TRANSIN GENE EXPRESSION: A METALLOPROTEINASE REGULATED BY EPIDERMAL GROWTH FACTOR AND NERVE GROWTH FACTOR

by

Cynthia M. Machida

A DISSERTATION

Presented to the Department of Cell Biology and Anatomy

and the Oregon Health Sciences University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

March 1989

APPROVED:

(Professors in Charge of Thesis)

(Chairman, Graduate Council)

DEDICATION

This thesis is dedicated to my parents,

HENRY T. MATSUYAMA

and the late

FLORENCE H. MATSUYAMA

Without their example, encouragement, and endeavor towards excellence,

I would have never embarked, continued or completed this work.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

bp	base pairs
CAT	chloramphenicol acetyl transferase
dbcAMP	dibutyryl cyclic adeno-mono phosphate
DEX	dexamethasone
DMEM	Dulbecco's modified Eagles' medium
DMSO	dimethylsulfoxide
DNA	deoxy-ribonucleic acid
DOG	L-sn-1,2 dioctanoyl glycerol
EGF	epidermal growth factor
aFGF	acidic fibroblast growth factor
bFBF	basic fibroblast growth factor
GAP-43	growth-associated protein
kb	kilobase
NGF	nerve growth factor
NF-M	neurofilament protein: middle molecular weight
PDGF	platelet-derived growth factor
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TGF-β	transforming growth factor beta
TPA	12-*O*-tetra-decanoyl phorbol-13-acetate

ACKNOWLEDGEMENTS

I am sincerely grateful and indebted to the following who made the work of this thesis possible:

-Members of the Magun and Ciment labs for their technical and intellectual support

-Various faculty members for their helpful suggestions, support, supplies and most of all, their time. They include **Drs. L. Baizer, J. Bissonnette, E. Lewis, B. Magun, R. Nishi, S. Planck, K. Rodland** and **G. Shipley**.

-My friends and family for their support and for showing me that there's a wonderful life B.B.G.S. (besides being a graduate student). My very special thanks to **Mehana, Peter, Rachel, Tom, Eileen, Bev, Cliff, Pauline, Jim, Sr., Irene, Mrs. Florence Knotts,** my pastor and his wife, **Mr. and Mrs. M. Baxter**, and many many others.

A special note of appreciation goes to my mentor, **Dr. Gary Ciment**, who in my opinion has been the "ideal advisor". He managed to survive the agony of reading my early drafts and has taught me the meaning of TOPIC SENTENCES.

Finally, my deepest appreciation goes to two very special people:

To my husband, **Curt**, for his example, love, understanding and support.

To my daughter **Cerina**, whose form of entertainment on many Saturday mornings was looking at "Mom's cells".

ABSTRACT

Extracellular proteinases are hypothesized to be involved in various morphogenetic events that occur during development and during neoplasia. These events include cell migration, regeneration, angiogenesis, neuronal differentiation, transformation and metastasis. Given the importance of these events, it seems likely that the expression of these proteases is under tight regulatory control.

One of the first proteinases to be cloned and characterized was the transformation-associated metalloproteinase, transin (Matrisian et al., 1985). Transin is known to be regulated by various growth factors in fibroblasts derived from a variety of sources (Matrisian et al., 1986b; Frisch and Ruley, 1987; Kerr et al., 1988). Recently, transin has been shown to be the rat homologue of stromelysin (Muller et al., 1988), a proteinase known to degrade various components of the extracellular matrix, including fibronectin, laminin and type IV collagen (Chin et al., 1985; Okada et al., 1986).

In initial work, I continued to characterize transin expression in Rat-1 fibroblasts. I found that transin mRNA is induced in these non-transformed fibroblasts by epidermal growth factor (EGF). This is in contrast to its constitutive expression in nearly all transformed cells, as well as in malignant mouse carcinomas. Induction of transin mRNA in Rat-1 cells requires protein synthesis, suggesting that EGF acts via an intermediary protein. Transforming growth factor type beta-1 (TGF-β1) was found to inhibit transin induction at both the transcriptional level and at the level of transin RNA accumulation. Although antagonistic interactions between EGF and TGF-β1 have been previously described, this was the first report that these growth factors act at the same molecular level.

More recently, I have examined the regulation of transin in PC12 cells -- a model system used extensively to study neuronal differentiation. Unlike its regulation in fibroblasts, transin is induced in PC12 cells by nerve growth factor (NGF) and by the fibroblast growth factors (FGF). The time course of transin induction by NGF is coinci-

dent with the neuronal differentiation of these cells, suggesting that these two phenomena may be linked. Induction of transin mRNA by NGF is inhibited by glucocorticoids at the transcriptional level. Finally, the NGF and glucocorticoid responsiveness appears to reside in 750 base pairs of the transin promoter region immediately adjacent to the transcriptional start site. This is the first reported case of transin's possible role in axon elongation.

The gene encoding the metalloprotease transin, therefore, appears to be differentially regulated by various growth factors in two distinct cell lines. Although other proteases have been associated with either neoplastic transformation or neuronal differentiation, the work reported here suggests that the transin may play a role in both of these phenomena. These data indicate that the regulated expression of proteases serve as important controls during both physiological and pathological morphogenetic events.

I. INTRODUCTION

Proteinases have been hypothesized to be involved in tissue remodeling during normal development and in various pathological states (Mullins and Rohrlich, 1983; Dano et al., 1985; Goldfarb and Liotta, 1986; Saksela and Rifkin, 1988). Various proteinases have been associated with angiogenesis (Herron et al., 1986), ovulation (Bicsak et al., 1989), embryonic implantation (Glass et al., 1983; Fisher et al., 1985), arthritis (Brinkerhoff et al., 1983; Okada et al., 1985; Okada et al., 1986), neoplasia (Goldberg, 1974; Sherman et al., 1976; Nakajima et al., 1984; Kao and Stern, 1986; Zeydel et al., 1986; Garbisa et al., 1987) and neuronal differentiation (Krystosek and Seeds, 1981a; 1981b;1986; Moonen et al., 1982), suggesting that the synthesis and secretion of these proteinases are well-regulated events. Some of these proteinases are induced by a variety of extracellular proteins, including growth factors (Chua et al., 1985; Bauer et al., 1985; Laiho et al., 1986; Frisch and Ruley, 1987). This thesis addresses the molecular mechanisms by which polypeptide growth factors affect the expression of the gene encoding the metalloproteinase transin.

A. Growth Factors

1. Epidermal Growth Factor (EGF)

Epidermal growth factor (EGF) is perhaps the best characterized growth factor (for review, see Carpenter and Cohen, 1978). The first description of EGF occurred in 1962 as a substance which could stimulate precocious eyelid opening and tooth eruption (Cohen, 1962). This bioassay for EGF activity led to its purification from mouse male submaxillary gland (Cohen and Carpenter, 1975; Gregory, 1975). Later, EGF became recognized for its mitogenic properties of epithelial cells as well as many other cell types (Carpenter and Cohen, 1973; Hollenberg and Cuatrecasa, 1973). EGF is first synthesized as a 128kDa precursor (Gray et al., 1983), and then processed into its mature form of 53 amino acids (6045 daltons) containing 3 internal disulfide bonds. These disulfide bonds are required for its biological activity (Taylor et al., 1972). Circular dichroic studies of the far-ultraviolet spectrum of EGF indicated that the molecule has a stable tertiary struc-

ture that is relatively compact and globular (Holladay et al., 1976). This compactness is mainly due to the many β pleated sheets, as well as the three disulfide bonds. In addition to its mitogenic properties in a number of cell types of ectodermal and mesodermal origin (Covellie et al., 1972; Armelin, 1973; Rose et al., 1975; Westermark, 1976; Rheinwald and Green, 1977), EGF has more recently been implicated as a neurotrophic molecule for neonatal neurons in the central nervous system (Morrison et al., 1987; also see below).

Nearly 2 decades after the first description of EGF, its receptor was purified from A-431 cells (Cohen et al., 1982) a cell line derived from human squamous carcinoma. This cell line has an increased number of EGF receptors (Fabricant et al., 1977), and therefore served as a good tissue source for the isolation of the EGF receptor. The 170-kDa receptor is an integral membrane protein that exhibits three domains (Cohen et al., 1982): 1) an extracellular ligand-binding domain, 2) a transmembrane domain and 3) an intracellular domain that contains tyrosine kinase function and binding sites for ATP phosphorylation substrates. In response to EGF binding, the receptor autophophorylates on its tyrosine residue (Ushiro and Cohen, 1980), the complex is internalized and subsequently degraded in lysosomal vesicles (Carpenter and Cohen, 1976; Gorden et al., 1978; Schlessinger et al., 1978; Stoschech and Carpenter, 1984; Matrisian et al., 1987a). Other growth factor receptors which resemble the EGF receptor with respect to its single transmembrane domain and repeating cysteine residues in the N-terminal extracellular domain are receptors for low density lipoprotein (Yamamoto et al., 1984), insulin (Ebina et al., 1985; Ullrich et al., 1985), and NGF (Chao et al., 1986; Johnson et al., 1986).

EGF immunoreactivity (Fallon et al., 1984) and precursor messenger RNA (mRNA) (Rall et al., 1985) have been identified in both forebrain and midbrain structures. In the developing mouse brain, the number of high affinity EGF receptors increased from embryonic day 11 to embryonic day 19 (Adamson and Meek, 1985). This suggests EGF may be involved in CNS development. More recently, EGF was found to promote the survival of neonatal rat CNS neurons and to stimulate their process outgrowth (Morrison et al., 1987). These data suggest that in addition to its mitogenic affect, EGF

promotes differentiation of subsets of neurons in the CNS much like NGF does in the peripheral nervous system (see below).

EGF acts through a number of effector systems, including the well-studied phosphoinositide pathway (for reviews, see Nishizuka, 1983;1984;1986; Berridge and Irvine, 1984; Majerus et al., 1985; Bell, 1986; Nahorski et al., 1986; Kikkawa and Nishizuka, 1986; Stryer and Bourne, 1986; Snyder, 1988; Benovic et al., 1988; Yarden and Ullrich, 1988). The first step of this pathway is the binding of a variety of agents, including EGF to specific cell surface receptors (Berridge et al., 1984; Brown et al., 1984; Vicentini and Villereal, 1984; Carney et al., 1985; Chu et al., 1985; Hasegawa-Sasaki, 1985; Sasaki and Hasegawa-Sasaki, 1985; Besterman, 1986). Receptor activation results in the activation of the enzyme phospholipase C. Phospholipase C, in turn, hydrolyzes the membrane lipid polyphosphatidyl-4,5-bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacyglycerol, both of which are believed to act as "second messengers". IP_3 is believed to elevate cytoplasmic calcium levels by inducing calcium release from intracellular stores, presumably in the endoplasmic reticulum (Berridge and Irvine, 1984). Diacylglycerol is believed to act as a potent activator of protein kinase C, thus catalyzing the phosphorylation of various substrate proteins (Nishizuka, 1984). Increased phosphoinositide metabolism has been implicated in activation of the Na^+/H^+ antiporter responsible for the cytosolic alkalinization ultimately associated with cellular proliferation (Vara et al., 1985; Muldoon et al., 1987). Activation of protein kinase C can in turn, activate other effector systems including the cyclic nucleotide pathway (Katada et al., 1985). Alternatively, activation of components of the cyclic nucleotide pathway including G proteins, and adenylate cyclase can feedback on various steps of the phosphoinositide pathway (Kaczmarek et al., 1980; Charest et al., 1983; Zavoico and Feinstein, 1984; Mullaney et al., 1988). Thus, the binding of EGF to its receptor activates several intracellular signals. None of these signals are mutually exclusive and may be acting in series or in parallel with one another.

Transforming growth factor-type beta 1 (TGF-β1) was originally isolated from conditioned media of transformed cells and purified on the basis of its ability to stimulate

the anchorage-independent growth of non-transformed fibroblasts in a reversible fashion (DeLarco and Todaro, 1978). This ability to confer a reversible transformed phenotype on non-transformed cells and the observation that many cells secrete increased levels of TGF-β1 following transformation (Tucker et al., 1983; Anzano et al., 1985), suggested that TGF-β1 might play an autocrine role in cancer development. In this sense, TGF-β1 may act to maintain tumor cells in a transformed state.

TGF-β however, apparently has other roles in the regulation of non-pathological conditions (Rizzino, 1988). Many normal cell types synthesize TGF-β1, for example, and virtually all types of cells have receptors for TGF-β1 (Cheifetz et al., 1986; Sporn et al., 1986; Ohta et al., 1987; Cochet et al., 1988; Sandberg et al., 1988). Additionally, TGF-β1 is generally more inhibitory than stimulatory to tumor cells, particularly those of epithelial or neuroectodermal origin (Moses et al., 1985). TGF-β1 appears to both antagonize and synergize the actions of EGF on cellular growth depending on the cell type (Roberts et al., 1985). TGF-β1 may modulate the effects of EGF at the level of the EGF receptor (Reynolds, Jr., 1981; Massague, 1985) or may affect the levels of various second messengers (Muldoon et al., 1988a,b).

Oncogenes, which encode acutely transforming genetic agents in viruses, have cellular counterparts termed "proto-oncogenes" (see Heldin and Westermark, 1984). Many of these proto-oncogenes share sequence similarities with the genes encoding a variety of known growth factors, growth factor receptors, and various components of their signal transduction pathways (see Figure 1; for reviews see Heldin and Westermark, 1984; Goustin et al., 1986; Hanley, 1988). For example, the cellular homolog of the *sis* oncogene is similar to the gene that encodes the B-chain of platelet-derived growth factor (PDGF). The activated PDGF receptor, in turn, induces the nuclear oncogenes c-*myc* and c-*fos* (Muller et al., 1984; Kruijer et al., 1984), the latter of which encodes a putative transcription factor for a variety of genes (for review see Curran and Franza, 1988). Constitutive activation of c-*myc* in some circumstances results in an apparently continuous stimulus to proliferate. The oncogenic erbB protein appears to correspond to a truncated EGF

receptor; the lack of a regulatory EGF binding domain may then be accompanied by constitutive activation (Downward et al., 1984).

In addition to their effect on proliferation, oncogenic proteins also mimic the differentiation capabilities of growth factors. For example, studies in PC12 pheochromocytoma cell line demonstated that the presence of viral *src* (v-*src*) (Alema et al., 1985; Casalbore et al., 1986), viral Kirsten and Harvey *ras* (Noda et al., 1985) and activated Ha- and N-*ras* proteins (Bar-Sagi and Feramisco, 1985; Guerrero et al., 1986) induced neuronal differentiation that was morphologically and biochemically indistinguishable from that induced by nerve growth factor (see below). Moreover, microinjection of anti-p21 (c-*ras*) antibodies in PC12 cells blocks NGF-induced, but not cyclic AMP-induced, neurite formation (Hagog et al., 1986), suggesting that *ras* may activate the same transduction pathway as NGF. Nonetheless, it is uncertain whether oncogenes activate the same molecular pathway as growth factors, or whether they act through different mechanisms, but converge on the same endpoint (i.e., proliferation or differentiation).

2. Nerve Growth Factor (NGF)

Nearly 40 years ago Levi-Montalcini and Hamburger demonstrated that cocultivation of mouse sarcoma tissue with sensory and sympathetic ganglia provoke an impressive outgrowth of nerve fibers from the ganglionic explants (Levi-Montalcini and Hamburger 1951;1953;1954). This classical bioassay was the basis of the isolation and purification of NGF from the submaxillary gland of the adult male mouse (Cohen, 1960). Since its initial isolation the biology and biochemistry of NGF has been well-studied (for reviews, see Greene and Shooter, 1980; Thoenen and Barde, 1980, Yanker and Shooter, 1982; Levi et al., 1988). The biologically active NGF molecule consists of two noncovalently linked, identical peptide chains each consisting of 118 amino acid residues (Greene et al., 1971; Angeletti et al., 1971; Angeletti and Bradshaw, 1971). Initial amino acid sequence analysis of this subunit demonstrated similarities with the proteins of the insulin group - proinsulin, insulin, the insulin-like growth factors and relaxin (Angeletti et al., 1971; Frazier et al., 1972).

NGF in known for its ability to support the survival of neurons and promote neuronal differentiation during the development of the nervous system (cf Thoenen and Barde, 1980; Yanker and Shooter 1982). One dramatic example of its biological role as a neurotrophic molecule was observed when injection of antibody to NGF resulted in the irreversible degeneration of the paravertebral and pre-vertebral sympathetic ganglia (Levi-Montalcini and Booker, 1960). In the adult rat, injection of NGF antibody or autoimmunization against NGF results in decreased sympathetic neuronal size and protein content as well as a decrese in tyrosine hydroxylase and dopamine β hydroxylase activities (Otten et al., 1979). The first evidence for an effect of NGF on neuronal differentiation was observed with the coculture of sarcoma tissue with sympathetic or sensory ganglia which brought about outgrowth of nerve fibers (Levi-Montalcini and Hamburger, 1951;1953). Since then, the development of a neuronal cell model, PC12 (see below) demonstrated that these premature chromaffin-like cells eventually stop dividing after a few days and extend neuritic processes in response to NGF (Greene and Tischler, 1976).

Injection of ^{125}I-NGF into the anterior eye chamber of the rat resulted in the subsequent appearance of label in the neuronal cell bodies of the superior cervical ganglia (Hendry et al., 1984; Dumas et al., 1975), demonstrating that there is a retrograde transport of NGF down the axon (see Figure 2 for NGF pathway). Uptake of NGF was found to be hormone-specific and receptor-mediated and was not quantitatively affected by simultaneous uptake and transport of a variety of lectins and toxins (Dumas et al., 1975). Moreover, the uptake of NGF was not stimulated by electrical activity as is the case with the nonspecific uptake of other macromolecules (Stoeckel et al., 1978). These initial studies provided evidence that the action of NGF is mediated by a specific receptor. More recently, the genomic and cDNA clones containing the entire coding region of the human NGF receptor have been isolated (Chao et al., 1986; Johnson et al., 1986). These studies showed the NGF receptor has structural similarities (i.e. a single transmembrane domain) to other membrane proteins including the receptors for low density lipoprotein (Yamamoto et al., 1984), EGF (Ullrich et al., 1984) and insulin (Ebina et al.,

1985; Ullrich et al., 1985). Detailed computer analysis revealed, however, these membrane receptors shares no sequence homologies and that the structural similarities were confined to repeating cysteine residues in the N-terminal extracellular domain. The extracellular region appears to be very acidic and contains two potential sites for N-linked glycosylation.

Kinetic studies reveal high affinity (0.2nM) and low affinity (5 nM) receptor subtypes, but only the high affinity receptors are responsible for internalization of NGF (Bernd and Greene, 1984). Internalization of NGF in PC12 cells is most likely to occur by receptor-mediated endocytosis (Levi et al., 1980) and is accompanied by the down regulation of receptors (Calissano and Shelanski, 1980; Layer and Shooter, 1983). The role of NGF as a retrograde messenger between peripheral target tissues and innervating neurons is supported by the observation that (a) the interruption of retrograde axonal transport has the same effects as the neutralization of endogenous NGF by anti-NGF antibodies and (b) the close correlation between the density of innervation by fibers of NGF-responsive neurons and the levels of NGF and NGF mRNA in their target organs (Korsching and Thoenen, 1983; see also Schwab and Thoenen, 1983).

Both nerve growth factor and its receptor are expressed throughout development. Although NGF mRNA was undetectable prenatally in total rat brain (Whittemore, et al., 1988; Aubuger et al., 1987), NGF protein was nearly at adult levels in prenatal brain tissue reaching peak expression (160% of adult levels) at 3 weeks postnatal (Whittemore et al., 1986). This discrepancy may reflect a rapid turnover of message and thus very low steady state level. NGF mRNA becomes detectable 1 day after birth and reaches adult levels by 3 weeks postnatal. The time course of NGF appearance in the brain closely parallels the maturation of synapses in cortex, hippocampus, as well as expression of hippocampal choline acetyl transferase (see Whittemore et al., 1988). Early chick embryo (E3.5-E12) expresses high levels of NGF receptor mRNA, particularly in the spinal cord, skeletal muscle and skin (Ernfors et al., 1988). These levels substantially decline later in development in all tissues tested except the brain. NGF receptor mRNA was also

detected in the lymphoid tissues, including the bursa Fabricius, thymus and spleen of chicken, and the lymph node, thymus, and spleen of adult rat (Ernfors et al., 1988) suggesting the NGF receptor may also serve a nonneuronal trophic function in the immune system.

The PC12 rat pheochromocytoma cell line is a well-established culture model for studying the events of neurogenesis induced by NGF (for review, see Greene and Tischler, 1982). In the absence of NGF, PC12 cells morphologically and biochemically resemble their in vivo counterpart, undifferentiated adrenal medullary cells (Greene and Tischler, 1976;1982). When NGF is added to the culture medium, however, PC12 cells begin to extend processes which are morphologically and biochemically indistinguishable from axons of sympathetic neurons (Luckenbill-Edds et al., 1979; Lee and Page, 1984). After several days of exposure to NGF, the PC12 cells complete their differentiation and assume many other properties of sympathetic neurons (Dichter et al., 1977; Rudy et al., 1987). This ability of PC12 cells to undergo metaplastic transformation into sympathetic neurons with exposure to NGF has also been shown to occur with normal adrenal chromaffin cells, as well (Unsicker et al., 1978; Aloe and Levi-Montalcini, 1979).

The various responses of PC12 cells to NGF have been divided into two distinct phases: early effects and late effects (Greene and Tischler, 1982). The early effects of NGF on PC12 cells are characterized by rapid transient changes in levels of putative second messengers and the levels of expression of certain genes. Within seconds to minutes of exposure to NGF, PC12 cells exhibit a very rapid increase in intracellular free Ca^{++} concentrations from both extracellular and intracellular stores (Pandiella-Alonso et al., 1986). This effect is not induced by either EGF or by an increased level of cAMP. It has also been shown that within minutes of binding to PC12 cells, NGF increases the incorporation of phosphate in phosphatidylinositol (Traynor et al., 1982); stimulates the turnover of phosphatidic acid (Traynor et al., 1984) and potentiates the bradykinin-stimulated accumulation of inositol phosphate (Van Calker and Heumann, 1987). The increased Ca^{++} mobilization and phospholipid turnover suggest the activation of protein

kinase C. In fact, the activity of several protein kinases including kinase C are rapidly induced in PC12 cells upon exposure to NGF (Hama et al., 1986; Blenis and Erikson, 1986; Matsuda et al., 1986; Rowland et al., 1987).

Within minutes of addition of NGF to PC12 cells, a number of genes are rapidly, but transiently induced including the proto-oncogenes c-*fos* and c-*myc*, actin, ornithine decarboxylase, and others (Greenberg et al., 1985; Curran and Morgan, 1985; Kruijer et al., 1985; Milbrandt, 1986; Kujubu, et al., 1987). In contrast to the later induced genes (see below), the induction of many of these early genes does not require protein synthesis. These early genes, however, do not specifically respond to only NGF, because other agents such as tumor-promoting agent tetradecanoyl phorbol acetate (TPA), cAMP, Ca^{++}, and EGF can induce them as well. Many of these early induced changes may be involved in the transient mitogenic effects of NGF which occurs within the first 24 hrs of exposure to NGF (Boonstra et al., 1983).

The late effects of NGF in PC12 cells are characterized by the acquisition of the differentiated neuronal phenotype. NGF-induced phenotypic changes in PC12 cells such as induction of electrical excitability, increase in sodium channels, muscarinic acetylcholine receptors, opiate receptors and neurite outgrowth occurs on the order of hours to days (for reviews, see Greene et al., 1985; Levi et al., 1988). The initial quest aimed at detecting proteins differentially expressed in NGF-treated and untreated PC12 cells using two-dimensional electrophoresis yielded a disappointing small number of minor changes (McGuire and Greene, 1980; Garrels and Schubert, 1979). The more sensitive molecular techniques such as differential hybridization and cDNA cloning showed that NGF induces the levels and/or synthetic rates of a number of genes including tubulin, microtubule associated proteins MAP-1, MAP-2 and tau, as well as vimentin, neurofilament proteins and others (Black et al., 1986; Drubin et al., 1985; Greene et al., 1983; Lee and Page, 1984; Levi et al., 1985; Leonard et al., 1987). It is unclear what intracellular signal transduction pathways are triggered by NGF because most of these late NGF-induced genes have high basal levels. These high basal levels make it difficult to assess whether

the activation of various pathways cause changes in the level of expression of these genes.

B. PROTEASES

1. Classification of proteases. Proteases are divided into two groups: 1) Exopeptidases which cleave polypeptides externally and 2) Endopeptidases or proteinases which cleave polypeptides internally. The four types of proteinases are classified on the basis of their catalytic mechanism. They are the serine, cysteine, aspartic and metallo-proteinases (see Barrett, 1980). Each of these subclasses have a different pH range for activity as well as different inhibitors. There are over 50 mammalian serine proteinases which include trypsin, elastase, coagulation factors, and plasminogen activators. They share homology in the amino acid sequences surrounding the reactive serine residues. The serine proteinases are particular sensitive to the specific inhibitor, diisopropyl fluorophosphate. The cysteine proteinases are thiol-dependent and include the cathepsin family and papain. The active site of aspartic proteinases appear to be the carboxyl group of aspartic acid residues. This subclass includes the pepsins and renin. Metalloproteinases depend upon the presence of a metal ion (usually Zn^{2+}) in their active site. They are inhibited by dithiothreitol and by chelating agents such as EDTA and 1,10-phenanthroline. Inhibition by dithiothreitol distinquishes a metalloproteinase from the cysteine proteinases. Metalloproteinases such as collagenase have been identified in a number of tissues involved in connective tissue breakdown such as the involuting uterus, rheumatoid synovial tissue, resorbing bone and wounded skin (see Evanson, 1971).

2. Proteinases in neoplasia.

Proteinases are believed to be involved in a number of phenomena associated with the metastatic process (see Liotta, 1986). These multi-step phenomena involve 1) detachment of malignant cells from the original tumor mass, 2) dissolution of the basement membrane, 3) migration of metastasized cells into (intravasation) and then out from (extravasation) capillary endothelial walls and 4) establishment of the metastatic foci at a distant site. Each of these steps have been shown to involve proteolytic enzymes such as collagenase, cathepsin B and/or plasminogen activator (Dano et al., 1985; Hearing et al.,

1988) which may activate one another in a proteolytic cascade (Mignatti et al., 1986). Tumor cells, moreover, were found to degrade both collagenous and noncollagenous components of the basement membrane (Liotta et al., 1977). Beyond these correlative data much less is known regarding the whether these proteinases are required for the metastatic process.

3. Proteases in neuronal differentiation.

Proteases may be involved in the growth of axons during development of the nervous system. One major feature of the developing nervous system is that differentiating neurons project their processes to their respective target tissues. At the leading edge of the growing axon is a specialized structure called the growth cone which is believed to mediate the events of axon elongation including a) adherence to the substratum, b) recognition of appropriate pathways, and c) movement (see Landis, 1983; Bray and Hollenbeck, 1988). Once viewed as a "battering ram", the growth cone may be more refined in its actions of attachment and detachment. For example, proteases are believed to be selectively released in the detachment process. Various proteinases have been reported to be released by growth cones of sympathetic and sensory neurons including the serine protease, plasminogen activator (Krystosek and Seeds, 1981a,b; 1986), and a calcium-dependent metalloprotease (Pittman, 1985). Moreover, inhibition of this metalloprotease activity results in a decrease in the rate of neurite outgrowth (Pittman and Williams, 1989), suggesting that protease activity is involved in process outgrowth.

There are several possible roles that proteinases may play in the process of axon elongation. First, these proteinases may simply degrade proteinaceous barriers in the extracellular matrix which would normally hinder the movements of growth cones. Conceivably, this would open up spaces in the matrix through which the growth cones and axons could then pass. Second, proteinases may degrade either substrate-attached neurotropic factors, such as NGF (see Gundersen, 1985) or molecules involved in axonal adhesion, such as laminin (see Rogers et al., 1983). From this scenario, proteases would serve to continuously regenerate concentration gradients of these guidance molecules,

thereby promoting directional growth of the axon towards higher concentrations of these molecules. Lastly, secreted proteinases may be involved in the detachment process by which proximal areas of the growth cone de-adhere from the substratum, allowing forward movement of the growth cone.

III. Transin

1. Isolation and characterization of transin.

The gene encoding rat transin has been cloned and characterized (Matrisian et al., 1985). The cDNA for rat transin was isolated by differential subtractive techniques between normal and transformed rat fibroblasts (PyT21), suggesting that transin may represent a transformation-specific protein. The protein contains 475 amino acids (53,000 daltons) deduced from its nucleic acid sequence. Electron microscopic analysis of the transin gene hybridized with its cDNA, together with restriction enzyme mapping and sequence analysis, revealed that the rat transin gene consisted of eight exons spanning a total of 14 kb (Matrisian et al., 1986b). The 1.9 kb rat transin mRNA may be the product of a single copy gene based on Southern blot analyses. The promoter region of the transin gene contains a classic TATA box at position -30 and a CAAT box at position -77 relative to the transcriptional start site (Matrisian et al., 1986b, see also Figure 6, Appendix).

The DNA sequences of several secreted metalloproteinases have subsequently become available and shows that transin is a member of this family. Rat transin was found to share 48% homology to human type-1 collagenase (Goldberg et al., 1986) and 75% homology to rabbit stromelysin (Whitham et al., 1986). The high homology to rabbit stromelysin suggested that transin may be the rat homologue of stromelysin. More convincing evidence that these two proteinases were the same molecule came from restriction enzyme mapping and sequence analysis of 33 clones from human tumors using the rat transin probe (pTR1) under low stringency conditions (Muller et al., 1988). Comparison of these data with previously published sequences of human stromelysin demonstrated that transin is the rat homologue of human stromelysin (Whitham et al., 1986; Wilhelm et al., 1987; Muller et al., 1988). Thus, the following four members of the metalloproteinase

family have been cloned and sequenced from human tissues: transin (stromelysin), collagenase, and two novel proteinases, stromelysin-2 and pump-1 (for putative metalloproteinase) (Muller et al., 1988). Amino acid analysis of human stromelysin, rat transin, and human collagenase revealed all three metalloproteinases contain a conserved arginine residue (at position 120) suggestive of N-glycosylation, as well as a conserved triplet of basic amino acids immediately preceding the site of trypsin cleavage during zymogen activation (Wilhelm et al., 1987)

Transin is a secreted proteinase that degrades extracellular matrix components. Stromelysin, (rat transin) has been isolated from the conditioned media of rabbit synovial fibroblasts (Chin et al., 1985) treated with 12-O-tetradecanoylphorbol 13-acetate (TPA) and from human skin fibroblasts (Wilhelm et al., 1987). Both rabbit and human stromelysin are secreted as preproenzymes based on zymogen activation followed by SDS polyacrylamide electrophoresis (Wilhelm et al., 1987) with apparent molecular weights of 51 and 54 kDa, respectively (Wilhelm et al., 1987). Purified stromelysin degrades casein, cartilage proteoglycans, fibronectin, laminin, elastin, type IV collagen, and gelatin, but does not degrade type I collagen (Chin et al., 1985; Okada et al., 1986; Wilhelm et al., 1987).

2. Transin gene expression

A number of agents associated with transformation induce transin gene expresion, suggesting expression of transin may be related to the the metastatic phenotype. For example, in fetal rat fibroblasts the transin gene is induced upon transformation with polyomavirus, Rous sarcoma virus, and the activated oncogene Ha-*ras* (Matrisian et al., 1985). Furthermore, transin mRNA is present at significantly higher levels in chemically induced skin carcinomas than in normal skin benign papillomas (Matrisian, et al., 1986a; Ostrowski et al., 1988). Many tumors that express transin are rapidly evolutive (head and neck carcinoma) or advanced-stage diseases (pooly differentiated lung squamous-cell carcinomas) (Muller et al., 1988), suggesting transin may contribute to the highly malignant state of epidermoid carcinomas. These findings are consistent with the notion that

increased expression of secreted proteases plays an important role in tumor invasion (Mignatti et al., 1986; Liotta, 1986). However, in contrast to these findings, expression of human stromelysin in normal and tumorigenic human cells does not consistently correlate with the metastatic phenotype (Wilhelm et al., 1987). This suggest that the role of transin (i.e. stromelysin) in tumor cell invasion may be cell-type specific and possibly varies among species.

Transin appears to be differentially regulated by different growth factors in various fibroblastic cell lines. In non-transformed Rat-1 fibroblasts transin mRNA expression is induced only upon treatment with epidermal growth factor. Other growth factors and agents including platelet-derived growth factor (PDGF), fibroblast growth factor, insulin and transforming growth factor-beta are not capable of eliciting any effect (Matrisian et al., 1986b). However, in mouse NIH 3T3 fibroblasts, transin RNA is induced by EGF, PDGF, and the phorbol ester tumor promoter, TPA (Kerr et al., 1988). Thus, not only do growth factors vary in their ability to induce transin depending on the cell type and perhaps species, but growth factors appear to regulate transin by both c-*fos*-dependent and c-*fos*-independent pathways (Kerr et al., 1988). In light of the recent evidence that transin may be the rat homologue of stromelysin (see above), it appears that 700 bases of the promoter region of this proteinase also confers induction by interleukin-1 and repression by dexamethasone in rabbit synovial fibroblasts (Frisch and Ruley, 1987). No report has yet been made regarding the possible induction of transin by nerve growth factor.

REFERENCES

Adamson, E.D. and Meek, J. (1984). The ontogeny of epidermal growth factor receptors during mouse development. Dev. Biol. *103*, 62-70.

Alema, S., Casalbore, P., Agostini, E. and Tato, F. (1985). Differentiation of PC12 pheochromocytoma cell induced by v-*src* oncogene. Nature *316*, 557-559.

Aloe, L. and Levi-Montalcini. (1979). Nerve growth factor-induced transformation of immature chromaffin cells in vivo into sympathetic neurons: Effect of antiserum to nerve growth factor. Proc. Natl. Acad. Sci. USA *76*, 1246-1250.

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

Angeletti, R.H., Bradshaw, R.A. and Wade, R.D. (1971). Subunit structure and amino acid composition of mouse submaxillary gland nerve growth factor. Biochemistry *10*, 463-469.

Anzano, M.A., Roberts, A.B., DeLarco, J.E., Wakefield, L.M., Assoian, R.K., Roche, N.S., Smith, J.M., Lazarus, J.E. and Sporn, M.B. (1985). Increased secretion of type β transforming growth factor accompanies vial transformation of cells. Mol. Cell. Biol. *5*, 242-247.

Armelin, H.A. (1973). Pituitary extracts and steroid hormones in the control of 3T3 cell growth. Proc. Natl. Acad. Sci. USA *70*, 2702-2706.

Aubuger, G., Heumann, R., Hellweg, R., Korshing, S. and Thoenen, H. (1987). Developmental changes of nerve growth factor and its mRNA in the rat hippocampus: Comparison with choline acetyltransferase. Dev. Biol. *120*, 322-328.

Barrett, A.J. (1980). Introduction: The classification of proteinases. In: Protein Degradation in Health and Disease. Ciba Foundation Symposium 75. Excerpta Medica. New York, pp.1-9.

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

Bauer, E.A., Cooper, T.W., Huang, J.S., Altman, J. and Deuel, T.F. (1985). Stimulation of in vitro human skin collagenase expression by platelet-derived growth factor. Proc. Natl. Acad. Sci. USA *82*, 4132-4136, 1985.

Bell, R.M. (1986). Protein kinase C activation by diacylglycerol second messengers. Cell *45*, 631-632.

Benovic, J.L. Bouvier, M., Caron, M.G. and Lefkowitz, R.J. (1988). Regulation of adenylyl cyclase-coupled β-adrenergic receptors. Ann. Rev. Cell Biol. *4*, 405-428.

Bernd, P. and Greene, L.A. (1984). Association of ^{125}I-nerve growth factor with PC12 pheochromocytoma cells: Evidence for internalization via high-affinity receptors only and for long-term regulation by NGF of both high- and low-affinity receptors. J. Biol. Chem. *259*, 15509-15516.

Berridge, M.J., Heslop, J.P., Irvine, R.F. and Brown, K.D. (1984). Inositol triphosphate formation and calcium mobilization in Swiss 3T3 cells in response to platelet-derived growth factor. Biochem. J. *222*, 195-201.

Berridge, M.J. and Irvine, R. (1984). Inositol triphosphate, a novel second messenger in

cellular signal transduction. Nature *312*, 315-321.

Besterman, J.M., Watson, S.P. and Cuatrecasas, P. (1986). Lack of association of epidermal growth factor-, insulin-, and serum-induced mitogenesis with stimulation of phosphoinositide degradation in BALB/c 3T3 fibroblasts. J. Biol. Chem. *261*, 723-727.

Bicsak, T.A. Cajander, S.B., Peng, X-R., Ny, T., LaPolt, P.S., Lu, J.K.H., Kristensen, P., Tsafriri, A. and Hsueh, A.J.W. (1989). Tissue-type plasminogen activator in rat oocytes: Expression during the periovulatory period, after fertilization, and during follicular atresia. Endocrinology *124*, 187-194.

Black, M.M., Aletta, J.M. and Greene, L.A. (1986). Regulation of MT composition and stability during NGF 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 J. *5*, 3441-3447.

Boonstra J., Mummery, C.L., Feyen A., DeHoog, W.J., VanderSaag, P.T. and DeLaat, S.W. (1987). Epidermal growth factor receptor expression during morphological differentiation of PC12 cells induced by olbcamp. J. Cell Physiol. *131*, 409-417.

Bray, D. and Hollenbeck, P.J. (1988). Growth cone motility and guidance. Ann. Rev. Cell Biol. *4*, 43-61.

Brinckerhoff, C.E., Coffey, J.W. and Sullivan, A.C. (1983). Inflammation and collagenase production in rats with adjuvant arthritis reduced with 13-*cis*-retinoic acid. Science 221, 756-758.

Brown, K.D., Blay, J., Irvine, R.F., Heslop, J.P. and Berridge, M.J. (1984). Reduction of epidermal growth factor receptor affinity by heterologous ligands: Evidence for a mechanism involving the breakdown of phosphoinositides and the activation of protein kinase C. Biochem. Biophys. Res. Comm. *123*, 377-384

Calissano, P. and Shelanski, M.L. (1980). Interaction of NGF with pheochromocytoma cells, evidence for tight binding and sequestration. Neuroscience *5*, 1033-1039.

Carney, D.H., Scott, D.L., Gordon, E.A. and LaBelle, E.F. (1985). Phosphoinositides in mitogenesis: Neomycin inhibits thrombin-stimulated phosphoinositide turnover and initiation of cell proliferation. Cell *42*, 479-488.

Carpenter, G. and Cohen, S. (1975). Human epidermal growth factor and the proliferation of human fibroblasts. J. Cell. Physiol. *88*, 227-238.

Carpenter, G. and Cohen, S. (1976). ^{125}I-labeled human epidermal growth factor (hEGF): Binding, internalization, and degradation in human fibroblasts. J. Cell Biol. *71*, 159-171.

Casalbore, P., Agostini, E., Tato, F., and Alema, S. (1986). Expression of the v-*src* oncogene induced differentiation in PC12 cells. In: Molecular Aspects of Neurobiology. (R. Levi-Montalcini, P. Calissano, E.R. Kandel, and A. Maggi, eds.). Springer-Verlag, Berlin, pp. 37-39.

Chao, M.V., Bothwell, M.A., Ross, A.H., Koprowski, H., Lanahan, A.A.. Bick, C.R. and Sehgal, A. (1986). Gene transfer and molecular cloning of the human NGF receptor. Science *232*, 518-520.

Charest, R., Blackmore, P.F., Berthon, B. and Exton, J.H. (1983). Changes in free

cytosolic Ca^{2+} in hepatocytes following α_1-adrenergic stimulation. J. Biol. Chem. *258*, 8769-8773.

Cheifetz, S., Like, B. and Massague, J. (1986). Cellular distribution of type I and type II receptors for transforming growth factor-β. J. Biol. Chem. *261*, 9972-9978.

Chin, J.R., Murphy, G. and Werb, Z. (1985). Stromelysin, a connective tissue-degrading metalloendopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase. J. Biol. Chem. *260*, 12367-12376.

Chua, C.C., Geiman, D.E., Keller, G.H. and Ladda, R.L. (1985). Induction of collagenase secretion in human fibroblast cultures by growth promoting factors. J. Biol. Chem. *260*, 5213-5216.

Chu, S.H., Hoban, C.J., Owen, A.J. and Geyer, R.P. (1985). Platelet-derived growth factor stimulates rapid polyphosphoinositide breakdown in fetal human fibroblasts. J. Cell. Physiol. *124*, 391-396.

Cochet, C., Feige, J.-J. and Chambaz, E.M. (1988). Bovine adrenocortical cells exhibit high affinity transforming growth factor-β receptors which are regulated by adrenocorticotropin. J. Biol. Chem. *263*, 5707-5713.

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

Cohen, S. (1962) Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J. Biol. Chem. *237*, 1555-1562.

Cohen S. and Carpenter, G. (1975). Human epidermal growth factor: isolation and chemical and biological properties. Proc. Natl. Acad. Sci., USA, *72*, 1317-1321.

Cohen, S., Ushiro, H., Stoscheck, C. and Chinkers , M. (1982). A native 170,000 epidermal growth factor receptor-kinase complex from shed membrane vesicles. J. Biol. Chem.*257*, 1523-1531.

Covelli, I., Mozzi, R., Rossi, R. and Friti, L. (1972). The mechanism of action of epidermal growth factor: Stimulation of the uptake of labeled precursors into RNA, DNA and proteins induced by EGF in isolated tumor cells. Hormones *3*, 183-191.

Curran, T. and Franza, B.R., Jr. (1988). Fos and Jun: The AP-1 connection. Cell *55*, 395-397.

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

Dano, K., Andreasen, P.A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L.S., L. Skriver. (1985). Plasminogen activators, tissue degradation, and cancer. Adv. Canc. Res. *44*, 139-227.

DeLarco, J.E. and Todaro, G.J. (1978). Growth factors from murine sarcoma virus-transformed cells. proc. Natl. Acad. Sci. USA *75*, 4001-4005.

Dichter, M.A., Tischler, A.S. and Greene, L.A. (1977). Nerve growth factor-induced increase in electrical excitability and acetylcholine sensitivity of a rat pheochromocytoma cell line. Nature *268*, 501-504.

Drubin, D.G., Feinstein, S.C., Shooter, E.M. and Kirschner, M.W. (1985). NGF induced neurite outgrowth in PC12 cells involves the coordinate induction of MT assembly and assembly prmoting factors. J. Cell Biol. *101*, 1799-1807.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., and Waterfield, M.D. (1984). Close similarity of epidermal growth factor receptor and v-*erb*-B oncogene protein sequences. Nature *307*, 521-527.

Dumas, M., Schwab, M.E. and Thoenen, H. (1975). Retrograde axonal transport of specific macromolecules as a tool for characterizing nerve terminal membranes. Brain Res. *88*, 37-43.

Ebina Y., Ellis, L., Jarnagin K., Edery M., Graf, L., Glauser, E., Ou, J.-H., Masiarz, F., Kan, Y.W., Goldfine, I.D., Roth, R.A. and Rutter, W.J. (1985). The human insulin receptor cDNA: The structural basis for hormone-activated transmembrane signalling. Cell *40*, 747-758.

Ernfors, P., Hallbook, F. Ebendal., T., Shooter, E.M., Radeke, M.J., Misko, T.P. and Persson H. (1988). Developmental and regional expression of beta-nerve growth factor receptor mRNA in the chick and rat. Neuron *1*, 983-996.

Evanson, J.M. (1971). Mammalian collagenase and their role in connective tissue breakdown. In: Tissue Proteinases (A.J. Barrett and J.T. Dingle, eds.). North-Holland Publishing Co., New York, pp.327-342.

Fabricant, R.N., DeLarco, J.E. and Todaro, G.J. (1977). Nerve growth factor receptors on human melanoma cells in culture. Proc. Natl. Acad. Sci. USA *74*, 565-569.

Fallon, J.H., Seroogy, K.B., Loughlin, S.E., Morrison, R.S., Bradshaw, R.A., Knauer, D.J. and Cunningham, D.D. (1984). Epidermal growth factor immunoreactive material in the central nervous system: Localization and development. Science *224*, 1107-1109.

Fisher, S.J., Leitch, M.S., Kantor, M.S., Basbaum, C.B. and Kramer, R.H. Degradation of extracellular matrix by the trophoblastic cells of first-trimester human placentas. J. Cell. Biochem. *27*, 31-41.

Frazier, W.A., Angeletti, R. H. and Bradshaw R.A. (1972). Nerve growth factor and insulin. Structural similarities indicate an evolutionary relationship reflected by physiological action. Science *176*, 482-488.

Frisch, S.M. and Ruley, H.E. (1987). Transcription from the stromelysin promoter is induced by interleukin-1 and repressed by dexamethasone. J. Biol. Chem. *262*, 16300-16304.

Garbisa, S., Pozzatti, R., Muschel, R.J., Saffiotti, U., Ballin, M., Goldfarb, R.H., Khoury, G. and Liotta, L.A. (1987). Secretion of type IV collagenolytic protease and metastatic phenotype: Induction by transfection with c-Ha-*ras* but not c-Ha-*ras* plus Ad2-E1a.

Garrels, J.I. and Schubert D. (1979). Modulation of protein synthesis by nerve growth factor. J. Biol. Chem. *254*, 7978-7985.

Glass, R.H., Aggeler, J., Spindle, A. Pederson, R.A. and Werb. Z. (1983). Degradation of extracellular matrix by mouse trophoblast outgrowths. A model for implantation. J. Cell Biol. *96*, 1108-1116.

Goldberg A.R. (1974). Increased protease levels in transformed cells: A casein overlay as-

say for the detection of plasminogen activator production. Cell *2*, 95-102.

Goldberg, G.I., Wilhelm, S.M., Kronberger, A., Bauer, E.A., Grant, G.A. and Eisen, A.Z. (1986). Human fibroblast collagenase: Complete primary structure and homology to an oncogene transformation-induced rat protein. J. Biol. Chem. *261*, 6600-6605.

Goldfarb, R.H. and Liotta, L. (1986). Proteolytic enzymes in cancer invasion and metastasis. Semin. Thromb. Hemost. *12*, 294-307.

Gorden, P., Carpentier, J.L., Cohen, S. and Orci, L. (1978). Epidermal growth factor: morphological demonstration of binding internalization and lysosomal association in human fibroblasts. Proc. Natl. Acad. Sci. USA *75*, 2659-2663.

Gospodarowicz, D. (1981). Epidermal and nerve growth factors in mammalian development. Ann. Rev. Physiol. *43*, 251-263.

Goustin, A.S., Leof, E.B., Shipley, G.D. and Moses, H.L. (1986). Growth factors and cancer. Canc. Res. *46*, 1015-1029.

Gray A., Dull T.J. and Ullrich, A. (1983). Nucleotide sequence of epidermal growth factor cDNA predicts a 128,000-molecular weight protein precursor. Nature *303*, 722-725.

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

Greene L.A., Aletta, J.M., Burstein, D.E., Drexler, S.A., Greenberg, M.F., Leonard, D. and Ziff, E.B. (1985). Gene regulation by nerve growth factor. In: Neurobiochemistry. (B.

Hamprecht and V. Neuhoff, eds.). Springer Verlag, New York, pgs 1-6.

Greene, L.A., Liem, 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.

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 pheochromocytomal cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA *73*, 2424-2428.

Greene, L.A. and Tischler, A.S. (1982). PC12 pheochromocytoma cultures in neurobiological research. In: Advances in Cellular Neurobiology, Volume 3 (S. Fedoroff, L. Hertz, eds.). Academic Press, New York, pp. 373-414.

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

Gregory, H. (1975). Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature *257*, 325-327.

Guerrero, I., Wong H., Pellicer, A., and Burstein, D. (1986). Activated n-*ras* gene induces neuronal differentiation of PC12 rat pheochromocytoma cells. J. Cell. Physiol. *129*, 71-76.

Gunderson, R.W. (1985). Sensory neurite growth cone guidance by substrate adsorbed nerve growth factor. J. Neurosci. Res. *13*, 199-212.

Hagag N., Halegoua, S., and Viola M. (1986). Inhibition of growth factor induced differentiation of PC12 cells by microinjection of antibody to *ras* p21. Nature *319*, 680-682.

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

Hasegawa-Sasaki, H. (1985). Early changes in inositol lipids and their metabolites induced by platelet-derived growth factor in quiescent Swiss mouse 3T3 cells. Biochem. J. *232*, 99-109.

Hearing, V.J., Law, L.W., Coreti, A., Appella, E. and Blasi, F. (1988). Modulation of metastatic potention by cell surface urokinase of murine melanoma cells. Canc. Res. *48*, 1270-1278.

Heldin, C-H. and Westermark, B. (1984). Growth Factors: Mechanism of action and relation to oncogenes. Cell *37*, 9-20.

Hendry, I.A., Stoeckel, K., Thoenen, H. and Iversen, L.L. (1974). The retrograde axonal transport of nerve growth factor. Brain Res. *68*, 103-121.

Herron, G.S., Banda, M.J., Clark, E.J., Gavrilovic, J. and Werb. Z. (1986). Secretion of metalloproteinases by stimulated capillary endothelial cells. J. Biol. Chem. *261*, 2814-2818.

Holladay, L.A., Savage, C.R., Jr., Cohen, S. and Puett, D. (1976). Conformation and unfolding thermodynamics of epidermal growth factor and derivatives. Biochemistry *15*, 2624-2633.

Hollenberg, M.D. and Cuatrecasas, P. Epidermal growth factor: receptors in human fibroblasts and modulation of action by cholera toxin. Proc. Natl., Acad. Sci. USA *70*, 2964-2968.

Johnson, D., Lanaha, 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.

Kaczmarek, L.K., Jennings, K.R., Strumwasser, F., Nairn, A.C., Walter, U., Wilson, F.D. and Greengard, P. (1980). Microinjection of catalytic subunit of cyclic AMP-dependent protein kinase enhances calcium action potentials of bag cell neurons in cell culture. Proc. Natl. Acad. Sci. USA *77*, 7487-7491.

Kao, R.T. and Stern, R. (1986). Collagenases in human breast carcinoma cell lines. Canc. Res. *46*, 1349-1354.

Katada, T., Gilman, A.G., Watanabe, Y., Bauer, S. and Jakobs, K.H. (1985). Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. Eur. J. Biochem. *151*, 431-437.

Kerr, L.D., Holt, J.T. and Matrisian, L.M. (1988). Growth factors regulate transin gene expression by c-*fos*-dependent and c-*fos*-independent pathways. Science *242*, 1424-1427.

Kikkawa, U. and Nishizuka Y. (1986). The role of protein kinase C in transmembrane signalling. Ann. Rev. Cell Biol. *2*, 149-178.

Korsching, S. and Thoenen, H. (1983). Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: Correlation with density of innervation. Proc. Natl. Acad. Sci. USA *80*, 3513-3516.

Kruijer, W., Cooper, J.A., Hunter, T. and Verma, I.M. (1984). Platelet-derived growth factor induces rapid but transient expression of the c-*fos* gene and protein. Nature *312*, 711-716.

Kruijer, W., Shubert, D. and Verma, M. (1985). Induction of proto-oncogene *fos* by nerve growth factor. Proc. Natl. Acad. Sci. USA *82*, 7330-7334.

Krystosek, A. and Seeds, N.W. (1981a). Plasminogen activator secretion by granule neurons in cultures of developing cerebellum. Proc. Natl. Acad. Sci. USA *78*, 7810-7814.

Krystosek, A. and Seeds, N.W. (1981b). Plasminogen activator release at the neuronal growth cone. Science *2213*, 1532-1533.

Krystosek, A. and Seeds, N.W. (1986). Normal and malignant cells, including neurons, deposit plasminogen activator on the growth substrata. Expt. Cell Res. *166*, 31-46.

Kujubu, D.A., Lim,R.W., Varnum, B.C. and Herschman, H.R. (1987). Induction of transiently expressed genes in PC12 cells. Oncogene *1*, 257-262.

Laiho, M., Saksela, O. and Keski-oja, J. (1986). Transforming growth factor β alters plasminogen activity in human skin fibroblasts. Exp. Cell Res. *164*, 399-407.

Landis, S.C. (1983). Neuronal growth cones. Ann. Rev. Physiol. *45*, 567-580.

Layer, P.G. and Shooter E.M. (1983). Binding and degradation of nerve growth factor by PC12 pheochrmocytoma cells. J. Biol. Chem. *258*, 3012-3018.

Lee, V.M.-Y. and Page, C. (1984). The dynamics of nerve growth factor-induced neurofilament and vimentin filament expression and organization in PC12 cells. J. Neurosci. *4*, 1705-1714.

Leonard, D.G.B., Gorham, J.D., Cole, P., Greene, L.A. and Ziff, E.B. (1988). A nerve growth factor-regulated messenger RNA encodes a new intermediate filament protein. J. Cell Biol. *106*, 181-193.

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. Molec. Cell. Biol. *7*, 3156-3167.

Levi, A, Biocca, S., Cattaneo, A. and Calissano, P. (1988). The mode of action of nerve growth factor in PC12 cells. Molec. Neurobiol. *2*, 201-226

Levi, A., Eldridge, J.D. and Paterson, B.M. (1985). Molecular cloning of a gene sequence regulated by nerve growth factor. Science *229*, 393-395.

Levi, A., Schechter, Y., Neufeld, E.J. 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. and Hamburger, V. (1951). Selective growth-stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J. Exptl. Zool. *116*, 321-362.

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

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

Levi-Montalcini, R. and Booker, B. (1960). Destruction of the sympathetic ganglia in mammals by an antiserum to nerve growth protein. Proc. Natl. Acad. Sci. USA *46*, 384-391.

Liotta, L.A. (1986). Tumor invasion and metastases - Role of the extracellular matrix: Rhoads Memorial Award Lecture. Canc. Res. *46*, 1-7.

Liotta, L.A., Goldfarb, R.H., Brundage, R., Siegal, G.P., Terranova, V. and Garbisa, S. (1981). Effect of plasminogen activator (urokinase), plasmin, and thrombin on glycoprotein and collagenous components of basement membrane. Cancer Res. *41*, 4629-4636.

Liotta, L.A., Kleinerman, J., Catanzara, P. and Rynbrandt, D. (1977). Degradation of basement membrane by murine tumor cells. J. Natl. Cancer Inst., 58, 1427-1439.

Luckenbill-Edds, L., Van Horn, C. and Greene, L.A. (1979). Fine structure of initial outgrowth of processes induced in a pheochromocytoma cell line (PC12) by nerve growth factor. J. Neurocytol. *8*, 493-511.

Majerus, P.W., Wilson, D.B., Connolly, T.M., Bross, T.E. and Neufeld, E.J. (1985). Phosphoinositide turnover provides a link in stimulus-response coupling. TIBS. *10*, 168-171.

Massague, J. (1985). Transforming growth factor-β modulates the high-affinity receptors for epidermal growth factor and transforming growth factor-α. J. Cell Biol. *100*, 1508-1514.

Matrisian, L.M., Glaichenhaus, N., Gesnel, M-C. and Breathnach, R. (1985). Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. EMBO J. *4*, 1435-1440.

Matrisian, L.M. Bowden, G.T., Krieg, P., Furstenberger, G., Briand, J.-P., Leroy, P. and Breathnach, R. (1986a). The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. Proc. Natl. Acad. Sci. USA *83*, 9413-9417.

Matrisian, L.M., Leroy, P., Ruhlmann, C., Gesnel, M.-C. and Breathnach, R. (1986b). Isolation of the oncogene and epidermal growth factor-induced transin gene: Complex control in rat fibroblasts. Mol. Cel. Biol. *6*, 1679-1686.

Matrisian, L.M., Davis, D. and Magun, B.E. (1987). Internalization and processing of epidermal growth factor in aging human fibroblasts in culture. Exp. Gerontol. *22*, 81-89.

Matsuda, Y., Nakanishi, N., Dickens, G. and Guroff, G. (1986). A nerve growth factor-sensitive S6 kinase in cell-free extracts from PC12 cells. J. Neurochem. *47*, 1728-1734.

McGuire, J.C. and Greene, L.A. (1980). Stimulation by nerve groth factor of specific protein synthesis in rat PC12 pheochromocytoma cells. Neuroscience *5*, 179-189.

Mignatti, P., Robbins, E., Rifkin, D.B. (1986). Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell *47*, 487-498.

Milbrandt, J. (1986). Nerve growth factor rapidly induced c-*fos* mRNA in PC12 rat pheochromocytoma cells. Proc. Natl. Acad. Sci. USA *83*, 4789-4793.

Misko, T.P., Radeke, M.J. and Shooter, E.M. (1988). The molecular aspects of the rat nerve growth factor receptor. In: Neural Development and Regeneration. (A. Gorio, J.R. Perez-Polo, J. deVellis, B. Haber, eds.). Springer-Verlag, New York, pgs. 257-283.

Moonen, G., Grau-Wagemans, M.P. and Selak, I. (1982). Plasminogen activator-plasmin system and neuronal migration. Nature *298*, 753-755.

Morrison, R.S., Kornblum, H.I., Leslie, F.M. and Bradshaw, R.A. (1987). Trophic stimulation of cultured neurons from neonatal rat brain by epidermal growth factor. Science *238*, 72-75.

Muldoon, L.L., Jamieson, G.A., Jr., Kao, A.C., Palfrey, H.C. and Villereal, M.L. (1987). Mitogen stimulation of Na^+-H^+ exchange: Differential involvement of protein kinase C. Am. J. Physiol. *253*, C219-C229.

Muldoon, L.L., Rodland, K.D. and Magun, B.E. (1988). Transforming growth factor β modulates epidermal growth factor-induced phosphoinositide metabolism and intracellular calcium levels. J. Biol. Chem. *263*, 5050-5033.

Muldoon, L.L., Rodland, K.D. and Magun, B.E. (1988). Transforming growth factor β and epidermal growth factor alter calcium influx and phosphatidylinositol turnover in

Rat-1 fibroblasts. J. Biol. Chem. *263*, 18834-18841.

Mullaney, J.M., Yu, M., Ghosh, T.K. and Gill, D.L. (1988). Calcium entry into the inositol 1,4,5-trisphosphate-releasable calcium pool is mediated by a GTP-regulatory mechanism. Proc. Natl. Acad. Sci. USA *85*, 2499-2503.

Muller, R., Bravo, R., Burckhardt, J. and Curran, T. (1984). Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. Nature. *312*, 716-720.

Muller, D., Quantin, B., Gesnel, M-C., Millon-Collard, R., Abecassis, J. and Breathnach, R. (1988). The collagenase gene family in humans consists of at least four members. Biochem. J. *253*, 187-192.

Mullins, D.E. and Rohrlich, S.T. (1983). The role of proteinases in cellular invasiveness. Biochim. Biophys. Acta *695*, 177-214.

Nahorski, S.R., Kendall, D.A. and Batty, I. (1986). Receptors and phosphoinositide metabolism in the central nervous system. Biochem. Pharmacol. *35*, 2447-2453, 1986.

Nakajima, M., Irimura, T., DiFerrante, N. and Nicolson, G.L. (1984). Metastatic melanoma cell heparanase. J. Biol. Chem, *259*, 2283-2290.

Nishizuka, Y. (1983). Phospholipid degradation and signal translation for protein phosphorylation. TIBS. *8*, 13-16.

Nishizuka, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature *308*, 693-698.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. Science *233*, 305-312.

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.

Ohta, M., Greenberger, J.S., Anklesaria, P., Bassols, A. and Massague, J. (1987). Two forms of transforming growth factor-β distinguished by multipotential haematopoietic progenitor cells. Nature *329*, 539-541.

Okada, K.I. Nagatsu, T., Fujita, K., Torikai, K., Hamamoto, T., Shibata, T., Maeno, Y. and Sakakibara S. (1985). Serum collagenase-like peptidase activity in rheumatoid arthritis and systemic lupus erythematosus. Clinica Chimica Acta *146*, 75-79.

Okada, Y., Nagase, H. and Harris, E.D., Jr. (1986). A metalloproteinase from human rheumatoid synovial fibroblasts that digest connective tissue matrix components. J. Biol. Chem. *261*, 14245-14255.

Otten, R., Goedert, M., Schwab, M. and Thibault, J. (1979). Immunization of adult rats against 2.5 nerve growth factor: Effects on the peripheral sympathetic nervous system. Brain Res. *176*, 79-90.

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 Lett. *208*, 48-51.

Pittman, R.N. (1985). Release of plasminogen activator and a calcium-dependent metalloprotease from cultured sympathetic and sensory neurons. Dev. Biol. *110*, 91-101.

Pittman, R.N. and Williams, A.G. (1989). Neurite penetration into collagen gels requires Ca^{2+}-dependent activity. Dev. Neurosci. (in press).

Rall, L.B., Scott, J., and Bell, GI. (1985). Mouse prepro-epidermal growth factor synthesis by the kidney and other tissues. Nature *313*, 228-231.

Reynolds, F.H.,Jr., Todaro, G.J., Fryling, C. and Stephenson, J.R. Human transforming growth factors induce phosphorylation of EGF receptors. Nature *292*, 259-262.

Rheinwald, J.G. and Green, H. (1977). Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. Nature *265*, 421-424.

Rizzino, A. (1988). Transforming growth factor-β: Multiple effects on cell differentiation and extracellular matrices. Dev. Biol. *130*, 411-422.

Roberts, A.B., Anzano, M.A., Wakefield, L.M., Roche, N.S., Stern, D.F. and Sporn, M.B. (1985). Type β transforming growth factor: A bifunctional regulator of cellular growth. Proc. Natl. Acad.Sci. USA *82*, 119-123.

Rogers, S.L., Letourneau, P.C., Palm, S.L., McCarthy, J.B. and Furcht, L.T. (1983). Neurite extension by peripheral and central nervous system neurons in response to substrate-bound fibronectin and laminin. Dev. Biol. *98*, 212-220.

Rose, S.P., Pruss, R.M. and Herschman, H.R. (1975). Initiation of 3T3 fibroblast cell division by epidermal growth factor. J. Cell. Physiol. *86*, 593-598.

Rowland, E.A., Muller, T.H., Goldstein, M. and Greene, L.A. (1987). Cell-free detection and characterization of a novel nerve growth factor-activated protein kinase in PC12 cells.

J. Biol. Chem. *262*, 7504-7413.

Saksela, O. and Rifkin, D.B. (1988). Cell-associated plasminogen activation: Regulation and physiological functions. Ann. Rev. Cell Biol. *4*, 93-126.

Sandberg, M., Vuorio, T., Hirvonen, H., Alitalo, K. and Vuorio, E. (1988). Enhanced expression of TGF-β and c-*fos* mRNAs in the growth plates of developing human long bones. Development *102*, 461-470.

Schlessinger, J., Schechter, Y., Cuatrecasas, P., Willingham, M.C. and Pastan, D. (1978). Direct visualization of binding, aggregation, and internalization of insulin and epidermal growth factor on living fibroblastic cellls. Proc. Natl. Acad. Sci. USA *75*, 2659-2663.

Schwab, M.E. and Thoenen, H. (1983). Retrograde axonal transport. In: Handbook of Neurochemistry. Vol 5. (A. Lajtha, Ed.), Plenum, New York, pp. 381-404.

Scott, J., Urdea, M., Quiroga, M., Sanchez-Pescador, R., Fong, N., Selby, M., Rutter, W.J. and Bell, G.I. (1983). Structure of a mouse submaxillary messenger RNA encoding epidermal growth factor and several related proteins. Science *221*, 236-240.

Sherman, M.I., Strickland, S. and Reich, E. (1976). Differentiation of early mouse embryonic and teratocarcinoma cells in vitro: Plasminogen activator production. Cancer Res. *36*, 4208-4216.

Snyder, S.H., Supattapone, S., Danoff, S., Worley, P.F. and Baraban, J.M. (1988). The inositol triphosphate receptor: A potpourri of second-messenger regulation. Cell. Molec. Neurobiol. *8*, 1-5.

Sporn, M.B., Roberts, A.B., Wakefield, L.M. and Assoian, R.K. (1986). Transforming growth factor-β: Biological function and chemical structure. Science *233*, 532-534.

Stoeckel, K., Dumas, M. and Thoenen, H. (1978). Uptake and subsequent retrograde axonal transport of nerve growth factor (NGF) are not influenced by neuronal activity. Neurosci. Lett. *10*, 61-64.

Stryer, L. and Bourne, H.R. (1988). G proteins: A family of signal transducers. Ann. Rev. Cell Biol. *2*, 391-420.

Taylor, J.M., Mitchel, W.M. and Cohen, S. (1972). Epidermal growth factor: Physical and chemical properties. J. Biol. Chem. *247*, 5928-5934.

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

Todaro, G.J., Fryling, C. and DeLarco, J.E. (1980). Transforming growth factors produced by certain human tumor cells: Polypeptides that interact with epidermal growth factor receptors. Proc. Natl. Acad. Sci. USA *77*, 5258-5262.

Traynor, A.E. (1984). The relationship between neurite extension and phospholipid metabolism in PC12 cells. Dev. Brain Res. *14*, 205-210.

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

Tucker, R.F., Volkenant, M.E., Branum, E.L. and Moses, H.L. (1983). Comparision of intra- and extracellular transforming growth factors from non-transformed and chemi-

cally transformed mouse embryo cell. Canc. Res. *43*, 1581-1585.

Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfeld, C., Rosen, O.M. and Ramachandran, J. (1985). Human insulin receptor and its relationship to tyrosine kinase family of oncogenes. Nature *313*, 756-761.

Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Liberman, T.A., Schlessinger J., Downward, J., Mayes, E.L.Y., Whittle, N., Waterfield, M.D. and Seeburg, P.H. (1984). Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermal carcinoma cells. Nature *309*, 418-425.

Unsicker, K., Krisch, B., Otten, U. and Thoenen, H. (1978). Nerve growth factor-induced fiber outgrowth from isolated rat adrenal chromaffin cells: Impairment by glucocorticoids. Proc. Natl. Acad. Sci. USA *75*, 3498-3502.

Ushiro, H., and Cohen, S. (1980). Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A431 cell membranes. J. Biol. Chem. *255*, 8363-8365.

Van Calker, D. and Heuman, R. (1987). Nerve growth factor potentiates the agonist-stimulated accumulation of inositol phosphates in PC12 pheochromocytoma cells. Eur. J. Pharmacol. *135*, 259-260.

Vara, F. and Rozengurt, E. (1985a). Stimulation of Na^+/H^+ antiport activity by epidermal growth factor and insulin occurs without activation of protein kinase C. Biochem. Biophys. Res. Commun. *130*, 646-653.

Vara, F., Schneider, J.A. and Rozengurt, E. (1985b). Ionic responses rapidly elicited by activation of protein kinase C in quiescent Swiss 3T3 cells. Proc. Natl. Acad. Sci. USA *82*, 2384-2390.

Vicentini, L.m. and Villereal, M.L. (1984). Serum, bradykinin and vasopressin stimulate release of inositol phosphates from human fibroblasts. Biochem. Biophys. Res. Comm. *123*, 663-670.

Werb, Z., Hembry, R.M., Murphy, G. and Aggeler, J. (1986). Commitment to expression of the metalloendopeptidases, collagenase and stromelysin: Relationship of inducing events to changes in cytoskeletal architecture. J.Cell Biol. *102*, 697-702.

Whitham, S.E., Murphy, G., Angel, P., Rahmsdorf, H-J., Smith, B., Lyons, A., Harris, T.J.R, Reynolds, J.J., Herrlich, P. and Docherty, A.J.P. (1986). Compariuson of human stromelysin and collagenase by cloning and sequence analysis. Biochem. J. *240*, 913-916.

Whittemore, S.R., Ebendal, T., Larkfors, L., Olson, L., Seiger, A., Stromberg, I. and Persson, H. (1986). Developmental and regional expression of nerve growth factor messenger RNA and protein in the rat central nervous system. Proc. Natl. Acad. Sci. USA *83*, 917-821.

Whittemore, S.R., Persson, H., Ebendal, T., Larkfors, L., Larhammar, D. and Ericsson, A. (1988). Structure and expression of beta-nerve growth factor in the rat central nervous system. In: Neural Development and Regeneration: Cellular and Molecular Aspects. (A. Gorio, J.R. Perez-Polo, J. deVellis, B. Haber, Eds.). Sprionger-Verlag, New York, pp. 245-256.

Wilhelm, S.M., Collier, I.E., Kronberger, A., Eisen, A.Z., Marmer, B.L., Grant, G.A., Bauer, E.A. and Goldberg, G.I. (1987). Human skin fibroblast stromelysin: Structure, glycosylation, substrate specificity, and differential expression in normal and tumorigenic cells. Proc. Natl. Acad. Sci. USA *84*, 6725-6729.

Yamamoto, T., Davies, C.G., Brown, M.S., Schneider, W.J., Casey, M.L., Goldstein, J.L. and Russell, D.W. (1984). The human LDL receptor: a cysteine-N rich protein with multiple Alu sequences in its RNA. Cell *39*, 27-38.

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

Yarden, Y. and Ullrich A. (1988). Growth factor receptor tyrosine kinases. Ann. Rev. Biochem. *57*, 443-478.

Zavoico, G.B. and Feinstein, M.B. (1984). Cytoplasmic Ca^{2+} in platelets is controlled by cyclic AMP: Antagonism between stimulators and inhibitors of adenylate cyclase. Biochem. Biophys. Res. Comm. *120*, 579-585.

Zeydel, M., Nakagawa, S., Biempica, L. and Takahashi, S. (1986). Collagenase and elastase production by mouse mammary adenocarcinoma primary cultures and cloned cells. Cancer Res. *46*, 6438-6445.

Figure 1

Involvement of proto-oncogene cell products in the growth factor-induced signal transduction pathways. Receptors are indicated as rectangles in the plane of the cell membrane. The phosphatidylinositol pathway (PI - PIP_2 - DAG - IP_3) is indicated in the plane of the membrane. Proto-oncogene products may share sequence similarities to growth factors (e.g. c-*sis*), growth factor receptors (e.g. c-*erb-B*, c-*fms*), elements of the signal transduction pathway (e.g. c-*ras*), or nuclear transcription factors (e.g. c-*fos*, c-*myc*). (Adapted from Goustin et al., 1986).

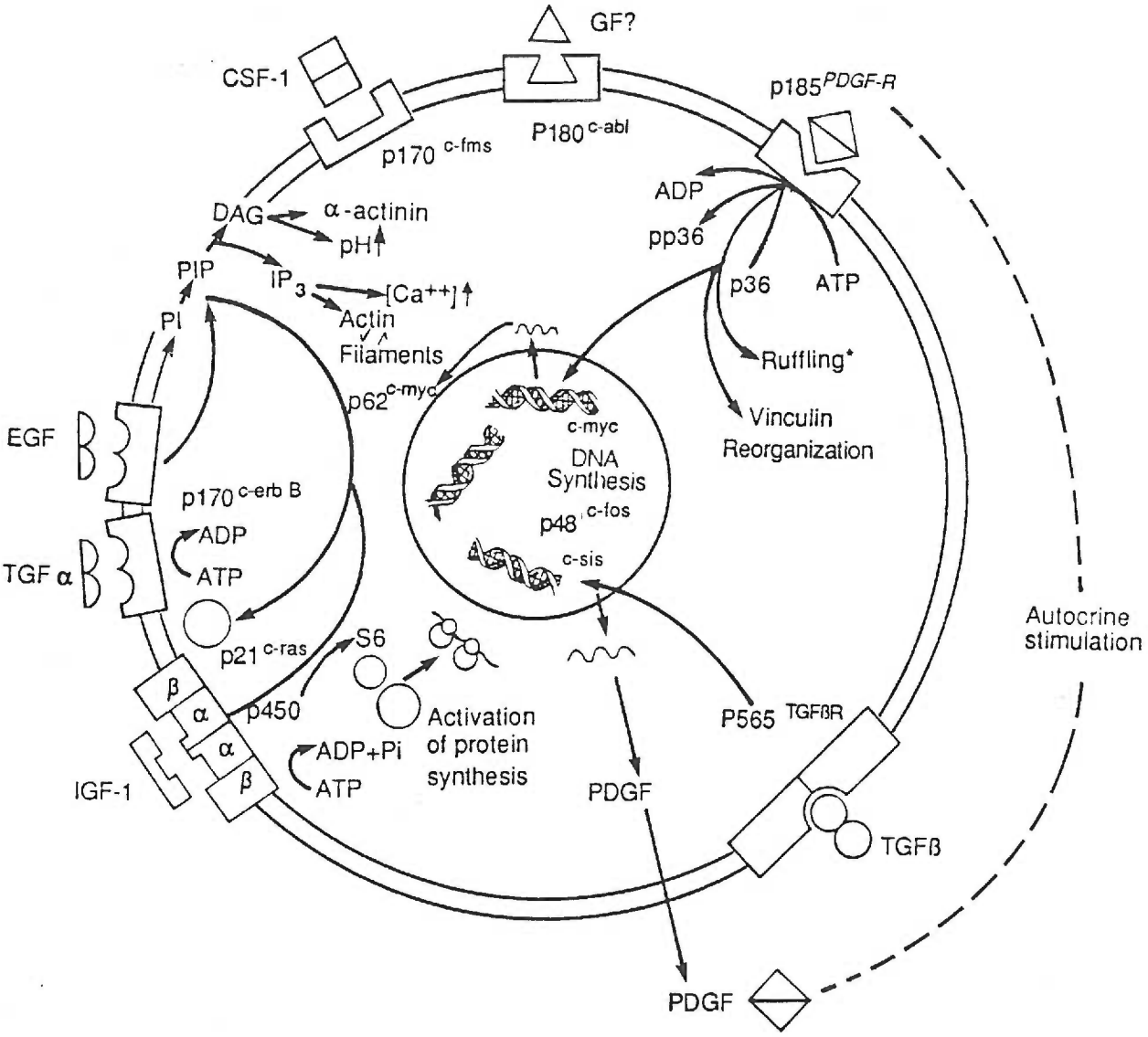
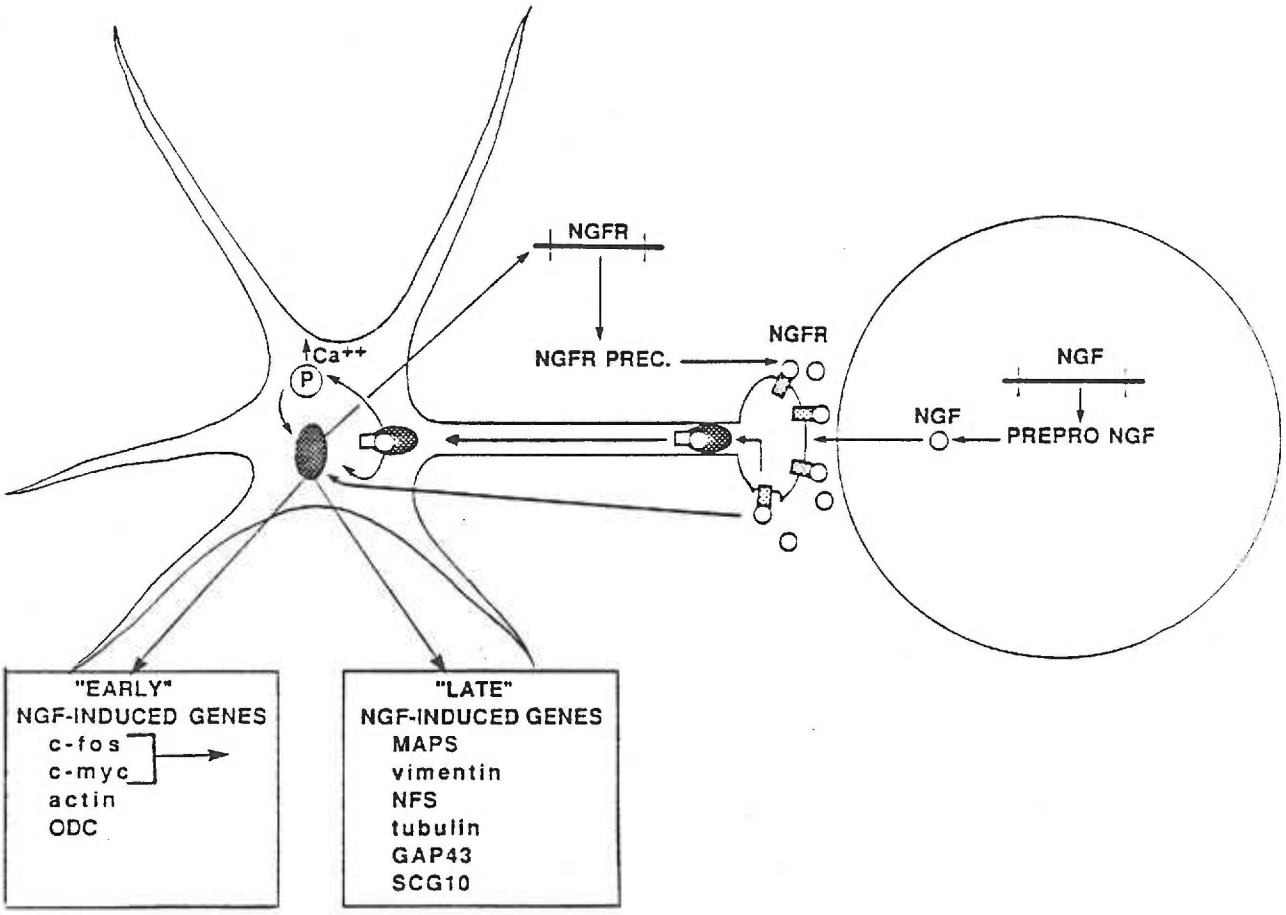

GF?
CSF-1
p170 c-fms
P180 c-abl
p185 PDGF-R
ADP
pp36
p36
ATP
Ruffling*
Vinculin
Reorganization
DAG
α-actinin
pH↑
PIP
IP3
[Ca++]↑
Actin
Filaments
PI
p62 c-myc
c-myc
DNA
Synthesis
p48 c-fos
c-sis
EGF
p170 c-erb B
ADP
ATP
TGF α
p21 c-ras
S6
β
α
α
β
p450
ADP+Pi
ATP
IGF-1
Activation
of protein
synthesis
P565 TGFßR
PDGF
TGFß
Autocrine
stimulation
PDGF

Figure 2

The NGF pathway. NGF is synthesized under the control of a single gene and is processed from two precursor proteins. It is secreted by the target tissue into the vicinity of the nerve terminal (i.e. growth cone). Binding to the NGF receptors on the nerve terminal initiates signal transduction and internalization. The internalized vesicles carrying NGF bound to the NGF receptors are transported along microtubules to the neuronal cell body. After the pH of the vesicles (endosomes) is lowered, NGF is transferred to lysosomes where it is degraded. The arrows emanating from the NGF-NGF receptor complex indicate locations at which intracellular signals might arise. These are (1) at the nerve terminal membrane (2) after internalization and/or retrograde flow up the axon and (3) in the neuronal cell body. The cascade of reactions which may include changes in intracellular calcium and phosphorylation levels, leads finally to modulation of gene expression. These NGF-activated genes include the "early" induced genes such as c-*fos* and c-*myc* and the "late" induced genes involved in the acquisition of the neuronal phenotype (see text) (adapted from Misko et al., 1988).

THE NGF PATHWAY



II. MANUSCRIPTS

A. Paper 1: **Transcriptional Modulation of Transin Gene Expression by Epidermal Growth Factor and Transforming Growth Factor β**

Machida C.M., Muldoon, L.L., Rodland, K.D. and Magun, B.E. (1988). Molec. Cell. Biol. *6*, 2479-2483.

ABSTRACT

The gene encoding the metalloproteinase transin is expressed constitutively in rat fibroblasts transformed by a variety of oncogenes, and in malignant mouse skin carcinomas but not benign papillomas or normal skin. It has been demonstrated that, in non-transformed Rat-1 cells, transin RNA expression is modulated positively by epidermal growth factor (EGF) and negatively by transforming growth factor beta (TGFβ); other peptide growth factors were found to have no effect on transin expression. Results presented here indicate that both protein synthesis and the continuous occupancy of the EGF receptor by EGF were required for the sustained induction of transin RNA. Treatment with TGFβ inhibited the ability of EGF to induce transin mRNA, whether assayed at the transcriptional level by nuclear run-on analysis or at the level of transin RNA accumulation by Northern blot analysis of cellular RNA. TGFβ both blocked the initial induction of transin transcription by EGF and halted the established production of transin transcripts during prolonged treatment. These results suggest that TGFβ acts at the transcriptional level to antagonize the EGF-mediated induction of transin gene expression.

INTRODUCTION

Transin is a secreted protease of Mr 53k (9,10) which shares significant amino acid homology with members of the metalloprotease family such as human stromelysin (4,15), human skin collagenase (5), and rabbit synovial cell activator (C. Brinckerhoff, personal communication). The transin gene was initially isolated by differential screening of a cDNA library from polyoma virus-transformed rat fibroblasts (9). Transin mRNA is constitutively expressed in significantly higher levels in rat cells transformed by polyoma virus, Rous sarcoma virus and the oncogene H-*ras* than in the normal parental cell lines (9). Transin mRNA was detected in mouse skin squamous cell carcinomas but not in normal mouse skin or in experimentally induced benign papillomas (11) suggesting a possible role in the tumorigenic capacities of transformed cells.

In non-transformed rat fibroblast cell lines, transin transcription is specifically induced by epidermal growth factor (EGF) but not by platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin, or transforming growth factor beta (TGFβ); TGFβ has been found to inhibit the expression of transin when analyzed several hours after simultaneous addition of EGF and TGFβ (10). Neither the molecular mechanisms responsible for the induction of transin by EGF nor the mechanisms responsible for the inhibitory effects of TGFβ are currently understood. In order to more fully characterize the respective roles of EGF and TGFβ as positive and negative effectors of transin expression, we conducted parallel studies on the cytoplasmic stability and nuclear transcription of transin RNA in response to these two effectors.

MATERIALS and METHODS

Cell Culture. Rat-1 fibroblasts (12) and their v-*src* transformed derivative RM2 (supplied by Michael Weber) were cultured in Dulbecco's modified Eagles' medium (DMEM) containing 10% defined calf serum (Hyclone) and appropriate antibiotics at 37°C in a humidified 95% air/5% CO_2 atmosphere. Confluent cells were serum deprived with unsupplemented DMEM 16-24 hours prior to stimulation with the various biological modifiers.

Northern blot analysis of RNA. Total cellular RNA was extracted and analyzed by Northern analysis as previously described (13). Cellular RNA was fractionated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde. The RNA was transferred to nitrocellulose membranes (Schleicher and Schuell) and prehybridized in the presence of a buffer containing 0.75 M NaCl, 75 mM sodium citrate and 50% formamide at 42°C for 16 hours as previously described (13). The prehybridization solution was replaced with hybridization solution containing the plasmid pTR1 labelled with ^{32}P by nick translation to a specific activity greater than $2x10^8$ cpm/μg. pTR1 contains a 1.6 kb EcoRI fragment representing most of the full length rat transin cDNA (10) (kindly supplied by L. Matrisian). Following hybridization for 16 hours at 42°C, the membranes were washed twice for 5 min each in 2X SSC, 0.1% SDS at room temperature followed by two washes in 0.1X SSC, 0.1% SDS at 50°C for 30 min each wash. The hybridized membranes were then exposed to Kodak X-Omat X-Ray film with intensifying screens for 1-2 days at -70°C.

Transcriptional Run-on Analysis. Nuclei were prepared as described by Brown et al. (3) and all preparations and materials were kept at 4°C. The final nuclear pellet was resuspended and stored at -80°C in 20 mM Tris-HCl pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 8.5 mM dithiothreitol, 0.125 mM phenylmethylsulfonyl fluoride and 50% glycerol. Initiated transcripts were elongated in the presence of 120 mM Tris-HCl pH 7.8, 50 mM

NaCl, 0.35 M ammonium sulfate, 4 mM manganese chloride, 0.24 mM EDTA, 1 mg/ml heparin and 1 mM each ATP, GTP, UTP, and 20-25 μCi of [α-^{32}P] CTP (NEN, 800 Ci/mmol). Radiolabeled nuclear RNA was isolated by four consecutive phenol-chloroform extractions and counted in order to obtain an identical amount of radioactivity (typically 10^7 cpm from an extract representing four 10 cm dishes). The RNA was then hybridized for 4 days at 42°C as described by Brown et al. (3) against transin cDNA immobilized on diazobenzyloxymethylcellulose (DBM) filters (Schleicher & Schuell). The filters were prepared by electrophoresis of EcoRI digested pTR1 in a 0.8% agarose gel followed by transfer onto activated DBM paper as described by Alwine et al. (1). After hybridization the filters were washed 3X in 2X SSC, 0.1% SDS for 10 min at 42°C, treated with RNase A (50 μg/ml) and washed again in 2X SSC. Final washes were then performed in 0.1X SSC, 0.1% SDS at 42°C. The filters were exposed overnight to Kodak X-Omat X-ray film at -70°C using intensifying screens.

Chemicals and Reagents. EGF was prepared from mouse submaxillary glands essentially as described by Savage and Cohen (14) and further purified to homogeneity as previously described (8). TGFβ was prepared from human platelets as described by Assoian et al. (2) with the addition of a final purification step consisting of reversed-phase HPLC on C3 columns and elution with an acetonitrile-trifluoroacetic acid gradient. 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) was purchased from Pharmacia and dissolved in dimethylsulfoxide (DMSO). L-*sn*-1,2 dioctanoyl glycerol (DOG) was purchased from Avanti Polar Lipids, Inc., Birmingham, Ala. Actinomycin D and anisomycin were both obtained from Sigma Chemical Co., St. Louis, Mo. Anti-EGF IgG was purified from rabbit antisera as described previously (6).

RESULTS

Effects of EGF and protein kinase C activators on transin induction. In order to determine whether the activation of protein kinase C might be involved in the induction of transin by EGF, we tested both EGF and activators of protein kinase C for their ability to induce transin RNA in Rat-1 cells. Confluent 10 cm plates were serum deprived for 24 hours prior to the addition of the putative inducing agents. Cells were harvested at 2, 4, or 6 h following stimulation, and total cellular RNA was extracted for gel electrophoresis and Northern blot analysis.

Stimulation of serum-deprived cells with EGF resulted in an elevation of transin RNA levels which gradually increased over the 6 h time course (Figure 1). Transin was essentially undetectable in non-stimulated cells (lane 1). In contrast to the stimulatory effects of EGF (lanes 2-4), neither TPA (lanes 5-7), DOG (lanes 8-10), nor the vehicular control DMSO (lane 11) produced any induction of transin RNA over the 6 hr time period studied. Thus, under these conditions, induction of transin in non-transformed cells does not appear to be mediated by the activation of protein kinase C.

EGF receptor occupancy and transin expression. In order to determine whether the continued presence of EGF was required for the sustained maintenance of transin RNA expression, we first treated cells with EGF for 10 h to establish high levels of transin RNA expression. EGF was then removed by rinsing the cells in serum-free medium and the cells were subsequently treated with anti-EGF IgG to prevent the rebinding of any EGF released from cell surfaces. The optimal concentration of IgG was derived from the concentration of IgG which maximally inhibited binding of [^{125}I]-EGF to cell surfaces (data not shown). Cells were harvested for RNA extraction and Northern analysis at different times following removal of EGF and the addition of anti-EGF IgG. Transin levels gradually decreased over a 6 h time period following the removal of EGF as shown in Figure 2. Graphic analysis of the densitometric signal measured at 0, 2, 4, and 6 h after

anti-EGF IgG treatment indicated that the half-life of transin RNA under these conditions was approximately 4 hours. Experiments measuring the decrease in cellular transin RNA levels following treatment with the RNA synthesis inhibitor actinomycin D produced similar estimates for the half-life of transin RNA (data not shown). Thus the sustained expression of transin for 10 to 12 h observed in these and earlier experiments (9) appeared to result from the continued binding of EGF to EGF receptors throughout the experimental exposure.

Transcriptional regulation of transin by EGF. In order to determine the temporal characteristics of transin transcription following EGF stimulation, transcription was monitored by nuclear run-on analysis. Following serum-deprivation for 24 h, nearly confluent Rat-1 cells were exposed to EGF for periods ranging from 0 to 10 h. Cells were harvested and nuclei were isolated for nuclear transcriptional run-on analysis. Newly synthesized transin transcripts were evident as early as 30 min following EGF stimulation, and transcription continued for at least 10 hours (Figure 3A). Nuclei from cells which had not been exposed to EGF failed to produce detectable transcripts. The ability of EGF to induce transin transcription was inhibited by the addition of the protein synthesis inhibitor anisomycin for 15 min prior to EGF stimulation (Figure 3B). Hybridization to vector DNA sequences immobilized on the same DBM strips was neglible under all experimental conditions.

Effects of TGFβ on induction of transin by EGF. In order to more fully define the interaction between EGF and TGFβ in modulating transin expression, we pretreated serum-deprived Rat-1 cells with EGF for varying times before adding TGFβ. Two hours after addition of TGFβ the cells were harvested and total RNA was extracted for Northern analysis. A two hour exposure to TGFβ was sufficient to decrease substantially the cellular levels of transin RNA, regardless of the length of pretreatment with EGF (Figure 4). Transin RNA was not detected in the control cells containing the diluent for TGFβ addi-

tions.

Nuclear run-on analyses of transcription were performed on serum-deprived Rat-1 cells following co-treatment with EGF and TGFβ. Nuclei from cells exposed to EGF alone for 2 h displayed a high level of transin transcription (Figure 5A, lane B), while nuclei from cells exposed to either TGFβ alone or TGFβ followed by EGF (lanes C and D) produced no discernible transin transcripts, although transcription of the ubiquitously expressed gene p1B15 (Danielson, P.E., Forss-Petter, S., Brow, M.A., Calavetta, L., Douglass, J., Milner, R.J., and Sutcliffe, J.G., DNA, *in press*) appeared to be unaffected by either EGF or TGFβ treatment (Figure 5A). Treatment of Rat-1 cells with TGFβ completely blocked the ability of EGF to promote transin transcription, whether TGFβ and EGF were added simultaneously (Figure 5B, lane 3) or whether TGFβ was added subsequent to establishment of transin induction by a 2 h pre-treatment with EGF (Figure 5B, lane 5). Thus, nuclear run-on analysis demonstrated that TGFβ could inhibit the initiation of transin transcription whether or not the transin gene was being actively transcribed at the time of TGFβ addition.

DISCUSSION

The combination of transcriptional run-on analysis and Northern analysis of total transin RNA have provided specific information regarding the interactions between EGF and TGFβ which coordinately result in modulation of transin expression. Northern analyses of the decline in transin RNA levels following the removal of EGF from the medium indicated that continuous occupancy of the EGF receptor is required for the maintenance of transin transcription. Addition of TGFβ caused an abrupt cessation of transin transcription, similar to that observed when EGF was removed, whether monitored at the level of transcription or cellular RNA accumulation. This observation strengthens the evidence indicating that continuous positive stimulation by EGF is necessary for active transin transcription. The minimal reduction in EGF binding observed in Rat-1 cells following TGFβ treatment (L.L. Muldoon, K.D. Rodland, and B.E. Magun, J. Biol. Chem., *in press*) was not sufficient to account for the extent of the observed inhibition. The dependence of transin RNA expression on protein synthesis demonstrated in the anisomycin experiments suggests, but does not prove, that TGFβ may modulate transin expression by inducing the synthesis or transcription of a separate gene product required for the induction of transin transcription.

Although both EGF and protein kinase C are independently capable of inducing the expression of the VL30 gene in serum-deprived Rat-1 cells (13), neither exogenous nor endogenous activators of protein kinase C (TPA and DOG respectively) were capable of inducing transin expression. This differential responsiveness of transin RNA expression to TPA and EGF parallels the differential mitogenic response of serum-deprived Rat-1 cells to TPA and EGF; i.e. EGF, but not TPA, is mitogenic in Rat-1 cells (7).

In the non-transformed Rat-1 cells, transin expression appeared to be tightly regulated by the coordinated actions of EGF and TGFβ, suggesting that growth factor modulation of this gene may be important *in vivo*. While the physiological role of transin in normal cells is currently unknown, evidence derived from sequence homology at both the

nucleotide and amino acid levels suggests that transin may be a member of a broadly defined metalloprotease family (4,5,15), and transin appears to be closely related to human stromelysin (15). Constitutive expression of transin appears to be a corollary of the transformed state, as transin expression is observed in a variety of oncogene-transformed cell lines (9) and in malignant mouse skin carcinomas but not benign papillomas nor normal mouse skin (11). While the molecular mechanisms involved in this constitutive expression are currently unknown, enhanced understanding of the normal interactions between EGF induction and TGFβ inhibition may lead to identification of the specific defect(s) responsible for the constitutive expression of transin in transformed cells. The ability to sort out the relative importance of positive and negative effectors in transformation-induced changes in gene regulation may contribute to a better understanding of the role of growth factors in modulating the expression of specific genes in neoplastic cells.

Acknowledgements

We thank J. Aschenbach, T-H. Dinh, and A. Gammie for expert technical assistance and J. Robinson and L. Scavone for manuscript preparation.

This work was supported by Public Health Service grant CA-39360 and CA-39181 from the National Cancer Institute. C.M.M. was supported by Public Health Service training grant HDO 7133-09.

LITERATURE CITED

1. **Alwine, J.C., D.J. Kemp, B.A. Parker, J. Reiser, J. Renart, G.R. Stark, and G.M. Wahl.** 1979. Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. Methods in Enzymol. **68**:220-242.

2. **Assoian, R.K., A. Komoriya, C.A. Meyers, D.M. Miller and M.B. Sporn.** 1983. Transforming growth factor in human platelets. Identification of a major storage site, purification and characterization. J. Biol. Chem. **258**:7155-7160.

3. **Brown, A.M.C., J-M. Jeltsch, M. Roberts, P. Chambon.** 1984. Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF-7. PNAS. **81**:6344-6348.

4. **Frisch, S.M., E.J. Clark, Z. Werb.** 1987. Coordinate regulation of stromelysin and collagenase genes determined with cDNA probes. PNAS. **84**:2600-2604.

5. **Goldberg G.I., S.M. Wilhelm, A. Kronberger, E.A. Bauer, G.A. Grant, A.Z. Eisen.** 1986. Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein. J. Biol. Chem. **261**:6600-6605.

6. **Magun, B.E., S.R. Planck, and H.N. Wagner, Jr.** 1982. Intracellular processing of ^{125}I-epidermal growth factor in rat embryo fibroblasts. J. Cell Biochem. **20**:259-276.

7. **Matrisian, L.M., G.R. Bowden, and B.E. Magun.** 1981. Mechanisms of synergis tic induction of DNA synthesis by epidermal growth factor and tumor promoters. J. Cell. Physiol. **108**:417-425.

8. **Matrisian L.M., B.R. Larsen, J.S. Finch, and B.E. Magun.** 1982. Further purification of epidermal growth factor by high performance liquid chromatog raphy. Anal. Biochem. **125**:339-351.

9. **Matrisian L.M., N. Glaichenhaus, M-C. Gesnel, and R. Breathnach.** 1985. Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. EMBO **4**:1435-1440.

10. **Matrisian, L.M., P. Leroy, C. Ruhlmann, M.-C. Gesnel, and R. Breathnach.** 1986. Isolation of the oncogene and epidermal growth factor-induced transin gene: complex control in rat fibroblasts. Mol. Cell. Biol. **6**:1679-1686.

11. **Matrisian, L.M., G.T. Bowden, P. Krieg, G. Furstenberger, J.-P, Briand, P. Leroy, and R. Breathnach.** 1986. The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. PNAS **83**:9413-9417.

12. **Prasad, I., D. Zouzias, and C. Basilico.** 1976. State of the viral DNA in rat cells transformed by polyoma virus. I. Virus rescue and the presence of non-integrated viral DNA molecules. J. Virol. **18**:436-444.

13. **Rodland, K.D., S.F. Jue, and B.E. Magun.** 1986. Regulation of VL30 gene expression by activators of protein kinase C. J. Biol. Chem. **262**:5029-5033.

14. **Savage C.R., Jr., and S. Cohen.** 1972. Epidermal growth factor and a new derivative: rapid isolation procedures and biological and chemical characterization. J. Biol. Chem. 247:7601-7611.

15. **Whitham, S.E., G. Murphy, P. Angel, H.J. Rahmsdorf, B.J. Smith, A. Lyons, T.J.R. Harris, J.J. Reynolds, P. Herrlich, and A.J.P. Docherty.** 1986. Comparison of human stromelysin and collagenase by cloning and sequence analysis. Biochem. J. 240:913-916.

Figure 1

Effect of EGF, TPA, and DOG on accumulation of transin RNA. Confluent Rat-1 cells (5 x 10^6 cells/10 cm plate) were serum-deprived for 16 h in DMEM, then exposed to EGF (10 ng/ml), TPA (100 ng/ml), DOG (100 μM), or the vehicular control DMSO (0.1% v/v). Cells were then harvested and total cellular RNA prepared. Total cellular RNA (10 μg/lane) was size fractionated by electrophoresis in formaldehyde gels and transferred to nitrocellulose membranes. The transin cDNA pTR1 was labelled with ^{32}P to a specific activity of 5-10 x 10^7 dpm/μg and hybridized to the immobilized RNA in a 50% formamide-5X SSC hybridization buffer at 42°C for 16 h. A stringent wash for 1 h using 0.1X SSC, 0.1% SDS at 50°C was followed by a 1-2 day exposure to Kodak X-Omat X-ray film using intensifying screens. Lane 1 shows the results in unstimulated control cells. Lanes 2, 3 and 4 show the increasing transin mRNA levels observed after exposure to EGF for 2, 4 and 6 h respectively. Neither TPA for 2, 4 and 6 h (lanes 5-7) nor DOG for 2, 4 and 6 h (lanes 8-10), produced any detectable induction of transin RNA. Lane 11 represents the results obtained in cells exposed to the TPA and DOG solvent DMSO for
6 h.

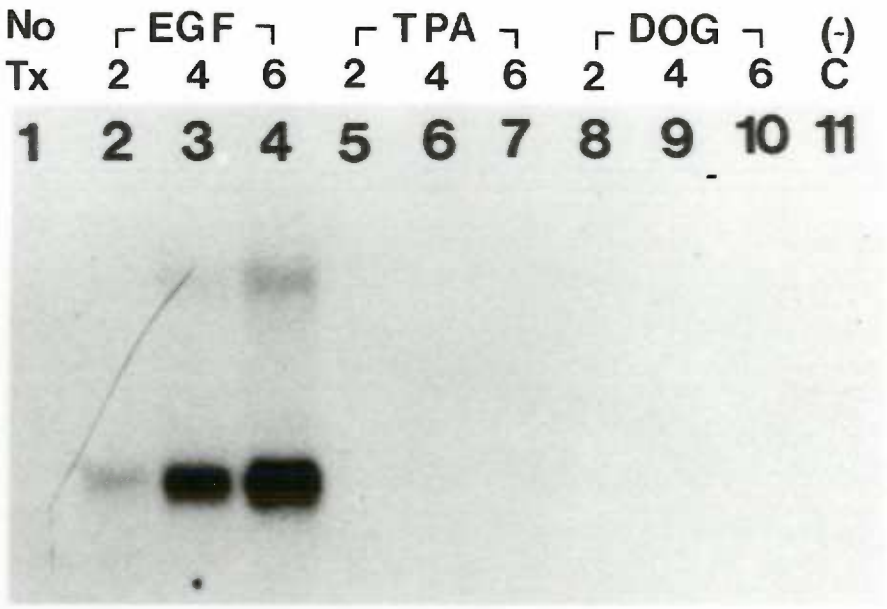

No
Tx
EGF
TPA
DOG
2
4
6
2
4
6
2
4
6
(-)
C
1
2
3
4
5
6
7
8
9
10
11

Figure 2

Transin RNA expression following the removal of EGF from the extracellular medium. Confluent Rat-1 cells were serum-deprived for 16 h in DMEM, then exposed to EGF for 10 h. The EGF-containing medium was removed and replaced with fresh DMEM containing levels of anti-EGF IgG sufficient to block EGF binding to the EGF receptor. Cells were incubated for an additional 2, 4, or 6 h, then harvested and total cellular RNA prepared as described in the text. Gel electrophoresis and Northern blot analysis were performed as described in Figure 1, using ^{32}P-labelled pTR1 as the hybridization probe. Lanes 1 through 4 respectively show the levels of transin RNA observed at 0, 2, 4 and 6 h after removal of EGF. Lane 5 represents RNA from RM-2 cells which express transin constitutively and was therefore used as a positive control.

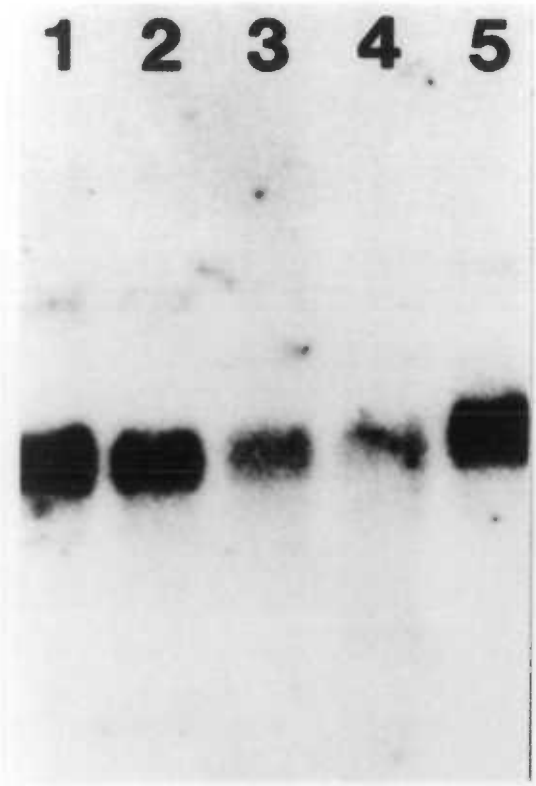

1 2 3 4 5
0 2 4 6 (+)
Time (hrs) after removing EGF

Figure 3

Nuclear run-on analyses of transin transcription in Rat-1 cells.

Panel A: Effect of EGF on transin transcription. Rat-1 cells were serum deprived and exposed to EGF for varying times. Cells from four 10 cm dishes were then harvested and nuclei were isolated for transcriptional nuclear run-on analysis as described in Materials and Methods. Lanes 1 - 5 show the hybridization signal produced by nuclear RNA extracted from cells exposed to EGF for 0, 0.5 h, 1 h, 6 h, and 10 h, respectively.

Panel B: Effect of anisomycin on EGF-induced transin transcription. Rat-1 cells were exposed to EGF for 30 min in either the absence (lane 2) or presence (lane 3) of 5 μg/ml anisomycin, added 15 min before the EGF stimulation; untreated control Rat-1 cells are represented in lane 1. Transin sequences wre released from the vector by EcoRI digestion, and the resulting fragments were size fractionated by gel electrophoresis before capillary transfer to DBM paper. Non-specific hybridization to vector sequences present on the DBM strips was negligible under all experimental conditions. The autoradiograph shows the hybridization signal obtained from RNA transcripts obtained from nuclear extracts as described in Materials and Methods.

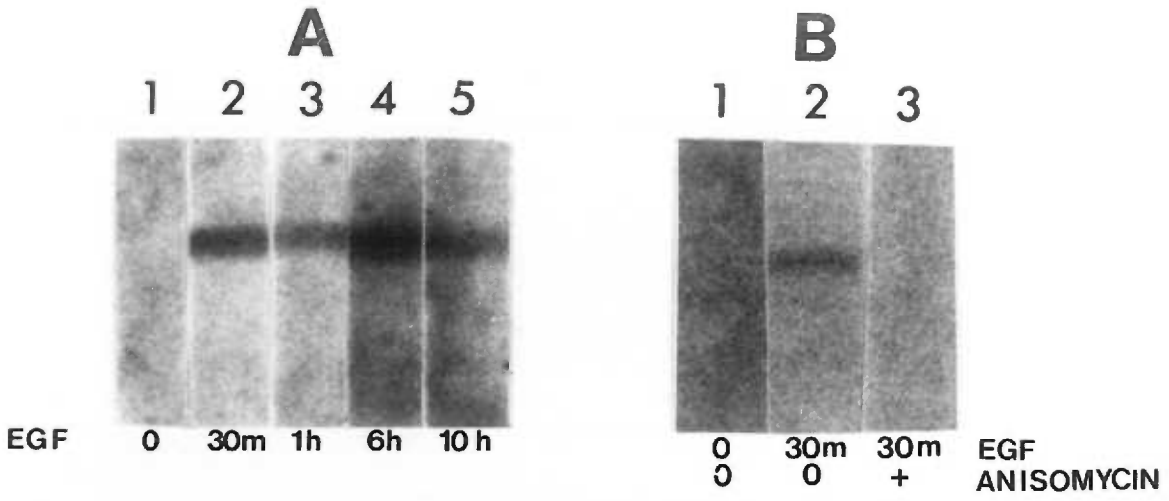

A
1 2 3 4 5
EGF 0 30m 1h 6h 10h
B
1 2 3
0 30m 30m EGF
0 0 + ANISOMYCIN

Figure 4

Northern analysis of transin RNA expression in response to EGF and TGFβ. Confluent Rat-1 cells were serum-deprived for 16 h in DMEM, then exposed to either EGF alone (10 ng/ml) or to EGF plus TGFβ (10 ng/ml). Cells were harvested 2 h after the addition of TGFβ or the TGFβ vehicle, and total cellular RNA prepared as described. Gel electrophoresis and Northern blot analysis were performed as described in Figure 1, using ^{32}P labelled pTR1 as the hybridization probe. Rat-1 cells were exposed to either EGF alone for 2 h (lane 1) or to both EGF and TGFβ for 2 h (lane 2). In lanes 3 and 4, transin expression was induced by a 2 h exposure to EGF. At this point, either TGFβ (lane 4) or the TGFβ vehicle (lane 3) was added to the EGF-containing medium and the cells were incubated for an additional 2 h before harvesting. Lane 5 represents cells which were exposed to the TGFβ vehicle (20% acetonitrile, 0.1% trifluoroacetic acid) for 4 h.

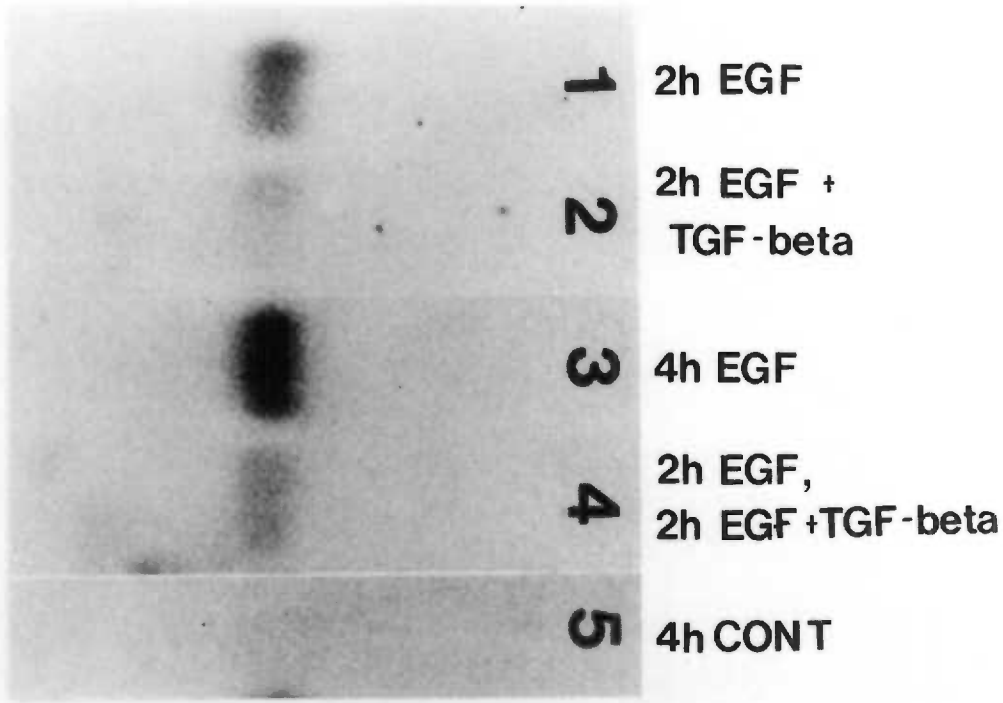

1
2h EGF
2
2h EGF +
TGF-beta
3
4h EGF
4
2h EGF,
2h EGF +TGF-beta
5
4h CONT

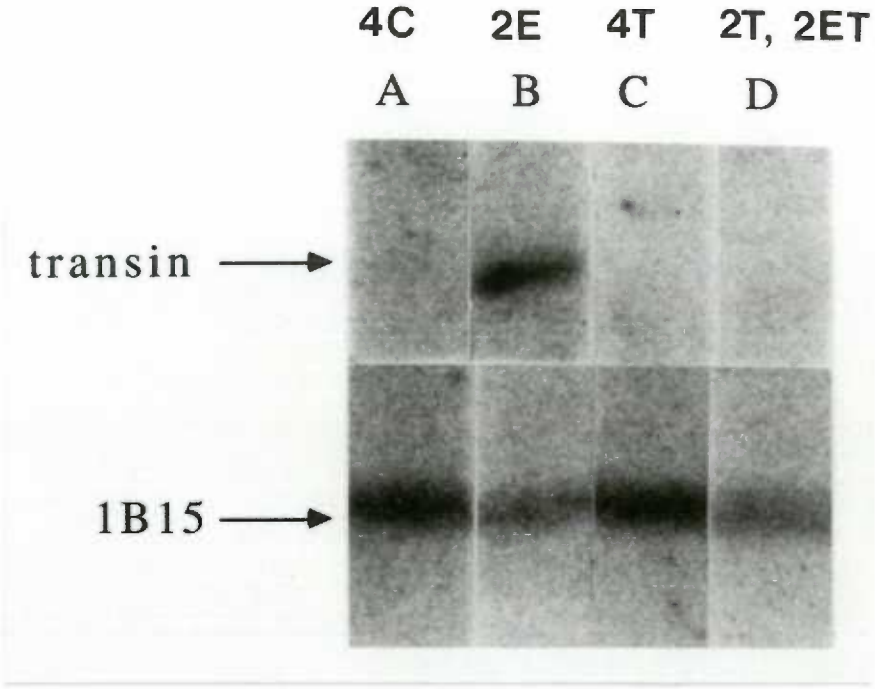
Figure 5

Nuclear run-on assay of transin transcription in response to EGF and TGFβ.

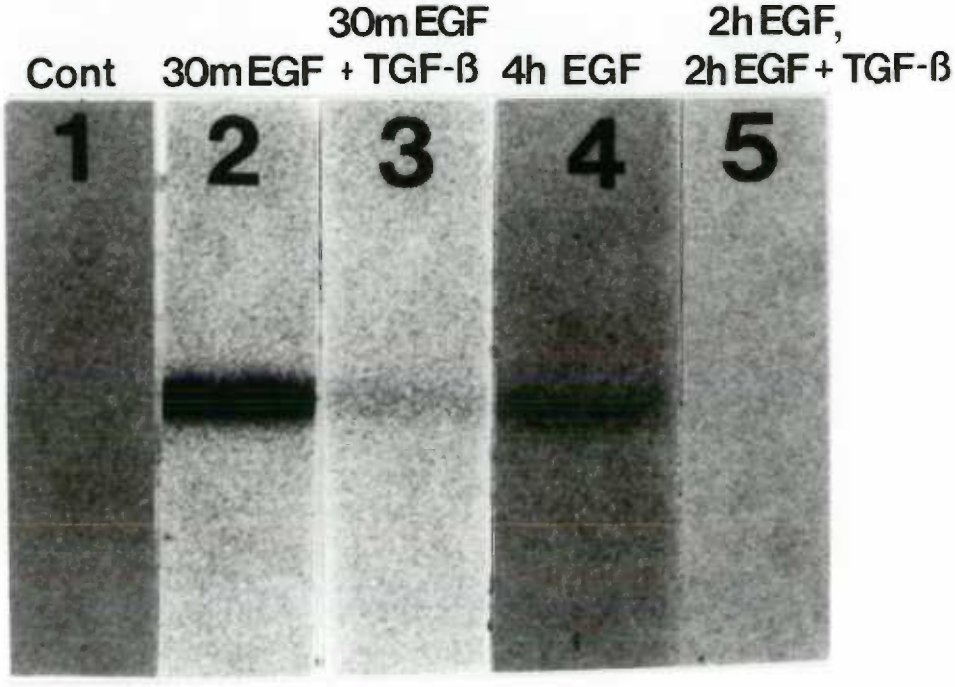
Panel A: Effect of TGFβ pretreatment on transin induction by EGF. Confluent 10 cm plates of Rat-1 cells were serum-deprived for 16 h in DMEM, then exposed to either the TGFβ vehicle (lanes A and B) or TGFβ for 4 h (lanes C and D). EGF was added to lanes B and D during the final 2 h of the incubation period. Transcripts obtained from nuclear extracts as described in Materials and Methods were hybridized to DBM strips containing either the 1.6 kb EcoRI transin insert (upper strips) or the ubiquitously expressed clone p1B15 (lower strips).

Panel B: Effect of TGFβ on an established induction of transin by EGF. Confluent 10 cm plates of Rat-1 cells were serum-deprived for 16 h in DMEM, then exposed to either EGF alone (10ng/ml) or to EGF plus TGFβ (10 ng/ml) for various times as described below. Rat-1 cells exposed for 30 min to EGF alone or in the presence of TGFβ are represented in lanes 2 and 3, respectively. Cells represented in lanes 4 and 5 were pre-treated with EGF for 2 h to establish the induction of transin RNA. At that point either TGFβ (lane 5) or the TGFβ vehicle (lane 4) was added and the cells were incubated for an additional 2 h before harvesting. Untreated control cells are represented in lane 1. Transcripts prepared from these nuclear extracts were hybridized to the 1.6 kb EcoRI fragment of pTR1 immobilized on DBM paper.

a


4C
2E
4T
2T, 2ET
A
B
C
D
transin
1B15

b


Cont
30m EGF
30m EGF + TGF-ß
4h EGF
2h EGF, 2h EGF + TGF-ß
1
2
3
4
5

II. MANUSCRIPT

B. Paper 2: Induction of the gene encoding the metalloproteinase transin accompanies differentiation in PC12 cells: Characterization of NGF and dexamethasone effects. Machida, C.M., Rodland, K.D., Matrisian, L., Magun, B.E. and Ciment, G. (Accepted for publication in **Neuron**, June 1989).

ABSTRACT

Various proteases have been found to be released by the growth cones of developing neurons in culture, and have been hypothesized to play a role in the process of axon elongation. We report here that nerve growth factor (NGF) induced the gene encoding the metalloprotease transin in PC12 cells with a time course coincident with the neuronal differentiation of these cells. Acidic and basic fibroblast growth factor also stimulated transin mRNA expression and neurite outgrowth, whereas various other agents had no effects on either of these phenomena. In contrast, dexamethasone was found to inhibit the induction of transin mRNA when added with, or following, NGF treatment. Finally, we show that sequences contained within 750 base pairs in the 5'-untranscribed region of the transin gene confers responsiveness to NGF and dexamethasone.

INTRODUCTION

The process of axon elongation by differentiating neurons is critical to the morphogenesis of the developing nervous system. At the tip of growing axons is a specialized structure called the growth cone which mediates axon elongation by sending out filopodia which adhere to the substratum, thereby providing a grip for traction (for reviews, see Letourneau, 1987; Bray and Hollenbeck, 1988; Patterson, 1988). It has been found that various proteases are released at the growth cone of developing neurons in culture (Krystosek and Seeds, 1981a,b; Pittman, 1985), and these presumably participate in the process of axon elongation (for reviews, see Patterson, 1985; Monard, 1988). One prediction of this hypothesis is that genes encoding proteases would be expressed by neuronal cells during early stages of their differentiation.

One in vitro model system used extensively to study neuronal differentiation is the rat pheochromocytoma cell line, PC12. In the untreated state, these cells resemble their normal counterparts, immature adrenal chromaffin cells. Following addition of nerve growth factor (NGF), PC12 cells begin to express characteristics of sympathetic neurons (Greene & Tischler, 1976), including the appearance of neurites and the enhanced expression of various neuronal genes (Lindenbaum et al., 1988; Federoff et al., 1988). More recently, it has been shown that the fibroblast growth factors (FGF) also induce neurite extension in PC12 cells (Togari et al., 1985; Rydel and Greene, 1988). Similar morphological changes are observed when normal neonatal rat adrenal medullary cells are exposed to either NGF or FGF (Unsicker et al., 1978; Aloe and Levi-Montalcini, 1979; Doupe et al., 1985; Claude et al., 1988), suggesting that the growth factor-induced differentiation of PC12 cells reflects developmental properties of their normal counterparts (see also Anderson and Axel, 1986).

Glucocorticoids have been shown to antagonize many of the effects of NGF. Induction of various gene products in PC12 cells, for example, is inhibited by simultaneous treatment with glucocorticoids, including the induction by NGF of the growth cone-associated proteins GAP-43 (Federoff et al., 1988) and SCG10 (Stein et al., 1988a,b), as

well as various other gene products (Leonard et al., 1987). Glucocorticoids have also been shown to inhibit the NGF-mediated neurite outgrowth from normal chromaffin cells (Unsicker et al., 1978; see also Doupe et al., 1985), suggesting that glucocorticoids antagonize the effects of NGF actions on normal neural crest-derived cells.

Transin is a secreted metalloprotease of 53,000 daltons (Matrisian et al., 1986a) that shares significant DNA sequence similarity with human skin collagenase (Goldberg et al., 1986; Muller et al., 1988), and appears to be the rodent version of stromelysin (Frisch et.al.,1987; Breathnach et al., 1987). In rat fibroblasts, transin RNA is induced by epidermal growth factor (EGF) (Matrisian et al., 1985, 1986b) and inhibited at the transcriptional level by transforming growth factor type-beta (TGF-β1) (Machida et al., 1988). Recently, it has been shown that platelet-derived growth factor and EGF regulate transin gene expression by both c-*fos*-dependent and c-*fos*-independent pathways in mouse NIH 3T3 fibroblasts (Kerr et al., 1988). Although much is known about the regulation of transin by growth factors in fibroblasts, little is known about its possible role in neuronal cells.

In this paper, we report that the gene encoding transin is highly regulated in PC12 cells. Unlike its regulation in fibroblasts, however, transin is induced in PC12 cells by NGF, and this induction is inhibited by glucocorticoids. The time course of transin induction is coincident with the neuronal differentiation of these cells and is also seen following treatment with fibroblast growth factor, suggesting an association between transin expression and axon elongation.

RESULTS

NGF Induction of Transin mRNA

To determine whether the protease transin is induced in PC12 cells, we first cultured cells in the presence or absence of NGF for 24 hrs, and then measured transin mRNA accumulation by RNA blot analysis using a rat transin cDNA probe (Matrisian et al., 1985). Figure 1a shows the characteristic 1.9 kb transin mRNA band in the lane from NGF-treated, but not from the untreated, PC12 cell cultures. To confirm that similar amounts of RNA were added to both lanes, this RNA blot was stripped and then reprobed for mRNA encoding cyclophilin, a ubiquitous protein whose expression appears to be constitutive for many mammalian cells (Danielson et al., 1988). Figure 1b shows that similar amounts of cyclophilin mRNA were present in both lanes.

Comparison of the Time Courses of Induction

If transin induction is involved in the process of axon elongation, then these two phenomena should have similar time courses. In order to compare directly the time course of transin mRNA induction by NGF with morphological changes, parallel cultures were either photographed (see below) or harvested for RNA. Figure 2a shows that transin mRNA levels became detectable as early as 2-4 hours following NGF treatment, reached a peak at around 24-72 hours, and then decreased over the next few days (even though fresh NGF-containing medium was added every 2-3 days). Transin mRNA was undetectable in any of the RNA prepared from cultures of non-treated PC12 cells. To confirm the presence of equivalent amounts of RNA in each of the lanes, this blot was stripped and reprobed for cyclophilin mRNA (Figure 2b).

Figures 3a and 3b are photomicrographs of NGF-treated and untreated cells, respectively, from the same experiment described in Figure 2, and show the difference in morphologies present 4 days after addition of NGF. Figure 3c represents a morphometric analysis of the proportion of PC12 cells possessing neurites as a function of time, and shows that the first neurite-bearing PC12 cells could be seen as early as 10 hours following addition of NGF. The proportion of PC12 cells possessing neurites increased steadily

thereafter, until nearly all cells possessed neurites at day 7. Similar time courses were observed when either the number of neurites per cell or the average length of neurites was plotted as a function of time (data not shown). These data indicate that transin mRNA accumulates to detectable levels immediately prior to neurite initiation in NGF-treated PC12 cells and remains at these high levels for several days.

The middle molecular weight neurofilament protein, NF-M, is a major component of the cytoskeleton of axons and has also been shown to be induced by NGF in PC12 cells (Lee and Page, 1984; Dickson et al., 1986; Lindenbaum et al., 1988). To compare the time course of appearance of transin mRNA with this cytoskeletal marker of axons, a duplicate RNA blot to that in Figure 2a was probed with a partial cDNA for murine NF-M (Julien at al., 1986). Figure 2c confirms that non-treated PC12 cells express NF-M transcripts constitutively, and that NGF-treatment increased this expression several-fold. This figure also shows that the time course of NF-M mRNA induction by NGF parallels that of transin mRNA. Although the high basal levels of NF-M transcripts make it difficult to determine precisely when induction first occurs, NF-M mRNA levels clearly reached a peak about 24-48 hours following NGF addition, and then declined somewhat over the next few days. When this blot was reprobed for cyclophilin mRNA, roughly equal amounts of RNA were found to have been loaded in each of the lanes (Figure 2d).

Nuclear Run-on Analysis

The appearance of transin mRNA in response to NGF may result from either an increased rate of transcription or an NGF-associated increase in the stability of transin transcripts. To determine whether the increase in transin mRNA levels was caused by an increased rate of transcription, nuclear run-on assays of RNA transcription were performed. Figure 4 shows that transcription of transin was undetectable in untreated cultures (i.e., at t=0) and became detectable as early as 2 hours after the addition of NGF (upper lanes). In contrast, the rate of cyclophilin transcription did not change over the same time period (lower lanes). These data indicate that the increased transin mRNA accumulation seen following NGF treatment reflects increases in <u>de novo</u> transcription of

transin.

Effects of Other Growth Factors

In order to determine whether the induction of transin RNA was specific to NGF, a number of other growth factors and agents were tested for their ability to induce transin mRNA. The RNA blot in Figure 5a shows that neither basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), transforming growth factor type beta (TGF-β1), dibutyryl cyclic AMP (dbcAMP) nor the phorbol ester, 12-*O*-tetra-decanoyl phorbol-13-acetate (TPA), were able to elicit any detectable increase in transin mRNA expression or to affect cyclophilin expression in this particular subclone of PC12 cells. It should be noted, however, that none of these agents were able to induce neurite outgrowth, either (data not shown).

In contrast to these findings, other groups have reported that treatment with either acidic FGF (aFGF) or basic FGF (bFGF) induces neurite outgrowth in PC12 cells (Togari et al., 1985; Rydel et al., 1987). Since the subclone of PC12 cells used in these experiments appeared to be non-responsive to FGF, we obtained another subclone of PC12 cells known to undergo morphological differentiation in response to FGF. These cells were then cultured for 48 hours in the presence of either NGF, aFGF, bFGF or heparin. The upper panel in Figure 5b shows that all three growth factors induced transin mRNA in this PC12 subclone. Neither the nontreated nor heparin-treated cells contained any detectable levels of transin RNA. The lower panel in Figure 5b shows that none of these growth factors had any effect on the level of cyclophilin mRNA. The neurites induced by aFGF and bFGF were morphologically indistinguishable from those of NGF-treated cells (data not shown).

Inhibition of Transin Induction by Cycloheximide

To determine whether the induction of transin mRNA by NGF required the synthesis of intermediary proteins, PC12 cells were incubated with NGF in the presence or absence of the protein synthesis inhibitor, cycloheximide, for various periods of time and then the cultures were assayed for transin mRNA levels. The upper panel in Figure 6

shows that at all times tested, the induction of transin mRNA by NGF was blocked or inhibited by co-treatment with cycloheximide, suggesting that NGF does not induce transin mRNA directly. The lower panel of Figure 6 shows there was no change in the level of cyclophilin mRNA with any of these treatments.

Inhibition of Transin Induction by Dexamethasone

Since glucocorticoids are known to antagonize the effects of NGF in various neural crest-derived cell types, we tested whether the synthetic glucocorticoid, dexamethasone, could also inhibit the NGF-induction of the transin gene. In these experiments, PC12 cells were treated with either NGF alone, NGF plus dexamethasone, or dexamethasone alone for 24 or 48 hours. The upper panel in Figure 7 shows that at both time points, dexamethasone substantially reduced NGF induction of transin (compare lanes b and c, or lanes e and f). Densitometric analysis showed, moreover, that the inhibition by glucocorticoids was down to 1-2% (data not shown). Pretreatment of the PC12 cells with NGF for 24 hrs, followed by a 24 or 48 hr exposure to both NGF and dexamethasone, also reduced transin mRNA levels (e.g. compare lanes e and h). The lower panel in Figure 7 shows dexamethasone had no effect on the levels of cyclophilin mRNA. Thus, dexamethasone specifically inhibited the induction of transin by NGF when added either simultaneously or following NGF treatment.

To determine whether the inhibition by dexamethasone occurred at the transcriptional level, nuclear run-on experiments were performed. Figure 8 is an autoradiograph showing that transcription of the transin gene was inhibited when dexamethasone was added either with NGF (compare lane "4ND" with "4N", upper row) or following NGF treatment (compare lane "3N-1ND" with "4N", upper row). In contrast, cyclophilin transcription was not affected by dexamethasone treatment (lower bands). Dexamethasone, therefore, appears to inhibit the induction by NGF of the transin gene at the transcriptional level.

Transfection Studies Using the Transin 5'-Untranscribed Region

The effects of NGF and dexamethasone on transin gene transcription were further

tested by determining whether the 5'-flanking region of the transin gene was able to confer NGF and dexamethasone responsiveness. For these experiments, PC12 cells were transfected with the recombinant plasmid, p750TRCAT (Kerr et al.,1988), containing 750 base pairs of the 5'-untranscribed region of the transin gene fused to the bacterial reporter gene, chloramphenicol acetyltransferase (CAT). The cells were then grown in the presence of either NGF alone, NGF plus dexamethasone, or dexamethasone alone for 8, 24 or 48 hours. NGF treatment of PC12 cells transfected with p750TRCAT produced a significant increase in CAT activity, shown in Figure 8a. This induction of CAT activity was approximately six-fold over non-induced cells at 24 and 48 hrs after addition of NGF (Figure 8b). In contrast, when dexamethasone was included with the NGF, the extent of induction was reduced by more than half at both 24 and 48 hrs. These data indicate that this 750 bp sequence of the transin promoter region conferred responsiveness to both NGF and glucocorticoids.

DISCUSSION

The observations that various proteases are released from the growth cones of developing neurons in culture (e.g., Krystosek and Seeds, 1981a,b; Pittman, 1985 Pittman and Williams, 1989) have led to the notion that secreted proteases may play some role in the process of axon elongation. Growth cone motility requires numerous adhesion as well as de-adhesion events, and proteases may be involved in altering the microenvironment by permitting penetration of the growth cone into the surrounding tissue (for review see Monard, 1988). In any case, the notion that proteases are involved in growth cone movements leads to the prediction that genes encoding specific proteases would be activated at the time when axons first appear. We report that the gene encoding the metalloprotease transin is induced by NGF and FGF and, furthermore, the time course of this induction is coincident with neurite outgrowth.

NGF Induces Expression of Transin mRNA

We found that levels of the transin mRNA in PC12 cells increased several-fold within 24 hours in response to NGF, making transin one of the most highly NGF-responsive gene products described (Leonard et al., 1987; Federoff et al., 1988; Stein et al., 1988; c.f., Milbrandt, 1987; 1988). Since we were unable to detect any basal levels of transin expression in untreated cells, however, this conservative figure is based on estimates of the minimum signal that was detectable on the densitometric tracings of the autoradiographs.

Unlike transin, various other NGF-inducible genes in PC12 cells have detectable basal levels of expression. These include GAP-43 (Federoff et al., 1988); the neurofilament proteins, NF-M and NF-H (Dickenson et al., 1986; Lindenbaum et al., 1987); SCG10 (Stein et al., 1988b); and various other gene products (Leonard et al., 1987; c.f., Milbrandt, 1987; 1988). It is difficult to conclude, however, whether the undetectable basal level of transin expression indicates that transin is involved only with the morphological differentiation of PC12 cells, or whether it results from our use of serum-free medium instead of the more commonly used serum-containing culture media. Perhaps

combinations of factors in serum induce low levels of expression of some of these other gene products. In any event, the undetectable basal levels of transin expression and its high level of NGF-responsiveness make transin a potentially useful gene for studying molecular mechanisms underlying NGF action.

It seems likely that most of the NGF-induced increase in transin mRNA levels was due to de novo synthesis. This conclusion is based largely on the nuclear run-on studies which show that transcription of transin in PC12 cells was undetectable until 2 hrs following addition of NGF to the culture medium. If, for example, the major effects of NGF on transin induction were due to post-transcriptional events (e.g., Lindenbaum et al., 1987; Cho et al., 1989), we would expect to find relatively higher basal levels of transin transcription. For instance, it is unlikely that NGF could alter the stability of transin mRNA when no basal levels were detected in the first place. Further evidence that NGF increases the rate of de novo transcription comes from the transfection studies using the p750TRCAT plasmid, which contains 750 base pairs of the 5'-untranscribed region of the transin gene fused to the CAT reporter gene. These studies show that this region of the transin gene confers NGF-responsive induction to this heterologous gene product.

Glucocorticoids Inhibit the Induction of Transin by NGF

We have also found that dexamethasone inhibits transin mRNA induction by NGF, and that this inhibition occurs at the level of de novo transcription. Although dexamethasone has previously been shown to inhibit the induction of various NGF-inducible genes in PC12 cells (e.g., Leonard et al., 1987), the extent of this inhibition was much less than with transin. Stein et al. (1988b) found, for example, that dexamethesone inhibited the NGF-induced increase in SCG10 mRNA to about 30% of NGF-treated cultures and this occurred only after several days of treatment with dexamethasone. In contrast, transin mRNA levels were inhibited to 1-2% when dexamethasone was included, and that significant inhibition of de novo transin transcription occurred within 1 hour after the addition of dexamethasone. It is interesting to note, that glucocorticoids have been shown to inhibit the growth factor-induced expression of a variety of proteases in

other systems, including various serine proteases (Medcalf et al., 1988) and the metalloprotease stromelysin (Frisch and Ruley, 1987), suggesting that proteases may be susceptible to a common inhibitory mechanism by glucocorticoids.

Interestingly, dexamethasone did not seem to inhibit the NGF-induced morphological differentiation of PC12 cells--an observation that has been noted by others (Stein et al., 1988b; Federoff et al., 1988). Although this observation would seem to suggest that the various NGF-inducible gene products are not involved in the process of neurite extension, other explanations are also possible. NGF may, for example, induce an excess of these gene products, and dexamethasone may simply act to moderate this induction. Alternatively, these gene products may serve functions associated with neurite outgrwoth which are not normally observable in two-dimensional tissue culture dishes. If the role of transin is to degrade proteinaceous barriers in a 3-dimensional extracellular matrix, for example, the lack of such barriers in the culture dish may obviate transin's function.

The Transin 5'-Untranscribed Region Confers Responsiveness to NGF and Dexamethasone

The transient transfection studies showed that a 750 base pair region of the transin gene confers inducibility by NGF to a heterologous gene. This region (Matrisian et al., 1986b) is just 5' to the transcription start site, and contains the TATAA and CAAT boxes, as well as an AP1 consensus binding site (Angel et al., 1987). It seems unlikely, however, that this region is directly responsive to the primary intracellular signals generated by NGF. We have shown, for example, that inhibitors of protein synthesis can block the induction of transin mRNA expression when included with NGF (Figure 6). These data would suggest, therefore, that the primary effect of NGF is to induce synthesis of a protein or proteins, which subsequently induce(s) expression of the transin gene. Likely candidates for such intermediary proteins include the proto-oncogenes c-*jun* and c-*fos*. This latter proto-oncogene, for example, has been reported to be induced in PC12 cells shortly after NGF treatment (Greenberg et al., 1985; see also Visvader et al., 1988), and is

known to associate with c-*jun* to form a complex which may recognize the AP1 consensus binding site present in the transin gene (for review, see Curran and Franza, 1988). Further circumstantial evidence for a causal role of c-*fos* in the induction of transin by NGF comes from comparisons of the time course of appearance of these two gene products. Induction of c-*fos* has been shown to occur within minutes following exposure of PC12 cells to NGF (Greenberg et al., 1985), whereas transin mRNA was first detected after 2 hours. On the other hand, it has recently been shown that transin may be induced by both c-*fos* dependent and c-*fos* independent pathways following addition of EGF and platelet-derived growth factor to cultures of mouse NIH 3T3 fibroblasts (Kerr et al., 1988). Therefore, the identity of the proteins mediating the induction of transin by NGF in PC12 cells and the exact location of NGF-responsive elements within the 5'-flanking region of the transin gene awaits more complete analysis.

The transfection studies also show that the inhibition of transin expression by dexamethasone is mediated by this 5' region. This observation, however, does not necessarily mean that dexamethasone directly modulates transcription of the transin gene. In fact, the effects of dexamethasone may be at any one of a number of pre-transcriptional levels, including inhibition of the primary intracellular signal induced by NGF, or inhibition of induction of the intermediary protein(s). Indeed, inhibition at these pre-transcriptional levels may explain the more global effects of dexamethasone in inhibiting the actions of NGF in PC12 cells.

Transin mRNA Induction Correlates with Neurite Outgrowth

These data provide two lines of evidence to suggest that expression of transin is associated with neurite outgrowth. First, the time course of transin mRNA induction precedes, and is coincident with, the initial appearance of neurites. This time course also correlates with that of a known cytoskeletal marker for neurites, NF-M. The short delay between the appearance of transin and NF-M transcripts and the appearance of neurites, moreover, may simply reflect the time necessary for translation to occur. Second, both transin mRNA expression and neurite formation are induced in some subclones of PC12

cells by basic and acidic forms of FGF. The finding that these other growth factors also induce transin suggests that expression of this protease is not necessarily linked with NGF, but correlates closely with the morphological changes in PC12 cells.

The identification of transin as the rat homologue of stromelysin (Whitman et al., 1986; Muller et al., 1988) suggests that transin may be involved in the degradation of various extracellular matrix components, including proteoglycans, fibronectin, laminin, elastin and possibly collagen type IV (Chin et al., 1985). The notion that transin would normally function extracellularly is supported by our observations that the transin protein is secreted into the culture medium of NGF-treated, but not untreated, PC12 cells (unpublished observations, Machida and Ciment). These data are consistent, therefore, with the notion that expression of this metalloprotease is involved with the process of axon elongation during neuronal development.

EXPERIMENTAL PROCEDURES

Cell Culture

Stock cultures of PC12 cells were grown in L15 basal culture medium supplemented with 6 mg/ml D-(+)-glucose (Sigma, St. Louis, MO.) and the following Gibco (Long Island, N.Y.) products: fetal bovine serum (5%), horse serum (5%), glutamine (2mM), penicillin (50 U/ml), and streptomycin (50 mcg/ml). Approximately 5 x 10^5 cells were plated onto 10 cm Primaria dishes (Falcon) which were pre-coated overnight at 37°C with 2μg/ml laminin (Collaborative Research) in L15 basal medium. After 24 hours, the cells were washed and cultured in N_2 serum-free defined medium (Bottenstein and Sato, 1979) for an additional 24 hours. At the beginning of each experiment, the medium was again changed to fresh N_2 medium containing either NGF (50 ng/ml); kindly provided by Dr. L. Baizer (Oregon Health Sciences University), or various other agents. The original PC12 cell line (subclone RG-5) was obtained from Dr. Rae Nishi (Oregon Health Sciences University); the FGF-responsive PC12 cells were obtained from Dr. Bradley Olwin (University of Wisconsin).

RNA blot analysis

Cells were harvested and total cellular RNA was isolated, as previously described (Machida et al., 1988). Briefly, total cellular RNA (20 μg/lane) was fractioned by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde, and then blotted onto Nytran membranes (Schleicher and Schuell, Keene, N.H.). Prehybridization and hybridization were performed as described previously (Machida et al., 1988), with the hybridization buffer containing 10^8cpm/μg of either the ^{32}P-labeled nick-translated pTR1 plasmid (containing a 1.6 kb transin insert); pNF-M plasmid (containing a 660 bp insert); or p1B15 plasmid (containing a 700 bp cyclophilin insert). After hybridization for 16 h at 42°C, the membranes were washed briefly in 2x SSC- 0.1% sodium dodecyl sulfate (SDS) at room temperature, followed by two 30 min washes in 0.2x SSC-0.1% SDS at 60 °C. The hybridized membranes were then exposed to X-Omat X-ray film (Kodak) with intensifying screens for 1-2 days at -70°C. For stripping, the RNA blots were allowed to

decay for several half-lives and then they were treated with the prehybridization solution at 42°C for a minimum of 2 hours.

Transcriptional run-on analysis

Preparation of nuclei and transcriptional run-on reactions were carried out as described previously (Machida et al., 1988). Briefly, nuclei containing identical amounts of radioactivity (2×10^7 cpm/0.5 ml) were hybridized for 4 days at 42°C with either transin or cyclophilin cDNAs immobilized on diazobenzyloxy-methyl paper (Schleicher and Schuell). After hybridization, the filters were washed two times in 2x SSC-0.1% SDS at room temperature and then washed in 0.1x SSC at 42°C. The filters were then washed 2x for 30 min in 2.5 mg/ml RNAse A at 37°C followed by a final wash in 0.1x SSC- 0.1% SDS at 42°C. The filters were exposed for 3 days to X-ray film at -70°C with intensifying screens.

CAT assays

PC12 cells were plated at a density of 3×10^4 cells per 6 cm plate one day before transfections. Calcium phosphate-DNA precipitates containing 10 μg of p750TRCAT (Kerr et al., 1988) were prepared by the method of Gorman et al. (1985). Cells were incubated with these DNA-calcium phosphate precipitates for 4 hr, then incubated in 15% glycerol in Hank's balanced salt solution (Gibco) for 2 min. The cultures were washed 2x in N_2 medium, and then grown overnight in fresh N_2 medium. One day following transfection, fresh N_2 medium containing NGF and/or dexamethasone was added for 24 or 48 hours. Cell extracts were then measured for either protein content (Smith et al. 1985), or for CAT activity using the method of Gorman et al. (1985) as modified by Seasholtz et al. (1988). After autoradiography, spots corresponding to acetylated and non-acetylated forms of chloramphenicol were excised and counted in a scintillation counter.

Chemicals and reagents

EGF was prepared from mouse submaxillary glands as described by Savage and Cohen (1972), and further purified to homogeneity, as previously described (Matrisian et al., 1982). TGF-β1 was prepared from human platelets as described by Assoian et al.,

(1983) with the addition of a final purification step consisting of reversed-phase high-performance liquid chromatography on C3 columns and elution with an acetonitrile-trifluroroacetic acid gradient. Basic FGF was obtained from Dr. G. Shipley (Oregon Health Sciences University) and acidic FGF was obtained from Dr. R. Nishi (Oregon Health Sciences University). 12-*O*-tetra-decanoyl phorbol-13- acetate (TPA) was purchased from Pharmacia and dissolved in dimethyl sulfoxide. Dexamethasone, cycloheximide, and dibutyryl cyclic AMP were purchased from Sigma.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. J.-P. Julien for his gift of the NF-M plasmid, Dr. J. Douglass for his gift of the cyclophilin plasmid, Dr. L. Baizer for his gift of NGF, Dr. R. Nishi for her helpful suggestions in the preliminary stages of this project, and Dr. K. Stocker for her critical reading of the manuscript. This work was supported by NIH grants to L.M. (CA 48643), B.E.M. (CA 39360, CA 47404) and G.C. (DE 07625, NS23883) and by NIH training grant stipend to C.M.M. (HDO 7133-10).

REFERENCES

Anderson, D.J. and Axel, R. (1986). A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoids. Cell *47*, 1079-1090.

Aloe, L. and Levi-Montalcini, R. (1979). Nerve growth factor-induced transformation of immature chromaffin cells in vivo into sympathetic neurons: Effect of antiserum to nerve growth factor. Proc. Natl. Acad. Sci. USA *76*, 1246-1250.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P. and Karin, M. (1987a). Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated trans-acting factor. Cell *49*, 729-739.

Assoian, R.K., Komoriya, A., Meyers, C.A., Miller, D.M. and Sporn, M.B. (1983). Transforming growth factor in human platelets. Identification of a major storage site, purification and characterization. J. Biol. Chem. *258*, 7155-7160.

Bottenstein, J. and Sato, G. (1979). Growth of rat neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. USA *76*, 514-517.

Bray, D. and Hollenbeck, P.J. (1988). Growth cone motility and guidance. Ann. Rev. Cell Biol. *4*, 43-61.

Breathnach, R., Matrisian, L., Gesnel, M-C., Staub, A. and Leroy, P. (1987). Sequences coding for part of oncogene-induced transin are highly conserved in a related rat gene. Nuc. Acids Res. *15*, 1139-1151.

Chin, J.R., Murphy, G. and Werb, Z. (1985). Stromelysin, a connective tissue-degrading metalloendopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with

collagenase. J. Biol. Chem. *260*, 12367-12376.

Cho, K-O, Skarnes, W.G., Minsk, B., Palmieri, S., Jackson-Grusby, L. and Wagner, J.A. (1989). Nerve growth factor regulates gene expression by several distinct mechanisms. Molec. Cell. Biol. *9*, 135-143.

Claude, P., Parada, I.M., Gordon, K.A., D'Amore, P.A. and Wagoner, J.A. (1988). Acidic fibroblast growth factor stimulates adrenal chromaffin cells to proliferate and to extend neurites, but is not a long-term survival factor. Neuron *1*, 783-790.

Curran, T. and Franza, B.R., Jr. (1988). Fos and Jun: The AP-1 connection. Cell *55*, 395-597.

Danielson, P.E., Forss-Petter, S., Brow, M.A., Calavetta, L., Douglass, J., Milner R.J. and Sutcliffe, J.G. (1988). p1B15: A cDNA clone of the rat mRNA encoding cyclophilin. DNA *7*, 261-267.

Dickson, G., Prentice, H., Julien 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 J. *5*, 3449-3453.

Doupe, A.J., Landis, S.C. and Patterson, P.H. (1985). Environmental influences in the development of neural crest derivatives: glucocorticoids, growth factors and chromaffin cell plasticity. J. Neurosci. *5*, 2119-2142.

Federoff, H.J., Grabczyk, E. and Fishman, M.C. (1988). Dual regulation of GAP-43 gene expression by nerve growth factor and glucocorticoids. J. Biol. Chem. *263*, 19290-19295.

Frisch, S.M., Clark, E.J. and Werb Z. (1987). Coordinate regulation of stromelysin and collagenase genes determined with cDNA probes. Proc. Natl. Acad. Sci. USA *84*, 2600-2604.

Frisch, S.M. and Ruley H.E. (1987). Transcription from the stromelysin promoter is induced by interleukin-1 and repressed by dexamethasone. J. Biol. Chem. *262*, 16300-16304.

Goldberg, G.I., Wilhelm, S.M., Kronberger, A., Bauer, E.A., Grant, G.A. and Eisen, A.Z. (1986). Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation-induce rat protein. J. Biol. Chem. *261*, 6600-6605.

Gorman C., Moffat, L.F. and Howard, B.H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. *2*, 1044-1051.

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

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.

Julien, J-P., Meyer, D., Flavell, D., Hurst, J. and Grosveld, F. (1986). Cloning and developmental expression of the murine neurofilament gene family. Molec. Brain Res. *1*, 243-250.

Kerr, L.D., Holt, J.T. and Matrisian, L.M. (1988). Growth factors regulate transin gene

expression by c-*fos*-dependent and c-*fos*-independent pathways. Science *242*, 1424-1427.

Krystosek, A. and Seeds, N.W. (1981a). Plasminogen activator secretion by granule neurons in cultures of developing cerebellum. Proc. Natl. Accad. Sci. (USA). *78*, 7810-7814.

Krystosek, A. and Seeds, N.W. (1981b). Plasminogen activator release at the neuronal growth cone. Science. *213*, 1532-1533.

Lee, L.M-Y and Page, C. (1984). The dynamics of nerve growth factor-induced neurofilament and vimentin filament expression and organization in PC12 cells. J. Neurosci. *4*, 1705-1714.

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. Molec. Cell. Biol. *7*, 3156-3167.

Letourneau, P.C. (1987). What happens when growth cones meet neurites: attraction or repulsion? Trends Neurosci. *10*, 390-393.

Lindenbaum, M.H., Carbonetto S., Grosveld, F., Flavell, D. and Mushynski, W.E. (1988). Transcriptional and post-transcriptional effects of nerve growth factor on expression of the three neurofilament subunits in PC-12 cells. J. Biol. Chem. *263*, 5662-5667.

Machida, C.M., Muldoon, L.L., Rodland K.D. and Magun B.E. (1988). Transcriptional modulation of transin gene expression by epidermal growth factor and transforming growth factor beta. Molec. Cell. Biol. *8*, 2479-2483.

Matrisian, L.M., Larsen B.R., Finch J.S. and Magun, B.E. (1982). Further purification of epidermal growth factor by high performance liquid chromatography. Anal. Biochem. *125*, 339-351.

Matrisian, L.M., Glaichenhaus, N., Gesnel, M.-C. and Breathnach, R. (1985). Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. EMBO J. *4*, 1435-1440.

Matrisian, L.M., Bowden, G.T., Krieg, P., Furstenberger, G., Briand, J.-P., Leroy, P. and Breathnach, R. (1986a). The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. Proc. Natl. Acad. Sci. USA *83*, 9413-9417.

Matrisian, L.M., Leroy, P., Ruhlmann, C., Gesnel, M.-C. and Breathnach, R. (1986b). Isolation of the oncogene and epidermal growth factor-induced transin gene: Complex control in rat fibroblasts. Mol. Cell. Biol.*6*, 1679-1686.

Medcalf, R.L., Van den Berg, E. and Schleuning, W-D. (1988). Glucocorticoid-modulated gene expression of tissue- and urinary-type plasminogen activator and plasminogen activator inhibitor 1 and 2. J. Cell Biol. *106*, 971-978.

Milbrandt, J. (1987). Nerve growth factor rapidly induces a gene which encodes a possible transcriptional regulatory factor. Science *2388*, 797-799.

Milbrandt, J. (1988). Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. Neuron. *1*, 183-188.

Monard, D. (1988). Cell-derived proteases and protease inhibitors as regulators of neurite outgrowth. Trends Neurosci. *11*, 541-544.

Muller, D., Quantin, B., Gesnel, M-C., Millon-Collard, R., Abecassis, J., and Breathnach, R. (1988). The collagenase gene family in humans consists of at least four members. Biochem J. *252*, 187-192.

Patterson, P.H. (1985). On the role of proteases, their inhibitors and the extracellular matrix in promoting neurite outgrowth. J. Physiol. (Paris) *80*, 207-211.

Patterson, P.H. (1988). On the importance of being inhibited, or saying no to growth cones. Neuron *1*, 263-267.

Pittman, R.N. (1985). Release of plasminogen activator and a calcium-dependent metalloprotease from cultured sympathetic and sensory neurons. Dev. Biol. *110*, 91-101.

Pittman, R.N. and Williams, A.G. (1989). Neurite penetration into collagen gels requires Ca^{2+}-dependent metalloproteinase activity. Dev. Neurosci. (in press).

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. Neurosc. *7*, 3639-3653.

Savage, C.R., Jr. and S. Cohen. (1972). Epidermal growth factor and a new derivative: Rapid isolation procedures and biological and chemical characterization. J. Biol. Chem. *247*, 7601-7611.

Seasholtz, A.F., Thompson, R.C. and Douglass, J.O. (1988). Identification of a cyclic

adenosine monophosphate-responsive element in the rat corticotropin-releasing hormone gene. Molec. Endocrin. *2*, 1311-1319.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk D.C. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. *150*, 76-85.

Stein, R, Mori, N., Matthews, K., Lo, L-C. and Anderson, D.J. (1988a). The NGF-inducible SCG10 mRNA encodes a novel membrane bound protein presen in growth cones and abundant in developing neurons. Neuron *1*,463-476.

Stein, R., Orit, S. and Anderson, D.J. (1988b). The induction of a neural-specific gene, SCG10, by nerve growth factor in PC12 cells is transcriptional, protein synthesis dependent, and glucocorticoid inhibitable. Dev. Biol. *127*, 316-325.

Togari, A., Dickens, G., Kuzuya, H. and Guroff, G. (1985). The effect of fibroblast growth factor in PC12 cells. J. Neurosci. *5*, 307-316.

Unsicker, K., Krisch, B., Otten, U. and Thoenen, H. (1978). Nerve growth factor-induced fiber outgrowth from isolated rat adrenal chromaffin cells: Impairment by glucocorticoids. Proc. Natl. Acad. Sci. USA *75*, 3498-3502.

Visvader, J., Sassone-Corsi, P. and Verma, I.M. (1988). Two adjacent promoter elements mediate nerve growth factor activation of the c-*fos* gene and bind distinct nuclear complexes. Proc. Natl. Acad. Sci. USA *85*, 9474-9478.

Whitham, W.E., Murphy, G., Angel, P., Rahmsdorf, H.J., Smith, B.J., Lyons, A., Harris, T.J.R., Reynolds, J.J., Herrlich, P. and Docherty, A.J.P. (1986). Comparison of human

stromelysin and collagenase by cloning and sequence analysis. Biochem. J. *240*, 913-916.

Figure 1: *RNA blot showing induction of transin mRNA by NGF.*

(a) PC12 cells were treated in the absence (CONT) or presence (NGF) of nerve growth factor for 24 hrs and then RNA blots were prepared. Note the characteristic 1.9 kb transin band present only in the lane from NGF-treated PC12 cells. "18S" and "28S" refer to positions of the ribosomal RNA bands.

(b) The same blot was stripped and reprobed for the 1.0 kb cyclophilin mRNA. Note that NGF had no effect on levels of this constitutively expressed gene product.

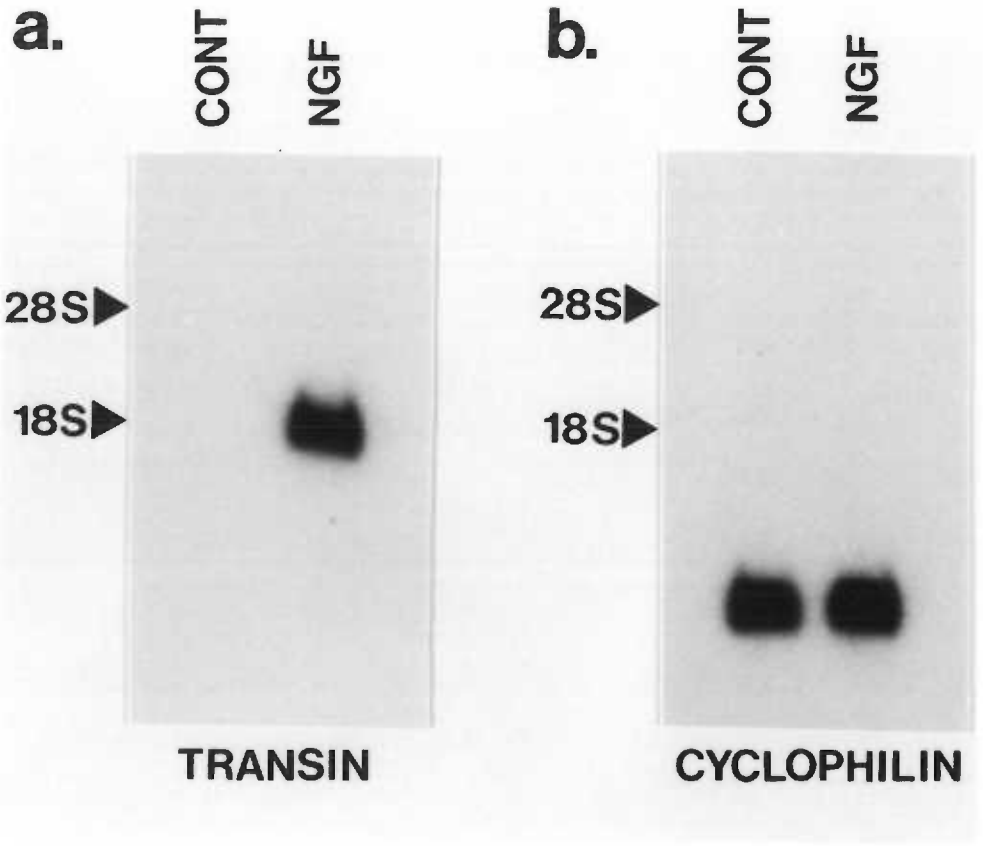

a.
CONT
NGF
28S
18S
TRANSIN
b.
CONT
NGF
28S
18S
CYCLOPHILIN

Figure 2: *RNA blots showing the time course of transin and NF-M mRNA induction.*

(a) PC12 cells were treated in the absence (C) or presence (N) of NGF and then harvested at various times for RNA blotting analysis of transin mRNA levels. The numbers over each lane refer to the duration of treatment (hrs) in culture. Note that transin mRNA became detectable by 2-4 hrs, reached maximal expression after 24-72 hrs, and then declined.

(c) An RNA blot similar to the one in panel a was probed for NF-M mRNA. Note that the induction of this transcript follows a similar time course as transin mRNA induction.

(b,d) The same RNA blots as in panel a and c, respectively, were stripped and reprobed for cyclophilin mRNA and shows the relative amounts of RNA present in each lane.

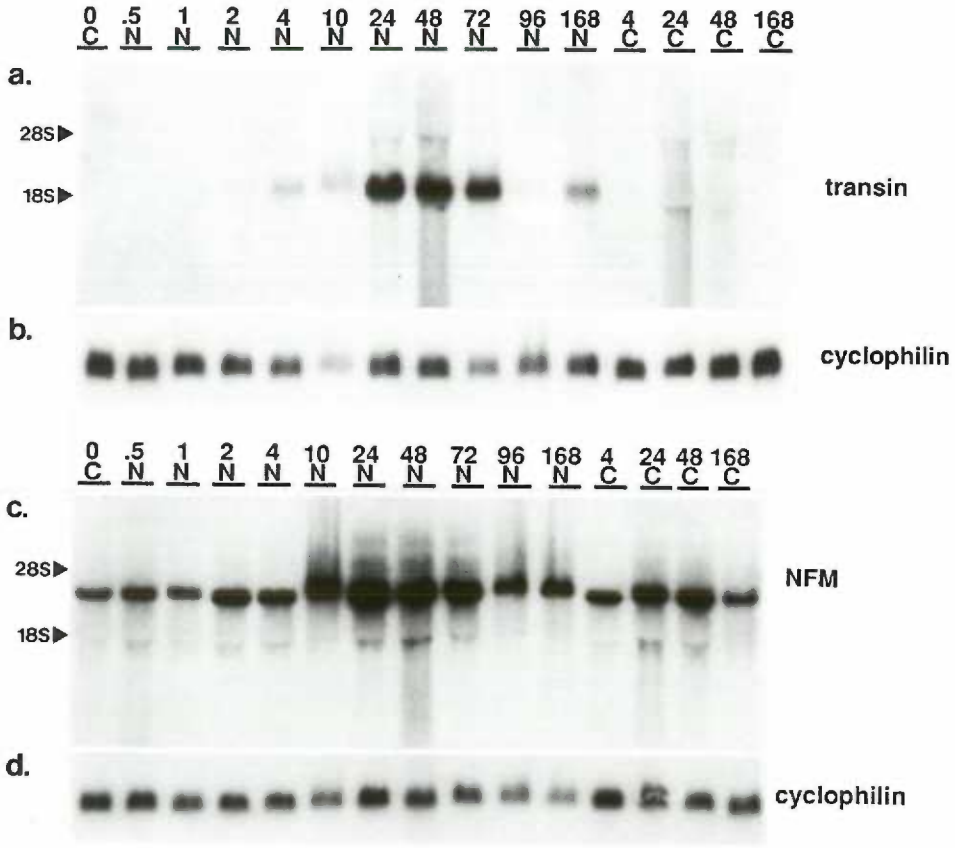

0 C
.5 N
1 N
2 N
4 N
10 N
24 N
48 N
72 N
96 N
168 N
4 C
24 C
48 C
168 C
a.
28S
18S
transin
b.
cyclophilin
0 C
.5 N
1 N
2 N
4 N
10 N
24 N
48 N
72 N
96 N
168 N
4 C
24 C
48 C
168 C
c.
28S
18S
NFM
d.
cyclophilin

Figure 3: *Morphological analysis of PC12 cells treated with NGF.*

Parallel cultures to those in Figure 2 were photographed at various times. Panels a and b are representative photographs of PC12 cells treated in the presence (a) or absence (b) of NGF for 96 hrs. Panel c shows the time course of neurite extention from PC12 cells treated with (●) or without (O) NGF. A neurite was operationally defined as a cell process greater than 2 cell diameters (approximately 12 μm). Note the initial appearance of neurites between 4 and 10 hours following the addition of NGF.

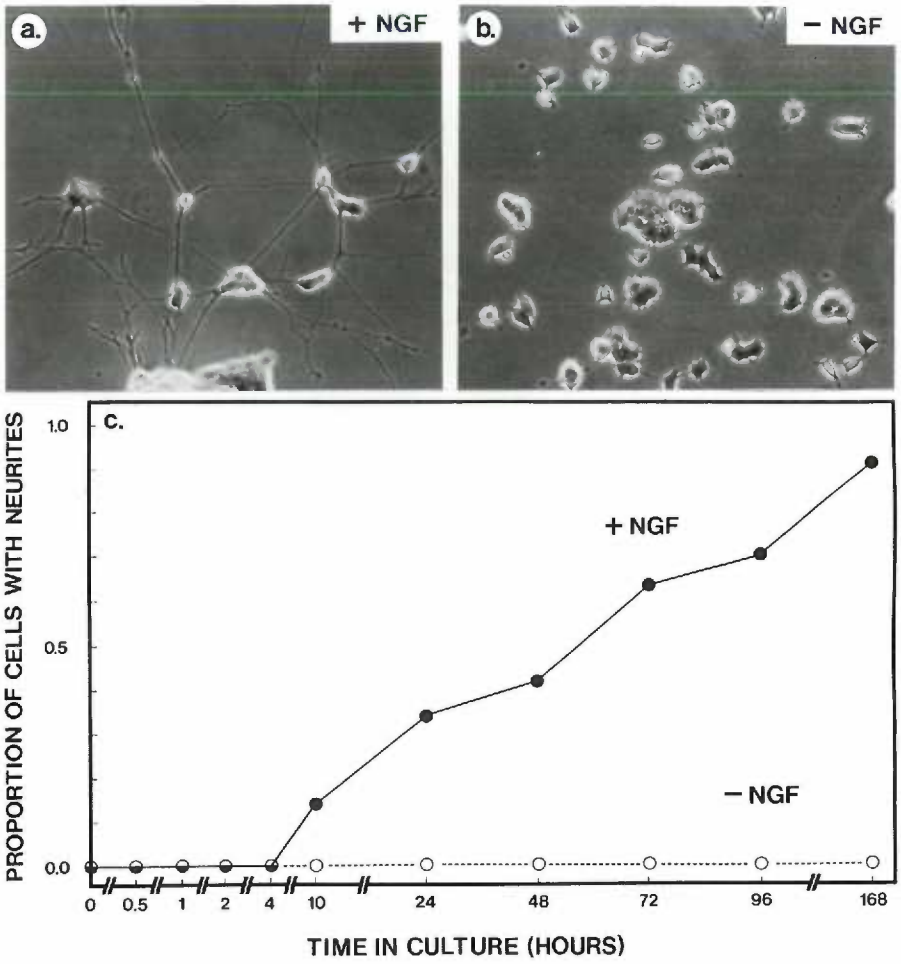

a.
+ NGF
b.
− NGF
c.
PROPORTION OF CELLS WITH NEURITES
1.0
0.5
0.0
+ NGF
− NGF
0
0.5
1
2
4
10
24
48
72
96
168
TIME IN CULTURE (HOURS)

Figure 4: *Transcriptional run-on analysis showing transin mRNA induction by NGF.*

PC12 cells were treated with NGF for the various times indicated (0-4 hrs) and then nuclei were isolated for nuclear run-on analysis of transin transcription (upper lanes) or cyclophilin transcription (lower lanes). Note that transin nuclear RNA was detectable by 2 hours after the addition of NGF (upper lanes).

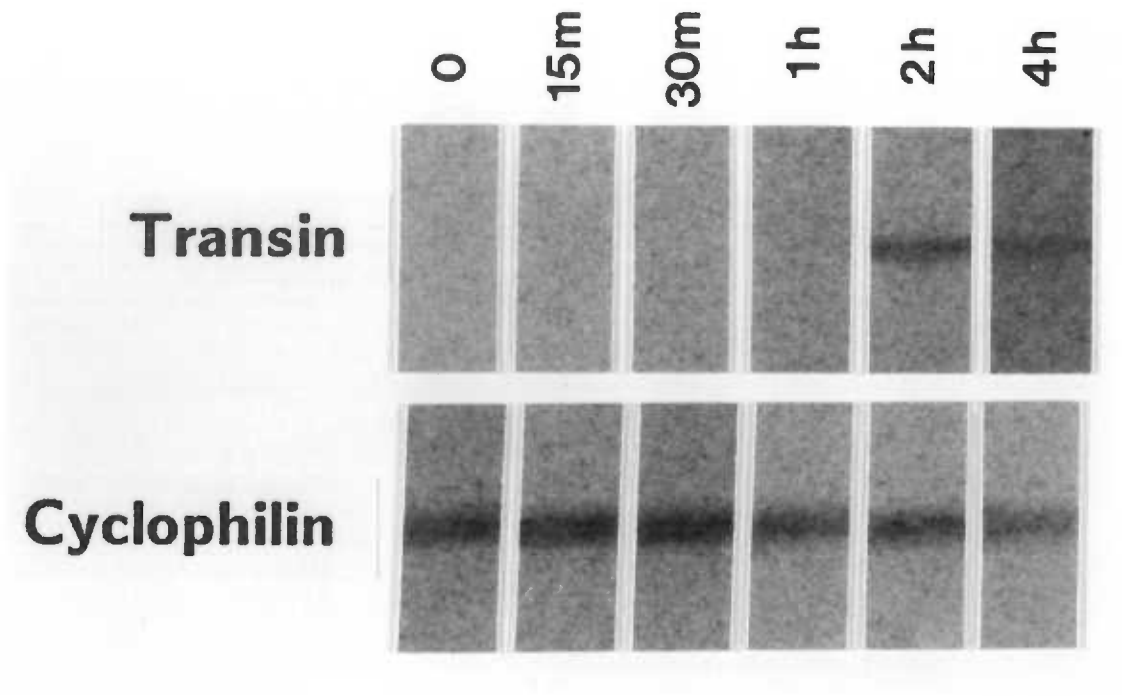

0
15m
30m
1h
2h
4h
Transin
Cyclophilin

Figure 5: *RNA blots showing the effects of various growth factors and agents on transin mRNA expression in two different subclones of PC12 cells.*

(a) The original subclone of PC12 cells was exposed for 24 hrs to one of the following: NGF (50 ng/ml), bFGF (5 ng/ml), EGF (5 ng/ml), TGF-β1 (5 ng/ml), dbcAMP (1 mM) or TPA (1.6 μM). RNA blots were then prepared and probed for transin and cyclophilin mRNA expression. Note that only NGF was able to induce transin mRNA in this particular PC12 subclone.

(b) A second subclone of PC12 cells that was responsive to FGF was treated for 48 hrs in culture with either NGF (50 ng/ml), aFGF (20 ng/ml), bFGF (5 ng/ml) or heparin (2 μM). [Heparin was included in the cultures treated with aFGF to stabilize this growth factor]. The lower panel shows control cyclophin mRNA expression in the same lanes. Note that all three growth factors specifically induced transin mRNA in this subclone of PC12 cells.

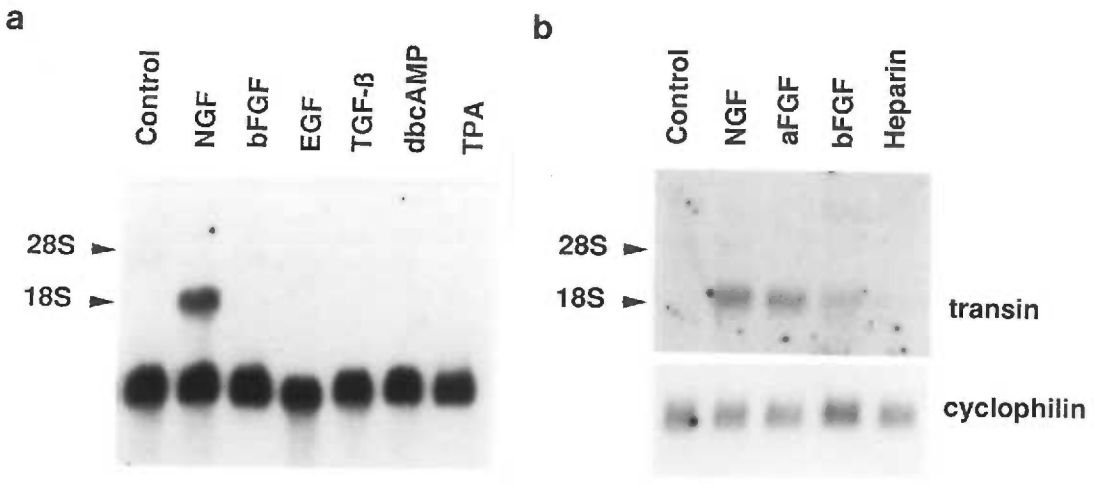

a
Control
NGF
bFGF
EGF
TGF-ß
dbcAMP
TPA
28S
18S
b
Control
NGF
aFGF
bFGF
Heparin
28S
18S
transin
cyclophilin

Figure 6: *RNA blot showing the effect of cycloheximide on the induction of transin mRNA by NGF.*

PC12 cells were treated with NGF for the times indicated, or pretreated for 15 min with cycloheximide (CHX; 25 μg/ml) followed by co-treatment with both cycloheximide and NGF. RNA blots were prepared from these cultures and probed for transin mRNA (upper panel) and then cyclophilin mRNA (lower panel). Note that co-treatment with cycloheximide blocked or inhibited the induction of transin by NGF at all time points.

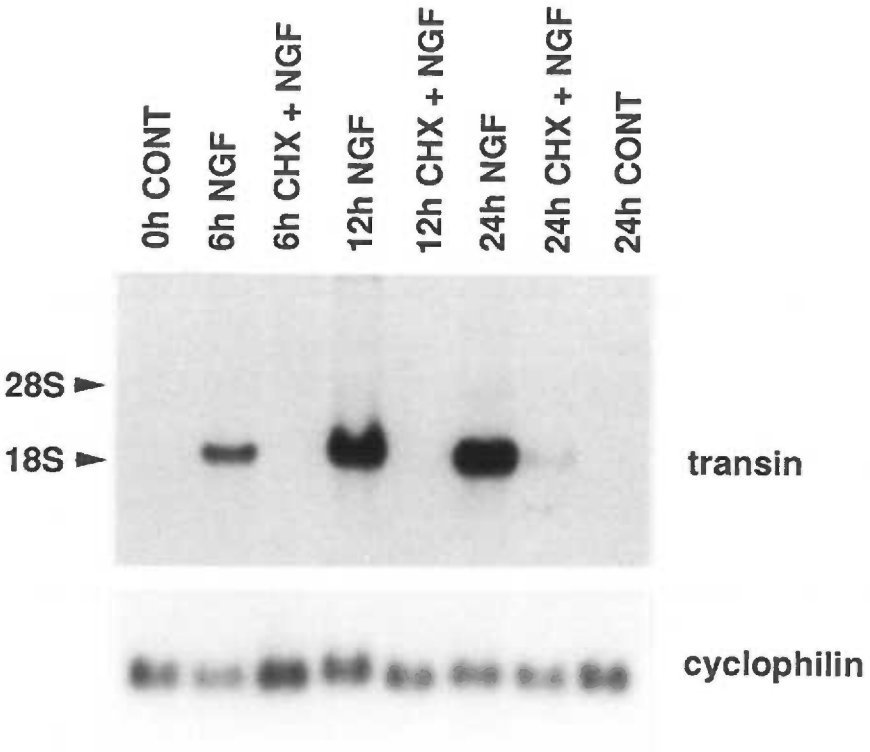

0h CONT
6h NGF
6h CHX + NGF
12h NGF
12h CHX + NGF
24h NGF
24h CHX + NGF
24h CONT
28S
18S
transin
cyclophilin

Figure 7: *RNA blot showing the effect of dexamethasone on transin mRNA induction by NGF.*

PC12 cells were treated with NGF alone, (b,e); NGF and dexamethasone simultaneously (c,f) or dexamethasone (DEX) alone (d,g) for 24 or 48 hrs. In the upper panel, note that dexamethasone inhibited the induction of transin by NGF at both time points (compare b with c, or e with f) but had no effect on transin expression by itself (d,g). Also note that dexamethasone reduced the levels of transin mRNA when the cultures were pretreated for 24 hrs with NGF (h,i). Reprobing the same blot with cyclophilin showed that similar amounts of RNA were present in all the lanes (lower panel).

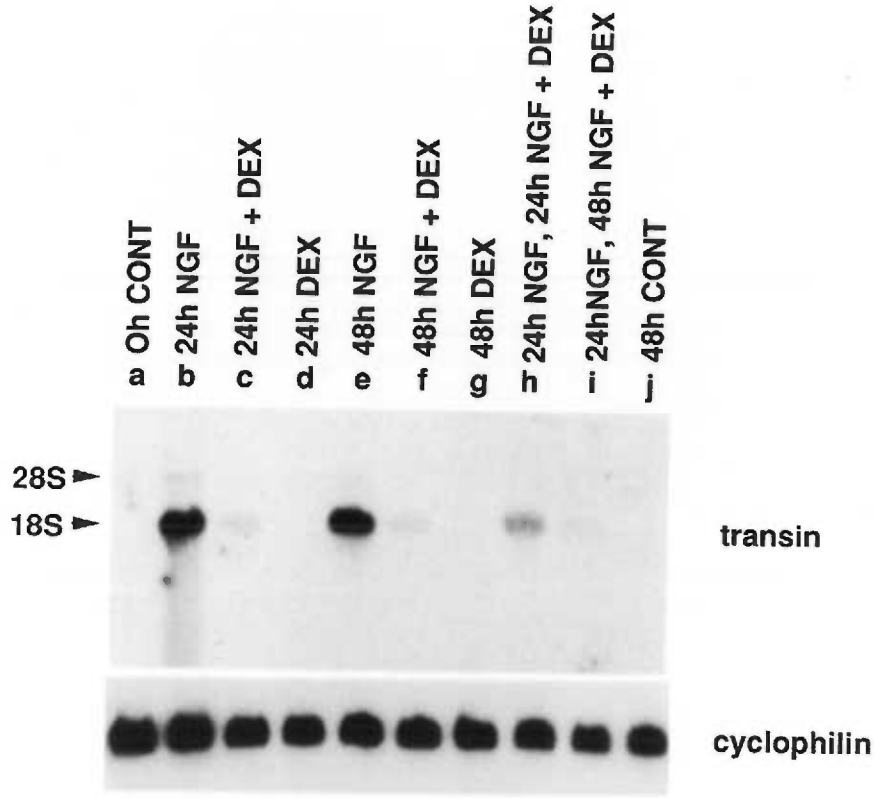

a Oh CONT
b 24h NGF
c 24h NGF + DEX
d 24h DEX
e 48h NGF
f 48h NGF + DEX
g 48h DEX
h 24h NGF, 24h NGF + DEX
i 24hNGF, 48h NGF + DEX
j 48h CONT
28S
18S
transin
cyclophilin

Figure 8: *Transcriptional run-on analysis showing inhibition of transin induction by dexamethasone.*

PC12 cells were treated for 4 hrs in the absence ("4C") or presence of NGF alone ("4N"), NGF and dexamethasone ("4ND") or dexamethasone alone ("4D"), as well as for 3 hrs with NGF followed by a 1 hr co-treatment with NGF and dexamethasone ("3N, 1ND"). Nuclei were then isolated and prepared for nuclear run-on analysis. The upper panel shows that transin induction by NGF at 4 hrs was inhibited by simultaneous treatment with dexamethasone (compare "4N" and "4ND"). Note also that 1 hr of exposure to dexamethasone inhibits the induction of transin RNA after 3 hrs of exposure to NGF ("3N,1ND"). The lower panel shows that cyclophilin transcription was unaffected by these various treatments.

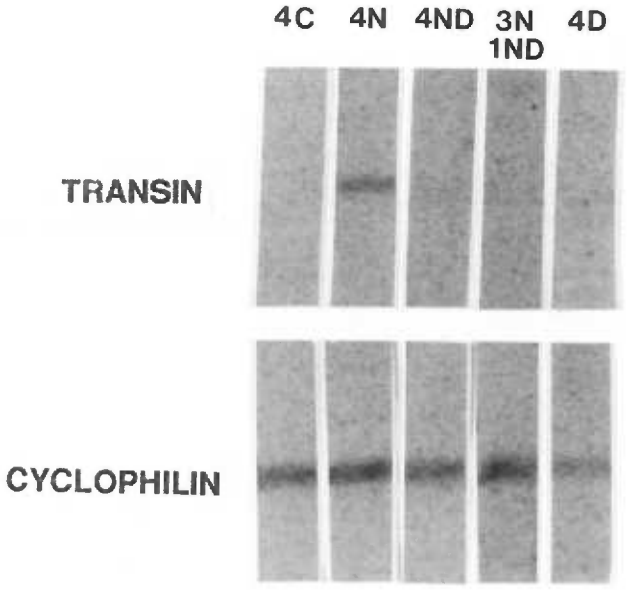
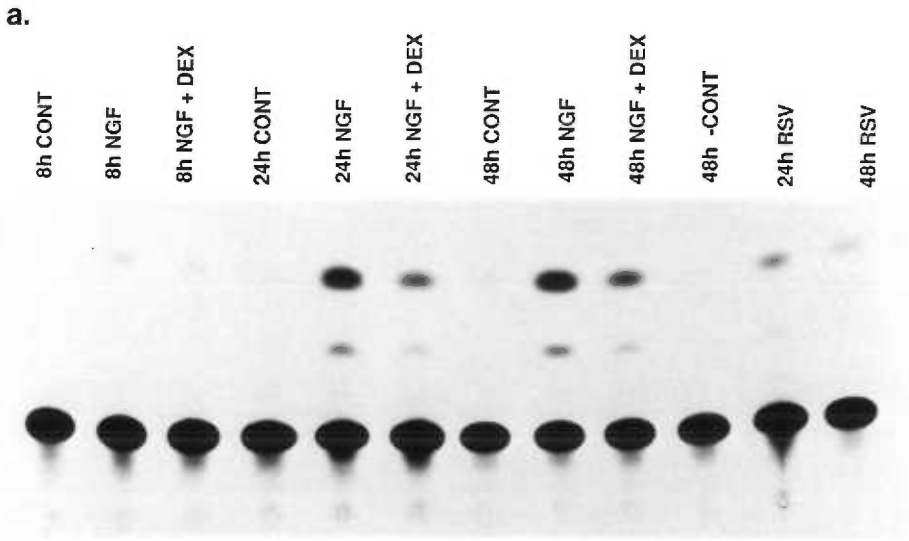

4C
4N
4ND
3N
1ND
4D
TRANSIN
CYCLOPHILIN

Figure 9: *Transient transfection studies showing that the 5'-untranscribed region of the transin gene confers NGF and dexamethasone responsiveness.*

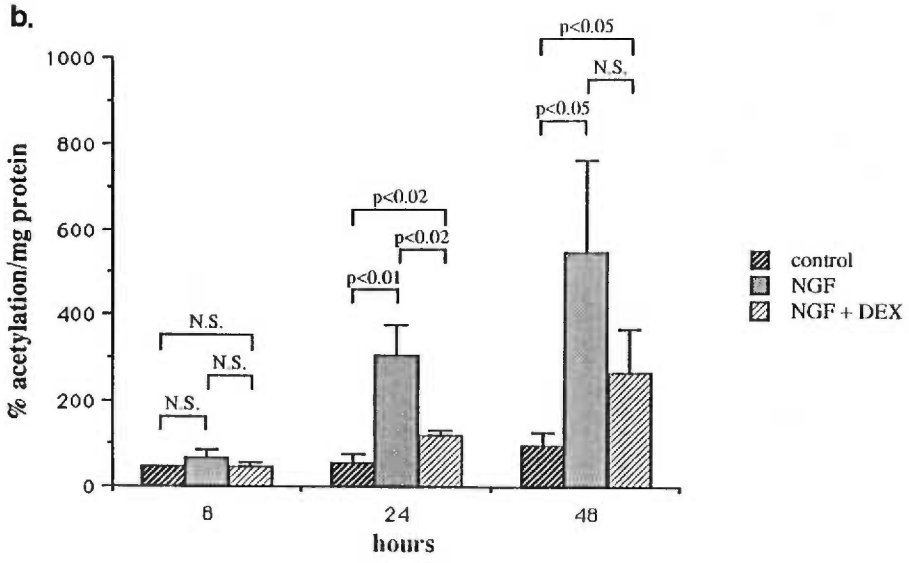
(a) PC12 cells were transfected with either p750TRCAT containing the 750 bp transin promoter region, pVL30CAT (a negative control) or pRSVCAT (a positive control). Note that the 750 bp transin 5'region induced CAT activity by 24 and 48 hrs., and that dexamethasone reduced this induction.

(b) Radioactive spots from each plate (n=3) were excised and counted in a scintillation counter to determine the CAT specific activity (% acetylation/mg protein) as a function of time. Panel b shows that NGF was able to induce at least a 6-fold increase in CAT activity over non-treated (Control) levels at either 24 or 48 hrs. Note that dexamethasone reduced this induction by half at both time points.

a.


8h CONT
8h NGF
8h NGF + DEX
24h CONT
24h NGF
24h NGF + DEX
48h CONT
48h NGF
48h NGF + DEX
48h -CONT
24h RSV
48h RSV

b.


% acetylation/mg protein
1000
800
600
400
200
0
N.S.
N.S.
N.S.
p<0.01
p<0.02
p<0.02
p<0.05
N.S.
p<0.05
8
24
48
hours
control
NGF
NGF + DEX

IV. SUMMARY AND DISCUSSION

A. Summary of Principal Findings

The gene encoding the metalloproteinase transin is regulated by growth factors in fibroblasts derived from a variety of sources (Matrisian et al., 1985; Frisch and Ruley, 1987; Kerr et al., 1988). In non-transformed Rat-1 fibroblasts, for example, transin mRNA is specifically induced by EGF and inhibited by TGF-β1 (Matrisian et al., 1986b). Prior to the work reported in this dissertation, characterization of the induction of transin by EGF had not been reported. Although antagonistic responses of EGF and TGF-β1 have been reported in other experimental systems (Roberts et al., 1985; Chiang and Nilsen-Hamilton, 1986; Whitsett et al., 1987), the mechanism of this interaction was also virtually unknown. In addition, expression of transin in cell types other than fibroblasts, in particular, neuronal cells, had not been studied. This thesis addressed all these points and the summary of these findings are as follows:

Characterization of transin induction by EGF in Rat-1 fibroblasts.

1. Induction of transin by EGF does not directly involve the protein kinase C pathway.
2. Transin mRNA has a stable half-life of 4 hours.
3. Induction of transin is an early (< 30 min.) but not direct consequence of EGF activation.
4. Continuous occupancy of the EGF receptor is required for maintenance of transin transcription.

Characterization of the inhibition of transin by TGF-β1 in Rat-1 fibroblasts.

1. TGF-β1 inhibited the induction of transin by EGF when added either simultaneously or following EGF treatment.
2. TGF-β1 inhibited the induction of transin by EGF at the transcriptional level.

Induction of transin mRNA in PC12 cells, a neuronal cell model system.

1. Transin mRNA was specifically induced by NGF and FGFs.

2. Induction of transin mRNA by these growth factors correlated with the appearance of neurites.

Characterization of transin induction by NGF in PC12 cells.

1. Induction of transin mRNA by NGF accompanies the expression of neurite outgrowth.
2. Transcription of the transin gene is induced by 2 hours following NGF treatment.
3. A 750 base pair sequence immediately 5' to the transcriptional start site of the transin gene confers responsiveness to NGF.

Characterization of the glucocorticoid inhibition of transin induction in PC12 cells.

1. Glucocorticoids inhibit the induction of transin by NGF when added either simultaneously or following NGF treatment.
2. Glucocorticoids inhibit the NGF induction of transin at the transcrip tional level.
3. The 750 base pair sequence in the transin promoter region also confers responsiveness to glucocorticoids.

B. Discussion of Findings

Molecular studies of the regulation of transin expression by growth factors are relevant from two perspectives. First, they may elucidate the events prior to gene activation; namely, the steps involved in the signal transduction pathways induced by the various growth factors. Second, they may provide insights of the events following gene activation; that is, the various morphogenetic events possibly associated with the proteolytic activity of transin such as metastases and axon elongation. This thesis characterized the specific induction of transin by EGF and NGF in two cell types, non-transformed Rat-1 fibroblasts and the neuronal cell model, PC12.

The correlation of transin expression with the metastatic phenotype (Matrisian et al., 1986a; Muller et al., 1988; Ostrowski et al., 1988) suggest that regulation studies in

non-transformed cells are fundamentally important for understanding the control mechanisms that may be altered during transformation. In non-transformed rat fibroblast cell lines, transin transcription was specifically induced by EGF, but not by PDGF, FGF, insulin or TGFβ (Matrisian et al., 1985). The studies presented in this thesis demonstrated that continuous activation of the EGF receptor was required for maintenance of transin transcription. Conceivably then, one possible difference between benign and metastatic tissues may be the EGF receptor is constantly activated in the transformed state. In fact, mutations in various domains of the EGF receptor may be accompanied by constitutive activation (see Downward et al., 1984; Prywes et al., 1986). It has been postulated that the constitutive release of a mitogenic growth factor like EGF can lead to uncontrolled proliferation and cell transformation. In fact, when normal fibroblasts were transfected with an expression vector which directed constitutive synthesis and release of EGF, the cells were phenotypically changed to transformed cells (Stern, et al., 1987).

Although in other experimental systems EGF is known to transduce its signal via the protein kinase C (pkC) pathway (Downward et al., 1985; Hunter et al., 1985), the data presented in this thesis showed transin mRNA was not induced by activators of pkC. EGF is known to activate other intracellular signals, however, which include altering the levels of intracellular Ca^{++}, pH, and phosphorylation (Moolenaar et al., 1983, 1984; Hesketh et al., 1985; Muldoon et al., 1988b). It is unknown whether transin induction by EGF involves any one of these early membrane events. Moreover, the observation reported here that induction of transin by EGF is dependent on the synthesis of other proteins coupled with the recent findings that transin induction by EGF did not involve activation of c-*fos* (Kerr et al., 1988) suggest that activation by EGF may involve some other, as yet unidentified secondary messenger(s).

In Manuscript 1, we showed that TGFβ modulated the EGF induction of transin at the transcriptional level (Machida et al., 1988). This observation, however, does not necessarily mean TGFβ directly modulates transcription of the transin gene. In fact, the

effects of TGFβ may be at any one of a number of pre-transcriptional levels, including inhibition at the level of the receptor (see Massague 1985), inhibition of the primary intracellular signal induced by EGF (see Muldoon et al., 1988a;b), or inhibition of induction of the intermediary protein(s). In fact, inhibition at any of these pre-transcriptional levels may explain the more global effects of TGFβ in inhibiting the action of EGF (Roberts et al., 1985; Chiang et al., 1986; Whitsett et al., 1987). The influence of TGFβ as a negative effector may be altered during the transformation process of normal tissues into metastatic cells, permitting the constitutive expression of this protease.

Studies on the regulation of transin by growth factors in fibroblasts and its possible role in metastasis as well as other pathological conditions are being conducted in many laboratories. For example, the reciprocal interactions between EGF and TGFβ at levels preceding transcription, in particular, early membrane-associated events such as calcium influx, are being continued in the laboratory of B. Magun (Oregon Health Sciences University). Identification of *cis*-acting elements and *trans*-acting factors that may influence the level of transin transcription in fibroblasts as well as their mechanism of interaction are currently being investigated in the laboratories of B. Magun and L. Matrisian (Vanderbilt University). Finally, the possible functional role of transin in neoplasia [R. Breathnach (Institute de Chimie Biologique, Strasbourg, FR.) and T. Bowden (University of Arizona)] and in rheumatoid arthritis [Z. Werb (University of California, San Francisco) and H. Ruley (Washington University) are also being investigated.

In Manuscript 2, I reported that NGF induced transin mRNA at least 100-fold in PC12 cells, making transin one of the most highly NGF-responsive gene products described (Leonard et al., 1987; Federoff et al., 1988; Stein et al., 1988). Transin is therefore, a potentially useful gene for studying the molecular mechanisms underlying NGF action. In fact, the paucity of putative NGF second messengers may be due to the lack of suitable NGF-inducible genes which do not have high basal levels.

One direction, therefore, that this research might be continued would be to

address the question of what are the secondary messengers of the NGF signal transduction pathway? This thesis provided some possible clues. For example, NGF and FGFs, but not EGF or TPA were capable of inducing transin mRNA (See Manuscript 2, Figure 5). All four of these agents, however, induce transcriptional activation of the *fos* gene in PC12 cells or other systems (Gilman et al., 1986; Prywes and Roeder, 1986; Treisman, 1986; Greenberg et al., 1987; Sheng et al., 1988; Norman et al., 1988; Visvader et al., 1988). These data suggest that fos may be necessary but not sufficient for the induction of transin in PC12 cells. Moreover, the inability of either dibutyryl cyclic AMP or TPA to induce transin mRNA expression (Manuscript 2, Figure 5) suggests that activation of transin may involve pathways besides adenylate cyclase or protein kinase C pathways. Alternatively, the combination of several second messenger pathways could be involved in transmitting the NGF message to the nucleus. This is supported by the observation that changes in ion fluxes and activation of protein kinase C have been associated with fos induction and/or neurite outgrowth (Schubert et al., 1978; Traynor, 1984; Kruiger et al., 1985; Greenberg et al., 1985; Morgan and Curran, 1986; Blackshear et al., 1987; Hall et al., 1988). Clearly, elucidation of NGF second messenger pathway(s) for transin induction will need to address the following points:

1) Whether the AP1 *cis*-acting sequences are necessary for NGF induction of transin expression and neurite outgrowth.
2) Whether transin induction by NGF is dependent on the presence of fos.
3) Whether other factors besides fos are necessary for transin expression by NGF.

The products of the so-called "late genes" are believed to be associated with the acquisition of the neuronal phenotype (see Section IA-3; for reviews see Greene et al., 1985; Levi et al., 1988). There are several lines of evidence suggesting transin is a "late" NGF-inducible gene. First, the time-course of induction showed transin was induced on the order of hours and not minutes. Second, the time-course also showed that the duration of transin mRNA induction continued for several days in contrast to the transiently

induced "early" genes. Third, transin induction by NGF required the synthesis of other protein(s). Induction of early NGF-induced genes such as c-*fos* and c-*myc* is not blocked by protein synthesis inhibitors (see Green et al., 1985). Thus, characterization of transin mRNA expression by NGF suggest it is a late-induced gene involved in acquiring the neuronal phenotype.

There are three additional lines of evidence to suggest that transin expression may be involved in the process of axon elongation in NGF-treated PC12 cells: (1) The time course of expression of transin mRNA following addition of NGF was coincident with the morphological appearance of neurites. (2) The time course of transin mRNA expression was similar to the expression of a biochemical marker for neurite outgrowth, NF-M. (3) Fibroblast growth factors also induced both neurites and transin mRNA, but various other growth factors and agents do not. This latter observation indicates that the induction of transin is not simply an NGF effect, but is associated with the initiation and production of neurites.

The identification of transin as the rat homologue of stromelysin (Muller et al., 1988) suggests that transin may be involved in the degradation of various extracellular matrix components including, including proteoglycans, fibronectin, laminin and elastin (Chin et al., 1985). Several lines of evidence suggest the basement membrane glycoprotein laminin, may be a likely substrate for transin in the nervous system. First, laminin is produced by the Schwann cells surrounding axons of the peripheral nervous system (Cornbrooks et al., 1983; McGarvey et al., 1984). Second, although absent from adult brain glia, laminin is transiently produced by astrocytes both in culture (Liesi et al., 1983) and in vivo after brain injury (Liesi et al., 1984a). Third, when presented as a substrate molecule, laminin promotes neurite outgrowth of both peripheral and central neurons (Rogers et al., 1983; Manthrope et al., 1984; Liesi et al., 1984b; Edgar et al., 1984). Fourth, laminin appears in immature brain cells during CNS development, and its presence coincides with phases of neuronal migration (Liesi, 1985). Although laminin

may be a likely candidate for degradation by transin, other extracellular matrix components such as fibronectin, proteoglycans and elastin may also serve as substrates for transin. In any case, these observations indicate that proteolysis of laminin and other extracellular matrix molecules by transin may play a role in normal rat nervous system development.

NGF and its receptor are expressed throughout development in both peripheral and central nervous systems (Whittemore et al., 1986;1988; Enfors et al., 1988). Recent evidence suggest, however, it may not be the only growth factor involved in axon elongation. In fact, the observation that NGF synthesis does not precede the advent of the first trigeminal nerve fibers in the target area together with the fact that these fibers do not yet express NGF receptors (Davies et al., 1987) is unambiguous evidence that NGF does not act as a chemotactic agent guiding the trigeminal sensory fibers to their targets. It is plausible, however, that the outgrowth of these fibers may be induced by other growth factors such as FGF (see Walicke et al., 1986; Rydel and Greene, 1987; Morrison, et al., 1986;1987), EGF (see Fallon et al., 1984; Morrison et al., 1987) or other polypeptide factors (see Risau, 1986; Wagner and D'Amore, 1986), all of which may mimic NGF in its ability to induce genes involved in the acquisition of the neuronal phenotype. FGFs, for example, induced both neurite outgrowth and transin mRNA expression in PC12 cells (see Figure 5, Manuscript 2).

Clearly, additional work is required in order to determine whether the expression and release of transin is directly involved in the process of axon elongation. Such future endeavors will aim:

1) to determine where the transin protein is localized and released in PC12 cells or other neuronal cell model systems.
2) to determine whether experimental manipulations of transin levels influence axon outgrowth in neuronal cells
3) to determine when and where during normal nervous system development transin

is expressed.

4) to determine what may be the endogenous substrate(s) of transin in the nervous system.

REFERENCES

Blackshear, P.J., Stumpo, D.J., Huang, J-K., Nemenoff, R.A. and Spach, D.H. (1987). Protein kinase C-dependent and -independent pathways of proto-oncogene induction in human astrocytoma cells. J. Biol. Chem. *262*, 7774-7781.

Chiang, C-P. and Nilsen-Hamilton, M. (1986). Opposite and selective effects of epidermal growth factor and human platelet transforming growth factor-β on the production of secreted proteins by murine 3T3 cells and human fibroblasts. J. Biol. Chem. *261*, 10478-10481.

Cornbrooks, C.J., Carey, D.J., McDonald, J.A., Timpl, R. and Bunge, R.P. (1983). In vivo and in vitro observations on laminin production by Schwann cells. Proc. Natl. Acad. Sci. USA *80*, 3850-3854

Davies, A.M., Bandtlow, C., Heumann, R., Korsching, S., Rohrer, H. and Thoenen, H. (1987). Timing and site of nerve growth factor synthesis in developing skin in relation to innervaton and expression of the receptor. Nature *326*, 353-358.

Downward, J., Waterfield, M.D. and Parker, P.J. (1985). Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor. Effect on tyrosine kinase activity and ligand binding affinity. J. Biol. Chem. *260*, 14538-14546.

Enfors, P., Hallbook, F., Ebendal, T., Shooter, E.M., Radeke, M.J., Misko, T.P. and Persson, H. (1988). Developmental and regional expression of β-nerve growth factor receptor mRNA in the chick and rat. Neuron *1*, 983-996.

Fallon, J.H., Seroogy, K.B., Loughlin, S.E., Morrison, R.S., Bradshaw, R.A., Knauer, D.J.

and Cunningham, D.D. (1984). Epidermal growth factor immunoreactive material in the central nervous system: Location and development. Science *224*, 1107-1109.

Frisch, S.M. and Ruley, H.E. (1987). Transcription from the stromelysin promoter is induced by interleukin-1 and repressed by dexamethasone. J. Biol. Chem. *262*, 16300-16304.

Gilman, M.Z., R.N. Wilson and Weinberg, R.A. (1986). Multiple protein-binding sites in the 5'-flanking region regulate c-*fos* expression. Mol. Cell. Biol. *6*, 4305-4316.

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

Greenberg, M.E., Siegfried, Z. and Ziff, E.B. (1987). Mutation of the c-*fos* gene dyad symmetry element inhibits serum inducibility of transcription in vivo and the nuclear regulatory factor binding in vitro. Mol. Cell. Biol. *7*, 1217-1225.

Greene, L.A., Aletta, J.M., Burstein, D.E., Drexler, S.A., Greenberg, M.E., Leonard, D. and Ziff, E.B. (1985). Gene regulation by nerve growth factor. In: Neurobiochemistry. (B. Hamprecht and V. Neuhoff, eds.). Springer Verlan, New York, pgs. 1-6.

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

Hanley, M.R. (1988). Proto-oncogenes in the nervous system. Neuron *1*, 175-182.

Hesketh, T.R., Moore, J.P., Morris, J.D.H., Taylor, M.V., Rogers, J., Smith, G.A. and Metcalfe, J.C. (1985). A common sequence of calcium and pH signals in the mitogenic stimulation of eukaryotic cells. Nature *313*, 481-484.

Hunter, T., Ling, N. and Cooper, J.A. (1985). Protein kinase C phosphorylation of the EGF receptor at a threonine reside close to the cytoplasmic face of the plasma membrane. Nature *311*, 480-483.

Kerr, L.D., Holt, J.T. and matrisian, L.M. (1988). Growth factors regulate transin gene expression by c-*fos*-dependent and c-*fos*-independent pathways. Science *242*, 1424-1427.

Kruijer, W., Shubert, D. and Verma, M. (1985). Induction of proto-oncogene *fos* by nerve growth factor. Proc. Natl. Acad. Sci. USA *82*, 7330-7334.

Levi, A., Biocca, S., Cattaneo, A. and Calissano, P. (1988). The mode of action of nerve growth factor in PC12 cells. Molec. Neurobiol. *2*, 201-226.

Liesi, P. (1985). Do neurons in the vertebrate CNS migrate on laminin? EMBO J. *4*, 1163-1170.

Liesi, P. Dahl, D. and Vaheri, A. (1983). Laminin is produced by early rat astrocytes in primary culture. J. Cell Biol. *96*, 920-924.

Liesi, P., Kaakkola, S., Dahl, D. and Vaheri, A. (1984a). Laminin is induced in astrocytes of adult brain by injury. EMBO J. *3*, 683-686.

Liesi, P., Dahl, D. and Vaheri, A. (1984b). Neurons cultured from developing rat brain attach and spread preferentially to laminin. J. Neurosci. Res. *11*, 241-251.

McGarvey, M.L., Baron-Van Evercooren, A., Kleinman, H.K. and Dubois-Daleq, M. (1984). Synthesis and effects of basement membrane components in cultured rat Schwann cells. Dev. Biol. *105*, 18-28.

Manthrope, M, Engvall, E., Ruoslahti, E., Longo, F.M., Davis, G.E. and Varon, S. (1983). Laminin promotes neuritic regeneration from cultured peripheral and central neurons. J. Cell Biol. *97*, 1882-1890.

Massague, J. (1985). Transforming growth factor-β modulates the high-affinity receptors for epidermal growth factor and transforming growth factor-α. J. Cell Biol. *100*, 1508-1514.

Matrisian, L.M., Glaichenhaus, N., Gensel, M-C. and Breathnach, R. (1985). Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. EMBO J. *4*, 1435-1440.

Matrisian, L.M. Bowden, G.T., Krieg, P., Furstenberger, G., Briand, J.-P., Leroy, P. and Breathnach, R. (1986a). The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. Proc. Natl. Acad. Sci. USA *83*, 9413-9417.

Matrisian, L.M., Leroy, P., Ruhlmann, C., Gesnel, M.-C. and Breathnach, R. (1986b). Isolation of the oncogene and epidermal growth factor-induced transin gene: Complex control in rat fibroblasts. Mol. Cel. Biol. *6*, 1679-1686.

Moolenaar, W.H., Tsien, R.Y., van der Saag, P.T. and de Laat, S.W. (1983). Na^+/H^+ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. Nature. *304*, 645-648.

Moolenaar, W.H., Tertoolen, L.G.J. and deLaat, S.W. (1984). Growth factors immediately raise cytoplasmic free Ca^{2+} in human fibroblasts. J. Biol. Chem. *259*, 8066-8069.

Morgan, J.I. and Curran, T. (1986). Role of ion flux in the control of c-fos expression. Nature *322*, 552-555.

Morrison, R.S., Sharma, A., deVellis, J. and Bradshaw, R.A. (1986). Basic fibroblast growth factor supports the survival of cerebral corticol neurons in primary culture. Proc. Natl. Acad. Sci. USA *83*, 7537-7541.

Morrison, R.S. (1987). Fibroblast growth factors: Potential neurotrophic agents in the central nervous system. J. Neurosci. Res. *17*, 99-101.

Morrison, R.S., Kornblum, H.I., Leslie, F.M. and Bradshaw, R.A. (1987). Trophic stimulation of cultured neurons from neonatal rat brain by epidermal growth factor. Science *238*, 72-75.

Muldoon, L.L., Rodland, K.D. and Magun, B.E. (1988a). Transforming growth factor β modulates epidermal growth factor-induced phosphoinositide metabolism and intracellular calcium levels. J. Biol. Chem. *263*, 5050-5033.

Muldoon, L.L., Rodland, K.D. and Magun, B.E. (1988b). Transforming growth factor β

and epidermal growth factor alter calcium influx and phosphatidylinositol turnover in Rat-1 fibroblasts. J. Biol. Chem. *263*, 18834-18841.

Muller, D., Quantin, B., Gesnel., M-C., Millon-Collard, R., Abecassis, J. and Breathnach, R. (1988). The collagenase gene family in humans consists of at least four members. Biochem. J. *253*, 187-192.

Norman, C., Runswick,M., Pollack, R. and Treisman, R. (1988). Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-*fos* serum response element. Cell *55*, 989-1003.

Ostrowski, L.E., Finch, J., Krieg, P., Matrisian, L., Patskan, G., O'Connell, J.F., Phillips, J., Slaga, T.J., Breathnach, R. and Bowden, G.T. (1988). Expression pattern of a gene for a secreted metalloproteinase during late stages of tumor progression. Molec. Carc. *1*, 13-19.

Prywes, R., Livneh, E., Ullrich, A. and Schlessinger, J. (1986). Mutations in the cytoplasmic domain of EGF receptor affect EGF binding and receptor internalization. EMBO J. *5*, 2179-2190.

Prywes, R. and Roeder, G. (1986). Inducible binding of a factor of the c-*fos* enhancer. Cell *47*, 777-784.

Risau, W. (1986). Developing brain produces an angiogenesis factor. Proc. Natl. Acad. Sci. USA *83*, 3855-3859.

Roberts, A.B., Anzano, M.A., Wakefield, L.M., Roche, N.S., Stern, D.F. and Sporn, M.B.

(1985). Type β transforming growth factor: A bifunctional regulator of cellular growth. Proc. Natl. Acad. Sci. USA *82*, 119-123.

Rogers, S.L. Letourneau, P.C., Palm, S.L., McCarthy, J. and Furcht, L.T. (1983). Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. Dev. Biol. *98*, 212-220.

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. Neurosci. *7*, 3639-3653.

Sanes, J.R. (1982). Laminin, fibronectin, and collagen in synaptic and extra-synaptic portions of muscle fiber basement membrane. J. Cell Biol. *93*, 442-451.

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

Sheng, M., Dougan, S.T., McFadden, G. and Greenberg, M.E. (1988). Calcium and growth factor pathways of c-*fos* transcriptional activation require distinct upstream regulatory sequences. Mol. Cell. Biol. *8*, 2787-2796.

Stern, D.F., Hare, D.L., Cecchini, M.A. and Weinberg, R.A. (1987). Construction of a novel oncogene based on synthetic sequences encoding epidermal growth factor. Science *235*, 321-324.

Traynor, A.E. (1984). The relationship between neurite extension and phospholipid metabolism in PC12 cells. Dev. Brain Res. *14*, 205-210.

Treisman, R. (1986). Identification of a protein-binding site that mdiates transcriptional response of the c-*fos* gene to serum factors. Cell *46*, 567-574.

Visvader, J., Sassone-Corsi, P. and Verma, I.M. (1988). Two adjacent promoter elements mediate nerve growth factor activation of the c-*fos* gene and bind distinct nuclear complexes. Proc. Natl. Acad. Sci. USA *85*, 9474-9478.

Wagner, J.A. and D'Amore, P.A. (1986). Neurite outgrowth induced by an endothelial cell mitogen isolated from retina. J. Cell Biol. *103*, 1363-1367.

Walicke, P., Cowan, W.M., Ueno, N., Baird, A. and Guillemin, R. (1986). Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. Proc. Natl. Acad. Sci. USA *83*, 3012-3016.

Whitsett, J.A., Weaver, T.E., Lieberman, M.A., Clark, J.C. and Daugherty, C. (1987). Differential effects of epidermal growth factor and transforming growth factor-β on synthesis of M_r=35,000 surfactant-associated protein in fetal lung. J. Biol. Chem. 262, 7908-7913.

Whittemore, S.R., Ebendal, T., Larkfors, L., Olson, L., Seiger, A., Stromberg, I., Persson, H. (1986). Developmental and regional expression of nerve growth factor messenger RNA and protein in the rat central nervous system. Proc. Natl. Acad. Sci. USA *83*, 817-821.

Whittemore, S.R., Persson, H., Ebendal, T., Larkfors, L., Larhammar, D. and Ericsson, A. (1988). Structure and expression of β-nerve growth factor in the rat central nervous system. In: Neural Development and Regeneration. Springer-Verlag, Berlin, pp.245-256.

IV. APPENDIX

A number of experiments were conducted in addition to those reported in the manuscripts. These experiments dealt with peripheral issues surrounding the regulation of transin and are reported in this Appendix. They include (1) cloning of the mouse transin gene, (2) establishing culture conditions for PC12 cells, and (3) characterizing the induction of neuronal intermediate filament (NF-M), a cytoskeletal component of neurites.

A. Cloning of the mouse transin gene

Using the rat transin cDNA probe, pTR1 (Matrisian et al., 1985), I screened a mouse Balb/C genomic library prepared in Charon 4A by utilizing standard techniques (Maniatis et al., 1982). Seven positive clones were identified from 2 x 10^5 recombinant clones after three rounds of screening. One of these clones has subsequently been subcloned by members in Dr. B. Magun's laboratory. The original 18 kb insert yielded a single strongly hybridizing band of 10 kb when cut with EcoRI. Most of the bands obtained from restriction digest of the insert using a variety of "six-cutters" (PstI, BamHI, BglII and HindIII) hybridized to the transin cDNA probe. This suggest that the coding sequences for mouse transin occupy approximately 10 kb of genomic DNA. Recently, 150 bp of the promoter region of this mouse genomic clone has been sequenced and found to contain a CAAT box and TATA box sequences. Isolation of this mouse transin gene permits further study of the mechanism of transin regulation by growth factors in numerous normal and transformed mouse cell-lines that are widely available.

B. Establishment of the cell culture conditions for PC12 cells

The wide use of serum-containing medium for NGF studies of the neuronal differentiation of PC12 has several disadvantages. First, the presence of serum can cause aggregation of the cells, thereby, making morphological analysis of neurite outgrowth difficult, if not impossible. Second, the complete neuronal differentiation of these cells is delayed by serum for as much as 2 weeks. This delay make such studies costly and time-

consuming. Third, the undefined nature of serum complicates the assessment of the effect(s) of regulatory agents. Furthermore, these unknown factors in serum may vary between different batches depending on the donor age, sex, nutrition, and physiological state and may affect reproducibility of the experiments.

For these reasons, I screened a variety of media on the basis of whether they would support the survival of PC12 cells and permit the cells to extend neurites in response to NGF. I found that the defined medium N2, originally established for growth of a rat neuroblastoma cell line (Bottenstein and Sato, 1979) was found to be the medium of choice based on these criteria. The basal medium of N2 consists of a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium and contains the following additives: insulin (5 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (100 μM), selenium (30 nM), $NaHCO_3$ (1.2 g/l), Hepes buffer (15 mM), penicillin (40 mg/l), ampicillin (8 mg/l), and streptomycin (90 mg/l).

In order to determine the effects of serum and defined medium on the aggregation of PC12 cells, the cells were plated at the same densities on laminin coated (2 μg/ml) Primaria plates and allowed to grow overnight in L15 basal medium containing 5% horse serum and 5% fetal bovine serum. The medium was washed several times and then changed to N2 medium with or without serum for 24 hours. NGF was then added to the cultures and fresh medium and additives were changed every 2-3 days for this and subsequent experiments. Figure 1 shows photomicrographs of the cells after 4 days in culture. Although neurites were visible from NGF-treated cells in the presence of serum (second panel, first row), the cells were highly aggregated. Such clumping prevented the accurate assessment of the number of cells containing neurites. The bottom row shows PC12 cells grown in N2 without serum in the presence or absence of NGF. The individual cells in this serum-free defined media are clearly distinquishable and the cells treated with NGF have neurites that are easily counted.

In order to test the effects of serum on transin mRNA expression, PC12 cells were

plated as described above and the medium changed to L15 serum-containing medium or N2 medium in the presence or absence of serum and NGF. The cells were harvested after 8 days and prepared for RNA blotting analysis. Figure 2 shows transin RNA was expressed in all cells treated with NGF (lanes 3,5,7) whether or not serum was present. However, the extent of transin expression induced by NGF was much greater in cells grown in defined medium (compare lane 7 with lane 5). A factor(s) in serum, therefore, appears to suppress the expression of transin RNA induced by NGF.

Figure 3 are photomicrographs of PC12 cells grown in N2 defined medium over various periods of time following NGF treatment. These are representative pictures of the ones used to generate the data in Figure 3, Manuscript 2. Clearly, neurites are beginning to appear following 24 hrs of exposure to NGF. Note, that by 7 days, nearly all the cells express neurites. Figure 4 shows morphometric analysis of this data in terms of the number of neurites per cell over time (open boxes) and in terms of the number of neurites that cross a 1 μm^2 grid used as an index of neurite length (closed circles). These data correspond to the data in Figure 3, Manuscript 2, which were plotted as the number of cells with neurites over time in culture with NGF. Both neurite length and the number of neurites per cell increase linearly between 10 hours and 7 days following NGF treatment.

All of the studies reported in Manuscript 2 examined expression of transin mRNA. To determine whether the transin protein was also induced by NGF treatment and secreted into the medium, conditioned medium from NGF-treated and non-treated cells was collected, subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions, blotted onto nitrocellulose, and then probed with an antibody generated to a synthetic peptide corresponding to the carboxy-terminal portion of the predicted amino acid sequence of transin (Matrisian et al., 1986a). Figure 5 shows a single band at about 110,000 daltons in the lane containing non-concentrated conditioned medium from NGF-treated cells, but not in the lane from untreated cells. Since this molecular weight is approximately twice that of transin (i.e., about 53,000 daltons), transin may possibly exist as

a homodimer. Clearly, more rigorous reducing conditions are required to test this hypothesis.

C. Plasmid p750TRCAT

An expression vector was prepared in the laboratory of our collaborator, Dr. L. Matrisian (Vanderbilt University) and was used in the CAT expression studies (see Manuscript 2, Figure 9). The DNA sequence of the transin promoter region is shown in Figure 6a (Matrisian et al., 1986b). A 750 base pair sequence from the mRNA cap site (indicated by the star) was cloned in the p750TRCAT (Figure 6b). This region has both CAAT and TATA sequences (underlined) and was used in all the transient transfection studies. The 750 bp Taq I - Taq I fragment of the transin promoter (position -1 to -750) was subcloned into the Cla I site of pBR322 in which the Hind III site was replaced with a Bgl II linker. The promoter was then removed by restriction with Eco Ri and Bgl II, the Eco RI site blunt-ended, and the fragment inserted in Sm I- Bgl II - restricted pPFCAT containing the poly linker Hind III/Sal I/Sma I/Sac I/Bgl II/Kpn I inserted into the Hind III site of pSVOCAT. The resulting plasmid, p750TRCAT, thus contains 750 bp of the transin prmoter, CAT coding sequences, and SV40 splice and polyadenylation sites (Figure 6b).

D. Induction of NF-M RNA by grwoth factors and hormones.

Neurofilaments are the intermediate filament subclass expressed specifically in neurons (Shaw and Weber, 1982; Trojanowski et al., 1986; Lee et al., 1987). Mammalian neurofilaments are composed of three subunits having apparent molecular weights of 70 kDa (NF-L), 150 kDa (NF-M), and 200 kDa (NF-H) (Hoffman and Lasek, 1975). Neurofilament proteins can be detected in cells with an identifiable neuronal phenotype at early stages of embryogenesis (Tapscott et al., 1981; Cochard and Paulin , 1984). NF-M mRNA can be detected in developing mouse brains as early as 11 days of gestation (Julien et al., 1986). These data suggest that neurofilaments are good markers of neuronal dif-

ferentiation. The induction of NF-M mRNA in PC12 cells by various growth factors and agents was studied in order to compare and contrast the expression of this neuronal marker with transin mRNA expression.

In order to determine which agents could induce or inhibit the expression of NF-M mRNA, PC12 cells were exposed to various growth factors or agents for 24 hours and then harvested for RNA analysis. The top panel in Figure 7 shows that NGF, but not TGF-β, hydrocortisone, or dibutyryl cyclic AMP (dbcAMP) was able to induce NF-M mRNA above basal levels. Hydrocortisone reduced the basal level of NF-M mRNA to about half (data not shown) while neither TGF-β nor dbcAMP had any effects. None of these agents had any effect on cyclophilin mRNA expression when this blot was stripped and reprobed with the labeled cDNA of this constitutive protein. Most of the agents had similar effects on transin mRNA expression (see Figure 5, Manuscript 2); however, the effects of hydrocortisone on transin mRNA expression were not assessed.

In order to determine the effects of the synthetic glucocorticoid, dexamethasone, on NF-M mRNA expression, PC12 cells were exposed for 24 or 48 hours in presence of NGF alone, NGF plus dexamethasone, or dexamethasone alone and then harvested for RNA analysis. As in Figure 7 above, NGF induced NF-M mRNA above basal levels at both time points (compare lanes a and b, or j and e of Figure 8). Dexamethasone inhibited the induction by NGF (compare lanes b and c, or e and f) while exposure to dexamethasone alone, lowered NF-M mRNA expression to below basal levels (compare lanes a and d, or j and g). Pretreatment of the PC12 cells with NGF for 24 hours, followed by a 24 or 48 hr exposure to both NGF and dexamethasone, also reduced NF-M mRNA levels (e.g. compare lanes e and h). The lower panel in Figure 8 shows dexamethasone had no effect on the levels of cyclophilin mRNA expression.

As noted in Manuscript 2, dexamethasone did not seem to inhibit the NGF-induced morphological differentiation of PC12 cells -- an observation that has been noted by others (Stein et al., 1988; Federoff et al., 1988). This observation conflicts with the

notion that that NF-M is a necessary cytoskeletal component of growing neurites (see Drager and Hofbauer, 1984; Cargen et al., 1987) and suggests that it is not involved in the process of neurite extension. Alternatively, the inability of dexamethasone to inhibit the NGF-induced neurites of PC12 cells may be an anomaly of this tumor cell line and may not reflect the inhibitory effects of glucocorticoids observed in normal chromaffin cells (Unsicker et al., 1978; Doupe et al., 1985). The inhibition of NF-M mRNA expression by dexamethasone, and the lack of inhibition of neurite outgrowth parallels the the similar studies of transin mRNA expression (see Figure 7, Manuscript 2). Thus, the lack of association between transin expression and neurite outgrowth does not necessarily mean the two phenomena are not linked because NF-M, a known component of neurites, followed a similar expression pattern.

REFERENCES

Bottenstein, J.E. and Sato, G.H. (1979). Growth of a neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. USA *76*, 514-417.

Carden M.J., Trojanowski, J.Q., Schlaepfer, W.W. and Lee, V.M-Y. (1987). Two-stage expression of neurofilament polypeptides during rat neurogenesis of early establishment of adult phosphorylation pattern. J. Neurosci. *7*, 3489-3504.

Cochard, P. and Paulin, D. (1984). Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo in vivo. J. Neurosci. *4*, 2080-2094.

Doupe, A.J., Landis, S.C. and Patterson, P.H. (1985). Environmental influences in the development of neural crest derivatives: Glucocorticoids, growth factors and chromaffin cell plasticity. J. Neurosci. *5*, 2119-2142.

Drager, U.C. and Hofbauer, A. (1984). Antibodies to heavy neurofilament subunit detect a subpopulation of damaged ganglion cells in retina. Nature *304*, 624-626.

Federoff, H.J., Grabczyk, E. and Fishman, M.C. (1988). Dual regulation of GAP-43 gene expression by nerve growth factor and glucocorticoids. J. Biol. Chem. *263*, 19290-19295.

Hoffman, P.N. and Lasek, R. J. (1975). The slow component of axonal transport. Identification of major structural polypeptide of the axon and their generality among mammalian neurons. J. Cell Biol. *66*, 351-366.

Julien, J.-P., Meyer, D., Flavell, D., Hurst, J. and Grosveld, F. (1986). Cloning and

developmental expression of the murine neurofilament gene family. Molec. Brain Res. *1*, 243-250.

Lee, V.M.-Y., Carden, M.J., Schlaepfer, W.W. and Trojanowski, J.Q. (1987). Monoclonal antibodies distinguish several differentailly phosphorylated states of the two largest rat neurofilament subunits (NF-H and NF-M) and demonstrate their existence in the normal nervous system of adult rats. J. Neurosci. *7*, 3474-3483.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). The identification of recombinant clones. In: Molecular Cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, pp. 309-361.

Matrisian, L.M., Glaichenhaus, N., Gesnel, M-C. and Breathnach, R. (1985). Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. EMBO J. *4*, 1435-1440.

Matrisian, L.M., Bowden, G.T., Krieg, P., Furstenberger, G., Briand, J-P., Leroy, P. and Breathnach, R. (1986a). The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. Proc. Natl. Acad. Sci. USA *83*, 9413-9417.

Matrisian, L.M., Leroy, P., Ruhlmann, C., Gesnel, M-C. and Breathnach, R. (1986b). Isolation of the oncogene and epidermal growth factor-induced transin gene: Complex control in rat fibroblasts. Molec. Cell. Biol. *6*, 1679-1686.

Shaw, G. and Weber, K. (1982). Differential expression of neurofilament triplet proteins in brain development. Nature *298*, 277-279.

Stein, R., Orit, S. and Anderson, D.J. (1988). The induction of a neural-specific gene, SCG10, by nerve growth factor in PC12 cells is transcriptional, protein synthesis dependent, and glucocorticoid inhibitable. Dev. Biol. *127*, 316-325.

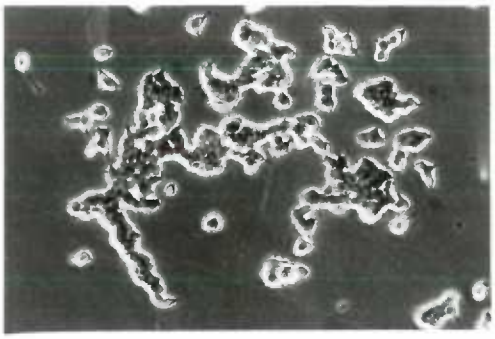
Tapscott, S.J., Bennett, G.S. and Holtzer, H. (1981). Neuronal precursor cells in the chick neural tube express neurofilament proteins. Nature *292*, 836-838.

Trojanowski, J.Q., Walkenstein, N. and Lee, V.M.-Y. (1986). Expression of neurofilament subunits in neurons of the central and peripheral nervous system: An immunohistochemical study with monoclonal antibodies. J. Neurosci. *6*, 650-660.

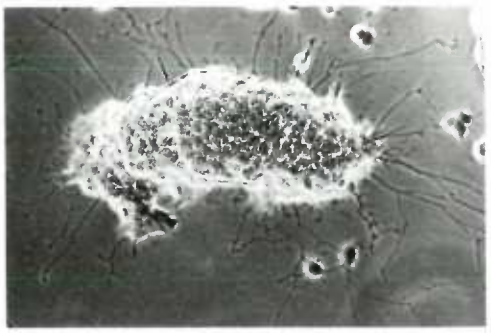
Unsicker, K., Krisch, B., Otten, U. and Thoenen, H. (1978). Nerve growth factor-induced fiber outgrowth from isolated rat adrenal chromaffin cells: Impairment by glucocorticoids. Proc. Natl. Acad. Sci. USA *75*, 3498-3502.

Figure 1: *Morphology of NGF-treated PC12 cells grown in the presence or absence of serum.*

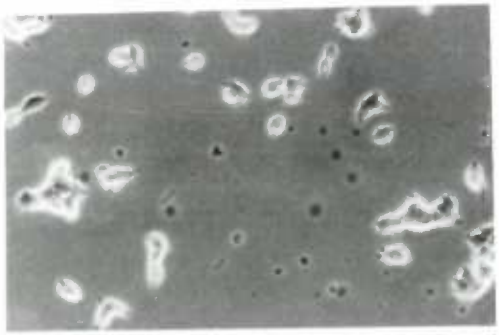
PC12 cells were grown in N2 medium in the presence or absence of 5% horse serum and 5% bovine serum and treated with or without NGF for 4 days at which time these photomicrographs were taken. Note that although both NGF-treated cultures expressed neurites at this time, the cultures containing serum had large aggregates of cells.



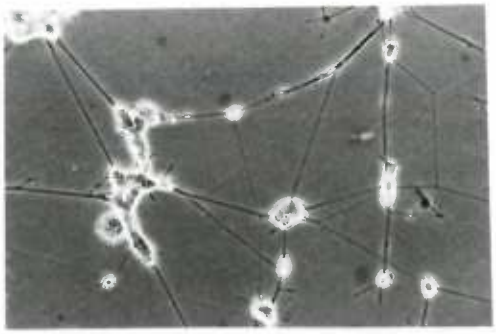
Control, + Serum



NGF, + Serum

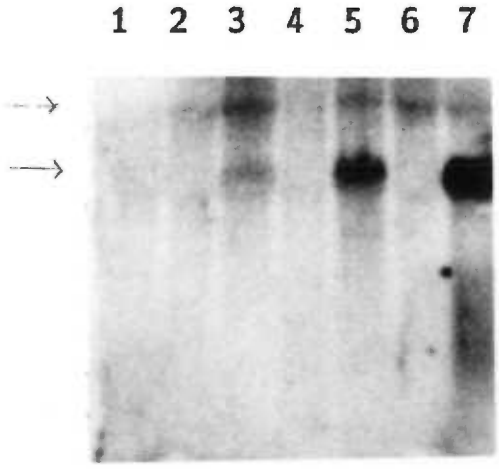


Control, - Serum



NGF, - Serum

Figure 2: *RNA blot showing the effect of serum on transin mRNA induction.* PC12 cells grown in L15 serum-containing medium (lanes 2 and 3), N2 serum-containing medium (lanes 4 and 5) or N2 medium without serum (lanes 6 and 7) were treated in the absence (lanes 2,4,6) or presence of 50 ng/ml NGF (lanes 3,5,7). All NGF treated cultures expressed transin mRNA but induction was more pronounced in the serum-free condition (lane 7).



1 0 d Control

2 L15-CO_2 Control

3 L15-CO_2 NGF

4 N_2 + Serum Control

5 N_2 + Serum NGF

6 N_2 Control

7 N_2 NGF

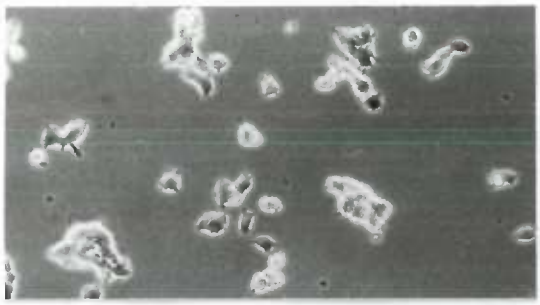
Cells harvested on day 8

Probe: rat transin

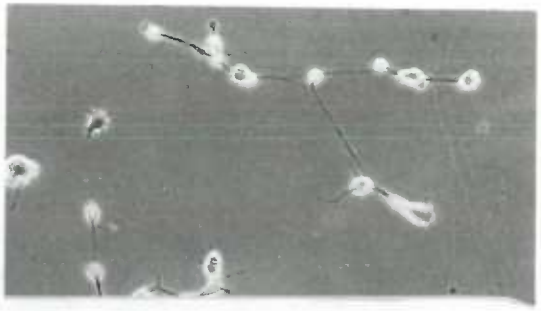
Figure 3: *Morphology of PC12 cells grown in N2 define medium in the presence of NGF for 7 days.*

PC12 cells grown in N2 define medium were photographed following various times of exposure to NGF. Fresh medium and NGF were added every 2-3 days. Note the absence of neurites in the untreated cells (control) while NGF-treated cells express increasing number of neurites over time.

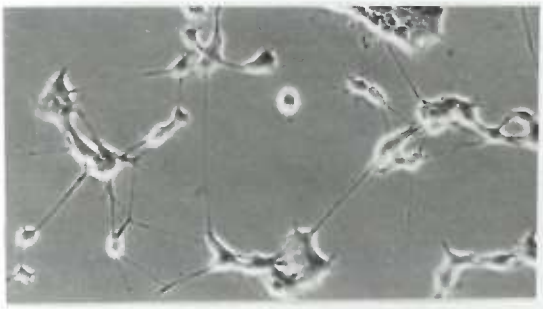
Control



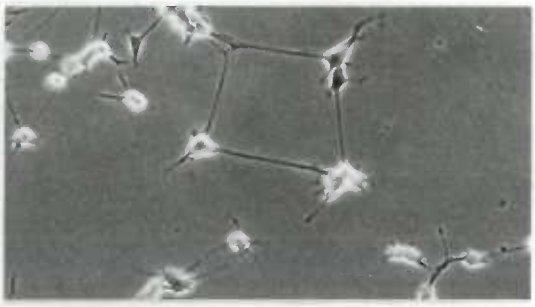
1 d NGF



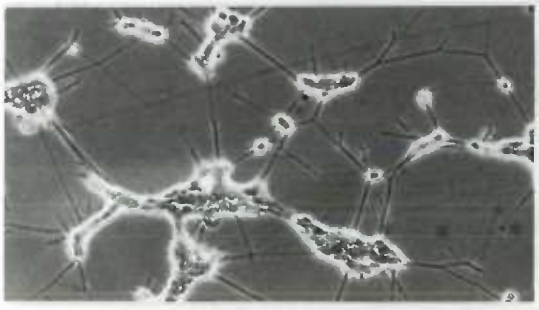
2 d NGF



3 d NGF



4 d NGF



7 d NGF

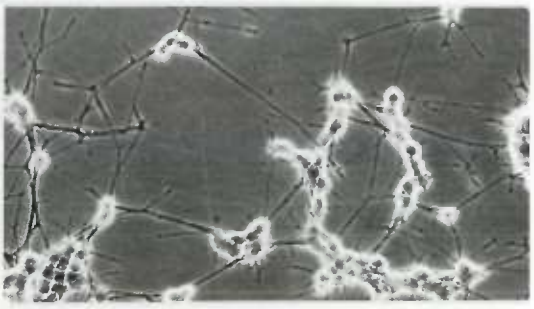


Figure 4: *Morphological analysis of PC12 cells treated with NGF.*

PC12 cells treated as described in Figure 3 were photographed ($n \geq 6$) and analyzed over time for the number of neurites per cell (open boxes) as well as the length of neurites (closed circles). A neurite was operationally defined as a cell process greater than two cell diameters (approximately 12 ϕm). Note that both the number of neurites per cell and length of neurites increase linearly from 10 hours to 7 days following exposure to NGF.

TIME COURSE OF INDUCTION OF NEURITES BY NGF

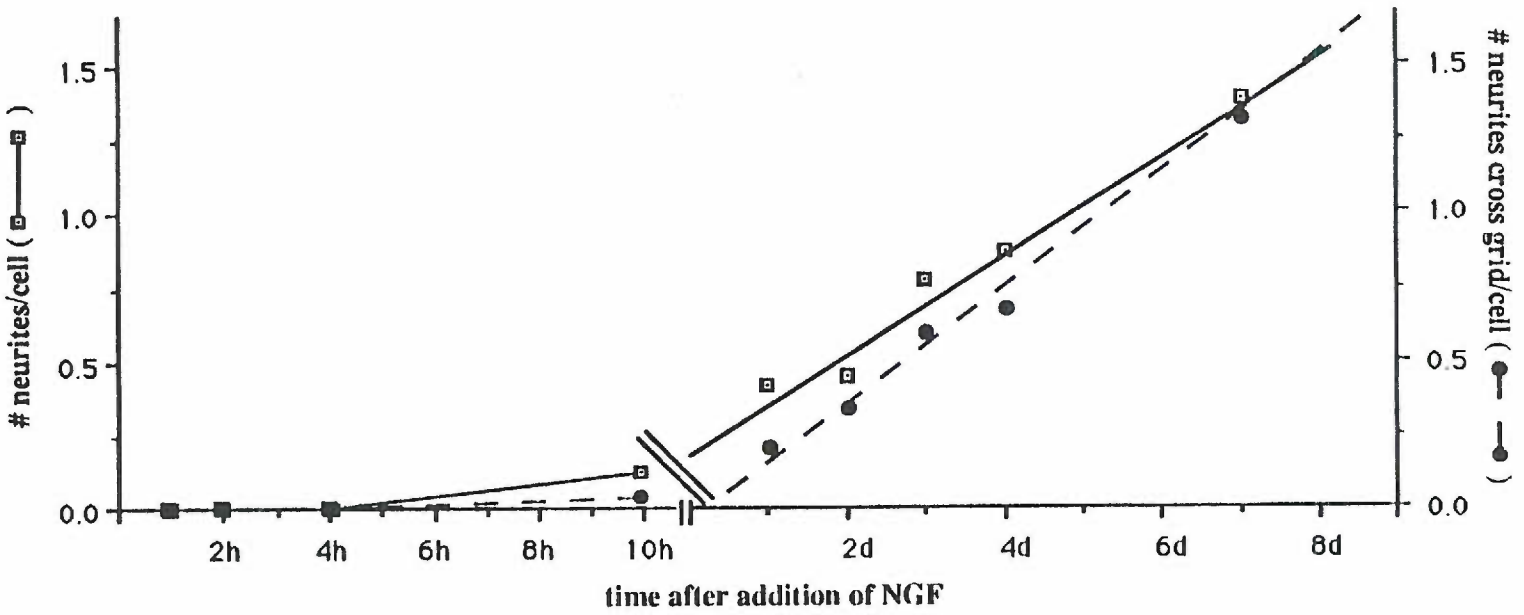


Figure 5: *Protein blot of conditioned medium from PC12 cells in the absence or presence of NGF.*

Conditioned medium (20 ϕl; non-concentrated) from PC12 cell cultures treated in the absence (C) or presence (N) of NGF was subjected to SDS-polyacrylamide gel electrophoresis and then blotted onto nitrocellulose filter paper. Following blocking with seru, the filters were incubated overnight with anti-transin antisera (1:100 dilution), washed, incubated for one hour with alkaline phosphatase conjugated anti-rabbit antibody, and then washed again. Phosphatase activity was then visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate (ProMega). Note the presence of a single band (110 kDa) of transin immunoreactive material in conditioned medium from NGF treated, but not from non-NGF-treated PC12 cells.

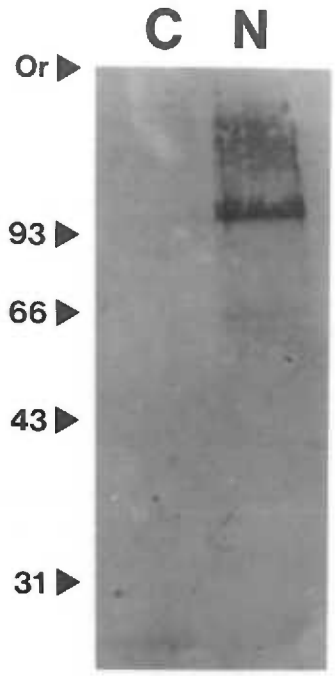

C
N
Or
93
66
43
31

Figure 6: *p750TRCAT expression vector containing 750 bp of the transin promoter region linked to the chloramphenicol acetyl transferase reporter gene.*

The promoter region of the rat transin gene was sequenced and reported by Matrisian et al (1986b). A 750 base pairs sequence proximal to the transcriptional start site of the transin promoter region containing CAAT and TATA elements (panal a) was cloned into the polycloning site of chloramphenicol acetyl transferase reporter vector, pSVOCAT.

The resulting plasmid, p750TRCAT (panel b), contained 750 base pairs of the transin promoter, CAT coding sequences and SV40 splice and polyadenylation sites and was used in the transfection studies described in Manuscript 2.

a

NUCLEIC ACID SEQUENCE OF THE TRANSIN PROMOTER REGION

-751

CGATGCCCCAGTGTATGAGAATGTTAGGGGCATGAGGTAGGAGTGAGTTG
GTGAGTGGGAGAGCATCCTCATAGAACCAGGGGAGGGAGGACTGGATAGG
GGGTTTGCAAAGGGAAAACCACAAAAGCGGATAACATTTGAAATGTAAAA
AAAAAAAAAAAATAACCAATAAAAAATATCATAAAATAAGAAAGACAAAT
CTGACAGAAAAGATATAAGATAAAACCAGGCCATTCTACTTCAGTAAATC
ATATCAATTATATGAGCCTTTATAGAAAAAACTATTATAGACCCATCTCC
TTTTAATATATGGGGTACAGAATGTGGGTCGTAGTGACAGACAGCGTGGG
CTGTGGCTACAGAGGCACACCTTGTCCTTACCTCATCTCACTGTATTCAG
CTTTGACTTCTGGAAGTTCTTTGTACAATTTGGACTTTTTACCAAGTTAG
GCCACTACTATCCAAGTCATAAACATTACAGCTTCTGAAGGATAGTTACA
TTTTCCAAAGTAGAAAAAAATGCCCCAGTTTTCTCTTTTGCTAAGGCAGG
AAGCATTTCCTGGAGATTAATCACCATTCGCTTTGCAAAATTAAGAAGGT
TTGAAGAACTGAGTAAAGAAGATTATATCACTCTTCTGATTTTTAATTTT

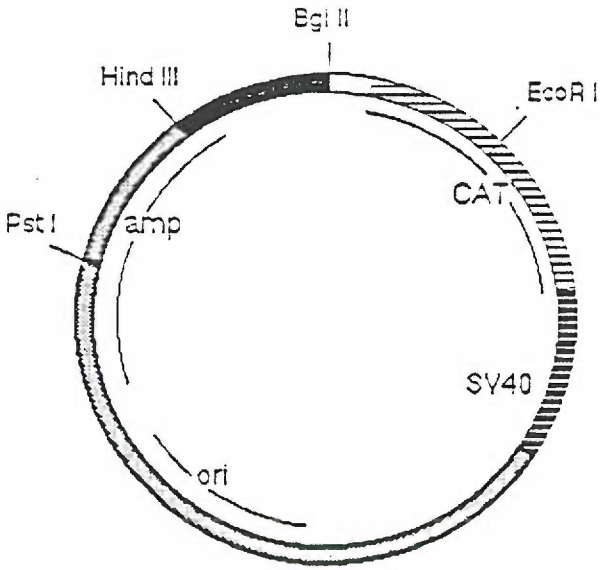
--AP1->

TGGAAATGGTCCCATTTGGATGGAAGCAATTATGAGTCAGTTTGCGGGTG

-1

ACTCTGCAAATACTGCCACTCTATAAAAGTTGGGCTCAGAAAGGTGGACCTCGA

b



transin promoter DNA

pBR322 DNA

Chloramphenicol acetyltransferase DNA (CAT)

SV40 DNA (with a polyadenylation site)

Figure 7: *RNA blot showing the effects of various growth factors and agents on NF-M mRNA expression.*

PC12 cells were grown in N2 medium and exposed for 24 hours to one of the following: NGF (50 ng/ml), TGF-β (10 ng/ml), hydrocortisone (HC)(1 nM), or dbcAMP (1 mM). RNA blots were then prepared and probed for NF-M or cyclophilin mRNA expression. Note that only NGF was able to induce NF-M mRNA while only hydrocortisone was able inhibit the basal expression of NF-M mRNA.

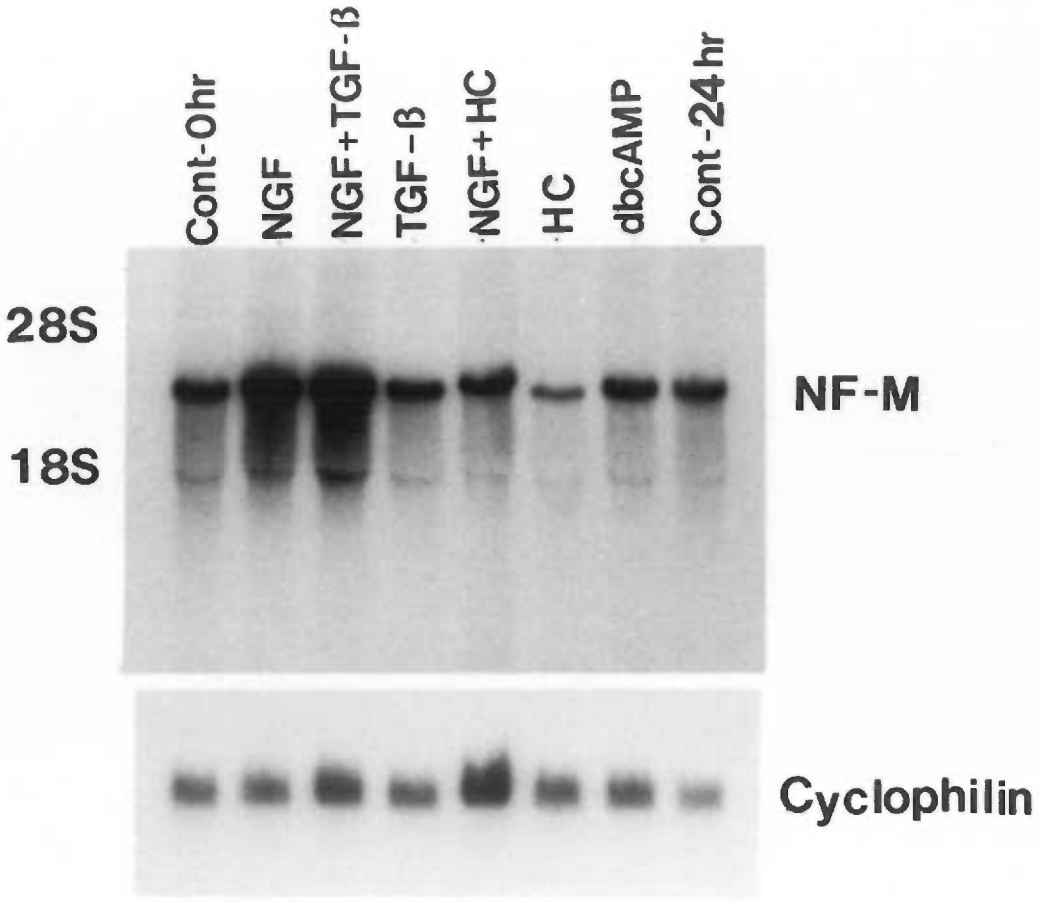
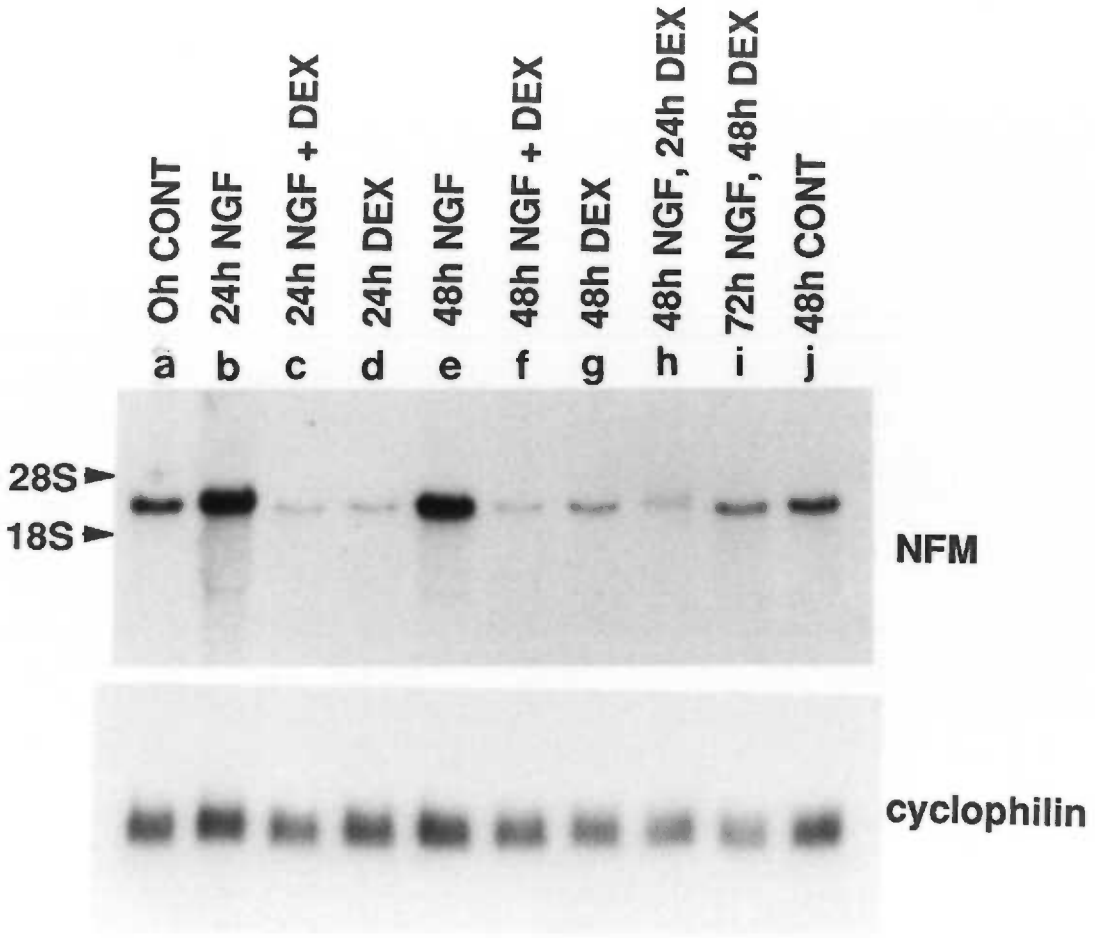

Cont-0hr
NGF
NGF+TGF-β
TGF-β
NGF+HC
HC
dbcAMP
Cont-24hr
28S
18S
NF-M
Cyclophilin

Figure 8: *RNA blot showing the effect of dexamethasone on NF-M mRNA induction by NGF.*

PC12 cells were treated with NGF alone (b,e); NGF and dexamethasone simultanesously (c,f); or dexamethasone (DEX) alone (d.g) for 24 or 48 hours. In the upper panel, note that dexamethasone inhibited the induction of transin by NGF at both time points (compare b with c, or e with f) but had no effect on transin expression by itself d,g). Also note that dexamethasone reduced the levels of NF-M mRNA when the cultures were pretreated for 24 hours with NGF (h,i). Reprobing the same blot with cyclophilin showed that similar amounts of RNA were present in all the lanes (lower panel).


a Oh CONT
b 24h NGF
c 24h NGF + DEX
d 24h DEX
e 48h NGF
f 48h NGF + DEX
g 48h DEX
h 48h NGF, 24h DEX
i 72h NGF, 48h DEX
j 48h CONT
28S
18S
NFM
cyclophilin