Subtractive Cloning of cDNAs from Motor Neuron-Like Hybrid Cells: A Strategy for Cloning cDNAs from Rare Cell Types

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A Thesis Submitted to The Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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(Abbreviated Title for Binding and Library Use)

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Abstract

Although motor neurons represent an important and well defined neuronal population, little is known about their unique gene expression. Because of the difficulties of purifying and culturing large numbers of primary motor neurons, we adopted a cloning strategy based on the NSC34 motor neuron-like hybrid cell line. This cell line was developed by fusing dissociated E14 mouse spinal cord cells with N18TG2 neuroblastoma cells and expresses a host of motor neuron markers and traits. We examined a cDNA library of the NSC34 cell line using a subtractive screening strategy in order to isolate cDNA copies of mRNAs preferentially expressed in the hybrid cells with respect to the N18 parents. Of 8 cDNAs isolated, two encode known neural genes, namely chromogranin B (a component of neurosecretory vesicles) and GAP43 (important for growth cone extension) and a third clone corresponds to a sequence which has been proposed to be the glutamate binding subunit of an NMDA receptor. One novel cDNA was isolated which appears to be preferentially expressed in brain and spinal cord (as determined by semi-quantitative PCR), and is clearly detectable in motor neurons by in situ hybridization. The remaining 4 cDNAs encode cytochrome oxidase subunit II, the mouse IAP element, and the mouse b1 element (2 copies). We conclude that this surategy is effective in isolating novel genes from rare cell types, but that large numbers of library phage must be examined and that hybrid cell-specific probes should be generated by subtraction with other hybrid cells (rather than the N18 parent) because of the large number of genes non-specifically activated by cell fusion.

Résumé

Les neurones moteurs représentent une population neuronale importante et bien définie. Cependant, très peu est connu sur leur expréssion génique spécifique. En raison de la difficulté d'isolation et de purification de ces cellules, nous avons adopté une stratégie de clonage basée sur la lignée cellulaire NSC34, issue de la fusion entre des cellules de moëile épiniere murine, E14, et des cellules de neuroblastome, N18TG2. Cet hybridôme (NSC34) exhibe plusieurs marqueurs propres aux neurones moteurs. Nous avons ensuite utilisé la technique d'hybridisation soustractive pour chercher dans une librairie d'ADNc dérivée de transcipts d'NSC34 des gènes préférentiellement exprimés dans cette lignée, comparativement à ceux du parent N18. De 8 ADNc isolés, deux représentent des gènes neurals (or neuraux) connus, tels que la chromogranine B, une composante des vésicules neurosécrétives, et GAP43, une protéine impliquée dans l'expansion des cônes. Un troisième clone correspond a la sequence présumée de la sous-unité du recepteur du NMDA responsable de la liaison avec la glutamate. De plus, nous avons trouvé un ADNc n'ayant aucune homologie avec des genes connus. Ce gene, préférentiellement exprimé dans le système nerveux central, est facilement détectable dans des neurones moteurs par hybridisation in situ. Les 4 derniers ADNc que nous avons isolés représentent la cytochrome oxidase II, l'IAP, ainsi que deux copies de l'élément b1. En conclusion, en dépit de l'efficacité de la strategie d'hybridization soustractive pour l'isolation de genes spécefiques à des types cellulaires rares, l'usage de cette technique pour le clonage de genes issus d'un hybridôme requiert l'examen d'un grand nombre de clones. Il est probable que l'usage de sondes dérivées d'une soustraction avec un autre hybridôme que le parent N18 augmenterait le rendement de cette technique.

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5-Aza	5-azacytidine
CO II	cytochrome oxidase subunit II
db-cAMP	dibutryl cyclic-adenosine monophosphate
DEPC	diethyl pyrocarbonate
EDTA	ethylene diamine teraacetate
GAP43	Growth Associated Protein 43
N18TG2	The neuroblastoma parent cell line
NI-1	An N18TG2 X rat pancreatic islet cell hybrid which secretes insulin
NO22	An N18TG2 X oligodendrocyte hybrid which expresses galactocerebroside and myelin basic portion.
NSC19	A sister hybrid of NSC34, which came from the same fusion experiments and also expresses choline acetyltransferase, extends processes, and supports action potentials.
NSC34	An immortalized motor neuron-like hybrid cell generated by fusing N18 cells with discociated mouse E14 spinal cord cells.

Claims to Originality

In performing these experments, I demonstrated:

- A novel strategy for isolating genes expressed in rare cell types, using subtractive screening on a cDNA library constructed from an immortalized motor neuron-like hybrid cell.

- The isolation, and tissue expression pattern of a novel cDNA, which is expressed primarily in brain and spinal cord, and is expressed in motor neurons.

- The actual tissue expression pattern of the glutamate binding subunit of an NMDA receptor, which was previously considered to be neuron specific.

Publications That Include My Work Done in the PhD Program

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Chapter 1

General Introduction

Overview of Motor Neuron Biology

Motor neurons are a population of large cholinergic neurons (α motor neurons are greater than 50 μm in diameter) which reside in the anterior horn of the spinal cord and in several nuclei of the brain stem. These neurons receive input from cortical structures (such as motor cortex via the corticospinal tract), brainstem structures (such as vestibular and red nuclei via the vestibulospinal tract and the rubrospinal tract, respectively) as well as spinal interneurons (such as the Renshaw cell) and monosynaptic and polysynaptic sensory input. They project large axons (up to 10 μm in diameter) out of the central nervous system to innervate skeletal muscle. With axons up to about a meter long (i.e. from the lumbar motor column to the muscles of the toe), motor neurons are amongst the largest cells in the body. Because of their role in integrating signals from these diverse systems, motor neurons are often referred to as the "final common pathway" for the control of voluntary muscle by the CNS (Burke, 1981; Shepherd, 1979).

Motor neurons arise very early in neural development, within days of the closure of the neural tube in humans. The birthdays of motor neurons have been estimated by a number of techniques to be embryonic day 10-11 in mice (Snider and Pereira-Smith, 1990) and rats (Mandler et al, 1990, Phelps et al, 1990) and embryonic day 2-3 in chicks (Leber et al, 1990; McCobb et al, 1990). In humans, motor neurons arise sometime between neural tube closure (embryonic day 24) and the first outgrowth of neurites from the ventral surface of the embryonic spinal cord (about embryonic day 28; Moore, 1982; Shinohara et al, 1990). It is interesting to note that, in rat brainstem at least, this is about 2 days earlier than other populations of large cholinergic neurons such as the neurons of the pedunculopontine tegmental nucleus or the laterodorsal tegmental nucleus (Phelps et al, 1990). Retroviral labeling experiments in chick embryos have shown that motor

neurons are not the daughter cells of a specific dedicated precursor. Instead, clones of neuroepithelial cells which give rise to motor neurons also give rise to other neuronal and non-neuronal components of the central nervous system (Leber et al, 1990). While labeling studies of chick optic tectum produced similar results (i.e. labeled clones of cells containing both astrocytes and neurons; Galileo et al, 1990), studies of mouse cerebral cortex suggest that neurons and glia arise from separate lineages in that retrovirally labeled clones virtually always contain a single cell type (Lushkin et al, 1988). This difference in neurogenesis between chick and mouse brain suggests that there may be specific neuronal (and perhaps motor neuronal) lineages in mice.

Soon after they arise, motor neurons send axons out of the central nervous system. The growth cones of these axons navigate through the periphery and make contact with specific peripheral targets. This navigation is remarkably precise in that motor neurons of a given level will almost always innervate their appropriate muscle (Landmesser, 1988). What is more, this navigation proceeds correctly even when regions of the spinal cord are reversed or displaced, as long as motor axons arrive at the correct nerve plexus (Lance-Jones and Landmesser, 1980a; Lance-Jones and Landmesser, 1980b). In addition, motor neurons are capable of finding their correct paths in the developing limb bud, even when it is rotated or reversed by transplantation in stage 16 chick embryos. (Ferguson, 1983) One important molecule which has been implicated in axonal guidance is polysialic acid (PSA), which is conjugated to the neural cell adhesion molecule (N-CAM). PSA has been shown to be differentially expressed in the chick motor neurons, with motor pools projecting to dorsal muscle masses tending to possess higher levels of sialation than those projecting to ventral muscle masses. Furthermore, in ovo injections of endoneuraminidase (which cleaves polysialic acid) increases the number of motor neuron pathfinding errors (Tang, 1992). The fact that many motor neurons still find their appropriate target muscle after endoneuraminidase treatments implies that other mechanisms of axonal pathfinding are also involved.

During the period in which motor axons make initial contact with their targets (around E6-9 in chick and E14-15 in mouse) and begin to establish functional synapses, as much as 40-60% of the motor neuron population dies. This large scale cell death has been proposed to be a mechanism by which appropriate numbers of neurons are matched with peripheral target muscles. Furthermore, it is believed that motor neuron cell death is an active developmental process in which motor neurons must compete for limited concentrations of "trophic factors" which assist their survival and/or enhance their phenotype. Many factors have been shown to have trophic effects on motor neurons in vivo and/or in vitro including retinoic acid (Wuarin et al, 1990) transforming growth factor $ß_1$ (TGF-$ß_1$; Martinou et al, 1990), insulin, insulin like growth factors 1 and 2 (IGF 1 and 2), cilliary neurotrophic factor (CNTF), basic Fibroblast Growth Factor (bFGF; Arakawa et al, 1990), ChAT Development Factor (CDF; McManaman et al, 1990), interleukin 6 (Il-6) and Cholinergic Differentiation Factor (CDF, also referred to as Leukemia Inhibitory Factor, LIF; Kushima and Hatanaka, 1992), Brain-Derived Neurotrophic Factor (BDNF; Oppenheim et al; 1992; Yan et al, 1992; Sendtner et al, 1992, Lohof et al, 1993; Henderson et al, 1993), Neurotrophin 3 (NT-3; Lohof et al, 1993; Henderson et al, 1993) and Neurotrophin 5 (NT-5; Henderson et al, 1993). In addition, there is evidence that the effects of some of these compounds (CNTF, bFGF, and IGF1) are additive (Arakawa et al, 1990). The requirement for trophic support seems to be important throughout the life of an animal and it has been suggested that failures in this trophic system may account for motor neuron cell death in motor neuron diseases (Crews and Wigston, 1990).

For neurons which survive the period of cell death, the established pattern of muscle innervation in adults appears to be carefully controlled. Studies have found that small motor neurons tend to innervate muscle fibers that are physiologically and biochemically characterized as slow (slow contraction time, low tetanic tension, predominantly oxidative metabolism, non fatiguable) and larger motor neurons tend to innervate muscle

fibers characterized as fast (rapid contraction, high tension, glycolytic metabolism, fatiguable). Because the input resistance of motor neurons is inversely related to cell body size, small motor neurons tend to be more easily excited (recruited) than larger neurons. Together, these two facts allow slow (non-fatiguable) motor units to be activated first by relatively low levels of stimulus. Higher levels of stimuli to a motor pool result in the activation of larger motor neurons and therefore the recruitment of faster muscle fibers. It is not known how this elegant scheme for matching muscle and nerve types and sizes is orchestrated, but it seems to require communication both from the nerve to the muscle and from the muscle to the nerve (Burke, 1981).

Human Motor Neuron Disease

A number of diseases are known to selectively affect motor neurons in humans. The best known of these is Amyotrophic Lateral Sclerosis (ALS) which has an incidence of about 1-2/100,000/year and a prevalence of 4-6 per 100,000. ALS is characterized by progressively increasing muscle weakness and atrophy, apparently due to death of spinal and bulbar motor neurons, and spasticity, brisk reflexes and pyramidal signs which are thought to reflect the degeneration of upper motor neurons (such as the giant Betz cells of the motor cortex). This disease has a mean age of onset of 55; the mean survival time is 3 years, although 10% of patients can survive for more than 10 years (Mitsumoto et al, 1988; Tandan and Bradley, 1985a; Tandan and Bradley, 1985b). The spinal muscular atrophies (SMA) are a similar group of diseases characterized by lower motor neuron loss. This group has an acute form, which is severe and often fatal in the first year of life (usually by age 4), and chronic forms with varying ages of onset and severity (from mild weakness to severe paralysis; Brzustowicz et al, 1990, Mitsumoto et al, 1988)

While exact causes of these diseases are undetermined, both are known to have heritable forms. In the case of ALS, as many as 5-10% of all cases are familial (Mitsumoto et al, 1988; Tandan and Bradley, 1985a; Tandan and Bradley; 1985b) and susceptibility in some kindreds is linked to chromosome 21 (Siddique et al, 1990).

Recently, this chromosomal locus has been identified as being in the Cu/Zn superoxide dismutase (SOD 1) gene (although the effects of this mutation are unknown; Rosen et al, 1993). The fact that some involvement of chromosome 21 has been ruled out in other kindreds, along with he widespread variability of penetrance (and perhaps the mode of inheritance) of the disease suggest that ALS may be a multigenic disease (Williams, 1992). In SMA, several genetic forms have been identified with recessive, dominant, and X-linked inheritance patterns. In fact, the childhood onset SMA is the leading cause of heritable infant mortality, with the acute form affecting 1 in 20,000 live births (with a carrier frequency of 1 in 60 to 1 in 80) and the chronic forms of SMA (which are clinically heterogeneous) affecting about 1 in 24,000 live births. Interestingly, both acute and chronic autosomal recessive SMA have recently been mapped to 5q11.1-13.3, implying that they may represent different allelic defects in a single gene (Brzustowicz et al, 1990, Melki et al, 1990).

Mouse Models of Heritable Motor Neuron Disease

A number of mouse lines are currently under study as models of heritable motor neuron disease. These include mice bearing the autosomal recessive mutations wobbler (Mitsumoto and Dradle, 1982, Kaupmann et al, 1992), wasted (Lutsep and Rodriguez, 1989) and Progressive Motor Degeneration (Schmalbrusch et al, 1991), the autosomal dominant mutation Motor Neuron Degeneration (Callahan et al, 1991), and two transgenic mouse lines which over-express particular neurofilament subunits (Cote et al, 1993, Zuoshang et al, 1993). Although all of these models may eventually help elucidate aspects of motor neuron cell death, none of these models is an exact mimic of ALS. Nevertheless, theses models all indicate that single genetic lesions can result in damage to motor neurons, and the underlying mechanisms involved are likely to be of great interest to motor neuron biology.

Motor Neuron Markers

The unique susceptibility of motor neurons to familial diseases suggests the existence of motor neuron-specific gene products. A number of divergent strategies have been used to identify such gene products, both as markers for the cell type (for use in future studies) and as a possible causes of motor neuron disease. To date, however, all of the proteins/antigens identified have also been found to be expressed in other cell types.

In 1981, Weil and McIlwain compared the protein expression profile of dissected bovine motor neurons and proximal dendrites with that of ventral gray matter (which contains motor neurons as well as other cell types) by 2-D gel electrophoresis. They found that of the 50 most abundant proteins, 6 were found to be more abundant in the motor neuron proteins by a factor of 17 or more as compared to that of the ventral gray matter (Weil and McIlwain, 1981). Similar analysis on the dorsal and ventral spinal roots (consisting of motor and sensory axons, respectively) showed no such differences (Weil et al, 1981). While none of the putative motor neuron-specific proteins uncovered in these studies were ever characterized, this was the first indication that motor neuron specific genes may exist (Hastings, 1992). Recently it has also been shown that differences exist in the ceramide component of the gangliosides in motor and sensory axons (Agawa-Goto et al, 1990). This suggests that there may be processing enzymes unique to or enriched in motor neurons.

At least one group has attempted to directly isolate human spinal cord specific cDNAs by making a library of a human spinal cord and screening it with a spinal cord specific probe, made by subtractive hybridization with brain mRNA. None of the isolated cDNA clones were spinal cord specific, although three clones were isolated which were enriched in spinal cord with respect to brain. The identities of these clones were never published (Kobayashi et al, 1991).

One potentially motor neuron specific protein was originally identified in the electric organ of Torpedo as a biological activity which caused the aggregation of acetylcholine

receptors on cultured myotubes. This protein, named agrin, was purified biochemically and has been shown by immunohistochemistry to be a component of the extracellular matrix in the muscle synaptic cleft of frogs and chickens as well as in the cytoplasm of motor neurons (Magill-Solc and McMahan, 1988, Nitkin et al, 1987, Reist et al, 1989). Agrin-like activity and immunoreactivity has since been found to be widespread throughout the CNS and the PNS (Leber et al, 1990). A similar type of activity/protein has been purified from chick brain which induces the synthesis and membrane insertion of acetylcholine receptors and has been named Acetylcholine Receptor Inducing Activity (ARIA; Usdin and Fischbach, 1986). A cDNA comprising ARIA has recently been cloned from a chick brain library. This cDNA encodes a 632 aa protein which is homologous to the rat Neu differentiation factor (which is known to stimulate DNA synthesis in Schwann cells) and is expressed in the spinal cord where it is concentrated in motor neurons. Although it's expression in brain precludes ARIA from being a motor neuron-specific molecule, it may still prove to be a useful marker of motor neurons in the spinal cord (Falls et al, 1993).

The development of motor neuron specific antibodies has been attempted by using the electric organ of Torpedo as a model. In one set of experiments, antisera raised against the electric organ of torpedo have been found to be specific for cholinergic neurons. This antiserum, designated Chol-1, has been found to bind to two different gangliosides in rat and human brain/spinal cord (Obrocki and Borroni, 1988). In another set of experiments, a library of 141 monoclonal antibodies was developed against the electric organ of Torpedo and screened for reactivity with human muscle tissue sections. Of the 141 original antibodies, 13 were found to stain nerve tissue (Bjornskov et al, 1988). One of these monoclonals (Tor-23) has been further characterized as binding to a presynaptic form of acetylcholinesterase in Torpedo (Kusher et al, 1987). Unfortunately, Tor-23 has been found in a number of different neuronal types (Stephenson and Kushner, 1988), so none of the Torpedo generated antibodies appear to be motor neuron specific.

The creation of libraries of monoclonal antibodies against whole brain provided another tool for the investigation of the development of specific neuronal cell types. The monoclonal antibody, Cat-301, was originally found to be expressed in an activity dependent fashion in neurons of the cat visual cortex. Suturing shut the eye of a newborn cat (but not at adult cat) resulted in the failure to develop expression of this antigen, concomitant with the inability develop the physiological pathways necessary for sight (Hockfield et al, 1983). This antigen has also be found to appear early in the development of motor neurons and to be similarly dependent upon the establishment of synaptic activity with muscle and spinal afferents to motor neurons in young (postnatal day 14) hamsters. Crushing of the sciatic nerve or thoracic hemicordectomy abolishes Cat-301 immunoreactivity of lumbar motor neurons in a young (postnatal day 7) hamster, but not in an adult hamster (Kalb and Hockfield, 1988a, Kalb and Hockfield, 1988b). Expression of this antigen may therefore reflect the formation of intact motor control circuits. The antigen which Cat-301 recognizes has been shown be an extracellular matrix chondriotin sulfate proteoglycan (Zaremba et al, 1989). While Cat-301 may prove useful in studying the development of motor neurons and motor circuits, its expression in the visual cortex indicates that it is not a motor neuron specific antigen.

Monoclonal antibodies have been also been raised against spinal cord and/or sciatic nerve. One such monoclonal antibody, SC-1, has been found to be developmentally regulated on motor neurons, being maximally expressed in the chick embryo at stage 23-24 and then declining. This antibody also stains spinal cord ventral epithelial cells, dorsal root ganglia, sympathetic ganglia, dorsal funiculus and dorsal and ventral roots (Tanaka and Obata, 1984). The SC-1 antigen has been cloned using monoclonal antibodies to screen a chick E5 lambda gt11 library. The cDNA isolates encode a 588 amino acid glycoprotein, of approximately 100 kd which, by sequence homology, belongs to the immunoglobulin superfamily. When transfected into human embryonic kidney cell line 293 under the control of a constitutive promoter, SC-1 can be shown to be localized

to the cell surface where it results in increased intracellular cell adhesion. Using in-situ hybridization, SC-1 RNA can be detected in chick E5 motor neurons as well as the floor plate of the spinal cord, the dorsal root ganglia. This expression disappears by day E9. Northern analysis of day E10 embryos demonstrates widespread expression of SC-1 throughout the embryonic tissues, including tectum, skeletal muscle eye, lung, heart and kidney. Because of its transient expression early in development, its relationship with the immunoglobulin superfamily, and its apparent function as an intracellular adhesion molecule, SC-1 has been proposed to be involved with early axonal guidance in the spinal cord (Tanaka et al, 1991). The widespread expression of SC-1 indicates that it is not a motor neuron specific marker, although it may prove useful for the study of motor neurons during its window of expression.

Another group has successfully raised a monoclonal antibody against human embryonic motor neurons by first tolerizing mice to posterior horn homogenate using the immunosuppressant cyclophosphamide, and then immunizing them with embryonic dorsal horn. The resulting monoclonal antibody A8A4 appears to stain the perinuclear region of a sub-population of motor neurons (the first row of cell adjacent to the marginal layer) during a transient period of development, peaking at week 9-10. Attempts to detect proteins (by immunoblot) or gangliosides and neutral lipids (by thin layer chromatography immunoblot) with this antibody have been unsuccessful, and the antibody does not appear to recognize antigens in other species such as rat, chicken or monkey. A8A4 can also be shown to stain the muscosa lining the lumen of the stomach, as well as brainstem and dorsal root ganglion cells (Erkman et al, 1992). To date, the most promising example of a motor neuron specific antibody was the development of the monoclonal antibody, MO-1, which recognizes cell bodies and proximal axons of brain stem and spinal cord motor neurons in the rat. This antibody has also been found to weakly stain neurons of the red nucleus (Urakami and Chiu, 1990). Neither of the antigens of A8A4 or MO-1 have been fully characterized.

The Immortalization of Cells with Differentiated Phenotypes

General Strategies for Immortalization

At least 4 strategies have been employed to generate immortalized cell lines bearing a neuronal phenotype, in order to study cell lineage in the CNS or the characteristics of rare neuronal cell types. These methods include, oncogene transfer mediated by retroviruses, infection with DNA tumor viruses, genetically targeted tumorigenisis in transgenic mice, and cell fusion. While each of these techniques has been shown to be capable of generating immortalized cells with neuronal properties, each has its own advantages and disadvantages.

Retroviruses have been shown to be effective in producing immortalized cells with neural properties. The Rous sarcoma virus, for example was used successfully to immortalize quail embryonic neuroretinal cells. The resulting cell lines possess a neuronal morphology, electrically excitable membranes, and expressed glutamic acid decarboxylase (GAD) enzymatic activity, which is a marker for amacrine and ganglion cells (Pessac et al, 1983). Engineered retroviruses, bearing a number of different oncogenes, have also been demonstrated to be effective in immortalizing neural precursors. These precursors can then be differentiated into cell lines bearing neuronal or glial markers by variations in culture conditions (such as the addition of dibutryl cyclic AMP or retinoic acid) to study cell lineage in different regions of the CNS. The major drawback of this technique is that only mitotically active cells may integrate retroviral sequences into their genomes and become immortalized. Purified populations of differentiated (post-mitotic) neurons may not be immortalized, and neuronal cell lines must be derived from an immortalized precursor. Generation of immortalized cells with specific neuronal phenotypes might therefore require the isolation and infection of specific neuronal precursors (Ryder et al, 1989, Frederikson, et al, 1990).

DNA tumor viruses, such as SV40, are well known for their ability to immortalize non-neuronal cells (Linder and Marshall, 1990). Unlike RNA viruses, DNA viruses exist in their host cells as non-integrating episomes, obviating the necessity of proliferation dependent integration, and in principle should be able to immortalize post-mitotic neurons. In one set of experiments, immortalized cell lines were generated from mouse hypothalamic neurons using SV40 virus. These cell lines possess a number of the characteristics of neurosecretory cells including a well developed golgi and secretory apparatus, and the ability to synthesize neurophysin and vasopressin, which are characteristic hypothalamic neuropeptides. Unfortunately these lines do not bear a neuronal morphology, being devoid of axons and axon termini, and are therefore described as immortalized neural precursors (De Vitry et al, 1974). Another group also attempted to immortalize neuronal cells using SV40 in the mouse striatum and mesencephalon. All cell lines examined bore only astrocytic markers such as GFAP and β-alanine sensitive uptake of GABA (Moura-Neto et al, 1986). Although DNA viruses have the advantage that mitosis is not required for immortalization, it has yet to be demonstrated that they are capable of producing neuronal cell lines with mature phenotypes. It should be noted that slower growing cell lines with differentiated phenotypes might have been produced in these studies and overgrown by faster growing, poorly differentiated or astrocytic clones.

A third strategy used to generate immortal neuronal cell lines, referred to as genetically targeted tumorigenesis, involves making transgenic mice bearing a transforming oncogene driven by a cell type specific promoter. Genetically targeted tumorogenisis has been used successfully to generate immortalized pancreatic β-cell lines from tumors induced in transgenic mice by the SV40 large T antigen driven by an insulin promoter. These immortalized lines resemble β-cells in that they secret insulin in response to glucose. It is possible, however, that these lines may represent β-cell precursors because they also express glucagon (Efrat et al, 1988). To date, the most

successful use of this technique in a neuronal system has been the generation of cell lines from gonadotropin releasing hormone (GnRH) neurons of the hypothalamus, by making transgenic mice bearing the SV40 T-antigen driven by the GnRH promoter. The cell lines generated from these mice possess a neuronal morphology, extend neurites, and express neuron specific enolase (NSE), neurofilaments and the secretory marker chromogranin B. These cell lines also express GnRH mRNA and secrete GnRH upon electrical stimulation (Melon et al, 1990). Another group has used this technique to produce SV40 T antigen expression in mouse retina and adrenal medulla using a construct consisting of the large T antigen linked to the promoter of the human phenylethanolamine N-methyltransferase gene (hPNMT; the enzyme responsible for the last step in catecholamine biosynthetic pathway). These transgenic mice possessed the predicted distribution of T antigen immunohistochemistry and developed tumors of the adrenal medulla and eye. Unfortunately, cell lines were not subsequently cloned out and evaluated (Baetge and Hammang, 1988). A fourth group has also tried to generate neuronal cell lines using the promoter for the hypothalamic peptide growth hormone-releasing factor (GRF) linked to the SV40 large T antigen. Mice made transgenic with this construct failed to express T antigen in their hypothalamus or brain, which is puzzling because this same promoter is capable of driving appropriate expression of other cDNAs such as NGF. These mice did not develop hypothalamic tumors and instead experienced massive thymic hyperplasia, resulting physical occlusion of their upper respiratory system and death. No hypothalamic tumors arose in these mice, even when these transgenic mice were thymectomized early in life and therefore did not die of respiratory failure (Botteri et al, 1987). The generation of cell lines using genetically targeted tumorigenesis in transgenic mice is a potentially powerful tool. The major drawback of this technique is that the neuronal population of interest must be known to express a particular cell type specific gene, and this gene must possess a strong promoter which is well restricted in its expression patterns. Because no such gene has been

characterized in motor neurons, this technique is not likely to be useful for the isolation of motor neuron cell lines.

All of these techniques have been shown to be effective in generating neural cell lines and could in principle be used to generate immortalized motor neuron cell lines. The main concern with all of these techniques is that they often fail to immortalize cells with mature phenotypes. This may be because precursor cells are often the targets of the techniques either intentionally, as in the case of retroviral infection, or unintentionally in the case of genetically targeted tumorigenesis in which it is not possible to control the exact stage of activation of an inserted oncogene. Because we hypothesized that mature motor neurons possess important cell type specific gene expression, it was important to adopt a strategy which would be more likely to yield such cell lines.

Immortalization by Cell Fusion

An immortalization strategy which might be more likely to produce cell lines with mature neuronal phenotypes is the generation of hybrid cells by cell fusion. Hybrid cells have been studied for many years as models of somatic cell genetics and cellular differentiation, although they have only relatively recently been used specifically for the immortalization of neuronal cells. Viable hybrid cells may be produced between virtually any cells (even between cells of different species) and depending upon the lineage of the parental cells, hybrid cells may express a variety of parental and non-parental traits. By fusing cells of similar or differing lineage and studying the resulting phenotype, it is possible to make inferences about regulatory genes active in parental cells (Harris, 1965; Davidson, 1974; Lewin, 1974). In addition, if one of the parents is immortal, the resulting hybrid cells may also be immortalized, although this depends upon the immortalizing lesion in the parent; the fusion of mortal and immortal cells may also "mortalize" the resulting hybrids, presumably by complementing the genetic lesion responsible for parental immortalization (Smith and Pereira-Smith, 1990). Because

hybrid cells were first developed and characterized in non-neuronal systems, it is in these systems that hybrid cell properties are best understood.

The production of hybrid cells involves fusing the cell membranes of two (or more) cells in a process mediated either by inactivated Sendai virus (Harris, 1965; Lewin, 1974) or polyethylene glycol (Fournier, 1981). If one of the host cells possesses a selectable marker, it is possible to detect and clone out resulting cell lines (Littlefield, 1964). The immediate result of cell fusion is a heterokaryon - a cell with two or more independent nuclei sharing a single cytoplasm. These nuclei may then interact to activate or inactivate RNA transcription or chromosomal replication in each other. The individual nuclei undergo nuclear mitosis which may be independent of each other and of the cell cycle; during cell division, whole nuclei may be mitotically unstable. Heterokaryon nuclei initially divide with a time period reflecting that of their cell of origin. Over time, individual nuclei tend to synchronize both with respect to their division rates and initiation times. Nuclei which enter cell cycle at the same time may then fuse into a single nucleus. A hybrid cell in which all nuclei have fused to form a single nucleus is referred to as a synkaryon. (Harris, 1965; Davidson, 1974; Lewin, 1974).

The most extreme example of the ability of individual nuclei to influence each other is in fusions between cells with active and completely inactive nuclei. Chick erythrocytes, unlike their enucleated mammalian counterparts, retain small compacted nuclei which are biosynthetically inactive. When fused with HeLa cells to form heterokaryons, chick nuclei undergo a rapid expansion, increasing their cross sectional area by a factor of 10 and their dry mass by a factor of 5 within in the first 41 hours after fusion. This enlargement coincides with the reactivation of transcriptional activity and nuclear size can be directly correlated with ^{3}H-uridine incorporation into RNA in the chick nuclei. The nuclear enlargement also precedes DNA replication, and chick nuclei can be seen to enter nuclear mitosis after about 4 days. Three days after fusion, nucleoli appear in the chick nuclei, and chick specific antigens begin to appear after 8 days. These experiments

demonstrate that even totally inactive nuclei can be "resuscitated" by cell fusion and returned to a biosynthetically active state (Harris et al, 1967; Lewin et al, 1974).

Hybrid cells have been used extensively to address questions related to dominance of gene expression and phenotype and to address the mechanisms of gene regulation. In general, fusion between any two cells may result in three different outcomes for any particular gene. The expression of a gene may be unaffected by the formation of a hybrid cells, its expression may be activated in the hybrid cells, or its expression may be extinguished in the hybrid as compared to its parents. (Weiss, 1980, Gourdeau and Fournier, 1990). In the case of cell fusions between cells of different lineage (inter-typic hybrids), differentiated characteristics are often extinguished while constitutive gene expression remains unchanged. This can be seen in intertypic fusion experiments between either immunoglobulin secreting B cells or growth hormone secreting GH3 cells with L cells (a fibroblastic cell line), in which the resulting hybrid cells no longer secrete their respective products; their differentiated traits have been extinguished (Junker et al, 1990, McCormick et al, 1988). Similarly, the intertypic fusion of mouse erythroid Friend cells expressing β-globin and lipoxygenase with neuroblastoma cells extinguishes the expression of these traits while intratypic fusion of Friend cells with T cells does not (Affara et al, 1985).

The time course of extinction can be studied in order to draw inferences about its underlying mechanisms. Fusing rat FAO-1 hepatoma cells (which secrete albumin) with mouse fibroblasts, results in the extinction of albumin production. After 8-12 days in culture, however, many clones of hybrid cells activate rat albumin synthesis and secretion. In these cells, the mouse albumin gene is re-expressed several days later. In hybrid cells bearing a genetic complement of 2 rat hepatoma genomes to one mouse fibroblast genome, albumin reactivation and reexpression occur significantly faster, implying that gene dosage has an important effect on gene regulation in hybrid cells. (Mével-Ninio and Weiss, 1981). In similar experiments with rat hepatoma X mouse

fibroblast fusions, it was possible to correlate hybrid cell karyotype with cell phenotype. These hepatoma X fibroblast cells have a fibroblastic morphology and can be shown to no longer express five liver specific enzymes (alanine amino transferase (AAT), alcohol dehydrogenase (ADH), Aldehyde Dehydrogenase (AHD), aldolase B (Ald B) and tyrosine amino transferase (TAT)) while possessing normal expression of all monitored constitutive markers (mannose phosphate isomerase, purine nucleoside phosphorylase, malate oxidoreductase and peptidase). As these hybrid cells were passaged in culture, they were documented to lose mouse (fibroblast) chromosomes and to shift their morphology from fibroblastic to hepatic. By following the karyotype of these hybrids over time, it was possible to correlate extinction of all 5 liver specific enzymes assayed with the presence of mouse chromosomes 8,9,10,11, and 13. Furthermore, it was also possible to show that hybrid cells that had lost all mouse chromosomes except chromosome 11 expressed all of the markers except tyrosine amino transferase (TAT). In fact, by constructing rat hepatoma microcell hybrids bearing only mouse chromosome 11, it was possible to demonstrate the existence of a trans-dominant repressor (referred to as Tissue Specific Extinguisher 1 or TSE1) residing on chromosome 11 which is responsible for extinguishing TAT (Killary and Fourier, 1984). A second extinguisher locus, TSE2, residing on chromosome 1 can also be demonstrated to extinguish the expression of the liver specific genes serum albumin and alcohol dehydrogenase (Gourdeau and Fournier, 1990). These results indicate that constitutive markers tend to be unaffected in hybrid cells, while markers representing a differentiated phenotype tend to be extinguished, but may be re-expressed if specific extinguishing loci are lost.

In contrast to intertypic hybrids, intratypic hybrids do not (in general) exhibit extinction, but rather can be shown to activate tissue specific genes in each other. If one of the parents is immortal, then intratypic hybrid cells may be expected to be immortal in addition to expressing parental traits. The most famous example of the production of a cell bearing a specific immortalized phenotype can be found in monoclonal antibody

technology. In this technology, immortalized antibody secreting cells are produced by fusion of immortal myeloma cell lines with isolated antibody secreting B cells (Kohler and Milstein, 1975). It is also possible to produce immortal myoblast hybrids by fusion of rat L6 myoblast cells with human fetal myoblasts. The resulting immortal cells express a variety of human and rat muscle markers and unlike the parental L6 cells, are capable of forming myotubes which exhibit spontaneous contraction (Quinn et al, 1981). In addition, rat hepatoma cells (which express α-fetoprotein (AFP) but no longer express the adult serum proteins albumin, transferrin or C3) may be fused with adult mouse hepatocytes (which produce adult serum proteins but very little AFP). The resulting hybrid cells all express mouse and rat serum proteins but only the rat form of AFP, implying that rat and mouse parent cells are capable of inducing some, but not all of their normal gene complement in these hybrid cell. (Szpirer et al, 1980). It should be noted that the cells of interest in these experiments were carefully cloned out; many other cells are produced in these fusion which probably do not have the desired characteristic phenotypes, presumably due to variability in fusion and chromosomal loss.

In a few cases, the mechanism of extinction or activation of specific genes in hybrid cells has been investigated. For example, the Tissue Specific Extinguisher 1 (TSE1) locus on chromosome 11 which controls extinction of liver specific TAT has been cloned and found to encode a regulatory subunit of protein kinase A (Jones et al, 1991, Boshart et al, 1991). In addition, at least two groups have investigated the extinction of differentiated cell traits (immunoglobulin expression in B cells fused with fibroblasts and growth hormone expression in GH3 cells fused with L cells) and concluded that the phenomenon was caused by the extinction of upstream cell type-specific transcription factors NF1 and GHF respectively. (Junker et al, 1990, McCormick et al, 1988). Other mechanisms of gene regulation in hybrid cells have also been demonstrated. When rat hepatoma cells are fused with either human fetal liver cells or human fibroblasts, it is possible to demonstrate that hepatoma X fetal liver fibroblast hybrids (but not the

hepatoma X skin fibroblast hybrids) activate the liver specific gene α_1-antitrypsin. The ability to activate α_1-antitrypsin expression in hepatoma X fibroblast hybrids can be shown to correlate with hypomethylation of the α_1-antitrypsin gene in expressing hybrids and hypermethylation of this gene in non-expressing hybrids. Both the human fetal liver and human adult skin fibroblasts possess intermediate levels of DNA methylation at this locus and did not express α_1-antitrypsin. DNAse sensitivity of the α_1-antitrypsin gene was the same in the parents and in the hybrids at this locus. It should be noted that it is impossible to tell, in these experiments, whether the observed methylation difference is the cause or the consequence of altered α_1-antitrypsin transcription. (Barton and Francke, 1987). When mouse erythroid Friend cells expressing β-globin and lipoxygenase were fused with either mouse T-cell lymphoma or a neuroblastoma to produce expressing and non-expressing hybrid cells respectively, it was found that erythroid cell-specific DNAse 1 hypersensitivity sites were lost in the non-expressing Friend cell X neuroblastoma hybrids (Affara et al, 1985). These results indicate that gene expression in hybrid cells (and presumably in wild-type cells) can be influenced by specific genetic loci, as well as by highly non-tissue type-specific variations in cAMP responsiveness, DNA methylation and chromatin structure.

Neural Hybrid Cells

Hybrid cells have also been used to study expression of genotype and phenotype in neural cells. Because the parental cells play an important role in defining what gene a hybrid cell will ultimately address, these studies have hinged upon the development of immortal neural cell lines. Such cell lines may be derived from spontaneously arising tumors of both the peripheral and central nervous system. In spontaneously arising PNS tumors (neuroblastomas, ganglioneuroblastomas and ganglioneuromas) which are thought to arise from neural crest cells, it is possible to demonstrate a wide range of neuronal and neuroendocrine markers. These include the pan-neuroendocrine markers such as synaptophysin and chromogranin A as well as neuronal markers such as neurofilament

isoforms and microtubule associated proteins (Molenaar, et al, 1990). The starting point for many in neural hybrid projects is the C1300 mouse neuroblastoma which arose spontaneously in 1940 and was carried by animal injection until 1969, when subclones were transferred to tissue culture (Augusti-Tocco and Sato, 1969). From the original C1300 isolate, it was possible to clone out cell lines with specific neural phenotypes, generating cell lines bearing cholinergic, adrenergic or neither phenotype. Presumably, clonal variations in C1300 derived cells are due to chromosomal loss or gene inactivation (Amano et al, 1972).

With the establishment of neural cell lines with defined phenotype, it was possible to make hybrids to study neural gene expression and the regulation of differentiated phenotype. For example, the fusion of neuroblastoma and glioma cell lines (neither of which express choline acetyl transferase or possess excitable membranes) results in hybrid cells which are high expressors of choline acetyltransferase (ChAT) as well as possessing membranes which were more excitable than the parent neuroblastoma (Amano et al, 1974). In addition, a number of studies utilized fusion of neuroblastoma cells with L cells (a fibroblastic cell line) to produce NL hybrid cells with a variety of neuronal properties ranging from highly differentiated to relatively undifferentiated. In any given neuroblastoma X L cell fusion experiment, it is possible to clone NL cell lines which vary in choline acetyltransferase (CAT) activity, or in the type of neuritic process that they form (i.e. long highly branched processes which form dense networks and stain by silver impregnation, long processes which rarely branch and do not stain with silver, and cells with no processes; Minna et al, 1972).

Because of the inherent variability of the NL hybrids, it was possible to use these cell lines to dissect some aspects of neuronal function. By making electrophysiological recordings of individual NL cells, it was possible to observe cells which possessed a type "A" response (an increased rate of voltage change early in depolarization) caused by early entry of sodium, a type "B" response (delayed rectification late in depolarization) caused

by late exit of potassium, A^+B^+ cells, and cells which were A^-B^- or not excitable (Minna et al, 1971). These experiments were early indications of the existence of discrete membrane channel proteins for sodium and potassium.

Hybrid cells have also been used to functionally dissect the neuromuscular junction. By examining a battery of neuroblastoma and neuroblastoma hybrid cells (crossed with a variety of neural and non-neural cell types) it was possible to identify cell lines deficient in different aspects of neuromuscular junction formation. In general, it is possible to classify these cell lines into three categories: those not synthesizing acetylcholine, those synthesizing ACh but making few or no synapses on co-cultured myotubes, and those synthesizing Ach and making many synapses on co-cultured myotubes. Hybrid cells which are capable of ACh secretion but not of synapse formation can be further subdivided into cells with voltage sensitive $Ca2^+$ channel defects (blocking voltage stimulated secretion), blockage in a further downstream secretion step, and absence of large dense core vesicles, whose presence correlates with acetylcholine receptor aggregating function (Bussis et al, 1984).

Some authors have looked for coordinate control of phenotype expression in neuronal hybrids. After examining the phenotype and karyotype of a battery of mouse and human neuroblastoma X mouse or human fibroblast cell lines, McMorris and Ruddle proposed that neuronal morphology (judged by the size and number of processes), and the extinction of fibroblast channel function (termed hyperpolarization activation or type C channel function) correlated well with the logarithm of both Acetylcholinesterase levels and the chromosomal number (McMorris and Ruddle, 1974). Other authors have also reported cosegregation of phenotypic traits. When NS20 neuroblastoma cells, which express choline acetyltransferase (CAT) and acetylcholine esterase (AChE), are fused with B82 fibroblasts (which express neither of these markers), hybrids with 4 phenotypes can be isolated. Of 25 hybrid analyzed, 11 (44%) were high expressers of AChE, and 14 (56%) were high expressers of CAT. Specifically, 9 (36%) were high expressers of both

and 10 (40%) were low expressers of both, leaving about 10% in each of the high-low phenotypes. This tendency for the NL hybrid cells to either possess or lose both traits together implies coordinate regulation of NL cell phenotype (Minna and Yavelow, 1975).

As with non-neuronal cell types, cell fusion has been used to generate immortalized cell lines as tissue culture models of specific neuronal cell types. For example, N18TG2 cells (deficient in choline acetyl transferase activity, tyrosine hydroxylase activity, neurite extension, and having electrically passive membranes) have been used as fusion partners with sympathetic neurons. The resulting immortal hybrid cell lines possess neuronal morphology, excitable membranes, tyrosine hydroxylase activity, and are capable of synthesizing dopamine (Greene et al, 1975). Other groups have used N18TG2 cells as fusion partners with dorsal root ganglion (DRG) cells. In fusion experiments with human DRG cells, it was possible to isolate cell lines displaying neurite outgrowth, and expressing the human Thy-1 and A2B5 antigens (neuronal markers) as well as mouse neurofilaments. In these cell lines, the expression of human neurofilament protein was inducible by db-cAMP (Dickson et al, 1983). Similar fusion experiments with rat embryonic dorsal root ganglion cells also produced immortalized cells which extended neuronal processes, expressed the neuronal ganglioside A2B5, and supported action potentials. Interestingly, a small number of these clones expressed substance-P, suggesting that their parents might correspond to the small pain-sensitive substance-P containing neurons which comprise about 20% of the cells of the DRG (Platika et al, 1985).

Cell fusion has also been used to generate immortalized neural cells from central nervous system neurons. By fusion of mouse embryonic septal forebrain cells with N18TG2 cells, it was possible to produce cell lines which extended neurites, expressed neurofilaments, and possesed choline acetyltransferase activity (Hammond et al, 1986). These fusion experiments have also been performed with adult septal forebrain cells to produce cell lines which extended neurites, expressed neurofilament proteins, possessed

choline acetyltransferase activity and expressed NGF (Lee et al, 1990). In addition, rat mesencephalon X N18 hybrids have been produced which (unlike the N18 parents) express tyrosine hydroxylase and synthesize dopamine (Crawford et al., 1992). These results demonstrate that cell fusion is capable of producing immortal cell lines from post-mitotic central and peripheral neurons. In addition this technique makes it possible to clone out, grow, and characterize immortal cells presumably representing small neuronal populations which would be otherwise inaccessible.

In general, many of the hybrid cell studies seem unsatisfactory. On the one hand, it is apparent that each individual hybrid cell may be unique, and may express a variety of traits as a result of the vagaries of cell fusion and chromosomal loss or inactivation. This problem is compounded if the parental cells represent a mixed population, and it then becomes impossible to say with certainty that resulting hybrids of interest express their phenotype as a result of the fusion of the intended parents. On the other hand, these experiments suggest that the determinants of phenotype are much more complex than was originally suspected. Although there are instances in which the mechanisms of activation or extinction of individual genes can be explained, it seems likely that developing an understanding of the coordinate regulation of all of the genes which make up a cell phenotype will be a daunting task; how many genes does it take to generate a neuronal process? Even in the cases where the mode of cell type-specific gene regulation has been elucidated, the mechanisms discovered have either consisted of the (unexplained) extinction of other cell type-specific transcription factors, or by disturbingly non-cell type-specific mechanisms such as protein kinase A regulation, DNA methylation, and rearrangement of chromatin structure. It would be more reassuring if less intangible mechanisms were found to regulate cell type-specific gene expression.

These problems are compounded in many of the studies of neural hybrid cells. From fusion experiments between neuroblastoma and non-neuronal cells, we must conclude that properties such as excitable membranes, neuronal morphology and cholinergic

phenotype, are not difficult to establish in neuroblastoma hybrid cell lines. In fact, because we would not *a priori* expect NL hybrids to express a neuronal phenotype (they should extinguish differentiated neuronal characteristics), we must also conclude that the expression of neuronal phenotypes in these cells might be the result of nonspecific neuronal gene activation as a result of fusion, perhaps by providing lost or unmethylated genomic material to the neuroblastoma cells, which must still possess some active neuronal transcription factors. The evaluation of N18 neuroblastoma-derived hybrids therefore cannot be based on morphology, excitable membranes, or cholinergic phenotype, because these may be general properties of neuroblastoma hybrids.

Based on this reasoning, the only way to evaluate the success or failure of previous attempts to generate immortalized neuronal cell lines must be to assay them for the expression of markers not observed in N18 cells or in N18-non-neural cell hybrids. Thus, while morphology alone cannot be relied upon to determine the success or failure of N18 X sympathetic neuron hybrids, the establishment of cells bearing specific sympathetic neuronal traits of tyrosine hydroxylase activity and dopamine secretion (Greene et al, 1975) might be suggestive of fusion of a neuroblastoma cell with a sympathetic neuron. Similarly the expression of human Thy-1 and A2B5 antigens (neuronal markers) by N18 X human DRG hybrids (Dickson et al, 1983), and neuronal ganglioside A2B5 and substance P by N18 X rat DRG hybrids (Platika et al, 1985) might also be taken as indicative of the establishment of neuronal hybrid cells. Of the fusion experiments with central cholinergic neurons (Lee et al, 1990), only the expression of NGF might be taken as proof that neuronal hybrids were in fact produced. While it seems safe to conclude that it is possible to use cell fusion to produce hybrid cells with specific neural phenotypes based upon expression of markers specific to only a subset of neurons, an important caveat is that NL hybrid cells have never been evaluated for their expression of many of these markers.

Even given all of these limitations, it is apparent that cell fusion can be an effective method of producing immortalized cells with desired characteristics. For studying rare post-mitotic cell types, there may be no alternatives to hybrid cell technology if large numbers of cells are desired. The key to success in these immortalization strategies seems to be to choose an immortal parent which is not likely to extinguish the desired traits, and then to vigorously screen for resulting hybrids which bear the phenotype of interest, concentrating on traits likely to be expressed only in the target cell. In addition, it is necessary to monitor the phenotype of the hybrid cell; loss of chromosomes during passage in culture can lead to alterations in hybrid cell behavior.

Our Hybrid Cell Based Cloning Strategy

Because of the difficulties involved with isolating large numbers of motor neurons in pure culture, addressing the molecular biology of motor neurons has proven to be challenging. Since there are no unique markers for motor neurons, it is difficult to obtain large numbers of motor neurons by fluorescence activated cell sorting, although small numbers may be prepared by injecting mouse peripheral muscle with fluorescent dyes and sorting the spinal cord cells which are labeled by retrograde transport (O'Brien and Fischbach; Eagleson and Bennet 1983; Cashman, 1992). Recent efforts have demonstrated the ability to purify rat motor neurons by panning with an antibody to the low affinity NGF receptor (which appears to be relatively motor neuron specific in embryonic day E15 spinal cord), yielding cultures of motor neurons with greater than 90% purity (Camu and Henderson, 1992). Because there are only about 23,000 motor neurons in a mouse or 200,000 motor neurons in a human (Shepherd, 1979), it will be difficult to obtain enough motor neurons to perform any meaningful molecular biology (i.e. for northern analysis or the creation of a library) even if effective techniques are developed for the purification of motor neurons. PCR based techniques may offer some assistance in analyzing the expression of genes already cloned.

In order to circumvent these problems, immortalized cell lines with motor neuron properties were developed in the laboratory of Dr. Cashman. To accomplish this, dissociated E14 spinal cord cultures enriched for motor neurons (but nonetheless containing other cell types) were fused with the HAT sensitive N18TG2 neuroblastoma cells. In 5 fusion experiments, 43 hybrid cells (immortal cells which were HAT resistant) were produced, and these were subcloned and screened for motor neuron properties. Of the 43 fusion products, 30 possessed a neuronal phenotype, (adherent, multipolar cells which extend long processes), 8 possessed a triangular morphology with short processes and 5 possessed adherence independent growth characteristics. Of these 30 neuronal hybrids, 10 possessed Chat activity, and 7 adhered to myotubes in co-culture. Two clones, NSC34 and NSC19, which possess both of these characteristics were selected for further study.

On the basis of morphology and the expression of traits expected in a motor neuron cell line, the NSC34 hybrid cell line was selected for our molecular cloning experiments. Unlike the parental neuroblastoma line, this cell line was found to support action potentials, to synthesize and package acetylcholine and to secrete acetylcholine in an excitation dependent manner. In co-culture experiments with myotubes, NSC34 cells are capable of making stable contact with myotubes, aggregating myotube acetylcholine receptors, and making the myotubes twitch. NSC34 cells express neuron specific enolase, choline acetyltransferase, NCAM, and neurofilaments. In addition, NSC34 cells have been shown to possess an S-laminin receptor. S-laminin is a protein with laminin homology which is present in the basal lamina at the neuromuscular junction and is believed to be a signal for extending motor axons to stop and elaborate the motor nerve terminal. The expression of an S-laminin receptor by NSC34 cells is particularly reassuring because it is expressed on only a subset of neurons and has not been detected in any other neural cell lines to date (Hunter et al, 1991). Given that NSC34 cells expresses all of the traites which we expected in a motor neuron hybrid cell line, these

hybrid cells cells may therefore be the result of the fusion of a neuroblastoma cell with a motor neuron, making them a unique resource for the isolation of motor neuron specific genes (Cashman et al, 1992).

Given an immortal cell line bearing motor neuron characteristics, and a parental neuroblastoma lacking them, we are in the position to address the question of what genes must be activated in the N18 cell line to produce the motor neuronal phenotype of the NSC34 cell line. An initial question that can be asked is what genes do NSC34 cells express that N18 cells do not. A number of groups have addressed similar questions by making cell type-specific probes of their cell lines of interest by means of subtractive hybridization and using these probes to screen whole (or subtracted) libraries. These probes are made by producing a ^{32}P-labeled cDNA probe from the cells of interest and allowing it to hybridize with a large excess of mRNA from cells not bearing desired traits. Probe molecules corresponding to mRNAs shared between the two cell lines hybridize to form heteroduplexes and are unavailable for hybridization with library DNA (Natzel at al 1986). This technique has been successfully used to clone the myogenic factor MyoD (Davis et al, 1987), the T-cell receptor (Hendrick et al, 1984), and to identify the B-cell specific A^d_α gene of the murine class II major immunohistocompatibility complex (Davis et al, 1984).

For my PhD thesis, I assessed the utility of the NSC34 cell line as a research tool for the isolation of motor neuron specific genes. Our strategy involved a 2 tiered approach to the cloning of motor neuron specific genes. In the first phase of the project, I isolated cDNAs representing genes specific to the NSC34 cell line with respect to the N18TG2 neuroblastoma parent. This was performed by making NSC34 specific probes by subtractive hybridization with N18 mRNA and using this probe to screen a cDNA library of the NSC34 cell line. The isolated clones were sequenced and screened for tissue specific expression by northern analysis, ribonuclease protection, or reverse transcription primed PCR using RNAs from different mouse organs. The exact identities of the cells

expressing some of these clones was established by in situ hybridization. In this way we proposed to assess whether genes expressed in NSC34 cells, but not in the neuroblastoma parent are, or include, genes expressed in motorneurons.

Chapter 2

Materials and Methods

Materials

Cultured Cells and Animal Tissues

Cultured cells were prepared with the assistance of Suzanne Boulet, Ivan Shaw, and Geralyn Trudel. All cells were grown in DMEM (Gibco/BRL) supplemented with 10% Fetal Bovine Serum on standard tissue culture plastic. Treated cells were prepared by 2 day incubation with either 3μM 5-azacytidine (5-Aza; Bartolucci et al, 1989) or 1mM dibutryl cyclic-AMP (db-cAMP; Minna et al, 1972; Ono et al, 1991). The NSC34 hybrid cell line was produced in the laboratory of Dr. N. Cashman (Cashman et al, 1992a), as were the control hybrid cell lines NSC19 (a sister hybrid of NSC34, which came from the same fusion experiments and also expresses choline acetyltransferase, extends processes, and supports action potentials), NO22 (an N18TG2 X rat oligodendrocyte hybrid which expresses galactocerebroside and myelin basic protein; Sugama 1989) and NI-1 (an N18TG2 X rat pancreatic islet cell hybrid which secretes insulin). Hybrid cell lines were grown for 4-6 days before harvesting. Mouse tissues were prepared from CD1 mice (Charles River Labs). Rats were Sprague Dawley Rats (Charles River Labs).

Enzymes and Reagents

Enzymes for molecular biology were obtained from Promega Biochemicals (Fischer Scientific), New England Biolabs, Bethesda Research Labs (Gibco/BRL) or Pharmacia, depending upon price and availability. Enzymes were used according to manufacturer's directions. Whenever possible, reaction buffers were obtained from the manufacturer.

Unless specifically noted, all materials and reagents were purchased from Sigma, Gibco/BRL, or Fischer Scientific depending upon price and availability.

RNA Isolation

RNA Isolation from Cultured Cells

RNA was isolated from cultured cells using a slightly modified form of the guanidinium isothiocyanate RNA isolation technique (Chomczynski et al, 1987). Media was removed from tissue culture plates and the cells were harvested by gently scraping with a rubber policeman in ice cold phosphate buffered saline (PBS). Cells in PBS were collected in 50ml Falcon tubes and pelleted by centrifugation at 1000 rpm (approximately 500g) for 5 minutes at 4°C in a tabletop centrifuge. The PBS was removed and the cells were washed twice by gently resuspending them in ice cold PBS, re-centrifuging, and discarding the PBS wash. The cell pellet was then disrupted by briefly vortexing and the cells were lysed by the addition (100µl per 10^6 cells) of 4M guanidinium HCl, 25mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1M β-mercaptoethanol and vigorous vortexing. After the initial lysis, 0.1 volumes of 2M sodium acetate pH 4.5, 1.0 volumes water equilibrated phenol, and 0.2 volumes chloroform were added. The mixture was shaken or vortexed vigorously incubated on ice for 15 minutes. Phenol and water phases were separated by centrifugation for 20 minutes in a Tabletop Clinical Centrifuge at setting 6 or in a Sorvall Superspeed centrifuge (SS34 rotor) at 10,000 rpm and 4°C. The aqueous phase was then re-extracted once with phenol/chloroform (1vol:0.2vol) and once with 1 volume of chloroform. RNA was precipitated with 1 volume of isopropanol at -20 C for 1 hour and collected by centrifugation for 20 minutes either in a tabletop Clinical Centrifuge at setting 6 or in a Sorvall Superspeed centrifuge (with an SS34 rotor) at 10,000 rpm at 4°C. The resulting RNA pellet was air dried, resuspended in water and re-precipitated with 0.1 volume 3M sodium acetate and 3 volumes ethanol in a 15ml corex tube either at -70 °C for 1 hour or at -20 °C overnight. The final sample was collected by centrifugation at 10,000 rpm for 20 minutes at 4°C in a

Sorvall centrifuge, dried, and resuspended in DEPC treated water or 0.05% sodium dodecyl sulfate. Nucleic acid concentration was determined either by measuring the optical density (OD_{260}) or by using a DNA Dipstick (Invitrogen) following manufacturers directions.

Extraction of RNA from Organ Tissues

RNA was isolated from tissue using a slightly modified form of the Guanidinium Isothiocyanate RNA isolation technique (Chomczynski et al, 1987). Tissue was removed from sacrificed mice or rats, and frozen on dry ice. Frozen tissue samples were then ground to powder using a mortar and pestle. This grinding step was omitted for very small samples. RNA was extracted from the frozen powder by homogenization in 100µl/mg of tissue of 4M guanidinium, 25mM sodium citrate pH 7.0, 0.5% sarcosyl and 0.1M β-mercaptoethanol using a Poly-Tron Homogenizer. Homogenization was performed several times at setting 6 (medium power) for 30 seconds, alternately chilling the samples on ice and homogenizing. After the initial homogenization, 0.1 volumes 2M sodium acetate pH4.5, 1.0 volumes water equilibrated phenol, and 0.2 volumes chloroform were added, and the mixture was homogenized for a further 30 seconds, transferred to a corex tube (or a snap-cap polypropylene tube) and iced for 15 minutes. The phenol and water phases were separated by centrifugation for 20 minutes in a Sorvall Superspeed centrifuge (SS34 rotor) at 10,000 rpm (approx. 12,000g) at 4 °C. The aqueous phase was then re-extracted once with phenol/chloroform (1vol:0.2vol) and once with 1 volume of chloroform. RNA was precipitated with 1 volume of isopropanol for 1 hour at -20 °C and collected by centrifugation for 20 minutes either in a tabletop Clinical Centrifuge at setting 6 or in a Sorvall Superspeed centrifuge (SS34 rotor) at 10,000 rpm at 4 °C. The RNA pellet was air dried, resuspended in water, and re-precipitated with 0.1 volume 3M sodium acetate and 3 volumes of ethanol in a 15ml corex tube either at -70 °C for 1 hour or at -20 °C overnight. The final sample was collected by centrifugation at 10,000 rpm for 20 minutes in a Sorvall centrifuge, dried, and resuspended in 0.05%

sodium dodecyl sulfate (SDS). Nucleic acid concentration was estimated by measuring the optical density at 260 nm and by ethidium bromide-agarose gel electrophoresis.

Isolation of mRNA

The messenger RNA fraction of total RNA was isolated by chromatography on oligo-dT cellulose (Pharmacia) as described in Molecular Cloning: A Laboratory Manual (Sambrook 1989, Sambrook 1992). Because mRNA constitutes a very small portion of total RNA, we found that this technique was only practical when relatively large amounts (>400µg) of total RNA starting material were use. Oligo-dT cellulose (0.1 g) was hydrated and suspended in Loading Buffer (100mM NaCl, 50mM Tris pH 7.5, 10mM EDTA pH 8.0, 0.05% SDS) and this slurry was loaded into a baked glass pasture pipette which had a small plug of siliconized glass wool. About 4 column volumes of loading buffer were passed through the column to ensure proper packing. Total RNA (>400µg) was added either to 10 volumes of Loading Buffer or 1 volume of 2X Loading Buffer and denatured at 65 C for 10 minutes. After cooling, this solution was allowed to drip through the column. If the sample ran though in less than 10 minutes, it was collected and passed through the column again. The column was washed with 4-5 volumes of Loading Buffer and messenger RNA was eluted with Elution Buffer (0.1 M Tris pH7.5, 10mM EDTA, 0.5% SDS) into 100 ml fractions. Fractions were assayed for nucleic acids by spotting small aliquots (2µl) onto a petri dish containing solid 1% agarose, containing 10mM M Tris pH 7.5, 1mM EDTA pH 8.0 and 1µg/ml ethidium bromide. After a 20 minute incubation at room temperature, the plate was examined for ethidium bromide induced florescence under UV light. Fractions containing mRNA were collected and their nucleic acid concentration was determined either by optical density at 260 nm or by using the DNA Dipstick (Invitrogen).

Library Screening with a Subtracted Probe

Production of cDNA Probes by Reverse Transcriptase

cDNA probes were synthesized using oligo dT12-18 (Pharmacia) primed reverse transcriptase as described in Molecular Cloning: A Laboratory Manual, Second Edition (Sambrook 1992). Briefly, 2µg of mRNA and 2µg of oligo-dT 12-18 were combined in a small volume (usually 4µl) and denatured at 65°C for 2 min. The samples cooled on ice and then brought to 20µl in 50mM Tris pH 8.3, 10mM MgCl, 10mM DTT, 0.015mM dCTP, and 0.15mM each of dATP, dGTP and dTTP. 10 µCi of [α-^{32}P]dCTP (ICN) and 2.5 units of AMV reverse transcriptase (Pharmacia) were added and the reaction was incubated at 43°C for 2 hours in an air incubator. The reaction was stopped and the RNA template was degraded by bringing the reaction to final volume of 45µl and a final concentration of 20mM EDTA, 0.1mg/ml denatured salmon sperm DNA (carrier), and 30mM NaOH and incubating the samples at 43°C for 1 hour in an air incubator. The cDNA probe was purified away from unreacted nucleotides by size exclusion gel filtration chromatography on a 3 ml Biogel P-60 (Biorad) column in 10mM Tris pH 7.5 and 1mM EDTA pH 8.0.

Preparation of Subtracted Probes

Probe subtraction was performed according to the protocols of Natzle *et al.* (1986) by hybridizing the ^{32}P-labeled cDNA probe produced from NSC34 mRNA with a 50-100 fold excess of N18TG2 neuroblastoma mRNA. Although it is possible to perform subtraction using total RNA, we found that it was very difficult to resuspend the required amount of RNA in the small volumes required for hybridization. In a typical experiment, 500ng of cDNA probe was mixed with 25µg of N18TG2 mRNA and precipitated with 1/10 volume 3M sodium acetate and 3 volumes of ethanol at -70 °C for 1 hour. The probe/RNA mix was spun for 10 minutes in a microfuge, vacuum dried, and resuspended in 5µl of diethyl pyrocarbonate (DEPC; Sigma) treated water. This probe-mRNA mixture

was then brought to a final volume of 15µl and the final composition 50% formamide, 2.67X SSC (20X SSC is 3M NaCl, 0.3M sodium citrate), 30mM sodium phosphate buffer pH 6.8 and 0.1% SDS. The cDNA probe was allowed to hybridize for 72 hours and then added directly to the hybridization solution for screening library plaque lifts.

Plating the Library/Plaque lifts

Library phage were plated and plaque lifts were performed using protocols supplied by Amersham with their Lambda gt10 cDNA Cloning System. E. Coli (strain NM514) bacteria were prepared for infection by growth to logarithmic phase (OD_{600} = 0.5-0.6) in LB broth (1% bactotryptone, 0.5% yeast extract and 1% NaCl) with 0.4% maltose followed by centrifugation for 10 minutes at 5000 rpm in a Sorvall SS34 rotor and resuspended in 0.4 volumes 10mM $MgSO_4$. 100 µl of cells were infected with 100 µl of library phage diluted in SM buffer (0.1M NaCl, 10mM $MgSO_4$ 5mM Tris pH 7.5 and 0.1% gelatin) at 37 °C for 15 minutes, mixed with molten (55 °C) 0.7% LB agarose and plated on LB-agar plates. After overnight incubation at 37 °C, the plates were overlaid with nitrocellulose filters (Amersham) for 1 minute, with successive filters (duplicate plaque lifts) overlaid for an additional minute. The filters were marked and transferred to denaturing solution (0.5M NaOH, 1.5M NaCl) for 1 minute, neutralized in 0.5M Tris HCl pH 7.0, 1.5M NaCl for 1 minute, and rinsed briefly in 2X SSC. The filters were then dried at room temperature and vacuum baked at 80 °C for 2 hours to cross link nucleic acids to the filters. Triplicate plaque lifts were taken from each phage plate, with the first and third hybridized with subtracted probe, and the second hybridized with unsubtracted probe.

Filters were prehybridized in 50% formamide, 0.75M NaCl, 0.15mM Tris pH 8.0, 10mM EDTA, 0.2% each of Ficoll 400, polyvinylpyrrolidone and bovine serum albumin, 0.5mg/ml denatured salmon sperm DNA, and 0.5% SDS overnight at 42°C in sealed bags with gentle agitation. Hybridization was performed in a similar buffer (substituting 0.02% each of Ficoll 400, polyvinylpyrolidone and bovine serum albumin and 0.1mg/ml

denatured salmon sperm DNA) with 10^6 cpm/ml of denature probe, overnight at 42°C in sealed bags with gentle agitation. After hybridization, filters were rinsed twice with room temperature wash solution (0.1X SSC, 0.1%SDS) and washed for 4 hours in 65°Cwash solution, changing the solution every hour, and flipping the filters by hand every 15 minutes. The washed filters were then wrapped with saran wrap, mounted on card board, and exposed to Kodak XAR film for 1-3 days at -70°C with 2 intensifying screens.

Positive clones were identified by aligning autoradiograms of the hybridized filters with petri dishes, and isolated by removing a plug of agar using a pasteur pipette. Phage were eluted from agar plugs into 400 µl of SM buffer (0.1M NaCl, 10mM $MgSO_4$ 5mM Tris pH 7.5 and 0.1% gelatin). Initial screens examined 30,000 plaques on 150mm petri dishes, with positives rescreened 2-3 times at lower density (200-500 plaques/100 mm petri dish) to purify them.

Rapid Analysis of Phage Isolates by PCR

Inserts of cloned bacteriophage were amplified using synthetic oligonucleotides (25mers prepared courtesy of Dr. R. Sekaly of the Institute de Recherché Clinique) complementary to sequences flanking the EcoR1 (cloning) site of the lambda gt10 phage, using published protocols (Higuchi 1989). For each PCR reaction, 5-20 µl of cloned phage (eluted from agarose plugs) were digested in a 25 µl reaction containing 0.45% Tween 20, 0.45% NP 40 and 1µg of proteinase K (Sigma) at 55 degrees C for 1 hour. The proteinase K was inactivated by heating the samples to 95 °C for 10 minutes. These samples are then brought to 100 µl in PCR buffer with a final concentration of 10mM Tris 9.5, 50mM KCl, 1.5mM MgCl, 0.1% Triton, 0.1% gelatin, 100 picomoles of each oligonucleotide, 200 µM each of dATP, dCTP, dGTP, and dTTP and 2 units of Taq Polymerase (Promega). Samples were amplified in a Perkin Elmer/Cetus DNA Thermocycler for 30 cycles set for 1 minute transit to 94 °C, 1 minute at 94 °C to denature templates, 1 minutes transit to 55 °C to anneal the oligos, 1 minute at 55 °C, 1 minute transit to 72 °C to extend the oligos and 1 minute at 72 °C. Results of PCR reactions were

analyzed by running 10-20 µl on a 1.5% agarose gel. If the products of the PCR reaction were to be subsequently radio-labeled by random priming, unreacted primers and nucleotides were removed by spin dialysis though Millipore Ultrafree UFC 30,000 ultrafiltration tubes (30,000 NMWL) 4 times (diluting with 10mM Tris pH 7.5, 1mM EDTA each time) in a Millipore Personal Microfuge.

Oligonucleotide sequences were as follows:

Oligo #879 (Sal1 linker in bold):

GGTCGACAGTATTTCTTCCAGGGTA

Oligo #880 (BamH1 linker in bold):

CGGATCCAAGTTCAGCCTGGTTAAG

Phage Growth and Extraction of DNA

Small amounts of bacteriophage were grown up from the initial isolates using a mini-lysate technique using protocols supplied by Amersham with their Lambda gt10 cDNA Cloning System. E. Bacteriophage in 200 µl SM (eluted from an agarose plug) were added to 200 µl bacteria from an overnight culture and allowed to adsorb for 15 minute at 37°C. 5ml of LB broth was added the culture was grown in a shaking 37°C incubator until there was evidence of lysis (suspended cell debrs) in the culture - usually 4 - 9 hours. A few drops of chloroform were added and the culture was shaken for an additional 10 minute. Cell debris was removed by centrifugation at 3000 rpm in for 10 minutes and the lysate was transferred to a clean sterile tube, and stored at 4 °C.

For large scale preparation of bacteriophage, 10^{10} cells of an overnight bacterial culture (E. coli strain NM514) were pelleted at 3000 rpm for 10 minutes and resuspended in 3 ml LB. Approximately $5 * 10^7$ bacteriophage were added, and the cell suspension was incubated for 20 minutes at 37 °C. Infected bacteria were then added to 500 mls of pre-warmed LB and shaken overnight at 37 °C (or until suspended cell debris appeared in the culture indicating the lysis of bacteria). Lysis of the bacteria was then completed by the addition of 10 mls of chloroform and a further 30 minutes of shaking. Cultures were

cooled to room temperature and 1µg/ml of crude pancreatic DNAse was added. After a 30 minute digestion at room temperature, the cultures were brought to 1 M NaCl and incubated on ice for 1 hour. Bacterial debris was removed by spinning the culture at 11,000g for 10 minutes at 4 °C in a Sorvall Superspeed centrifuge (GS3 rotor). Polyethylene glycol (50g) was dissolved in the supernatant by vigorous agitation, and phage particles where precipitated by incubation on ice for 1 hour. Phage particles were recovered by centrifugation at 11,000g for 10 minutes at 4 °C in a Sorvall Superspeed centrifuge. The phage/PEG pellet was resuspended in 8 ml SM and extracted once with 8 ml of chloroform. The aqueous phase was recovered by centrifugation in a tabletop clinical centrifuge for 10 minutes (setting 6) and 0.5g/ml cesium chloride was added. This CsCl/phage solution was layered on a block gradient with layers consisting of 3.56 M CsCl, 3.97 M CsCl, and 5.65 M CsCl (all made up in SM) in a Beckman Ultraclear ultracentrifuge tube, and spun at 22,000 rpm in an SW41 rotor for 2 hours.

The phage were recovered using a pasteur pipette at the interface between the 3.56 M CsCl and the 3.97 M CsCl layers. Phage were dialyzed against 2 changes of 50 mM Tris 7.5, 10 mM NaCl and 10 mM $MgSO_4$ for 1 hour and overnight respectively. EDTA was then added to a final concentration of 20 mM and the phage particles were digested by the addition of proteinase K (final concentration 50 µg/ml) and SDS (final concentration 0.5%) and incubation at 65 °C for 1 hour. The digested phage were extracted twice with phenol/chloroform and once with chloroform, and ethanol precipitated by the addition of 1/10 volumes sodium acetate and 2 volumes ethanol and incubation overnight at -20 °C. DNA was recovered by centrifugation for 10 minutes in a microfuge, and was then air dried, and resuspended in TE.

Subcloning Inserts into Bluescript SK II

Inserts were cut out of isolated phage using the restriction enzyme Eco RI using manufacturers recommended buffers, and purified by electrophoresis on a 1% agarose gel. Ethidium bromide stained bands were visualized under UV light and excised with a

razor blade. DNA was extracted from the agarose bands using a Sephaglas band prep kit (Pharmacia) following manufacturers directions. Concentration of the isolated DNA was estimated by analyzing an aliquot of the purified DNA on a second agarose gel. Digested, gel purified vector (100 ng) was mixed with isolated DNA fragment in various molar ratios (typically 1:1, 1:3 and 1:10) in ligation buffer (0.066 M Tris HCl, 5mM MgCl, 5 mM DTT and 1mM ATP) with 1 µl T4 ligase (BRL) and allowed to react overnight at either 4 °C or 12 °C.

Sequencing of Isolated cDNAs

DNA was sequenced using either a Sequenase kit (U.S. Biochemical Corporation) for cloned DNAs or a Cycle Sequencing kit (New England Biolabs) for sequencing cloned DNAs and uncloned PCR products, following manufacturers directions. For the Sequenase kit, supercoiled plasmids (2-3µg in about 5µl TE) were denatured by the addition of 100µl 0.2M NaOH at room temp for 5 min. Plasmid DNA was then precipitated with 25µl 5M ammonium acetate pH 7.5 and 400µl ethanol at -70 C for 10 minutes, and recovered by centrifugation in a microfuge for 10 minutes. Sequencing primer (0.5 picomoles) was annealed to the template in and annealing buffer containing 10mM Tris-HCl pH7.5, 20mM $MgCl_2$ and 50mM NaCl by briefly heating the sample tubes to in a beaker of water at 65 °C and then allowing them cool very slowly to 37°C along with the water in the beaker, when the beaker was placed at root temperature. The primers were briefly extended in the presence of 0.2µM dCTP, dGTP, and dTTP, 6.7mM DTT, 0.5µl of Ci/mMole [α-^{32}P]dATP or [α-^{35}S]dATP (Amersham) and 3 units of Sequenase for 5 minutes at room temp. 1/4 of each reaction was then added to 2.5 µl of termination mix containing 80mM of each nucleotide, 8mM of the corresponding dideoxy nucleotide, and 50mM NaCl and incubated for 15 minutes at 37°C. Reactions were stopped with the addition of 4 µl of 95% formamide, 20mM EDTA, 0.05% bromophenol

blue, 0.05% xylene cyanol FF, denatured at 75 °C for 2 minutes, and analyzed by electrophoresis through a denaturing (7M urea) polyacrylamide gel.

Uncloned PCR products and some cloned cDNAs were sequenced using a PCR-based Cycle Sequencing Kit (New England Biolabs). Oligonucleotides were end labeled by combining 7µl of [γ-^{33}P]ATP (New England Nuclear) with 10 pmoles of oligonucleotide. This was brought to a volume of 25 µl, and a concentration of 50mM Tris HCl pH 7.6, 10 mM $MgCl_2$ and 5 mM DTT. 1 µl of T4 polynucleotide kinase (BRL) was added and the reaction was incubated at 37 °C for at least 30 minutes. Phosphorylated oligonucleotides were used directly (without further purification), or were stored -20 °C for use within a few days. For each sequencing reaction, 1.2 pmol labeled primer was added to 10 ng or more of double stranded template DNA, 1.5 µl of 10X sequencing buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl pH 8.8 and 50 mM $MgSO_4$), 1 µl of Triton buffer (3% Triton X-100), and brought to 14 µl with water. After the addition of 1 µl (2 units) Taq DNA polymerase, 3.2 µl of this reaction was aliquoted into each of 4 tubes containing 3 µl the nucleotide reaction/chain termination solutions, and overlaid with a drop of mineral oil. The sequencing reactions were performed in a Perkin Elmer/Cetus thermal cycler set for 1 minute to 94 °C, 20 seconds at 94 °C, 1 minute to 55 °C, 20 seconds at 55 °C, 1 minute to 72 °C, and 20 seconds at 72 °C. The reaction proceeded for 20 cycles, after which 4µl of stop solution (formamide containing 0.3% xylene cyanol, 0.3% Bromophenol blue and 0.37% EDTA), was added to each tube. Reactions were heated to 94 °C for 2 minutes before electrophoretic analysis on a 7 M urea/5% polyacrylamide sequencing gel.

Detection of Transcripts by Northern Analysis

Production of Double Stranded DNA Probes by Random Priming

DNA probes were synthesized using a random priming kit (Pharmacia) following the manufacturer's directions. Probes were purified by precipitation in 10mM Tris HCl pH 7.5, 0.1mM EDTA, 0.1mg/ml denatured salmon sperm DNA and 20mM spermine on ice for 45 minute followed by 10 min. centrifugation in a microfuge. Probes were resuspended in 200µl 0.5M NaCl, 10mM Tris HCl pH7.5, 0.1mM EDTA and a 2µl aliquot was counted in a scintillation counter. The purified probes were denatured by boiling for 10 minutes and chilling on ice before addition to any hybridization reaction.

RNA Fractionation and Transfer

RNA samples (typically 1µg of mRNA or 20-40µg total RNA) in 5 µl were brought to a total volume of 24 µl in a buffer containing 0.04M MOPS, 10mM sodium acetate, 1mM EDTA, 55% deionized formamide, 14% formaldehyde and 0.2mg/ml ethidium bromide and heated to 55°C for 15 minutes. 1 µl of loading dye (0.4% bromphenol blue, 0.4% xylene cyanol, 50% Ficoll 400) was then added and the samples were electrophoresed through a 1.5% agarose slab gel containing 0.04M MOPS, 10mM sodium acetate, 1mM EDTA and 18% formaldehyde for approximately 3 hours. The gel was trimmed and soaked in distilled water for 20-30 minutes (with gentle agitation) and then photographed under UV light. RNA was transferred to Nytran (charge modified nylon membranes- Amersham) with a Vacugene vacuum transfer apparatus (Pharmacia) using 10mM NaOH, 50mM NaCl for 20 minutes, 0.1M Tris 7.5 for 20 minutes followed by 10X SSC for 3 hours. Nucleic acids were then crosslinked to the nylon membrane by exposure to UV light in a Stratalinker cross linker (Stratagene) set for 1200 µJoules ("Auto-Crosslink").

Probe Hybridization

Filters were prehybridized in 50% formamide, 0.75M NaCl, 0.15mM Tris pH 8.0, 10mM EDTA, 0.2% each of Ficoll 400, polyvinylpyrolidone and bovine serum albumin, 0.5mg/ml denatured salmon sperm DNA, and 0.5% SDS overnight at 42°C either in sealed bags with gentle agitation or in a screw top plastic tube in a hybridization rotisserie-type air incubator. Hybridization was performed in a similar buffer (substituting 0.02% each of Ficoll 400, polyvinylpyrolidone and bovine serum albumin, 0.1mg/ml denatured salmon sperm DNA) with 1-5*10^6 cpm/ml of denature probe. Dextran Sulfate was often added to a concentration of 10% to increase the sensitivity of hybridization. Filters were incubated either in sealed bags overnight at 42°C with gentle rocking or in a screw top plastic tube in a hybridization rotisserie-type air incubator. Filters were rinsed twice in wash buffer (0.1X SSC, 0.1% SDS) and washed at 65°C for 2 to 4 hours. Washed filters were exposed to Kodak X-Omat film overnight with 2 intensifying screens.

Synthesis of Radiolabeled cRNA Probes (Riboprobes)

Riboprobes were synthesized using T3 and T7 promoters flanking the cloning region of the Bluescript SK+ II plasmid, using protocols supplied by Stratgene for use with their Bluescript SK+ II plasmid. The restriction enzyme BssH II, which cuts at sites flanking the multiple cloning site region of the plasmid and the T3 and T7 RNA polymerase promoters, was used to release a transcriptional cassette from insert containing Bluescript II plasmids. This cassette was gel purified using Pharmacia Sephaglas gel purification system following manufacturer's directions. DNA was added to a 25µl (final volume) reaction containing 40mM Tris-HCl (pH 7.5), 6mM $MgCl_2$, 2mM spermine HCl, 5mM NaCl, 10mM dithiolthreitol and 100µg/ml BSA. Depending upon the intended use of the probe, 5µl (5 µCuries) of ^{35}S or ^{32}P α-labeled CTP (Amersham) or ^{33}P α-labeled UTP (New England Nuclear) was included in the reaction, and the reaction was brought to 400

µM with unlabeled nucleotides. Unlabeled CTP or UTP (matching the label used) was usually added to 40 µM to increase the number of full length probe molecules. One µl of RNA Guard (Pharmacia) was added and the reaction was initiated by the addition of 1 µl (10 units) and 1 µl (5-7 units) of T3 or T7 polymerase. The reaction was incubated at room temperature for 1-2 hours. While higher temperatures increased the rate of nucleotide incorporation, it also increased the rate of early chain termination, reducing the number of full length molecules. The DNA template was degraded by the addition of 1 µl (10units) Promega RQ1 RNAse-free DNAse at 37 °C for 15 minutes.

Probes for use in in-situ hybridization were purified by spin dialysis in Millipore UFC 30,000 ultrafiltration tubes to concentrate probe and remove unreacted nucleotides. 200 µl of 1mM EDTA, (with 1mM DTT for ^{35}S-labeled probes) was added to the labeling reaction and the reaction was transferred to an ultra filtration tube. The tubes were spun in a Millipore "Personal Microfuge" for about 5 minutes or until the sample volume was reduced to about 50 µl. The dialysis was repeated twice after the addition of 400 µl 1mM EDTA, +/-1mM DTT. Aliquots were take for scintillation counting before and after the ultrafiltration.

Probes to be used in Ribonuclease Protection Assays were assayed for radiolabel incorporation by trichloracetic acid (TCA) precipitation and then gel purified to assure homogeneous probe size. Briefly, 1 µl of reach probe reaction was spotted on duplicate glass fiber filters (Millipore) and allowed to dry. One filter was added directly to a scintillation vial containing 5 mls of Cytosol scintillation fluid (Amersham). The second filter was rinsed in 3 changes of ice cold 10% trichloracetic acid, once in ice cold ethanol, and then allowed to air dry before being placed in a scintillation vial with scintillation fluid. Both samples were counted in a scintillation counter. The remaining probe was mixed with 25 µl of loading dye, boiled for 2 minutes, and run on a 5% polyacrylamide gel at 250 volts for 1 - 2 hours. The gel was then dismantled, wrapped in saran wrap and exposed to Kodak XAR film (in a darkroom) for 5-10 minutes with the corner of the film

taped to the glass plate. At the end of the exposure time, the film was cut (leaving the taped corner on the glass for alignment) and developed. The band of gel containing full length probe was then excised from the gel, using the autoradiograph and a hand held geiger counter. Probe was eluted from the acrylamide overnight in elution buffer. An aliquot of the eluted probe was also counted in a scintillation counter in 5 ml Cytosol scintillation fluid.

Detection of RNA Transcripts by Ribonuclease Protection

Ribonuclease Protection Analysis were performed using kits purchased from Ambion and following manufacturer's directions. RNA to be analyzed (1-40µg) was combined with 1-5 $*10^5$ cpm of cRNA probe (calculated to be a 4 fold excess for a high abundance message), ethanol precipitated and resuspended in 30µl of hybridization buffer consisting of 80% formamide, 40mM PIPES pH 6.4, 1mM EDTA and 0.4M NaCl. This mixture was incubated at 85°C for 10 minutes to denature all RNA secondary structures and then allowed to anneal for 8-12 hours at approximately 44°C. The mixture was then cooled to room temperature, and unhybridized RNAs were digested by the addition of 300µl of a solution containing 300mM NaCl, 10mM Tris-HCl (pH 7.4), 5mM EDTA, 2µg/ml RNAse T1 and 40µg/ml RNAse A and incubation at 37°C for 60 minutes. RNAses were inactivated by the addition of 20µl of 10% SDS and 10µl of 10mg/ml of proteinase K and incubating the reaction at 37 °C for 30 minutes. The reaction products were extracted in phenol/chloroform and ethanol precipitated after the addition of 20µg of carrier tRNA. Digestion products were resuspended in 10µl of loading buffer (80% formamide, 10mM EDTA pH 8.0, 1mg/ml xylene cyanol and 1mg/ml bromophenol blue), denatured for 5 minutes at 95 °C and analyzed by denaturing polyacrylamide gel electrophoresis. In a later version of this kit (RPA II), RNAse was inactivated and RNA was precipitated in a single step by the addition of a proprietary solution.

Detection of mRNA Transcripts using RT-PCR

Detection of target clones was performed by semi-quantitative Reverse Transcriptase Primed - Polymerase Chain Reaction (RT-PCR). RNA from cells of tissues to be examined (usually 0.5 µg) was added to PCR tubes in duplicates. For each sample, 50 µl of buffer was prepared which containing standard PCR buffer (10mM Tris pH 9.5, 50mM KCl, 1.5mM MgCl, 0.1% Triton, 0.1% gelatin; Sambrook 1989) , 100 picomoles of each oligonucleotide, 200mM each of dATP, dCTP, dGTP, and dTTP and 2 units of Taq DNA Polymerase (BRL). In addition, radiolabel (0.2µCi ^{32}P-dCTP/reaction) was added as a tracer. This sample buffer was added to the duplicate RNA samples either with or without 2 units of Reverse Transcriptase(BRL) and the reactions were sealed with 100µl of mineral oil. Samples were placed in a Perkin Elmer/Cetus DNA Thermocycler and the Reverse Transcription reaction was allowed to proceed for 15 minutes at 50 °C, followed by a standard 3 stage PCR (1 minute transit to 94 °C , 1 minute at 94 °C, 1 minute transit to 55 °C, 1 minute at 55 °C, 1 minute transit to 72 °C, and 1 minute at 72 °C). An aliquot of each PCR reaction (usually 10-20µl) was analyzed by polyacrylamide gel electorphoresis, and the gel was dried exposed either to film or to a PhosphorImager exposure plate. The number of cycles for the PCR reaction was determined by creating a standard amplification curve, plotting amplification cycle number vs. radioactivity incorporation, and choosing a cycle number such that amplification is in the log-linear range. Oligonucleotides were designed to amplify 150 and 120 base products of Clones 8 and 110 respectively and synthesized by the Sheldon Biotechnology Center. Actin primers were synthesized from the published sequence (Tokunaga 1986) to amplify a 611 base band and were provided courtesy of Dr. Trevor Owens.

Oligonucleotide sequences were as follows:

Clone 8 Reverse:	GGTACAACTTCTAGACAACT
Clone 8 Forward:	GACTCAGACTTCAGTAACAA
Clone 110 Reverse:	GGTACAACTTCTAGACAACT
Clone 110 Forward:	ATGTCTGTATGTTCTTGTTT
Actin Reverse:	CAGGCAGCTCATAGCTCTTCT
Actin Forward:	TGGGTCAGAAGGACTCCTATG

In Situ Hybridization

Tissue preparation

Tissue blocks were prepared for sectioning in three different ways: fresh frozen, paraformaldehyde perfused, and paraformaldehyde perfused/sucrose embedded. For fresh frozen tissue blocks, mice were killed by cervical dislocation and rapidly dissected. Tissues were mounted on polypropylene sectioning blocks with 7% tricanthum gum and frozen in liquid nitrogen cooled isopentane. To prepare tissue blocks from perfused animals, mice were anesthetized by intraperitoneal injection of 5% chloral hydrate (0.01ml/gram body weight) and their thoracic cavity was opened to give a wide exposure of the heart. Their right atrium was nicked with fine dissecting scissors, and 40 mls of ice cold 4% paraformaldehyde in PBS was injected slowly in to the left ventricle through a 1/2 inch 22 gauge needle. Perfusion was judged successful if the animal's tail became rigid. Tissues were dissected and emersed in ice cold 4% paraformaldehyde in PBS for at least one hour. Tissues to be sucrose embedded (for cryoprotection) were placed in ice cold 30% sucrose in PBS and left overnight at 4 °C.

Samples which were not sucrose embedded, were left in PBS at 4 °C overnight if there was not enough time to freeze them on the day of dissection. Tissues were mounted on polypropylene sectioning blocks with 7% tricanthum gum and/or Histotech Mounting

Solution (Fisher Scientific) and frozen in liquid nitrogen cooled isopentane. Frozen tissue blocks were kept in 50ml falcon tubes in isopentane at -20 °C for short times (<1 week) or at -80 °C for long term storage.

Glass microscope slides were prepared for sections by TES (aminopropyltriethoxysilane; Sigma) coating, which allows the formation of covalent bonds between glass and tissue sections. Other coating methods have been tested extensively (by Holly Bradshaw in our laboratory) and found to be inadequate for bonding sections to slides through the In Situ procedure. To coat slides, slides in slide racks were immersed in a 2% TES solution in dry acetone (BDH) for 30 seconds, and then dipped slowly for 10 times. Slide racks were then dipped in 2 changes of acetone, 2 changes of distilled water, and dried in a 42 °C incubator.

Sectioning was performed in a Zeiss Microm Cryostat at -12 to -16 °C. Tissue blocks (in isopentane) were warmed to the cryostat temperature for 1 hour before sectioning. Section were cut at 10 - 14 microns, depending on the condition of the particular tissue block and thaw mounted on prepared slides. Slides were stored at -20 °C until use.

The *In Situ* Hybridization Protocol

Protocols for *in situ* hybridization are adopted from a protocol developed by Dr. Ken Hastings (Shoubridge et al, 1990) and modified for the use with cRNA probes (riboprobes) using published procedures (Bendotti 1991, and Valentino 1987).

Slide mounted fresh frozen tissue sections were fixed in ice cold 4% paraformaldehyde in PBS for 2 minutes and then rinsed 3 times in distilled water. (This pre-fix step was omitted with fixed tissues.) Sections mounted on glass slides were hydrated in PBS at room temperature for 10 minutes and then transferred to a solution containing 0.1mg/ml Pronase (Pharmacia) in 50mM Tris HCl pH 7.5, 5mM EDTA for 5 minutes at room temperature. After rinsing once with room temperature distilled water, slides were transferred to a freshly made solution of 0.2% HCl for 5 minutes, rinsed again in room temperature distilled water, and post-fixed in ice cold 4%

paraformaldehyde in PBS for 2 minutes. Slides were then rinsed 3 times in room temperature distilled water and transferred to pre-warmed prehybridization solution.

Prehybridization was performed for 3-6 hours at 42° C in 50% deionized formamide, 0.02M sodium phosphate buffer (pH 6.8), 0.75M NaCl, 0.075M sodium citrate, 0.1mg/ml denatured salmon sperm DNA, 1% glycine, and 0.1% each of Ficoll 400, polyvinylprolidone (PVP) and BSA. For ^{35}S labeled probes, 0.1% DTT was added to the prehybridization and hybridization mixes. Hybridization was performed by overlaying 100 µl of hybridization solution (50% formamide, 0.75M NaCl, 0.075 M sodium Citrate, 1mg/ml denatured salmon sperm DNA, 10% dextran sulfate, 50mM sodium phosphate pH 6.8 0 and 0.25% each of Ficoll 400, PVP, and BSA) with 10^7 cpm/ml of probe and covering with a cover slip. Sections were then incubated for 4 - 12 hours at 42 °C.

After hybridization was complete, coverslips were removed under 4X SSC/0.08% β-mercaptoethanol (β-Me) and slides were placed in racks. Slide racks were incubated twice for 10 minutes in 4X SSC/0.08% β-Me and transferred to RNAse buffer (0.5M NaCl, 10mM Tris HCl pH 8.0 and 1mM EDTA) containing RNAse A at 20µg/ml in for 30 minutes at 37 °C. The slide rack were then transferred to RNAse buffer without RNAse and incubated a further 30 minutes at 37 °C.

Final washes were performed in 2X SSC (with 0.08% mercaptoethanol) for 30 minutes at room temp., 0.1X SSC (with 0.08% mercaptoethanol) at 42 °C for 30 minutes. Sections were then dehydrated in graded ethanols (30%, 60% and 90%) with 1.7% ammonium acetate, and 95% ethanol and then air dried for 1 hour at room temperature. The dried slides were exposed to Kodak XAR film for at least 4 days.

Dipped Emulsion Autoradiography

Kodak NTB2 emulsion was slowly melted at 42 °C and mixed with distilled water (to 50%) with occasional stirring. Emulsion was transferred to slide dipping apparatus and allowed to equilibrate to 37 °C for 30 minutes. Slides were dipped 4 at a time and slowly

removed from the emulsion by the dipping apparatus. Dipped slides were allowed to dry at room temperature for 1 hour standing upright. Slides were then packed in light tight containers and exposed for 3-8 weeks at 4°C.

After exposure, slides were placed in racks and developed by immersion in D-170 solution (2.5% sodium sulfite, 0.1% sodium bromide, 0.45% 2,4-diaminophenol hydrochloride) for 6 minutes, rinsed for 30 seconds in 1% acetic acid, and fixed for 3 minutes in 24% sodium thiosulfate. Slides were then washed in bath of gently running tap water for 10 minutes. All solutions were at 18 °C (in a water bath of running tap water). Slides were not allowed to dry out before staining.

Staining Slides with Hematoxylin and Eosin

Protocols for Hematoxylin and Eosin staining were obtained from the Neuropathology Department of the Montreal Neurological Institute, as modified from the published protocols. (Lillie, 1954; Gilbert and Nuttal, 1965). Harris' Hematoxylin was prepared by dissolving 1 gm hematoxylin (Sigma) in 10ml absolute ethanol, and 20 grams of aluminum alum in 200 ml distilled water (both over heat). As soon as both reagents had dissolved, the solutions were mixed. The solution was brought to a rapid boil and 0.5 grams of mercuric oxide (Sigma) was added. The solution was cooled and glacial acetic acid was then added to 4%. This solution has a very long shelf life, but must be filtered before every use (or once a day).

Eosin counterstain was prepared by mixing 150ml of 2% Eosin Y with 30ml saturated picric acid (1gm/78ml water) and 20 ml 95% ethanol. This solution must also be filtered before use (or once a day).

Sections were stained in racks, by immersion in Harris Hematoxylin for 3 minutes, rinsing in 3 changes of distilled water, and then placed in 0.3% ammonium hydroxide until the sections turn blue (about 30 seconds). Section racks were then rinsed once in distilled water and placed in the eosin stain for 30 seconds. Eosin dipped slide racks were

washed in 3 changes of 95% ethanol, cleared in xylene and then mounted under coverslips with Permount (Fischer Scientific).

Chapter 3
Results

The Library Screen

Preliminary Characterization of the Library

The creation of the NSC34 cell line, as well as the synthesis and initial characterization of the NSC34 cDNA library were performed by Drs. Neil Cashman and Ken Hastings before my arrival in the laboratory. The library initially contained approximately $4.3 * 10^5$ independent phage, making it 2.5 times larger than the $1.7 * 10^5$ phage theoretically required to have 0.99 probability of including cDNA copies of all low abundance (<14 copies/cell) mRNAs (Sambrook et al, 1989). From early plaque hybridization experiments, in which about 10^4 phage plaques were examined, about 10^3 showed detectable hybridization with NSC34 bulk cDNA probes. Of these, approximately 2% showed significantly greater hybridization with NSC34 cDNA than with N18 cDNA and these appear to contain cDNAs encoding RNA messages which are more abundant in the hybrid cells than in the parent N18 cells by a factor of 10 or more.

Subtractive Hybridization Screen

In order to create an NSC34-specific cDNA probe, we prepared 32P-labeled cDNA probe from NSC34 mRNA and allowed this probe to hybridize in solution with N18 mRNA according to the protocols of Natzle et al (1986) to a theoretical Rot (extent of hybridization) of 1047 (calculated for 25µg of N18 poly (A)+ RNA in a volume of 15 µl, allowed to anneal for a period of 72 hours). This "subtracted" probe was then used to screen $1.5 * 10^5$ plaques of the NSC34 cDNA library. Plaque lifts were performed in triplicate with the first and the third filters probed with subtracted probe and the second

filter probed with bulk (unsubtracted) cDNA probe. All filters were washed together in the same wash solution in order to reduce filter-to-filter variations in washing, which we found to greatly affect the interpretations of the screen. The attenuation of almost all of the most prominent signals on the filters screened with subtracted probe (by comparison with the bulk cDNA probe) was taken to indicate successful probe preparation and subtraction.

Positive clones were identified as possessing the same signal intensity on all three triplicate plaque lifts. This was an important criterion because phage plaques corresponding to very abundant transcripts often gave still visible, albeit much attenuated, hybridization signals on the autoradiographs of filters examined with the subtracted probe. Using this technique, 40 agar plugs including positive clones were isolated for further study. Phage isolated in these plugs were re-plated at low titers and rescreened using a differential hybridization screen, comparing the signals generated by NSC34 and N18 bulk cDNA probes. Phage plaques showing stronger relative hybridization with the NSC34 probe than the N18 probe were picked. Differential hybridization was used in these rescreening steps because of its technical simplicity as well as the fact that subtractive screening on the scale required would consume impractically large quantities of mRNA. After 2-3 rounds of differential screening, 8 insert containing phage were successfully purified.

It is not clear why only 8 of 40 cases were differentially hybridizing phage. Many of the originally-identified phage may have been false positives due either to the noise in the screen or to particular technical complications. (For example, it is possible to imagine that if the probe complexity is small (the average probe size is short), some phage might actually produce a higher signal with the subtracted probe by forming probe-mRNA-phage DNA hybrids; these false positives would be discarded in subsequent differential screens.) In addition, because isolating the initial clones required exact alignment of nitrocellulose filters, autoradiographs and petri dishes, any slippage in this alignment

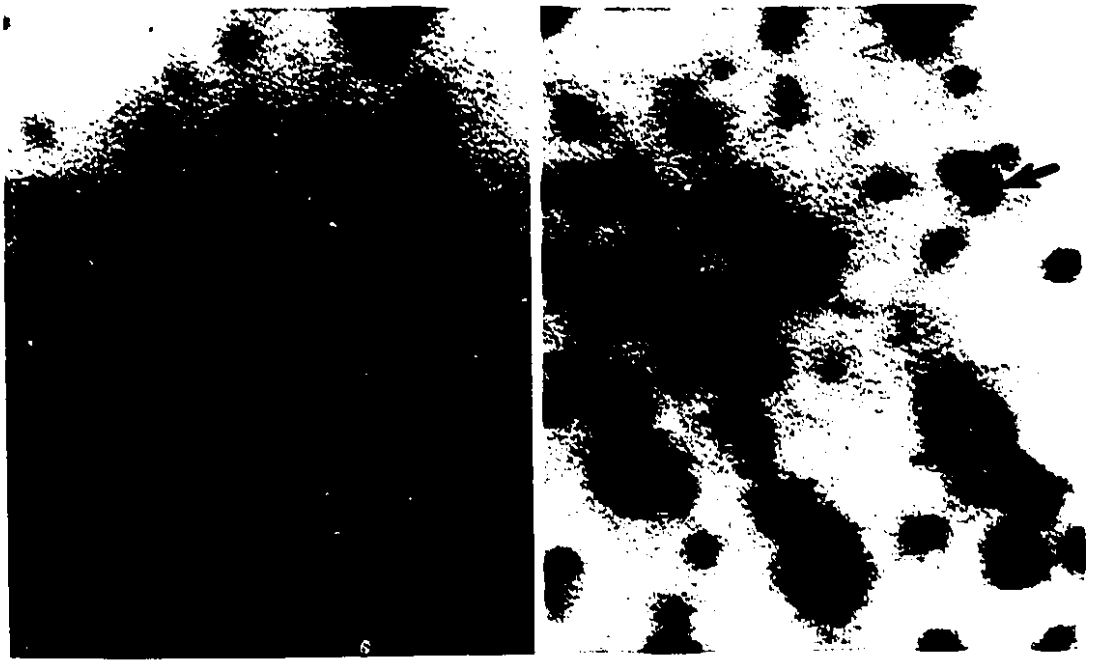

Figure 3-1. The preliminary differential screen. Comparison of autoradiograms of duplicate plaque lifts of the NSC34 library screened with bulk N18 cDNA probe (left) and bulk NSC34 cDNA probe (right). Positive phage, such as the one at the arrow, are phage which are identified by the NSC34 probe but not by the N18 probe.

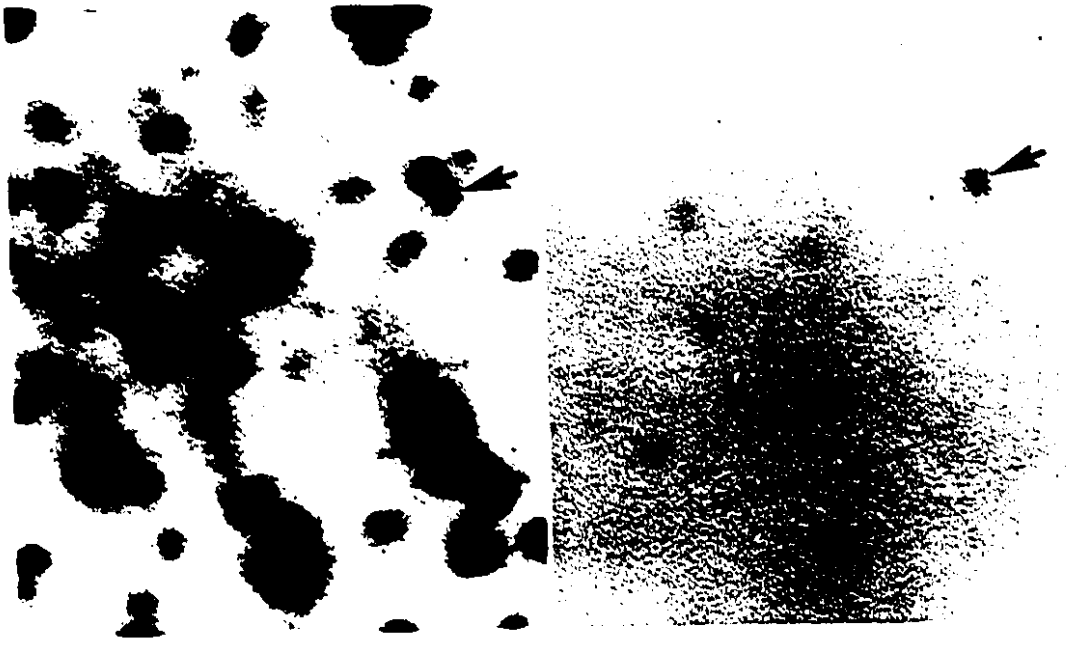

Figure 3-2. The preliminary subtractive screen. Comparison of autoradiograms of duplicate plaque lifts of the NSC34 library screened with total (left) and subtracted (right) NSC34 probe. Positive phage, such as the one at the arrow, are phage which are identified by the total probe and maintain their signal intensity with the subtracted probe. Note that many phage which give strong signals with the total probe still produce some signal with the subtracted probe, but the signal intensity is greatly reduced.

might result in failure to isolate a positive phage. Finally, because the differential screen was often difficult to interpret, by nature of the fact that there were many more high abundance signals on the autoradiogram (many more phage hybridize with the bulk cDNA probes than with the subtracted probe), it is possible that true positives may have been passed over in favour of higher abundance transcripts if some of those transcripts possessed even a modest amount of preferential expression in NSC34 cells.

Initial Characterization of Clones

After isolating and purifying differentially hybridizing phage, our immediate concerns were 1) demonstrating that the cDNA clones actually correspond to mRNAs which were more abundant in the hybrid cells than in the parent neuroblastoma and 2) determining if any of our isolates encoded previously identified transcripts. The first concern was addressed by showing that the isolated phage did indeed possess cDNA inserts and that these inserts hybridized in Northern blots with mRNA whose expression was higher in the NSC34 than in the N18 cell line. In order to prepare Northern blot probes, we used PCR to amplify cDNA inserts from some of our phage using primers for the lambda gt10 phage arms. The expression patterns of several clones which did not generate detectable Northern blot hybridization signals were examined by ribonuclease protection experiments and where necessary, semi-quantitative PCR. For this purpose phage cDNA inserts were subcloned into a Bluescript SK II+ plasmid (Stratagene). This plasmid contains 2 RNA polymerase promoters flanking its cloning site allowing easy production of sense and antisense cRNA probes.

Our second concern, the initial identification of our isolates, was addressed by sequencing the ends of the cDNA inserts and using these sequences to perform homology searches against the GenBank DNA sequence database. Early sequence comparison work

was performed using the FASTA program from the University of Wisconsin GCG package running on a MicroVAX at the Montreal Neurological Institute. Later sequence searches were performed by electronic mail (Email), using the BLAST program network server at the National Center for Biotechnology Information (NCBI) of the American National Institutes for Health. In addition to being much faster and providing more comprehensive search options, sequence searching by Email allowed comparison with a large number of databases (including GenBank) which were updated on a daily basis.

After initial characterization of our cDNA clones, the expression of their corresponding mRNAs was examined in NSC34 as well as in a number of control cell lines, to investigate the role cell hybridization played in their expression. These cell lines include N18TG2 (the parental cell line), db-cAMP treated N18TG2 (which extend small processes; Minna et al, 1972; Ono et al, 1991), N18TG2 treated with 5-azacytidine (which de-methylates genomic DNA; Bartolucci et al, 1989), NSC19 (a sister hybrid of NSC34, which came from the same fusion experiments and also expresses choline acetyltransferase, extends processes, and supports action potentials), NO22 (an N18TG2 X oligodendrocyte hybrid which expresses galactocerebroside and myelin basic protein) and NI-1 (an N18TG2 X rat pancreatic islet cell hybrid which secretes insulin).

Finally, the expression patterns of novel clones were examined in northern blot experiments or semi-quantitative PCR experiments with mouse tissue RNAs. Clones which appeared to have a neuronal pattern of expression were then used as probes in situ hybridization experiments in mouse brain and spinal cord. This allowed us to specifically assay the expression of our isolates in motor neurons.

Based on sequence data, we categorize our 8 isolated cDNAs (from both the preliminary and large scale screens) into 5 distinct classes:

- Transcripts encoded by the mitochondrial genome (1)

  Clone 5.21: Cytochrome Oxidase subunit II (CO II)

- Transcripts consisting of small RNAs, presumably transcribed by RNA polymerase III (2)

  Clones 1.91 and 1.32: the mouse b1 element (2 independent isolates)

- Known cDNAs encoding genes with widespread tissue distribution (1)

  Clone 1.92: the mouse IAP element

- Known cDNAs representing RNAs with neural distribution (2)

  Clone 22: Chromogranin B
  Clone 3.72: GAP-43

- cDNAs representing novel sequences (2)

  Clone 3.82
  Clone 1.10

Mitochondrial Transcripts

In our screen, we isolated a cDNA representing the mitochondrially encoded gene cytochrome oxidase subunit II. This cDNA, clone 5.21, comprises the 3'- 120 bases of the COII sequences and includes 18 bases of polyadenylation. Single pass sequencing yields a 98% homology with the published mouse sequence (Figure 3-3; Bibb et al, 1981). Northern blotting experiments showed that CO II mRNA was indeed expressed at significantly higher levels in NSC34 cells than in control N18 cells (Figure 3-4). In addition, when NSC34 cells are compared to N18 cells by electron microscopy, hybrid cells do in fact appear to possess about 10 times more mitochondria than the parent neuroblastoma (A. Beaudet and N. Cashman, unpublished results). Therefore our screening methodologies were effective in isolating cDNAs representing mRNA transcripts which are, in fact, enriched in the hybrid cells.

```
LOCUS       MITOMM      16295 bp    DNA   Circular  ROD
DEFINITION  Mouse mitochondrial genome- from: 7000  to: 7700
ACCESSION   V00711

 to: Cln521.Seq  check: 1642  from: 1  to: 120

 Percent Similarity: 98.305   Percent Identity: 98.305


7577 CCAGGGTTATTCTATGGCCAATG.CTCTGAAATTTGTGG.ATCTAACCAT 7624
     |||||||||||||||||||||||| |||||||||||||| ||||||||||
   1 CCAGGGTTATTCTATGGCCAATGACTCTGAAATTTGTGGAATCTAACCAT 50

7625 AGCTTTATGCCCATTGTCCTAGAAATGGTTCCACTAAAATATTTCGAAAA 7674
     |||||||||||   ||||||||||||||||||||||||||||||||||||
  51 AGCTTTATGCC..ATGTCCTAGAAATGGTTCCACTAAAATATTTCGAAAA 98

7675 CTGATCTGCTTCAATAATTTAAT 7697
     |||||||| |||||||||||| |
  99 CTGATCTG.TTCAATAATTTATT 120
```

Figure 3-3. Sequence alignment of our isolated clone 5.21 with the mouse mitochondrial genome (Output from the Program BESTFIT).

We examined the expression of cytochrome oxidase II mRNA in our series of control cell lines. Figure 3-4 demonstrates that in addition to being expressed at higher levels in the NSC34 hybrid cells than in the N18 cells, CO II mRNA expression is upregulated significantly by db-cAMP treatment of N18 cells and to a lesser extent by 5-Aza. Furthermore CO II mRNA is expressed at similar levels in the NSC34, NSC19 and NO22 cell lines and at significantly lower levels in the NI-1 cell line.

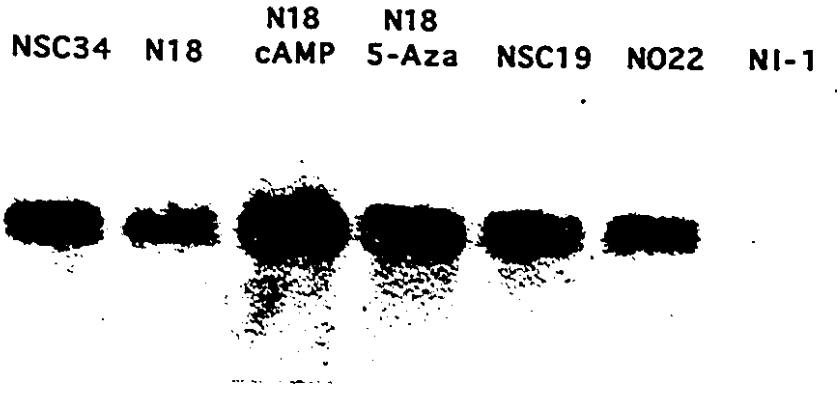

Figure 3-4. Northern blot showing the expression of cytochrome oxidase subunit II in hybrid and control cell lines. The lanes are loaded (from left to right) with 20 µg of total RNA from NSC34 cells, N18 cells, N18 cells treated with db-cAMP, N18 cells treated with 5-Azacytidine, NSC19 cells, NO22 cells, and the NI-1 cells. The blot was washed at high stringency (0.1X SSC/0.1% SDS at 65 °C for 2 hours) and exposed to Kodak XAR film overnight with 2 intensifying screens.

Transcripts encoding small RNAs (the Mouse b1 element).

We isolated 2 independent cDNA clones, clone 1.32 and clone 1.91, which hybridizes to a very small, diffuse band on a Northern blot (at approximately 100-200 bases) of NSC34 poly A+ RNA, but not in that of N18 (Figure 3-5). The clone 1.32 contains a cDNA encoding 45 bases of sequence and 80-100 bases of polyadenylation. The clone 1.91 comprises 120 bases of sequence and 17 bases of polyadenylation. The sequence of these isolates corresponds to the mouse b1 element (Coggins et al, 1982) and is shown in Figure 3-6.

This ds-RNA species is homologous to the human Alu repeat element (the major class of human repetitive sequence DNA) and the 7SL RNA of the signal recognition particle (Kalb et al, 1983; Schmid and Jelinek, 1982). These elements are believed to have expanded in copy number from 7SL RNA by acting as a "retrotransposon," that is by reverse transcription of an RNA intermediate and insertion into the genome. Alu/b1 repeats are present in a number of species, and the higher intra- than inter-species homologies of Alu family members suggest that sequence expansion is a relatively recent event in evolution. In addition, the existence of subfamilies of Alu sequence gives the impression that sequence expansion might occur in waves with only a few of the individual elements being actively "retrotransposed" during any expansion (Quentin, 1988; Howard and Sakamoto 1988). The presence of at least one example of a human genetic disease (neurofibromatosis type 1) caused by the insertion of an Alu sequence indicates that Alu sequences in the human, and presumably b1 sequences in the mouse, may still be actively expanding in the genome (Wallace et al, 1991).

In addition to Alu/b1 elements, many other families of short interspersed repetitive sequences (SINEs) have also been described in species as diverse as silkworm, newt, salmon and sea urchin. While some of these families possess homology to Alu sequences, some may also resemble other RNAs such as tRNAs or have uncertain

Figure 3-5. Northern blot of our isolate of the mouse b1 element in mRNA from N18 and NSC34 cells. One µg of poly A + RNA was run in each lane. The blot was washed for 2 hours at high stringency at 65 °C and exposed for 1 week to Kodak XAR film. The blot was stripped and reprobed with Actin as a control for lane loading (Lower Panel). Arrows indicate the position of the 18 and 28S Ribosomal bands.

homology and origin (Deinger et al, 1986; Howard et al, 1990). What SINEs have in common is that they are transcribed by RNA polymerase III. Transcripts of various SINE elements can be detected in normal mouse tissues (Limborska et al, 1987) and their transcription in tissue culture models can be shown to increase following transformation (i.e. by SV40; Singh et al, 1985) or by transition from quiescent to proliferative states (Lania et al, 1987; Carey et al, 1986).

```
 LOCUS       MUSRSB1H      250 bp ds-DNA
DEFINITION  Mouse B1 repetitive sequence DNA.
ACCESSION   M24152

 to reverse of: Cln191.Seq  check: 5447  from: 1  to: 140

 Percent Similarity: 89.922   Percent Identity: 89.922

 B1h.Seq x Cln191.Seq

  40 GCCAGGCGTTGGTGGCACACT.CCTTTAATCCCAGCACTCGGGAGGCAGA 88
     |||||||  |||||||  |||  |||||||||||||||| |||||  |||
 243 gccaggc..tggtggctcacacccttaatcccagcactagggagtaaga 196

  89 GGCAGGCAGATTTCT.GAGTTCGAGGCCAGCCTGGTCTACAGAG.TGTGT 136
     ||||||||||| |||  |  ||||||||||||||||||||||||    ||
 195 ggcaggcagatctctataactcgaggccagcctggtctacagagcaaggt 146

137 TCCAGG.ACAGCTAGGGCTACACAGAGAAAACCTGTC 172
    ||||||  |||||  |||||||||||||  ||||||||
145 tccaggaacagccagggctacacagag..aacctgtc 111
```

Figure 3-6. Sequence alignment of our isolated clone 1.91 with another isolate of the mouse b1 element (Output from the Program BESTFIT).

The Mouse IAP element.

One of the cDNAs isolated in the subtractive screen is about 330 bases in length (including 26 bases of polyadenylation) and possesses 92% homology to the published sequence of the mouse intracisternal A particle genome (IAP; Kuff et al, 1983; Mietz et al, 1987), as shown in Figure 3-7. Because IAP transcripts could not be readily detected by Northern blotting, IAP expression in NSC34 and in our control cell lines was examined using ribonuclease protection assays (Figure 3-8). Using this technique, IAP transcripts were detected at higher levels in the NSC34 hybrid cell than in the N18 parent.

```
LOCUS       MUSFLIAP     7117 bp ds-DNA
      Mouse full-length intracisternal A-particle encoding
      gag and pol proteins, complete cds.

ACCESSION   M17551 K01572 K01573

 to: Cln192.Seq  from: 1  to: 246

  Percent Similarity: 92.531   Percent Identity: 91.286
                .         .         .         .         .
494 CTTTTCAGCTGGGGAACGAGAGTACCAGTGAGTACAGCTTTACGAGGTAA 543
    ||||||||||||||||||||||||||||||||||
  1 cttttcagctggggaacgagagtaccagtgagta.............. 34
                                .
                                .
                                .
                .         .         .         .         .
594 AGTGGAGAAAATGTTTGGCCTTGAATTTTTTCTAGTGTTAGAAGCCCTTT 643
              |||||  ||||||||||||||| |||||||  | ||||||
 35 ...........tgttttgccttgaatttttctggtgttaggaacccttt 73
                .         .         .         .         .
644 TGTTCCTTTTCACATGTTATCAAGTGGTTAAG.GCAGGGCGG...ATTCT 689
    ||||  ||||||||||| |||||||| |||||  ||||||  |   |||||
 74 tgtt.cttttcacatgctatcaagtgattaagatcagggctgaaaattct 122
                .         .         .         .         .
690 AGATGAAATTCAGGACAAGCTATCAGAAGTAAAGCGGGGAGAGAGAGTAG 739
    |||  ||||||||||||||||||||||||||||||||  |||  |||||||
123 agaggaaattcaggacaagctatcagaagtaaagcgggaagaaagagtag 172
                .         .         .         .         .
740 GAACAAAGAGGAAATATGGTA.CACAAAATAAGTATACAGGCCTTTCCAA 788
    ||||||||||  |||||||::: ||||||||||||||||  |||||||||
173 gaacaaagagaaaatatgxxxtcacaaaataagtatacaa.cctttccaa 221
                .         .         .
789 GGGTCTTGAACCCGAGGAAAAGTTAAGGTTA 819
      |   ||||||  |     ||||||||||||
222 ..gatctgaaccagg....aagttaaggtta 246
```

Figure 3-7. Sequence alignment of our isolated clone 1.92 with the Full length IAP Element (Out put of the Program BESTFIT)

However, IAP transcripts were also readily detectable in N18 cells treated with db-cAMP, or 5-Aza as well as in NSC19, NO22 and NI-1 cell lines. The IAP element is an endogenous non-infectious retrovirus which, by sequence homology, is related to the murine mammary tumor virus. This virus is present in approximately 1000 copies per

haploid mouse genome and is transcribed in normal mouse tissues (with some developmental and tissue specificity; Gaubatz et al, 1991). The full length genome is about 7.2 kb long, but several subtypes of the IAP genome exist which are classified based upon the presence of insertions or deletions in the full length sequence. In cells which possess high levels of IAP RNA transcripts, it is possible to detect IAP particles by immunoblotting and it is possible to observe viral particles budding into the endoplasmic reticulum (by electron microscopy). Reverse transcriptase activity has been found associated with these viral particles (Kuff and Lueders, 1988) and IAP elements have been demonstrated to behave as retrotransposons, able to be copied and integrated into the genome by means of an RNA intermediate (Heidmann and Heidmann, 1991).

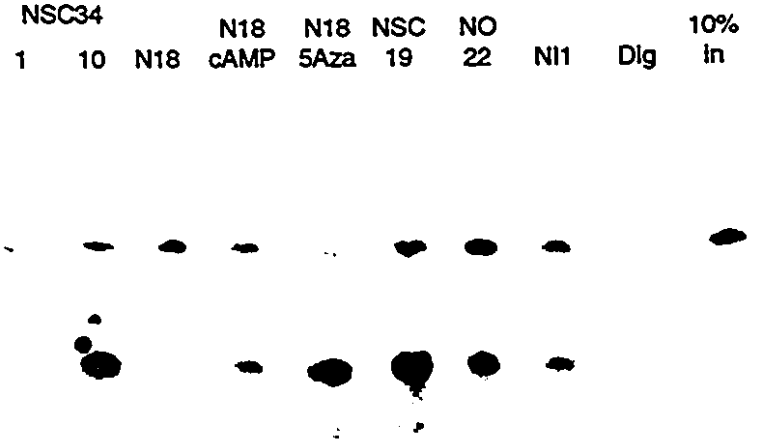

Figure 3-8. Ribonuclease Protection Assay of IAP expression in NSC34 and control cell lines. The lanes are loaded (from left to right) with 1 and 10µg of NSC34 RNA, and 10 µg of RNA from N18 cells, N18 cells treated with db-cAMP, N18 cells treated with 5-Azacytidine, NSC19 cells, NO22 cells, and the NI-1 cells. The remaining 2 lanes contain the total input probe digested (Dig), and 10% of the input probe undigested (10% in). The upper band appears in the digested probe alone and is therefore an artifact.

Known cDNAs representing RNAs with neural distribution

Two of the cDNAs which we isolated correspond to mRNAs that have been previously identified in the literature and reported to possess a neural tissue distribution, namely GAP-43 and chromogranin B. The cDNA of clone 3.72 is 656 bases long and spans the region from bases 308 to 964 of the sequence of GAP43 (P-57; Cimler et al, 1987). The homology of this sequence alignment (from single pass sequencing) is 98%. Clone 22 contains a cDNA bearing a 98% homology to the 3' 238 of mouse chromogranin B (Linard et al, 1990). Sequence alignments are shown in Figure 3-9 and 3-10. Using the ribonuclease protection assay, GAP43 and chromogranin B are detectable in 10 µg of NSC34 total RNA but not in N18 RNA (Figure 3-11). Furthermore, NSC19 and NO22 but not NI1 also express these transcripts (N18 and NI-1 cells may possess a low level of expression of GAP43). GAP43 but not chromogranin B transcription is inducible in N18 cell by treatment with db-cAMP which induces process formation in these cells. The demethylating agent 5-azacytidine was not found to have any effect on the expression of either of these mRNAs in N18 cells.

GAP43

GAP-43 (also referred to as P57, F1, B50, and neuromodulin) is a gene which is believed to be important in axonal growth cone extension and regulation. A major constituent of the axonal growth cone, GAP43 accounts for approximately 1% of the growth cone protein content. (Goslin et al, 1990). Although the amino acid sequence of GAP-43 is highly hydrophilic (Basi et al, 1987), the protein is tightly coupled to the growth cone membrane by fatty acylation (Skene and Virag, 1989). GAP43 can be shown to interact with a number of regulatory systems including calmodulin (Alexander et al, 1988), G-proteins (Strittmatter et al, 1990) and protein kinase C (Lovinger et al, 1985; Skene, 1989). Although GAP-43 would seem to interact with a number of

regulatory systems in the growth cone, its exact function is unclear. While transfected GAP43 appears to stimulate the elaboration of filipodial extension in NIH 3T3 cells and COS cells (Zuber et al, 1990), PC12 cell mutants which are deficient in GAP43 still attain a differentiated phenotype, extending neurites in response to NGF or db-cAMP (Baetge et al, 1991). Given the apparent regulatory interactions of GAP-43, it must now be presumed that GAP43 might play a role in axonal navigation, axon terminal remodeling or synaptic plasticity.

```
LOCUS       MUSCBPNS     1227 bp ss-mRNA
DEFINITION  Mouse neural specific calmodulin-binding protein
            P-57 mRNA, complete cds.
ACCESSION   J02809

 to: Cln372f.Seq  from: 1  to: 196

 Percent Similarity: 98.0

 Muscbpns.Rodent x Cln372f.Seq

308 GAAGGGTGATGCACCAGCTGCTGAGGCCGAGGCCAAGGAGAAGGATGATG 357
    |||||||||||||||||||||||||||||||||||||||||||||||||||
  1 gaagggtgatgcaccagctgctgaggccgaggccaaggagaaggatgatg 50

358 CTCCCGTTGCTGATGGTGTGGAGAAGAAGGAGGGAGATGGCTCTGCTACT 407
    |||||||||||||||||||    |||||||||||||||||||||||||||
 51 ctcccgttgctgatggtgtgg...agaaggagggagatggctctgctact 97

408 ACCGATGCAGCCCCAGCCACCAGCCCCAAGGCTGAGGAGCCCAGCAAGGC 457
    |||||||||||||||||||||||||||||||||||||||||||||||||||
 98 accgatgcagccccagccaccagccccaaggctgaggagcccagcaaggc 147

458 AGGAGAT.GCACCTTCTGAGGAGAAGAAGGGTGAAGGGGATGCGGCCCCC 506
    |||||||   |||||||||||||||||||||||||||||:|||||||
148 aggagatcagaccttctgaggagaagaagggtgaaggggatxcggcccc. 196
```

Figure 3-9. Sequence alignment of a portion of our isolated clone 3.72 with the mouse gene for GAP43 (Output of the Program BESTFIT).

Chromogranin B

Chromogranin B is one of a family of 3 proteins, referred to as chromogranins, which were originally described as major components of the adrenal chromaffin granules and which are secreted along with the contents of the granules. All three proteins (chromogranin A, B, an C) are highly acidic, glycosylated, tyrosine sulfated and proteolytically processed. Expression of chromogranins is not confined to the adrenal, but is widespread throughout the nervous system and the neuroendocrine system (but absent from the exocrine system), with each cell type possessing its own unique ratio of chromogranin A:B:C. (Somogyi et al, 1984; Lassman et al, 1986; Nolan et al, 1985; Fischer-Colbrie et al, 1985; Fischer-Colbrie et al, 1987).

Chromogranin B has been cloned in the mouse and humans. In both species, this protein bears significant homologies to chromogranin A in the N and C terminal domains, and contains a non-homologous internal sequence. This non-homologous region is highly hydrophilic with numerous acidic amino acids and potential dibasic cleavage sites. In the mouse, chromogranin B is encoded by a 2337 nucleotide message, with a theoretical translation product of 655 aa or 75 kd (Forss-Petter et al, 1989; Benedum et al, 1987; Pohl et al, 1990). The actual molecular mass of chromogranin B has been estimated by SDS-PAGE electrophoresis to be 100 Kd, probably reflecting post-translational modification (Winkler et al, 1986; Fischer-Colbrie et al, 1987).

Although the role of chromogranins is unknown, the fact that these proteins are proteolytically processed and secreted suggests that they may play a role as peptide hormones. In fact the proposed peptide hormone pancreastatin, which suppresses insulin secretion in response to glucose in isolates of pancreatic islet cells, has a high degree of homology to chromogranin A (Huttner and Benedum, 1987; Eiden et al, 1987; Tatemoto, et al, 1986). Alternatively, it has been proposed that chromogranins might aid the packing of secretory granule contents, stabilize secretory granules by regulating osmotic

pressure, or buffer granule pH by virtue of its low pI which matches the pH of secretory granules (Winkler et al, 1986; Fischer-Colbrie et al, 1987).

```
LOCUS       MMCHRB       2340 bp    RNA              ROD
DEFINITION  Mouse mRNA for chromogranin B (secretogranin I)
ACCESSION   X51429

 to: Cln22.Seq  check: 7611  from: 1  to: 238

 Percent Similarity: 98.707   Percent Identity: 98.707

1739 TTCCCAGAGTACAACTATGACTGGTGGGAGAGAAGGCCCTTCTCAGAGGA 1788
     ||||||||||||||||||||||||||||||||||||||||| |||||||||
   1 ttcccagagtacaactatgactggtgggagagaaggccctcctcagagga 50

1789 TGTGAATTGGGGATATGAGAAGAGAAGCTTTGCCAGGGCCCCTCAGCTCG 1838
     |||||||||||||||| |||||||||||||||||||||||| |||||||
  51 tgtgaattggggata.gagaagagaagctttgccagggccccacagctcg 99

1839 ACTTGAAACGGCAATATGATGGAGTGGCCGAGTTGGACCAGCTGCTTCAC 1888
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 100 acttgaaacggcaatatgatggagtggccgagttggaccagctgcttcac 149

1889 TACAGGAAGAAGGCAGACGAATTT..CCCGATTTCTACGAC.TCGGAGGA 1935
     ||||||||||||||||||||||  ||||||||||||||| ||||||||
 150 tacaggaagaaggcagacgaatttagcccgatttctacgacatcggagga 199

1936 GCAGATG...GGGCCTCACCAGGAGGCAAACGATGAAAA 1971
     |||||||   || ||||||||||||||||||||||||||
 200 gcagatgggaggacctcaccaggaggcaaacgatgaaaa 238
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Figure 3-10. Sequence alignment of our isolated clone 22 with the mouse gene for chromogranin B (Output of the Program BESTFIT).

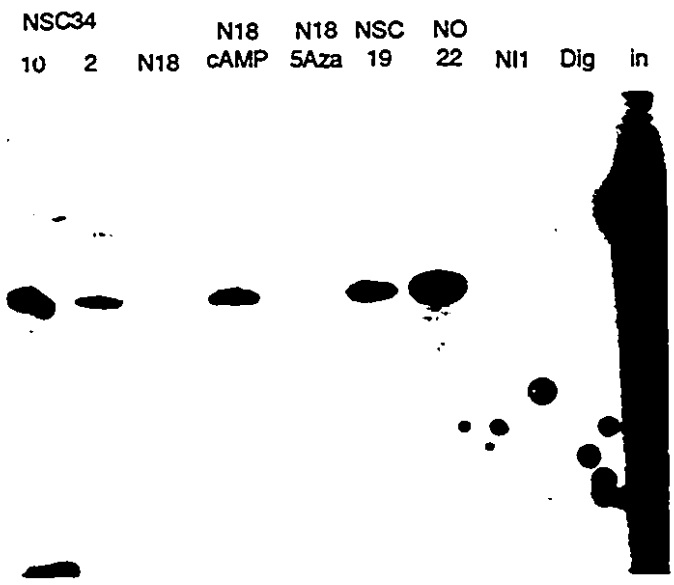


Figure 3-11. Ribonuclease Protection Assay of GAP43 expression in NSC34 and control cell lines. The lanes are loaded (from left to right) with 10 and 2 µg of NSC34 total RNA, and 10 µg of RNA from N18 cells, N18 cells treated with db-cAMP, N18 cells treated with 5-Azacytidine, NSC19 cells, NO22 cells, and the NI-1 cells. The remaining 2 lanes contain the total input probe digested (Dig), and the input probe undigested (in). Ribonuclease Protection Assay of Chromogranin B produces similar results, except that Chromogranin B expression is not inducible in the N18 cell line with db-cAMP.

cDNAs representing novel sequences

Clone 3.82

One of the cDNAs which we isolated did not match any sequences in GenBank at the time of isolation. This cDNA was about 896 bases long (including 41 bases of polyadenylation) and corresponded to an mRNA that was easily detected in Northern blots, giving a single 2300 base band in all of our cell lines (Figure 3-12). This transcript was expressed at higher levels in NSC34 cells than in the parent neuroblastoma cells, and expression was upregulated by db-cAMP in the N18 cell and reduced in by 5-Aza. This sequence was also expressed at slightly higher levels in the control hybrid cell line than in the N18 parent. Northern blots probed with our clone 3.82 cDNA detected a 2300 base band in every tissue examined, with the highest levels of expression in kidney and spinal cord (Figure 3-13).

More than a year after its initial isolation, the sequence of clone 3.82 was identified in a routine search of GenBank as being identical to the rat glutamate binding subunit of an NMDA receptor (GBP; Kumar et al, 1992) at 86% of the 533 bases sequenced (corresponding almost entirely to putative protein coding sequence). Part of this alignment is shown in Figure 3-14. This suggests that our isolate encodes the mouse homologue of the glutamate binding subunit of an NMDA receptor. This glutamate binding protein was originally purified from a protein complex of approximately 4 different proteins (of molecular weight 71,000, 63,000, 43,000 and 36,000) which, when reconstituted into liposomes, could confer glutamate and NMDA (but not kainate or quisqualate)-gated channel function. The glutamate/NMDA binding subunit of this protein complex was further purified by binding to glutamate conjugated affinity matrix and subsequent elution with NMDA. The individual purified NMDA binding subunit has a molecular weight of 71 Kd and can be shown to bind glutamate and aspartate but not to confer NMDA-gated channel function (Kumar et al, 1991).

Antibodies were raised against this purified 71 Kd protein which are capable of blocking glutamate binding to purified GBP (Chen et al, 1988) and glutamate-gated ion influx into liposomes (Ly and Michaelis, 1991). These antibodies were reported to bind to post synaptic membranes (Eaton et al, 1990) and to stained the surface of cultured hippocampal cells and protect these cells against NMDA induced excitoxicity (Mattson et al, 1991).

Kumar et al used these anti-GBP antibodies to clone a GBP cDNA from an expression vector library of rat brain. The cloned sequence is about 1800 bp and contains an open reading frame of 516 aa. The conceptual translation of this protein possesses 4-8 transmembrane domains and does not bear homology to any previously described protein (Kumar et al, 1991) or with the functional NMDA-gated ion channel (NMDA-R1) described by Moriyoshi et al (1991). Despite this, antisense-oligonucleotides directed against the GBP mRNA sequence have been reported protect cultured neurons against NMDA induced excitotoxicity (Kumar et al, 1992).

In the published report of the sequence of GBP, the authors reported that their cDNA identified at ~1700 base band present in brain, but not in liver, lung or muscle (Kumar et al, 1992). This data is in contrast with our own (Figure 3-13). Because our isolate encoded only the 3' 790 bases of the mouse homologue of GBP and the published Northern blot was performed with the 5' 550 bases of the published (rat) sequence, we hypothesized that there may be alternate splice products of the GBP, possibly possessing different tissue distributions. To investigate this possibility, we made PCR primers from the published sequence in order to amplify a 550 base product (corresponding to the fragment used to probe their Northern blot) and a 900 base fragment, which includes this 550 base product and spans the intervening sequence to overlap our isolate. Both PCR products were successfully generated by Reverse-Transcriptase primed PCR from rat brain RNA and their identities were confirmed by directly sequencing the ends of the

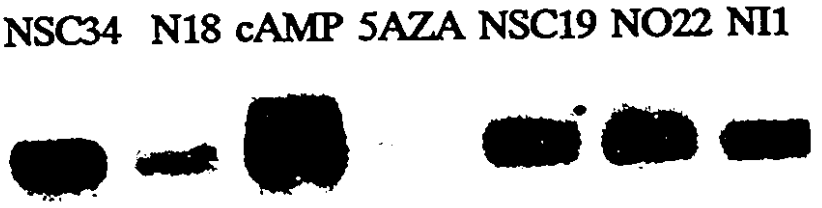


Figure 3-12. Northern blot of clone 3.82 expression in NSC34 and control cell lines. The lanes are loaded (from left to right) 40µg of total RNA from NSC34 cells, N18 cells, N18 cells treated with db-cAMP, N18 cells treated with 5-Azacytidine, NSC19 cells, NO22 cells, and the NI-1 cells. The blot was washed for 2 hours at high stringency at 65 °C and exposed to Kodak XAR film for 48 hours with two intensifying screens.

PCR products. When these PCR fragments are used to probe Northern blots of RNAs from rat and mouse, the results are similar to Figure 3-13.

Given the fact that GBP does not possess homology to any known ligand gated channel, and may have a broad tissue distribution, it seems unlikely that this protein represents a bona fide neuronal NMDA channel. Nevertheless, it is impossible to rule out the possibility that GBP interacts with other (perhaps neuronal) proteins to form a hetero-oligomeric channel complex. It could therefore play a role in NMDA mediated effects such as learning/memory (Coltman et al, 1988) or excitotoxicity (Rothman and Olney, 1987; Beal, 1992).

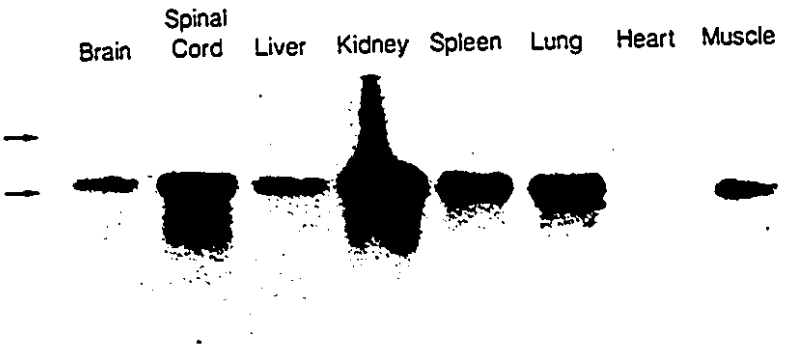


Figure 3-13. Northern blot of clone 3.82 expression in mouse tissues. 40µg of RNA was loaded in each lane. The blot was washed for 2 hours at high stringency at 65 °C and exposed for 48 hours to Kodak XAR film with 2 intensifying screens. Arrows indicate the position of the 18 and 28S Ribosomal bands.

```
LOCUS       S61973       1742 bp    mRNA;
DEFINITION  NMDA receptor glutamate-binding subunit 1742
ACCESSION   S61973

 to: Cln382f.Seq  check: 4839  from: 1  to: 292


 Percent Similarity: 93.993   Percent Identity: 93.286

 Gbp.Seq x Cln382f.Seq


 887 CAGACCCGCTACGACTTCACCTCGTGCATGGGCGTGCTCCTGGTGAGCGT 936
     ||||||| ||| |||  |||||||||||||||||||||||||| |  ||
   1 cagacccccctatgacggcacctcgtgcatgggcgtgctcctggtcactgt 50

 937 GGTGGTGCTCTTCATCTTCGCCATACTCTGCATCTTCATCCGGAACCGC. 985
     |||||||||||||||||||||| ||||||||||||||||||||||||
  51 tgtggtgctcttcatcttcgccatcctctgcatcttcatccggaaccgct 100

 986 ATCCTGG.......AGATCGTGTACGCCTCGCTGGGCGCTCTGCTCTTCA 1028
     |||||||       |||| || ||||||||||||||||||||||||||
 101 atcctggagattgtagattgtatacgcctcgctgggcgctctgctcttca 150

1029 CCTGCTTCCTGGCTGTGGACACCCAGCTGCTCCTGGGGAACAAGCAACTG 1078
     |||||||||||||| |||||||||||||| |||||||||||||||| |||
 151 cctgcttcctggcagtggacacccagctactcctggggaacaagcagctg 200

1079 TCCCTGAGCCCAGAAGAATATGTGTTTGCGGCCCTGAATCTGTACACGGA 1128
     | |||||| ||||||||||||||||||:|:|||||||| ||||||||||
 201 t.cctgag.ccagaagaatatgtgtttxcxgccctgaacctgtacacgga 248

1129 CATCATCAACATCTTCCTATACATTCTCACCATCATTGGCCGT 1171
     |||||||||||||||||||| |||||||||||||||| ||||
 249 catcatcaacatcttcctatatattctcaccatcattgcccgt 291
```

Figure 3-14. Sequence alignment of our isolated clone 3.82 with the rat gene for the glutamate binding subunit of an NMDA receptor (Output of the Program BESTFIT).

Clone 1.10

We isolated one additional cDNA (clone 1.10) corresponding to a gene whose complete sequence has never been published. Clone 1.10 consists of a cDNA 176 bases long (including 56 bases of polyadenylation) which possesses homology to two short (300-650 base) uncharacterized cDNAs published in two collections of Expressed Sequence Tags of human brain, with reported frequencies of 2 in 1000 and 1 in 3400 (Khan et al, 1992; Adams et al, 1993). The alignment of our isolate with one of these ESTs in shown in Figure 3-15. Despite the apparent high abundance of this transcript in brain, we were unable to detect clone 1.10 mRNA using northern blotting or ribonuclease protection assays. We now believe that this failure to detect clone 1.10 message could be due to the high A-T content (and therefore low Tm) of our cDNA, which might cause probe-mRNA hybrids to denature in what would be considered normal high stringency conditions.

Because of the perceived low abundance of these transcripts, we used semi-quantitatitve PCR assays to examine the expression of these in mouse tissue RNAs. To do this, we first established PCR standard curves to determine the rate of amplification of our products in the PCR reaction (Figure 3-16). From these standard curves, amplification parameters were chosen which would ensure that amplification of PCR products would remain within the linear range (17 cycles). The expression of Clone 1.10 and actin is mouse tissues is displayed in Figure 3-17. Clone 1.10 is expressed predominately in brain and spinal cord, suggesting that it may encode a neuron-specific gene. A number of attempts were made to detect Clone 1.10 in our control cell lines using the RT-PCR detection system. These were, unsuccessful in that RNA of several of the cell lines did not produce reproducible results, possible due to SDS contamination of our RNA samples.

The expression pattern of clone 1.10 was further examined by in-situ hybridization. In the spinal cord, clone 1.10 was clearly expressed in motor neurons. In the brain, clone 1.10 transcripts were detectable in the hippocampal formation, the cortex, and the cerebellar granule cells but not in Purkinje cells (Figures 3-18 and 3-19). From this experiment, we can conclude that although our hybrid cell-cDNA cloning strategy did not yield a motor neuron specific gene, we were in fact, capable of isolating a cDNA which was expressed prominently in motor neurons in the spinal cord.

```
LOCUS       T03322        656 bp ss-mRNA            EST
DEFINITION  IB1241 Homo sapiens cDNA clone IB1241.
ACCESSION   T03322

 to reverse of: Cln11022.Seq  check: 9955  from: 1  to: 121

 Percent Similarity: 89.908   Percent Identity: 89.908

656 GAAGAGAAAATCTCTAATAA..TTTATTGACCTTCAGTTT.CACATTGTG 610
    |||||||||   ||||||||  ||||||||||||||||||| ||||| |||
  1 gaagagaaa...tctaataaattttattgaccttcagtttacacatcgtg 47

609 AAAAAAAAAAAAAATAACAGTTTTACAAAACCTCAAAATGTAGTCATAGC 567
    |||||||       ||||||||||||||||||| ||||||||||    ||
 48 aaaaaaa.......taacagttttacaaaaccttaaaatgtagtagatgc 97

566 AAACAAGTACATATGACCATGA 545
    ||||||| |||||    |||||
 98 aaacaagaacatacagacatga 119
```

Figure 3-15. Nucleic acid sequence of Clone 1.10 aligned with the sequence of Expressed Sequence Tag IBI1241 (Output of the Program BESTFIT).

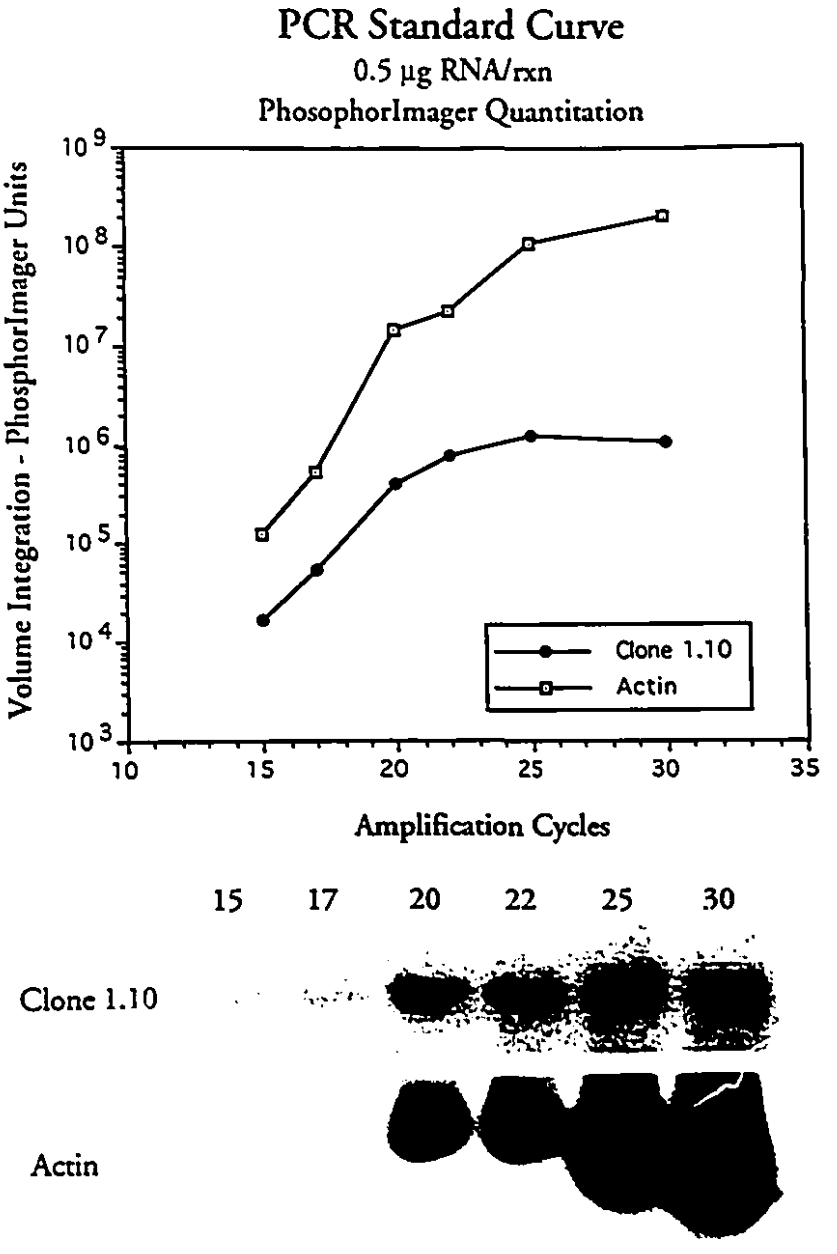


Figure 3-16. PCR Standard Amplification Curves. Reverse Transcriptase Primed PCR reactions starting with 0.5 µg of NSC34 RNA and using Clone 1.10 and Actin oligonucleotides were run for varying numbers of cycles and analyzed by polyacrylamide gel electrophoresis. Dried polyacrylamide gels were exposed overnight to PhosphorImager exposure plates (for quantitation) or Kodak XAR film (for photography).

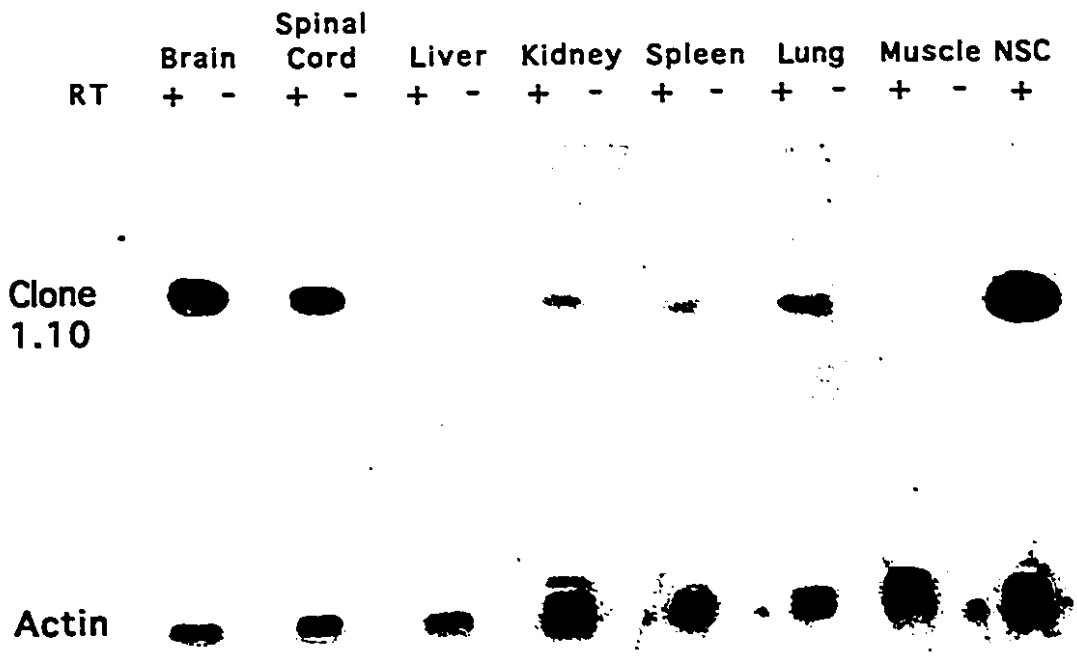


Figure 3-17. Determination of tissue distribution of novel sequences by semi-quantitative PCR. Top panel shows RT-PCR reactions primed with oligonucleotides for clone 1.10. Bottom panels shows parallel reaction primed with actin oligonucleotides, as a control. Reactions were performed with 0.5 µg of RNA from each tissue type either with or without the addition of Reverse Transcriptase (RT). PCR amplification proceeded for 17 cycles. Reactions were analyzed by polyacrylamide gel electrophoresis. Gels were dried and exposed overnight to Kodak XAR film.

Figure 3-18. In situ hybridization studies with Clone 1.10. in mouse spinal cord. Panels A and B show mouse spinal cord sections probed with anti-sense Clone 1.10 cRNA, with motor neurons prominently labeled (at 100X and 400X respectively). Panels C and D show mouse spinal cord sections probed with Clone 1.10 sense control probe (at 100X and 400X respectively).

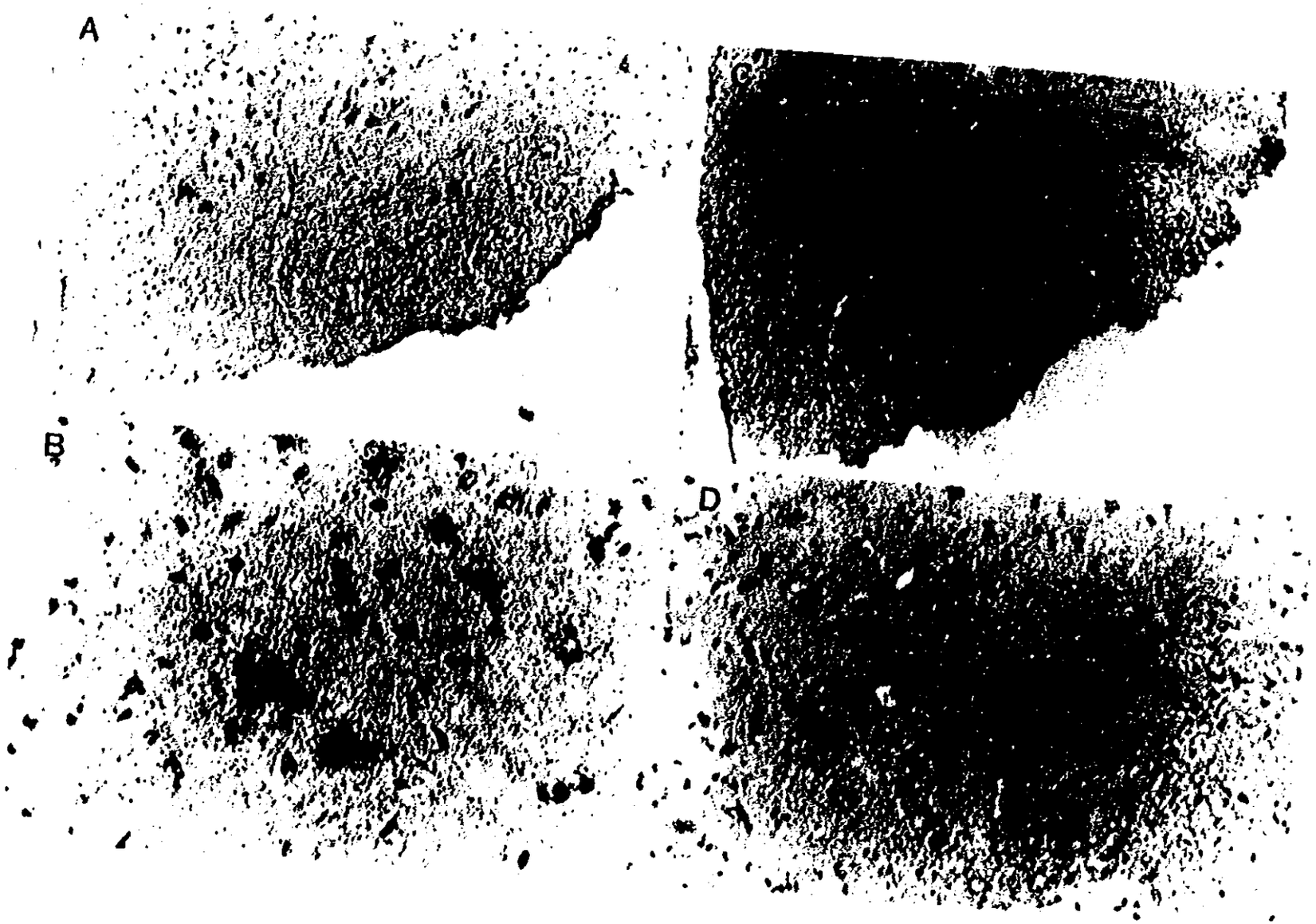
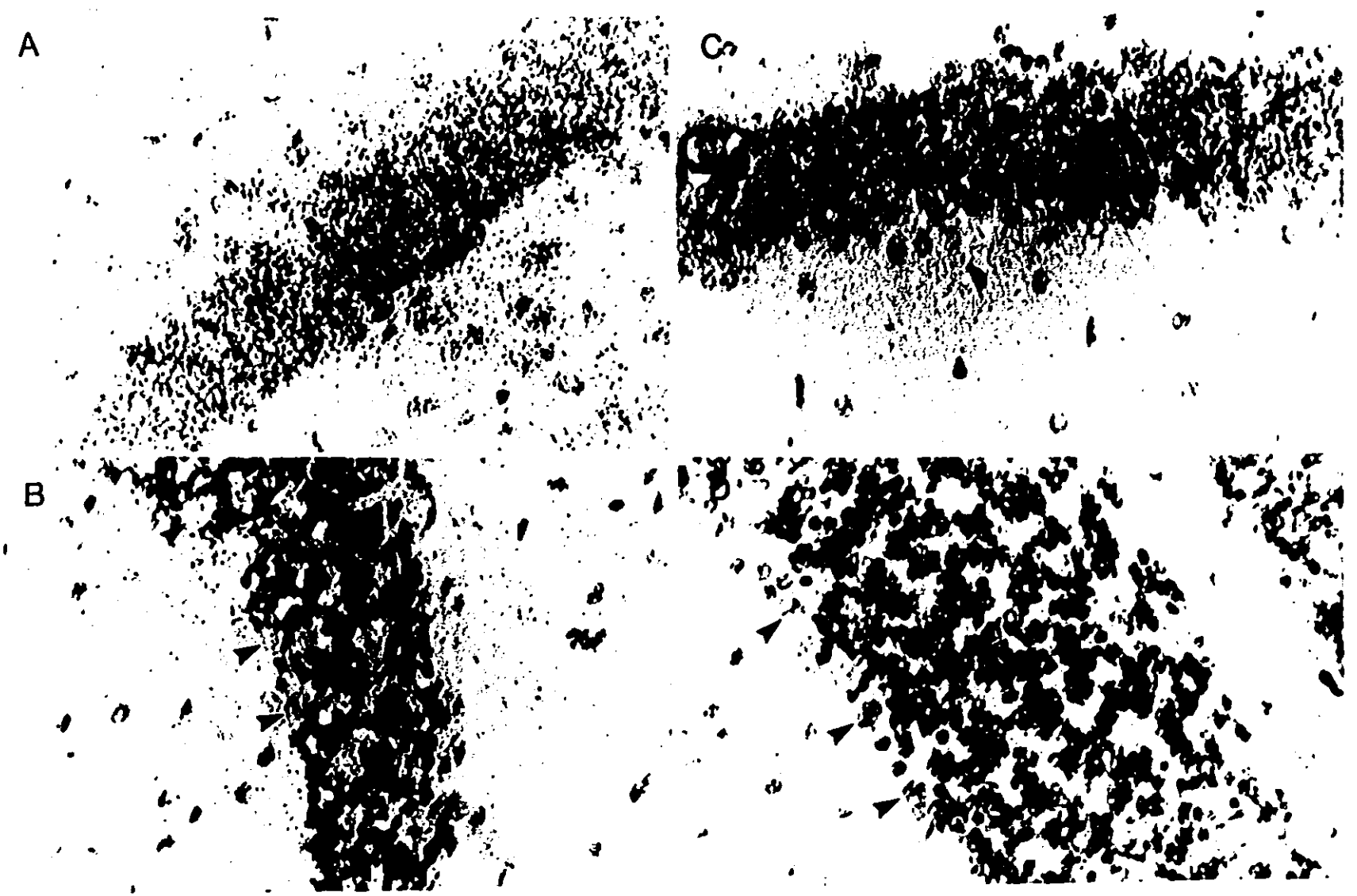

A
B
C
D

Figure 3-19. In situ hybridization studies with Clone 1.10. in mouse brain. Panels A and B show mouse hippocampus and cerebellum sections probed with anti-sense Clone 1.10 cRNA (400X). The dentate granule cells of the hippocampus (as well as other cells) are labeled whereas the cerebellar Purkinje cells are not. Panels C and D show mouse hippocampus and cerebellum control sections probed with Clone 1.10 sense probe (400X). Arrows indicate Purkinje cell bodies.


A
C
B
D

Chapter 4
Discussion

In this project, we began with a hybrid cell line (NSC34) which was selected to possess many motor neuron characteristics, both in terms of the expression of specific biochemical markers (i.e. neurofilaments and an S-laminin receptor) and in terms of the ability to exhibit specific neuronal traits such as process extension, and synapse formation with co-cultured myotubes. We then used a subtractive screening strategy to identify and clone genes expressed in this cell line but not in the parental N18TG2 neuroblastoma cell line. Using this strategy, we isolated 8 cDNAs, 6 of which have been previously described. Of the previously described cDNAs, 2 encode known neural genes (GAP-43 and chromogranin B) and 4 encode genes with no neural specificity (cytochrome oxidase subunit II, the intracisternal A particle, and 2 isolates of the mouse b1 element). We also provide evidence that an additional gene, an NMDA binding subunit of a glutamate an NMDA (GBP), which was isolated contemporaneously by another group, is not neuron specific as has been previously reported. In addition, we have isolated 1 novel cDNA which is expressed preferentially in mouse CNS and can be shown to be expressed in motor neurons by in situ hybridization.

Genotype and Phenotype in the NSC34 and N18 Cell Lines

Our cloning strategy was designed to identify differences in gene expression between the NSC34 cell line, which has a distinct motor neuron like phenotype, and its parent cell line N18, which does not. The underlying assumption of such an experiment is that the differences in phenotype between these two cell lines should be directly reflected by

differences in gene expression. While this assumption must be true for the expression of specific assayable markers, the results of our subtractive screen indicate that it might not be entirely valid with respect to the net gene expression in our hybrid cells. Of 8 cDNAs which we isolated in our screen, only 3 represent genes which we can say have neural pattern of expression and presumably relate to the motor neuron-like phenotype of the hybrid cells. These consist of GAP43 and chromogranin B, which are known to play roles in neuronal process extension and neuroendocrine secretion respectively, as well as clone 1.10, which is preferentially expressed in neural tissues and is expressed in motor neurons. The remaining 5 cDNAs encode cytochrome oxidase II, the mouse intracisternal A particle, two copies of the mouse b1 element, and GBP. None of these five cDNAs encode genes which exhibit neuronal specificity in their expression pattern or would be expected to make a contribution to the motor neuron-like phenotype of the hybrid cells. The isolation of these cDNAs in our screening experiments was unexpected. We must conclude from these results that about half of the differentially expressed genes in NSC34 (vs. N18) do not contribute directly to the motor neuron-like phenotype of the hybrid cell. Furthermore, the unexpected differentially expressed genes do not fall into any one single category (e.g. pol II, pol III transcribed genes) suggesting that several modes of gene activation are operative in the hybrid cells. Although the fusion of a dissociated spinal cord cell with a neuroblastoma does induce the expression of neuronal genes in the resulting hybrids, the net gene expression of the hybrid cells must be seen as reflecting a combination of cell type-specific (relative to the phenotype of the mortal spinal cord parent cell) and non-cell type-specific regulatory phenomenon.

Table 1. Expression of isolated cDNAs in NSC34 and control cell lines.

	NSC34	N18	N18 db-cAMP	N18 5-Aza	NSC19	NO-22	NI-1
Cln 5.21 (Cyt. Oxidase Subunit 2)	++	+	+++	++	++	+	+/-
Cln 1.92 (IAP)	++	-	++	++	++	+	++
Cln 3.72 (GAP43)	+	-	+	-	+	++	-
Cln 22 (Chrm B)	+	-	-	-	+	++	-
Cln 3.82 (GBP)	++	+	++	+/-	+	+	+
Cln 1.32 Cln 1.91 (b1)	+	-	ND	ND	ND	ND	ND
Cln1.10	ND	ND	ND	ND	ND	ND	ND

The Origins of Pol II Transcribed Gene Expression in NSC34 Hybrid Cells.

There are a number of mechanisms which might be important for the activation of gene expression in the NSC34 hybrid cells as compared to the parental N18 cells. The expression of any given gene requires that both the gene itself and its cis-acting regulatory sequences are present and intact, and that the genes for any required trans-acting factors are also properly expressed. Thus, one might propose that the N18 neuroblastoma parent cell does not express a particular gene for one of two reasons:

physical loss (or inactivation) of the gene in long term culture, or absence (or loss) of the trans-acting factors which regulate the gene. Expression of a given gene in a hybrid cell might therefore be the result of the introduction by cell fusion either of an intact functional copy of the gene itself or of a required trans-activating system.

It is easy to imagine that there are a large number of genes which were expressed in the original isolate of the N18 neuroblastoma and whose expression might have been subsequently extinguished in culture. These genes might include many "housekeeping genes" required for normal growth but not required for growth in culture (i.e. some growth factor receptors or the genes and enzymes which synthesize any agents found in tissue culture media) as well as genes required for cell type-specific functions only required in an intact animal. In the absence of selective pressure, the expression of these genes might be preferentially inactivated by deletion, gene rearrangement, or methylation. In this case, required trans-activating factors might still be expressed in the neuroblastoma parent. Expression of the gene could therefore be "restored" in the neuroblastoma cells merely by introducing an intact functional copy of the gene by cell fusion, and this restoration should be independent of the phenotype of the mortal parent cell.

Alternatively, it is possible that whole developmental programs required in an intact animal (but not in cell culture) might be rendered inoperable by the inactivation of key trans-acting factors such as transcription factors or regulatory factors involved in intracellular signaling pathways. The activation of a given gene in hybrid cells might therefore be accomplished by supplying intact, active copies of genes encoding missing transcription or signal transduction factors. In this case, only a limited subset of mortal cell types might be expected to be executing a given differentiation program and be able to convey the required combination of transcription factors and signaling systems for gene activation. Genes in this category would therefore be expected to be expressed only in hybrid cells made with parents of specific phenotypes or lineages; the expression of the genes in this category are parent cell-dependent.

This distinction between cis- and trans- activated gene expression in the neuroblastoma hybrids is therefore experimentally accessible by comparing the expression of a given gene in a panel of hybrid cells made with mortal parents of differing lineage, but sharing the same immortal parent. Genes which are not expressed in N18 due to cis-acting defects in the N18 genome (due to the direct loss or inactivation) should be re-expressed when functional copies are introduced by cell fusion with any lineage or phenotype that has not also inactivated the gene. On the other hand, genes which are intact in the N18 parent but require cell type-specific trans-acting factors for expression should have differing expression patterns in different hybrid cells, depending upon the phenotype (or lineage) of their mortal parent.

In both of these scenarios, providing a functional gene or transcription/signaling factor to a neuroblastoma cell by cell fusion might also result in the *de novo* activation of genes never expressed in the N18 cell line. In cases where, like the IAP, we find elevated expression in a range of hybrid lines having different types of partner cells, it seems likely that what we are seeing is the restoration of a non-essential housekeeping-like gene. However it is less clear in cases where the gene in question is expressed in some hybrid cell lines, but not in others. This could represent either restoration of a lost non-essential cell-type-specific function or the introduction of novel cell-type-specific gene expression by the partner cell. In the case of the induction of insulin gene expression by fusion of N18 cells with pancreatic islet cells (cell line NI-1), there is no reason to doubt that this represents novel parent-cell-specific gene expression (although we would have to assay insulin production in a range of hybrid cell to know for sure). However, things are far less clear cut in the case of neuronal or ectodermal cell specific gene expression, because the original N18 cell presumably had some definite, though unclear relationship to neural cells.

Mechanisms of Gene activation in the NSC34 Hybrid Cells

One of our cDNAs, clone 1.92 (IAP), is interesting because it demonstrates the presence of a number of different modes of gene activation in the NSC34 hybrid cells. Clone 1.92 (IAP) mRNA is more abundant in the NSC34 RNA than in N18 mRNA. However it is similarly abundant in NSC19, NO22 and NI-1 RNA. Because IAP RNA is expressed at similar levels in all N18 hybrid cell lines including those derived from neural/ectodermal (NO22) and pancreatic/endodermal (NI-1) partner cells, its elevated expression in NSC34 does not appear to reflect any specific feature of the spinal cord cells that were the diploid fusion partner. Rather, IAP expression is induced non-specifically in the N18 cells by the cell fusion/hybridization procedure.

There could be several possible mechanisms for this non-specific elevation of IAP expression in hybrids made with N18. It may be that N18 cells possess a cis-acting or genetically recessive "defect" in IAP expression (e.g. deletion, mutation or methylation of IAP genomes themselves, rendering them transcriptionally incompetent) or in a factor required for IAP expression. Although there are about 1000 copies of the IAP element per genome, the long culture history of N18 cells makes it conceivable that all of the IAP elements might have been effected. The defects in the N18 line could have arisen at any point in its long culture history because IAP expression is apparently not necessary for cell growth in culture and there would be no selective pressure to maintain IAP gene expression in N18 cells. Under these conditions, upon fusion with other cells that express IAP, the N18 contribution to the hybrid cell would not extinguish IAP expression in the partner cell and therefore the hybrid cell (e.g. NSC34) would express IAP at higher levels than in the N18 parental cell line.

Further insight into possible gene regulatory mechanisms emerges from our ability to restore IAP gene expression in N18 cells either by treatment with cAMP agonists or by 5-azacytidine (5-Aza). These inductions prove that at least one IAP genome as well as any

transcription factors specifically required for IAP expression have not been lost from N18 by outright deletion or other irreversible mutation. The inducibility by 5-Aza implicates DNA methylation as a mechanism for the loss of IAP expression. Again, this could be due to methylation of either IAP DNA itself or of any gene whose expression is required to support IAP transcription. This methylation is likely to be an event that happened during N18 cell culture history and because of the heritability of methylation, has been maintained since. It is interesting to note that other authors have identified methylation as an important regulatory factor in IAP expression in other experimental systems such as Syrian hamster BHK21 cells, primary embryo fibroblasts (Lesser et al, 1986) and F9 embryonal carcinoma cells (Kuff and Lueders, 1988). It does not appear that repression by DNA methylation accounts for a large fraction of NSC34 enriched sequences because none of the other RNAs isolated in the subtractive screening approach show induction in N18 by 5-Aza.

In addition to activation by 5-Aza, IAP expression is inducible by the cAMP agonist db-cAMP. This suggests either that some component of the apparatus required for IAP transcription is expressed in N18 cells but is under cAMP regulation. It is not obvious how cAMP agonists could overcome the apparent methylation block of IAP expression revealed by the 5-Aza induction. One possible idea is that some key gene (perhaps the IAP genome) can be transcribed from two promoters, one of which is cAMP responsive and is not methylation-inhibited (or does not tend to become methylated) and another that is not cAMP-dependent but which is methylation inhibited and does tend to become methylated. Alternatively, it is possible that elements in the cAMP synthesis/transduction pathway are affected by DNA methylation or that cAMP agonists may lead to an effect on methylation, perhaps by interfering with the maintenance methylase.

Just as the 5-Aza induction of IAP expression suggests the possibility that there may be some "defect" in N18 cells related to DNA methylation, the cAMP agonist induction suggests that there may be some defect in the cAMP second messenger system in N18

cells. That is, perhaps the basal levels of cAMP or the factors responsible for synthesizing or responding to cAMP are lower in N18 cells than they are in most cells and cAMP agonists might override these defects. While cAMP induction does not prove this idea, it suggests that there might be a large number of genes under cAMP regulation that are expressed at far lower levels in the N18 cells than in db-cAMP treated N18 cells. If this defect is not in a cell-type-specific gene, then these cAMP dependent genes might be expected to behave like the IAP in that they would be expressed at elevated levels in all hybrid cells regardless of fusion partner.

Because the cAMP second messenger system is only one of several different cell regulatory systems that effect gene expression, these findings suggest the existence of other groups of genes controlled by other signal transduction systems, whose expression would be elevated (non-specifically) in N18 hybrid cell lines. Although we have no evidence for such systems in our clones, it is interesting to note that other authors have described similar regulation of IAP by other signal transduction systems. For example, IAP expression has been shown to be elevated during the retinoic acid induced differentiation of F9 embryonal carcinoma cells into parietal endoderm-like cells (Howe and Overton, 1986), the differentiation of a B-cell lymphoma into an antibody secreting cell by the addition of lipopolysacharides (Wiest et al, 1989), as well as when pancreatic β cells are stimulated by glucose to produces insulin (Leiter et al, 1986). Thus it appears that IAP gene expression may be activated by a wide variety of signal transduction systems, any of which might also be defective in N18 cells.

It is also interesting to note that cAMP agonists stimulated expression of all RNAs cloned in our subtractive screening approach with one exception, chromogranin B (mRNAs corresponding to clone 1.10, 1.32 and 1.91 were not investigated). From this we can say that a model based on cAMP regulatory/transduction factors could account for most, but not all NSC34-enriched gene expression. However, among those genes that are cAMP stimulated, there is in many cases an element of cell-type specificity. For example

CO II RNA (presumably reflecting mitochondrial accumulation) and GAP43 mRNA are not expressed at high levels in the NI-1 hybrid line though they are expressed in the NSC19 and NO22 cell lines. This suggests that elevated expression of these mRNAs depends not only on a normal cAMP signal transduction system but also on other factors characteristic of neural/ectodermal cells and not of pancreatic/endodermal cells.

Regulation of Neuronal Gene Expression in the NSC34 Hybrid Cells

While we can say that generalized (and perhaps non-specific) gene activation does take place in the NSC34 cells, some neuron specific genes are expressed in the hybrids presumably as a result of the introduction of neuronal trans-acting factors. The most convincing example of this is the expression pattern of chromogranin B. This mRNA is expressed only in the NSC34 cells, in its sister hybrid NSC19 and in the N022 hybrid cell line. Because chromogranin B expression is not inducible in the N18 cells, we can conclude that the expression of this gene is independent of any cAMP effects observed with the IAP. In addition, the specificity of expression in cell lines based on neural/ectodermal fusion partners makes it seem unlikely that we are simply complementing a defect in some other non-cell type-specific second messenger system in N18 cells, since we should expect NI-1 should also do this. Instead, it seems probable that chromogranin B expression depends upon specific factors that are characteristic of neural/ectodermal cells and one or more of these factors are absent or inoperative in N18 cells.

As discussed above, this could be for two reasons. It could be that all factors and conditions required for chromogranin B expression were present in the initial "wild type" N18 cell line but were lost during the long culture history of the line. This would correspond to a restoration hypothesis. Alternatively, it is possible that the chromogranin

B gene never was expressed in the "wild type" N18 ancestor (or its developmental precursors) and that its expression in the hybrid lines NSC34, NSC19, and NO22 is a direct reflection of chromogranin B gene expression in the mortal parent. Our ability to detect chromogranin B transcripts in the NO22 cell line (an N18 X rat oligodendrocyte hybrid) by RNAse protection with a mouse clone suggests that the (mouse) N18 chromogranin B gene is intact and functional. Transcription activation of chromogranin B might therefore be due to the introduction of a cell type specific transcription factor by cell fusion.

Although chromogranin B expression has never been specifically examined in motor neurons, its widespread expression throughout the neuroendocrine system coupled with the fact that chromogranin A is known to be synthesized in motor neurons, and to undergo rapid axonal transport to the motor end plate (Bööj et al, 1989, Lahr et al, 1992) indicate that chromogranin B is almost certainly present in motor neurons. The expression of chromogranin B in the NSC34 cell line could therefore be as a result of the fusion of motor neuron with a neuroblastoma cell. In addition, the expression of chromogranin B in the NO22 cell line might reflect the fact that oligodendrocytes and neurons arose from a common lineage (Goldman, 1992) or the possibility that subsets of glia do express chromogranins; the Bergman glia have been reported to contain chromogranin B-like immunoreactivity (McAuliffe and Hess, 1990).

On the other hand we might have expected that we should also find chromogranin B expression in N18 cells, since these are a tumor of neuroblasts. It is possible that, as a tumor of neuroblasts, N18 cells correspond to neural precursors in which the chromogranin B gene has not yet been switched on in the neuroectodermal lineage. Alternatively, chromogranin B might be expressed in primitive neuroectodermal cells and then later developmentally switched off in certain neuronal and glial cell types including the cell type which became the precursor to N18. Finally, it is possible that chromogranin B was expressed in the wild N18 cell and its expression was lost due to

mutation during culture of the line due to inactivation of a required trans-acting factor. Thus chromogranin B expression in the NSC34, NSC19 and NO22 hybrid lines could be both a reflection of chromogranin B expression in the fusion partners cells and a restoration of chromogranin B expression that had been switched off during N18 culture or developmental history.

It is more difficult to interpret the expression pattern of clone 3.72, which encodes the neuronal gene GAP43. The expression of GAP43 in our cell lines appears to be parent cell-dependent in that it, like chromogranin B, is expressed when a neuroblastoma cell is fused with a neural cell (as in the case of NSC34, NSC19 and NO22) but not a non-neural cell, as in NI-1 cell. Unlike chromogranin, however, GAP43 expression is also induced when N18 are induced to differentiate and elaborate processes by db-cAMP.

Thus, GAP43 expression in the hybrid cell might be dependent upon a cell-type specific factor present in the parent cell, or might represent the complementation of a defect in the cAMP regulation/transduction system in the N18 cells (as discussed for IAP expression). Given this data, it is impossible to distinguish between these two possibilities. *A priori*, it might be unlikely that an important cell-type specific gene could be regulated primarily by such a non-specific system as cAMP transduction. However, other groups working on gene extinction of cell type specific genes in hepatoma hybrid cells have demonstrated that extinction of cell-type-specific genes is linked to a protein kinase A regulatory subunit (a cAMP dependent kinase; Jones et al, 1991; Boshart et al, 1991). Expression of GAP43 in the N18 might therefore be the direct result of the introduction of a neural transcription factor into the N18 cells or complementation of an N18 defect in cAMP regulation or transduction.

In any case, GAP43 expression in the NSC34 hybrid cells is not a surprising finding. GAP-43 is known to be expressed at high levels in motor neurons, particularly when they are elaborating processes (Linda et al, 1992), and GAP43 expression in NSC34 and NSC19 hybrid cells but not in NI-1 cells is consistent the idea that a neuron (perhaps a

motor neuron) was a parent of NSC34. The expression of GAP43 in the NO-22 cells was initially viewed with surprise, as GAP43 was traditionally view as a neuron specific protein. A number of recent studies have documented the expression of GAP43 in oligodendrocytes, type 2 astrocytes, and the O-2A progenitor cells which give rise to these cells (da Cunha et al, 1990, Deloume et al, 1990).

Regulation of Mitochondrial Transcripts in NSC34 Hybrid Cells

The discussion so far has focused on the regulation of polymerase II transcribed genes. It is important to note that 2 of our clones, encoding COII and the b1 element, have their own regulatory systems which are distinct from that of Pol II transcribed genes. COII, for example is transcribed from the mitochondrial genome. Although each of the three mitochondrial promoters possesses its own characteristic transcription activation efficiency, the overall level of transcription of mitochondrial RNAs does not appear to be directly regulated but rather linked to the copy number of the mitochondrial genome. In normal developmental systems in which mitochondrial RNA levels have been found to rise there is a concurrent rise in mitochondrial DNA copy number (Attardi, et al, 1989, Clayton, et al, 1991). This proportional relationship can be shown, for example, in a variety of striated muscle fiber types (Williams, 1986) as well as in rat liver whose mitochondrial gene expression and copy number rise over ten fold in the first 2 postnatal weeks (Canttore et al, 1986). The only known exception to the linkage between mitochondrial copy number and mitochondrial RNA expression is that transformed cells (i.e. with polyoma large T, adenovirus E1A, or myc oncogenes) possess elevated levels of mitochondrial RNAs, with no variation in mitochondrial copy number (Glaichenhause, 1986). We might therefore expect that the elevated levels of mitochondrial message in the NSC34 cells might genuinely reflect higher numbers of mitochondria, but may also reflect the introduction of an oncogene into a normal somatic cell by cell fusion.

One source of the high levels of mitochondria in the NSC34 cells might be their direct introduction during cell fusion, by fusion of a neuroblastoma cell with a cell (or cells) rich in mitochondria. The oxidative capacity of motor neurons (the presumed parent cell) has been studied by means of histochemical stains which identify the biochemical activities of succinate dehydrogenase (a citric acid cycle enzyme) and NADH diaphorase (NADH-D; an enzyme in the oxidative transport chain). From these studies, it seems apparent that motor neurons possess a greater oxidative capacity than their surrounding glial cells, although there is considerable debate as to the relative importance of neuronal size on the oxidative capacity of motor neurons (Robinson, 1969; Campa and Engel, 1970; Campa and Engel, 1971; Ishihara et al, 1990; Ishihara et al, 1991; Sickles and Oblak, 1984; Miyata and Kawai, 1992; Hirofumi et al, 1992). In addition interpretation of these studies is made more difficult because the authors do not relate these mitochondrial levels to other cell types and because by studying cell soma staining, they systematically neglect the large number of mitochondria in dendrites (Burke, 1981) and the terminal axon (Alnaes et al, 1975). Given the data, however, it seems reasonable to conclude that motor neurons possess large numbers of mitochondria and that this could be the source of elevated mitochondrial gene expression in the NSC34 hybrid cells.

Elevated levels of mitochondrial transcripts in the NSC34 cell line might also reflect the normal metabolic regulation in the parent cells. Unlike some sensory systems (such as the avian auditory neurons of the nucleus magnocellularis), motor neurons do not appear to modify their oxidative capacity in response to chronic over- or under-use. Chronic axotomy of the motor nerve root, however, results in a 30% drop in succinate dehydrogenase activity in the motor neuron cell soma. It therefore seems reasonable to conclude that for motor neurons, axonal maintenance consumes a large portion of oxidative/metabolic requirements (Chalmers et al, 1992). From this, we might expect that the act of extending or maintaining neuronal processes by NSC34 cells would be sufficient to result in elevated expression of mitochondrial transcripts.

Thus, although cytochrome oxidase is a housekeeping gene, which we would not *a priori* expect to demonstrate differential expression between hybrid cells, differences could have arisen because of the mitochondrial content of the diploid (mortal) parent(s), the introduction of an oncogene into the diploid parent by cell fusion, or be the result of normal energy metabolism regulation of the diploid parent.

The Implications of Pol III Transcripts in NSC34 cells

Two of our cDNAs encode RNAs transcribed by RNA polymerase III from a highly repetitive fraction of the mouse genome. These short transcripts of the mouse b1 element belong to a group of elements referred to collectively as short interspersed repetitive elements (SINEs). Although SINE transcription is known to be increased by transformation or by transition from quiescent to proliferative states, the mechanism of this regulation is unknown. While the significance of SINEs and SINE transcription is unknown, many hypothesis have been proposed, including a role in the regulation of tissue specific gene expression. For example SINE sequences may be found in the in 5' or 3' untranslated regions of many Pol II transcripts and many authors have reported the isolation of groups of genes that exhibit developmental regulation and contain copies of the various SINEs. This SINE mediated regulation has been suggested in L6 myotubes (Herget et al, 1986), differentiating mouse embryos, embryonal carcinoma cells (Murphy et al, 1983; Vasseur et al, 1985) and brain (McKinnon et al, 1988). While these observations are interesting, it is difficult to judge the statistical significance of the small numbers of gene reported.

Many other biological roles have been proposed for SINE elements. For example, there is evidence that transcribed SINE elements play a role in regulating cell growth (Sakamoto et al, 1991) or in RNA transport into neuronal dendrites (Tiedge et al, 1991). In addition, pol III transcription of various SINE elements has been demonstrated to

regulate the activity of nearby Pol II promoters. Specifically, SINE elements have been demonstrated to behave as enhancers of the SV40 promoter in hepatoma cell lines (Oliviero and Manaci, 1988), and in PC12 cells (in an orientation and position specific manner; McKinnon, et al, 1986), and to override the existence of known transcriptional suppressor in the ε-globin gene in HeLa cells (Wu et al, 1990). SINE transcripts may also play a role in post transcriptional regulation, by hybridizing to SINE containing pol II transcripts and increasing their stability (Glaichenhaus and Cuzin, 1987; Clemens et al, 1987). Given all of these observation, it is possible that SINE elements might an play an important role in the regulation of large scale coordinate gene expression. Much more study will be needed to determine the role and importance of SINE elements.

Gene Expression in N18 Hybrids: Considerations for Subtractive Screening

It is interesting to note the great similarity of gene expression between NSC34, NSC19 and NO22. In fact, all of our NSC34-enriched and specific mRNAs are also expressed in NO22. Given the fact that NSC34 cells and NO-22 cells possess distinct phenotypes, both with respect to morphology and the expression of specific markers, these are surprising findings. One possibility which might explain this similarity of gene expression is that all neurocctodermal cells, when fused with an N18 neuroblastoma cell, may form the same basic cell type, regardless of pre-existing phenotypic specializations. This could happen if, for instance, the transformed aspect of N18 cells tended to drive highly differentiated fusion partner cells into a developmentally more primitive, less differentiated condition, perhaps resembling a general purpose neuroectodermal precursor cell. We know from a review of the literature that even fusion of fibroblasts with neuroblastoma cells (NL cells) results in the production of cells with some neuronal traits. Thus it seems that all neuroblastoma hybrid cells might possess a similar pattern of net

gene expression. As with previously described neuroblastoma hybrids, we know that it is possible to generate hybrids with specific traits by carefully choosing fusion parent cells and rigorous selection. Therefore it might seem reasonable to conclude that neuroblastoma hybrids may express different genes, but these unique genes are in the minority; the vast majority of genes might be similarly expressed in all neuroblastoma hybrids.

These conclusions have serious implications for our subtractive screen. We know from our own experiments that on the one hand, fusion of diploid parent cells with neuroblastoma cells results in the activation of a large number of genes, many of which have little to do with the phenotype of the hybrid cell. On the other hand, we know that there are very few differences between the gene expression of these hybrid cells, especially if neural precursor cells are used in the cell fusion. The fact that the NSC34 cells express an S-laminin receptor (which is known to be expressed on motor neurons) and the NO22 cells express myelin basic protein and galactocerebroside, indicate that there are at least some differences between these two cell types. The solution to the problem of identifying genes in hybrid cells, might therefore be to subtract one hybrid cell only from another hybrid cell and not from the N18 parent.

In other words, instead of using N18 RNA to make a subtracted NSC34-specific probe, perhaps we should have used NO22 instead. This strategy, of course, would depend upon the numbers of neuron-specific genes expressed in NO22. If NO22 cells are found to express many neuronal markers non-specifically, then using them in the subtraction might yield no results. If there are a subset or motor neuron-specific transcripts expressed in NSC34 but not expressed in NO-22, however, then subtraction with NO-22 mRNA (as opposed to subtraction with N18 mRNA) might be a much more efficient means of cloning those transcripts, because it would eliminate neural parental-dependent genes from our screen. The NO-22 cell line must be further characterized for its expression of neuronal and glial markers before such a strategy is recommendable.

Another important conclusion of our screen is that we probably did not examine enough of our library. Because all of the clones which we isolated are expressed in both NSC34 and NO22, this implies that if high abundance genes are responsible for phenotypic differences between NSC34 and NO-22 cells, then we have failed to clone them. Furthermore, our sample size is much too small to conclude that there are no high abundance NSC34-specific genes. In our screen, we isolated two copies of the b1 element. If there were only 10 high abundance NSC34-specific genes, we might expect to get duplicates of others as well. Although it is impossible to say with certainty the odds of picking 8 clones, and having only 1 duplicate (the sample size is two small for statistics), it must be concluded that there are probably more than 10 high abundance NSC34-specific genes and perhaps less than 100. From this reasoning, we must conclude that any library screening experiment must be much larger (perhaps by a factor of 10) in order to successfully identify NSC34-specific cDNAs.

Chapter 5

Conclusions

Summary

In this thesis, we describe a strategy for the isolation of cell-type specific cDNAs from very rare cell types. This strategy is based upon generating immortalized hybrid cells bearing a phenotype of interest. Such hybrid cells would be generated by fusing dissected and purified target cells with an immortal cell line and vigorously selecting for immortalized lines which bear the desired characteristics. It is then possible to grow large numbers of these immortalized cells in tissue culture for study using standard biochemical or molecular biological techniques. Because the gene expression of a hybrid cell line might be expected to reflect the gene expression of both parents, by screening a cDNA library of the hybrid cells with a screening technique designed to subtract out the gene expression of the immortal parent, it should be possible to isolate genes which are specific to the target cell of interest.

We applied this strategy towards isolating cDNAs representing mRNAs which are expressed principally in motor neurons. To do this, hybrid cells were generated by fusion of motor neuron-enriched mouse E14 dissociated spinal cord cells with the mouse N18 neuroblastoma (which has been previously demonstrated as an adequate fusion partner for the immortalization of cells with neuronal characteristics). The resulting hybrid cells were then screened extensively for traits expected in an immortalized motor neuron cell line. One of the resulting hybrid cell lines, NSC34, possesses many motor neuron-like traits which the N18 neuroblastoma parent lacks, including the ability to support action potentials, to synthesize and package acetylcholine, and to secrete acetylcholine in an excitation dependent manner. In addition, NSC34 cells are capable of making stable contact with myotubes in co-culture experiments, aggregating myotube acetylcholine

receptors, and to making the myotubes twitch. NSC34 cells also express the markers neuron specific enolase, choline acetyltransferase, NCAM, and neurofilaments, and is the only cell line known to posses an S-laminin receptor (which is expressed in primary motor neurons). The expression of this constellation of neuronal traits, in addition to an S-laminin receptor, make the NSC34 cell line a good candidate cell line for expression of motor neuron-specific genes.

A cDNA library of the NSC34 cell line was constructed and examined by differential and subtracted screening strategies designed to identify genes expressed in the hybrid cells but not in the parent neuroblastoma cell line. In all, 8 cDNAs were isolated which can be divided into 5 classes based upon sequence:

1) Transcripts of encoded by the mitochondrial genome (1)
2) Transcripts encoding small RNAs, presumably transcribed by RNA polymerase III (the mouse b1 element) (2)
3) Previously described cDNAs encoding genes with widespread tissue distribution, corresponding to the mouse IAP element (1)
4) Previously described cDNAs representing RNAs with neural distribution (2) corresponding to Chromogranin B, and GAP-43
5) cDNAs representing novel sequences (2) - Clone 3.82 (GBP) and Clone 1.10.

To examine the parental contribution to the expression of these cDNAs in the NSC34 hybrid cell line, we compared the expression of these cDNAs in NSC34 to a battery of control cell lines using northern blotting, ribonuclease protection assays, and semi-quantitative PCR. These cell lines consisted of N18TG2 (the parental cell line), db-cAMP treated N18TG2 (which extends small processes), N18TG2 treated with 5-azacytidine (which de-methylates genomic DNA), NSC19 (a sister hybrid of NSC34, which came from the same fusion experiments and also expresses choline acetyltransferase, extends processes, and supports action potentials), NO22 (an N18 X rat

oligodendrocyte hybrid which expresses galactocerebroside and myelin basic protein) and NI-1 (an N18 X rat pancreatic islet cell hybrid which secretes insulin).

From the expression pattern of our isolates in these control cell lines, we can conclude that although neural gene expression is indeed activated in the NSC34 cell line, as evidenced by the expression of GAP-43 and chromogranin B, the majority of the previously described differentially expressed transcripts do not make obvious contribution to the motor neuron phenotype of the hybrid cells. Future screens employing a similar strategy would therefore have to be much larger (to examine larger numbers of phage) and/or be modified to increase the likelihood of cloning neuronal genes.

Finally we examined the expression of GBP and clone 1.10 in normal mouse tissues. In contrast to published reports, we found that the glutamate binding protein was widely expressed throughout mouse and rat tissues, with highest levels of expression in kidney. Using semi-quantitative PCR, we found that Clone 1.10 was expressed principally in brain and spinal cord. Finally, we turned to in situ hybridization to examine the cellular distribution of clone 1.10 in mouse brain and spinal cord. Although, we found Clone 1.10 expression throughout the nervous system, we found high levels of expression in motor neurons. The isolation of a cDNA for a gene expressed in motor neurons makes this project a qualified success in that we have isolated a cDNA which might be a useful marker in the study of motor neuron biology.

Strategy Considerations for Future Screens

Can this strategy be altered to improve that chances of success in isolating a true motor neuron specific gene? There are two basic concerns with the strategy that need to be addressed in any recommendations for future investigation. The first set of considerations relate to the patterns of gene expression in hybrid cells, and these limitations may be the most serious because little can be done to alter them. The second

set of recommendations pertain to technical points in subtractive screening, which can be expected to lessen over time with the improvement of molecular biological techniques.

In principle, the genes expressed in hybrid cells may represent gene activation as a result of the interactions of the specific parent cells involved in the hybrid cross, or as a result of less (or non-) specific interactions such as chromosomal loss or the introduction of unmethylated DNA into the immortal parent. If hybrid cells are to be used effectively as models of differentiated cells in subtractive screening experiments, the numbers of diploid parent-dependent genes expressed in a given hybrid cell would seem to be an important determinant of success.

Of the isolates which we were previously characterized, only 2 out of 6 cDNAs from our library screen encode genes which clearly make a contribution to the motor neuron phenotype of the hybrid cells, and even these are expressed in the NO-22 hybrid cells. This has important implications for the application of our hybrid cell based subtractive cloning strategy to other systems. To start with, these results suggest that the expression of many genes are elevated in hybrid cells, even though they may have no apparent relationship to the differentiated phenotype of the hybrid cell line. The net result is that larger numbers of library phage must be screened in order to isolate true parental cell-dependent cDNAs; you only get 2 neural cDNAs of interest for every 6 that are isolated.

Furthermore, a review of the literature indicates that in a number of successful subtractive screening experiments, such as the isolation of MyoD (Davis et al, 1987), the T-cell receptor (Hendrick et al, 1984) or A^d_α gene of the murine class II major immunohistocompatibility complex (Davis et al, 1984), the target gene was one of only a few major transcripts differentially expressed between the two cell lines of interest. In cases in which a large number of genes are differentially expressed, subtractive screening has proven to be much more difficult. For example, a subtractive screen of scrapie infected hamster brains (suffering from a spongiform encephalopathy) isolated cDNAs encoding metallothioneine II, the B chain of a-crystallin, and GFAP, all of which are

believed to be elevated as a result of stress response and scar formation rather than the disease process (Durguid et al, 1988). In addition, a group studying differentiation of promyelomonocytic cell line cloned 8 promyelocyte-specific cDNAs, all of which turned out to encode the mouse IAP (Takayama et al, 1981). Ideally, cells or cell lines being used in a subtractive screen should have only one high abundance gene of interest differentially expressed. The large number of non-neural genes which are differentially expressed between N18 and NSC34 probably made the interpretation of our subtractive screen much more difficult.

A possible solution to these problems might by to screen hybrid cell libraries with probes that are subtracted with other related hybrid cells. For example, the use of mRNA from db-cAMP treated N18 cells in the subtraction would eliminate process- (or N18 differentiation)- specific genes as well as the IAP element. In addition, subtracting the NSC34 probe with mRNA derived from NO-22, would eliminate all of the previously characterized cDNAs from our screen, and presumably leave only parental cell-dependent cDNAs which are also not present in closely related hybrid cells. This would seem to be a better library screening strategy, but it assumes that the chosen control hybrid cell for the subtraction does not also express genes which are the target of the cloning experiment. In our case, it is not immediately clear that the NO-22 cell line does not express the whole complement of neuronal genes (in addition to oligodendrocyte genes) which would result in a failure of a subtractive screen. This would indicate that either the phenotypic differences observed between these two cells might be generated by low abundance cell type-specific genes or (possibly minor) quantitative differences in a common set of genes shared between the two cell lines. Before embarking on a subtractive screening strategy using NO-22, it would be necessary to assay this cell line for as many neuronal markers as possible, to convince ourselves that the gene expression patterns of NSC34 and NO-22 are indeed different.

Technical Considerations for Future Screens.

It is also possible that many more library phage contain NSC34-specific cDNAs and they were not identified because of technical imperfections in our screening methodology. A number of possibilities exist for improving the success of our library screen by reducing the number of background positive clones. For example, the inclusion of mitochondrial DNA and sheared chromosomal DNA would eliminate the mitochondrially encoded genes and the cDNAs representing highly repeat sequences. In addition, phenol emulsion-driven hybridization might be used to further drive the hybridization reaction in order to isolate lower abundance transcripts using smaller amounts of starting materials (Travis and Sutcliffe, 1988).

Other strategies for improving our library screen would require the remaking of our library. The application of newer library synthesis techniques which utilize "directed cloning" into vectors with phage origins of replication (which can therefore be propagated as single stranded phage) would be particularly beneficial. Using these techniques, libraries would be made of both N18 and NSC34 cell lines. Cell type specific probes can then be made directly from the libraries and probe made from one library can be subtracted using the other library. In addition, it is possible to produce subtracted libraries from the whole cell libraries (Rubenstein et al, 1990). These library systems offer a number of advantages over the techniques we used. The most important advantage is that because the probes are being made from the library itself, the probe is guaranteed to match the library in sequence complexity. Because our probes were made from RNAs extracted from cultured cells, it is possible that as the cells "evolved" in culture, their gene expression was modified such that genes of interest present in the library might not be reflected in the probe. A second advantage to these techniques is that they require far less cellular RNA; once the initial libraries are made, no additional RNA

is needed. The library phage themselves can be grown to large numbers using relatively easy and inexpensive bacteriological techniques and reagents. The subtractive screening strategy we used required large amounts of tissue culture cells to obtain sufficient RNA both to make and subtract probes. These tissue culture techniques are more difficult and much more costly in terms of both time and money. Finally, using PCR based techniques, it might be possible to make a library from very small amount of starting material such as a petri dish of purified motor neurons, making it possible to study real motor neurons instead of model cells (Timblin et al, 1990). Needless to say, at the time this project was initiated, none of these vectors or technologies where easily available.

Conclusion

In conclusion, we have described a strategy based on subtractive screening of hybrid cell line cDNA libraries to isolate genes expressed in or specific to rare cell types. This technique may offer the ability to isolate genes from very rare cell types which can not be cultured, or which cannot be effectively purified. Using a cDNA library of the motor neuron-like hybrid cell line NSC34, we performed subtractive screening experiments designed to isolate cDNAs encoding genes expressed in the hybrid cells but not in the N18 neuroblastoma parent. We examined 160,000 recombinant phage and isolated 8 cDNA clones, one of which is preferentially expressed in neural tissues as well as in motor neurons in the spinal cord, but is also expressed by many other neuronal types throughout the CNS. Of the 8 cDNAs isolated, 5 encode genes which are unlikely to contribute to the phenotype of interest (i.e. that of motor neurons). In addition, several of these cDNAs demonstrate no parental cell specificity at all, suggesting that their expression in the NSC34 cell line is a non-specific result of cell fusion. We conclude that it is possible to isolate novel cDNAs which are expressed in the diploid parent using the strategy described here. In addition, we believe that this strategy would be more effective

if a related hybrid cell rather than the neuroblastoma parent was used in the probe subtraction. We also believe that additional clones of interest would have been isolated had the size of the screen been increased by an order of magnitude.

Chapter 6

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