Vol. 89, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 13, 1979 Pages 943-950

CELL-FREE SYNTHESIS OF PUTATIVE NEUROPHYSIN PRECURSORS FROM RAT AND MOUSE HYPOTHALAMIC POLY(A)-RNA

C. Lin*, P. Joseph-Bravo*, T. Sherman*, L. Chan** and J.F. McKelvy*

* Department of Biochemistry, University of Texas Health Science Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235 and ** Departments of Cell Biology and Medicine, Baylor College of Medicine, Houston, Texas

Received June 29,1979

SUMMARY

Poly(A)-containing RNA has been isolated from rat and mouse hypothalamic tissue and 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 and [^{3}H]-L-proline. Translation products were subjected to immunoprecipitation using an antiserum to rat neurophysin proteins. Following purification of the immunoprecipitates by protein A-Sepharose chromatography, sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed a single polypeptide species of molecular weight 17,500 derived from both cell-free systems.

INTRODUCTION

Neurohypophyseal hormones and their neurophysins are synthesized in the perikarya of neurosecretory neurons of the supraoptic and paraventricular nuclei whose axons project to a neurohemal release site in the neural lobe. Sachs et al, (1) postulated the existence of a common prohormone for both neurophysins and their associated neurophyseal hormones. Recently, Gainer et al (2) provided evidence for the existence of such putative prohormones in vivo in the rat. They have identified two neurophysin-like proteins which have a molecular weight (20,000) greater than that of mature neurophysins (12,000). Since their 20,000 dalton species were detected 20 min after [^{35}S]-L-cysteine injection into the supraoptic nucleus, it is possible that such studies may not reveal the initial form of neurophysin that was synthesized in vivo. An alternative method to study the initial translation product of neurophysin involves the isolation of the specific mRNA from the hypothalamus and its translation in a heterologous system in vitro. Here

0006-291X/79/150943-08$01.00/0

we report on the first studies involving the detection in cell-free translation systems of a precursor form of neurophysin proteins, translated from both rat and mouse Poly(A)-RNA, which exhibits a molecular weight of 17,500 daltons for both species.

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. L-[2,3,4,5-^{3}H] proline at 110 Ci/mmole was from Amershan, L-[^{35}S]-cysteine at 527 Ci/mmole was from New England Nuclear. Rabbit anti-rat neurophysin, which recognizes all three rat neurophysins, was a gift from Dr. Alan Robinson. Affinity-purified rabbit anti-pyruvate kinase was a gift from Dr. Larry Cottam. Rat liver mRNA was a gift from Raymond DuBois, Jr. Female Sprague-Dawley rats, weight 250g, were purchased from Holtzman, mice of mixed strains and of both sexes were donated by The University of Texas Health Science Center at Dallas. Purified rat neurophysins (I, II, and C) were prepared according to Dr. David Sunde by affinity chromatography on a lysine^8-vasopressin-Sepharose 4B column, using a cyanogen bromide procedure (3), and preparative polyacrylamide gel electrophoresis in the presence of bromophenol blue (4).

METHODS

Isolation of Hypothalamic mRNA

Total cytoplasmic RNA from the hypothalami of both rats and mice was extracted by a guanidine-HCl procedure (5) with the following modifications. All centrifugation steps were carried out at 10,000 rpm and the salt precipitation steps using 3M sodium acetate were omitted.

Poly(A)-RNA was purified from total cytoplasmic RNA by two passages over oligo(dT)-cellulose using the procedure described by Aviv and Leder (6).

Translation of Poly(A)-RNA in Cell-free Translation Systems

Wheat germ extracts were prepared and the translation assays performed as described by Roberts and Paterson (7) without a preincubation step. The assay system was supplemented with 100 µCi/ml of either [^{3}H]-L-proline or [^{35}S]-L-cysteine. Poly(A)-RNA, at 0 to 100 µg/ml, was incubated for 90 min at 25°. Translation of mRNA in rabbit reticulocyte lysate was performed as described by Pelham and Jackson (8) using a New England Nuclear Translation Kit with 50 µCi/ml [^{35}S]-L-cysteine. Assay of amino acid incorporation was a described in the brochure accompanying the Translation Kit.

Immunoaffinity Chromatography

IgG fractions of normal rabbit serum and rabbit anti-rat neurophysin were purified from whole serum with Protein A-Sepharose according to Miller and Stone (9). Translation products from either wheat germ extract or

---

‡Abbreviations: PMSF, phenylmethyl-sulfonyl Fluoride; TCA, Trichloroacetic acid; PBS, Phosphate-buffered Saline; SDS, Sodium Dodecyl Sulfate; IgG, Immunoglobulin G; AVP, Arginine Vasopressin.

reticulocyte lysate were subjected to immunological studies by the addition of an equal volume of 2X Buffer A (Buffer A: 0.05 M Na phosphate, pH 8.0 containing 0.15 M NaCl, 0.002% sodium azide, 2% Triton, 1% sodium deoxycholate, 0.002 M PMSF) followed by centrifugation at 12,000 rpm for 20 min at 4° in microcentrifuge. The supernatant was then incubated with Protein A-purified IgG for 90 min in the presence or absence of rat neurophysins. The incubation mixture was then passed through a 20 µl column of Protein A-Sepharose which had been washed with 1M acetic acid and pre-equilibrated with 1X Buffer A containing 2.5 mg/ml bovine serum albumin. The column was washed with 1X Buffer A until the eluted radioactivity stabilized, and an additional 1 ml PBS (0.05 M Na phosphate, pH 8.0, 0.15 M NaCl) was passed through the column before the elution with 1M acetic acid. 100 µl fractions of eluate from incubation mixture, Buffer A washings, PBS washings and acetic acid eluates were collected, and 5 µl from each fraction was counted in 10 ml of a mixture of: Omnifluor (New England Nuclear) 17g, BBS-3 (Beckman Bio-Solve) 83 ml, and toluene to 4 l. The acetic acid fractions were then pooled and lyophilized.

SDS-Polyacrylamide Slab Gel Electrophoresis

The lyophilized samples were subjected to SDS-polyacrylamide slab gel electrophoresis (12.5% or 15% acrylamide) according to a previously published procedure (10). Fluorography was then carried out using the procedure of Laskey and Mills (11). Catalase (60,000), aldolase (40,000), carbonic anhydrase (29,500), β-lactoglobin (18,400), myoglobin (17,200) and cytochrome c (12,000) were used as a molecular weight standards.

RESULTS AND DISCUSSION

From 10 g of mouse hypothalami, we routinely obtained approximately 1 mg of poly(A)-RNA. The yield for rat hypothalamic poly(A)-RNA was about 0.5 mg when starting with the same amount of tissue. When translated in both the wheat germ and rabbit reticulocyte cell-free systems, mRNAs from both species stimulated incorporation of labelled amino acids into TCA-insoluble material. As shown in Fig. 1, rat hypothalamic mRNA at 5 µg/50µl incubation volume gave rise to a 14-fold stimulation of incorporation of [^{3}H]-L-proline into TCA-insoluble material in the wheat germ system. The stimulation of incorporation of [^{35}S]-L-cysteine was 5.5 fold (data not shown). The optimal potassium and magnesium ion concentrations were 90 mM and 2.8 mM respectively. It is interesting to note that mouse hypothalamic RNA showed virtually the same characteristics in the wheat germ system.

After translation over a 60 minute time course, the products were purified by immunoaffinity chromatography and subjected to SDS-polyacrylamide slab gel electrophoresis to identify the immunoreactive species. Fig. 2 shows the kinetics of rat hypothalamic mRNA directed incorporation of [^{35}S]-L-

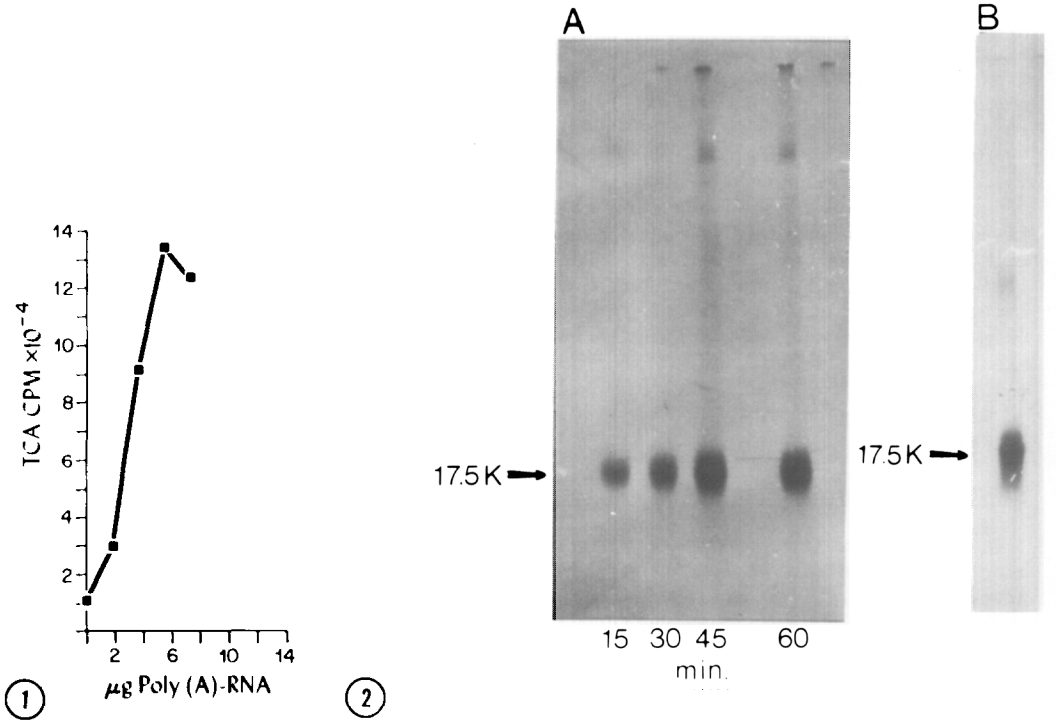


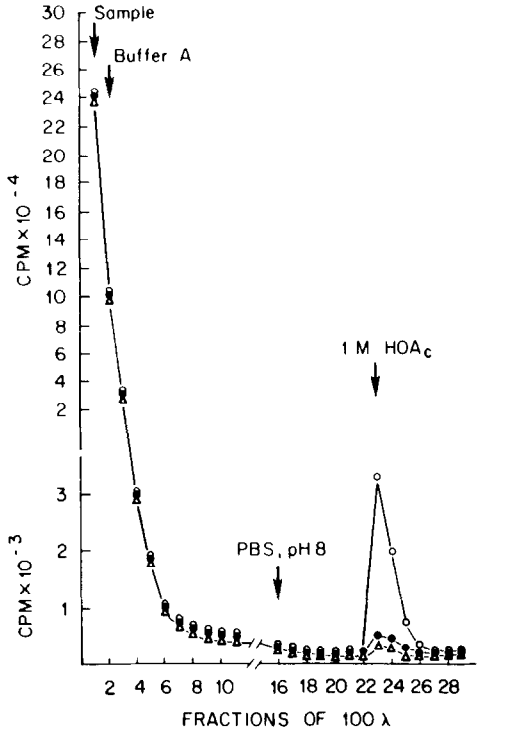
Figure 1: Incorporation of [^{3}H]-proline into TCA-insoluble compounds as directed by rat hypothalamic poly(A)-RNA in wheat germ translation system. Conditions were described in Methods.

Figure 2: Fluorograph of the SDS-polyacrylamide slab gel (12.5% acrylamide). Rat hypothalamic poly(A)-RNA in optimal concentration was used to direct the ^{35}S-cysteine incorporation to total translation products at different time periods. IgG from rabbit anti-rat-neurophysin was added to the translation products and immunoaffinity chromatography was carried out as described in Methods. The 1 M acetic acid eluates were pooled, lyophylyzed and subjected to electrophoresis. A: The kinetics of [^{35}S]-cysteine incorporation in wheat germ system at different time periods. B: The immunoreactive band obtained in reticulocyte lysate translation system after 60 min translation. Exposure time was 12 hours.

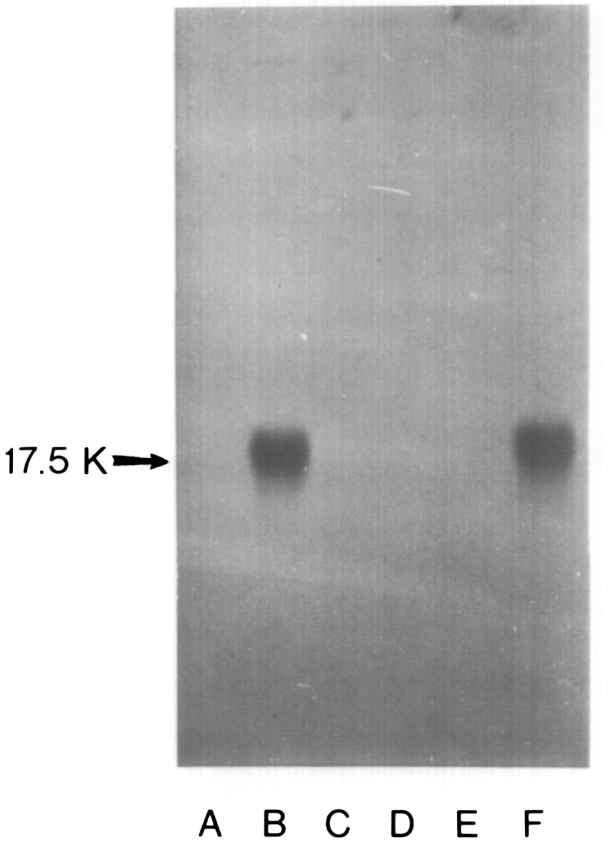
cysteine into the immunoreactive species as revealed by fluorography of the gel. There is only one major immunoreactive band present between β-lactoglobin and myoglobin, whose molecular weight is estimated to be 17,500 (17.5K), higher than that (ca 12,000) of mature rat neurophysin. The presence of a very faint smear of slower moving radioactive species was also noted, but densitometric analysis of the fluorograph revealed that greater than 95% of the total area of the electrophoretogram was represented by the 17.5K

species. It should be noted that this film was subjected to a long exposure time (12 hr) to probe for other species. Fig. 2 also shows that a single major species of 17.5K molecular weight was also demonstrated when rat hypothalamic mRNA was translated in a second cell-free system, the rabbit reticulocyte lysate system. Although not shown here, we also observed a single product of 17,500 molecular weight using [^{3}H]-L-proline as a precursor amino acid. Since only a single immunoreactive product of identical molecular weight was observed in both the wheat germ and reticulocyte lysate systems, even with prolonged exposure times upon fluorography, it is unlikely that the 17,500 molecular weight immunoreactive species is a cleavage product resulting from the early translation of an even higher molecular weight precursor. To determine if this 17,500 immunoreactive species is neurophysin-related and immunospecific, we carried out control experiments using protein A-purified IgG from normal rabbit serum and affinity-purified rabbit anti-pyruvate kinase as substitutes for anti-rat neurophysin IgG. In addition, we tested the ability of affinity-purified total rat neurophysins and cytochrome c (20 µg each) to displace the bound 17.5K species. Finally, we translated rat liver mRNA and the products were incubated with anti-rat neurophysin IgG and subjected to immunoaffinity chromatography. The results of these experiments are shown in Fig. 3, which represents the Protein A-Sepharose elution profiles, and Fig. 4, which shows the slab gel fluorograph.

As can be seen, neither the use of non-immune serum, nor of a heterologous antiserum, against rat hypothalamic mRNA-directed translation products give rise to detectable bands on the gel (Fig. 4A,D). Moreover, the use of anti-rat neurophysin antibody against rat liver mRNA-directed translation products does not result in a detectable [^{35}S]-labeled species (Fig. 4E). Thus, the appearance of the 17.5K species upon gel electrophoretic analysis of immunoaffinity isolated translation products is both antibody and mRNA specific. Finally, the inclusion of excess purified rat neurophysins in the rat hypothalamic mRNA translation products completely displaced the 17.5K



3



4

Figure 3: Immunoaffinity chromatography of wheat germ translation products with Protein A-Sepharose 4B. [^{35}S]-Cysteine was used to label the products. Procedures and conditions were described in Methods. The incubation mixtures contained: ● —— ●, IgG from normal rabbit serum; o —— o, IgG from rabbit anti-rat-neurophysin; Δ —— Δ, IgG from rabbit anti-rat-neurophysin plus rat neurophysins.

Figure 4: The specificity of the immunochemical demonstration of the neurophysin precursor. Poly(A)-RNA was translated in wheat germ system for 60 min and immunoaffinity chromatography was carried out and followed by SDS-polyacrylamide slab gel electrophoresis (15% acrylamide). In this experiment the following components were used: A, Rat hypothalamic mRNA and IgG from normal rabbit serum; B, Rat hypothalamic mRNA and anti-rat-neurophysin IgG. C, Rat hypothalamic mRNA and anti-rat-neurophysin IgG plus rat neurophysins; D, Rat hypothalamic mRNA and anti-pyruvate kinase IgG; E, Rat liver mRNA and anti-rat-neurophysin IgG; F, Rat hypothalamic mRNA and anti-rat-neurophysin IgG plus cytochrome c. Exposure time was 3 hours.

species from the antibody (Fig. 4C), while the inclusion of cytochrome c, a protein of similar charge and molecular weight, did not. (Fig. 4F).

When mouse hypothalamic mRNA was translated employing the same methods described above, and using both [^{35}S]-L-cysteine and [^{3}H]-L-proline as precursors, virtually identical results to those found for the rat were obtained. Preliminary experiments using anti-AVP IgG showed only two bands at 17.5K and 16.5K on an SDS-polyacrylamide gel (data not shown). The relationship between these species and the neurophysin precursor is being studied.

We conclude that the primary cell-free translation product for neurophysins in both the rat and the mouse is a polypeptide of molecular weight 17,500. This is in close agreement with the molecular weight of 20,000 for neurophysin precursors reported by Gainer et al (2) on the basis of *in vivo* studies, and which have recently been reported to by glycosylated (12), while our cell-free products are probably not. It is, however, not clear whether the *in vitro* translation product contains an extra NH_2-terminal peptide, as compared to the *in situ* product, as has been reported for a number of secretory proteins (13). This aspect of neurophysin biosynthesis is currently under investigation.

Acknowledgements

This work was supported by a research grant from the National Science Foundation (GB-40434). JFM is the recipient of NIH Research Career Development Award K04-AM 00331. L.C. is an established investigator of the American Heart Association. P.J.B. is a fellow from Instituto Investigaciones Biomedica, U.N.A.M., Mexico. T.S. was supported by NIH National Service Award GM 07062 from the NIGMS. We acknowledge LaVerne K. Snave for donating the mice and Barbara Lewis for her skilled secretarial assistance.

References

1. Sachs, H. and Takabatake, Y. (1964) Endocrinology 75, 943-948.
2. Gainer, H., Sarne, Y. and Brownstein, M. (1977) J. Cell. Biol. 73, 366-378.
3. March, S., Parikh, I. and Cuatrecasas, P. (1974) Anal. Biochem. 60, 149-152.
4. Burford, G. and Pickering, B. (1972) Biochem. J. 128, 941-944.
5. Deeley, R.G., Cordon, J.I., Burns, A.T.H., Mullimix, K.P., Binastein, M. and Coldberger, R.F. (1977) J. Biol. Chem. 252, 8310-8319.
6. Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1408-1412.
7. Roberts, B.E. and Paterson, B.M. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2330-2334.

8. Pelham, R.B. and Jackson, R.T. (1976) Eur. J. Biochem. 67, 247-256.
9. Miller, T.J. and Stone, H.O. (1978) J. Immunol. Methods 24, 111-125.
10. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
11. Laskey, R.A. and Mills, A.D. (1975) Eur. J. Biochem. 56, 335-341.
12. Gainer, H. In: The Role of Peptides in Neuronal Function, J. Barker and T. Smith (Eds.), Raven Press, New York, in press.
13. Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 852-862.