Neurochemical Research, Vol. 26, No. 10, October 2001 (©2001), pp. 1101–1105

Cloning and Identification of Differentially Expressed Transcripts in Primary Culture of GABAergic Neurons

Zhi Li,[1] Qiang Li,[1] Chun Xiao Sun,[1] Leif Hertz,[2] and Albert Cheung Hoi Yu[1,2,3,4]

(Accepted June 15, 2001)

A RNA based arbitrarily primed polymerase chain reaction (RAP-PCR) was used to identify differentially expressed transcripts in primary cultures of cerebral cortical neurons prepared from E16 mouse cerebral cortex. The majority of neurons found in this culture preparation are known to be GABAergic. Different primer combinations were used, and the PCR products were separated on PAGE. Visualization by silver staining revealed a high resolution RNA fingerprint pattern with a total of about 200 transcripts. Six differentially expressed cDNA fragments were recovered, cloned and sequenced. The results of a NCBI database search showed that 6 clones were highly homologous to known genes and expressed sequence tags (ESTs), and that they were either up-regulated or down-regulated during development. Among these clones, Clone 3.1.7 shared 99% sequence homology to mouse Reelin, a neuronal migration and positioning related protein. Clone 4.6.2 shared 91% homology to Rat prepro bone morphogenetic protein-3 mRNA. Clone 6.10.2 had 90% homology to a novel orphan gene of calcium-independent alpha-latrotoxin receptor, which stimulates presynaptic neurotransmitter release. Northern blot analysis confirmed the up-regulated expression profile of Clone 6.10.2 in neuron from Day 2 to 7 during stages of differentiation and development.

KEY WORDS: Neurons; RAP-PCR; development; ESTs; gene discovery.

INTRODUCTION

The course of neuronal development and differentiation is highly regulated by a program of differential gene expression (1). This study was carried out to more fully understand the molecular mechanism that underlies the differentiation of cultured cerebro-cortical interneurons, a GABAergic preparation (2).

RNA based arbitrarily primed polymerase chain reaction (RAP-PCR) was used as a tool to identify genes, which were differentially expressed at different ages. Differential expression analysis involves comparing many mRNAs, expressed in two or more cell populations, by separating their transcribed PCR products in adjacent lanes on a suitable gel (3,4). This technique allows the analysis of changes in specific mRNA levels at different developmental stages and under various pathologic conditions (5). This type of analysis has been used to isolate a number of differentially expressed genes in a variety of experimental systems (6,7,8).

The RAP-PCR system has been modified and improved many times. The conventional method separated PCR products on a sequencing gel and used radioactive labeling to visualize the differentially expressed

[1] Brain Research Institute, Shanghai Research Center of Life Sciences, Chinese Academy of Sciences, Shanghai, China.
[2] Department of Biology, the Hong Kong University of Science and Technology, Hong Kong, China.
[3] Central Laboratory of Human Genome Research, Research Center of Biotechnology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences.
[4] Address reprint requests to: Albert C. H. Yu, Ph.D., Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China. Tel: (852) 2358-7303; Fax: (852) 2358-1559; E-mail: boachy@ust.hk

0364-3190/01/1000–1101$19.50/0

transcripts (9). However, the resolution of the separated bands was poor and created difficulties in recovering the differentially expressed transcripts. Mathieu-Daude et al. (10) resolved the isolated cDNA fragments by single strand conformation polymorphism gels to separate the regulated gene transcripts from the putative unregulated species with the same length on the initial PCR gel.

In this study, specially designed primers for RAP-PCR were used. The RAP-PCR products were separated on 9% polyacrylamide gel and the bands were visualized by a silver staining method for improved resolution. This modified method facilitates the study of neuronal development and differentiation in cultures of cerebral cortical neurons of different ages. The ages of 2-, 4-, 7-day-old cultures were chosen (Fig. 1), because neurons in culture for 2 days have only a few processes, whereas process formation is more extensive after 4 days, and cells in culture for 7 days have migrated to form neuronal aggregates and release GABA when depolarized by elevated K^+ (2).

EXPERIMENTAL PROCEDURE

Preparation of Neuronal Cultures. Primary cultures of cerebral cortical neurons were prepared from ICR mice, as described by Yu et al. (2,11) with minor modifications. Dissociated cells from cerebral cortex of 16 day (E16) ICR mice embryo were seeded in polylysine-coated tissue culture dishes, at a concentration of 9×10^6 cells per 60-mm dish (Becton Dickinson Labware, NJ, USA) in modified Eagle's Minimum Essential Medium (MEM) (2) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Life Technologies Inc., NY, USA) and 2 mM glutamine. On day 3, 40 μM cytosine arabinoside was added to the culture to inhibit astrocytic growth. After 24 h, half of the medium was replaced with freshly prepared medium of similar composition as before.

mRNA Isolation and cDNA Synthesis. Cells of different developmental stages (D2, D4, and D7) were collected with a disposable scraper, and mRNA was isolated using the QuickPrep® Micro mRNA Purification System (Pharmacia Biotech, Sweden). RAP-PCR was performed with the arbitrary 18-mer primers shown in Table I. In brief, 1 μg of mRNA was incubated at 37°C for 1 h with 0.125 U/μl DNase (Promega, WI, USA) and 1.4 U/μl RNasin (Promega, USA), and subsequently re-isolated from the mixture by phenol/chloroform extraction. Reverse transcription was performed at 70°C for 10 min with 2.5 ng/μl Random Hexamer (Promega, USA), then at room temperature for 10 min and at 42°C for 2 min with 1.4 U/μl RNasin (Promega, USA), 0.01M DTT (Gibco BRL, USA) and 0.5 mM dNTPs, followed by incubation at 42°C for 50 min and then at 70°C for 15 min with 10 U/μl Superscript II RTase (Gibco BRL, USA). RNA was removed from RNA-DNA hybrid with 3 U/μl RNase H (Gibco BRL, USA) by incubation at 37°C for 20 min. PCR was performed by first denaturing the sample at 94°C for 2 min, followed by 45 cycles at 94°C for 1 min, at 32°C for 1 min and at 72°C for 1 min. The final step was extension at 72°C for 10 min.

Cloning, Sequencing, and Database Searching. The PCR products were separated on PAGE gel and visualized by silver staining. The differentially expressed fragments were excised from the polyacrylamide gels and re-amplified. DNA fragments were cloned into pGEM-T Easy vector and pBlueScript vector using pGEM-T Easy Kit (Promega, USA). The fragments were sequenced using the Auto-Read™ 100 Sequencing Kit (Pharmacia Biotech, Sweden) with an automated fluorescent Pharmacia LKB A.L.F. DNA Sequencer (Pharmacia Biotech, Sweden). The DNA sequences of the clones were compared with the National Center for Biotechnology Information (NCBI) database (12).

Northern Blot Analysis. Total RNAs from neurons at different stages (D2, D4, D7) were prepared using TRIzol Reagent (Gibco BRL, USA). Thirty micrograms of total RNA were electrophoresed on formaldehyde-1.2% agarose gel and transferred onto a nitrocellulose membrane (Hybond-C, Amersham LIFE SCIENCE). Hybridization was performed using random-^{32}P-labeled purified DNA probe (Promega, USA) at 42.5°C for 24 h in phosphate buffer. After hybridization, the membrane was washed twice at 42°C for 15 min in 2X SSC/0.1% SDS, followed by 30 min at 55°C in 0.2X SSC/0.1% SDS twice. The membrane was exposed to X-Omat Film (Kodak, Japan) with an intensifying screen at −70°C for 2–7 days. Equal loading was determined by EtBr staining of total RNA.

RESULT AND DISCUSSION

Primary cultures of cerebral cortical neurons have been widely used for the investigation of neuronal biochemistry and physiology at both the cellular and molecular levels (13–16). In the culture system used in the present study, most of the cells isolated from E16 mouse cerebral cortex were neuronal precursor cells which were able to undergo various stages of development after attachment to the culture dish (Fig. 1). It had been shown in previous studies that in culture these cells develop and become functionally mature between D4 to D7, as indicated by potassium-induced GABA release (2). Thus, this culture system simulates neuronal differentiation and provides a useful tool to investigate gene expression in neurons undergoing development and differentiation.

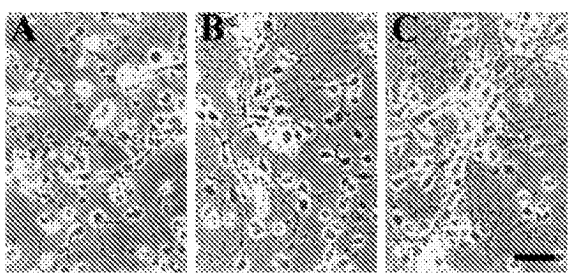

Fig. 1. Phase contrast micrographs of cerebro-cortical mouse neurons. The primary cultures of neuron were prepared from 16-day-old embryos, and cultured for 2 days (A), 4 days (B) and 7 days (C). Bar = 25 μm.

Table I. Analysis of Differentially Expressed Fragments Based on Sequence Database Search

Clone	Size (bp)	Primer combinations***	Expression pattern*	Result of Blastn Search (HSP)** against GenBank(nr)
1.3.4	303	B3 + B3	Down	*Mus musculus* cadherin-related neuronal receptor 1. (99%; 283/285)
3.1.7	234	A2 + A3 + A5	Up	*Mus musculus* Reelin. (99%; 220/221)
4.6.2	451	B3 + B3	Down	*Rattus norvegicus* mRNA for prepro bone morphogenetic protein-3. (91%; 369/402)
5.1.4	356	B3 + B3	Up	*Mus musculus* hexokinase (Hk-1). (98%; 328/331)
5.4.7	468	B2 + B2	Down	*Rattus norvegicus* neurabin II. (96%; 248/257)
6.10.2	296	B3 + B3	Up	*Rattus* calcium-independent alpha-latrotoxin receptor (CIRL). (90%; 225/248)

Note. Sequences were compared with those in Genbank (nr), using the BLAST program.
*Differential expression patterns were demonstrated during RAP-PCR studies (from day 2 to day 7).
**The DNA sequence similarity of HSPs was shown in parentheses.
***Primer sequences:
A2: 5′AATCTAGAGCTCCAGCAG
A3: 5′AATCTAGAGCTCTCCTGG
A5: 5′AATCTAGAGCTCCCTCCA
B2: 5′CCTACACGCGTATACTCC
B3: 5′CATACACGCGTATACTGG

To identify the genes that were regulated during neuronal development and differentiation, mRNA was extracted from neurons in cultures for 2, 4 and 7 days for RAP-PCR analysis. The PCR products were separated on 9% non-denaturing polyacrylamide gel and visualized by silver staining. A high-resolution pattern of 30–40 main amplified products was obtained for each sample, with about 200 different transcripts being identified in total. The RAP-PCR product patterns obtained were primer combination specific, and reproducible. The differentially expressed fragments were identified by parallel comparison of RAP-PCR products of D2, D4 and D7 cultures (Fig. 2). Re-amplification, cloning and sequencing of the fragments revealed the 6 clones described in Table I.

After searching the GenBank(nr) database, Clone 1.3.4 was found to have 99% (283/285) DNA sequence homology to *Mus musculus* cadherin-related neuronal receptor (CNR). CNR belongs to the novel cadherin superfamily which is localized in synaptic junctions. This receptor plays a central role in establishing specific neuronal connections and in signal transduction at synaptic junctions (17). Clone 3.1.7 had 99% (220/221) sequence similarity to *Mus musculus* Reelin, an extracellular matrix protein which plays a crucial role in the histogenesis of several laminated structures during postnatal brain development (18). Recent research suggested that Reelin acts via very low-density lipoprotein receptor (VLDLR) and apoE receptor 2 (ApoER 2) to regulate Disabled-1 (Dab 1) tyrosine phosphorylation and microtubule function in neurons (19). Clone 4.6.2 had 91% (369/402) homology to *Rattus norvegicus* prepro bone morphogenetic protein-3 mRNA.

Comparing the sequence of Clone 5.1.4 with the database shows that it has 98% (328/331) sequence

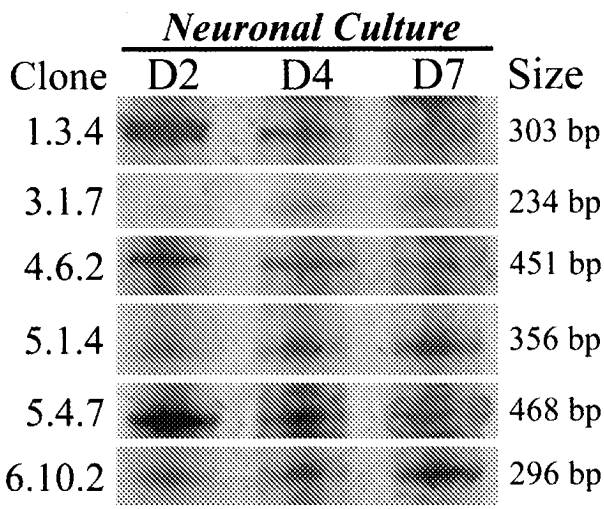

Fig. 2. Typical gels obtained by RAP-PCR analysis of cultured neurons of different ages. The PCR products were separated on 9% PAGE gel and visualized by silver staining. D2, D4 and D7 indicate 2-day-old, 4-day-old and 7-day-old cultures, respectively. The names of the gene products shown on the left correspond to those described in Table I. Molecular sizes are indicated on the right.

similarity to hexokinase, a key enzyme during glycolysis. Clone 5.4.7 had 96% (248/257) homology to neurabin (also called spinophilin) mRNA. Neurabin is a novel component at cadherin-based cell-cell adherens junctions and plays important roles in the formation of complex neural networks (20). Clone 6.10.2 was found to have 90% (225/248) DNA sequence homology to the rat calcium-independent alpha-latrotoxin receptor (CIRL) gene. CIRL appears to be a novel orphan G-protein-coupled receptor—a member of the secretin receptor family—found in brain and enriched in the striatum and cortex (21). This receptor is involved in secretion and stimulation of presynaptic neurotransmitter release (22).

We investigated the relative expression profiles of cloned cDNAs in neuronal culture by Northern blot analysis. Gene specific primers were designed and radioactive-labeled probes were prepared to detect their correspondent transcripts in cells. As shown in Figure 3, Clone 5.4.7 has a down-regulated expression pattern in neuronal culture from D2 to D7, suggesting that the gene plays a role in a relatively early stage of neuronal development. However, its homologous gene, which encodes spinophilin, participates in neural network formation at a relatively late developmental stage. The differential expression profiles of Clone 6.10.2 revealed an up-regulated pattern with the highest expression level on D7 during the culture stages. All 6 ETSs characterized in Table I were differentially expressed during neuronal development by RAP-PCR studies. Of the 6 clones, 3 clones were up-regulated and the other 3 were down-regulated.

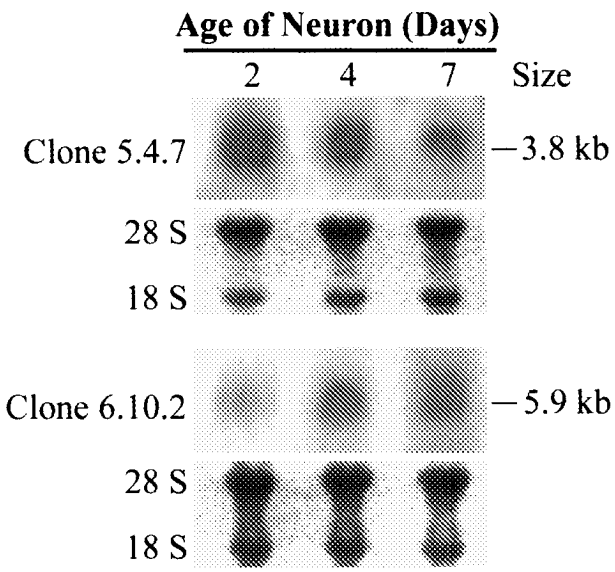

Fig. 3. Northern blot analysis of Clone 5.4.7 and Clone 6.10.2. Total RNAs (30 μg/lane) were isolated from primary neuronal cultures of different ages (D2, D4, and D7) and were hybridized to [^{32}P]-labeled cloned cDNA probes. Sizes of mRNAs are shown on the right in kilobases. The intensity of 28S and 18S bandstaining indicates equal loading of each product.

In conclusion, we have identified 6 differentially expressed transcripts, which play functional roles during neuronal development and differentiation. The identification of some of the genes isolated in this study confirms that this culture system is a suitable tool for analyzing genes related to neuronal development and differentiation. Further dissection of the precise functions subserved by these genes, and identifying other genes involved in the process of neuronal development and differentiation should facilitate the understanding of the molecular mechanisms underlying this process.

ACKNOWLEDGMENTS

We would like to thank Dr. Richard Collins from Hong Kong DNA Chips Limited for his assistance in the preparation of this manuscript. This study was supported by grants from the Shanghai Commission of Science & Technology Grant 99JC14024, Shanghai Research Center of Life Sciences, Chinese Academy of Sciences, Research Grant Council (H.K.) HKUST6177/97M, HKUST/CAS Joint Laboratory Scheme and the North American Medical Association Foundation (Hong Kong) NAMA 94/95.SC01 to ACHY.

REFERENCES

1. Mannsfeldt, A. G., Carroll, P., Stucky, C. L., and Lewin, G. R. 1999. Stomatin, a MEC-2 like protein, is expressed by mammalian sensory neurons. Mol. Cell. Neurosci. 13:391–404.
2. Yu, A. C., Hertz, E., and Hertz, L. 1984. Alterations in uptake and release rates for GABA, glutamate, and glutamine during biochemical maturation of highly purified cultures of cerebral cortical neurons, a GABAergic preparation. J. Neurochem. 42:951–960.
3. Liang, P. and Pardee, A. B. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257:967–971.
4. Welsh, J., Chada, K., Dalal, S. S., Cheng, R., Ralph, D., and McClelland, M. 1992. Arbitrarily primed PCR fingerprinting of RNA. Nucleic Acids Res. 20:4965–4970.
5. Micheli, M. R. and Bova, R. 1996. Fingerprinting Methods Based on Arbitrarily Primed PCR, pp. 256–268. Springer Press, New York.
6. Lafontaine, D. A., Mercure, S., and Perreault, J. P. 1998. Identification of a Crohn's disease specific transcript with potential as a diagnostic marker. Gut. 42:878–882.
7. McClelland, M., Mathieu Daude, F., and Welsh, J. 1995. RNA fingerprinting and differential display using arbitrarily primed PCR. Trends Genet. 11:242–246.
8. Schweitzer, B., Taylor, V., Welcher, A. A., McClelland, M., and Suter, U. 1998. Neural membrane protein 35 (NMP35): A novel member of a gene family which is highly expressed in the adult nervous system. Mol. Cell. Neurosci. 11:260–273.

9. Melia, M. J., Bofill, N., Hubank, M., and Meseguer, A. 1998. Identification of androgen-regulated genes in mouse kidney by representational difference analysis and random arbitrarily primed polymerase chain reaction. Endocrinology 139:688–695.
10. Mathieu Daude, F., Cheng, R., Welsh, J., and McClelland, M. 1996. Screening of differentially amplified cDNA products from RNA arbitrarily primed PCR fingerprints using single strand conformation polymorphism (SSCP) gels. Nucleic Acids Res. 24:1504–1507.
11. Yu, A. C., Chan, P. H., and Fishman, R. A. 1986. Effects of arachidonic acid on glutamate and gamma-aminobutyric acid uptake in primary cultures of rat cerebral cortical astrocytes and neurons. J. Neurochem. 47:1181–1189.
12. Xie, T. 1998. *Bioinformatics: Is it new?* http://www.sibc.ac.cn/bio/newgene.htm
13. Chiu, F. C., Potter, P. E., and Rozental, R. 1998. Purification of human fetal hippocampal neurons by flow cytometry for transplantation. Methods 16:260–267.
14. Ohkura, N., Hijikuro, M., Yamamoto, A., and Miki, K. 1994. Molecular cloning of a novel thyroid/steroid receptor superfamily gene from cultured rat neuronal cells. Biochem. Biophys. Res. Commun. 205:1959–1965.
15. Said, S. I., Dickman, K., Dey, R. D., Bandyopadhyay, A., De Stefanis, P., Raza, S., Pakbaz, H., and Berisha, H. I. 1998. Glutamate toxicity in the lung and neuronal cells: Prevention or attenuation by VIP and PACAP. Ann. NY Acad. Sci. 865:226–237.
16. Williams, K., Russell, S. L., Shen, Y. M., and Molinoff, P. B. 1993. Developmental switch in the expression of NMDA receptors occurs in vivo and in vitro. Neuron 10:267–278.
17. Sugino, H., Hamada, S., Yasuda, R., Tuji, A., Matsuda, Y., Fujita, M., and Yagi, T. 2000. Genomic organization of the family of CNR cadherin genes in mice and humans. Genomics 63: 75–87.
18. Schiffmann, S. N., Bernier, B., and Goffinet, A. M. 1997. Reelin mRNA expression during mouse brain development. Eur. J. Neurosci. 9:1055–1071.
19. Ringstedt, T., Linnarsson, S., Wagner, J., Lendahl, U., Kokaia, Z., Arenas, E., Ernfors, P., and Ibanez, C. F. 1998. BDNF regulates reelin expression and Cajal-Retzius cell development in the cerebral cortex. Neuron 21:305–315.
20. Mizoguchi, A. 1999. Role of vesicle fusion mechanism and cell adhesion molecules in growth cone elongation and synaptogenesis. Kaibogaku Zasshi. 74:473–483.
21. Krasnoperov, V. G., Bittner, M. A., Beavis, R., Kuang, Y., Salnikow, K. V., Chepurny, O. G., Little, A. R., Plotnikov, A. N., Wu, D., Holz, R. W., and Petrenko, A. G. 1997. alpha-Latrotoxin stimulates exocytosis by the interaction with a neuronal G-protein-coupled receptor. Neuron 18:925–937.
22. Elrick, D. B. and Charlton, M. P. 1999. alpha-Latrocrustatoxin increases neurotransmitter release by activating a calcium influx pathway at crayfish neuromuscular junction. J. Neurophysiol. 82:3550–3562.