Genes expression monitoring using cDNA microarray: Protocol and Application

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Abstract

The major issue in the post genome sequencing era is determination of gene expression patterns in variety of biological systems. A microarray system is a powerful technology for analyzing the expression profile of thousands of genes at one experiment. In this study, we constructed cDNA microarray which carries 2,304 cDNAs derived from oligo-capped mouse cDNA library. Using this hand-made microarray we determined gene expression in various biological systems. To determine tissue specific genes, we compared Nine genes were highly-expressed in adult mouse brain compared to kidney, liver, and skeletal muscle. Tissue distribution analysis using DNA microarray extracted 9 genes that were predominantly expressed in the brain. A database search showed that five of the 9 genes, MBP, SC1, HiAT3, S100 protein-beta, and SNAP25, were previously known to be expressed at high level in the brain and in the nervous system. One gene was highly sequence similar to rat S-Rex-s/human NSP-C, suggesting that the gene is a mouse homologue. The remaining three genes did not match to known genes in the GenBank/EMBL database, indicating that these are novel genes highly-expressed in the brain. Our DNA microarray was also used to detect differentiation specific genes, hormone dependent genes, and transcription-factor-induced genes. We conclude that DNA microarray is an excellent tool for identifying differentially expressed genes.

Introduction

In higher eukaryotes, biological processes such as cellular growth organogenesis, and tumorigenesis are mediated by programs of differential

gene expression. To understand the molecular regulation of biological processes, the relevant subsets of differentially expressed genes of interest have been isolated, and studied extensively. The conventional standard techniques of molecular biology have been successfully used to identify genes involved in regulation of the processes. However, these methods are highly focused, targeting only several specific genes, and do not provide insight into global gene expression. In the near future, complete genome sequence for all of the model organisms and human will be available. As for the next step towards a detail understanding of the human genome, the implementation of the sophisticated methods for gene expression analysis and gene discovery is required.

Recently new methods to define gene expression patterns, including differential display (1), suppression subtractive hybridization (2), cDNA microarray (3, 4), and serial analysis of gene expression (SAGE) (5) have been developed. The cDNA microarray system is one of powerful technologies for analyzing expression profile of thousands of genes in a wide range of biological systems (6-10). In this method, DNA probes representing cDNA clones are arrayed onto glass slides and interrogated with fluorescently labeled cDNA targets. The diagnosis of the disease using the microarray has been also recently carried out (11, 12).

This current study was designed to determine the feasibility of our in-house cDNA microarray system as a means of obtaining differentially expressed genes. For this purpose, we made a cDNA microarray system using a set of 2,304 cDNA clones derived from oligo capped mouse brain cDNA (MNCb) library (13, 14), and as the first trial, attempted to identify novel mouse genes highly-expressed in the brain. Our results showed that the microarray data was selected nine genes highly-expressed in the brain, and consistent with the tissue distribution analysis by RT-PCR. Furthermore, we identified four of novel mouse genes highly-expressed in the brain. Thus, we conclude that our cDNA microarray system is an efficient tool for identifying differentially expressed genes in the brain. The search for new genes highly-expressed in brain may provide an initial

step for the cerebral functional research.

MATERIALS AND METHODS

mRNA Preparation

Mouse poly (A)+ RNAs (brain, kidney, liver, and skeletal muscle) were purchased from CLONTECH Lab. Inc.

Preparation of cDNA Microarray

A cDNA microarray chip consisted of 2,304 cDNA was made as previously described (3,4) with several modifications. Briefly, 2 mg/ml of products amplified with PCR were mixed at 1:1 with 4 mg/ml nitrocellulose in dimethylsulfoxide (DMSO) just before printing, and then spotted onto carbogiimide-coated glass slides (Nisshinbo) using a robotics (SPBIO-2000, Hitachi Software Engineering Co.). Murine b-actin was also spotted on the same array to serve as an internal control. After drying printed PCR products were crosslinked with ultraviolet light with the use of a UV crosslinker (60 mJ/cm2, Amesham Pharmacia Biotech.), and stored at room temperature.

Fluorescent Probe

Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia Biotech.) was incorporated during reverse transcription of poly(A)+ RNA, primed by a oligo(dT) primer. Two mg of poly(A)+RNA and 4.5 mg of oligo (dT) primer was dissolved in 15.4 ml of ddH2O, heated to 70℃ for 10 min, and immediately chilled on ice. Six ml of 5 x SuperScript II buffer, 3 ml of 0.1 M DTT (GIBCO BRL), 0.6 ml of 50 x dNTP (25 mM dATP, dCTP and dGTP, and 10 mM dTTP), and 3 ml of 1mM Cy3-dUTP and Cy5-dUTP were serially provided to the RNA. Finally 400 U of SuperScript II (LIFE Technologies) was added to the RNA, and then incubated at 42℃ for 1 hour. One hour after, 200 U SuperScript II was further added to the reaction mixture, and the mixture was incubated at 42℃ for additional 1 hour. The RNA was degraded by adding 1.5 ml of 1 N NaOH/20 mM EDTA followed by a 10-min incubation at 65℃. The samples were neutralized by addition of 270 ml of TE (pH 8.0) and 1.5 ml of 1 N HCl.

Unincorporated fluorescent nucleotides were removed by concentrating the mix to below 10 ml, using Centricon-30 microconcentrators (Millipore/Amicon). Twenty mg of salmon sperm (GIBCO BRL) and 500 ml of TE were provided into the probe, and after concentrated to 10 ml, the fluorescent probes were collected into a new tube.

Hybridization and Scanning

The fluorescent probe was mixed with 50 mg of yeast RNA (Sigma Chemical Co.), 50 mg of poly (A) (Roche Diagnostics Co.), 4.25 ml of 20 x SSC and 0.75 ml of 10 % SDS, and adjusted to 25 ml of total volume with ddH2O. Twenty-five ml of probe mixture was heated at 100℃ for 2 min, and incubated for 30 min at room temperature before use. Prior to hybridization, microarray was incubated with blocking buffer containing 3 % BSA, 0.2 M NaCl, 0.1 M Tris/HCl (pH 7.5) and 0.05 % Triton X-100 for 30 min at room temperature, and then thoroughly dried at 37℃. After wash three times with TE, the array was dried by centrifugation at 1,000 rpm for 1 min. The 25-ul probe mixture was applied onto the microarray surface and covered with cover slip (24 x 40 mm, Matsunami). The array was transferred to a hybridization chamber and incubated at 65 ℃ overnight under a humidified condition. The array was washed twice for 5 min at room temperature in low-stringency wash buffer (2 x SSC and 0.1 % SDS), washed twice for 5 min at 40℃ in high-stringency wash buffer (0.2 x SSC and 0.1 % SDS), and then rinsed with 0.2 x SSC. The array was centrifuged at 1,000 rpm for 1 min, and then scanned with the use of a fluorescence laser scanning device (ScanArray4000, GSI lumonics).

Tissue Distribution Analysis

The templates of the murine tissues of poly(A)+RNAs (brain, heart, lung, liver, kidney, skeletal muscle, thymus, spleen, stomach, intestine, testis, and skin) were purchased from Sawady Technology. The cDNA templates for RT-PCR were synthesized from 1 mg of poly(A)+RNA, using 200 U Superscript II reverse transcriptase and oligo (dT) primer. PCR was carried out in a final volume of 10 ml containing 1 x LA-PCR buffer, 200 mM each dNTP, 0.01 units of LA-Taq DNA polymerase

(TaKaRa Biomedicals), 2 mM each primer, and template cDNA. Temperatures and time schedules were: 25 to 35 cycles of 96℃ for 20 s and 62-64℃ for 1 min. PCR products were separated on 2.0 % Nusieve 3:1 agarose gel (FMC BioProducts) with a 100-kb ladder DNA marker (GIBCO BRL).

RESULTS AND DISCUSSION

cDNA microarray analysis

To determine the feasibility of our in-house cDNA microarray system, we arrayed a set of 2,304 cDNA clones from oligo-capped mouse brain cDNA (MNCb) library on glass slides using robotic printing, and attempted to identify differentially expressed genes in mouse brain compared to kidney, liver and skeletal muscle. A house keeping gene, b-actin (accession number: X03672), was also printed on the same array to serve as an internal control, and human Cot I DNA was used as a negative control. The microarray was subsequently hybridized with cDNA probes labeled with fluorochromes. Probes were prepared with poly (A)+ RNA from mouse brain, kidney, liver, and skeletal muscle. An example of one such hybridization was showns the cDNA probe derived from brain is labeled with Cy-3 fluorochrome (red) and the cDNA probe from kidney is labeled with Cy-5 fluorochrome (green). Red and Green fluorescent signals indicate greater relative expression in brain and kidney, respectively The yellow fluorescent signal indicates that both of the RNA is equal expression level.

The detection was carried out in each combination brain vs kidney, brain vs liver and brain vs skeletal muscle using the cDNA microarray. We defined highly-expressed genes in brain when the fluorescent ratio was 4 times higher compared to other tissues. First, the cDNA microarray was hybridized with fluorescent-labeled probes prepared from brain- and kidney derived poly (A)+ RNAs. Hybridization with probes selected 31 cDNA clones, which are considered highly-expressed in brain compared to kidney. Based on the one-pass sequence analysis, these 31 cDNA clones were categorized to 13 independent genes. Next, to confirm the

microarray data, further hybridizations were also carried out in combinations of brain vs liver and brain vs skeletal muscle. We found that nine of the 13 genes overlapped. Thus, we further analyzed these 9 genes.

A database search revealed that five (MNCb-0942, -1173, -1334, -2231, -0168) of the 9 genes were previously described genes: extracellular matrix associated protein (Sc1) (accession number: U64827), hippocampus abundant transcript 3 (HiAT3) (accession number: AB007912), S100 protein-beta (accession number: NM-009115), synaptosomal associated protein 25 (SNAP-25) (accession number: M22012), and major basic protein (MBP) (accession numbers: M15060) or the related (Golli-mbp) (accession numbers: L07507) genes. MNCb-2936 showed 98 % and 97 % identities to rat S-Rex-s (accession number: U17603) and human neuroendocrine-specific protein-C (NSP-C) (accession number: L10335) at the amino acid level, respectively, suggesting that the gene is murine S-Rex-s /NSP-C homologue (UniGene database: www. ncbi. nlm. nih. gov/UniGene). The remaining three genes did not match to sequences in the GenBank/EMBL database, indicating that these may be novel mouse genes. MNCb-0879 and MNCb-1002 were moderately similar to human ZWINT (81 %) (accession number: AF067656) and human PAK5 (88 %) (accession number: AB040812) genes at the nucleotide level, respectively. The remaining one gene (MNCb-0950) did not resemble to known genes in public database.

Analysis of tissue distributions by RT-PCR

To confirm the results of our microarray selection, we analyzed tissue distributions of the 9 genes by RT-PCR method using messenger RNAs from twelve types of mouse tissues. RT-PCR analysis showed predominant expression in brain compare to other tissues, for Sc-1 (MNCb0942), HiAT3 (MNCb-1173), S100 protein-beta (MNCb-1334), SNAP-25 (MNCb-2231), and MBP (MNCb-0168) genes (Table 2). Sc1 displays 70 % similarity to mouse secreted protein acidic and rich in cysteine (SPARC) (accession number: NM-009242), and is expressed at high level in mouse brain, while expressed at moderate levels in heart,

adrenal gland, and lung, and at low levels in kidney, liver, spleen, and testis (15). HiAT3 has high similarity to SCG10 at amino acid level, and is a neuron-specific negative regulator of microtubule dynamics during neurite outgrowth. The expression is limited to neurons (16). Murine S100 protein-beta is expressed at high level in glial than in non-glial cells. This gene is under complex transcriptional regulation involving tonic negative control exerted by combination of multiple cis-acting regulatory elements including cell type-specific elements (17). SNAP-25 is widely, but differentially expressed by diverse neuronal subpopulations of the mammalian nervous system and is suggested to play an important role in the synaptic function of specific neuronal systems (18). The mbp-related gene, Golli-mbp, which is a transcription unit of 105 kb that encompasses the mouse mbp gene, have been described to play a possible role in oligodendrocyte differentiation prior to myelination (19).

In this study, the microarray data identified four of novel genes, among which one is a mouse homologue of rat S-REX-s gene. All novel genes were confirmed to be differentially expressed in mouse brain by RT-PCR method, indicating that our microarray data are consistent with the results of tissue distribution analysis. Rat S-REX-s mRNAs had different regional distributions in the developing and mature rat nervous system (20), while the murine homolog (MNCb-2936) displayed restricted expressions in brain. The MNCb-0879 gene was relatively expressed at high level in brain compared with other tissues, while the MNCb-1002 gene showed brain-restricted distribution. The remaining one gene (MNCb 0950) was predominantly expressed in the brain.

In conclusion, we made a in-house cDNA microarray carrying 2,304 cDNA clones derived from oligo-capped mouse brain cDNA library. By using this, differential gene expression was examined for brain vs kidney, liver and skeletal muscle. We could detect nine independent genes that were preferentially expressed in the brain, among which 4 genes were novel. These results demonstrate that our cDNA microarray system is an efficient tool for identifying differentially expressed genes in mouse brain

and contributes the expression profile of the various mouse tissues.

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