GSBS Dissertations and Theses   Graduate School of Biomedical Sciences

1988-08-01

MYC and E1A Oncogenes Alter the Response of PC12 Cells to Nerve Growth Factor and Block Differentiation: A Thesis

Susan C. Schiavi
University of Massachusetts Medical School

Let us know how access to this document benefits you.

Repository Citation

MYC AND E1A ONCOGENES ALTER THE RESPONSE OF PC12 CELLS TO NERVE GROWTH FACTOR AND BLOCK DIFFERENTIATION

A Thesis Presented

By

Susan C. Schiavi

Submitted to the faculty of the
University of Massachusetts Medical School in partial
fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

Biochemistry

AUGUST   1988
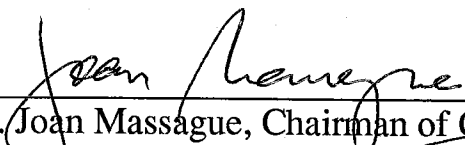
MYC AND E1A ONCOGENES ALTER THE RESPONSE OF PC12 CELLS TO NERVE GROWTH FACTOR AND BLOCK DIFFERENTIATION

A Thesis

By

Susan C. Schiavi

Approved as to style and content by:

Dr. Joan Massague, Chairman of Committee

Dr. Anthony Carruthers, Member

Dr. Frank Chlapowski, Member

Dr. Daniel Kilpatrick, Member

Dr. Paul Dobner, Member

Dr. Thomas B. Miller, Jr.,
Dean of Graduate Studies

Dr. Gary L. Johnson,
Thesis Advisor

Department of Biochemistry
August   1988

ABSTRACT

PC12 rat pheochromocytoma cells respond to nerve growth factor (NGF) by neuronal differentiation and partial growth arrest. Mouse c-myc and adenovirus E1A genes were introduced into PC12 cells to study the influence of t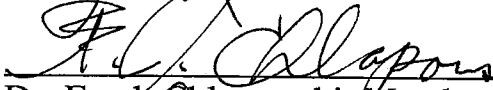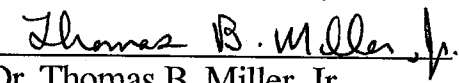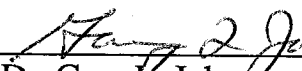hese nuclear oncogenes on neuronal differentiation. Expression of myc and E1A blocked morphological differentiation and caused NGF to stimulate rather than inhibit cell proliferation. NGF binding to cell surface receptors, activation of ribosomal S6 kinase, and ornithine decarboxylase induction were similar in myc and E1A expressing clones compared with wild-type PC12 cells, suggesting that changes in the cellular response to NGF were at a post-receptor level. The ability of myc and E1A expression to block the transcription-dependent induction of microtubule associated proteins by NGF further suggested that these genes may inhibit differentiation by interfering with NGF's ability to regulate transcription. These results illustrate that NGF can promote either growth or differentiation of PC12 cells, and that myc or E1A alter the phenotypic responses to growth factors

ACKNOWLEDGEMENTS

I would like to express my appreciation for the support given by my thesis advisor, Dr. Gary L. Johnson, as well as other members of his laboratory. I would like to specifically extend my warm appreciation to Dr. Ellen Weiss and Dr. Sela Chefeitz for their encouragement, scientific discussions and most importantly, for their friendships. I also thank Denise Basset for her extreme patience, kindness, and flawless skills in typing this thesis. In addition, I wish to thank my father for showing me how to dream and my mother for teaching me to work hard to achieve my goals. Finally, I wish to extend my most special thanks to my husband, David St. Laurent, for his continued encouragement, support, love, and belief in my abilities.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABREVIATIONS

NGF   Nerve Growth Factor

EGF   Epidermal Growth Factor

CT   Cholera Toxin

DME   Dulbecco's Modified Eagle's Medium

CHAPTER I

INTRODUCTION

HISTORICAL PERSPECTIVE

Since its discovery in the early 1950's, nerve growth factor (NGF) has become the prototype of neurotrophic factors (reviewed: Thoenen and Barde, 1980; Yanker and Shooter, 1982; Thoenen and Edgar, 1985; Levi-Montalcini, 1987). The identification of NGF was a consequence of early experiments performed by Bueker (1948) who demonstrated the invasion of sensory neurons from the dorsal root ganglia of a chick embryo into a mouse sarcoma transplanted into the body wall of the embryo adjacent to the ganglia. Levi-Montalcini and Hamburger extended this work to show that sympathetic ganglia also participated in the nerve ingrowth to the tumor and most significantly, that ganglia not in direct connection with the tumor also underwent extreme hyperplasia (Levi-Montalcini and Hamburger, 1951 and 1953). These observations led to the suggestion that the sarcoma tissue released a diffusible factor into the circulation which had nerve growth promoting effects. Direct evidence supporting this hypothesis came from in vitro tissue culture experiments in which co-cultivation of sensory or sympathetic ganglia with sarcoma tissue resulted in marked nerve fiber outgrowth from the ganglia explants (Levi-Montalcini et al., 1954).

Stanley Cohen was instrumental in the continued progress of NGF research. Through the identification of potent sources of NGF, Cohen was able to partially purify NGF and subsequently produce NGF antibodies (Cohen, 1960). The resulting destruction of the peripheral sympathetic

system after injection of these NGF antibodies into neonatal mice provided strong evidence for a physiological role of NGF (Cohen, 1960). These pioneering studies laid the foundation for further research which demonstrated participation of NGF in at least three processes of the developing nervous system: cell survival, axonal orientation, and neuronal differentiation.

One of the most dramatic processes in neural morphogenesis is the regression of neurons by cell death. Transplantation and ablation experiments indicate that survival of developing neurons is determined by innervation of their target tissues. It has been suggested that release of neurotropic factors from these target tissues determines neuronal survival. In support of this proposal, NGF has been shown to be required for the survival of developing sympathetic and sensory neurons both in vivo and in vitro (Thoenen and Barde, 1980; Yankner and Shooter, 1982).

The accurate formation of synaptic connections requires that growing axons be oriented in the proper direction. A chemotactic effect of NGF has been demonstrated both in vivo and in vitro by the rapid reorientation of the axonal growth cone to a gradient of NGF (Levi-Montalcini and Hamburger, 1951 and 1953). Release of NGF from specific tissues may be directly responsible for the growth of neurons to that tissue.

Finally, NGF appears to be directly involved in the neuronal differentiation of several neural crest-derived tissues including sympathetic and certain sensory neurons, as well as cells of the adrenal medulla (Thoenen and Barde, 1980; Yankner and Shooter, 1982). NGF's capacity to modulate phenotypic expression has been most thoroughly characterized in the

pheochromocytoma cell line, PC12 (Greene and Tischler, 1976; Tischler and Greene, 1978). Unlike primary neuronal cultures, PC12 cells do not require NGF when grown in serum containing medium. Under these conditions they lack neurites, replicate, and possess the phenotypic properties of their non-neoplastic counterpart, adrenal chromaffin cells. These properties include the presence of chromaffin granules and the capacity to synthesize, store and release catecholamines (Greene and Rein, 1977). Upon exposure to NGF, PC12 cells partially growth arrest and acquire several properties of sympathetic neurons, including the outgrowth of long branching neurites, the appearance of evocable sodium action potentials, the presence of synaptic-like vesicles; and the enhanced synthesis of several neuronal proteins.

Because PC12 cells can be grown in the absence of NGF but undergo a well characterized differentiation program upon its exposure to NGF, these cells provide an excellent model system for studying the initial responses to NGF. Thus, PC12 cells are valuable for studying early signalling mechanisms activated by the binding of NGF to its receptor and for identifying rapid events committing the cell to differentiation. In addition, since these cells replicate they can be used to genetically dissect NGF's mechanisms either by somatic mutation or by introducing a specific gene into the cells through transfection.

BIOLOGICAL ACTIONS OF NGF IN PC12 CELLS

An outline of the temporal events that are known to occur during the differentiation program of PC12 is given in Table 1. The initial step in the differentiation of PC12 cells is the binding of NGF to specific cell surface

Table 1: Summary of biological events known to occur in PC12 cells in response to NGF

TIME	BIOCHEMICAL EVENT	REF
0	Binding of NGF to its receptor Conversion from a low affinity monomer to a high affinity dimer	Buxser et al., 1985a
5-15 m	Plasma membrane ruffling Increased activity Na+/K+ and Na+/H+ pumps	Schubert et al., 1978 Varon and Skaper, 1983
	Increase in cytosolic Ca2+ Increased metabolic transport	Pandiella-Alonso et al., 1986 Greene and Tischler, 1982
	Stimulation phosphoinositol turnover	Traynor et al., 1983
	Activation Protein Kinase C Activation S6 protein kinase Phosphorylation of specific proteins	Hama et al., 1986 Halegoua and Patirck, 1980
15m-2h	Induction of transcription: c-fos, c-myc, actin and ODC	Greenberg et al., 1985
24 h	2-fold increase mRNA, tRNA, rRNA Increase in somatic size Increase in cell adhesion	Gunning et al., 1981 Greene and Tischler, 1982 Schubert and Whitlock, 1977
	Down-regulation of EGF receptor Short neurite processes	Lazarovica et al., 1987 Greene and Tischler, 1976
3d	Induction of neurotransmitters & related enzymes	Greene and Tischler, 1982
	Induction of HMW MAPs, tau proteins	Drubin et al., 1986
	Increase in microtubule polymers Stable neurites	Drubin et al., 1986 Greene and Tischler, 1976
7d	Induction of tubulin Long branching neurites Cessation of DNA synthesis	Drubin et al., 1986 Greene and Tischler, 1976 Greene and Tischler, 1976

receptors on the plasma membrane (reviewed, Buxser et. al , 1985a). Kinetic and equilibrium binding studies have revealed two apparent affinity states of the receptor (Yanker and Shooter, 1979; Landreth and Shooter, 1980; Bothwell and Schecter, 1981; Buxser et al., 1983). The high affinity NGF-receptor complex is trypsin resistant and has an apparent K_d of 0.3 nM. The second NGF-receptor state has a lower affinity for ^{125}I-NGF (K_d 5.2 nM) and is trypsin sensitive. Furthermore, photoaffinity crosslinking experiments with ^{125}I-NGF (Buxser et al., 1983), and analysis of highly purified NGF-receptor by SDS-PAGE electrophoresis (Puma et al., 1983) reveal two proteins with molecular weights of 80 and 200 kDa. Partial proteolytic peptide mapping indicates that the 200 and 80 kDa NGF binding proteins are indistinguishable (Puma et al., 1983).

A model to explain the molecular interactions between NGF and its receptor based on the structure and binding properties of the NGF receptor and the dimeric structure of β-NGF, has been recently proposed (Buxser et al., 1985b). β-NGF is composed of two identical 13 kDa peptides which are non-covalently associated to form a 26 kDa molecule (Angelletti and Bradshaw, 1971; Greene et al., 1971). The dimeric structure appears to be important functionally, since attempts to produce biologically active monomeric β-NGF have not been successful. The dimeric structure of β-NGF may allow each 13 kDa monomer of the molecule to bind one 80 kDa receptor. The hypothesis further proposes that low affinity rapidly dissociating binding results when one of the subunits of β-NGF associates with a single 80 kDa receptor molecule. Binding of the second subunit of β-NGF to a separate receptor molecule produces a dimeric receptor structure

bridged by the dimeric β-NGF (200 kDa complex). Since dissociation of NGF from two receptors simultaneously is significantly less probable than dissociation from a single receptor, the resulting 200 kDa complex has a slower dissociation rate and a higher apparent affinity. Consistent with this model, Green and co-workers (1986), have isolated chemically mutagenized PC12 cells whose failure to respond to NGF is correlated with the absence of high affinity binding sites. These results suggest that the high affinity NGF receptor mediates the biological actions of NGF.

In spite of the rapid progress in defining the kinetic and structural features of the NGF receptor, it is still not known how the receptor "signals" the cell to undergo neuronal differentiation. Recent cloning of the receptor (Johnson et al., 1986) has provided no further clues to a possible signalling mechanism. While the NGF receptor shares general structural features with other growth factor receptors, there is no homology with growth factor receptors containing intrinsic tyrosine kinase activity or with other known tyrosine or serine/threonine specific protein kinases. This is consistent with the failure of several laboratories to demonstrate an intrinsic tyrosine kinase activity of the purified NGF receptor (Boonstra et al., 1985). In addition, the predicted amino acid sequence does not contain structural features common to receptors which serve as ion channels. Finally, the receptor lacks amino acid homology with proteins which are presently known to interact with GTP-binding proteins (G-proteins), as well as the conserved amino acid residues involved in GTP binding to G-proteins. This indicates that the receptor is not a G protein but does not exclude the possibility that the receptor interacts with an unknown G-protein.

Recent experiments predict that the NGF signalling pathway may indeed include the interaction of the receptor with a GTP-binding protein. Microinjection of the v-ras protein (p21) into PC12 cells (Bar-Sagi and Feramisco, 1985) or infection of these cells with ras-containing retrovirus (Noda et al., 1985) leads to apparent growth arrest and morphological differentiation. Moreover, microinjection of the monoclonal antibodies to Ha v-ras p21 inhibits NGF-induced neurite outgrowth (Hagag et al., 1986). While the simplest interpretation of these results is that the ras proto-oncogene mediates NGF's actions, one must be cautious about such a conclusion. First, microinjection of c-ras into PC12 does not produce the same effects on cellular differentiation as does injection of v-ras (Bar-Sagi and Feramisco, 1985). It is perhaps not surprising that the expression of exogenous v-ras produces different effects in PC12 cells since v-ras p21 may escape the normal regulatory controls that c-ras p21 is subjected to. Second, the ability of the anti-ras p21 antibody to inhibit NGF function in PC12 cells may be explained by cross-reactivity of this antibody in vivo with a yet unidentified G-protein. The antibody used in this study does in fact bind weakly to G_s and G_i proteins (Beckner et al., 1985). Third, there may be multiple pathways for induction of neuronal differentiation in PC12. Clearly many agents are capable of inducing cell proliferation within a given cell type, therefore it is not unreasonable to speculate that a variety of agents may also induce a specific differentiation state. This is illustrated by the fact that fibroblast growth factors (FGFs) mimic many of NGF's effects in PC12 cells (Rydel and Greene, 1987; Togari et al., 1983) even though FGFs apparently utilize a different receptor system (Neufeld and Gaspadarowica, 1986).

Furthermore, infection of PC12 cells with Rous sarcoma virus containing the v-src oncogene also induces morphological alterations associated with neuronal differentiation (Alemo et al., 1985). Thus, while these studies support a possible role of G-proteins in the NGF-induced pathway of neuronal differentiation they do not provide conclusive evidence that c-ras is the G-protein involved.

It has also been postulated that cAMP mediates NGF's effects in PC12 cells. This hypothesis is based on the observation that cAMP and NGF elicit similar protein phosphorylation patterns (Halegoua and Patrick, 1980; Cremins et al., 1986) and on the ability of cAMP to mimic NGF's induction of neurites (Schubert et al., 1977; Schubert et al., 1978). However, cAMP-induced neurites are clearly distinct from those induced by NGF. The transient neurites produced in response to cAMP are short and unbranched. In contrast, NGF produces long, branched neurites which are maintained in the continued presence of NGF. Furthermore, several lines of evidence indicate that NGF and cAMP elicit their effects on neurite outgrowth by dissimilar mechanisms. First, RNA synthesis inhibitors block NGF's induction of neurites but do not interfere with the ability of cAMP to direct neurite extension (Burstein and Greene, 1978; Greene et al., 1982). Second, NGF and cAMP act synergistically to produce neurites that are longer and more branched than those produced by either agent alone (Gunning et al., 1981; Heidemann et al., 1985). In addition, NGF does not appear to require cAMP as a second messenger since cAMP antagonists do not block NGF-induced morphological differentiation (Richter-Landsberg and Jastroff, 1986). Finally, NGF does not activate adenylate cyclase (Race and Wagner,

1985) or cAMP-dependent protein kinase (L. Heasley, unpublished observations) indicating that NGF's actions are not mediated through the classic adenylate cyclase system.

Internalization of NGF and retrograde transport of the molecule along the axons to the cell bodies of differentiated neurons is well documented (Levi et al., 1980; Stockel et al.,1974; Stockel and Thoenen, 1975). While several lines of evidence suggest that these mechanisms may be involved in some of NGF's actions, NGF does not act as its own second messenger to promote neuronal differentiation. This is illustrated by the failure of microinjected NGF to promote neurite outgrowth as well as by the inability of microinjected anti-NGF antibodies to block NGF-induced morphological differentiation in PC12 cells (Heuman et al., 1981; Seeley et al., 1983). Furthermore, the time course for internalization is too slow to account for early cellular responses to NGF (Buxser et al., 1983). In PC12 cells, the major pool of internalized NGF appears to be targeted to the lysosome for degradation (Kasaian and Neet, 1988).

Perhaps the most significant reason for the difficulty in defining NGF's signalling mechanism is the inability to identify early events which contribute directly to the neuronal phenotype. As shown in Table 1, all the events known to occur rapidly in response to NGF are those typically elicited by mitogenic factors rather than differentiation-promoting agents. Early events occuring within minutes of NGF binding include membrane ruffling (Schubert, et al., 1978; Connolly et al., 1979 and 1981), increased levels of cytosolic calcium (Pandiella-Alonso et al., 1986), and activation of the Na^+/K^+ and Na^+/H^+ pumps (Boonstra et al., 1981 and 1983; Varon and

Skaper, 1983). NGF also enhances the turnover of phosphoinositols resulting in an increased production of diacyglycerol and increased activity of protein kinase C (Contreras and Guroff, 1987; Traynor et al., 1983; Hama et al., 1986). Several proteins are phosphorylated rapidly in response to NGF (Halegoua and Patrick, 1980; Landreth and Rieser, 1985; McTigue et al., 1985), and at least one of these, tyrosine hydroxylase is phosphorylated in vitro by protein kinase C (Cremins et al., 1986). Nerve growth factor also stimulates within minutes of its addition the S6 protein kinase and correspondingly, an increased phosphorylation of its known substrate, the ribosomal S6 protein (Blenis and Erikson, 1986; Matsuda and Guroff, 1987).

In addition to these non-transcriptional events, NGF also induces rapid and transient increases in the transcription of several genes including c-fos , c-myc, actin and ornithine decarboxylase genes (Greenberg et al., 1985; Feinstein et al., 1985; Curran and Morgan, 1985). c-fos transcription is increased greater than 100-fold within 5 minutes of NGF addition, peaks between 15-30 min and is rapidly shut-off by 60 minutes. Similarily, actin transcription is also induced by 5 minutes, but transcription peaks within 30-60 min and returns to its basal level by 2 hours. In contrast to c-fos and actin transcription, the level of c-myc transcription peaks within 1-2 h after NGF treatment and remains high for several hours. Transcription of the gene for ornithine decarboxylase, the rate limiting enzyme in polyamine synthesis, is induced by 2 hours and reaches its greatest level by 4 hours after NGF addition. The corresponding mRNA's for each of these genes are induced with time courses similar to their rates of transcription.

EGF elicits early responses in PC12 cells which are identical to these rapid events known to be induced by NGF (Landreth and Reiser, 1985; McTigue et al., 1985; Greenberg et al., 1985; Feinstein et al., 1985). This is paradoxical since EGF acts as a mitogen in PC12 cells (Huff and Guroff, 1981) while NGF promotes differentiation of the cells into a neuronal phenotype. The question then arises as to the importance of these initial events in the phenotypic changes associated with differentiation of PC12 cells into a sympathetic-like neuron.

While early events occur within minutes to a few hours of NGF addition, events which are directly associated with differentiation along the neuronal pathway do not occur until after a lag of about 24 hours. At this time the cells have flattened considerably and short neurites are observed which elongate over several days to form long branching extensions (Greene and Tischler, 1976). Concurrent with this morphological change is the elevation of several neuronal specific proteins including microtubule associated proteins, tau and high molecular weight MAPs (Greene et al., 1983; Black et al., 1986; Drubin et al., 1985); neurofilament proteins, NG68 (Dickson et al., 1986); cell surface glycoproteins, thy-1 and NILE (Dickson et al., 1986; McGuire et al., 1978); and neurotransmitter-related proteins, acetylcholinesterase, choline acetyl transferase, neurotensin, and tyrosine hydroxylase (Greene and Rukenstein, 1981; Goodman et al., 1980; Tischler et al., 1986). Recently, several of these proteins have been shown to be regulated at the transcriptional level (Dickson et al., 1986;

The events leading to neuronal specific changes in PC12 have not been defined and it is not known if the rapid events which have been identified are

necessary for the delayed differentiation-specific events. This thesis attempts to address the role of early responses on long term phenotypic alterations by specifically examining the influence of one of these events, transcriptional activation of the proto-oncogene c-myc, on the cell's ability to undergo neuronal differentiation.

CHAPTER II
MATERIALS AND METHODS

Materials

Cholera toxin was purchased from List Biological Laboratories, Inc. (101),3-isobutyl-1-methyl xanthine was purchased from Sigma (I-5879), Dexamethasone was purchased from Sigma (D-1756). Taxol was a gift from Dr. M. Suffness, National Cancer Institute.

NGF Purification

ß-NGF was purified according to the method of Mobley et al., (1976). All steps were performed at 4°C. Submaxillary glands from 50, 8 week old CD-1 male mice (Charles River Breeding Laboratories) were homogenized in 50 mls of distilled water for 1 min in a Waring blender. The homogenate was centrifuged at 25,000 x g for 60 min. The supernatant was dialyzed against two, 4 liter lots of 0.02 M phosphate buffer (pH 6.8) for 16 h and then loaded onto a CM-cellulose (Whatman) column (2.5 x 12 cm) which has been equilibrated with 0.02 M phosphate buffer (pH 6.8). Two ml fractions of the NGF-containing eluate were collected. Fractions with absorbance greater than 0.5 at 280 nm were pooled and dialyzed against two, 4 liter lots of 0.25 mM phosphate buffer (pH 6.8). 0.5 M sodium acetate buffer (pH 4.0) was added to a final concentration of 0.05 M and sodium chloride was added to a final concentration of 0.4 M. The solution was centrifuged at 25,000 xg for 30 min. The supernatant was then loaded onto a second CM-cellulose column (1.5 x 30 cm) which had been equilibrated with 0.05 M sodium acetate, 0.4 M

NaCl (pH 4.0). The column was washed consecutively with 0.05 M sodium acetate, 0.4 M NaCl (pH 4.0), 0.05 M sodium acetate (pH 4.0), and 0.05 M Tris -HCl (pH 9.0) making sure absorbance at 280 nM reached zero during each wash step. NGF was eluted with 0.05 M Tris-HCl, 0.4 M NaCl (pH 9.0) and 1.0 ml fractions were collected. Fractions were analyzed by electrophoresing 30 to 40 μl aliquots of each fraction on a 15% SDS-polyacrylamide gel and observing the presence of a band at 13,000 daltons. NGF containing fractions were pooled and dialyzed against two changes of PBS for 16 h. The sample was concentrated using an Amicon concentrator with a YM5 filter. The concentration of NGF was estimated by reading absorbance at 280 nm and calculated using an extinction coefficient of 1.64 ml/mg cm. NGF was typically stored at -80°C in 300 μl aliquots at a concentration of 150 μg/ml.

Cell Culture

PC12 and variant clones were grown in an atmosphere containing 7.5% CO_2 on uncoated plastic culture dishes (Falcon Primeria) in Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum (FCS) and 10% heat inactivated horse serum (HS). Antibiotics were not typically added to the growth medium. Maintenance cultures were not allowed to reach a cell density greater than 5×10^6 cells per 100 mm^2 dish.

In general, cells were grown in culture for a maximum of one month and then replenished by thawing a frozen ampule from a large stock stored in liquid nitrogen. Cells were frozen by resuspending 5×10^6 cells in 1 ml of growth medium containing 10% DMSO and 1 mM Hepes and placed in a

cryostat vial. The vials were chilled by incubating 30 min at 4°C followed by an overnight incubation at -80°C. Vials of frozen cells were placed in liquid nitrogen the following day for long-term storage.

NGF treated cells were grown in the above medium supplemented with 100 ng/ml NGF for appropriate incubation times. Cells treated with cholera toxin were grown in medium containing 1 μg/ml cholera toxin and 1 μg/ml 3-isobutyl-1-methyl xanthine. Dexamethasone was added to cell cultures at a concentration of 1 μM. Supplemented medium was changed every 2 or 3 days.

Plasmids

pKO neo (Figure 1) contains aph coding sequences that utilize the SV40 early region transcription and RNA processing signals (Van Doren et al., 1984). aph encodes an aminoglycoside phosphotransferase which enables eukaryotic cells to express resistance to the antibiotic G-418 (Colbere-Garapin et al., 1981). p1A neo (Figure 1) contains adenovirus-5 E1A sequences (nucleotides 1-1834) and aph from pKO neo. pM myc neo (Figure 1) contains the two coding exons of a myc gene isolated in a murine plasmacytoma (Stanton et al., 1983). myc and aph were expressed from SV40 early region and mouse metallothionein-I (Durnam et al., 1980) promoters, respectively.

pMTV Xba myc and pMTV Hd3 myc (Figure 2) were obtained from Dr. Phillip Leder (Harvard Medical School, Boston, MA) (Steward et al., 1984). The constructions were produced by linking the mouse mammary

Figure 1. Construction of myc and E1A plasmids. pKO neo contains the aminoglycoside transferase gene (aph) derived from Tn5 cloned into pKO neo, a pBR322 derivative containing SV40 early region promoter and RNA processing signals (D. Hanahan, unpublished). p1A neo contains Ad5 E1A sequences (from nt 1 to nt 1834, shaded) and the aph gene from pKO neo. The two genes were inserted in opposite transcriptional orientations into pUC 18. pM myc neo contains exons 2 and 3 of the mouse myc gene expressed from the SV40 early promoter (shaded), and aph sequences from pKO neo , joined to the mouse metallothionein-promoter. The two genes were inserted in opposite orientations into pUC18.

pKOneo

SV40 promoter

aph

SV40 small t intron

SV40 poly A

pBR322

p1Aneo

SV40 promoter

aph

SV40 small t intron

SV40 poly A

pUC18

Ad E1A

pMmycneo

SV40 promoter

Metallothionein promoter

aph

SV40 small t intron

SV40 poly A

pUC18

Mouse myc
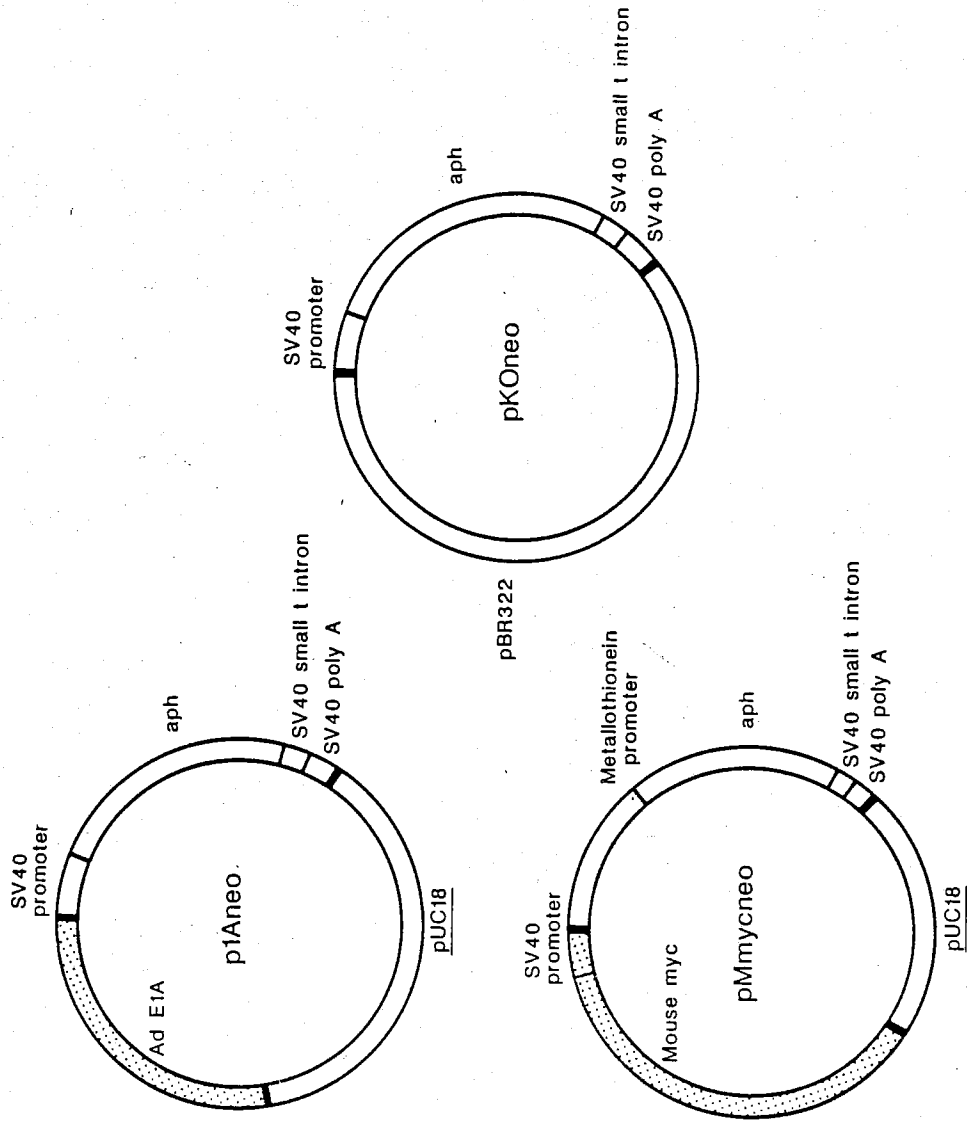
Figure 2. Diagrammatic representation of the murine c-myc gene and the MTV-c-myc hybrid genes. This figure was modified from a diagram previously published (Leder et al., 1986). The three exons of the gene are indicated as numbered boxes. The two normal promoters of the c-myc gene are indicated as arrows within exon 1. Sites used in constructing pMTV Hd3 myc or pMTV Xba myc are marked. The heavy line beneath each gene map represents the mRNA expected to be produced from each potentially active promoter. The dark hatched area in the fusion gene is the MTV LTR; the light hatched area is an unrelated DNA fragment carried over from one of the parental constructs (pA9; Huang et al., 1981) used in making the fusion gene.

Genomic c-myc

MTV Hd3 myc
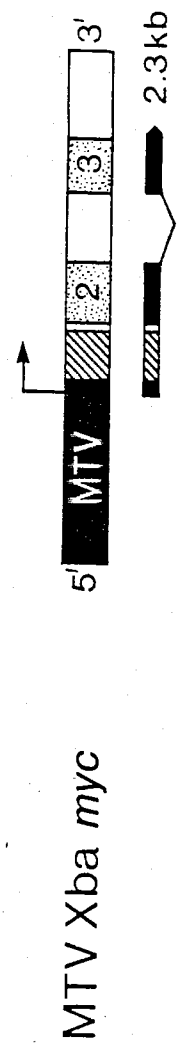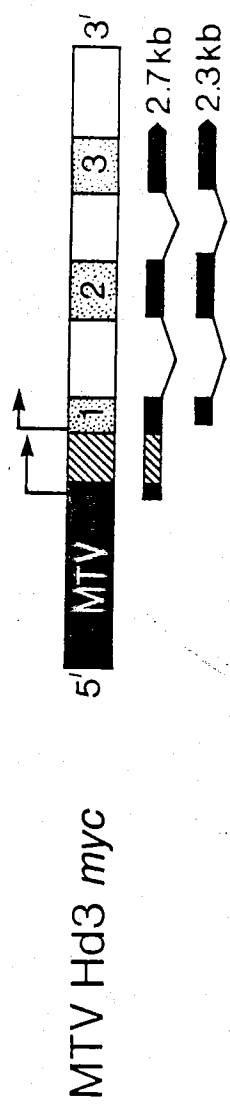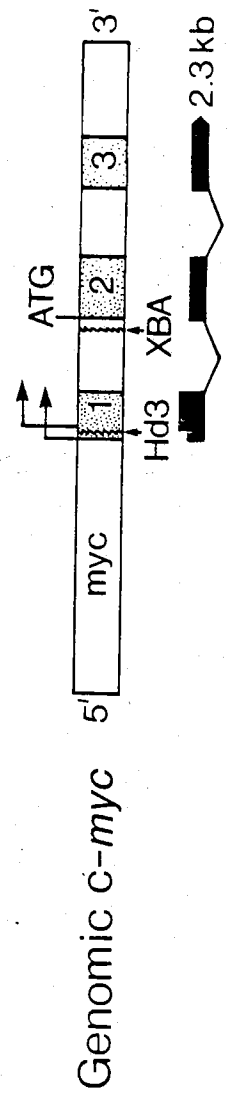
MTV Xba myc

tumor virus promoter (Huang et al., 1981) to portions of the genomic mouse c-myc gene. pMTV Xba myc lacks the first exon which contains the two transcription start sites and first intron (Bernard et al., 1983; Stanton et al., 1983) but includes the entire protein coding sequence. pMTV Hd3 myc possesses a 5' delection within the first exon of c-myc which eliminates the myc cap site of the longer transcript.

DNA Transfections

Plasmid DNAs pM myc neo, p1A neo and pKO neo were introduced into PC12 cells by calcium phosphate co-precipitation. DNA precipitates (0.5 ml) containing 0.5 μg plasmid DNA and 10 μg rat genomic carrier DNA in 125 mM $CaCl_2$, 0.7 mM $NaPO_4$, 20 mM Hepes, 150 mM NaCl, pH 7.1 were added dropwise to a 60 mm^2 dish containing 5×10^6 PC12 cells in 5 mls DME plus 5% FCS, 5% HS. Sixteen hours after transfection, the medium was decanted and replaced with new medium. After an additional 24 hours the cells were split 1:3 into growth medium containing 0.5 μg/ml G-418. G-418 resistant colonies were isolated and further characterized. Established clones were maintained in growth medium without G-418.

Plasmid DNAs, pMTV Xba myc and pKO neo were co-transfected into PC12 cells by protoplast fusion (Sandri-Goldin et al., 1981). Protoplasts were prepared as follows: 10 mls of chloramphenicol amplified bacteria (4 x 10^8 cells/ml) were centrifuged at 3000 rpm for 10 min. The cell pellet was resuspended in 0.5 ml of 20% sucrose in 0.05 M Tris-HCl (pH 8.0). Lysozyme was added to a final concentration of 1 mg/ml and cells incubated 5 minutes on ice. 0.2 ml of 0.5 M Tris-HCl (pH 8.0) was added and cells

were incubated at 37°C for 10 minutes. 4 ml of DME containing 10% sucrose, 10 mM MCL$_2$, was added. 5 ml of protoplast suspension prepared from bacteria containing pMTV Xba myc and 0.5 ml of protoplast suspension prepared from bacteria containing pKO neo, were fused simultaneously to 5 x 10^6 PC12 cells using 48.8% PEG (KOCH Chemicals, Ltd.). Cells were incubated in normal growth media for 48 hours after which the medium was replaced with fresh medium containing 0.5 mg/ml G-418 (Gibco). Resistant colonies were trypsinized within cloning cylinders, expanded to mass culture, and subcloned by limited dilution.

RNA Purification

Total RNA from PC12 cells was purified by the lithium chloride method (Segerson et al., 1987). PC12 and variant clones were washed 2 times with PBS at 4°C without detaching cells from the dishes. A solution containing 6 M urea, 3 M lithium chloride, 10 mM vanadyl ribonucleoside complex in DEPC-treated water was added directly to the dish and the cells removed by scraping. Typically 2 mls of this solution was added to each 100 mm^2 dish containing 5 x 10^6 - 1 x 10^7 cells. The cell suspension was passed five times through a 20 cc syringe and five times through a 22 cc syringe, followed by an overnight incubation at 0°C. RNA was pelleted by centrifugation at 16,000 x g for 3 min at 0°C. The pellets were resuspended in 10 mM Tris-HCl (pH 7.5), 5mM EDTA, 0.1% SDS and extracted twice with phenol/chloroform (1:1) and twice with chloroform. RNA was recovered by ethanol precipitation from 0.1M NaCl and quantitated by reading absorbance at 280 nm.

RNA Protection Assays

myc and E1A mRNA transcribed from the SV40 promoter were analyzed by RNase protection as described by Zinn et al., 1983. myc (Eco RI to Sst I fragment from pSVM mycneo) and E1A (Eco RI to Xba I fragment from p1A neo) sequences were subcloned downstream from the SP6 RNA polymerase promoter of pSP62-PL to construct pSPMmyc and PSP1AZO, respectively. Template plasmids were linearized by restriction enzyme cleavage (pSPMmycS with Rsa I and pSP1AXO with Nae I) prior to in vitro transcription. Transcription reactions (20 μl) contained 100 μCi of ^{32}P-CTP (600 Ci/mmol), diluted with CTP to a final concentration of 15 μM, and 0.5 μg or template DNA. 20 μg of total cellular RNA was hybridized to 10^4 Cerenkov cpm of RNA probe, and RNase resistant fragments were fractionated on 5% polyacrylamide-8 M urea gels.

Poly A$^+$ Selection of mRNA

Total RNA (typically 500-1000 μg) was resuspended in 500 μl ETS (0.01M EDTA, 0.01 M MOPs (pH 7.4) 0.2% SDS), heated at 65°C for 5 min, followed by cooling on ice for 5 min. 12 μl of 5 M NaCl was added and the suspension was applied directly to a 500 μl poly-uridine Sepharose 4B (Pharmacia) column which had been prewashed twice with ETS, once in ETS plus 90% formamide, six times in ETS, and six times in NETS (ETS + 0.1M NaCl). After loading RNA, the column was washed three times with each of the following solutions: NETS, ETS, and ETS plus 10% formamide. Poly A$^+$ mRNA was eluted with 1.5 ml of 90% formamide in ETS. RNA was ethanol precipitated overnight at -20°C using 0.05 M lithium chloride and 20

μg of RNase-free E. coli tRNA (Boehringer Mannheim). Precipitated RNA
was collected by centrifuging at 15,000 x g for 30 min at 4°C. The pellet was
resuspended immediately in 30 μl H_2O and 60 μl MIX (500 μl formamide, 83
μl formaldehyde, 100 μl 0.2M MOPs (pH 7.0) 0.05 M sodium acetate, 0.01
M EDTA).

Northern Analysis of myc RNA

Poly A^+ mRNA resuspended in MIX as described above was heated to
60°C for 5 min, cooled on ice, and loaded directly onto a 1.0% formaldehyde
agarose gel. The gel was electrophoresed at 50 volts using 20 mM Mops (pH
7.0) 5 mM sodium acetate, 1 mM EDTA as the running buffer. Following
electrophoresis the gel was washed for 1 h in 3 changes of water and
transferred overnight to nitrocellulose with 20X SSC (3 M NaCl, 0.3 M
sodium citrate, pH 7.0) using the capillary method. The filter was baked at
80°C for 90 min and prehybridized overnight at 42°C in 1% glycine, 50%
formamide, 5x SSC, 50 mM sodium phosphate, (pH 6.5) 5x Denhardts (0.1%
Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin and 250
μg/ml salmon sperm DNA. Hybridization was at 42°C overnight in 50%
formamide, 2x SSC, 40 mM sodium phosphate (pH 6.5) 0.8% Denhardts,
10% dextran sulfate, 10 mM EDTA, 0.1% SDS, and 200 μg/ml salmon
sperm DNA and $1-3x10^6$ CPM c-myc probe/ml. c-myc probe was a 1.0 kb
pst I cut insert containing the 3rd exon of myc (Stanton et al., 1984) which
had been labelled by random priming using a commercial kit (Boehringer
Mannheim). Specific activity of the probe was typically $1-2x10^9$ CPM/mg of
insert. Following hybridization, the filter was washed at 60°C for 20 min

consecutively in 2X SSC, 0.1% SDS; 1X SSC, 0.1% SDS; 0.5X SSC, 0.1% SDS; and 0.25X SSC, 0.1% SDS. Filter was autoradiographed at -80°C using 2 intensifying screens.

Emulsion Autoradiography

Cells were labelled for 2 hours in growth medium (as described in the text) containing 1 μCi ml ^{3}H-thymidine, washed with phosphate buffered saline (PBS), fixed with 3.7% formaldehyde in PBS, washed with distilled water and dried. Dishes, coated with NTB-2 photographic emulsion (Kodak), were placed in light tight boxes containing dessicant. Autoradiographs were exposed for three days at 4°C and developed. Cell counts were performed using a hemocytometer.

^{125}I-EGF Binding Assay

Mouse EGF and ^{125}I-labeled EGF were a gift from Dr. Roger Davis and A431 membranes were provided by Dr. Joaquin Toquido. A431 (2 μg) membranes were incubated in 200 μl of binding buffer (50 mM Hepes (pH 7.5), 125 mM NaCl, 5 mM KCl, 1.2 mM MgSO$_4$, 1.2 mM CaCl$_2$, 0.5% bovine serum albumin) containing 70 pM ^{125}I-EGF in the presence or absence of unlabeled EGF or NGF at 22°C. After 50 min of incubation, samples were placed on ice, diluted with 4.0 ml of ice-cold binding buffer, and filtered through 0.2 μM cellulose acetate/nitrate filter (Amicon). The filters were washed with three 1 ml washes of binding buffer and counted on a gamma counter.

Ornithine Decarboxylase Assay

Ornithine decarboxylase was assayed according to Guroff et al.,
(1981). Logarithmically growing cells were treated with 100 ng/ml NGF for
5 hours and harvested by scraping into PBS. Cells were centrifuged and
resuspended twice in PBS and once in 20 mM Tris-HCl (pH 7.4), 2 mM
$MgCl_2$ and 100 µM pyridoxal phosphate. Cell extracts were frozen and
thawed twice, centrifuged at 10,000 xg for 15 min., and supernatants were
assayed for ornithine decarboxylase. Assay mixtures contained 50 µl of
supernatant and 200 µl reaction buffer: 125 mM Tris-HCl (pH 7.5) 100 µM
pyridoxal phosphate, 12.5 µM dithiothreitol, 10 mM EDTA, and ^{14}C-
ornithine (0.5 µCi per 0.04 mmol unlabelled ornithine). Reactions were
performed in rubber stoppered tubes with hanging center wells containing
filter paper saturated with 200 µl protosol:toluene (1:1) to trap liberated
$^{14}CO_2$. After 1 hour at 37ºC, reactions were terminated by injecting 300 µl
of 2N H_2SO_4 through the stoppers into the reaction mixtures. After an
additional hour at 37ºC, trapped $^{14}CO_2$ was counted in Omnifluor
scintillation fluid.

^{125}I-NGF Binding Assay

NGF was radioiodionated using immobilized glucose oxidase and
lactoperoxidase as described previously (Buxser et al., 1983). 20 µg of NGF
was added to 50 µl of reconstituted Enzymo-beads (BioRad) with 2 mCi of
Na ^{125}I. The reaction was initiated by adding ß-D-glucose to a final
concentration of 0.1% and proceeded for 10 min, after which a second
aliquot of ß-D -glucose was added followed by an additional 10 min

incubation. Guanidine-HCl was added to a final concentration of 4 M to disrupt aggregates and to increase the release of NGF which adheres to the Enzyme-beads. Free ^{125}I was separated from proteins incorporated ^{125}I by chromatography on a short Sephadex G-25 column. Specific activities of ^{125}I-NGF were between 2.25 x 10^6 and 3 x 10^6 cpm/pmol.

^{125}I-NGF binding was measured by a centrifugation assay. Cells were detached by incubating in Puck's buffer containing 0.5 mM EDTA for 5 min., centrifuged and resuspended in Hepes buffered (pH 7.4) Kreb's Ringer solution containing 0.5 mg/ml albumin and 0.5 mg/ml glucose. 1 x 10^6 cells were incubated with varying concentrations of ^{125}I-NGF for 1 hour at room temperature. Triplicate 100 µl samples were centrifuged (10,000 x g) for 1 min. Supernatants were aspirated and the bottom of each tube was counted in a gamma counter. Non-specific binding was determined by parallel incubations in the presence of 10 µg/ml unlabeled NGF.

Indirect Immunofluorescence Microscopy.

Cells were stained essentially as described (Bloom et al., 1984). Briefly, PC12 cells were grown on poly-L-lysine coated coverslips and treated under the appropriate conditions as described in figure legends. Coverslips containing attached cells were rinsed 2 times in PBS at 37°C. The cells were fixed by placing coverslips in 100% methanol at -20°C for 5 min followed by rehydration in TBS (0.05 M Tris-HCl (pH 7.4) 0.15M NaCl). Coverslips were then incubated for 30 min in the appropriate antibody-conditioned hybridoma media in a 37°C humidified chamber and washed 4 times in TBS. Secondary antibody was applied by incubating the coverslips

at 37°C for 30 min with rhodamine-conjugated rabbit anti-mouse IgG antibodies (Cappel) at a 1:1000 dilution in TBS. After washing 4 times in TBS and 1 time in distilled water, the coverslips were sealed to slides with elvanol (Monsanto Corp).

The slides were observed on a Zeiss IM-35 inverted microscope equipped with an epifluorescent illuminator, fluorescein and rhodamine filters, and the following objectives: 40X phase 3Na 1.0 Aprochromat, 63X phase 3 NA 1.4 Planapochromat.

Taxol-Dependent Purification of Microtubules and MAPs.

PC12 cells were washed two times with PBS by centrifugation. The pellet was resuspended in ten volumes of ice cold buffer containing 1 mM $MgSO_4$, 1 mM EGTA (pH 6.8) 2 mM PMSF, 1 KU/ml aprotinin, 5 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 µg/ml chymostatin. Cells were immediately centrifuged at 1000 rpm for 2 min and supernatant was removed. This step was repeated until the cell pellet had swelled to twice its original volume (usually 2 wash steps). The swelled pellet was incubated on ice for 15 minutes and then passed five times through a 1 cc tuberculin syringe and the homogenate was centrifuged at 30,000 x g for 15 min at 2°C. The supernatant from this centrifugation was re-centrifuged for 90 min at 150,000 x g at 2°C. The supernatant was collected and incubated for 5 min at 37°C in the presence of 20 µM taxol and 1 mM GTP and then underlayed with a 5% sucrose cushion containing 20 µM Taxol and 1 mM GTP. The sample was then centrifuged at 7000 RPM in a Sorvall HB-4 rotor and the

pellet was resuspended in Laemmli sample buffer (2% SDS, 2% glycerol, 25 mM Tris, pH 6.8) or PBS. Samples were stored at -80°C.

Immunoblots.

Laemmli (1970) on 6% or 7.5% polyacrylamide gels to resolve HMW MAPs or tubulin and tau proteins, respectively. Electrophoretic transfer of proteins was performed by a modification of Towbin et al. (1979). After electrophoresis, the gels were transferred to nitrocellulose paper for 8 hours (MAPs) or 2 hours (tubulin and tau) at 350 mAmps in 0.25 M Tris, 0.192 M glycine, 0.1% SDS, and 20% methanol. Transfer of MAPS appeared to be quantitative under these conditions. For analysis of HMW MAPs and tubulin, the nitrocellulose filters were incubated at room temperature for 30 min in TBS containing 20% gamma-globulin free horse serum. The blots were incubated at room temperature overnight in a 1:200 dilution of monoclonal antibody for MAP 1A, MAP 1B, MAP 2, or tubulin. Immunoblots were washed four times in TBS plus 0.1% Tween-20 followed by a 2 hour incubation in a 1:500 dilution of rabbit anti-mouse IgG (Cappel). Washed blots were probed with 1 μCi/ml of ^{125}I-protein A (Amersham) then washed four times and autoradiographed.

For immunoblot analysis of tau proteins, blots were incubated for 30 minutes in 10 mM Borate-buffered saline, pH 7.6 (BBS) containing 5% nonfat dry milk. Blots were probed overnight with a 1:50 dilution of tau monoclonal antibodies (provided by Dr. L. Binder) and then incubated sequentially for 1 hour with each of the following antibodies: 1:2500 dilution of rabbit anti-mouse IgG, a 1:1000 dilution of sheep anti-rabbit IgG,

and a 1:500 dilution of rabbit PAP antibody. All antibodies were diluted in BBS and 5% nonfat dry milk except for rabbit PAP antibody which was diluted in 10mM Tris-HCl (pH 7.5). Blots were washed with four changes of TBS plus 0.1% Tween-20 between antibody incubations. After the final antibody incubation, blots were washed three times in TBS plus 0.1% Tween-20, once in TBS, and once in 10 mM Tris-HCl (pH 7.5). The peroxidase color reaction was performed in 50 mls of 10 mM Tris-HCl (pH 7.5) containing 5 mg dimethoxybenzidine dihydrochloride (Kodak), 5% methanol, and 0.9% H_2O_2. This procedure was modified from the procedure of Sternberger et al., (1970).

Protein Kinase Assays

For assay of protein kinases, PC12 cells and transfected clones were seeded into poly-L-lysine coated 96-well microtiter plates at a density of 5,000 to 10,000 cells/well and cultured for 3 days. For incubations with NGF, the growth medium was aspirated and the attached cells were rinsed with 100 µl of a buffered salt solution (137 mM NaCl, 5.4 mM KCl, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 1 mM calcium chloride, 1 mg/ml glucose and 20 mM HEPES (pH 7.2) at 30°C. The cells were incubated for indicated times at 30°C in 40 µl of the buffered salt solution with or without 100 µg/ml NGF. To initiate protein kinase assays, hormone solutions were aspirated and replaced with 40 ml of the buffered salt solution supplemented with 50 mg/ml digitonin, 10 mM magnesium chloride, 25 mM β-glycerophosphate and 100 µM [γ-^{32}P] ATP (~ 5000 cpm/pmol). Further additions for assay of specific kinases were: cAMP-dependent protein

kinase, 100 µM Kemptide (Kemp et al., 1977), 0.5 mM EGTA with or without 3 µM cAMP or 25 µg/ml IP-20 (Cheng et al., 1986); ribosomal S6 kinase, 250 µM RRLSSLRA peptide (Pelech and Krebs, 1987); 1 mM EGTA, 10 mM NaF, 20 µM sodium vanadate, 25 µg/ml IP-20; MLL peptide kinase, 100 µM KKRPQRATSNVFS peptide (Kemp et al., 1983; Pearson et al., 1985), 10 mM NaF, 20 µM sodium vanadate, 25 µg/ml IP-20 and 1 mM EGTA. Reactions proceeded for 10 minutes at 30°C and were terminated upon addition of 10 µl of 25% (w/v) trichloroacetic acid. Aliquots (45 µl) of the acidified reactions were spotted on phosphocellulose squares (P81) and phosphopeptides quantified as previously described (Heasley and Johnson, 1988).

Preparation of High Molecular Weight Genomic DNA

Approximately 5, 100 mm^2 dishes of cells were washed twice with PBS. The cellular pellet was recovered by centrifugation at 1000 x g and resuspended in 10 ml of DNA lysis buffer (0.32 M sucrose, 10 mM Tris (pH 7.5), 5 mM MgCl$_2$, 1% Triton X-100 at 4°C. Nuclei were collected by centrifugation at 1000 x g for 10 min at 4°C and the nuclear pellets were resuspended in 4.5 mls of 0.075 M NaCl, 0.024M proeinase K (Boehringer Mannheim) to a final concentration of 200 µg/ml and the mixture was incubated overnight at 37°C. The solution was phenol/chloroform extracted 2-3 times followed by 2-3 chloroform extractions without vortexing. DNA was precipitated by adding 0.5 ml of 3 M sodium acetate (pH 5.2) and 11 mls of room temperature 100% ethanol and inverting the tube several times until a DNA clot formed. The DNA was removed with a large bore pasteur

pipette, rinsed in 70% ethanol, and dissolved at 4oC in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. The integrity of the DNA was visualized on a 0.8% DNA agarose gel as a single band which migrated slightly slower than the 22 kb Hind III fragment of lambda DNA marker (purchased from New England Biolabs). the concentration of DNA was determined by measuring the absorbance at 260 nm and calculated assuming that an OD_{260} reading of 1 contains 50 μg DNA/ml.

Southern Analysis

0.1 μg of plasmid DNA or 10-20 μg of genomic DNA were digested with the appropriate restriction enzymes according to the conditions specified for the enzyme. Typically plasmid DNA was digested at 37oC for 3 hours using approximately 1 unit of enzyme/μg of DNA and genomic DNA was digested overnight at 37oC using greater than 5 units of enzyme/μg of DNA. Digestions were terminated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 3 volumes of 95% ethanol following freezing in dry ice and centrifuging in an microfuge . The pellet was rinsed once in 70% ethanol, dried, and dissolved in 5% Ficoll 400, 5 mM EDTA (pH 8.0), 0.2% SDS, 0.01% bromphenol blue, 0.01% xylene cyanol. The DNA was run on a 0.8% agarose gel in 1X TAE (10 mM Tris (pH 7.8), 5 mM sodium acetate, 0.5 mM EDTA) at 20 volts. Following electrophoresis, the gel was washed in two, 10 min changes of 0.25 M HCl and twice for 10 min each in 1.5 M NaCl, 0.5 M NaOH. The gel was neutralized by soaking in two 10 min changes of 2X SSC and transferred overnight to nitrocellulose with 20X SSC using the cappillary method. The filters were baked, prehybridized and

hybridized as described for Northern analysis. Specific probes used were: a 1.0 kb Pst I insert containing the third exon of c-<u>myc</u> (Stanton <u>et</u> <u>al</u>., 1984), a 1.4 kb Sac I insert containing the second exon of c-<u>myc</u>, and a 2.2 kb Bam HI insert containing the MTV promoter (Stewart <u>et</u> <u>al</u>., 1984).

CHAPTER III
MYC AND E1A ONCOGENES ALTER THE RESPONSES OF PC12 CELLS TO NERVE GROWTH FACTOR AND BLOCK DIFFERENTIATION

Shortly after exposure to NGF, wild-type PC12 cells transiently enhance the transcription of genes characteristically induced in mitogenically stimulated cells such as myc, fos and ornithine decarboxylase (Greenberg et al., 1985). The role of these rapidly expressed gene products in NGF action is not understood. For this reason c-myc was introduced into PC12 cells to examine the influence of this gene product on NGF action and cell phenotype. In addition, the early region 1A (E1A) of human adenovirus was also introduced into PC12 cells because of its structural and functional similarities to myc.

Several observations suggest that oncogenes such as myc and E1A influence cell proliferation in response to signals induced by external stimuli. First, c-myc RNA levels frequently increase in mitogenically stimulated cells (Kelley et al., 1983; Armelin et al., 1984; Campisi et al., 1984). Second, myc and E1A reduce or eliminate the need for serum and other growth factors necessary for cell proliferation (Shiroki et al., 1979; Keath et al., 1984; Mougneau et al, 1984; Rapp et al., 1985). Third, c-myc levels decrease in several cell types induced to differentiate (Westin et al., 1982; Reitsma et al., 1983; Campisi et al., 1984; Gonda and Metcalf, 1984), and transcriptionally activated myc genes block terminal differentiation of erythroleukemia cells (Coppola and Cole, 1986). Finally, myc and E1A assist in a process whereby

primary cells escape senescence and grow indefinitely as established lines (Houweling et al., 1980; Mougneau et al., 1984, Ruley et al., 1984). Establishment may depend on activities which enable cultured cells to proliferate in response to serum growth factor signals and/or activities which promote growth at the expense of terminal differentiation (Martin et al., 1974 and 1975; Bell et al., 1978; Ruley et al., 1984).

Given that myc and E1A appear to affect both cellular responses to external stimuli and cell differentiation, the present study has investigated whether myc and E1A alter the responses of PC12 cells to NGF. These results show that both myc and E1A inhibit the differentiation promoting effects of NGF in PC12 cells and when expressed constitutively allowed NGF to act as a mitogenic stimuli.

RESULTS

Isolation of myc and E1A expressing PC12 cells

myc and E1A genes driven by the SV40 early promoter were introduced into PC12 cells by DNA-mediated gene transfer, followed by selection for G-418 resistance. Two G-418 resistant clones obtained following transfection of pKO neo (neo 2111 and neo 2212) and pMmyc neo (myc 41 and myc 42), and three clones derived following transfection of p1A neo (E1A 313, E1A 314 and E1A 324) were characterized in the present study.

E1A and myc gene expression was measured in each clone. Total cellular RNA was annealed with ^{32}P-labeled anti-sense RNA generated in vitro by SP6 RNA polymerase, and fragments resistant to RNases A and T1

Figure 3. E1A and Mouse myc Expression in PC12 and Derivative Clones.
Cellular RNAs from the indicated clones were annealed to ^{32}P-labeled RNA
probes generated in vitro by SP6 polymerase. Fragments resistant to RNase
digestion were detected following gel electrophoresis and autoradiography.
(A) Probes complementary to E1A (between viral Xba I and Nae I cleavage
sites) protected fragments in E1A 324, E1A 313 and E1A 314 cellular RNAs
of 300, 162 and 107 nt. The protected fragments were appropriate in size to
be derived from E1A 13S and 12S transcripts. Size markers (end-labeled
Hinf I digested pAT153 DNA, lane M) and undigested RNA probe (lane P)
were run in parallel. (B) Probes complementary to mouse myc sequences
(spanning Sst I and Rsa I cleavage sites) protected a 129 nt fragment in myc
41 and myc 42 cellular RNAs. The protected fragment was the size expected
of mouse myc transcripts with properly spliced second exons.

A.

B.
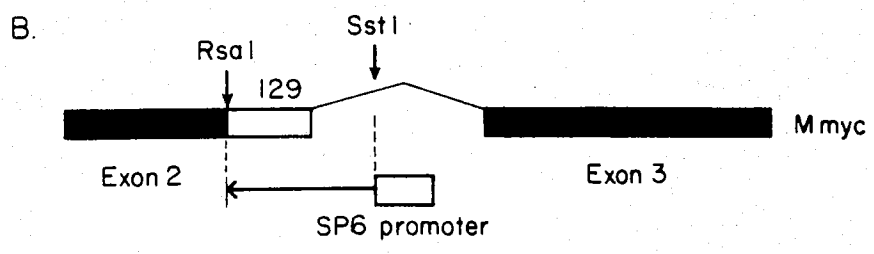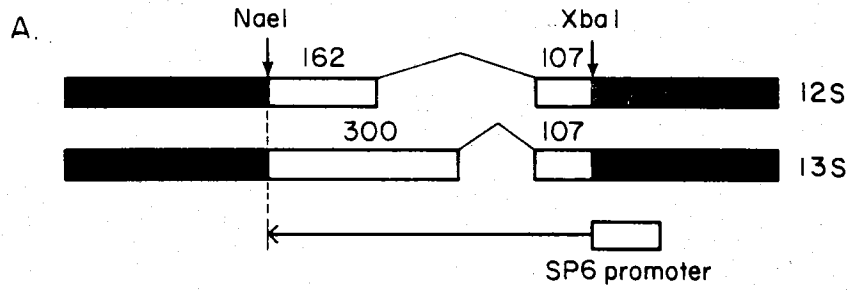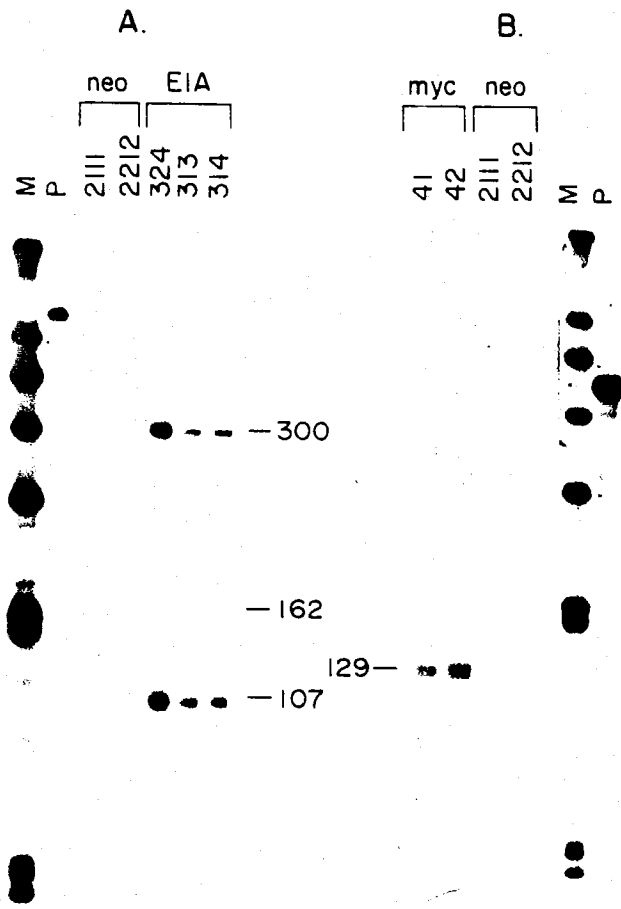
were analyzed by gel electrophoresis and autoradiography. As shown in Figure 3A and B, E1A clones 324, 313 and 314 expressed RNAs related to E1A 12S and 13S transcripts, and RNA from myc 41 and myc 42 cells protected myc probe sequences diagnostic of properly spliced exon 2. Probe sequences protected by myc 41 and myc 42 cellular RNAs resulted from mouse and not rat myc transcripts, since RNA from neo 2111 and neo 2212 cells failed to protect detectable segments of the mouse myc probe (Figure 3A).

EXPRESSION OF MYC OR E1A BLOCKS NGF-INDUCED PC12 CELL DIFFERENTIATION

PC12 cells normally grow as round, loosely adherent cells. NGF induced PC12 cells to flatten, increase cell substratum adhesiveness and extend neurite-like processes (Greene and Tischler, 1976; Schubert and Whitlock, 1977). Figure 4 shows that PC12 clones constitutively expressing either myc or E1A demonstrated a flattened morphology. Several independent isolated PC neo clones failed to demonstrate this altered morphology suggesting that myc, as well as E1A, were capable of inducing a flattened cell shape. While all the G-418 resistant clones not containing the c-myc or E1A cDNA insert responded normally to NGF with neurite extension, the myc and E1A expressing clones failed to extend long neurite-processes in response to NGF. Both myc and E1A expressing clones did demonstrate very small processes, however, these processes were not influenced by NGF and were not morphologically true neurite.

Figure 4. Phase Contrast Photomicrographs of PC12 and Derivative Clones in the Absence and Presence of NGF. Cells were cultured on plastic dishes for 7 days in the absence (-NGF; A, C, E and G) or presence (+NGF; B, D, F and H) of 100 ng/ml NGF. Medium was changed every 3 days. (A, B) PC12; (C, D) PC neo; (E, F) E1A 324; and (G, H) myc 41.

PC 12

PC neo

PC myc

PC 1A
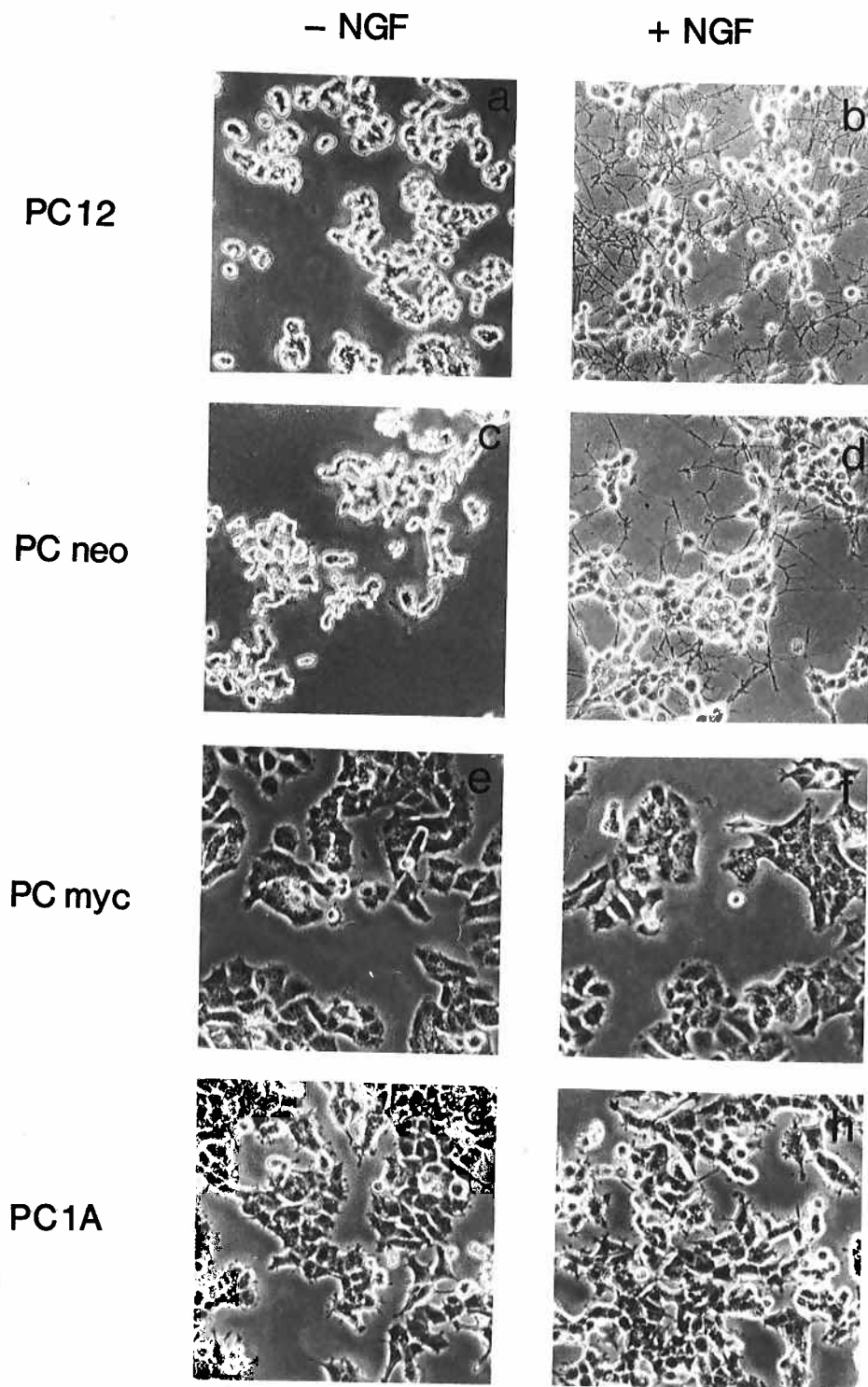
NGF Stimulates DNA Synthesis in PC12 Cells Expressing myc or E1A

NGF-induced differentiation of PC12 cells is accompanied by a reduced rate of cell proliferation (Greene and Tischler, 1976). Thus, the effects of myc and E1A on cell growth in the presence or absence of NGF were investigated. Replica cultures, treated with or without NGF, were labeled for 2 hours with ^{3}H-thymidine, and the percent of cells which synthesized DNA during the labeling period were counted after emulsion autoradiography.

Figure 5 shows the effects of myc and E1A on DNA synthesis in appropriate PC12 clones incubated in the presence or absence of NGF. All cell lines were labeled to a similar extent when grown in the absence of NGF, suggesting that myc or E1A had little effect on cell growth rates in normal medium. NGF caused a reduced rate of cell proliferation of PC12, neo 2111, and neo 2212 cells grown in high (10% fetal calf serum, 5% horse serum) or low serum (1% fetal calf serum), as evidenced by the decreased percentage of labeled nuclei during a two hour exposure to ^{3}H-thymidine. In myc or E1A expressing cells grown in high serum, NGF had little influence on the percentage of nuclei labeled during a two hour ^{3}H-thymidine pulse. However, myc and E1A expressing clones maintained in low serum responded to NGF with a 70-100% increase in labeled nuclei, whereas normal PC12 cells showed a 30-90% decrease in labeled nuclei. The failure to observe increased DNA synthesis with myc and E1A expressing clones in high serum is presumably due to the maximum mitogenic effect of other

Figure 5. ^{3}H-Thymidine Incorporation in PC12 and Derivative Clones in the Presence and Absence of NGF and in Medium Containing High and Low Serum. PC12 cells and derivative clones were grown and processed for emulsion autoradiography. Open bars indicate the percent of cells with labeled nuclei after 7 days in growth medium (DME, 10% fetal bovine serum, 5% horse serum) in the absence (-) or presence (+) of NGF. A similar analysis was performed on cells maintained in DME containing low serum (1% fetal bovine serum, hatched bars). In particular, cells were seeded in growth medium and one day later were changed to medium containing 1% serum. After 2 days, the medium was replaced with new medium containing 1% serum with (+) or without (-) 100 ng/ml NGF and after 2 additional days, cells were labeled with ^{3}H-thymidine and processed for autoradiography. Thin bars indicate the standard deviations of triplicate experiments.
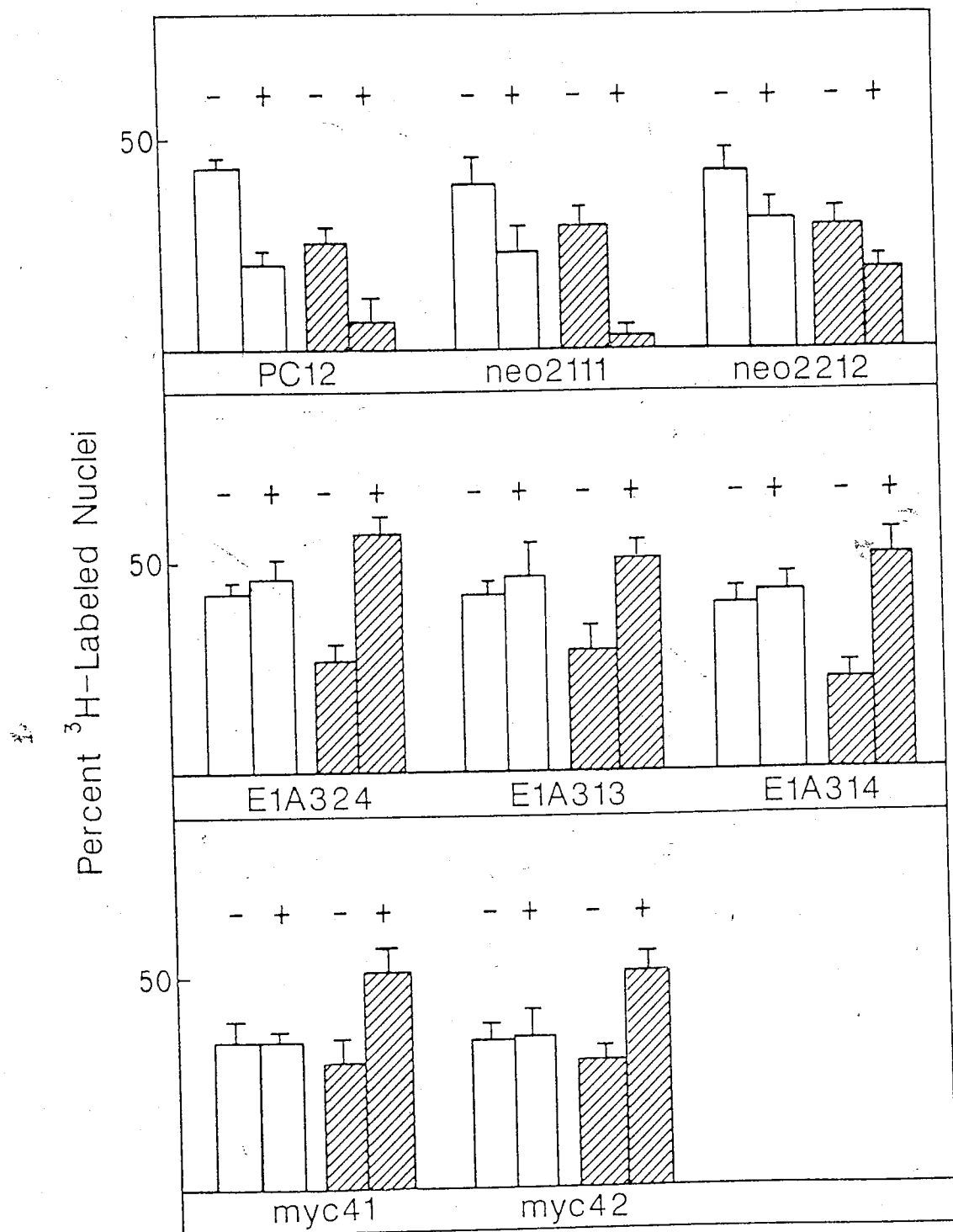
growth factors present in fetal bovine and horse serum. Growth of the clones in low serum minimized the action of other growth factors so that a clear NGF-stimulation of DNA synthesis was observed in cells expressing either myc or E1A.

While the response of neo 2212 to NGF was weaker than that observed with PC12 and neo 2111, the difference between neo 2111 and neo 2212 clones illustrates the range of responses observed with NGF-induced inhibition of DNA synthesis. NGF-induced inhibition of DNA synthesis in four additional PC neo clones fell within the range observed with neo 2111 and 2212 (not shown). In contrast, all of the clones expressing myc and E1A demonstrated increased DNA synthesis in response to NGF in low serum.

Figure 6 shows the growth curves for the various clones in low serum. Both myc and E1A expressing clones responded to NGF with an increased growth rate relative to control clones. Since DNA synthesis and cell number was increased in response to NGF in myc and E1A expressing clones, but not in PC neo clones, this response was a result of oncogene expression.

EGF has been shown to induce a similar increase in PC12 cell number (Boonstra et al., 1983). Figure 7 demonstrates that the observed affects was not due to contamination of EGF within the NGF preparation. NGF was tested for its ability to compete with the binding of ^{125}I-EGF to A431 plasma membrane. Nonlabeled EGF inhibited binding of ^{125}I-EGF with 50% inhibition occuring at 1 nM EGF. In contrast, NGF did not compete with the binding of ^{125}I-EGF even at concentrations as great as 10 nM NGF.

Figure 6. Growth Rates of PC12 and Derivative Clones in the Presence and Absence of NGF. 16 mm culture dishes were seeded with 2×10^4 cells in growth medium (DME, 10% fetal bovine serum, 5% horse serum) and 24 hours later the medium was replaced with fresh medium containing 2% fetal calf serum (o). After an additional 24 hours 100 μg/ml NGF was added to appropriate dishes (Day 0) (Δ). Cell numbers represent the means of duplicate determinations.

Cell Number (x10⁴)
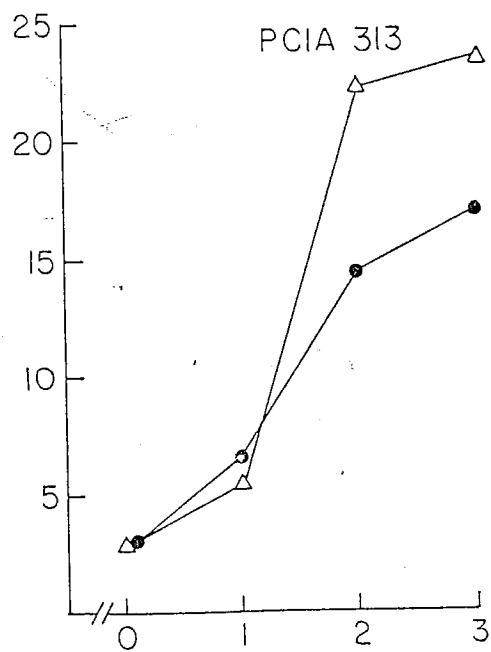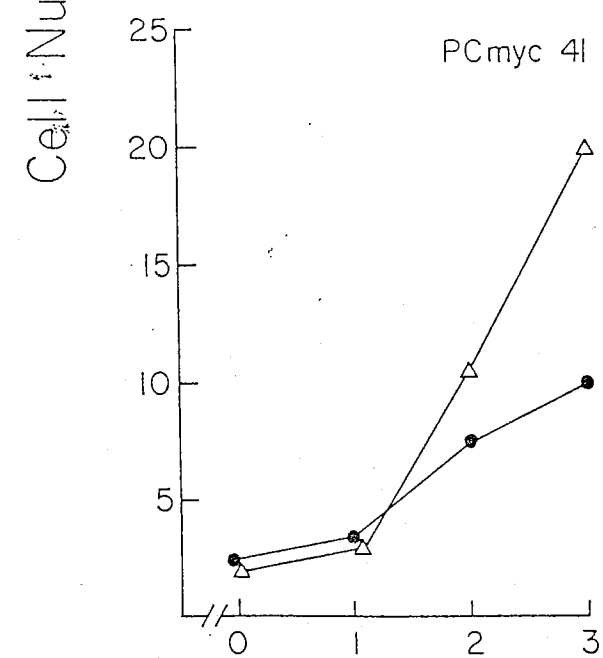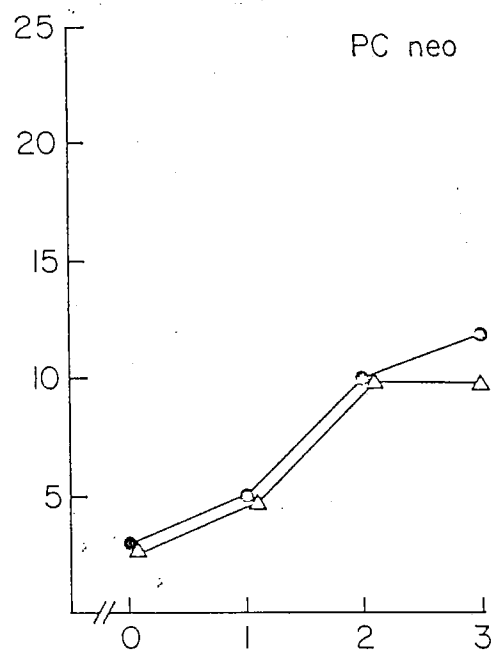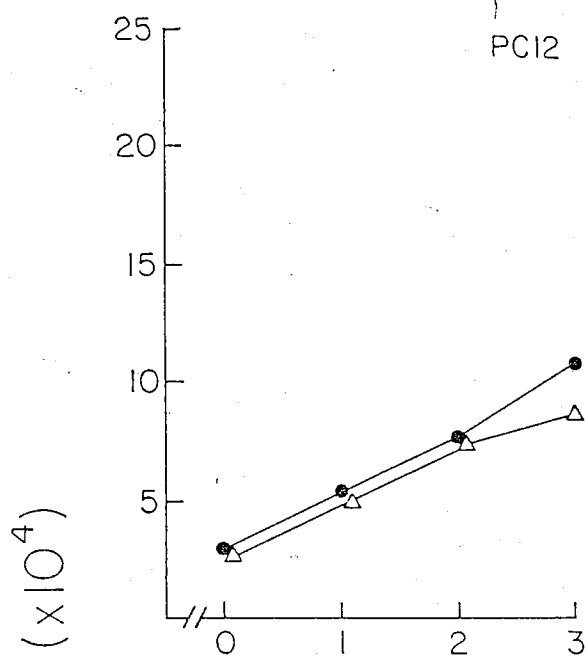
Days

Figure 7. Competitive inhibition of the binding of ^{125}I-EGF to A431 membranes by EGF but not NGF. A431 (2 µg) membranes were incubated at 22°C in the presence of 70 pM ^{125}I-EGF with the indicated concentrations of unlabeled EGF (O) or NGF (Δ) as described in methods. Data are expressed as percent fraction of specific binding to membranes incubated in the absence of unlabeled ligands. Data are the average of triplicate determinations. Standard deviations were within 10% of the mean.

^{125}I-EGF Bound (percent of control)

Unlabeled Ligand (nM)

NGF

EGF
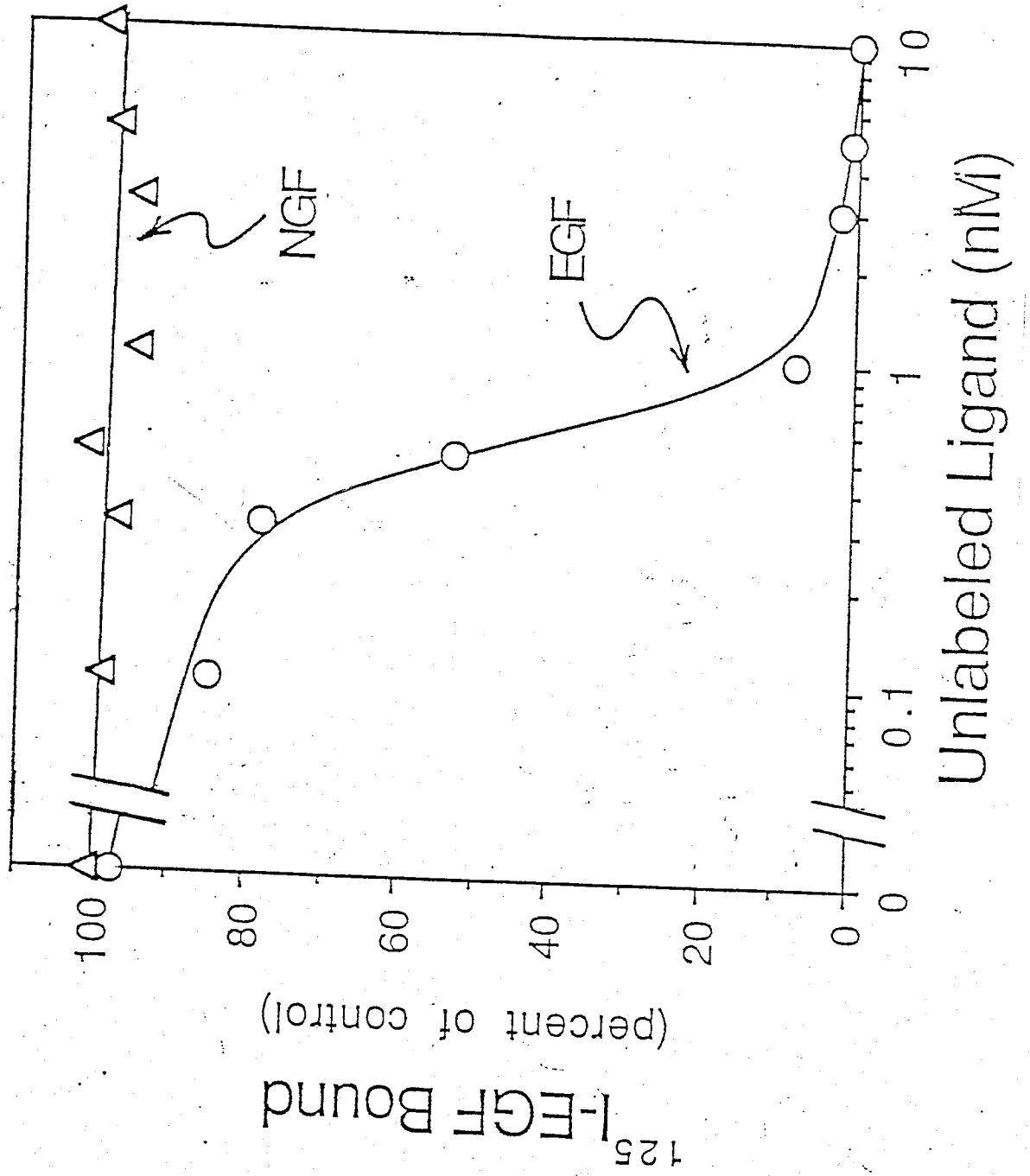
Expression of myc and E1A Do Not Alter NGF Receptor in PC12 Cells

The biological effects of NGF on PC12 cells are initiated by binding to surface receptors. myc and E1A could influence cellular responses to NGF by affecting the number of NGF receptors expressed in PC12 cells. Consequently, PC12 cells and derivative clones were analyzed with regard to NGF-receptor binding.

As summarized in Table 2, ^{125}I-NGF equilibrium binding properties were essentially identical in myc and E1A expressing clones as compared to normal PC12 cells. Scatchard analysis (Figure 8) revealed biphasic binding for all the cell lines demonstrating the characteristic high (K_d = 0.3 nM) and low (K_d = 5.2 nm) affinity binding sites. Each clone also expressed similar number of receptors per cell.

myc and E1A Do Not Block Ornithine Decarboxylase Induction

Failure of myc and E1A to alter NGF receptor number or ligand affinities suggested that myc and E1A affect either NGF receptor function or cellular responses to signals induced by NGF. To assess whether NGF receptors were activated by ligand binding, changes in ornithine decarboxylase (ODC, EC 4.1.1.17) levels were monitored in cells stimulated by NGF. ODC mRNA and enzyme activity are induced rapidly in PC12 cells, with enzymatic activities peaking 4 to 6 hours after NGF stimulation (Greene and McGuire, 1978; Hatanaka et al., 1978; Feinstein et al., 1985; Greenberg et al., 1985). ODC catalyzes a rate-limiting step required for polyamine biosynthesis, and increased activity correlates with cell growth and differentiation (for review see Heby, 1981). While inhibitors of ODC

Table 2. NGF-Receptor Equilibrium Binding. NGF receptor affinities and capacities were calculated from Scatchard analysis of ^{125}I-NGF binding (Figure 8). The values shown are the means of triplicate measurements from two experiments. Standard deviations were typically within 10% of the mean.

CELL LINE	K_d (nm)	Low Affinity Capacity (Molecules/Cell)	K_d (nM)	High Affinity Capacity (Molecules/Cell)
PC12	5.2	7.5×10^4	0.3	1.5×10^4
neo2111	5.2	7.6×10^4	0.3	1.5×10^4
myc41	5.0	7.8×10^4	0.3	1.5×10^4
E1A324	5.2	7.2×10^4	0.3	1.5×10^4

Figure 8. Binding of ^{125}I-NGF to PC12 cells. PC12 (A), PC neo (B), PC myc (C), PC1A, (D). Experimental conditions are described in methods. Scatchard plot and binding saturation curve (inserts) were calculated from data obtained from triplicate measurements of two independent experiments. Standard deviations were typically within 10% of the mean.
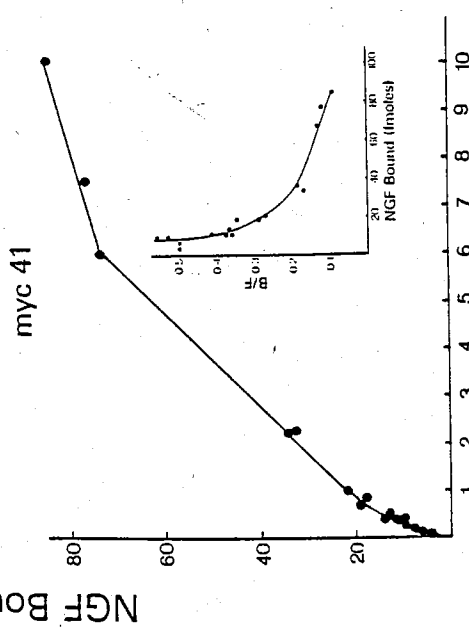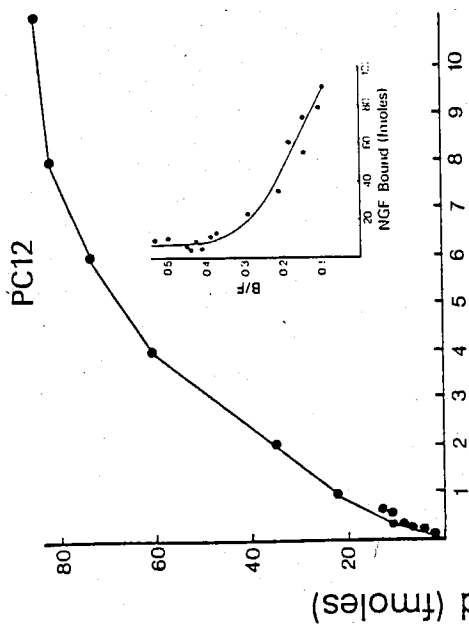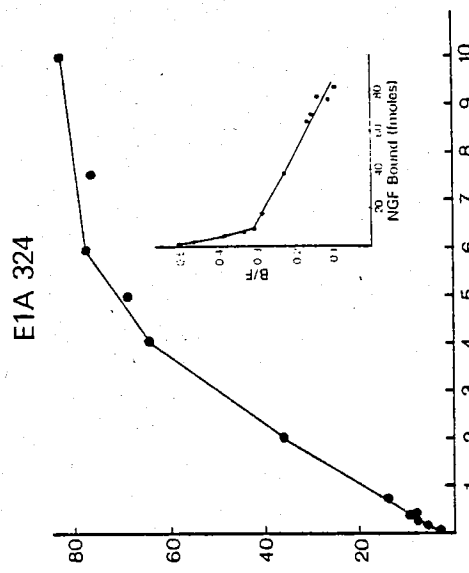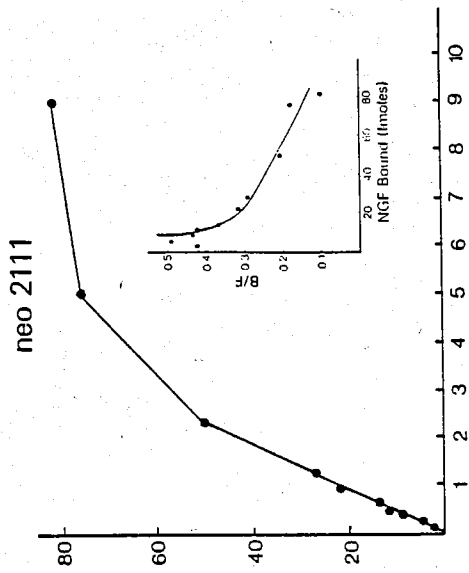
delay NGF-induced neurite outgrowth, the role of polyamines in PC12 differentiation remains unclear. Nevertheless, ODC provides a marker of an early, transcription-dependent response to NGF in PC12 cells.

ODC activity was measured in extracts from exponentially growing cells treated with 100 ng/ml of NGF for 5 hours. myc and E1A did not inhibit the ability of NGF to stimulate ODC activity, although basal and induced activity varied among different clones (Table 3). Since ODC induction and NGF receptor binding properties are similar in the various clones, myc and E1A alter the responsiveness of PC12 cells to NGF-induced signalling events at sites distal to receptor activation.

Table 3. Induction of Ornithine Decarboxylase by NGF in PC12 and Derivative Clones. Ornithine decarboxylase activities in cells untreated and treated with 100 ng/ml NGF for 5 hours are given. Values represent the means ± S.E.M. of triplicate measurements for three separate experiments.

Cell Line	Treatment	Ornithine Decarboxylase Activity pmol $^{14}CO_2/\mu g$ protein/hr
PC12	-NGF	1.67 ± 0.42
	+NGF	16.98 ± 3.30
neo2111	-NGF	2.57 ± 0.98
	+NGF	14.33 ± 2.60
neo2212	-NGF	1.87 ± 0.03
	+NGF	10.13 ± 4.44
myc41	-NGF	0.96 ± 0.18
	+NGF	$4.00 + 0.65$
myc42	-NGF	1.00 ± 0.44
	+NGF	8.60 ± 4.05
E1A313	-NGF	1.00 ± 0.35
	+NGF	9.00 ± 3.55
E1A324	-NGF	0.90 ± 0.33
	+NGF	4.00 ± 0.25

DISCUSSION

Mouse c-myc or adenovirus E1A altered the phenotypic response of PC12 cells to nerve growth factor. Whereas, NGF normally induces neuronal differentiation and growth arrest, PC12 cells expressing myc or E1A resist morphological differentiation and continue to proliferate. In addition, NGF can substitute for serum factors to stimulate growth of cells expressing myc or E1A.

PC12 cells normally respond to NGF with rapid and transient increases in transcription of c-fos, c-myc, actin and ODC (Greenberg et al., 1985). These early effects on cell gene expression resemble changes induced in several cell types stimulated by mitogens (Kelly et al., 1983). Reasons for similar responses to agents which promote cell proliferation and differentiation are unclear. Some genes induced by external stimuli may not contribute to resulting cell phenotypes. Indeed agents such as epidermal growth factor, insulin, and tumor promoting phorbol esters also induce fos, myc and ODC transcription in PC12 cells, but do not promote growth arrest or differentiation (Greenberg et al., 1985).

Alternatively, transcriptionally induced genes may indicate that overlapping signals participate in both differentiation and proliferation. Thus, NGF can stimulate the growth of PC12 variants which have been selected for their inability to growth arrest and differentiate (Burstein and Greene, 1982) and NGF may induce one replicative cycle in PC12 cells prior to differentiation (Boonstra et al., 1983). Finally, primary cultures of

normal chromaffin cells respond mitogenically to NGF before differentiating into a neuronal cell type (Lillian and Claude, 1985).

Decreased myc expression accompanies differentiation and growth arrest in a variety of cell types (Westin et al., 1982; Reitsma et al., 1983; Campisi et al., 1984; Gonda and Metcalf, 1984). In contrast, c-myc transcription and steady-state RNA concentrations are not reduced in differentiated PC12 cells (Greenberg et al., 1985). This may reflect the fact that PC12 cells do not terminally differentiate in response to NGF. Nevertheless, transfected c-myc genes alter the response of PC12 cells to NGF probably as a result of elevated or de-regulated myc expression. Our results indicated that myc and E1A uncouple morphological differentiation and growth arrest from the mitogenic effects of NGF.

Previous studies have demonstrated that myc can reduce or eliminate cellular requirements for external stimuli necessary for growth, including serum, platelet derived growth factor, IL-2 and IL-3 (Shiroki et al., 1979; Keath et al., 1984; Mougneau et al., 1984; Armelin et al., 1984; Rapp et al., 1985). Consequently, myc could participate in carcinogenesis by enabling cells to proliferate without appropriate external stimuli. However, the present study indicates that c-myc can play a larger role regulating cell growth than simply reducing or eliminating growth factor requirements. Thus, myc alters cellular responses to NGF by stimulating PC12 cells to proliferate in response to signals which normally promote growth arrest and differentiation.

The present study clearly demonstrates that a single receptor-activated signal can promote either differentiation and growth arrest or proliferation,

depending on the physiological state of the cell. These results provide one mechanism to explain how hormonal factors identified by virtue of growth promoting effects on one cell type often promote growth arrest or differentiation when assayed in a different context or in other cell types. The biochemical mechanism whereby myc and E1A regulate cellular responses to NGF is presently unknown. However, situations in which alternative cellular responses to external stimuli can be regulated by transfected genes should help delineate signalling pathways controlling cell growth and differentiation.

CHAPTER IV

CONSTITUTIVE EXPRESSION OF C-MYC OR E1A PREVENTS THE INDUCTION OF MICROTUBULE ASSOCIATED PROTEINS AND NEURITE OUTGROWTH IN PC12 CELLS

Agents that elevate cellular concentrations of cAMP such as cholera toxin, partially mimic the neurite outgrowth response observed with NGF (Schubert et al., 1977; Gunning et al., 1981; Heidemann et al., 1985). Cholera toxin induced neurites are qualitatively different from NGF induced neurites in that they are shorter and unbranched. However, when added together NGF and cholera toxin appear to be synergistic in their effects on neurite outgrowth. Under these conditions neurite extension is more rapid and the neurites formed are considerably longer and more branched than those produced by NGF alone (Gunning et al., 1981; Heidemann et al., 1985). These observations suggest that the mechanisms of NGF and cAMP-induced neurite outgrowth are not identical or that the short extensions induced by cAMP may represent early stages of neurite outgrowth that are incapable of progressing morphologically into true neurites. Furthermore, studies using inhibitors of transcription have suggested that cAMP induction of neurite-like processes is independent of transcription while NGF-induced neurite extension requires transcriptional events (Gunning et al., 1981; Burstein and Greene, 1978).

Given that NGF and cAMP differentially regulate neurite extension, the ability of c-myc or E1A expression to influence NGF and cAMP-directed

neurite outgrowth was examined. As shown in Figure 9, PC neo clones responded to both NGF and cholera toxin in a manner analogous to wild type PC12 cells. Neurites induced by NGF (Figure 9B) were long and branched while those processes produced by cholera toxin (Figure 9D) were unbranched and relatively short, less than 2 to 3 times the cell body diameter. Furthermore, PC neo cells grown in the presence of both NGF and cholera toxin (Figure 9C) possessed a more pronounced neurite network characteristic of the synergistic response normally observed in parental PC12 clones exposed to both agents (Gunning et al., 1981). In contrast to PC neo cells, the overall morphology of both PC myc and PC1A clones (Figure 9E and I) was dramatically altered. Cells constitutively expressing c-myc or E1A were larger and flatter than parental PC12 and PC neo clones and tended to adhere more tightly to each other and to the plastic culture dish. Furthermore, PC myc and PC1A failed to respond to NGF (Figure 9F and J), cholera toxin (Figure 9H an L) or to the simultaneous addition of NGF and cholera toxin (Figure 9G and K) with cell shape changes or the extension of neurites. These results suggest that the constitutive expression of c-myc or E1A interferes with both the NGF and cAMP-induced pathways for neurite outgrowth. The constitutively expressing c-myc or E1A clones were further examined in an attempt to define the mechanism by which these genes could interfere with NGF and cAMP. It was also of interest to determine whether NGF and cAMP action were affected in a similar manner in order to better understand the differences and similarities in the induction of neurite extension by these two agents.

Figure 9. Phase Contrast Photomicrographs of Derivative PC12 Clones in the Absence or presence of NGF and/or Cholera Toxin. 2×10^5 cells were plated per 35 mm^2 plastic dish and grown for 24 hours in DME plus 10% FCS and 10% HS before supplementing media with NGF or cholera toxin. PC neo (A-D), PC myc (E-H) and PC1A (I-L) were grown for 7 days in the absence (A,E,I) or presence of 100 ng/ml NGF (B,F,J), 0.5 μg/ml cholera toxin (D,H,L) or NGF plus cholera toxin (C,G,K).
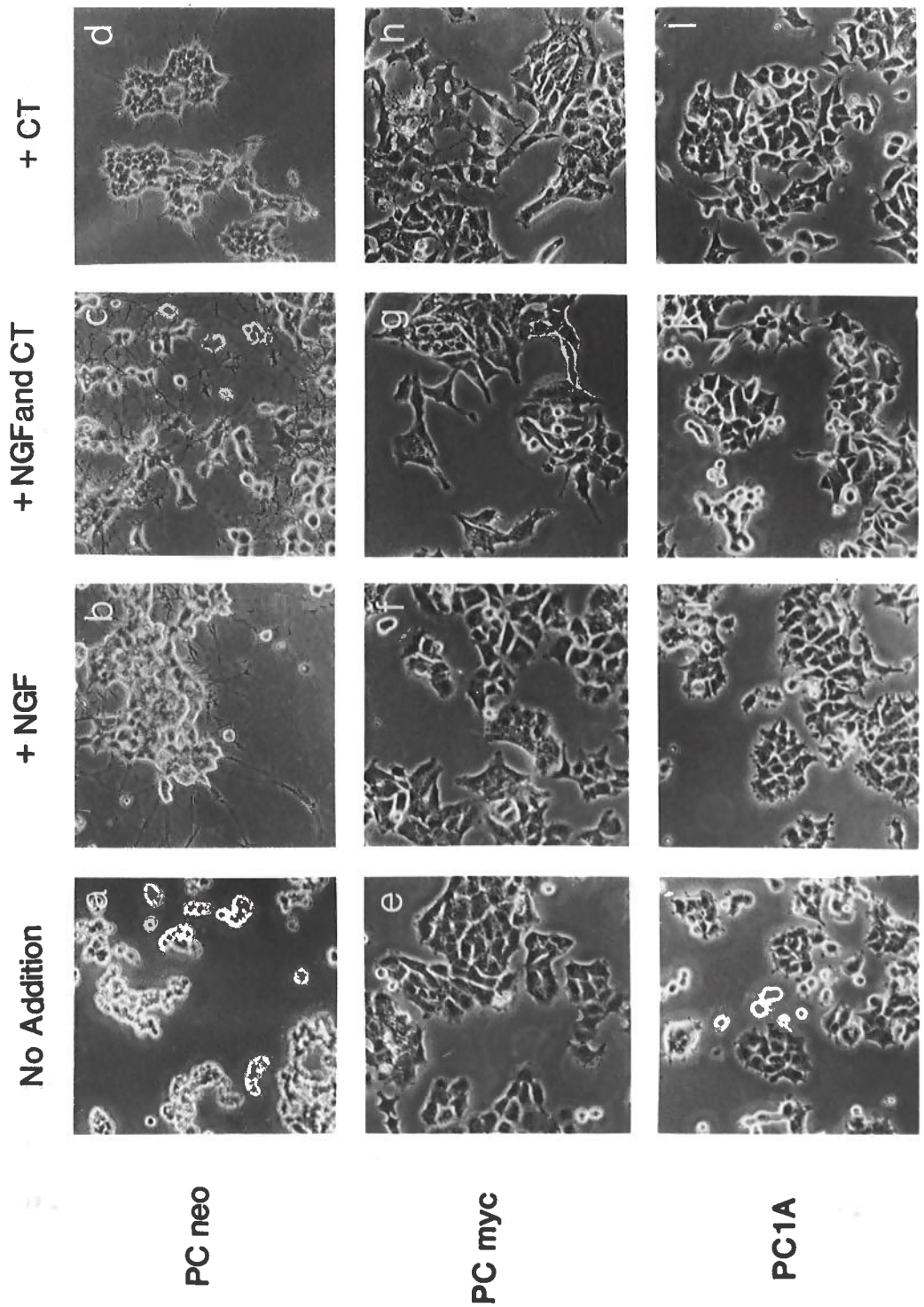
NGF AND cAMP SIGNALLING MECHANISMS ARE NORMAL IN PC12 CELLS EXPRESSING C-<u>MYC</u> OR E1A

c-<u>myc</u> or E1A expression did not alter NGF receptor binding properties in PC12 cells. To determine if the receptors were signalling normally, the activities of two NGF regulated protein kinases were measured in each of the various clones. The ribosomal S6 kinase is maximally activated within 15 minutes of NGF addition and is stably maintained for at least 24 hours (Blenis and Erickson, 1986; Heasley and Johnson, 1988). A novel serine/threonine protein kinase, identified by its ability to phosphorylate a peptide of the myosin light chain in a Ca^{2+}/calmodulin-independent manner, is maximally activated within 15 to 30 minutes of NGF addition and returns to its basal level within 3 to 5 hours (Heasley and Johnson, 1988). Table 4 shows that NGF stimulated both the MLC peptide kinase and the S6 kinase in PC12, PC neo, PC myc, and PC1A clones. Both the basal and NGF stimulated activities for MLC peptide kinase and S6 kinase were slightly elevated in PC myc clones when compared to the activities measured in PC12 and PC neo clones. PC1A cells appeared to have slightly lower basal and NGF stimulate MLC peptide kinase activities as well as a depressed response to NGF's stimulation of S6 kinase in comparison to PC12 and PC neo clones. However, none of these differences were statistically significant and there was no consistent trend in the ability of c-<u>myc</u> or E1A expression to alter kinase activities. These results indicate that receptor signalling mechanisms in PC myc and PC1A clones are similar to wild-type cells, but constitutive <u>myc</u> or E1A expression alters the response of PC12 cells to these signalling pathways.

Table 4. Protein kinase activities in PC12 cells and transfected clones

MLC peptide kinase and S6 peptide kinase activities were assayed as described in Materials and Methods following a 15 minute incubation with or without 100 ng/ml NGF while cAMP-dependent protein kinase was assayed in untreated cells. Basal cAMP-dependent protein kinase activity was defined as the Kemptide kinase activity inhibited by 25 μg/ml Ip-20 (Cheng et al., 1986) and total cAMP-dependent protein kinase was defined as the IP-20-sensitive activity measured in the presence of 3 μM cAMP. The data is the means ± SEM of three independent experiments. While both the basal and NGF stimulated activities for MLC peptide kinase and S6 kinase varied slightly among the clones, none of these differences were statistically significant and no consistent trend could be found in the ability of myc or E1A expression to alter kinase activities.

	MLC Peptide Kinase pmol/min/mg		S6 Peptide kinase pmol/min/mg		cAMP-Dependent Protein Kinase pmol/min/mg protein		
	Control	NGF-treated	Control	NGF treated	Basal	+cAMP	cAMP/+cAMP
PC12	30.7 ± 5.3	54.2 ± 1.3	45.6 ± 2.7	91.7 ± 10.3	293.3 ± 103.80	1381.5 ± 512.0	0.22 ± 0.014
PC neo	29.6 ± 7.1	56.3 ± 10.3	43.8 ± 9.8	107.3 ± 18.6	435.8 ± 60.3	1707.8 ± 421.5	0.28 ± 0.040
PC myc	45.5 ± 4.2	64.4 ± 14.2	79.9 ± 13.1	112.8 ± 20.8	252.8 ± 111.8	1457.7 ± 74.6	0.18 ± 0.02
PC1A	22.4 ± 0.6	32.0 ± 4.6	47.9 ± 7.5	60.6 ± 3.0	269.1 ± 86.0	1170.8 ± 195.6	0.22 ± 0.040

Since cAMP induces its biological effects through the activity of cAMP-dependent protein kinase (Edelman et al., 1987), the activity of this kinase was measured in each of the PC12 clones to determine if it could be altered by the expression of c-myc or E1A. As shown in Table 1, cAMP-dependent protein kinase was present with similar basal and cAMP-stimulated activities in each of the PC12 clones. These results indicate that neither c-myc or E1A expression interferred with cAMP-dependent protein kinase expression since the activities measured in PC myc and PC1A clones were similar to activities measured in parental PC12 and PC neo clones. Thus, the inability of cAMP to induce neurite extension in PC myc and PC1A clones cannot be explained by the loss of cAMP-dependent protein kinase. c-myc or E1A expression must therefore interfere with another component that is involved in the cAMP regulated pathway for neurite growth. One possibility is that the c-myc or E1A activities have influenced expression of a substrate for cAMP-dependent protein kinase which is required for cAMP regulated neurite extension. Alternatively, c-myc or E1A expression may alter the activities of yet another kinase, which is regulated by cAMP-dependent protein kinase.

HIGH MOLECULAR WEIGHT MAPS ARE REGULATED BY NGF IN PC12 CELLS

c-myc or E1A expression inhibits NGF induced neurite outgrowth without interfering with NGF's binding to its receptor (Table 2 and Figure 8) or the activation of ribosomal S6 kinase or MLC peptide kinase (Table 4). cAMP induced neurite extension is also blocked by the expression of these

genes even though cAMP-dependent protein kinase remains normal in the transfected cell lines. These results suggest that the expression of c-myc or E1A blocks the differentiation pathways distal to primary signalling events. The adenovirus E1A gene encodes two distinct nuclear proteins which have the ability to specifically activate or repress both viral and cellular genes (Berk, 1986). The nuclear localization of c-myc protein and structural similarities to E1A has been used to suggest a possible role for c-myc in regulation of transcription. myc or E1A expression could alter secondary events associated with neurite outgrowth by affecting expression of specific genes required for extension and/or stabilization of microtubules. Alternatively, c-myc or E1A could affect regulation of such gene products. To distinguish these possibilities, we examined NGF's regulation of MAPs.

Several observations suggest that MAPs may play a key role in regulating the extension and/or the stabilization of neurites. In vitro experiments have shown that MAPs promote microtubule polymerization (Cleveland et al., 1977; Murphy et al., 1975; Sloboda et al., 1976; Weingarten et al., 1975). Evidence also suggests that these proteins play a role in controlling spatial arrangements of microtubules (Bloom et al., 1984b; Brown and Berline, 1985; Kim et al., 1979; Murphy et al., 1985; Vallee and Davis, 1983).

Four immunologically distinct high molecular weight MAPs, classified as MAP 1A, 1B, 1C and 2, on the basis of mobility on SDS-acrylamide gel electrophoresis, have been detected in brain (Bloom et al.

Figure 10. Identification of high molecular weight microtubule associated proteins using the taxol purification procedure. Partial purification of tubulin and HMW MAPs is described in detail in methods. Briefly 1 mg of packed PC12 cells was hypotonically lysed and particulate matter was removed by high speed centrifugation. The resulting extract was incubated at 37°C in the presence of 20 µM taxol and 1 mM GTP followed by centrifugation. (A) Coomassie blue stained polyacrylamide gel of various fractions obtained. (B) Duplicate gel shown in A was transferred to nitrocellulose and immunoblotted with anti-HMW MAP antibodies as described in Methods. E, extract; S, supernatant obtained after centrifugation of taxol treated extract; P, taxol pellet.
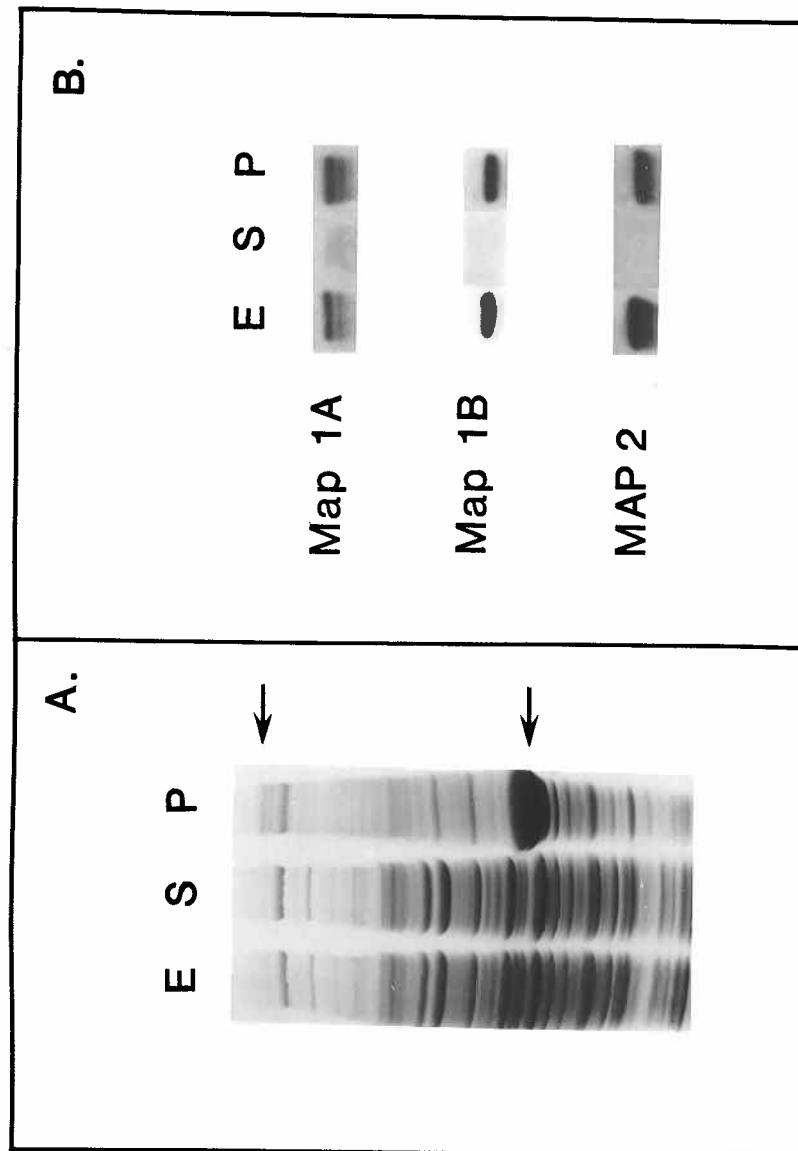
1984a,b and 1985). Differential localization and temporal expression during development suggests that these proteins may have specialized functions and may be differentially regulated. To determine if other HMW MAPs in addition to MAP 1B are present in PC12, tubulin and its associated proteins were partially purified using taxol, a drug which induces polymerization of microtubules (Vallee, 1982). The taxol procedure pelleted tubulin and a group of high molecular weight proteins of approximately 300-350 kilodaltons from a PC12 total cell extract (Figure 10A). To determine if these proteins are related to the high molecular weight MAPs previously identified in calf brain, the taxol fractions were immunoblotted using specific monoclonal antibodies against MAP 1A, 1B, 1C and β-tubulin (Figure 10B). Anti-MAP 1A, 1B, and 2 antibodies bound selectively to single bands within the molecular weight region of approximately 300-350 kilodaltons. Furthermore, antibodies bound to single bands within the total extract and taxol pellet fractions but did not bind to the supernatant fraction. Binding of tubulin antibody to a 55 kilodalton protein band was observed in the total extract and taxol pellet fraction but absent in the supernatant fraction. Precipitation of HMW MAPs using taxol was quantitative. These data show that three high molecular weight MAPs, MAP 1A, 1B and 2 are all present in PC12 cells. MAP 1C was not detected and is apparently not expressed in PC12 cells.

An important criterion used to define a protein as a functional MAP is to show association of the protein with microtubules in whole cells. Although most antibodies isolated against high molecular weight MAPs have been shown to bind to microtubules in situ (Bloom et al., 1984b), anti-MAP 1

Figure 11. Immunofluorescent detection of HMW MAPs in PC12 cells grown in the presence or absence of NGF. 1×10^5 PC12 cells were plated on poly-L-lysine coated glass coverslips placed in 35 mm^2 culture dishes. Cells were grown for 7 days in DME plus 10% horse serum and 10% fetal calf serum in the absence or presence of 100 ng/ml NGF. Coverslips were stained with anti-MAP 1A (C, D), anti-MAP 1B (E, F), anti-MAP 2 (G, H) or β-tubulin (A, B), followed by rhodamine sheep anti-rabbit IgG as described in Methods.

69
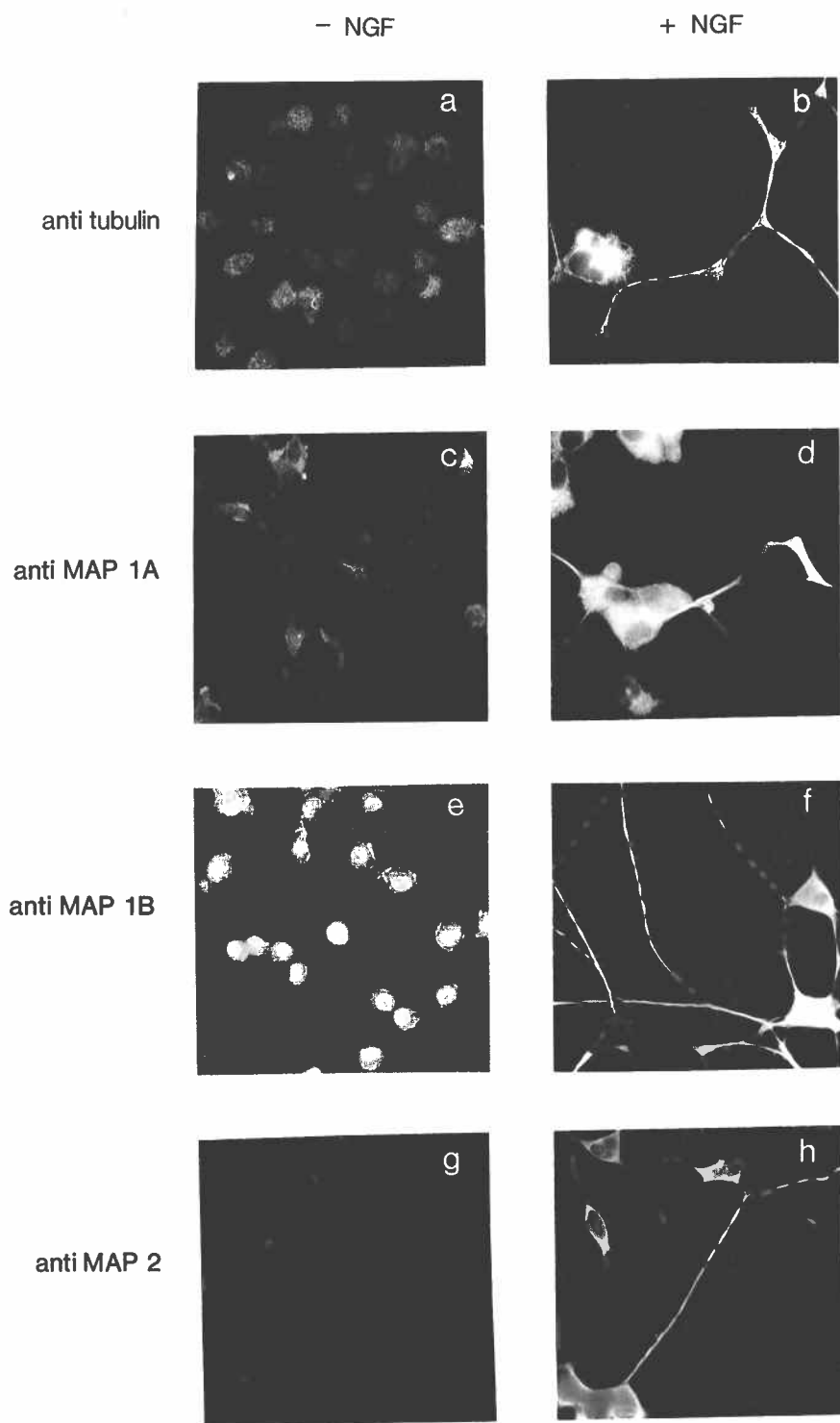
antibodies have also been isolated which associate with reassembled brain microtubules in vitro but stain stress fibers and not microtubules in fixed cells (Asai et al., 1985). These results suggest that MAP 1 polypeptides may be a group of heterogenous proteins that are structurally related but have different functions. Thus, it is necessary to show that a specific MAP protein is associated with microtubules in whole cells before assigning a role for that protein in regulation of formation or organization of microtubules. PC12 cells grown in the absence or presence of NGF for 7 days were stained with each of the four monoclonal antibodies (anti-beta tubulin, anti-MAP 1A, anti-MAP 1B, and anti-MAP 2), using indirect immunofluorescence methods. Anti-tubulin stained a diffuse pattern throughout the cytoplasm in control PC12 cells (Figure 11A). Upon differentiation induced by NGF, anti-tubulin staining was distributed in a punctate pattern along the neurite extensions as well as the cytoplasm (Figure11B). Anti-MAP 1B staining appeared to be concentrated around the nucleus in control PC12 cells (Figure 11E). This reflects the relative thickness of the rounded cells and the plane of focus. MAP 1B was found to be distributed throughout the cytoplasm when examined at several planes of focus in both control and NGF treated cells. Anti-MAP 1B stained neurites in a punctate pattern similar to anti-tubulin staining (Figure 11F). Anti-MAP 1A in control cells appeared to be diffusely distributed throughout the cytoplasm but was markedly absent within or around the nucleus (Figure 11C). This is in contrast to the pattern observed with anti-MAP 1B. Neurite staining with anti-MAP 1A was similar to that of anti-tubulin (Figure 11D). Anti-MAP 2 staining is virtually undetected in control PC12 cells (Figure 11G). However, after NGF-

induced differentiation anti-MAP 2 staining was distributed throughout the cytoplasm with a slightly higher density along the plasma membrane, and in a punctate pattern along neurite extensions (Figure 11H).

These results indicated that high molecular weight MAPS, 1A, 1B and 2, are associated with microtubules in differentiated PC12 cells since the pattern of immunoreactivity using antibodies to each of these proteins was similar to the staining pattern of anti-tubulin. In addition, the immunofluorescence data suggested a role for NGF in regulation of these proteins since an increase in immunoreactivity was observed in differentiated PC12 cells with antibodies to MAP 1A and MAP 2.

HMW MAPS ARE INDUCED COORDINATELY BY NGF BUT NOT BY CHOLERA TOXIN

To quantitatively examine the regulation of HMW MAP expression by NGF and cholera toxin, immunoblotting was performed using whole cell extracts from PC12 cells grown in the presence of NGF or cholera toxin for 0, 1, 3 or 7 days. The levels of MAP 1A, 1B and 2 detected immunologically in untreated PC12 cells appeared to be present in relatively equivalent abundance (Figure 12). The concentration of all three proteins increased concurrently in response to the addition of NGF. Quantitation of these results indicated that each of these proteins increased approximately 2 to 3-fold within 3 days of NGF treatment and were maintained at these new concentrations (Figure 13).

The time course for induction of HMW MAPs is consistent with a role of these proteins in neurite extension. Maximal induction of HMW MAPs

Figure 12. Effect of NGF and/or cholera toxin on the expression of HMW MAPs and tubulin. PC12 cells were grown on 100 mm plastic dishes in normal growth medium supplemented with 100 ng/ml NGF and/or 1.0 μg/ml cholera toxin for 0, 1, 3 or 7 days. Whole cell extracts were prepared and immunoblot analysis using monoclonal antibodies specific for MAP 1A, MAP 1B, MAP 2 or β-tubulin was as described in Methods. 100 μg of protein was loaded in each lane.
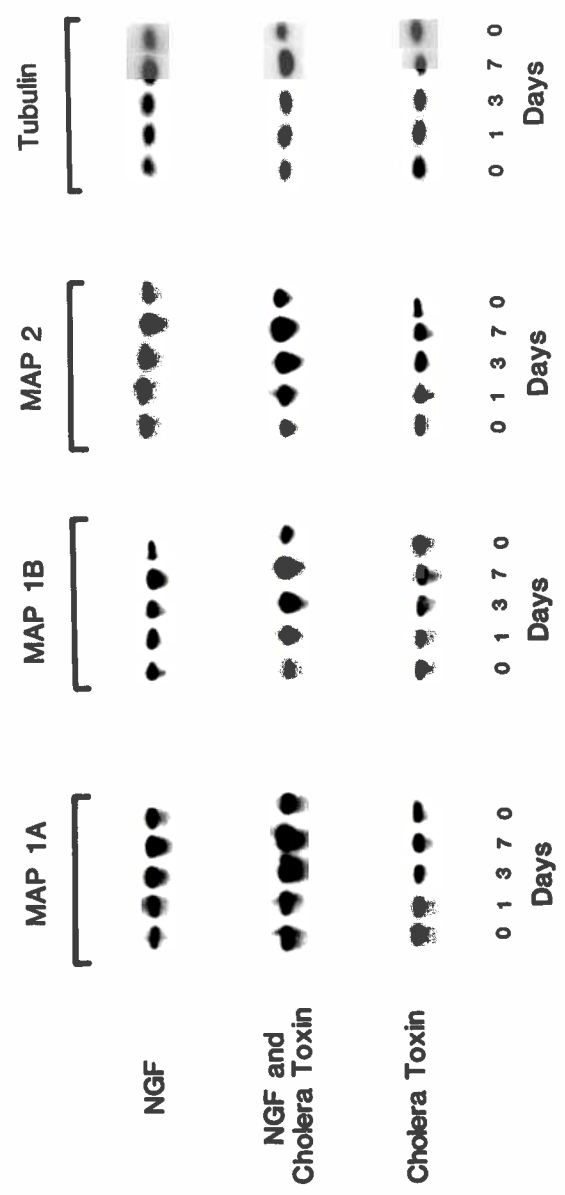
Figure 13. Time course of HMW MPA and β-tubulin expression in PC12
cells treated with NGF and/or cholera toxin. PC12 cells were grown on 100
mm plastic dishes in normal growth medium supplemented with 100 ng/ml
NGF and/or 1.0 μg/ml cholera toxin whole cell extracts were prepared and
immunoblot analysis was as described in Materials and Methods.
Quantitation was achieved by overlaying autoradiograph on the
nitrocellulose and excising appropriate bands from filter. The excised bands
were counted on a gamma counter. Values represent the mean from four
independent experiments including the experiment presented in figure 12.
(O) NGF, (O) NGF and cholera toxin, (Δ) cholera toxin.
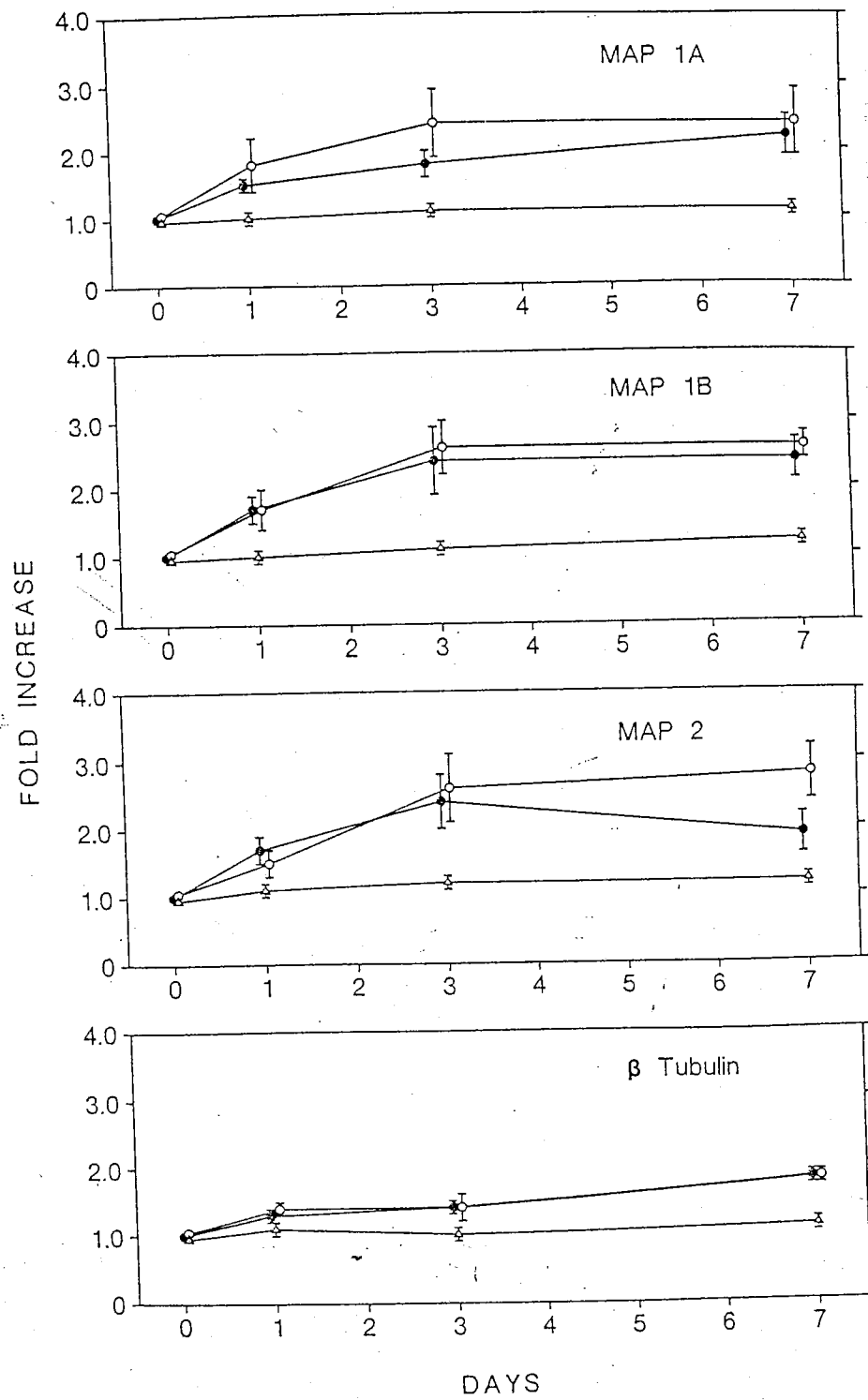
occurs on day 3 of NGF treatment, the time when significant increases in microtubule mass and neurite length begin (Black et al ., 1986; Drubin et al., 1986). During the first 2 to 3 days of NGF treatment, microtubule mass remains relatively constant. The short processes induced within 24 hours of NGF addition therefore apparently involves the reorganization of pre-existing microtubules rather than a net increase in microtubule polymerization. The coordinate induction of HMW MAPs with an increase in microtubule mass implies that increased MAP expression is not required for the initial re-organization of existing microtubules but rather may be important in the formation and/or stabilization of new microtubules.

The cellular concentration of tubulin was also regulated by NGF. A linear increase in tubulin levels occurred from 0 to 7 days of NGF exposure so that on day 7 there was an approximate two-fold increase in tubulin compared to controls (Fig 12 and 13). Similar results were obtained using a monoclonal antibody which specifically recognizes α-tubulin. These results agree with previous studies which report that the concentration of both α and β-tubulin increase 2 to 3-fold relative to total cellular protein (Black et al., 1986; Drubin et al., 1986).

In contrast to the effects of NGF on HMW MAPs and tubulin, cholera toxin did not alter the expression of MAP 1A, 1B, 2 or β-tubulin (Figure 12 and 13). Furthermore, cholera toxin did not enhance NGF's induction of HMW MAPs or tubulin when both agents were added simultaneously. Neurite processes formed in response to elevated cAMP are similar to early processes induced within the first 2-3 days of NGF exposure in that they involve re-organization of existing microtubules (Gunning et al., 1981) and

do not require increased expression of HMW MAPs or tubulin. The failure of cholera toxin to induce HMW MAP expression offers at least one plausible explanation for its inability to direct the formation of long, stable neurites similar to those produced by NGF.

C-MYC OR E1A EXPRESSION PREVENTS THE REGULATION OF HMW MAPS BY NGF

Expression of HMW MAP 1A, 1B and MAP 2 was measured in the variant PC12 cell lines grown in the presence or absence of NGF for 7 days to determine if c-myc or E1A expression could block neurite extension by interfering with the expression or regulation of these proteins (Figure 14). The relative concentrations of HMW MAPs were not significantly altered by c-myc or E1A expression in several replicative experiments. However, HMW MAPs were not induced by NGF in PC myc or PC1A clones while PC neo clones (PC neo) responded normally to NGF by inducing HMW MAPs (Figure 14). Although NGF's regulation of HMW MAPs has not been directly shown to occur at the level of transcription, mRNA levels for at least one MAP 1 species increases several-fold in response to NGF, similar to that observed for the proteins (Lewis et al., 1986b). Furthermore, NGF's induction of MAP 1B can be blocked by inhibitors of transcription (Greene et al., 1983). These results are consistent with the hypothesis that the inappropriate expression of c-myc or E1A interferes with neuronal differentiation by inhibiting transcription-dependent responses.

While blockage of HMW MAP induction provides a possible explanation for the ability of c-myc or E1A expression to interfere with

Figure 14. The effect of c-myc or E1A expression on the expression and regulation of HMW MAPs by NGF. PC neo, PC myc, and PC1A clones were plated at a density of 1×10^6 cells/100 mm^2 dish and grown in the presence or absence of 100 ng/ml NGF for 4 days. Whole cells extracts were prepared and immunoblot analysis using monoclonal antibodies specific for MAP 1A, MAP 1B, and MAP 2 was as described in methods. 100 μg of total cellular protein was loaded for PC neo lanes. 50 μg of total protein was loaded for each lane from PC myc and PC1A clones.
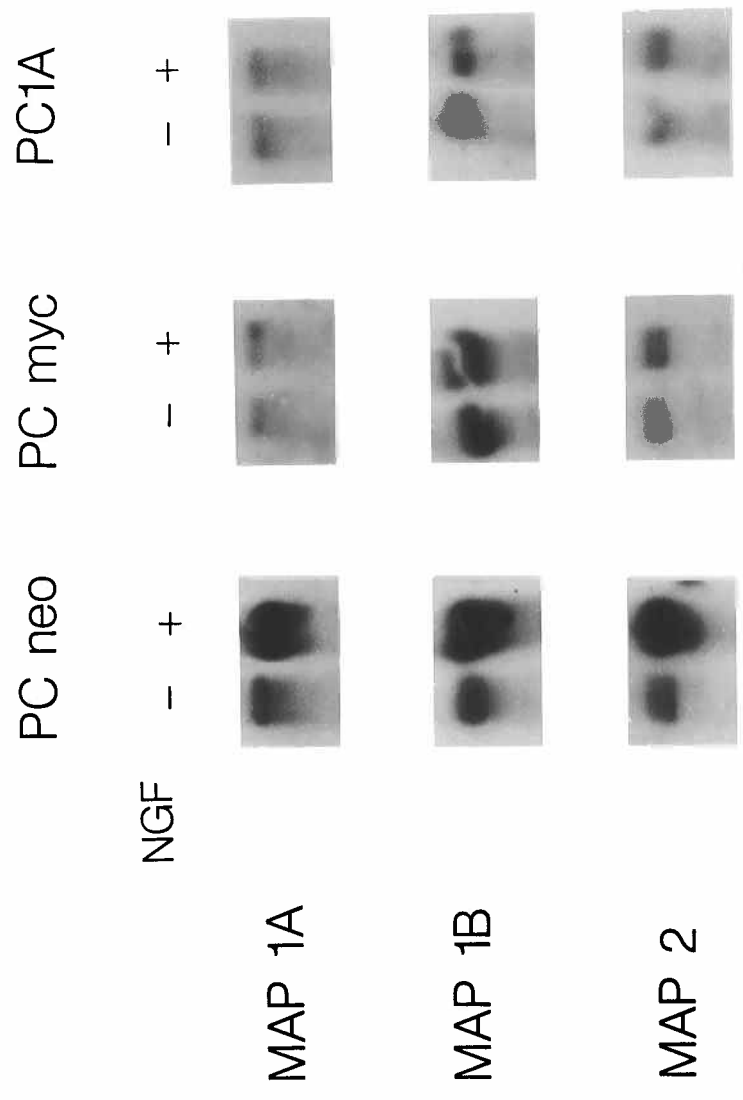
NGF-induced neurite growth it cannot account for their ability to inhibit cholera toxin regulated neurite extension, as cholera toxin does not appear to increase the expression of HMW MAPs. The basal expression of HMW MAPs (Figure 14) or the activity of cAMP-dependent protein kinase (Table 4) was not altered in PC myc and PC1A clones. It has been proposed that cAMP directs neurite extension through activation of cAMP-dependent protein kinase and its subsequent phosphorylation of MAPs. If this was the case, then expression of c-myc or E1A would not be expected to interfere with cholera toxin's induction of neurites. These results indicate that additional gene products other than HMW MAPs must be involved in the mechanism of cAMP-directed neurite extension.

C-MYC OR E1A EXPRESSION PREVENTS THE REGULATION OF TAU BY NGF

Two additional groups of MAPs, tau proteins and chartins, have been implicated in the participation of neurite extension within PC12 (Black et al., 1986; Drubin et al , 1986). Three tau species with apparent molecular weights of 61, 68 and 125 kDa have been previously identified in PC12 cells. The induction of these polypeptides upon NGF treatment is similar to that observed for HMW MAPs.

Immunoblot analysis of tau expression in the various clones was performed using whole cell extracts from cells grown in the presence or absence of NGF, cholera toxin, or NGF and cholera toxin for 4 days. As shown in Figure 15, 3 polypeptides of 61, 68 and 125 kDa were recognized by the anti-tau antibody in each of the clones. Furthermore, the relative

Figure 15. Regulation of tau proteins in response to NGF and/or cholera toxin in variant PC12 clones. PC neo, PC myc, and PC1A clones were plated on plastic culture dishes at a density of 1 x 10^6 cells/100 mm dish and grown in the absence or presence of 100 ng/ml NGF and/or 0.5 μg/ml cholera toxin for 4 days. Preparation of whole cell extracts and immunoblot analysis of tau was as described in Methods. 30 μg of protein was loaded in each lane.
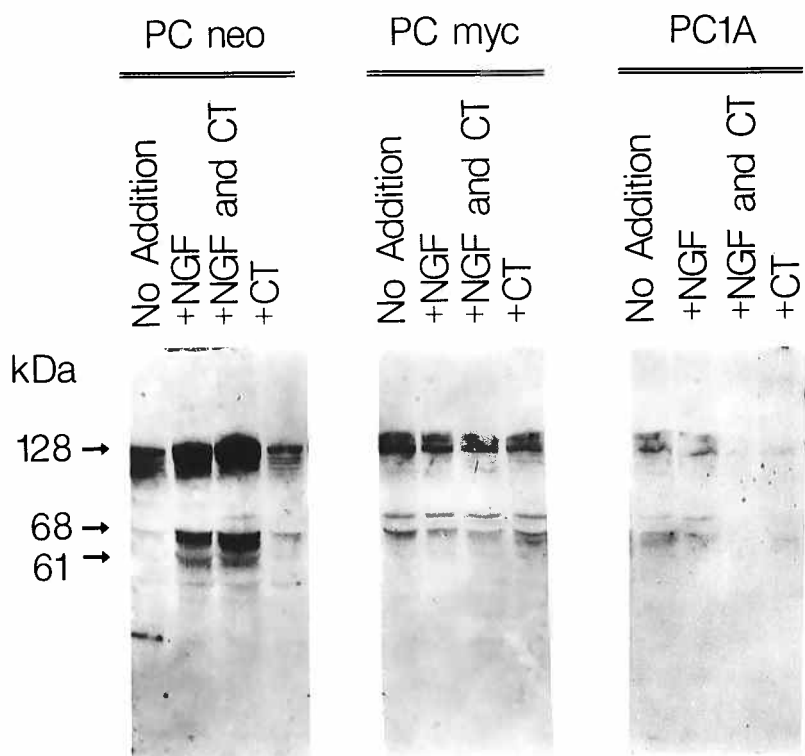
levels of these 3 polypeptides present in PC myc and PC1A were similar to the control PC neo clone in several experiments. NGF addition caused a marked stimulation of all 3 tau polypeptides in PC neo clones (Figure 15).

While PC12 clones constitutively expressing c-myc or E1A did not exhibit altered basal expression of tau, these clones failed to induce tau in response to NGF (Figure 15). Like HMW MAP induction, the increase in tau protein appears to reflect an elevation in tau mRNA concentrations (Drubin et al., 1988). Tau proteins are also elevated in NGF-primed cells further suggesting the involvement of a transcriptional event in their regulation by NGF (Drubin et al., 1986). Myc and E1A may interfere with NGF's stimulation of tau and HMW MAPs by influencing a pathway common to the regulation of both MAP classes.

As shown in Figure 15, cholera toxin did not increase the expression of tau in PC neo, PC myc, or PC1A clones, or enhance NGF's stimulation of tau in PC neo. A decreased expression of tau polypeptides was observed in PC1A clones treated with cholera toxin and NGF or cholera toxin alone. Similar results were obtained when cells were treated with dibutyryl cAMP instead of cholera toxin. The ability of PC12 and PC neo clones to extend processes in response to cholera toxin, without any increase in tau expression indicates that tau induction is not a necessary event in the cAMP directed reorganization of microtubules during neurite formation in PC12. Furthermore, the inability of PC myc and PC1A clones to extend neurites in response to cholera toxin cannot be due to any effects on the expression or the induction of tau proteins since tau proteins are expressed at normal

concentrations in these clones, and induction of tau is not a requirement for cholera toxin directed neurite outgrowth.

Recent studies suggest that a third class of MAPs, the chartins might also be regulated by NGF in PC12 cells during neurite extension (Pallas and Solomon, 1982; Black et al., 1986; Aletta and Greene, 1987). Alterations in the levels of chartins could potentially alter the ability of cells to form neurites in response to NGF and cAMP. However, preliminary finding (not shown) indicate that the chartins are also present in normal quantities in PC12, PC neo, PC myc, and PC1A clones.

These results indicate that c-myc and E1A do not block neurite extension by altering the basal steady-state expression of any of the three classes of microtubule associated proteins, HMW MAPs, tau, or chartins. Because cholera toxin-induced neurite extension is inhibited despite normal activities of cAMP-dependent protein kinase, these results further imply that reorganization of microtubules requires an additional step(s) besides simply phosphorylation of microtubule associated proteins by cAMP-dependent protein kinase.

The inability of NGF to elevate MAP expression in cells which do not extend neurites is consistent with a role of MAPs in either microtubule elongation or stabilization. However, the time course of induction of HMW MAPs and tau indicates that elevation of these proteins is not responsible for the early changes in microtubule organization induced by NGF. Expression of c-myc and E1A inhibited the early neurite extensions without altering the expression of several kinases or MAPs that have been proposed to be associated with NGF action. Our results indicate that additional gene

products, as yet unidentified, are involved in the initial regulation of neurite extension besides the HMW, tau and chartin MAPs. The myc and E1A expressing clones may be used in conjunction with parental PC12 cells in differential screening procedures to identify proteins whose expression is regulated by c-myc or E1A which are important in regulating neurite extension.

CHAPTER V
TRANSFECTION OF PC12 CELLS WITH
MTV/<u>MYC</u> FUSION GENES

The results presented in Chapters III and IV demonstrate that constitutive elevation of c-<u>myc</u> expression does not trigger neuronal differentiation or enhance NGF's ability to induce differentiation. Moreover, these results show that differentiation can not proceed while c-<u>myc</u> levels remain elevated suggesting that the temporal regulation of c-<u>myc</u> expression is critical for the NGF-directed differentiation program. The aim of the work presented in this chapter was to introduce an exogenous c-<u>myc</u> gene into PC12 cells under control of a promoter which could be regulated independently of NGF addition. This strategy would allow c-<u>myc</u> expression to be manipulated at different times during the NGF-induced differentiation program to define more precisely the influence of both the activation and the subsequent repression of c-<u>myc</u> transcription on NGF action.

PC12 cells were co-transfected using protoplast fusion with an exogenous mouse c-<u>myc</u> gene under control of the dexamethasone-inducible mouse mammary tumor virus promoter (pMTV Hd3 myc or pMTV Xba myc) and a plasmid containing the selectable marker, aph, (pKO neo). Clones were selected by their ability to grow in the presence of G-418 to insure that selection was neutral with regard to <u>myc</u> expression and differentiation potential. The efficiency of transfection using protoplast fusion was greater than the efficiency using the calcium phosphate method. While two and three G-418 resistant clones were isolated from 5 x 10^6 cells

transfected with pM myc neo or P1A neo, using the calcium phosphate procedure, approximately 200 G-418 resistant colonies arose from each 5 x 10^6 cells co-transfected with pMTV Hd3 myc and pKO neo or pMTV Xba myc and pKO neo using protoplast fusion.

Colonies were selected using cloning cylinders approximately 3 weeks after the transfection. Twenty-four colonies from cells co-transfected with pMTV Hd3 myc/pKO neo and twenty-seven colonies co-transfected with pMTV Xba myc/pKO neo were randomly selected and grown to mass culture. Fifteen neo resistant clones were also selected randomly from cells transfected only with pKO neo.

IDENTIFICATION OF CLONES WHICH DID NOT GROW NEURITES IN THE PRESENCE OF DEXAMETHASONE

Clones were initially screened by their ability to respond to NGF with the extension of neurites in the presence or absence of dexamethasone (table 5). The rationale for this screening procedure was based on the result that cells constitutively expressing c-myc did not grow neurites in the presence of NGF. It was assumed that a cell which contained an exogenous MTV-linked c-myc gene would extend NGF-induced neurites when grown in the absence of dexamethasone since only the endogenous gene was expressed. However, the addition of dexamethasone would theoretically activate transcription of the exogenous c-myc gene above the steady state level and block NGF's induction of neurite outgrowth. Five clones: PC MTV Hd3 13, PC MTV Hd3

Table 5: NGF-regulated neurite extension in PC MTV transfected PC12
clones in the presence or absence of dexamethasone. Duplicate 15 mm^2 poly-
L-lysine coated dishes were plated with 1 x 10^4 cells of each cell line in DME
plus 2% FCS and cultured for 24 hours. Dexamethasone was added to one
well of each duplicate to a final concentration of 1mM. After an additional 24
hours the medium was replaced with medium supplemented with 100 mg/ml
NGF (for wells not treated with dexamethasone) or 100 mg/ml NGF and 1
mM dexamethasone (for wells treated with dexamethasone). After 7 days the
cells were scored for neurite outgrowth "+++" represents neurite lengths
similar to wild-type PC12 while "-" represents no neurite extension. Cells
were not scored on the % of cells bearing neurites. 15, PC MTV Xba 4A3,
PC MTV Xba 1C3, and PC MTV Xba 4A2 , responded to NGF with normal
neurite outgrowth similar to parental PC12

	NGF	NGF/DEX		NGF	NGF/DEX
PC12	+++	+++			
			PC MTV Xba myc		
PC MTV Hd3 myc			1A4	+++	++
			4A3	+++	-
19B	++	+	2C3	+++	+++
16	+	+	4B1	++	+
19A	++	+	5B1	-	-
9	+	+	2C1	+	+
4	++	+	2C5	-	-
40	-	-	2D5	+++	+++
15	+++	+	1C3	+++	+
22	+++	++	1B2	-	-
35	+++	+++	5A1	++	+
25B	++	+++	4C3	-	-
221	++	+	5A2	+	-
42	++	+	4C2	+++	-
21	++	++	4A5	+++	++
6A	++	+	4B4	-	-
30	++	+	2B4	+	-
32	++	++	2C4	+++	+++
7	++	++	5B3	+	+
8	++	++	2A3	++	+
33	-	-	4B2	+++	+++
2	+++	+++	1B3	+++	+++
13	+++	+	2C4	+++	+++
			2B4	++	-

15, PC MTV Xba 1C3, PC Xba 4A3, and PC MTV 4C2 extended neurites in the absence of dexamethasone but failed to extend neurites in the presence of dexamethasone (table 5). This response is demonstrated in figure 16. Both PC neo and PC MTV Xba 4A3 were morphologically similar to parental PC12 cells in the absence of either dexamethasone or NGF (Figure 16 A, E). Dexamethasone caused a slight rounding of PC neo cells (Figure 16B) while in comparison, PC MTV Xba 4A3 cells flattened considerably in response to dexamethasone (Figure 16F). PC neo clones treated with dexamethasone for 2 days followed by treatment with dexamethasone and NGF for seven days extended neurites (Figure 16C). No difference in the length of neurites or the number of cells possessing neurites after seven days of NGF treatment was observed with PC neo cells grown in the presence of dexamethasone compared to those grown in the absence of dexamethasone (Figure 16 C, D). In contrast, PC MTV Xba 4A3 clones did not estend neurites when grown in the presence of dexamethasone and NGF (figure 16G). Both the PC neo clones and PC MTV Xba 4A3 clones extended neurites when treated with NGF alone (figure 16 D,H). Based on their morphological responsiveness to dexamethasone or NGF it was postulated that these five clones contained a dexamethasone inducible c-myc gene.

Several clones: PC MTV Hd3 40, PC MTVHd3 33, PC MTV Xba 5B1, PC MTV Xba 2C1, PC MTV Xba 2CD, PC MTV Xba 1B2, PC MTV Xba 4C3, PC MTV Xba 1B4, PC MTV Xba 4B4 and PC MTV Xba 5B3, failed to extend neurites in either the presence or absence of dexamethasone. One such clone, PC MTV Hd3 33 is shown in figure 17. These ten clones were

Figure 16. Phase contrast photomicrograph of PC neo cells and PC MTV
Xba myc 4A3 cells in the absence or presence of dexamethasone and/or NGF.
Cells were plated onto poly-L-Lysine coated 24-well dishes at a density of
2×10^4 cells/well in DME plus 2% FCS. Four wells were seeded per cell
types. After 24 hours, two wells were supplemented with 1 µM
dexamethasone. 48 hours later the medium was replaced with fresh medium
containing no addition, 100 µg/ml NGF, 1 µM dexamethasone or NGF and
dexamethasone. Medium was changed every 2 days. Cells were
photographed after seven days of NGF treatment.

Figure 17. Phase contrast photomicrograph of PC MTV Hd3 myc in the absence or presence of dexamethasone and/or NGF. 2×10^4 cells/well were plated in four wells in a poly-L-lysine coated 24 well dish in DME plus 2% FCS. After 24 hours, two wells were supplemented with 1 μM dexamethasone. 48 hours later, the medium was replaced with fresh medium containing no addition, 100 μg/ml NGF, 1 μM dexamethasone, or NGF and dexamethasone. Medium was changed every two days. Cells were photographed after seven days of NGF treatment.
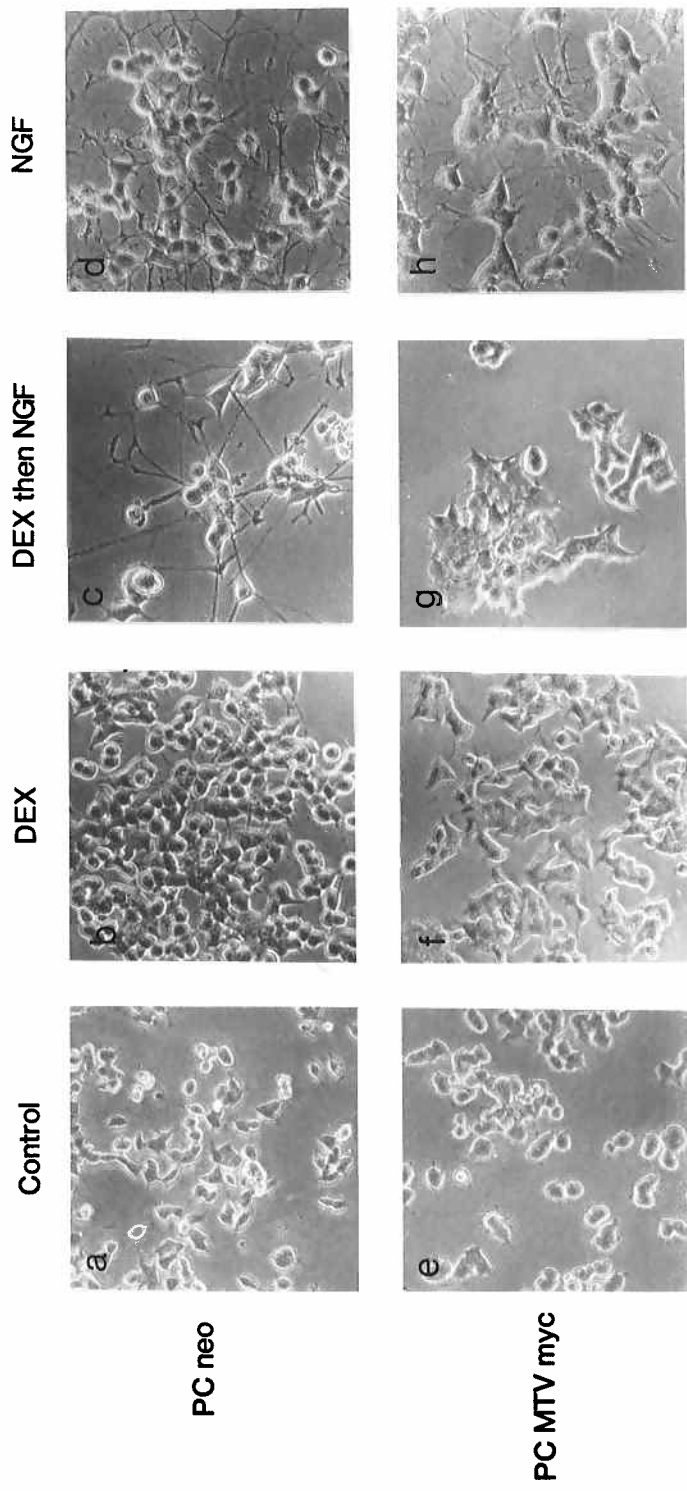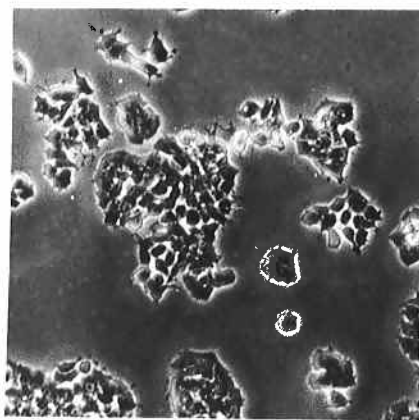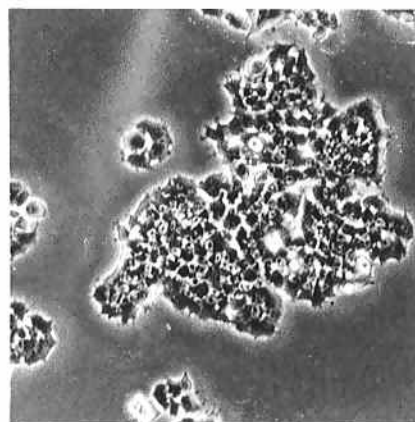
CONTROL +NGF
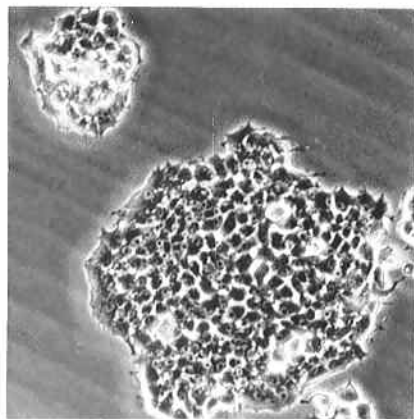
+DEX +DEX/NGF
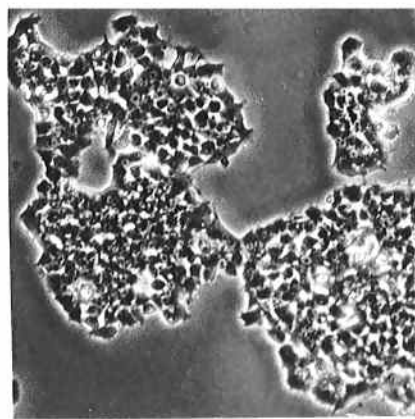
also notable in that they all showed grossly altered cellular morphology. Each of these clones were extremely flat and tended to grow in clumps in both the absence and presence of dexamethasone (compare PC MTV Hd3 33 cells in Figure 17 with PC neo cells in Figure 16A-D). Interestingly their morphology resembled that of the constitutive myc clones PC myc 41 and PC myc 42, suggesting the possibility that these clones also expressed exogenous c-myc constitutively. The remainder of clones isolated showed a range of dexamethasone-inhibited neurite outgrowth.

Eleven of the fifty-one clones screened responded to NGF in either the presence or absence of dexamethasone with extension of neurites similar to PC12. Because of the aph marker gene and the c-myc gene were introduced to the cells on separate plasmids, it was conceivable that these eleven clones represented cells which acquired only the aph gene. This was supported by the observation that fifteen clones which had been transfected with only the pKO neo cells also extended neurites in the presence of NGF and dexamethasone. The remainder of the clones co-transfected with the MTV-linked myc and the aph gene showed a range in the morphological responsiveness to NGF in either the absence or presence of NGF.

DNA BLOT ANALYSIS OF MTV-MYC TRANSFECTED PC12 CELLS

Figure 18 shows Southern blot analysis of genomic DNA isolated from individual clones probed with either the third exon of c-myc (A) or MTV-LTR-specific sequences (B). The endogenous c-myc gene appeared in all clones examined as an 8.8 kilobase band which hybridized to the c-myc specific probe (Figure 18A) but not the MTV-LTR-specific probe (Figure

Figure 18. Southern hybridization analysis of pMTV myc transfected cell lines. Genomic DNA extracted from the indicated cell lines was digested with Eco RI, (Eco RI does not cut within the MYV-linked myc gene. This DNA was electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose, and hybridized with a 1.0 kb pst I-pst I third exon myc probe (A) or a 2.2 kb Bam H1-Bam H1 MTV-LTR specific probe. (B) Specific details of this procedure are described in methods.
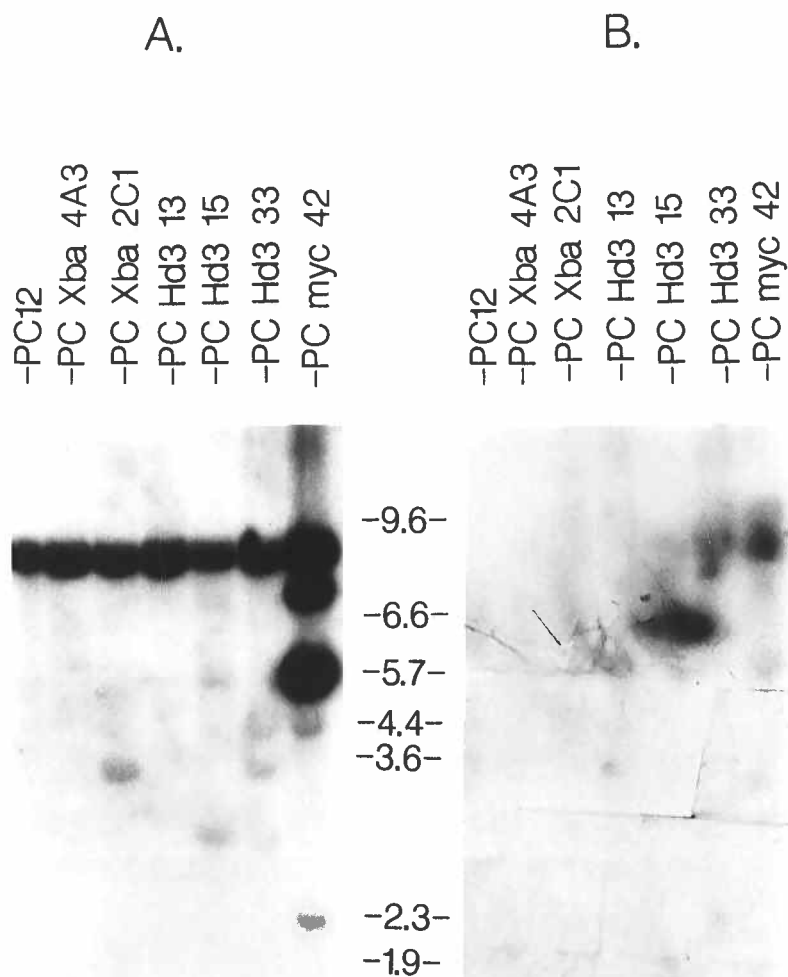
18B). PC Xba 2C1, PC Hd3 15, and PC Hd3 33 contained additional bands suggesting that these clones had acquired exogenous c-myc genes (Figure 18A). The molecular weight of the bands recognized specific sequences were different in the three cell lines demonstrating that they were independent clones. However, comparison of the intensities of the new bands relative to the intensity of the endogenous gene indicated that only a portion of cells within a given cell line contained copies of the exogenous band. In earlier experiments (not shown) the intensity of exogenous myc bands were equal to or greater than the intensity of the endogenous myc gene in these clones, indicating that the cell lines were losing copies of the exogenous gene. Homogenous cell lines could be obtained by re-cloning the cells and/or returning them to G-418 supplemented medium.

The MTV-myc encoding sequences for pMTV Hd3 myc and pMTV Xba myc are 9.4 and 7.0 kilobases, respectively. While none of the bands representing exogenous c-myc were large enough to encode the entire length of the hybrid MTV-myc genes, it was possible that gene copies which were the same size as the endogenous c-myc gene were incorporated into the clones. To differentiate between the endogenous c-myc gene and the hybrid MTV-myc genes, a duplicate DNA blot was probed with MTV-LTR-specific sequences. As shown in Figure 18B, no MTV-LTR were identified in any of the clones. The MTV LTR sequences appeared to be lost during integration of the plasmids into the genome. These results indicated that the block of neurite extension in the presence of dexamethasone in clone PC MTV Hd3 15

could not be due to the induction of the exogenous c-myc gene by dexamethason (Table 5).

PC MTV Xba 4A3 and PC MTV Hd3 13 DNA did not contain exogenous MTV-myc genes, as the myc genes, as the myc probe recognized only the endogenous c-myc gene (Figure 18A) and the MTV probe did not recognize any bands (Figure 18B). This result was unexpected since the ability of dexamethasone to inhibit neurite growth in these cells had suggested that they expressed an exogenous MTV-linked myc gene. There are at least two possible explanations for these findings. First, clones may have been selected which respond differently to dexamethasone in comparison to wild-type PC12 cells. While dexamethasone does not inhibit neurite growth in PC12 cells (Tischler and Green, 1980), it does inhibit NGF's regulation of neurite growth in primary adrenal chromaffin cells (Unsicker et al., 1978). The selection procedure may have selected cells whose phenotypic response to dexamethasone was clear to primary chromaffin cells than wild-type PC12. This possibility appears unlikely as dexamethasone did not inhibit neurite growth in any of the clones isolated which were transfected only with the aph gene. An alternative explanation is that these clones had initially acquired the hybrid MTV-myc gene but lost its copies over time. This possibility was supported by the observation that PC MTV Xba 4A3 and PC MTV Hd3 13 response to NGF and dexamethasone was similar to wild-type upon re-examination.

RNA ANALYSIS OF MTV-MYC TRANSFECTED PC12

Although clones PC MTV Hd3 15, PC MTV Xba 2C1, and PC MTV Hd3 33 did not contain DNA sequences long enough to encode the full length MTV-myc gene, they did contain myc sequences long enough to encode a full length protein. RNA blot analysis from poly A+ selected RNA was performed to determine whether exogenous myc transcripts were present. As shown in Figure 19B, dexamethasone treatment did not significantly alter the expression of c-myc RNA or its induction by NGF. PC MTV Hd3 15 cells expressed both a 2.2 kb and a 1.9 kb transcript recognized by the c-myc probe (Figure 19B). Only a 1.9 kb myc transcript was expressed in PC MTV Xba P2C1 and PC MTV Hd3 33 (Figure 19C,D). As expected by the absence of MTV-LTR-specific sequences within these clones, dexamethasone did not alter the expression of the myc transcripts in the transfected PC12 cell lines.

In summary, several G-418 resistant clones co-transfected with pKO neo/pMTV myc plasmids were isolated. While several clones contained myc-specific DNA sequences, the MTV-promoter sequences were not present in any of these. One possible explanation for this consistent loss of the MTV-LTR-specific sequences is that a hot spot for recombination existed within the MTV-myc hybrid gene. Because the isolated clones did not contain full-length copies of the MTV-myc gene they were not used for additional studies.

Figure 19. RNA analysis of MTV-myc transfected and wild-type PC12 cells.
Total RNA was isolated from untreated cells or cells treated for 2 hours with
100 µg/ml NGF, 1 µM dexamethasone, or a combination of dexamethasone
and NGF as described in methods. PolyA$^+$ selected RNA isolated from 1000
µg of total RNA was fractionated on a formaldehyde-agarose gel, transferred
to nitrocellulose and hybridized with a random primed ^{32}P-labeled third
exon c-myc probe. (A) PC12, (B) PC MTV Hd3 15, (C) PC MTV Hd3 33,
(D) PC MTV Xba 2C1.

QUIESCENT +SERUM   A.

CONTROL +DEX +DEX/NGF +NGF   B.

CONTROL +DEX   C.

CONTROL +DEX   D.

CONTROL +DEX   E.

28→

18→
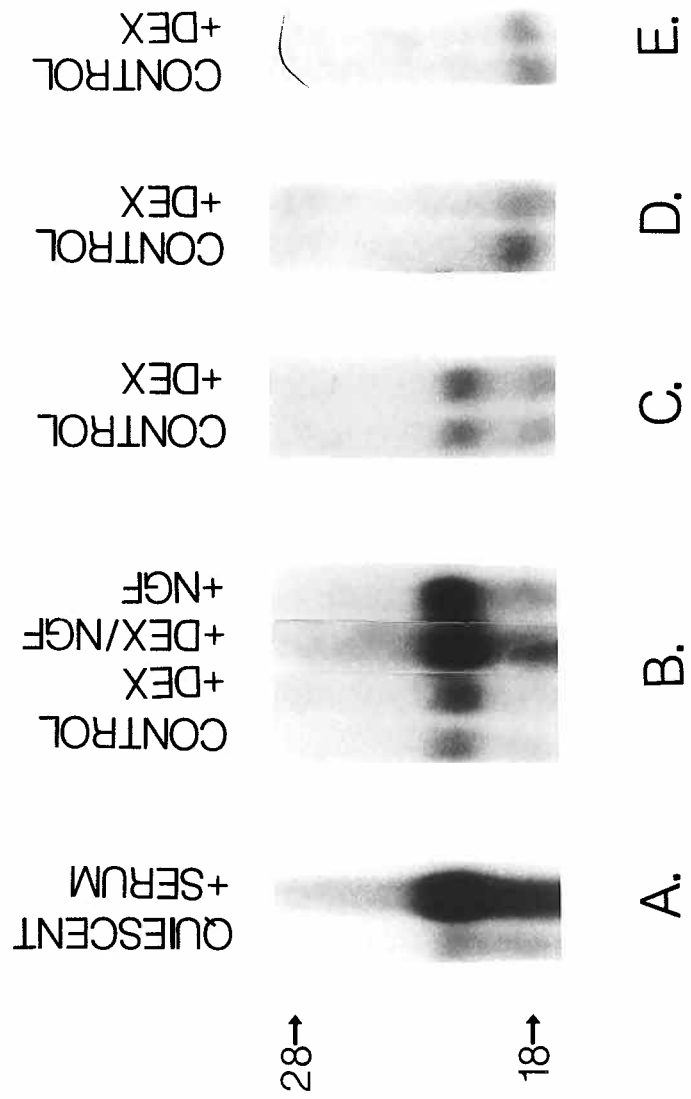
CHAPTER VII

PERSPECTIVES AND FUTURE DIRECTION

THE ROLE OF C-MYC IN NGF ACTION.

The ability of NGF to induce neuronal differentiation of PC12 cells requires long term changes in gene transcription and enhanced synthesis of neuronal proteins. In attempts to understand the primary regulatory mechanisms controlling these alterations in gene expression, several early responses to NGF have been identified. Among these responses is the rapid and transient increase in transcription of the genes for c-fos, c-myc, actin, and ornithine decarboxylase (Greenberg et. al., 1985). Because these events can be mimicked by several growth factors which do not promote the neuronal phenotype, it has been difficult to assess the importance of these early changes in relation to latent transcriptional events and subsequent differentiation. Results of the present study demonstrate that the temporal expression of the c-myc gene has direct influence on PC12 cells' decision to undergo neuronal differentiation.

Constitutive c-myc expression inhibits rather than enhances NGF's effects on neuronal differentiation in PC12 cells. This indicates that while the induction of c-myc transcription may be important in the the cell's response to NGF, the subsequent repression of c-myc transcription is critical for the cell's ability to undergo neuronal differentiation. Thus, one consequence of the transient induction of c-myc expression may be to delay the onset of differentiation.

PC12 cells normally respond to NGF by undergoing neuronal differentiation and growth arrest. However, the constitutive expression of c-myc causes these cells to respond mitogenically to NGF. This raises the possibility that the induction of c-myc expression by NGF that typically occurs in wild-type PC12 cells may also allow the cells to respond mitogenically to NGF before proceeding through the differentiation program. NGF has been shown to initially induce proliferation prior to differentiation in both PC12 cells (Boonstra et al., 1983) and primary chromaffin cells (Lillian and Claude, 1985). Proliferation has been shown to be a prerequisite for differentiation in a variety of cell types (Metcalf, 1985). It is not known whether proliferation is also a prerequisite for the neuronal differentiation regulated by NGF. Prevention of the transient elevation of c-myc by the introduction of anti-sense c-myc RNA into PC12 cells may clarify whether c-myc induction is required for the induction of DNA synthesis and the subsequent differentiation events which occur in response to NGF.

A clear understanding the role of c-mycin NGF action must await identification of its function. This requires a more complete characterization of the c-myc protein and its regulation by growth and differentiation factors. A significant step in this direction has been achieved by the recent finding that the two known c-myc proteins are derived from alternative translational initiations at the exon 2 AUG and at a non-AUG codon near the 3' end of exon 1, resulting in the production of two proteins with distinct N termini (Hann, et al., 1988). In Burkitt's lymphomas the absence of exon 1 in c-myc translocations correlates with suppression of synthesis of the larger protein. While the loss of exon 1 has been typically associated with de-regulation of

c-myc expression, these results raise the possibility that loss of this larger protein may contribute to the oncogenic activation of c-myc. The SV40-linked myc gene transfected into PC12 cells in this study is also lacking the first exon. Thus, differential expression of the two forms of proteins may provide an alternative mechanism for the block in differentiation caused by expression of the exogenous myc gene. Introduction of a mutated c-myc gene into PC12 cells which retains the first translational initiation site within the first exon but eliminates the second initiation site at AUG would test the effects of over-expression of the larger protein to determine whether these two proteins have differential affects on NGF action.

While the function of c-myc is not known, several observations have led investigators to suggest the possibility that c-myc might act as a regulator of transcription. The c-myc protein is localized in the nucleus and has been shown to bind to DNA in vitro (Donner et al., 1982). Futhermore, c-myc has been shown to activate transcription from the heat shock gene HSP70 promoter in a transient expression assay (Kingston et al.,1984) and c-myc protein levels are increased in cells exposed to heat shock (Luscher and Eisenman, 1988). The ability of constitutive c-myc expression to block the transcription-dependent induction of HMW MAPS and tau proteins is consistent with a possible role of c-myc in the regulation of transcription. Availability of cDNA clones encoding tau and HMW MAPS would allow transcriptional run-on assays to be performed with isolated nuclei from wild-type PC12 and clones expressing c-myc, or E1a to directly test whether the expression of these genes blocks regulation of MAPs at the transcriptional level. It has been recently shown that constitutive expression of E1A in

myoblasts suppressed myogenic differentiation and inhibited transcription from muscle-specific promoters (Webster et al.,1988).

FUTURE DIRECTIONS

NGF appears to be among a growing number of polypeptide growth factors which can have multifunctional activities. Growth factors may provide different functions depending on the cell type. For example, transforming growth factor-beta (TGF-ß) has been demonstrated to have proliferative effects on some fibroblasts and osteoblasts but anti-proliferative effects on other fibroblasts, osteoblasts, and epithelial cells (reviewed, Sporn and Roberts, 1988). Futhermore, the nature of a growth factor action may be dependent on the presence of other growth factors. For example, TGF-ß stimulates growth of NRK cells in monolayer culture in the presence of platelet-derived growth factor but inhibits their growth if EGF if present (Roberts et al., 1985). One potential mechanism for modulation of one growth factor's action by another is by altering receptor function. In this regard, it is interesting to note that NGF causes down-regulation of the EGF receptor in PC12 cells within 24 hours (Huff and Guroff, 1981).

All of the events which occur early in the NGF-induced differentiation program (Table 1) can be mimicked by EGF. To date no rapid signalling events have been identified which are unique to NGF action that may account for its ability to induce differentiation. However, binding of EGF to it's receptor activates an associated tyrosine kinase activity which is not regulated

by NGF. Thus, the ability of PC12 cells to differentiate in response to NGF may be dependent on the ability of NGF to inhibit EGF action through down-regulation of its receptor. This hypothesis may be tested by intoducing an exogenous EGF-receptor gene under control of a promoter that is independent of NGF regulation into PC12 cells. This strategy would induce overexpression of the EGF-receptor and if the hypothesis is correct, NGF induction of differentiation would be inhibited by the additon of EGF to these cells.

Constitutive expression of c-myc in PC12 cells appears to uncouple differentiation and growth arrest from the mitogenic effects of NGF action c-myc expression may allow NGF to function mitogenically by influencing pathways similar to EGF which have the ability to over-ride differentiation promoting events. This would further predict that additional gene products which function at multiple steps along mitogenic pathways would also block NGF action when introduced into PC12 cells.

Clearly no single experimental design can answer all questions regarding the mechanisms of NGF action. However, as classical biochemical techniques have so far failed to define the early NGF-induced signalling events regulating neuronal differntiation , new strategies are demanded. This thesis presents a novel approach for testing possible roles of specific gene products which are have been implicated in NGF action.

CHAPTER VII

REFERENCES

Alemo, S., Casalbore, Agostini, E. and Tato, F. (1985). Differentiation of PC12 phaeochromocytoma cells induced by v-src oncogene. Nature 316, 557-559.

Aletta, J.M. and Greene, L.A. (1987). Sequential phosphorylation of chartin microtubule-associated proteins is regulated by the presence of microtubules. J. Cell Biol 105, 277-290.

Angeletti, R.H. and Bradshaw, R.A. (1971). Nerve growth factor from mouse submaxillary gland: Amino acid sequence. Proc. Natl. Acad. Sci. USA 68:2417-2420.

Armelin, H.A., Armelin, M.C.S., Kelly, K., Stewart, T., Leder, P., Cochran, B.H. and Stiles, C.D. (1984). Functional role for c-myc in mitogenic response to platelet-derived growth factor. Nature 310, 655-660.

Asai, D.J., Thompson, W.C., Wilson, L., Dresden, C.F., Schulman, H. and Purich, D.L. (1985). Microtubule-associated proteins (MAPs): A monoclonal antibody to MAP 1 decorates microtubules in vitro but stains stress fibers and not microtubules in vivo. Proc. Natl. Acad. Sci. USA 82, 1434-1438.

Bar-Sagi, D. and Feramisco, J.R. (1985). Microinjection of the ras oncogene protein into PC12 cells induces morphological differentiation. Cell 42, 841-848.

Beckner, S., Hattori, S. and Shih, T. (1985). The ras oncogene product p21 is not a regulatory component of adenylate cyclase. Nature 317, 71-72.

Bell, E., Marek, L.F., Levinstone, D.S., Merrill, C., Sher, S., Young, I.T. and Eden, M. (1978). Loss of division potential in vitro: aging or differentiation. Science 202, 1158-1163.

Berk, A.J. (1986). Adenovirus promoters and E1A transactivation. Annu. Rev. Genet. 20, 45-79.

Bernard, O., Cory, S., Gerondakis, S., Webb, E. and Adams, S.M. (1983). Sequence of the murine and human cellular myc oncogenes and two modes of myc transcription resulting from chromosome translocation in B lymphoid tumors. EMBO J. 23, 2375-2383.

Bishop, J.M. (1985). Viral Oncogenes. Cell 42, 23-38.

Black, M.M., Aletta, J.M. and Greene, L.A. (1986). Regulation of microtubule composition and stability during nerve growth factor-promoted neurite outgrowth. J. Cell Biol. 103, 545-557.

Blenis, J. and Erikson, R.L. (1986). Regulation of protein kinase activities in PC12 pheochromocytoma cells. EMBO 5, 3441-3447.

Bloom, G.S., Luca, F.C. and Vallee, R.B. (1984a). Widespread cellular distribution of MAP 1A in the mitotic spindle and on interphase microtubules. J. Cell Biol. 98, 331-340.

Bloom, G.S., Luca, F.C. and Vallee, R.B. (1985). Microtubule-associated protein 1B: Identification of a major component of the neuronal cytoskeleton. Proc. Natl. Acad. Sci. USA 82, 5404-5408.

Bloom, G.S., Schoenfeld, T.A. and Vallee, R.B. (1984b). Widespread distribution of the major polypeptide component of MAP 1 in the nervous system. J. Cell Biol. 98, 320-330.

Boonstra, J., Moolenaar, W.H., Harrison, P.H., Moed, P., van der Saag, P.T. and de Laat, S.W. (1983). Ionic responses and growth stimulation induced by nerve growth factor and epidermal growth factor in rat pheochromocytoma (PC12) cells. J. Cell. Biol. 97, 92-98.

Boonstra, J., van der Saag, P.T., Moolenaar, W.H. and de Laat, S.W. (1981). Rapid effects of nerve growth factor on the Na^+/K^+ pump in rat pheochromocytoma cells. Exp. Cell Res. 131, 452-455.

Boonstra, J., van der Saag, P.T., Feijen, A., Bisschop, A. and de Laat, S. (1985). Epidermal growth factor, but not nerve growth factor, stimulates tyrosine-specific protein kinase activity in pheochromocytoma (PC12) plasma membranes. Biochimie 67, 1177-1183.

Bothwell, M.A. and Schecter, A.L. (1981). Nerve growth factor receptors on PC12 cells: Evidence for two receptor classes with differing cytoskeletal association. Cell 24, 867-874.

Brown, P.A. and Berlin, R.D. (1985). The packing volume of sedimented microtubules: Regulation and potential relationship to a potential intracellular matrix. J. Cell Biol. 101, 1492-1500.

Bueker, E.D. (1948). Implantation of tumors in the hind limb of the embryonic chick and the developmental response of the lumbrosacral nervous system. Anat. Rec. 102, 369-390.

Burstein, D.E. and Green, L.A. (1978). Evidence for RNA synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor. Proc. Natl. Acad. Sci. USA 75, 6059-6063.

Burstein, D.E. and Greene, L.A. (1982). Nerve growth factor has both mitogenic and antimitogenic activity. Dev. Biol. 94, 477-482.

Buxser, S.E., Kelleher, D.J., Watson, L., Puma, P. and Johnson, G.L. (1983a). Change in state of nerve growth factor receptor. J. Biol. Chem. 258, 3741-3749.

Buxser, S.E., Puma, P. and Johnson, G.L. (1985a). Purification and properties of the nerve growth factor receptor. In: Biochemical Actions of Hormones Vol. XII (USA: Academic Press) pp. 433-456.

Buxser, S., Puma, P. and Johnson, G.L. (1985b). Properties of the nerve growth factor receptor. J. Biol. Chem. 260, 1917-1926.

Buxser, S.E., Watson, L. and Johnson, G.L. (1983b). Multiple states of nerve growth factor receptors on PC12 pheochromocytoma and A875 melanoma cells: Comparison of structure and binding properties. J. Cell Biochem. 22, 219-233.

Campisi, J., Gray, H.E., Pardee, A.B., Dean, M. and Sonenshein, G.E. (1984). Cell-cycle control of c-myc but not c-ras expression is lost following chemical transformation. Cell 36, 241-247.

Cheng, H.-C., Kemp, B.E., Pearson, R.B., Smith, A.J., Misconi, L., Van Patten, S.M. and Walsh, D.A. (1986). A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. J. Biol. Chem. 261, 989-992.

Cleveland, D.W., Hwo, S. and Kirschner, M.W. (1977). Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin. J. Mol. Biol. 116, 207-225.

Cohen, S. (1960). Purification of a nerve-growth promoting protein from the mouse salivary gland and its neurocytotoxic anti-serum. Proc. Natl. Acad. Sci. USA 46, 302-311.

Cole, M.D., (1986). The myc oncogene: Its role in transformation and differentiation. Ann. Rev. Genet. 20:361-384.

Colbere-Garapin, F., Horodniveanu, F., Kourilsky, P. and Garapin, A.C. (1981). A new dominant hybrid selective marker for higher eukaryotic cells. J. Mol. Biol. 150, 1-14.

Connolly, J.L., Green, S.A. and Greene, L.A. (1981). Pit formation and rapid changes in surface morphology of sympathetic neurons in response to nerve growth factor. J. Cell Biol. 90, 176-180.

Connolly, J.L., Greene, L.A., Viscarello, R.R. and Riley, W.D. (1979). Rapid, sequential changes in surface morphology of PC12, pheochromocytoma cells in response to nerve growth factor. J. Cell Biol. 82, 820-827.

Contreras, M.L. and Guroff, G. (1987). Calcium-dependent nerve growth factor stimulated hydrolysis of phosphoinositides in PC12 cells. J. Neurochem. 48, 1466-1472.

Coppola, J.A. and Cole, M.D. (1986). Constitutive c-myc oncogene expression blocks mouse erythroleukaemia cell differentiation but not commitment. Nature 320, 760-763.

Cremins, J., Wagner, J.A. and Halegoua, S. (1986). Nerve growth factor action is mediated by cyclic AMP- and Ca^{2+}/phospholipid-dependent protein kinases. J. Cell Biol. 103, 887-893.

Curran, T. and Morgan, J.I. (1985). Superinduction of c-fos by nerve growth factor in the presence of peripherally active benzodiazepenes. Science 229, 1265-1268.

Dickson, G., Prentice, H., Jullien, J.-P., Ferrari, G., Leon, A. and Walsh, F.S. (1986). Nerve growth factor activates thy-1 and neurofilament gene transcription in rat PC12 cells. EMBO 5, 3449-3453.

Donner, P., Greiser-Wilke, I., and Moeling, K. (1982). Nuclear localization and DNA binding of the transforming gene product of avian myelocytomatosis virus. Nature. 296, 262-266

Drubin, D.G., Feinstein, S.C., Shooter, E.M. and Kirschner, M.W. (1986). Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly-promoting factors. J. Cell Biol. 101, 1799-1807.

Drubin, D.G., Kobayashi, S., Kellogg, D. and Kirscher, M. (1988). Regulation of microtubule protein levels during cellular morphogenesis in nerve growth factor-treated PC12 cells. J. Cell Biol. 106, 1583-1591.

Durnam, D.M., Perrin, F., Gannon, F. and Palmiter, R.D. (1980). Isolation and characterization of the mouse metallothionein-I gene. Proc. Natl. Acad. Sci. USA 77, 6511-6515.

Edelman, A.M., Blumenthal, D.K. and Krebs, E.G. (1987). Protein serine/threonine kinases. Ann. Rev. Biochem. 56, 567-613.

Feinstein, S.C., Dana, S.L., McConlogue, L., Shooter, E.M. and Coffino, P. (1985). Nerve growth factor rapidly induces ornithine decarboxylase mRNA in PC12 rat pheochromocytoma cells. Proc. Natl. Acad. Sci. USA 82, 5761-5765.

Gonda, T.J. and Metcalf, D. (1984). Expression of myb, myc and fos proto-oncogenes during the differentiation of a murine myeloid leukaemia. Nature 310, 249-251.

Goodman, R., Slater, E. and Herschman, H.R. (1980). Epidermal growth factor induces tyrosine hydroxylase in a clonal pheochromocytoma cell line, PC-G2. J. Cell Biol. 84, 495-500.

Greenberg, M.E., Greene, L.A. and Ziff, E.B. (1985). Nerve growth factor and epidermal grwoth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. J. Biol. Chem. 260, 14101-14110.

Green, L.A. and Mcguire, J.C. (1978). Induction of ornithine decarboxylase by nerve growth factor dissociated from effects on survival and neurite outgrowth. Nature 276, 191-194.

Greene, L.A. and Rein, G. (1977). Release, storage and uptake of catecholamines by a clonal cell line of nerve growth factor (NGF) responsive pheochromocytoma cells. Brain Res. 129, 247-263.

Greene, L.A. and Rukenstein, A. (1981). Regulation of acetylcholinesterase activity by nerve growth factor. J. Biol. Chem. 256, 6363-6367.

Greene, L.A. and Shooter, E.M. (1980). The nerve growth factor: biochemistry, synthesis and mechanism of action. Ann. Rev. Neurosci. 3, 353-402.

Greene, L.A. and Tischler, A.S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA 73, 2424-2428.

Greene, L.A., Burstein, D.E. and Black, M.M. (1982). The role of transcription-dependent priming in nerve growth factor promoted neurite outgrowth. Dev. Biol. 91, 305-316.

Greene, L.A., Liem, R.K.H. and Shelanski, M.L. (1983). Regulation of a high molecular weight microtubule-associated protein in PC12 cells by nerve growth factor. J. Cell Biol. 96, 76-83.

Green, S.H., Rydel, R.E., Connolly, J.L. and Greene, L.A. (1986). PC12 cell mutants that possess low- but not high-affinity nerve growth factor receptors neither respond to nor internalize nerve growth factor. J. Cell Biol. 102, 830-843.

Greene, L.A., Varon, S., Piltch, A. and Shooter, E.M. (1971). Substructure of the β subunit of mouse 7S nerve growth factor. Neurobiology 1, 37-48.

Gunning, P.W., Landreth, G.E., Bothwell and Shooter, E.M. (1981). Differential and synergistic actions of nerve growth factor and cAMP in PC12 cells. J. Cell Biol. 89, 240-245.

Guroff, G., Dickens, G. and End, D. (1981). The induction of ornithine decarboxylase by nerve growth factor and epidermal growth factor in PC12 cells. J. Neurochem. 37, 342-349.

Hagag, N., Halegoua, S. and Viola, M. (1986). Inhibition of growth factor-induced differentiation by microinjection of antibody to ras p21. Nature 319, 680-682.

Halegoua, S. and Patrick, J. (1980). Nerve growth factor mediates phosphorylation of specific proteins. Cell 22, 571-581.

Hall, F.L., Fernyhough, P., Ishii, D.N. and Vulliet, P. R. (1988). Suppression of NGF-directed neurite outgrowth in PC12 cells by spinghosine, an inhibitor of protein kinase C. J. Biol. Chem. 263, 4460-4466.

Hama, T., Huang, K.-P. and Guroff, G. (1986). Protein kinase C as a component of a nerve growth factor sensitive phosphorylation system in PC12 cells. Biochem. 83, 2353-2357.

Hann, R.S., King, M.W., Bentley, D.L. , Anderson, C.W., and Eisenman, R.N. (1988). A non-AUG translational initiation in c-myc exon 1 generates an N-terminally distinct protein whose synthesis is disrupted in Burkitt's lymophomas. Cell 52, 185-195.

Hatanaka, H., Otten, U. and Thoenen, H. (1978). Nerve growth factor-mediated selective induction of ornithine decarboxylase in rat pheochromatocytoma: a cyclic AMP-independent process. FEBS Lett. 92, 313-316.

Heasley, L.E. and Johnson, G.L. Dection of nerve growth factor and epidermal growth factor-regulated protein kinases in PC12 cells with selective peptide substrates. Submitted for publication.

Heby, O. (1981). Role of polyamines in the control of cell proliferation and differentiation. Differentiation 19, 1-20.

Heidemann, S.R., Joshi, H.C., Schecter, A., Fletcher, J.R. and Bothwell, M. (1985). Synergistic effects of cyclic AMP and nerve growth factor on neurite outgrowth and microtubule stability of PC12 cells. J. Cell Biol. 100, 916-927.

Heumann, R., Schwab, M. and Thoenen, H. (1981). A second messenger required for nerve growth factor biological activity. Nature 292, 838-840.

Houweling, A., van der Elsen, P.J. and van der Eb, A.J. (1980). Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. Virology 105, 537-550.

Huang, A.L., Ostrowski, M.C., Berard, D. and Hager, G.L. (1981). Glucocorticoid regulation of the Ha-MuSV p21 gene conferred by sequences from mouse mammary tumor virus. Cell 27, 245-255.

Huff, K.D., End and Guroff, G. (1981). Nerve growth factor induced alteration in the response of PC12 cells to epidermal growth factors. J. Cell Biol. 88, 189-198.

Johnson, D., Lanahan, A., Buck, C.R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M. and Chao, M. (1986). Expression and structure of the human NGF receptor. Cell 47, 545-554.

Kasaian, M.T. and Neet, K.E. (1988). Internalization of nerve growth factor by PC12 cells: A description of cellular pools. J. Biol. Chem. 263, 5083-5090.

Keath, E.J., Caimi, P.G. and Cole, M.D. (1984). Fibroblast lines expresssing activated c-myc oncogenes are tumorigenic in nude mice and syngeneic animals. Cell 39, 339-348.

Kelly, K., Cochran, B.H., Stiles, C.D. and Leder, P. (1983). Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35, 603-610.

Kemp, B.E., Graves, D.J., Benjamini, E., and Krebs, E.G. (1977). Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. J. Biol. Chem. 252, 4888-4894.

Kim, H., Binder, L.I. and Rosenbaum, J.L. (1979). The periodic association of MAP 2 with brain microtubules in vitro. J. Cell Biol. 80, 266-276.

Kingston,R.E., Baldwin, A.S., Sharp, P.A. (1984). Regulation of heat shock protein 70 gene expression by c-myc. Nature 312, 280282.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 27, 580-585.

Landreth, G.E. and Rieser, G.D. (1985). Nerve growth factor- and epidermal growth factor-stimulated phosphorylation of PC12 cytoskeletal associated protein in situ. J. Cell Biol. 100, 677-683.

Landreth, G.E. and Shooter, E.M. (1980). Nerve growth factor receptors on PC12 cells: Ligand-induced conversion from low- to high-affinity states. Proc. Natl. Acad. Sci. USA 77, 4751-4755.

Lazarovici, P., Dickens, G., Kuzuya, H. and Guroff, G. (1987). Long term, heterologous down-regulation of the epidermal growth factor receptor in PC12 cells by nerve growth factor. J. Cell Biol. 104, 1611-1621.

Leder, A., Pattengale, P.K., Kuo, A., Stewart, T.A. and Leder, P. (1986). Consequences of widespread de-regulation of the c-myc gene in transgenic mice: Multiple neoplasms and normal development. Cell 45, 485-495.

Leonard, D.G.B., Ziff, E.B. and Greene, L.A. (1987) Identification and characterization of mRNAs regulated by nerve growth factor in PC12 cells. Mol. Cell Biol. 7, 3156-3167.

Levi, A., Schechter, Y. and Schlessinger, J. (1980). Mobility, clustering and transport of nerve growth factor in embryonal sensory cells and in a sympathetic neuronal cell line. Proc. Natl. Acad. Sci. USA 77, 3469-3473.

Levi-Montalcini, R. (1987). The nerve growth factor: Thirty-five years later. EMBO J. 6, 1145-1154.

Levi-Montalcini, R. and Hamburger, V. (1951). Selective growth-stimulation effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J. Exp. Zool. 116, 32-362.

Levi-Montalcini, R., Hamburger, V. (1953). A diffusible agent of mouse sarcoma producing hyperplasia of sympathetic ganglia and hyperneurotization of viscera in the chick embryo. J. Exp. Zool. 123, 233-278.

Levi-Montalcini, R., Meyer, H. and Hamburger, V. (1954). In vitro experiments on the effects of mouse sarcoma 180 and 37 on the spinal and sympathetic ganglia of the chick embryo. Cancer Res. 14, 49-57.

Lewis, S.A., Villasante, A., Sherline, P. and Cowan, N.J. (1986a). Brain-specific expression of MAP 2 detected using a cloned cDNA probe. J. Cell Biol 102, 2098-2105.

Lewis, S.A, Sherline, P. and Cowan, N.J. (1986b). A cloned cDNA encoding MAP1 detects a single copy gene in mouse and a brain abundant RNA whose level decreases during development. J. Cell Biol 102, 2106-2114.

Lillien, L.E. and Claude, P. (1985). Nerve growth factor is a mitogen for cultured chromaffin cells. Nature 317, 632-634.

Luscher B., and Eisenman, R.N. (1988). c-myc and c-myb protein dergradation:Effect of metabolic inhibitors and heat shock. Mol. Cell.Biol. 8, 2504-2512.

Martin, G.M., Sprague, C.A., Norwood, T.H. and Pendergrass, W.R. (1974). Clonal selection, attenuation and differentiation in an in vitro model of hyperplasia. Am. J. Path. 74, 137-150.

Martin, G.M., Sprague, C.A., Norwood, T.H., Pendergrass, W.R., Bornstein, P., Hoehn, H. and Arend, W.P. (1975). Do hyperplastoid cell lines "differentiate themselves to death"? In Cell Impairment in Aging and Developmen, V.J., Cristofalo and E. Holeckova, eds. (New York: Plenum Press), pp. 67-90.

Marujama, K. Schiavi, S.C., Huse, W., Johnson, G.L. and Ruley, H.E. (1987). Myc and E1A oncogenes alter the responses of PC12 cells to nerve growth factor and block differentiation. Oncogene 1, 361-367.

Matsuda, Y. and Guroff, G. (1987). Purification and mechanism of activation of a nerve growth factor-sensitive S6 kinase from PC12 cells. J. Biol. Chem. 262, 2832-2844.

McGuire, J.C., Greene, L.A. and Furano, A.V. (1978). NGF stimulates incorporation of fucose or glucosamine into an external glycoprotein in cultured rat PC12 pheochromocytoma cells. Cell 115, 357-365.

McTigue, M., Cremins, J. and Halegoua, S. (1985). Nerve growth factor and other agents mediate phosphorylation and activation of tyrosine hydroxylase. J. Biol. Chem. 260, 9047-9056.

Mobley, W.C., Schenker, A. and Shooter, E.M. (1976). Characterization and isolation of proteolytically modified nerve growth factors. Biochemistry 15, 5543-5552.

Mougneau, E., Lemieux, L., Rassoulzadegan, M. and Cuzin, F. (1984). Biological activities of v-myc and rearranged c-myc oncogenes in rat fibroblast cells in culture. Proc. Natl. Acad. Sci. USA 81, 5758-5762.

Muller, R. (1986). Proto-oncogenes and differentiation. TIBS 11, 129-132.

Murphy, D.B. and Borisy, G.G. (1975). Association of high molecular weight proteins with microtubules and their role in microtubule assembly in vitro. Proc. Natl. Acad. Sci. USA 72, 2696-2700.

Neufeld, G. and Gospodarowicz, D. (1986). Basic and acidic fibroblast growth factors interact with the same cell surface receptors. J. Biol. Chem. 261, 5631-5637.

Noda, M., Ko, M., Ogura, A., Liu, D., Amano, T., Takano, T. and Ikawa, Y. (1985). Sarcoma viruses carrying ras oncogenes induce differentiation-associated properties in a neuronal cell line. Nature 318, 73-75.

Pallas, D. and Solomon, F. (1982). Cytoplasmic microtubule associated proteins: Phosphorylation at novel sites is correlated with their incorporation into assembled microtubules. Cell 30, 407-414.

Pandiella-Alonso, A., Malgaroli, A., Vicentini, L.M. and Meldolesi, J. (1986). Early rise of cytosolic Ca^{2+} induced by NGF in PC12 and chromaffin cells. FEBS Letters 208, 48-51.

Pelech, S.L., and Krebs, E.G. (1987). Mitogen-activated S6 kinase is stimulated via protein kinase C-dependent and independent pathways in Swiss 3T3 cells. J. Biol. Chem. 262, 11598-11606.

Prochownik, E.V. and Kukowska, J. (1986). Deregulated expression of c-myc by murine erythroleukemia cells prevents differentiation. Nature 322, 848-850.

Puma, P., Buxser, S.E., Watson, L., Kelleher, D.J. and Johnson, G.L. (1983). Purification of the receptor from A875 melanoma cells by affinity chromatography. J. Biol. Chem. 258, 3370-3375.

Race, H.M. and Wagner, J.A. (1985). Nerve growth factor affects cyclic AMP metabolism, but not by directly stimulating adenylate cyclase activity. J. Neurochem. 44, 1588-1592.

Rapp, U.R., Cleveland, J.L., Brightman, K., Scott, A. and Ihle, J.N. (1985). Abrogation of IL-3 and IL-2 dependence by recombinant murine retroviruses expressing v-myc oncogenes. Nature 317, 434-438.

Reitsma, P.H., Rothberg, P.G., Astrin, S.M., Trial, J., Bar-Shavit, Z., Hall, A., Teitelbaum, S.L. and Kahn, A.J. (1983). Regulation of myc gene expression in HL-60 leukaemia cells by a vitamin D metabolite. Nature 306, 492-494.
Roberts, A.B., Anzano, M.A., Wakefield, L.M., Roche, N.S., Stern, D.F., and Sporn, M.B. (1985). Type-ß transforming growth factor: A bifunctional regulator of cellular growth. Proc. Natl. Acad. Sci. USA 82, 119-123.

Richter-Landsberg, C. and Jastorff, B. (1986). The role of cAMP in nerve growth factor-promoted neurite outgrowth in PC12 cells. J. Cell Biol. 102, 821-829.

Ruley, H.E., Moomaw, J.F. and Maruyama, K. (1984). Avian myelocytomatosis virus myc and adenovirus early region 1A promote the in vitro establishment of cultured primary cells. In Cancer Cells 2, G.F. Vande

Woude, A.J. Levine, W.C. Topp and J.D. Watson, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 481-486.

Rydel, R.E. and Greene, L.A. (1987). Acidic and basic fibroblast growth factors promote stable neurite outgrowth and neuronal differentiation in cultures of PC12 cells. J. of Neurosci. 7, 3639-3653.

Sandri-Goldin, R.M., Goldin, A.L., Levine, M. and Glorioso, J.C. (1981). High-frequency transfer of cloned herpes simple virus type 1 sequences to mammalian cells by protoplast fusion. Mol. and Cell. Biol. 1, 743-752.

Schubert, D., La Corbiere, M., Whitlock, and Stallcup,W. (1978). Alterations in the surface properties of cells responsive to nerve growth factor. 273, 718-723.

Schubert, D. and Whitlock, C. (1977). Alteration of cellular adhesion by nerve growth factor. Proc. Natl. Acad. Sci. USA 74, 4055-4058.

Schubert, D., Heinemann, S. and Kidokoro, Y. (1977). Cholinergic metabolism and synapse formation by a rat nerve cell line. Proc. Natl. Acad. Sci. USA 74, 2579-2583.

Schulman, H. (1984). Phosphorylation of microtubule associated proteins by Ca^{2+}/Calmodulin-dependent protein kinase. J. Cell Biol. 99, 11-19.

Segerson (1987). Science 23, 78-80.

Seeley, P. Keith, C.H., Keith, M.L., Shelanski, and Greene, L.A. (1983). Pressure microinjection of nerve growth factor and anti-nerve growth factor into the nucleus and cytoplasm: Lack of effects on neurite outgrowth from pheochromocytoma cells. J. Neuroscience 3, 1488.

Shiroki, K., Shimojo, H., Sawada, Y., Uemizu, Y. and Fujinaga, K. (1979). Incomplete transformation of rat cells by a small fragment of Adenovirus 12 DNA. Virol. 95, 127-136.

Sloboda, R.D., Dentler, W.L. and Rosenbaum, J.L. (1976). Microtubule associated proteins and stimulation of tubulin assembly in vitro. Biochemistry 15, 4497-4505.

Sporn, M.B., and Roberts, A.B. (1988). Peptide growth factors are multifunctional. Nature 332, 217-219.

Stanton, L.W., Watt, R. and Marcu, K.B. (1983). Translocation, breakage and truncated transcripts of c-myc oncogene in murine plasmacytomas. Nature 303, 401-406.

Sternberger, L.A., Hardy, P.H., Cuculis, J.C. and Meyer, H.G. (1970). The unlabeled antibody enzyme method of immunohistochemistry. J. Histochem. Cytochem. 18, 315-333.

Stewart, T.A., Pattengale, P.K. and Leder, P. (1984). Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell 38, 627-637.

Stockel, K. and Thoenen, H. (1975). Retrograde axonal transport of nerve growth factor: Specificity and biological importance. Brain Res. 85, 337-341.

Stockel, K., Paravicini, U. and Thoenen, H. (1974). Specificity of the retrograde axonal transport of nerve growth factor. Brain Res. 76, 413-421.

Thoenen, H. and Barde, Y.-A. (1980). Physiology of nerve growth factor. Physiological Rev. 60, 1284-1335.

Thoenen, H. and Edgar, D. (1985). Neurotrophic Factors. Science 229, 238-242.

Tischler, A.S. and Greene, L.A. (1978). Morphological and cytochemical properties of a clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Laboratory Investigation 39, 77-89.

Tischler, A.S., Lee, Y.C., Costopoulos, D. , Slayton, V.W., Jason, W.J. and Bloom, S.R. (1986). Cooperative regulation of neurotensin content in PC12 pheochromocytoma cell cultures: Effects of nerve growth factor, dexamethasone, and activators of adenylate cyclase. J. of Neurosci. 6, 1719-1725.

Togari, A., Baker, D., Dicken, G. and Guroff, G. (1983). The neurite-promoting effect of fibroblast growth factor on PC12 cells. Biochem. Biophys. Res. Commun. 114, 1189-1193.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350-4354.

Traynor, A.F., Schubert, D. and Allen, W.R. (1982). Alterations of lipid metabolism in response to nerve growth factor. J. Neurochem. 39, 1677-1683.

Tsuyama, S., Bramblett, G.T., Huang, K.-P. and Flavin, M. (1986). Calcium/phospholipid-dependent kinase recognizes sites in microtubule-associated protein 2 which are phosphorylated in living brain and are not accessible to other kinases. J. Cell Biol. 261, 4110-4116.

Vallee, R.B. (1982). A taxol-dependent procedure for the isolation of microtubules and microtubule-associated proteins (MAPs). J. Cell Biol 92, 435-442.

Vallee, R.B. and Davis, S.E. (1983). Low molecular weight microtubule-associated proteins are light chains of microtubule-associated protein 1 (MAP 1). Proc. Natl. Acad. Sci. USA 80, 1342-1346.

Van Doren, K., Hanahan, D. and Gluzman, Y. (1984). Infection of eukaryotic cells by helper-independent recombinant adenoviruses: early region 1 is not obligatory for integration of viral DNA. J. Virol. 50, 606-614.

Varon, S. and Skaper, S.D. (1983). The Na^+, K^+ pump may mediate the control of nerve cells by nerve growth factor. TIBS 8, 22-25.

Webster, K.A., Muscat., O., and Kedes, L. (1988). Adenovirus E1A products suppress myogenic differentiation and inhibit transcription from muscle-specific promoters. Nature 332, 553--557.

Weingarten, M.D., Lockwood, A.H., Hwo, S.-Y. and Kirschner, M.W. (1975). A protein factor essential for microtubule assembly. Proc. Natl. Acad. Sci. USA 72, 1858-1862.

Westin, E.H., Wong-Staal, F., Gelmann, E.P., Favera, R.D., Papas, T.S., Lautenberger, J.A., Eva, A., Reddy, E.P., Tronick, S.R., Aaronson, S.A. and Gallo, R.C. (1982). Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. Proc. Natl. Acad. Sci. USA 79, 2490-2494.

Yanker, B.A. and Shooter E.M. (1979). Nerve growth factor on the nuclear membrane. Proc. Natl. Acad. Sci. USA 76, 1269-1273.

Yanker, B.A. and Shooter, E.M. (1982). The biology and mechanism of action of nerve growth factor. Ann. Rev. Biochem. 51, 845-868.

Yokoyama, K. and Imamoto, F. (1987). Transcriptional control of the endogenous myc protooncogene by antisense RNA. Proc. Natl. Acad. Sci. 84, 7363-7367.

Zinn, K., DiMaio, D. and Maniatis, T. (1983). Identification of two distinct regulatory regions adjacent to the human ß-interferon gene. Cell 34, 865-879.