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Gene Chips: Applications to Neuroscience

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Abstract

A convergence between molecular biological technique and the technology of miniaturization has produced the “gene chip” or microhybridization array. This device multiplies by several thousand fold the power of the northern blot for studying gene expression. Now, it is possible to survey simultaneously a large fraction of all genes in an experimental organism, and within a few years all of the approximately 140,000 human genes will be within reach of the technique. This capability is not only accelerating the rate of research into gene expression and function, it is changing the perspective of inquiry from single genes in isolation to networks of genes operating as a system. Many neurological diseases, from hydrocephalus to schizophrenia, have a genetic component, and individual responses to therapeutic drugs can vary with the genetic background of patients. In neurology and neurobiology, the ability to obtain “gene expression profiles” from nervous tissue promises to illuminate interactions between neuronal genes and the environment, development, disease, aging, and response to drugs and injury.

Keywords

Hybridization arrays; Gene chips; Gene expression profiling; cDNA; mRNA expression

Hybridization arrays are essentially southern blots, spotted with hundreds or thousands of DNA probes. (See Fig. 1.) These probes can be of two types: cDNA (1), or oligonucleotides synthesized from previously identified mRNA sequences (2). The spots of DNA are either placed on the substratum individually by a robotically controlled device (a quill, ring and pin, or ink jet) (3), or in the case of oligonucleotides, synthesized directly on the substratum through a photolithographic process (4). Robotics can be used to spot the probes onto nitrocellulose membrane or glass slides, but direct synthesis of oligonucleotides is accomplished on specially treated glass slides. mRNA from treated and control cells can be detected by either radioactive or fluorescence labeling of synthesized cDNA.

Several manufacturers offer ready-made hybridization arrays for genes of different species (5, 6). As a result of the successful effort to sequence the genome of humans and key experimental organisms, there is considerable sequence information, but the function of a large number of sequenced genes is unknown. Many commercial arrays contain thousands of unknown genes or partial gene sequences, termed expressed sequence tags (EST sequences),

initially identified from total mRNA. These sequences of unknown function enable researchers to discover the involvement of new genes in the process under investigation without prior hypotheses about which genes might be involved. Alternatively, the apparatus or institutional resources are becoming available to enable manufacturing custom arrays of cDNA derived from cells or genes of interest in a particular experiment (7).

Comparison among Alternatives

There are a number of alternatives to uncover genes expressed in a particular experimental paradigm, including screening cDNA libraries, differential display, and various types of gene arrays. Construction of cDNA libraries requires a high concentration of starting material (mRNA), and a laborious procedure of screening and sequencing each positive clone. Upon sequencing, one can discover that many of the clones are duplicates. For example, from 2287 clones isolated from four different libraries of macrophage progenitor cells, 1141 were uniquely represented and the rest were duplications (8). Finally, relative comparisons of message levels across libraries cannot be obtained.

Differential display (9) can circumvent some of the problems associated with cDNA libraries. This technique requires microliter amounts of mRNA; however, the nonlinear amplification of the PCR-based technique can introduce false positives and large uncertainty in relative comparisons of message level. Matsuo et al. (10) observed approximately 70,000 bands using 459 combinations of primer pairs on mRNA that was isolated from hippocampus after induction of LTP. This must include many duplicates and false positives, considering a reasonable estimate of 11% to 15% expression of all approximately 140,000 genes in a given cell. Finally, differential display requires sequencing the PCR products, whereas gene chips provide immediate identification of sequences in sample DNA by hybridization to known probes.

Hybridization arrays on nitrocellulose or charged nylon membranes are the easiest and most economical method. However, larger volumes of sample are required for hybridization on these membranes because of the porosity of the membrane and the larger size of the array as a consequence of the inability to produce as closely spaced probes as on arrays on solid supports. Furthermore, warping of the malleable matrix can cause artifacts and increase variability in comparison with solid matrices. Radioactive labeling is used for detection of hybridization onto membrane arrays, and fluorescent labeling of sample cDNA is used for detection on glass slides. An advantage of fluorescence detection is that experimental and control cDNA can be labeled with probes that fluoresce at different colors and therefore analyzed together on the array. Radioactive detection requires the use of separate hybridizations of control and experimental samples on separate arrays. Fluorescence detection may reduce experimental variation between control and experimental groups, but the method is less sensitive than radioactive detection. Full-length clones on robotically spotted arrays allow greater hybridization stringency and specificity, but single-base mismatches can be synthesized on oligonucleotide microarrays to provide specific controls.

Technical Considerations

DNA microhybridization arrays are an emerging technology, and as such the limitations of the technique are not well understood. The accuracy, reproducibility, and quantitative sensitivity are still being assessed. The technique is relatively simple in principle but made complex by the great number of steps involved in fabrication of the arrays, sample preparation, hybridization, and analysis. As a practical matter, this complexity multiplies the opportunities for mistakes. A number of artifacts must be recognized and excluded, including nonspecific radioactive or fluorescence smearing across several grids, variation in spotting size, shape and amount, and fluorescence bleed-through between detectors discriminating the color of fluorescence emitted by control and experimental samples. For these reasons, it is advisable to confirm the results with standard techniques of assessing gene expression levels. The current expense of the technology and the necessity for replication and independent confirmation are practical limitations at present, but this should improve as the technology matures.

Although the methodology is in its infancy, the technological issues are far less challenging than the analytical obstacles. Consider that a simple printout listing the genes on an array of 18,000 requires approximately 380 pages. How does one distill meaningful correlations and conclusions from changes in sets of hundreds of genes, perhaps representing several experimental groups? Pair-wise correlations are quite easily drawn, though subject to uncertainties of interpretation. Three-way correlations are considerably more difficult, requiring three-dimensional plots for representation and analysis. Multidimensional interactions and correlations among hundreds of variables (genes) at once require advanced statistical methods for grouping sets of genes into functional groups and specialized visualization techniques to display patterns of gene expression in multidimensional space (11, 12). Principal component analysis (13) and cluster analysis (14) have been applied to analyze data derived from microhybridization arrays.

Advances in bioinformatics and computerization are equally as important for this technique as advances in the technology to fabricate the arrays. Just as there are databanks for genes, peptides, and three-dimensional structure of protein, this technology would benefit from a databank where researchers could submit their array results. This could increase our understanding of how gene networks are affected by a particular phenomenon and how pathways converge and diverge in different biological paradigms. More powerful analytical software would be required to extract information from such a databank. The current method of database analysis is to search and examine each gene individually, but this involves a substantial data retrieval effort considering that a single array can show more than 200 changes. Expressed sequence tag (EST) sequences must be compared across Genbank to search for homology with any sequence identified since the EST was logged. Once genes are identified from an array result, there is a great deal of literature searching to be done to look for connections between the experiment and the genes identified. Sometimes these connections can be several times removed, and the investigator can miss an important link. One method of analysis, which shows promise, is data mining, as used in marketing research (11).

Hybridization arrays are most readily applied to simple cells, such as yeast or cell lines (15). Indeed, for these cells much progress has been made. For example, efforts using other techniques have identified roughly 150 genes involved in the sporulation cycle of yeast. In 1998, Chu et al. (16), using an array consisting of 6200 yeast genes from the completed yeast genome, showed 1000 significant changes during sporulation, which included the previously identified genes. In tissue, however, the problem of heterogeneity among cells becomes a major complicating factor. In developmental studies, for example, the observed changes in gene expression may be due to transcriptional regulation or changes in the number and type of cells in the sample at different stages of development due to cell migration, death, proliferation, or differentiation.

Special Considerations for Studies of the Nervous System

Studies of gene expression in the nervous system are especially difficult because of the small amount of starting material and the heterogeneity of the sample. Nervous tissue is an extremely complex assemblage of neuronal, glial, and nonneuronal cells. Neurons within the same region of the nervous system can be highly individualized in terms of phenotype, connections with other cells, level of excitability, and function. A single-cell approach may be required to analyze only the neurons directly participating in a particular function, such as CA1 neurons in studies of synaptic plasticity in the hippocampus, or small-type dorsal root ganglion neurons in studies of pain. This requires amplification methods that are linear and unbiased prior to analysis on hybridization arrays (17). Reducing the amount of starting material can lead to a decrease in the number of changes detected (18). Aspiration of individual neurons with a microelectrode or laser-capture (19, 20) of cells from histological sections followed by RNA amplification (17) have been used to derive expression profiles on microarrays in studies of the nervous system.

Second, many neuronal genes are present at low expression levels, and many neuronal gene families share high sequence homologies, such as neurotransmitter receptors or G-protein-linked receptors. Moreover, neuron-specific genes are underrepresented in public databases, in comparison to genes involved in the structure and function of cells in general; for example, genes involved in transcriptional regulation, mitosis, cellular structure and metabolism, and intracellular signaling pathways. This makes it difficult to obtain arrays of appropriate genes for neuronal studies and increases the experimental effort required for sequencing and characterizing novel genes.

Applications in Neurobiology

This technique is so new to the field that there are few publications in the neurosciences using it at present. However, the many potential applications in neurology and neurobiology may be glimpsed from the preliminary results covering a diverse range of studies. In basic neuroscience research, the technique is being used to characterize expression levels of 30,000 genes in the mouse retina (Y. Cao and L. Stryer, personal communication, November 4, 1999), to search for genes expressed differentially between the left and right hemispheres (21), and to monitor transcriptional changes during development or differentiation (22, 23). In biomedical research into the genetic basis and response to neurological diseases, such as

Alzheimer's disease (24), microarrays are being used to compare samples from normal and pathological cells. Microhybridization arrays are aiding the search for genetic markers of neuron progenitor cells (25), an important first step to developing transplantation methods to treat neurodegenerative diseases such as Parkinson's disease or spinal cord injury.

Four recent articles utilizing gene arrays give an idea of what to expect in the years to follow. The first two articles concern experiments that would have been difficult to attempt in any other way and that are contributing to a paradigm shift in signal transduction and gene regulation. The ability to monitor global expression levels in fibroblasts (26) and yeast (27) using oligonucleotide arrays reveals that intracellular signaling involves a network of signaling proteins that regulate gene transcription through a combinatorial activation of multiple promoters by many transcriptional activators and repressors and the transcriptional initiation apparatus on multiple genes, rather than a simple linear signaling pathway (28 for review). In fibroblasts stimulated with platelet-derived growth factor β , 66 immediate early genes on an array of 5938 oligonucleotides were induced at four different time points, a surprisingly diverse response to a single stimulus. Of these, 48% were previously identified, and of 10 the authors further investigated with standard techniques, all were expressed as observed on the arrays. Interestingly, mutation of various components in the receptor tyrosine kinase signaling pathway still allowed induction of 64 of these immediate early genes (IEGs), indicating diverse and broadly overlapping control of IEG induction.

The second example, from a recent article by Cirelli and Tononi (29), compared two approaches, differential display and gene arrays, to identify genes expressed during sleep and upon waking. When RNA isolated from the cerebral cortex was used for differential display, 11 genes were identified, most of which were subsequently shown to be sleep related. On the other hand, probing a cDNA array of 1,000 known genes and 18,000 ESTs with the same RNA revealed changes in 34 clones. However, when these clones were further investigated, only two were shown to be sleep related. The authors suggest that the high error may be related to using rat RNA to probe a mouse array.

With sufficient improvement in the technique, gene chips of the future may become powerful tools for diagnosis of neurological disease; not only for detecting genetic polymorphisms and mutations but also for identifying "signature gene profiles" indicative of specific neuropathologies. Using cDNA arrays of 1400 or 5000 genes on a glass slide, Whitney et al. (30) identified 29 genes that were overexpressed in plaques from multiple sclerosis patients compared with normal white matter. The changes were reproducible and several were validated by immunohistochemistry.

The most compelling use for this new technology is in basic research into how networks of genes are regulated and in uncovering genes involved in disease without the prior hypotheses of what genes may be involved. This is the type of information and approach that could shed light on why certain patients respond favorably to a certain drug whereas others do not (lithium for treatment of depression, perhaps), and why there is a genetic predisposition for certain medical conditions or behaviors, such as drug or alcohol abuse, or schizophrenia and other psychiatric disorders. Pursuing these types of important neurobiological questions one

gene at a time may become an obsolete strategy if the technical and analytical issues involving gene chips can be overcome.

Addendum

Several important developments and publications appearing while this review was in press make it clear that microhybridization arrays will have a major impact on neuroscience and cell biology. With the recent announcement by the National Center for Biological Information, the urgent need called for in this review (p. 312) for a central database storing gene array results has just been achieved (31). This database may be accessed over the internet at the following address: www.ncbi.nlm.nih.gov/geo. A technical improvement for isolating mRNA primed for translation has been described (32) by using a polysome fractionation technique prior to isolation. This offers a significant advantage to the gene array technology since not all mRNA transcripts isolated from cells are translated in a given cellular state. Three recent applications of gene arrays to neurology exemplify the power of this technique to improve our understanding of the basis of nervous system disease. This includes: a comparison of gene expression in normal versus trisomy (Down Syndrome) cell lines of mice (33); profiling the expression pattern of genes involved in human neuronal differentiation (34); and analysis of the transcriptional network controlled by the photoreceptor homeobox gene *Crx*, a transcription factor linked to congenital blindness or photoreceptor degeneration in humans (35).

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Fig. 1. Microhybridization arrays may be manufactured by two different methods: (A) from cDNA libraries or (B) from oligonucleotide synthesis on glass slides. *A*, Genes of interest are cloned in multiwell plates, amplified by PCR, purified and spotted onto membranes or slides with a high-speed robotic arm, which deposits approximately 5 nL droplets of DNA in a grid pattern. *B*, Oligonucleotide synthesis on glass slides is controlled by a sequential photolithographic process through a mask. Nucleotides are covalently linked in the desired sequence by light-activated deprotection, to generate arrays of oligonucleotides of approximately 25 bases in length. For expression profiling, RNA is extracted from

experimental and control cells and reverse transcribed into cDNA using radioactive or fluorescently tagged bases. The samples are allowed to hybridize on the microarray and then imaged. The use of fluorescently labeled tags of different colors allows co-hybridization of experimental and control samples on the same array. Fluorescent imaging is used to detect the ratio of hybridization of the two samples on each spot. Radioactive labeling requires separate hybridization of control and experimental samples. The results are entered into computer databases, analyzed by specialized software, and compared with information in public databases.

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