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Mini-Review

High Throughput Analysis of Gene Expression in the Human Brain

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The human brain is thought to have the greatest complexity of gene expression of any region of the body, reflecting the diverse functions of neurons and glia. Studies of gene expression in the human brain may yield fundamental information about the phenotype of brain cells in different stages of development, in different brain regions, and in different physiological and pathological states. As the human genome project nears completion, several technological advances allow the analysis of thousands of expressed genes in a small brain sample. This review describes available sources of human brain material, and several high throughput techniques used to measure the expression of thousands of genes. These techniques include expressed sequence tag (EST) sequencing of cDNA libraries; differential display; subtractive hybridization; serial analysis of gene expression (SAGE); and the emerging technology of high density DNA microarrays. Measurement of gene expression with microarrays and other technologies has potential applications in the study of human brain diseases, including cognitive disorders for which animal models are typically not available. Gene expression measurements may be used to identify genes that are abnormally regulated as a secondary consequence of a disease state, or to identify the response of brain cells to pharmacological treatments. J. Neurosci. Res. 59:1–10, 2000.

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The human brain is the most complex region of the body in terms of diversity of gene expression. The brain mediates cognition and emotional experience, and performs major functions including the acquisition of sensory input, encoding and storage of memories, homeostatic regulation of the body, and coordination of motor output. These various functions of the brain are accomplished by only a few basic cell types, and in particular neurons and glia (astrocytes, oligodendrocytes, and microglia). While most neurons share a highly similar structure (consisting of a cell body, dendritic tree, and an axonal process), there is a tremendous diversity of subtypes of these cells.

The functional properties of specific regions of the brain are determined in large part by the genes that are expressed in individual cells. This gene expression occurs in defined regions and developmental times in brain, and is dynamically regulated. As the DNA sequences of tens of thousands of genes become known through the human genome project, it is possible to determine the profile of their expression in biological samples (Strachan et al., 1997). This review describes several techniques useful for the investigation of gene expression in the human brain.

The human genome consists of ≈3 billion nucleotides of DNA, including approximately 100,000 expressed genes (URL ref. 1). Sequencing of the human genome is 13% complete as of August, 1999 (URL ref. 2), and is expected to be completed within the next several years. Approximately 75,000 human genes have been identified by the Unigene project of the National Center for Biotechnology Information (URL ref. 3). The function of the vast majority of the proteins encoded by these genes is not known. In any given cell, only a subset of genes (≈20,000 to 30,000; Wan et al., 1996) is expressed. Gene expression may be regulated developmentally, spatially (e.g., in a cell type-specific manner), environmentally (e.g., activity-dependent gene expression) and by disease states. Evidence in rodents suggests that the brain exhibits greater complexity of gene expression than any other region of the body. Hahn and Laird (1971) estimated the complexity of RNA in mouse brain by studying DNA-RNA hybridization and found that brain RNA is three times more complex than kidney or liver. In 1983, Milner and Sutcliffe performed Northern analyses on 191 randomly selected rat brain cDNA clones, and found that 30% were expressed in brain but not liver or kidney. They suggested that many messenger RNAs (mRNAs) present at low

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levels are expressed preferentially in brain, a finding that has been supported by analysis of large-scale sequencing of human brain cDNA libraries (see below).

The analysis of comprehensive gene expression profiles from the brain will be useful to address fundamental questions such as the following: (1) in a brain region or an individual brain cell, what is the number of genes that are expressed at detectable levels? (2) within an individual, what is the complexity of gene expression in different regions of the brain? For example, what are the differences in gene expression between cerebral cortex and cerebellum? Are there significant differences within one region, such as in adjacent sections of cerebellum? (3) how does the pattern of gene expression vary between brain and different parts of the body? (4) between individuals, what is the normal variation of levels of gene expression in corresponding brain regions? (5) between males and females, and between individuals at different developmental stages, what is the variation in gene expression? (6) what changes in gene expression accompany pharmacological interventions, changes in physiological state, or disease? Are there consistent changes in gene expression associated with factors such as cause of death?

In addition to these specific questions about patterns of gene expression in different states, places, and developmental stages, the generation and analysis of high throughput data on gene expression is likely to provide broad insight into flows of information transfer in the brain. This analysis will depend upon mathematical models that reflect the complexity and parallel processing capabilities of genetic information in the brain (Somogyi and Sniegoski, 1996).

The Acquisition of Human Brain Specimens and Brain RNA

Studies of gene expression in the human brain typically rely on postmortem samples. Alternative sources of RNA are less commonly available, and include biopsies of excised tumors or hemispherectomized tissue (Kukekov et al., 1999; Takeshima et al., 1994), biopsies of olfactory neurons (Johnson et al., 1994; Ensoli et al., 1998; Abrams et al., 1999), and human neuronal cell lines (Ronnett et al., 1994).

Postmortem human brains are available from a variety of brain banks. These include two that are supported by the National Institutes of Health: the Brain and Tissue Bank for Developmental Disorders at the University of Maryland at Baltimore (URL ref. 4) and at the University of Miami (URL ref. 5). Other banks include the Harvard Brain Tissue Resource Center (URL ref. 6), and the National Neurological Research Specimen Bank of Los Angeles (URL ref. 7). In addition, fibroblast and lymphoblast-derived cell lines from patients with neurological and other disorders are available from banks such as the Coriell Cell Repositories (URL ref. 8).

When collecting human postmortem samples, biopsy material, or cultured cell lines, there are several factors that should be taken into account (Ravid et al., 1992). It is essential to know the clinical histories of the patients from whom biological material is obtained. For example, if a patient diagnosed with autism was taking antiseizure or other medications at the time of death, comparison of that brain sample to a matched control could result in the identification of gene expression differences attributable to drug effects rather than to the disease. As another example, in a comparison of brain samples from patients with schizophrenia to age-, gender-, and regionally matched controls, if several of the schizophrenia cases were clinically atypical, then differences in gene expression between diseased and control samples might be obscured. An additional complexity is that multiple molecular etiologies may underlie a single clinical diagnosis.

In addition to clinical histories, it is important to control for factors which may affect mRNA integrity. Most important may be the agonal state (condition of the individual before death; Barton et al., 1993; Johnston et al., 1997) and in particular the pH of the brain (Harrison et al., 1995). For example, we have observed dramatic changes in gene expression in RNA derived from a patient who was alive but brain dead for 8 days prior to clinical death (Colantuoni et al., unpublished). Other factors that affect RNA integrity include the patient's medication history and other drug use, the postmortem interval, the number of times a stored sample is thawed and refrozen, and the length of time in storage (Leonard et al., 1993). Typically, a nonfetal human brain sample has a postmortem interval of 17 hours (Dr. R. Zeilke, personal communication), although the length of postmortem interval may not be a critical factor in obtaining RNA of high integrity (Schramm et al., 1999; Kobayashi et al., 1990). For cell lines, the number of cell passages, the cell confluency at the time RNA is harvested, and the cell culture conditions may all affect RNA integrity and/or composition.

Viruses often target the central nervous system (Kristensson, 1992), and postmortem human specimens as well as cell lines are routinely infected by a variety of viruses including hepatitis, herpes, and human immunodeficiency viruses (Shapshak et al., 1986; Bolay et al., 1996; Sanders et al., 1996; Petito and Roberts, 1995). By using the technique of subtractive hybridization (see below), we identified hepatitis C virus and a simian sarcoma virus variant in postmortem Rett Syndrome and autistic brains and transformed lymphocytes (O.-H. Jeon and J. Pevsner, unpublished observations). Viruses may induce apoptosis (Petito and Roberts, 1995) or other host responses, thus complicating gene expression studies. Laboratory procedures have been developed to handle high-risk viruses and transmissible human spongiform encephalopathies in neuropathology studies (Bell and Ironside, 1997), and these guidelines should be applied by researchers conducting gene expression studies with human samples. In particular, "universal precautions" must always be followed (Hutchins et al., 1994).

Once human brain material is obtained, RNA can be isolated for gene expression studies, and for techniques such as cDNA library construction (Sainz, 1993). RNA

has been isolated from autopsy material and shown to be of sufficient integrity for Northern (RNA blot) and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (Larsen et al., 1992; Schramm et al., 1999). Brain-specific transcripts lacking a polyadenine tract would not be represented by RNA amplification approaches relying on oligo d(T) priming, but such polyA- transcripts may merely represent polyA+ transcripts that are nicked during RNA isolation (Fung et al., 1991).

The heterogeneity of cell types in the human brain is so great that controlling for the brain region being studied is difficult. If gene expression in a region such as hippocampus is compared between two individuals, a discrepancy in the exact region that is dissected could lead to the identification of differences that are caused by regional variation rather than variation between individuals. In one approach to address the heterogeneity of brain samples, Eberwine and colleagues have isolated RNA from single cells of the postmortem human brain (Kacharmina et al., 1999) and have conducted gene expression studies. As an example, they analyzed 51 mRNAs from single cells associated with senile plaques and hippocampus from Alzheimer disease brains (Ginsberg et al., 1999). In another application, they isolated mRNA from single cells in paraffin-embedded tubers of tuberous sclerosis brain specimens (Crino et al., 1996). Single cells may also be isolated by laser capture microdissection for further RNA analysis (Emmert-Buck et al., 1996).

High Throughput Analysis of Gene Expression in the Human Brain: EST Sequencing

Expressed sequence tags (ESTs) are randomly selected clones sequenced from cDNA libraries. Each cDNA library is constructed from total RNA or poly(A) RNA derived from a specific tissue or cell, and thus the library represents genes expressed in the original cellular population. A typical EST consists of 300 to 500 base pairs of DNA, and is often deposited in a database as a "single-pass read" that is sufficiently long to establish the identity of the expressed gene. Adams et al. (1991, 1992, 1993a,b) pioneered the large-scale sequencing of ESTs from human brain libraries and identified thousands of genes expressed in brain. Often, new members of gene families were identified, such as a cDNA encoding a neural-cell adhesion molecule paralog (Adams et al., 1992). The analysis of EST data revealed answers to fundamental questions such as which genes were most abundantly expressed (e.g., β-actin, myelin basic protein, α and β-tubulin; Adams et al., 1992) and the amount of regional variation of gene expression within brain regions.

There are currently over 1.5 million human ESTs in the publicly available database of ESTs (dbEST) provide by the National Center for Biotechnology Information (NCBI; release 082799; URL ref. 9). These ESTs are derived from approximately 1,200 human cDNA libraries, 100 of which were generated from postmortem brains. By comparing genes that are expressed in these libraries, information can be obtained concerning regional distribution of gene expression (e.g., comparing genes expressed in fetal versus adult libraries, or hippocampal versus cerebellar; Carulli et al., 1998). Statistical analysis of thousands of cDNAs from hundreds or thousands of libraries can also allow a description of patterns of gene expression in different pathological or physiological states, depending upon the specific RNA that is isolated to generate each library. Walker et al. (1999) examined the expression of 40,000 human genes in 522 cDNA libraries and used an algorithm called Guilt-by-Association to identify five genes associated with schizophrenia and Parkinson's disease. Similar high throughput comparisons can be performed electronically by differential display or serial analysis of gene expression (SAGE; see below).

In addition to the public EST database, several companies have generated larger collections of ESTs. These companies include Human Genome Sciences (URL ref. 10), Incyte Pharmaceuticals (URL ref. 11), and Celera Genomics Group (URL ref. 12). Large sets of EST data from The Institute for Genomic Research (TIGR; URL ref. 13) and from a Washington University initiative (supported by Merck & Co. and the National Cancer Institute; URL ref. 14) have been deposited in the public database.

The EST approach to gene expression analysis has several fundamental limitations. Libraries are generated from RNA that is isolated at a specific developmental time and place, and many expressed genes may not be represented because only a limited number of libraries have been sequenced. All expressed genes will be identified in the next several years as genomic sequencing of human DNA is completed, but until then it will not be known how many expressed genes are not represented in currently available cDNA libraries. Complementary EST sequencing projects in other organisms such as mouse (Marra et al., 1999) are also useful in the analysis of genes expressed at specific stages in development. Another concern about the EST approach is the complexity of the cDNA library that is sequenced: libraries prepared from whole brain or from specific brain regions are likely to contain a heterogeneous population of neurons and several glial cell types. Additionally, data obtained from the sequencing of multiple libraries may not be comparable if they differ in normalization (Bonaldo et al., 1996). A non-normalized library reflects the complexity of the original RNA source, while a normalized library represents transcripts at comparable levels, minimizing the redundancy. Finally, libraries may be sequenced more or less exhaustively, and this may influence the number of rare cDNA transcripts that are identified.

High Throughput Analysis of Gene Expression in the Human Brain: Differential Display, Subtractive Cloning, and SAGE

Several techniques have been developed to measure gene expression in paired samples (reviewed in Watson and Margulies, 1993; Sagerstrom et al., 1997; Carulli et al., 1998; Vietor and Huber, 1997; Wan et al., 1996). In each case, total RNA or poly(A) RNA is isolated from two sources of interest, converted to cDNA by reverse transcription, and compared. In differential display, RT-PCR

is accomplished with oligo dT (for RT) and short, random primers (for PCR) that produce a series of products that can be compared between two samples. By using arbitrary primers, many PCR products are formed, and differences that are observed represent mRNAs that are selectively enriched in one population. This technique has been applied to neurons (reviewed in Livesey and Hunt, 1996) and used to identify genes differentially expressed in postmortem alcoholic brains (Fan et al., 1999), human brain tumors (Uchiyama et al., 1995; Sehgal et al., 1998b; Comincini et al., 1999), neuroblastoma cells (Kito et al., 1997), and preneuronal NT2 cells (Santiard-Baron et al., 1999). Experiments using differential display often result in the identification of novel or uncharacterized genes, as well as suggesting novel functions for known genes. For example, Uchiyama et al. (1995) performed differential display on mRNA from normal human brain and tumor specimens (glioblastoma mulitforme and anaplastic astrocytoma). In addition to identifying 20 novel sequences, they identified a kinesin heavy chain homolog. The differential regulation of this mRNA was confirmed by Northern blot analysis. In differential display (as well as other comparative techniques), it is necessary to independently confirm changes in gene expression using conventional methods such as quantitative PCR and RNA (Northern) blotting.

Another technique for the isolation of differentially expressed genes is subtractive cloning (reviewed by Sagerstrom et al., 1997). In a common form of subtractive hybridization (Diatchenko et al., 1996), two cDNA populations are generated from RNA sources. Adaptors are ligated to a "tester pool" of cDNA. A second pool of cDNA ("driver cDNA") is added in excess, and only genes that have been up-regulated in the tester pool can then be selectively amplified by PCR. This technique requires only small amounts of RNA, and it is best suited for identifying dramatically differentially expressed genes (e.g., 5- to 10-fold differences). Labudova et al. (1999a,b) applied this technique to postmortem brains of patients with Down's syndrome and identified an up-regulation in the mRNA for phosphoglycerate kinase and thyroid stimulating hormone receptor. In another application, Ligon et al. (1998) used subtractive hybridization to identify genes regulated in human glioblastoma cell lines. In our laboratory, comparison of normal and autistic or Rett syndrome brains resulted in the identification of both viruses (see above) and brain transcripts that may related to neuropathology and were independently confirmed using microarray technologies.

A technique related to subtractive hybridization that also relies on PCR is representational difference analysis (RDA). Although originally applied to genomic DNA (Lisitsyn et al., 1993), it has been used to compare differences between two cDNA populations (O'Neill and Sinclair, 1997). Dron and Manuelidis (1996) used cDNA RDA to isolate a rare viral transcript in Creutzfeldt-Jakob disease brains.

A third approach to measuring mRNA differences between two populations is SAGE (Velculescu et al., 1995). cDNA fragments are generated with short tags (typically 8 to 11 base pairs) at the 3' end of each transcript, concatenated, and sequenced. Analysis of tags allows the cataloging of thousands of genes expressed from a tissue source, including a quantitative estimate of gene expression. Zhang et al. (1997) used SAGE to analyze 300,000 transcripts derived from 45,000 genes.

It is now possible to analyze large quantities of data on genes expressed in the human brain by rapid, electronic versions of differential display and SAGE offered by the NCBI (URL refs. 15 and 16). For example, the differences in gene expression between 20 human brain libraries and dozens of nonbrain libraries can be compared by library differential display, resulting in the identification of genes selectively expressed in brain. Similarly, genes expressed in libraries derived from various brain regions, cell types, or developmental or disease states can be compared electronically. The results from such surveys are likely to provide invaluable information on gene expression in the brain.

High Throughput Analysis of Gene Expression in the Human Brain: cDNA Microarrays

High density DNA microarrays have been developed to analyze gene expression patterns in a variety of biological systems (Khan et al., 1999; Duggan et al., 1999; Brown and Botstein, 1999; these and other microarray articles published in Nature Genetics are available at URL ref. 17). A review of commercially available microarray technologies is provided by Bowtell (1999). Typically, hundreds or thousands of cDNAs or oligonucleotides (Lockhart et al., 1996; Lipshutz et al., 1999) corresponding to ESTs or known genes are immobilized on a solid support such as a nylon membrane or glass microscope slide. Gene expression is assessed by isolating total RNA or mRNA from a sample of interest, such as a postmortem human brain. The RNA is labeled as radioactive or fluorescent cDNA, and the array is probed. After hybridization, washing, and then imaging, the pattern of gene expression is quantitated. This technology represents one method of using the large amount of information accumulated by the Human Genome Project and other genome projects to assess complex patterns of gene expression in biological samples. Tens of thousands of cDNAs or oligonucleotides may be immobilized on microarrays, and so the amount of data generated is potentially large. Data are analyzed and stored in a database, although at present there is not a central repository for gene expression data generated from microarray experiments (Ermolaeva et al., 1998).

Most studies using high density DNA microarrays (i.e., cDNA- or oligonucleotide-based arrays) have not involved the brain. A focus has been the study of human cell lines (DeRisi et al., 1996; Iyer et al., 1999; Heller et al., 1997; Rhee et al., 1999), normal versus cancerous or other diseased samples (Hacia et al., 1996; Maniotis et al., 1999; Wang et al., 1999; Alon et al., 1999), and yeast samples (e.g., Lashkari et al., 1997; Spellman et al., 1998).

Several early studies addressed the diversity of gene expression in the human brain using high density microar-

rays. Zhao et al. (1995) generated an array of 2,505 cDNAs derived from human brain and probed it with radiolabeled cDNA from adult brain, fetal brain, or adult liver. For four cDNAs preferentially expressed in brain, subsequent Northern analysis revealed a distribution pattern consistent with the microarray findings. In a similar study, this group (Takahashi et al., 1995) immobilized ≈8,300 human cerebral cortex cDNA clones on a filter, then probed the arrays with cDNA made from RNA isolated from brain, kidney, or liver. Two hundred clones expressed preferentially in brain versus peripheral tissues, and 100 clones expressed preferentially in fetal versus adult brain, were partially sequenced. Many of these genes were novel, and the authors further studied the expression profile of the 300 sequenced cDNAs in several cell lines. In another approach Drmanac et al. (1996) immobilized ≈73,000 infant brain cDNA clones on membranes, probed the membranes with radiolabeled oligonucleotide probes, and determined a hybridization signature for each clone. Data analysis suggested that they identified about 14,000 genes, many of which were expressed at low levels. Such findings are consistent with the observations of Milner and Sutcliffe (1983) described above who performed 191 Northern blots on rat brain cDNAs and found that many transcripts expressed preferentially in brain are present in low abundance.

Gene Expression Studies in the Diseased Human Brain

High density microarrays may provide a useful tool for the study of human disease (Khan et al., 1999). Two major applications of microarray technology are to survey genome-wide expression profiles in normal versus diseased states, or to use oligonucleotide arrays to define mutations that occur in a single gene of interest. The earliest uses of microarrays in human disease have been to study cancer (Zhang et al., 1997) and inflammatory disease (Heller et al., 1997). While the pathophysiology of these diseases is complex, they are amenable to study using microarrays in part because relatively homogeneous cell lines are easily available. Other high throughput technologies are useful for the study of disease. Cossman et al. (1999) isolated single Reed-Sternberg cells from Hodgkin's disease and sequenced 27,000 cDNA sequences (11,000,000 bases of DNA). Data analysis of the expressed genes suggested an unusual lineage of these diseased cells.

Few studies have been published on gene expression in human brain using high density cDNA microarrays. Whitney et al. (1999) examined the expression of up to 5,000 genes on high density microarrays using brain tissue obtained at autopsy from a multiple sclerosis patient. Sixty-two genes were found to be significantly regulated in acute sclerotic lesions relative to unaffected white matter brain regions. Sehgal et al. (1998a) compared the expression of 588 genes on a CLONTECH microarray in normal brain tissue to glioblastoma multiforme tumor tissue. Fifty-two genes were overexpressed in tumor tissue, and 57 were down-regulated relative to a normal brain sample. The authors analyzed the expression levels of seven of these genes by RT-PCR and confirmed the abnormal regulation of five in tumors. In preliminary studies, Somogyi et al. (1999) have examined the expression profiles of 9,000 genes from control and Alzheimer's Disease postmortem brain amygdala and hippocampus using Incyte Pharmaceuticals microarrays. Many neuron-specific genes were down-regulated, while glial-specific genes were up-regulated.

In contrast to these studies, cell lines are not available for most brain disorders. Postmortem or biopsied human brain is thus necessary for the study of many pathological neurological processes. For hundreds of disorders of the human brain including schizophrenia, depression, autism, and mental retardation, the primary genetic defects are not known. Traditional genetic approaches have not yet succeeded in identifying genes responsible for many diseases, especially when adequate pedigrees are unavailable. For human disorders that affect cognition or other higher mental functions such as language skills, animal models may be inadequate because they cannot accurately represent the pathological changes. Readily available peripheral tissues (e.g., skin fibroblasts or blood cells) may not be affected in terms of gene expression or biochemical changes in disorders of the central nervous system. Thus, an important approach to studying human brain disorders is to analyze postmortem brain samples. The technology of high density cDNA microarrays can be used to measure gene expression in diseased and control human brains, even in studies of a clinically heterogeneous syndrome.

In our laboratory, we have investigated gene expression in autism, and these studies illustrate some of the technical challenges involved in studying a human neurological disorder. We measured the expression of 20,000 genes in 12 autistic brains and 12 controls matched for age, gender, and brain region (Purcell et al., unpublished). Autism is a pervasive developmental disorder that affects as many as 1 in 500 individuals, and it is characterized by a triad of deficits: (1) an individual's failure to have normal reciprocal social interaction, (2) impaired language or communication skills, and (3) restricted, stereotyped patterns of interests and activities (Rapin and Katzman, 1998). There are no known biological markers. In the simplest scenario, a single gene defect causes a disease such as classical infantile autism. In an ideal case, all the autistic cases we are studying are clinically authentic, and consistent changes in gene expression occur which will be informative to identify both the gene(s) responsible for autism and the genes whose products are relevant to a disrupted biochemical pathway in autistic brains. These genes may lead to a diagnostic test for autism, or to strategies for therapeutic intervention based upon correcting perturbed pathways.

In reality, autistic disorder may be caused by simultaneous defects in multiple genes. The primary genes, while defective, might not be expressed at abnormal overall levels, making them indistinguishable from controls by cDNA microarray analysis. For many diseases, such as X-linked adrenoleukodystrophy (Ligtenberg et al., 1995),

the defective gene contains mutations that do not alter the level of mRNA, and so most gene expression technologies would fail to detect the primary genetic defect in comparisons of diseased and normal samples. For autism, it is also possible that the clinical diagnosis is applied to a set of symptoms that result from a series of distinct gene disorders that share a common phenotypic expression. Thus, the 12 autistic brains we are studying may derive from patients with several distinct brain disorders. Furthermore, even those with classical infantile autism may be more or less severely affected. Thus, it is highly likely that the autistic samples we are studying will exhibit some degree of heterogeneity in their levels of gene expression. Finally, the region of brain we are studying (cerebellum), while implicated in autism based upon previous neuropathology and neuroimaging studies, may not express the changes in gene expression that are relevant to the pathophysiology of autism. The benefit of microarray technologies may lie in defining the consequences of primary defects on the expression of other genes in the brain.

Analysis of Gene Expression Data

A common feature of the various high throughput technologies available to measure gene expression is the generation of vast amounts of data. Assorted tools are being developed (Vingron and Hoheisel, 1999; Liang et al., 1998; Audic and Claverie, 1997) such as cluster analysis to visualize large-scale profiles of gene expression (Eisen et al., 1998; Michaels et al., 1998). Some of these approaches have been developed from quantitative analysis of individual genes (Roberts, 1994; Takahashi, 1992). The presentation of microarray and other high throughput data may take the form of a website supplied by individual laboratories, or an internet-based repository provided by a scientific journal or by an agency such as NCBI (Ermolaeva et al., 1998).

Future Prospects for Gene Expression Studies in the Central Nervous System

In the future, a complete catalog of gene expression in the human brain is likely to be generated in which the spatial and temporal expression pattern of each gene is determined. For an individual gene product, this may be accomplished with labor-intensive techniques such as in situ hybridization (at the mRNA level) and immunohistochemistry (at the protein level). Such studies will be complemented by parallel studies of gene expression in organisms such as the mouse (Woychik et al., 1998), fruit fly, plant (Schena et al., 1995), and nematode. Animal models may be useful for studies of gene expression in physiological states such as sleeping versus waking (Cirelli and Tononi, 1999), hibernation (O'Hara et al., 1999), thirst, and hunger. Additionally, organisms such as the rat, mouse, and monkey may be the most suitable organism to study the developmental (Wen et al., 1998) and regional profiles of gene expression in the mammalian brain (Gautvik et al., 1996; Usui et al., 1994; Miller et al., 1987; Bernal et al., 1990). It will be essential to study gene expression in the human brain for questions related to cognition, language, and other brain functions that are not developed in other mammals or primates. The human brain will be an especially important object of study for diseases. Neurological and psychiatric disorders are extremely common, and for most, the genetic basis and the effects of the disease on gene expression ("secondary effects") are unknown. High throughput studies of gene expression may result in the identification of genes that are differentially regulated in disease. This could be useful for two main reasons: (1) the genes could form the basis for a diagnostic test. For example, if some differentially regulated genes are expressed in peripheral tissues such as blood, then a test for a brain disorder could be developed. This could be helpful to allow families to begin early behavioral or pharmacological interventions for children likely to be at risk for a disorder; and (2) the genes that are abnormally regulated in the disease samples could be dynamically altered in response to the disruption of a particular biochemical pathway in affected brain cells. Thus, the genes that are altered in expression level may represent secondary changes in the disease state that reflect the essential pathway whose function has been compromised in the disease state. Such genes identified as abnormally regulated using microarray technology could indicate rational therapeutic targets.

There are many potential limitations to studies of gene expression in the human brain. The following issues must all be taken into consideration in attempting to identify abnormally regulated genes.

1. Can appropriate control samples be selected? For this, it is necessary to obtain control postmortem brain samples that are closely matched for gender, age of death, postmortem brain interval, and region of brain dissected. If the control tissue is from a different region of brain (e.g., a portion of cerebral cortex or hippocampus differing by only several millimeters), then the genes expressed in the control(s) may be different than in the diseased sample(s), and any differences observed could be due to regional variation in expression patterns and not the due to the disease. One approach to address this concern is to study as many brain samples as possible, and to characterize the cell types in each specimen.

2. What if the gene that causes a brain disease is expressed in only a specific brain region? Ideally, brain regions will be studied that have been shown to be affected based upon clinical, neuroimaging, neuropathological, or biochemical criteria.

3. What if the gene that causes a brain disease is expressed at only a specific age of development, and is not expressed in available postmortem samples? If this is the case, then the gene expression studies will not identify the causative gene(s). Rather, the studies may reveal secondary changes in gene expression. These secondary changes may still be crucial for understanding the effects of the disease on the brain and for the development of pharmacological therapies.

4. What if several genes contribute to a disease? An advantage of high throughput gene expression studies is

that they make no assumptions about how many genes are causative or to which chromosomes any candidate genes are localized.

5. Are gene expression techniques sensitive enough to detect significant changes in gene expression in rare transcripts? In principle, high density microarrays are extremely sensitive. A technique such as subtractive hybridization is useful to detect relatively large changes in gene expression between diseased and control samples, whether the genes in question are abundant or rare. The selective amplification of differentially expressed transcripts by using PCR for the construction of normalized cDNA libraries or in differential display ensures that even extremely rare messages may be identified.

6. Are most brain disorders heterogeneous at the molecular level, and if so, what impact will this have on gene expression studies? A wide range of clinical phenotypes is observed for the majority of central nervous system disorders. It is thus important that as many postmortem brain samples as possible are assayed. For example, if subtractive hybridization or large-scale cDNA array hybridization experiments suggest that a particular gene is significantly down-regulated in a disease, it is then crucial to verify this change. This verification should be performed with an independent technique, and preferably in an independent panel of diseased and control patient samples. Given the likely heterogeneity of central nervous system disorders, it is possible that five different brain samples will each exhibit differences in gene regulation of a subset of genes that are unique to each patient, and a different subset of genes may be significantly regulated in all (or most) patients examined. By obtaining a relatively large panel of patient and matched control brain samples, such as 6 to 12 samples in each category, it may be possible to focus on changes in gene expression that are relevant to the etiology of the disease. Such a large sample size may also be useful to delineate changes in gene expression that occur at different developmental stages of a disorder.

High density microarrays and other techniques for high throughput analysis of gene expression permit the analysis of up to tens of thousands of genes. In broad comparisons of gene expression between two samples, such as normal and diseased brain, it is likely that data analysis will routinely lead to the identification of genes that are differentially regulated in their expression. The next phase of the research project must be validation to confirm that the identified genes are in fact regulated. This validation may be performed at the RNA level (with techniques such as Northern blotting, RT-PCR, RNase protection, and in situ hybridization) and/or at the level of protein (e.g., with Western blotting and immunohistochemistry). In human brain studies, the amount of protein will not necessarily correlate with the amount of the corresponding mRNA; in the yeast Saccharomyces cerevisiae, mRNA levels are not consistently correlated with amounts of the corresponding proteins (Gygi et al., 1999). Nonetheless, once abnormally expressed genes are identified by high throughput analyses, they represent targets for subsequent, more focused molecular and biochemical analyses.

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