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Identification of Transcripts Expressed under Functional Differentiation in Primary Culture of Cerebral Cortical Neurons*

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In this study, we utilized primary cultures of cerebral cortical neurons and RNA arbitrarily primed polymerase chain reaction (RAP-PCR) to identify differentially expressed transcripts in neurons of different culture ages. Eleven cDNA fragments with high sequence similarity to known genes and Expressed Sequence Tags (ESTs) were cloned. From the National Center for Biotechnology Information (NCBI) sequence database, two clones were shown to be identical to known sequences, *Mus musculus* HP1-BP74 protein mRNA and *Mus musculus* KRAB-containing zinc finger protein, both were up-regulated. These genes have never before been shown to be involved in neuronal functional maturation. Among the remaining clones, clone 8-14 (239 bp) was very similar to *Rattus norvegicus* rS-Rex-b mRNA, which was further confirmed by sequencing its shortest isoform (1.5 kb) obtained by computer cloning. This study has identified eleven potential genes and transcripts, which might be involved in the development and differentiation of GABAergic neurons in culture.

KEY WORDS: GABAergic neurons; development; RNA fingerprinting; ESTs.

INTRODUCTION

The processes of development, differentiation and degeneration in the Central Nervous System (CNS) are regulated by a complex program of gene expression and suppression. Much effort has been put into identifying and characterizing stage-specific genes in order to understand their roles in the CNS (1). In this study, an attempt was made to identify differentially expressed genes and expressed sequence tags (ESTs) in cultured neurons of different ages, using a modified RAP-PCR technique. Primary culture of cerebral cortical neurons has been used for studying the physiological and biochemical functions of neurons (2–5). We have previously shown that the number of neurites and synapses in primary cultured neurons increased from day 2 and day 4. At day 4 to 7, neurons underwent a functional maturation, as indicated by the high-potassium-induced release of GABA (6). Thus, these neurons in culture provide a system for studying the complex program of gene expression and suppression during development without interference from other cell types in the brain.

Various strategies, including plus/minus hybridization, subtractive hybridization, differential display PCR (DD-PCR), and single cell amplification, have been employed in the search of novel gene expression (7).

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However, it has been shown that the reproducibility of this technique is relatively low and the percentage of false positives is high (8). RNA arbitrarily primed polymerase chain reaction (RAP-PCR) allows parallel analysis of several transcripts derived from different samples, and provides a rough estimation of their expression level (9–11). We modified the RAP-PCR technique by resolving RAP-PCR products on a non-denaturing polyacrylamide gel, where the bands were subsequently visualized by ethidium bromide staining. The method was rapid, economic, convenient and safe. In our study, we compared transcripts expressed in neurons at day 2, 4 and 7 after culture. We were able to isolate genes expressed differentially in these neurons at different developmental stages.

EXPERIMENTAL PROCEDURE

Primary Culture. Primary cultures of cerebral cortical neurons were prepared from ICR mice (6,12). Pregnant female mice carrying 16-day old fetuses were sacrificed by cervical dislocation. Under sterile conditions, the meninges and hippocampi of the fetuses were removed. The neopallia were cut into small pieces and incubated in 0.25% trypsin in Ca^{2+}- and Mg^{2+}-free HBSS (Gibco BRL, NY, USA) at room temperature for 5 min. The trypsinized tissue was filtered through a 70-μm sieve (Spectrum Medical Industries, Inc., TX, USA) and the cell suspension was plated in poly-D-lysine (12.5 μg/mL) pre-treated 60-mm Falcon tissue dishes (Becton Dickinson Labware, NJ, USA). All cultures were incubated in a 37°C incubator with 5%/95% CO_2/air (v/v) and 95% humidity. After 30 min of pre-incubation, the culture media was replaced with freshly prepared medium containing 10% fetal calf serum (Hyclone, UT, USA). The medium used was modified Eagle's minimum essential medium (MEM) with some modification (6,12). On day 3, 40 μM of cytosine arabinoside was added to the culture for 24 hr, and then half of the medium was replaced with fresh medium.

mRNA and Total RNA Extraction. mRNA was prepared from day 2, 4 and 7 neuronal cultures using a QuickPrep® Micro mRNA Purification Kit (Pharmacia Biotech, Sweden). To remove genomic DNA, mRNA was treated with RNase-free DNase (Promega, WI, USA) and purified by phenol-chloroform extraction and ethanol precipitation. The mRNA pellet was dissolved in DEPC-treated H_2O. Total cellular RNA was extracted with TRIzol Reagent (Gibco BRL, NY, USA) following the protocol provided.

cDNA Synthesis. The reaction volume was 20 μl and contained 5 μM random hexamer and 1 μg mRNA in DEPC-treated H_2O with 1X First Strand Buffer (Gibco BRL, NY, USA). The sample was denatured at 70°C for 10 min. RNasin (2 U/μl), 10 mM DTT and 0.5 mM dNTPs were added and kept at room temperature for 10 min followed by incubation at 42°C for 2 min. Finally, with addition of 10 U/μl Superscript™ II RTase, the mixture was incubated at 42°C for 50 min, then at 70°C for 15 min in order to stop the reaction. After the reverse transcription reaction, 1.1 U/μl of RNase H was added to the cDNA pool to remove the RNA strand from the DNA-RNA hybrid. All reagents for reverse transcription were from Gibco BRL (NY, USA), except for dNTPs (Pharmacia Biotech, Sweden).

Polymerase Chain Reaction. Each 20 μl of RAP-PCR reaction mixture contained 0.6 μl of cDNA pool, 1X PCR buffer, 1.2 mM $MgCl_2$, 200 μM of dNTPs, 0.04 U/μl *Taq* DNA polymerase, and 0.4 μM each of three oligonucleotide primers with arbitrary sequences and zinc-finger-like domain sequences selected from Table I. Different primer combinations were used in each amplification in order to develop different RNA fingerprints. Using a Robocycler Gradient 40 Thermocycler (Stratagene, Amsterdam, Holland), the reaction mixtures were denatured at 94°C for 2 min and cycled for 45 rounds with denaturation at 94°C for 1 min, annealing for 1 min at 32°C, extension for 1 min at 72°C, and elongation at 72°C for 10 min to end the amplification. The PCR products were separated on a 9% polyacrylamide gel in Tris-borate buffer. The bands were visualized by ethidium bromide staining.

Recovery and Reamplification. The differentially displayed bands were excised from the gel. Individual polyacrylamide gel slices were rinsed with 100 μl of double-distilled water and crushed in 20 μl of double-distilled water. After 30 min incubation at 37°C followed by 5 min centrifugation at 14,000 *g*, the supernatant was used for reamplification by conventional PCR. The amplification began with denaturation for 3 min at 94°C, followed by 15 cycles at 94°C for 30 s, 40°C for 2 min, 72°C for 1 min and a further 20 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, with an extension period of 7 min at 72°C (13). The products were separated on a 2% agarose gel. Bands of the appropriate size were excised, and purified with the GeneClean Kit (Bio101, CA, USA). The purified products were stored at −70°C for future analysis.

Cloning and Sequencing. Using a LigATor Kit (R&D Systems, UK) and pGEM-T Easy Kit (Promega, WI, USA), DNA fragments were cloned into pTAg and pGEM-T Easy vectors. Generally, the size of inserts was between 200 bp and 1 kb. The fragments were sequenced using an AutoRead™ 100 Sequencing Kit (Pharmacia Biotech, Sweden) with a Pharmacia LKB. A.L.F. DNA Sequencer (Pharmacia Biotech, Sweden).

Expressed Sequence Tag Analysis. After sequencing, all ESTs were compared with NCBI database (14). Vector sequences, repeats or low complexity region sequences were removed by error checking. A Blastx search against the SwissProt EST database was performed. Sequences containing a high score pattern (HSP) of more than 34 amino acids or 100 bp, and having more than 95% sequence similarity, were recognized as encoding known proteins or known protein domains. A Blastn search against the GenBank(nr) and dbest EST databases was also performed. Sequences containing at least one HSP longer than 100 bp, with sequence similarity greater than 95%, were not considered as novel. The identification of the EST in the UNIGENE database enabled computer cloning to be performed, using an online EST assembly machine (located at http://www.tigem.it/ESTmachine.html), allowing a longer contig to be generated.

RESULTS

Cortical neurons isolated from E16 mouse cortex would attach to the polylysine-coated culture dishes during the 30 min pre-incubation period. Unattached cells were removed when the medium was changed. Attached cells appeared bright under a phase contrast microscope. They started to send out short processes from 3 to 12 hr. These cells acquired neuronal-like morphology with many processes on day 2 and 4 (Fig. 1A, B). The cell bodies of these neurons varied in mor-

Table I. Primers Used in AP-PCR

Arbitrary Primers	B1	CTTGTACGCGTGTGCGAC
	B2	CCTACACGCGTATACTCC
	B3	CATACACGCGTATACTGG
	B4	ACGCACACGCACAGAGAG
	B5	CACACGCACACGGAAGAA
	Cr	CACACGCACACGGAAGAA
Zinc-finger-like Domain Sequences	Zn1	CAYACHGGRGAAAAACC
	Zn2	CAYACHGGRGAAAAGCC
	Zn3	CAYACHGGRGAGAAACC
	Zn4	CAYACHGGRGAGAAGCC
	ZnF1	TAACCCCACCGGAGAGAA

phology. Some appeared dark under phase contrast while others appeared phase bright. Many neurons sent out long, branching neurites. There were no astrocytes in the culture. In 7-day-old cultures, many neurons grouped together and formed clusters of cell bodies (Fig. 1C). A few astrocyte-like cells were identified (Fig. 1C, arrows). The neurites became more extensive and thicker. The neurons appeared intact until day 10, after which they began to deteriorate. In this study, mRNA extracted from cultures on day 2, 4 and 7 were compared.

RAP-PCR products were separated on 9% polyacrylamide gel (Fig. 2). After ethidium bromide staining, a reproducible RNA fingerprint was produced which was primer-specific. Generally, about 10 bands were observed in each lane, although the exact number of visualized bands was mainly dependent on the different primer combinations. The size of the PCR products was generally between 200 bp and 1 kb. Parallel comparison of the RAP-PCR patterns of day 2, 4 and 7 cultures showed bands either increasing or decreasing in their intensity, indicating that the fragments might be either up- or down-regulated, respectively.

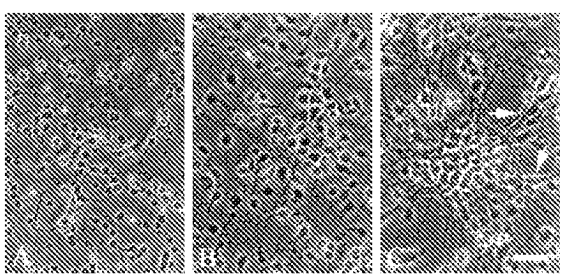

Fig. 1. Primary culture of neurons prepared from E16 mouse cerebral cortex. **A**) day 2 culture; **B**) day 4 culture; **C**) day 7 culture. Cultures were treated with cytosine arabinoside for 24 hr. No astrocytes were observed on day 4, but two astrocytes were observed on day 7 culture (arrows). Bar = 25 μm.

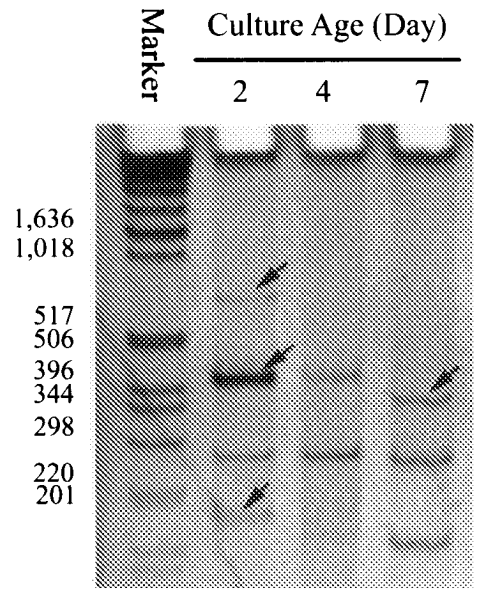

Fig. 2. RAP-PCR products resolved on 9% non-denaturing PAGE gel stained with ethidium bromide. mRNAs were obtained from day 2, 4 and 7 cortical neurons. The fingerprint pattern was primer specific. Different primer combinations were utilized for each PCR. About 10 bands were detected in each lane from each primer combination. Marker was 1 kb ladder. The size of bands ranged from 200 to 2,000 bp. Selected differentially expressed bands (arrows) from day 2, 4 and 7 cultures were excised for further analysis.

Selected bands with differential intensities from day 2, 4 to 7 cultures were excised from the gel for recovery and reamplification. In our study, we identified 8 up-regulated bands and 3 down-regulated bands (Fig. 3).

The reamplified PCR products from the selected bands were separated on 2% agarose gels (Fig. 4). Some of the amplified cDNA fragments were consistent in size with the original excised bands. These bands were purified and cloned into LigATor and pGEM-T Easy vectors. Some excised bands could not be reamplified and were not studied further.

Utilizing different primer combinations selected from Table I, II differentially expressed fragments were amplified and sequenced. All sequences were analyzed by Blast search of various sequence databases. The results are summarized in Table 2. A Blastx search against the SwissProt sequence database provided limited information. However, a Blastn search against the GenBank(nr) sequence database gave scores for clones 16-2 and 8-5 of 1314 and 541 and sequence similarities of 697/707 (98%) and 282/285 (98%), respectively. Thus, EST 16-2 and EST 8-5 represent two known sequences, *Mus musculus* KRAB-containing zinc finger protein mRNA and *Mus musculus* HP1-BP74 protein mRNA, respectively.

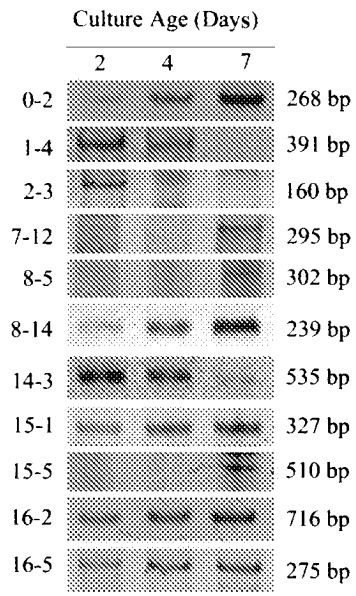

Fig. 3. Eleven differentially displayed DNA fragments were selected after visualization by ethidium bromide. In this study, 8 up-regulated fragments and 3 down-regulated fragments were selected for further analysis.

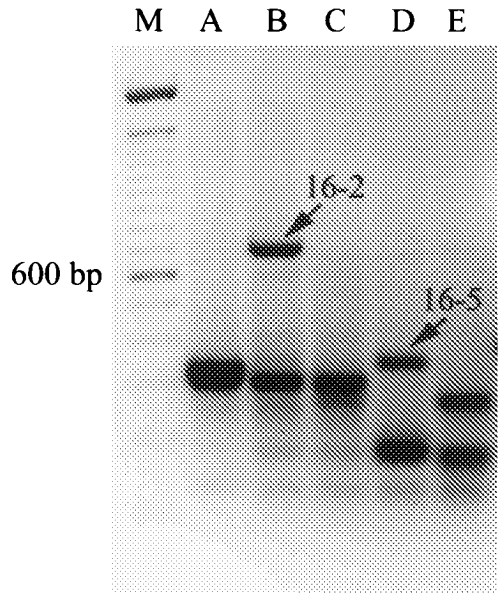

Fig. 4. Agarose gel (2%) analysis of PCR reamplified bands excised from polyacrylamide gel. Reamplification of all excised sequences was not possible. Reamplified products from clones 16-2 (lane **B**) and 16-5 (lane **D**) had a similar size as the original excised bands, although shorter sequences were also amplified at the same time. The reamplified gene fragments were excised and purified for cloning. Bands in lane **A**, **C** and **E** were not consistent in size with the original excised bands. Marker (**M**) was 100 bp ladder.

When clones 2-3, 7-12 and 16-5 were compared with the GenBank(nr), no matches were found (Table II). However, after searching against the dbest database, they were found to match known ESTs—mr76d04.x1 Stratagene mouse heart (#937316), mj54h12.r1 Soares mouse embryo NbME13.5 14.5 *Mus musculus* cDNA, and vs86a05.r1 Barstead mouse myotube MPLRB5 *Mus musculus* cDNA clone with sequence similarities of 134/135 (99%), 231/232 (99%) and 147/150 (98%), respectively (Table II).

Nine other clones were highly similar to either known genes or known ESTs. Using a Blastn search against the GenBank database, clone 0-2 was found to have a HSP with *Rattus* synaptic glycoprotein SC2 with an identity of 126/128 (98%). Similarly, using a Blastn search against the dbest database, clone 0-2 was found to have HSP with vs92d03.r1 Barstead mouse myotubes MPLRB5 *Mus musculus* cDNA clone 1153 733 5′, with an identity of 237/242 (97%) (data not shown). Furthermore, clones 1-4, 14-3, 15-1, 15-5, and 8-14 had high sequence similarity to known genes and known ESTs when they were searched in both databanks (Table II).

In order to acquire full-length sequences, clones 15-1, 7-12 and 8-14 were entered into an EST assembly machine. After computer cloning, the length of clone 15-1 was extended from 327 bp to 900 bp, clone 7-12 was extended from 295 bp to 1.8 kb, and clone 8-14 was extended from 239 bp to 1.5 kb. Based on these full-length sequences, primers were designed to amplify partial fragments. After PCR, appropriately sized fragments from clones 7-12 and 8-14 were detected and their identities were confirmed by sequencing (Fig. 5). However, the expected fragment from clone 15-1 was not detected.

DISCUSSION

Primary culture of cerebral cortical neurons has been widely used for the investigation of neuronal biochemistry and physiology at both cellular and molecular levels (2,4,5). In this culture system, most of the cells isolated from E16 mouse cerebral cortex were progenitor neurons, which would undergo various stages of development. These cells would send out processes within 12 hr after seeding. Morphologically, these cells were no different from mature neurons bearing long and branching neurites. It has been shown that these cells in culture undergo a functional maturation from day 4 to day 7 as indicated by their sensitivity to high-potassium-

Table II. ESTs Homolog Information Based on GenBank(nr) and dbest-Searches

Clone no.	Size (bp)	Primer combination	Expression pattern[a]	Result of Blastn search against GenBank (nr)/Result of Blastn search against dbest	
				High score pattern	Identity
0–2	268	Zn1 + B1 + B3	Up	*Rattus* Synaptic glycoprotein SC2	126/128 (98%)
1–4	391	B1 + B2 + B3	Down	Human mRNA for KIAA 0217 gene	137/165 (83%)
2–3	160	B2 + B3 + B4	Down	mr76d04.x1 Stratagene mouse heart (#937316)	134/135 (99%)
7–12	295	Zn4 + B1 + B3	Up	mj54h12.r1 Soares mouse embryo NbME13.5 14.5 *Mus musculus* cDNA	231/232 (99%)
8–5	302	Zn1 + Zn2 + Zn4	Up	*Mus musculus* mRNA for HP1-BP74 protein	282/285 (98%)
8–14	239	Zn1 + Zn3 + Zn4	Up	*Rattus norvegicus* rS-Rex-b mRNA	197/217 (90%)
14–3	535	ZnF1 + B2 + B3	Down	*Mus musculus* secreted frizzled-related sequence protein-4	197/201 (98%)
15–1	327	ZnF1 + B1 + B4	Up	*Mus musculus* mRNA for KRAB-containing zinc-finger protein KRAZ1	116/127 (91%)
15–5	510	B1 + B4 + Cr	Up	Mouse major histocompatibility complex region containing the Q region of Class I	120/142 (84%)
16–2	716	Zn1 + B5 + Cr	Up	*Mus musculus* KRAB-containing zinc finger protein mRNA	697/707 (98%)
16–5	275	Zn1 + B5 + Cr	Up	vs86a05.r1 Barstead mouse myotube MPLRB5 *Mus musculus* cDNA clone	147/150 (98%)

[a] Up, Up-regulation of fragment expression on day 7 compared with day 4 and day 2; down, Down-regulation of fragment expression on day 7 compared with day 4 and day 2; –: no significant match.

induced neurotransmitter (γ-aminobutyric acid, GABA) release (12). Clone 0-2 was found very similar to synaptic glycoprotein SC2, which was thought to play a role in the development and/or maintenance of synapses in the mammalian central nervous system (15). Those cDNA fragments having a high degree of sequence similarity with the known genes indicate that it might be a member of the same gene family. In the culture, it was more highly expressed on day 7 than day 2 and 4. This observation supports our previous finding that cerebral cortical neurons in primary culture exhibited high potassium-induced neurotransmitter release only on day 7, but not on day 4 (12).

RAP-PCR is a sensitive technique to identify cell-, tissue-, and process-specific differentially expressed gene (16). However, the drawback of this method is lack of reproducibility. In this study, we improved the reproducibility by separating the RAP-PCR products on a non-denaturing polyacrylamide gel and visualizing them by ethidium bromide staining. The RNA fingerprint pattern produced during these experiments was primer specific and reproducible. Differentially expressed bands were detected in a range from 200 bp to 1 kb. In this study, we utilized 6 different 18-mer arbitrary primers. To identify genes containing specific domains, a further 5 zinc finger domain sequences were designed. Three primers were randomly selected from the 6 arbitrary primers and the 5 zinc finger domain sequences for amplification. Three primers were used for each amplification. The RNA fingerprint obtained was reproducible and primer-specific; some primer combinations yielded

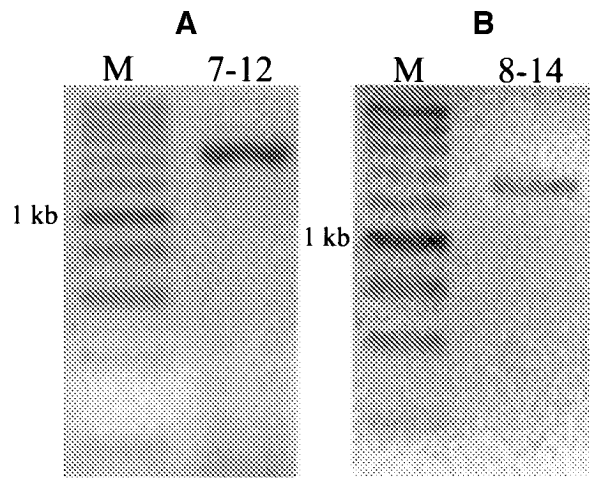

Fig. 5. Confirmation of sequences by computer cloning. **A**. A longer sequence (contig 7-12, 1.8 kb) was assembled by computer (see text) for clone 7-12. A pair of primers was designed based on the assembled sequence and used to amplify a partial fragment (1.5 kb). The expected band was detected as shown in panel A and the DNA sequence was confirmed by sequencing. **B**. A longer sequence (contig 8-14, 1.5 kb) was also assembled for clone 8-14. Another pair of primers was designed for amplification of a partial fragment (1.3 kb). The result of amplification is shown in panel B and the DNA sequence was confirmed by sequencing. Marker (M) was a 200 bp ladder and the arrow indicates the 1,000 bp band.

more cDNA fragments while other combinations amplified only a few bands (Fig. 2). Thus, the primer combination used was important in determining the RAP-PCR products and the number of visible bands harvested from each run.

In this study, we cloned 11 differentially expressed cDNA fragments. After searching through databases, cDNA fragments were identified that may be related to development and differentiation, such as clones 8-5 and 14-3. Clone 8-5 represents a known sequence, *Mus musculus* HP1-BP74 protein mRNA, which was originally identified using the yeast two-hybrid screen (17). The 70 C-terminal residues of HP1-BP74 showed significant similarity to the globular domains of histone H1 and its variants. In the yeast two-hybrid system, mouse heterochromatin protein 1 (HP1) interacted with HP1-BP74 protein and transcriptional intermediary factor 1 (TIF1). TIF1 was identified as a protein that interacted specifically with the ligand-binding domain of several nuclear receptors (18). Although it is generally accepted that nuclear receptors are transcriptional factors that control many aspects of development, differentiation and homeostasis, the role of HP1-BP74 involvement in HP1 and TIF1 is unknown. Our data indicated that clone 8-5 was up-regulated in cultured neurons from day 4-7, a stage when the GABAergic neurons began to exert a K+-induced neurotransmitter release. Thus, clone 8-5 may play a role in this functional differentiation.

Clone 14-3 was very similar to the Frizzled-related protein, *Mus musculus* secreted Frizzled-related sequence protein 4 (19). The Wnt/Frizzled cell signaling pathway has been implicated in playing an important role in embryonic induction, cell fate determination, polarity determination and malignant transformation (20–22). Frizzled is expressed in a variety tissues, including brain, heart, liver, and testis. Different Frizzled proteins have different patterns of expression (23). In our model, clone 14-3 may well be involved in the development of neurons.

Zinc finger proteins play important roles in many different processes. Following the identification of the zinc finger KARA-containing protein as a key regulator of early *Drosophila* development, more zinc finger proteins have been identified as important molecules involved in development (24). In order to identify zinc finger protein genes related to neuronal development, 5 zinc finger sequences were designed and used as arbitrary primers in this study (Table I). Clone 15-1 and 16-2 were isolated during these studies and found to contain typical zinc finger domains. Both clone 15-1 and clone 16-2 were up-regulated during neuronal development in culture. Although the functions of these as yet uncharacterized zinc finger proteins are unknown, their differential expression during neuronal differentiation suggests that they might play an important role in the process.

Some clones had no similarity to any known genes, but were similar to some known ESTs, such as clones 2-3 and 16-5. Bioinformatic techniques can be used to give an indication of the potential function of incomplete genes or those with unknown functions (25–29). Apart from the NCBI database, other databases, such as the Gene Expression Database (GXD) and the Mouse Genome Database (MGD), are available (30,31). Appropriate utilization of online databases will be helpful in studying and understanding sequence and function of novel genes. In this study, we attempted to acquire full-length sequences through computer cloning. Clone 8-14 (239 bp) was extended to a 1.5 kb contig whose sequence was confirmed by PCR. A database search using this 1.5 kb contig produced the same result as the original shorter clone 8-14, i.e. high similarity (90%) to *Rattus norvegicus* rS-Rex-b mRNA whose length was 1.5 kb. It is a homologue of human reticulon1-C (RTN1-C) which is known to express in nerve and neuroendocrine tissues (32). The contig sequence includes a start codon and poly-A tail. Compared with a cDNA library screen-

ing or Rapid Amplification of cDNA Ends (RACE), computer cloning allowed the rapid acquisition of the full-length gene sequence. In a similar manner, clone 7-12 was extended to 1.8 kb, although a database search did not reveal any HSP matches.

In summary, we identified 11 ESTs that were differentially expressed during neuronal maturation in culture. Eight were identified as known genes, and 3 were known ESTs. By computer cloning, two novel genes were constructed. One was very similar to a known gene, *Rattus norvegicus* rS-Rex-b mRNA; the other did not show any similarity to any known genes, and thus may be novel. These findings confirm the suitability of this culture system for mining and identifying known genes related to the GABAergic neuronal function maturation. Further functional study of these genes should facilitate the understanding of the molecular mechanisms underlying this process.

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