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Expression of β-nerve growth factor mRNA in rat glioma cells and astrocytes from rat brain

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A 50-base synthetic oligodeoxynucleotide complementary to a portion of mouse nerve growth factor (NGF) mRNA was used as a probe for analysis of the expression of NGF gene. Northern blot analysis showed the presence of a major 1.3 kb transcript, which was identical in size to mouse NGF mRNA, in both C6Bu1 cells and rat astrocytes cultured from newborn rat brain. Further, the rearrangement of DNA sequence in and around the NGF gene locus of C6Bu1 cells was not detected by Southern blot analysis. These results indicate the expression of NGF mRNA in both C6Bu1 cells and astrocytes from rat brain, suggesting that astrocytes may produce NGF protein in the rat brain, especially in developing rat brain.

Astrocyte; Nerve growth factor; mRNA; (C6Bu1 cell, Brain)

1. INTRODUCTION

β-Nerve growth factor (NGF) is essential for the development and maintenance of sympathetic and neural crest-derived sensory neurons in the peripheral nervous system (PNS) [1–3]. Recently, several researchers have reported the presence of NGF mRNA and a NGF-like protein in the rat central nervous system (CNS) as well as in the PNS, and suggested that NGF may function as a retrograde trophic factor for magnocellular cholinergic neurons in basal forebrain nuclei [4–7]. However, we have no information on the cell type(s) producing NGF protein in the brain and on the molecular mechanism that controls the expression of the NGF gene in the CNS. To clarify these problems, we cultured clonal cells derived from mammalian brains and astrocytes from newborn rat brains, and analyzed the levels of NGF mRNA in these cells by using a synthetic oligonucleotide as a probe for analysis. Here we describe the evidence for the expression of NGF mRNA in rat glioma C6Bu1 cells and astrocytes from rat brains.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

Rat astrocytes used in the present experiment were cultured as follows: cerebra without basal brains of 2-day postnatal rats were dissected out and dissociated with 0.25% trypsin (Difco). Cells were plated at a density of 5×10^6 cells in poly-L-lysine-coated dishes of 100 mm diameter, and cultured for 10 days in Dulbecco's modified Eagle's medium and F12 medium (1:1) containing 5% newborn calf serum (NCS) and 5% horse serum. Rat glioma C6Bu1 cells [8] were cultured in medium containing 10% NCS as described [9], and harvested at confluence.

2.2. *Preparation of RNA*

Cytoplasmic RNA was isolated from adult rat

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brains by a phenol-chloroform-isoamylalcohol extraction procedure [10]. Total RNA of male mouse salivary gland and cytoplasmic RNA from postnuclear fraction of cultured cells were extracted by using the guanidinium isothiocyanate/CsCl procedure [11]. Poly(A) RNA was isolated by oligo(dT) cellulose chromatography [12].

2.3. *Preparation of probe*

A synthetic oligodeoxynucleotide (3′-TTTGTG-ACCTTGAGTATGACGTGGTGCTGAGTGTG-GAAGCAGTTCCGCAA-5′) complementary to the regions of mouse β-NGF mRNA coding for amino acid residues (74–90) excluding the third base of the last Leu [13], was made by solid-phase synthesis on an Applied Biosystems DNA synthesizer. Subsequently the probe was purified on an 8% polyacrylamide/8 M urea gel. The purified probe was labeled using T_4 polynucleotide kinase and [γ-^{32}P]ATP. The nucleotide sequence of the synthetic probe was also confirmed by the procedure of Maxam and Gilbert [14]. The inserts of cloned S-100 protein (β-subunit) cDNA [10] and CCK cDNA [15], respectively, were also ^{32}P-labeled by nick-translation procedure and used as probes for Northern blot analysis.

2.4. *Northern blot hybridization analysis*

Poly(A) RNAs treated with 50% formamide were electrophoresed on a 1.3% agarose gel containing 2.2 M formaldehyde [10] and transferred onto nitrocellulose filters. The filters were hybridized with the ^{32}P-labeled synthetic probe essentially according to the procedure of Thomas [16]. Hybridization with the synthetic probe was performed at 42°C overnight. The filters were then washed 4 times for 5 min each in 2× SSC, 0.1% SDS at room temperature, and then twice for 15 min each in 2× SSC, 0.1% SDS at 42°C. Blot analysis using S-100 protein (β-subunit) cDNA and CCK cDNA was carried out according to the previous reports [10,15]. After washing, the filters were autoradiographed using an intensifying screen.

2.5. *Southern blot hybridization analysis*

Total genomic DNAs were isolated from the nuclei of rat brain and C6Bu1 cell according to Maniatis et al. [17]. The DNAs digested with endonucleases (*Eco*RI, *Hin*dIII and *Pst*I) were electrophoresed on a 0.8% agarose gel, transferred to a nitrocellulose filter for hybridization analysis. Hybridization and autoradiography were performed as described above.

3. RESULTS AND DISCUSSION

3.1. *Evidence for the expression of NGF mRNA in rat glioma C6Bu1 cells*

We used as a probe oligodeoxynucleotide complementary to a portion of mouse NGF mRNA coding for the amino acid sequence conserved evolutionally. In order to test whether the probe cross-hybridizes to rat NGF mRNA, poly(A) RNA from adult rat brain was analyzed by Northern blot analysis. As shown in fig. 1, a 1.3 kb transcript, which is identical in size to NGF mRNA in mouse salivary gland, was detected. In addition to the major 1.3 kb mRNA transcript, hybridization to a more slowly migrating band was also observed. These results were in agreement with data previously reported [5–7], which indicates that the probe specifically cross-hybridizes to the transcript of rat NGF gene. Therefore, we applied the synthetic probe to analyze the expression of NGF gene in C6Bu1 cells. Northern blot analysis showed that C6Bu1 cells contained transcripts identical in size to those detectable in rat brain using the probe (fig. 1). Furthermore, Southern blot analysis of rat genomic DNA and C6Bu1 cell

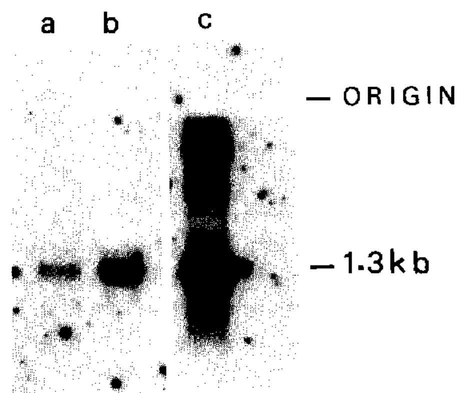

Fig. 1. Northern blot analysis of poly(A) RNA from rat brain and rat glioma C6Bu1 cells using a synthetic NGF cDNA probe. Lanes: (a) rat brain (20 μg); (b) C6Bu1 cell (20 μg); (c) male mouse salivary gland (4 μg).

genomic DNA digested with the restriction enzymes, *Eco*RI, *Hin*dIII and *Pst*I was carried out. The restriction patterns observed were identical for the genomic NGF locus in rat and C6Bu1 cell (fig. 2), implying that there is no change in the recognition sites for the restriction enzymes in the genomic NGF locus of C6Bu1 cell. We also analyzed poly(A) RNAs from a mouse neuroblastoma cell N18TG-2 and a hybrid cell NG108-15, which originated by fusion of N18TG-2 cell with C6Bu1 cell, by Northern blot analysis. We could detect the 1.3 kb mRNA in the neuroblastoma hybrid cells but not in N18TG-2 cells (not shown). Together these results indicate the expression of NGF mRNA in C6Bu1 cells. We could therefore use a rat glioma C6Bu1 cell and a hybrid cell NG108-15 as model systems for studies on the mechanism that controls the expression of NGF gene in the nervous system.

3.2. *Evidence for the expression of NGF mRNA in astrocytes from newborn rat brain*

It has been previously reported that adult rat brain astrocytes can promote the in vitro survival of NGF-dependent neurons, such as rat embryo

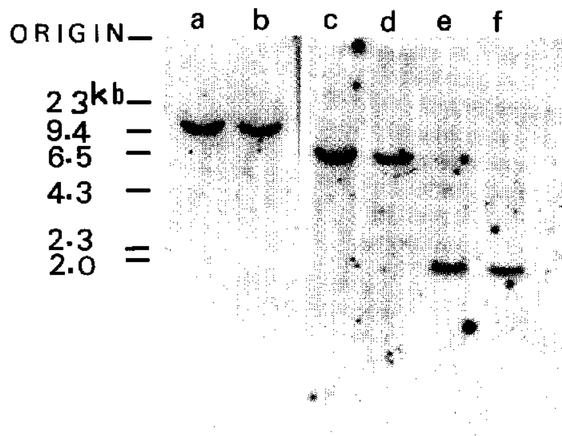

Fig. 2. Southern blot analysis of genomic DNAs from rat and C6Bu1 cells using a synthetic NGF cDNA probe. Aliquots (10 μg) of rat and C6Bu1 DNAs were digested with restriction endonucleases, electrophoresed on a 0.8% agarose gel, transferred to nitrocellulose filter, and hybridized: rat DNA (lanes a, c and e) or C6Bu1 DNA (lanes b, d and f) were digested with *Eco*RI (lanes a and b), *Hin*dIII (lanes c and d) or *Pst*I (lanes e and f).

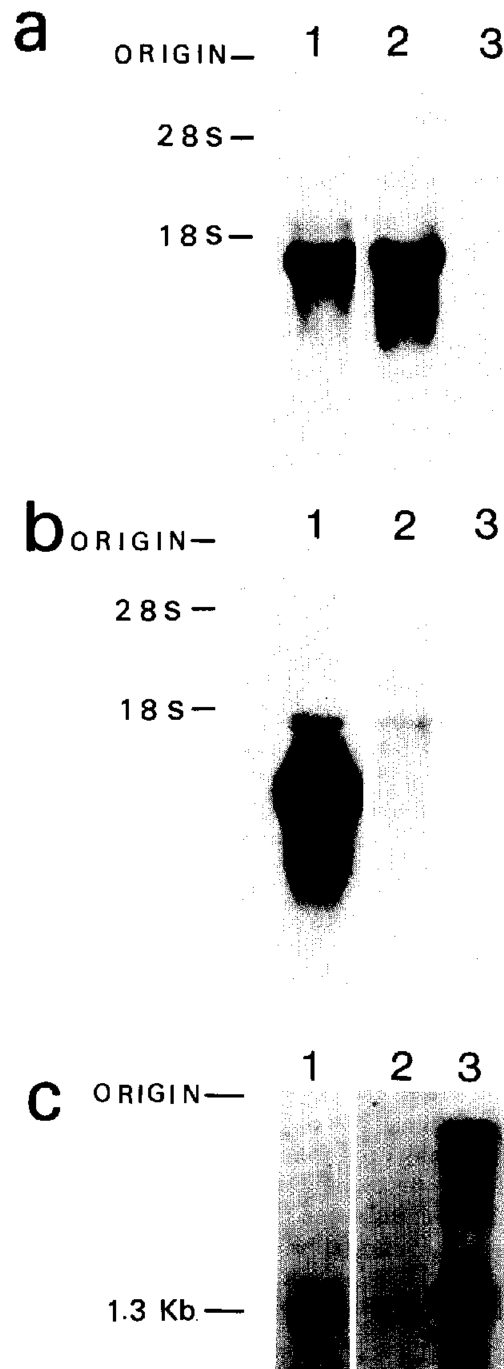

Fig. 3. Northern blot analysis of poly(A) RNA of the cells cultured from newborn rat brain using ^{32}P-labeled (a) rat S-100 protein (β-subunit), (b) rat CCK cDNA or (c) synthetic mouse NGF cDNA. Lanes: (1) rat brain (20 μg); (2) cultured cell from rat brain (20 μg); (3) mouse salivary gland (3 μg).

superior cervical ganglion cells [18]. Further, a recent report [7] has shown that in adult rat brain, cortex and hippocampus have the highest NGF mRNA, while NGF mRNA is undetectable in the hypothalamus, striatum and septum. Therefore, we cultured cells from the region of newborn rat brain including cortex and hippocampus. First, to identify the type of cells cultured from rat brain, the cells were immuno-stained with antibody to glial fibrillary acidic protein (GFAP) which is a specific marker protein for astrocyte [19]. The cells were more than 95% positive for the GFAP (not shown). We also analyzed poly(A) RNA from the cells by Northern blot analysis using S-100 protein (β-subunit) and CCK cDNAs. By Northern blot analysis, it was shown that the cells cultured from rat brain contained a 1.5 kb transcript which is similar in size to S-100 protein (β-subunit) mRNA detectable in rat brain (fig. 3a). CCK mRNA, the size of which is about 850 bases in length [11], however, was not detected in the cultured cells (fig. 3b). S-100 protein is a marker protein for astrocyte [20] in the CNS. CCK-like immunoreactivity is located in neurons of rat brain [21]. Additionally, Hasegawa et al. [22] have reported that high levels of CCK mRNA were found in the cortex and hippocampus of rat brain. We have therefore considered that astrocytes were predominant in the cells cultured from rat brain. Further, to examine the expression of NGF mRNA in the cultured cells, we analyzed poly(A) RNA from the cultured cell using the synthetic probe by Northern blot analysis. Fig. 3c shows that significant amounts of NGF mRNA were present in the cultured cells from rat brain, indicating the expression of NGF mRNA in rat astrocytes. These results suggest that astrocytes may produce NGF protein in rat brain, especially in developing rat brain. Furukawa et al. [23] have recently reported that astroglial cells cultured from mouse brain are able to synthesize and secrete NGF in culture. Our results support this observation. Our preliminary data also showed that treatment of rat astrocytes with epinephrin caused elevation of NGF mRNA level in the astrocytes (not shown), suggesting that expression of NGF gene may be controlled via neurotransmitter by neurons. Currently, study by in situ hybridization are under way to determine the cellular localization of NGF mRNA in the brain.

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REFERENCES

[1] Levi-Montalcini, R. and Booher, B. (1969) Proc. Natl. Acad. Sci. USA 49, 384-391.
[2] Johnson, E.M., Gorin, P.D., Brandeis, L.D. and Pearson, J. (1980) Science 210, 916-918.
[3] Goedert, M., Otten, U., Hunt, S.P., Bond, A., Chapman, D., Schlumpt, M. and Lichtensteiger, W. (1984) Proc. Natl. Acad. Sci. USA 81, 1580-1584.
[4] Korsching, S., Auburger, G., Heumann, R., Scott, J. and Thoenen, H. (1985) EMBO J. 4, 1389-1393.
[5] Shelton, D.L. and Reichardt, L.F. (1986) Proc. Natl. Acad. Sci. USA 83, 2714-2718.
[6] Large, H.T., Bodary, S.C., Clegg, D.O., Weskamp, G., Otten, U. and Reichardt, L.F. (1986) Science 234, 352-355.
[7] Whittemore, S.R., Ebendal, T., Larkfors, L., Olson, L., Seiger, A., Stromberg, I. and Persson, H. (1986) Proc. Natl. Acad. Sci. USA 83, 817-821.
[8] Amano, T., Hamprecht, B. and Kemper, W. (1974) Exp. Cell. Res. 85, 339-408.
[9] Hatanaka, H. and Amano, T. (1981) Brain Res. 215, 305-316.
[10] Kuwano, R., Usui, H., Maeda, T., Fukui, T. and Takahashi, Y. (1984) Nucleic Acids Res. 12, 7455-7465.
[11] Chirgwin, J.M., Przbvla, A.E., McDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294-5299.
[12] Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408-1412.
[13] Ullrich, A., Gray, A., Berman, C. and Thomas, J.D. (1983) Nature 303, 821-825.
[14] Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499-560.
[15] Kuwano, R., Araki, K., Usui, H., Fukui, T., Ohtsuka, E., Ikehara, M. and Takahashi, Y. (1984) J. Biochem. 196, 923-926.
[16] Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77, 5201-5205.
[17] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, pp. 280-281, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[18] Lindsay, R.M. (1979) Nature 282, 80-82.
[19] Eng, L.F. (1980) Proteins of the Nervous System, pp. 85-117, Raven, New York.

[20] Ghandour, M.S., Langley, O.K., Labourdetle, G., Vincendon, G. and Gombos, G. (1981) Dev. Neurosci. 4, 66–78.
[21] Innis, R.B., Correa, F.M.A., Uhl, G.R., Schneider, B. and Snyder, S.H. (1979) Proc. Natl. Acad. Sci. USA 76, 521–525.
[22] Hasegawa, M., Usui, H., Araki, K., Kuwano, R. and Takahashi, Y. (1986) FEBS Lett. 194, 224–226.
[23] Furukawa, S., Furukawa, Y., Satoyoshi, E. and Hayashi, K. (1986) Biochem. Biophys. Res. Commun. 136, 57–63.