) of 60 µM of unlabeled Met-enkephalin as described in Materials and Methods. Each profile represents immunoprecipitated radioactivity from 40% of the original eluate, not corrected for recovery (approximately 70%) from gel slices. Numbers and arrows at the top indicate molecular weights (x 10^{-3}) and elution positions of ^{14}C-labeled protein standards (Bethesda Research Labs).

provide suggestive evidence for the presence of at least one Leu-enkephalin sequence in a protein containing multiple Met-enkephalin sequences, as predicted from partial amino acid sequence data (4,5).

To determine the size of striatum proenkephalin mRNA, total RNA was fractionated according to chain length in agarose gels containing the denaturant methylmercuric hydroxide. As shown in Fig. 4, a single peak of putative proenkephalin mRNA was obtained with an average chain length of 1450 ± 200 nucleotides. In contrast to bovine adrenal medulla proenkephalin mRNA (10), a larger mRNA species of 4700 nucleotides was not observed.

DISCUSSION

Bovine striatum mRNA was used to direct the cell-free synthesis of enkephalin precursor protein in order to test the hypothesis that enkephalin of the central nervous system originates from a gene product different from that of enkephalin of adrenal chromaffin cells. Our results reveal similarities rather than differences in the enkephalin precursors of bovine striatum and adrenal medulla. Within experimental error identical molecular weights were obtained for the major proenkephalin gene product (M_r 31,000) and the major pro-

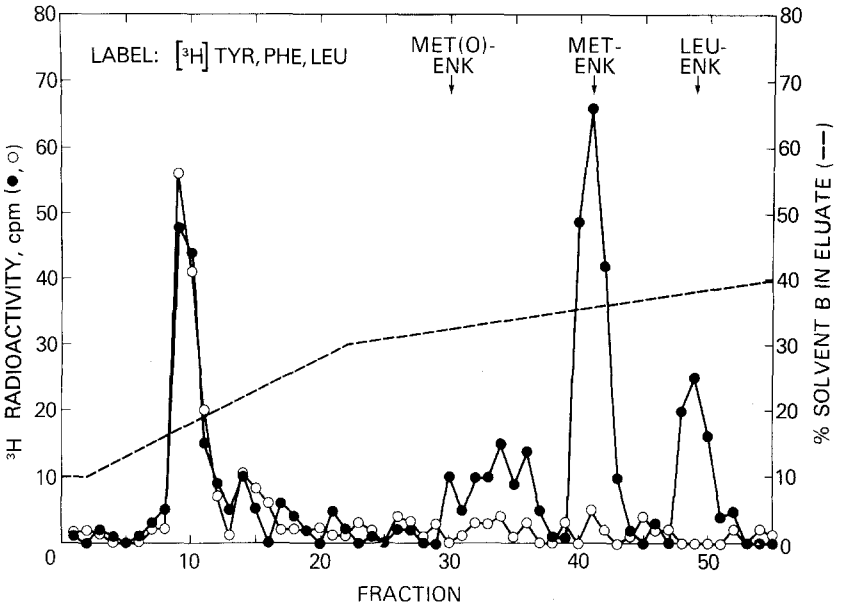

Fig. 3. HPLC profile of immunoprecipitated ^{3}H-labeled peptides derived from cell-free translated striatum proteins(s) of M_r 28-34,000. Striatum proteins were synthesized in the reticulocyte system (240 µl) containing 4 µg mRNA, [2,3,5,6-^{3}H]tyrosine (70 Ci/mmole), [2,3,4,5,6-^{3}H]phenylalanine (125 Ci/mmole), and [4,5-^{3}H]leucine (147 Ci/mmole) (all from Amersham) and 17 unlabeled amino acids. ^{3}H-Proteins (7.4 x 10^{6} cpm) and ^{14}C-protein molecular weight standards were fractionated by SDS-polyacrylamide gel electrophoresis in adjacent lanes. The lanes were sliced and proteins were eluted. Those of M_r 28000-34000 were pooled and digested with trypsin/carboxypeptidase B, then immunoprecipitated. The immunoprecipitated peptides were subsequently analyzed by HPLC, and radioactivity was counted at 2σ error of 5-7%. Each profile represents peptides derived from 36% of a reaction mixture, immunoprecipitated in the absence (●) or presence (○) of 80 µM unlabeled Met-enkephalin. No correction was made for losses. The C_{18} µBondapak HPLC column used was different from that used for the experiment shown in Fig. 1.

enkephalin mRNA (1450 nucleotides). The ratio of Met-enkephalin to Leu-enkephalin estimated by us for striatum proenkephalin (approximately 5) is in agreement with the ratio (approximately 6) determined for proenkephalin extracted from bovine adrenal medulla (4). The size of proenkephalin mRNA determined by us is also in agreement with the values of 1450-1500 nucleotides (16,17) obtained by blot hybridization of oligodeoxynucleotide probes to bovine adrenal medulla mRNA. These results suggest that the same proenkephalin gene may be expressed in bovine brain and adrenal medulla; however, nucleotide sequence analysis is required to substantiate this hypothesis.

In the previous study (10) as well as the present one, proenkephalin was identified by proteolysis to release immunoreactive enkephalin pentapeptides, because of the unavailability of a satisfactory antiserum recognizing intact proenkephalin. However, in preliminary experiments an antiserum recognizing proenkephalin specifically immunoprecipitated a protein of M_r 31,000 syn-

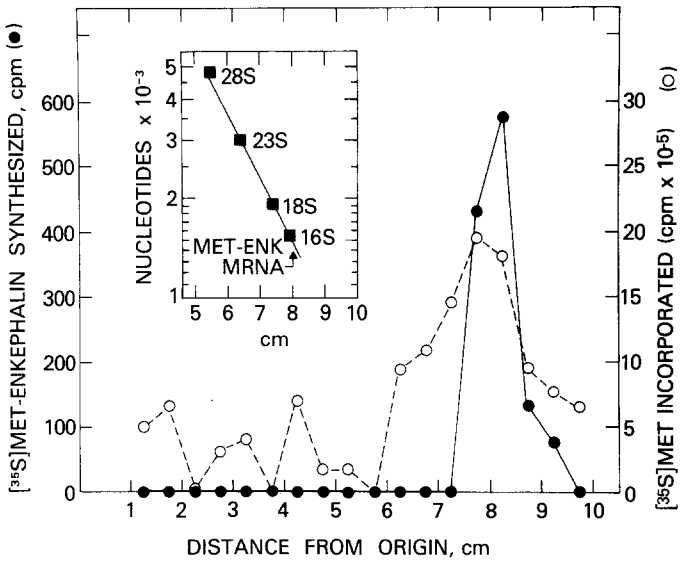

Fig. 4. Analysis of the size of striatum mRNA coding for proenkephalin. Poly(A)-RNA (12 μg) was electrophoresed in a methylmercuric-hydroxide agarose gel and subsequently eluted as described in Materials and Methods. Eluted RNA was translated in the presence of [^{35}S]methionine in the wheat germ system. The volume of each reaction mixture was 300 μl per RNA fraction in order to reduce the concentration of soluble agarose-derived inhibitors of translation; little or no inhibition was found under these conditions in control studies. ^{35}S-Labeled translation products were digested with trypsin and carboxypeptidase B, and [^{35}S]Met-enkephalin was immunoprecipitated in the presence and absence of 100 μM unlabeled Met-enkephalin and subsequently purified by HPLC. Symbols: (○) total [^{35}S]methionine incorporation into protein; (●) immunocompeted [^{35}S]Met-enkephalin recovered, corrected to represent Met-enkephalin recoverable from original translation mixtures. Inset: Migration of standard rRNA markers as a function of chain length.

thesized in reaction mixtures programmed with either bovine adrenal medulla or bovine striatum mRNA (unpublished results).

The size of the putative proenkephalin is similar to that of the common precursor to corticotropin and β-endorphin, which contains one Met-enkephalin sequence within its primary structure (18,19). However, it is unlikely that the M_r 31,000 protein identified by us is the corticotropin-endorphin precursor. Peptides related to this precursor are present in low concentrations compared to enkephalins in bovine adrenal medulla (3) and striatum (20). Furthermore, treatment of 5 μM β-endorphin with trypsin and carboxypeptidase B under the standard conditions used by us converted only 0.3% to immunoreactive Met-enkephalin (unpublished results). Finally, a peptide resembling Leu-enkephalin, absent in the corticotropin-endorphin precursor, was recovered from protein comigrating with that containing Met-enkephalin sequences (Fig. 3).

In spite of the low abundance of proenkephalin mRNA detected in adrenal medulla (10,16,17) and striatum (this study), our results indicate that quantitation of functional proenkephalin mRNA is feasible by the use of cell-free

translation. The method should be useful for studies on the regulation of enkephalin biosynthesis.

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