REGULATION OF STROMELYSIN-1 GENE EXPRESSION BY NERVE GROWTH FACTOR

by

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ABSTRACT

The neurotrophin nerve growth factor (NGF) is a target derived factor that is critical for the maintenance and survival of sensory and sympathetic neurons of the peripheral nervous system. An *in vitro* culture system, the rat pheochromocytoma PC12 cell line, has been widely used to study the effect of NGF on neuronal precursor cells. In response to NGF, PC12 cells differentiate into a sympathetic neuronal-like phenotype that is distinguishable morphologically by the appearance of neurites and is typified by the expression of several neuronal-specific genes. Several labs have investigated the regulation of these NGF-inducible genes in order to understand the molecular mechanisms underlying NGF's actions. One such gene, which is the primary focus of the work presented here, is the stromelysin-1 gene.

Stromelysin-1 (ST-1) is an extracellular matrix metalloproteinase whose mRNA levels increase several hundred fold in response to NGF in PC12 cells. Since this increase is due to to *de novo* transcription, we examined the proximal ST-1 promoter for *cis* regulatory elements that are responsive to NGF. We found that mutations in the AP1 sequence within this promoter strongly affected basal levels of ST-1 gene transcription but had no effect on NGF-responsiveness. However, 5' deletion mutational analysis revealed that sequences between -247 and -315 bp upstream of the transcriptional start site were necessary for NGF-responsiveness. Nuclear proteins from both untreated and NGF-treated PC12 cells were able to bind to this region in a specific and saturable manner. In addition, this region of the ST-1 promoter, when placed in the promoter of a gene that is not normally responsive to NGF, conferred NGF-inducibility to that promoter.

Using a concatemerized probe containing this NGF-responsive element, we screened a PC12 cell λgt11 expression library and identified interferon response element binding factor-1 (IREBF1) as the transcription factor that binds to the NGF-response element of the ST-1 promoter. We showed that IREBF1 is constitutively present in the nuclei of PC12 cells and can mediate the NGF induction of ST-1. Overexpression of IREBF1 was shown to augment NGF-responsiveness whereas overexpression of a DNA binding-defective mutant of IREBF1 had the reverse effect on ST-1 transcription but not on another promoter, the *fos* promoter. In addition, neither of these mutants had any significant effect on basal levels of ST-1 transcription indicating that IREBF1 is mainly affecting NGF-induced promoter activity.

These results indicate that in addition to the AP1 site, NGF-induction of ST-1 occurs through a defined sequence in the proximal promoter. The bZIP transcription factor, IREBF1 binds to this sequence and is directly involved in ST-1 induction by NGF. This analysis of the mechanism of how NGF mediates gene transcription allows us to undertand, on a molecular level, how NGF may mediate the survival and differentiation of neuronal cells.

I

INTRODUCTION

A. NERVE GROWTH FACTOR

Historical Perspective

In the 1950's and early 1960's, Rita Levi-Montalcini, Viktor Hamburger and Stanley Cohen performed a whole series of experiments to investigate the role of a "nerve growth promoting factor" which they subsequently called nerve growth factor (NGF). The detection of this factor was an unexpected and fortuitous discovery in an experiment originally designed by Bueker in 1948 to show that the development of the spinal motor and sensory systems was directly dependent on the amount of peripheral target tissue (1). In these studies, Bueker showed that the transplantation of mouse sarcoma tissues into the body wall of young chick embryos caused a marked increase in the size of the sensory ganglia supplying the region of the transplants. Only the sensory nerve fibres but not the motor neurons from adjacent dorsal root ganglia had gained access into the tumor leading Bueker to conclude that the fast-growing sarcoma offered a favorable field for the growth of sensory fibres. In addition to sensory neurons, the transplantation of mouse sarcomas had an even greater increase in sympathetic ganglia (2). It was Levi-Montalcini and Hamburger who realized that not only the ganglia adjacent to the tumor, but also those further away from it were affected, leading them to suggest that the sarcoma tissue released a diffusible factor into the circulation that was responsible for the observed effects (2). The same effect was seen when the mouse sarcoma tissue was grafted

directly onto the chorioallantoic membrane of the chick embryo (3). The most direct and convincing evidence for the existence of a diffusible factor was provided by a simple *in vitro* assay that involved the co-cultivation of sarcoma tissue or tumor extracts with sensory and sympathetic ganglia under well-controlled experimental conditions. This co-cultivation provoked an impressive outgrowth of nerve fibers from the ganglionic explants (4). This phenomenon of fiber outgrowth from chick sensory ganglia is still the basis of the most commonly used bioassay for NGF or NGF-like activity.

The fortuitous character of this research continued when biochemist Stanley Cohen, in attempting to characterize the NGF activity present in sarcoma tissues, used snake venom as a source of phosphodiesterase to degrade nucleic acids and was surprised to find that the snake venom alone showed an effect on neuronal fiber outgrowth that was even higher than that seen with sarcoma tissue extract (5). This discovery prompted a screening of a variety of tissues in many different species which led to the detection of a particularly rich source of NGF in the submaxillary gland of the adult male mouse (6) and also in the prostrate gland of guinea pigs (7). NGF from mouse submaxillary gland was also shown to have growth promoting activity on chick sympathetic ganglia both *in vitro* and *in vivo* (8). These naturally occurring, highly concentrated sources of NGF facilitated the efforts of biochemists to purify NGF.

NGF is a complex of three subunits -- α, β and γ in the ratio 2:1:2 respectively - and the molecular weight of this complex is 130,000 daltons (9). The biological activity of NGF resides entirely in the β-subunit (10) and is a 26.5 kd dimer of two identical chains held together by noncovalent forces. Each monomer consists of 118 amino acids (11) and shows sequence similarity with proteins of the insulin group including proinsulin, insulin, insulin-like growth factors and relaxin (12). Subsequently, the mouse (13), human (14), bovine (15), and chick (16) genes coding for NGF were cloned. The human NGF gene is located on the proximal short arm of chromosome 1 and codes for a

large polypeptide of 307 aa, which is cleaved to give rise to the mature 118 aa βNGF (17) More recently, the complete structure of the 2.5S β-subunit of NGF has been crystallized and the structure visualized (18).

NGF and neurotrophins in the development of the nervous system.

The isolation and purification of NGF led to the pioneering introduction of immunological procedures into neurobiological research. The production of antibodies proved to be a vital tool in determining the role of NGF in the nervous system. The destruction of the peripheral sympathetic ganglia caused by NGF antibodies provided strong evidence for a physiological role for NGF (6, 19, 20). The mechanism of NGF's actions i.e. how NGF reached its target cells and what were its sources of production, were determined by studies using both pharmalogical and surgical approaches. When drugs such as 6-hydroxydopamine, which targets adrenergic neurons, or vinblastine, which blocks axonal transport, were applied to neonatal rodents, a majority of the sympathetic neurons did not differentiate or survive (21). The degeneration of the vetebral sympathetic ganglia seen with these chemicals was similar to that seen with anti-NGF antibodies. Surgical transection of post-ganglionic axons of the superior cervical ganglion also resulted in death of about 90% of immature sympathetic cells in this ganglion (22). In all these instances, nerve cell death was prevented by an exogenous supply of NGF, indicating the vital role of this molecule in the survival and differentiation of these neurons (23-25). Observations that labelled NGF is taken up by nerve endings of both sympathetic (26) and sensory (27) neurons and is retrogradely transported to the neuronal cell body, further supported the theory that NGF acts as a trophic factor. This led to the "neurotrophin hypothesis" which defines neurotrophins as

target-derived factors that are present in limiting amounts and thereby regulate the developmental innervation of the target tissue.

In the peripheral nervous system, NGF acts as a survival factor in culture for all sympathetic neurons and a subpopulation of sensory neurons (28, 29). *In vivo*, it has been shown that sensory neurons develop NGF dependency soon after their growth cones reach appropriate targets in the periphery, and that they require the continued presence of NGF throughout their lifetime (30, 31). In addition to the well characterized trophic response of peripheral nervous system (PNS) neurons (both sympathetic and sensory) to NGF, a few neuronal populations of the central nervous system (CNS) and some non-neuronal cells such as mast cells are also targets for NGF In the CNS, adrenergic and subpopulations of cholinergic neurons in the basal forebrain, corpus striatum, septum and the nucleus diagonal band of Broca are all responsive to NGF (32-36) . In the adult nervous system, NGF can rescue both the survival and function of neurons after damage during axonal regeneration. In the case of Alzheimer's disease, where selected cholinergic neurons in the basal forebrain degenerate, it has been demonstrated that NGF can rescue both the survival and function of these neurons (37-39).

Less than a decade ago, NGF was the only identified neurotrophin. These intervening years have seen the discovery of an entire family of related neurotrophins such as brain derived neurotrophic factor (BDNF) (40), neurotrophin-3 (NT-3) (41, 42), neurotrophin-4 (NT-4) (43), neurotrophin-5 (NT-5) (44) and neurotrophin-6 (NT-6) (45). All these neurotrophins, including NGF have specific effects on different and overlapping populations of the nervous system (reviewed in Table 1, page 00). For example, BDNF, in addition to septal cholinergic neurons, can support the survival of dopaminergic neurons in the substantia nigra where as NGF and NT3 cannot (46, 47). NT-4 has a trophic effect on sensory neurons in the dorsal root ganglion but not on sympathetic ganglia where as both those neuronal populations can be support by NT-5 (43, 44).

TABLE 1: The effects of various neurotrophic factors on neuronal cells.*

	NGF	BDNF	NT-3	NT-4	NT-5	CNTF	LIF	bFGF
Peripheral Nervous System (PNS)								
Parasympathetic (ciliary ganglion)	--	--	-?	...	--	+	--	+
Sensory (dorsal root ganglion)	+	+	+	+	+	+	+	+
Sensory (Nodose ganglion)	--	+	+	...	--	--	...	...
Sympathetic chain ganglia	+	--	+?	--	+	+	+	+
Pheochromocytoma cells (PC12)	+	--	?	--	+	...	...	+
Central Nervous System (CNS)								
GABAergic neuron (basal forebrain)	--	+	...	...	...	+	...	+
Granule cell (cerebellum)	--	+	--	...	...	...	...	+
Dopaminergic neuron (substantia nigra)	--	+	--	...	...	...	...	--
Cholinergic neuron (basal forebrain)	+	+	--	...	...	+	--	+
Motoneuron	-?	+	+	...	--	+	+	+
Pukinje cell (cerebellum)	+	...	...	...	...	...	...	...
Neuronal precursor cells								
Chromaffin precursor	+	...	...	...	...	...	...	+
Neural crest cell	--	+	+	...	...	...	+	+
Neuroepithelial stem cell	+	...	...	...	...	...	...	+
Sensory ganglion precursor	+	+	+	...	...	...	+	+
Sympathetic ganglion precursor	--	--	...	...	...	+	...	--

Key: + indicates a positive biological response; -- indicates the lack of a response; ? indicates controversial findings; and ... stands for not determined.
* Adapted from Korsching, 1993. *J Neuroscience* 13:2739 and references therein.

There are also a range of structurally unrelated proteins which also have effects on neurons from both the peripheral and central nervous system (Table 1). These include ciliary neurotrophic factor (CNTF) which supports the survival of the ciliary and sympathetic ganglion in the PNS and motoneurons and cholinergic neurons of the basal forebrain in the CNS (48-51); leukemia inhibitory factor/cholinergic differentiation factor (LIF/CDF) which effects both sympathetic, sensory neurons, and motor neurons (52-54); the fibroblast growth factors (FGFs) which have trophic effects on several neuronal precursor cells and several cell types in the CNS such as hippocampus, thalamus, some cortical neurons and granule cells of the cerebellum (55-59); glial cell line-derived neurotrophic factor (GDNF) which is a potent survival factor for motor neurons and dopaminergic neurons (60); insulin-like growth factors which can enhance growth of neurons from embryonic chicken brains *in vitro* (61, 62); and epidermal growth factor which exerts a trophic effect on subneocortical telancephalic neurons from embryonic rats (63).

The PC12 cell line.

In addition to the cell types discussed above, NGF can also induce chromaffin cells from the rat fetal adrenal medulla, a neural crest-derived tissue, to differentiate into neurons (64). This phenomenon is recapitulated *in vitro* by the PC12 cell line, isolated and developed by Llyod Greene and Arthur Tischler in 1976 (65). The PC12 cell line is a clonally derived cell line from a rat pheochromocytoma and iswidely used to study neuronal differentiation and NGF-signaling pathways. These cells are the neoplastic counterparts of adrenal chromaffin cells. In the undifferentiated state, PC12 cells resemble their normal counterparts, the neural crest-derived precursor cells, but in the presence of glucocorticoids, they resemble adrenal chromaffin cells. Upon NGF stimulation, however, PC12 cells undergo a diverse set of molecular and gross

morphological changes and carry out many physiological processes similar to sympathetic neurons, including the extension of neurites, which are visible 1-2 days following NGF exposure, the development of electrical excitability and the expression of genes encoding neuronal cell-specific proteins (65-68). The growth factor-induced differentiation of PC12 cells appears to reflect the developmental properties of their normal counterparts since similar morphological changes have been observed when normal neonatal rat adrenal medullary cells are exposed to NGF (64, 69). This differentiation of PC12 cells caused by NGF is a transcriptionally dependent transformation into a neuronal phenotype (70, 71) and is a phenomenon that has been exploited by many labs to understand how NGF regulates neuronal-specific gene expression.

Aside from NGF, PC12 cells also respond in a very different manner to another growth factor, epidermal growth factor (EGF). EGF is a mitogen and causes PC12 cells to proliferate, rather than differentiate. The receptor for EGF is very similar to the NGF receptor as both have tyrosine kinase intracellular domains and can induce similar cytoplasmic events (discussed below). In addition, no major differences with respect to substrate phosphorylation and induction of early response genes have been detected (72). The question as to how specificity in different signaling pathways arises is still unanswered, though our understanding has increased tremendously in the last few years. Since the biological effects of these two growth factors on PC12 cells are so vastly different, it makes this model system a good one for determining the specificity of the NGF-signaling pathway.

The NGF signal transduction pathway.

The PC12 cell line has also been extremely useful in the elucidation of the NGF-signaling pathway i.e. how a signal from the extracellular environment is communicated to the nucleus resulting in a change in gene expression. Many of the players in this signaling cascade, including the receptors and cytoplasmic second messengers, have recently been identified.

The neurotrophins recognize two different classes of receptors, the *trk* family of tyrosine protein kinases and the low-affinity receptor, p75 (Fig.1, page 00). The role of p75 in neurotrophin fuction is still unresolved (73, 74), but may facilitate the interaction of NGF with its signaling receptor *trk*A, perhaps by increasing the concentration of NGF in the vicinity of *trk*A receptors (75). Neurotrophin function is primarily mediated by the *trk* family of tyrosine kinase receptors. The *trk* receptors were first identified in 1986 when the *trk* gene (now known as *trk*A) was found to be part of an oncogene isolated from a human colon carcinoma (76). However, it was not until 1991 that their physiological role was unveiled when *trk*A tyrosine kinase was shown to be the signaling receptor for NGF (77, 78). Members of the *trk* receptor family are single transmembrane domain proteins that have two extracellular immunoglobulin-like domains and an intracellular tyrosine kinase domain (Fig.1) . Additional members of the *trk* receptor family have been identified that bind other members of the neurotrophin family. *Trk*A principally binds NGF, whereas *trk*C principally binds NT-3 (79). *Trk*B is the most promiscuous member of this receptor family and can bind both BDNF and NT-4/5 with roughly equal affinity (80, 81). Whether any of these *trk* kinases also serves as a receptor for the recently discovered NT-6 remains to be determined. The precise contribution of the two types of receptors to biological function is still controversial. While *trk*A is clearly able to transduce NGF signals by itself in some cells (81-83) indications of a combined function for *trk*A and p75 have also been seen (84). p75 could be involved in

more subtle aspects of signal modulation such as increasing overall sensitivity, altering dose-response or tyrosine-phosphorylation activity, or discriminating between the neurotrophins (75).

The role of oncoproteins in the NGF signaling pathway was initially demonstrated by Bar-Sagi and Feramisco in 1985 when an oncogenic form of *ras* injected into PC12 cells caused these cells to differentiate into a neuronal-like phenotype in a similar timecouse as that seen with NGF (85). Subsequently, other oncoproteins such as *src* and *raf* were also shown to be involved in the differentiation signal leading to the acquisition of a neuronal phenotype. Expression of the activated, oncogenic forms of *src*, *ras* and *raf* in PC12 cells can mimic NGF's effects including neurite extension and expression of genes encoding neuronal cell-specific proteins (85-89). Further evidence for the role of these oncoproteins as cellular mediators of differentiation comes from studies using dominant negative mutant proteins and antibodies that block the NGF-induced differentiation in PC12 cells (90, 91). Using various PC12 cell lines that are either deficient in or overexpress *src*, *ras* and *raf*, the sequence of activation of these cellular proteins involved in neuronal differentiation has been determined (92, 93). Though these molecules were shown to be directly involved in the NGF signaling pathway, the exact mechanism of activation of these molecules and their interaction with one and other were unclear until quite recently.

Binding of NGF to *trk* stimulates homodimerization of the receptor and activates its intrinsic kinase activity leading to the cross-phosphorylation of specific tyrosine residues in the carboxy terminus of the receptor (94-96). The *trk* receptor autophosphorylation is the initial step in an elaborate kinase cascade that transduces the signal through the cytoplasm (Fig. 2, page 00). Several proteins have been found to form complexes with the cytoplasmic domain and be substrates for activated *trk* (97-99). These molecules mediate their interaction with the activated *trk* receptors via their *src*

homology (SH2) domains that recognize specific phosphotyrosine residues (100). Upon receptor binding, these molecules also become phosphorylated on tyrosine residues, a step required for their activation.

The family of receptor tyrosine kinases (which includes the PDGF, EGF, NGF and insulin receptors) have all been shown to activate extracellular signal-regulated kinases, also known as mitogen-activated protein (MAP) kinases (101, 102). How these receptors mediate activation of MAP kinases has been elucidated recently (103-105). For example, the EGF receptor, when activated, binds GRB-2, thereby linking the receptor to the guanine nucleotide-exchange protein, son of sevenless (SOS) (106). SOS replaces *ras*-bound GDP with GTP, resulting in the activation of *ras*. The GTP-bound *ras* protein has been shown to interact with *raf* (107), a serine threonine kinase. Activated *raf* can then activate MAP kinase kinase (also know as MEK), which finally activates MAP kinase (108). The NGF receptor, *trk*A, does not bind GRB-2. NGF treatment of PC12 cells results in the rapid phosphorylation of the adaptor protein SHC on tyrosine residues (Fig.2), allowing SHC to interact with the GRB-2-SOS complex via its SH2 domain, leading to the subsequent activation of the MAP kinase pathway (109). Moreover, it was shown that overexpression of SHC leads to the neuronal differentiation of PC12 cells, suggesting that SHC is an important mediator of NGF signalling (110).

A second, *ras*-independent pathway for NGF signaling (Fig.2) has been proposed that involves phopholipase Cγ (PLCγ) and protein kinase C (PKC) (111, 112). Upon activation, PLCγ catalyzes hydrolysis of phophatidylinositol-4,5-biphosphate (PIP_2) into the second messengers diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 induces the release of Ca^{2+} from intracellular stores and, together with DAG, activates certain PKC isotypes (113, 114) Activated PKC is then able to activate *raf* (115, 116), allowing activation of MEK and MAP kinase.

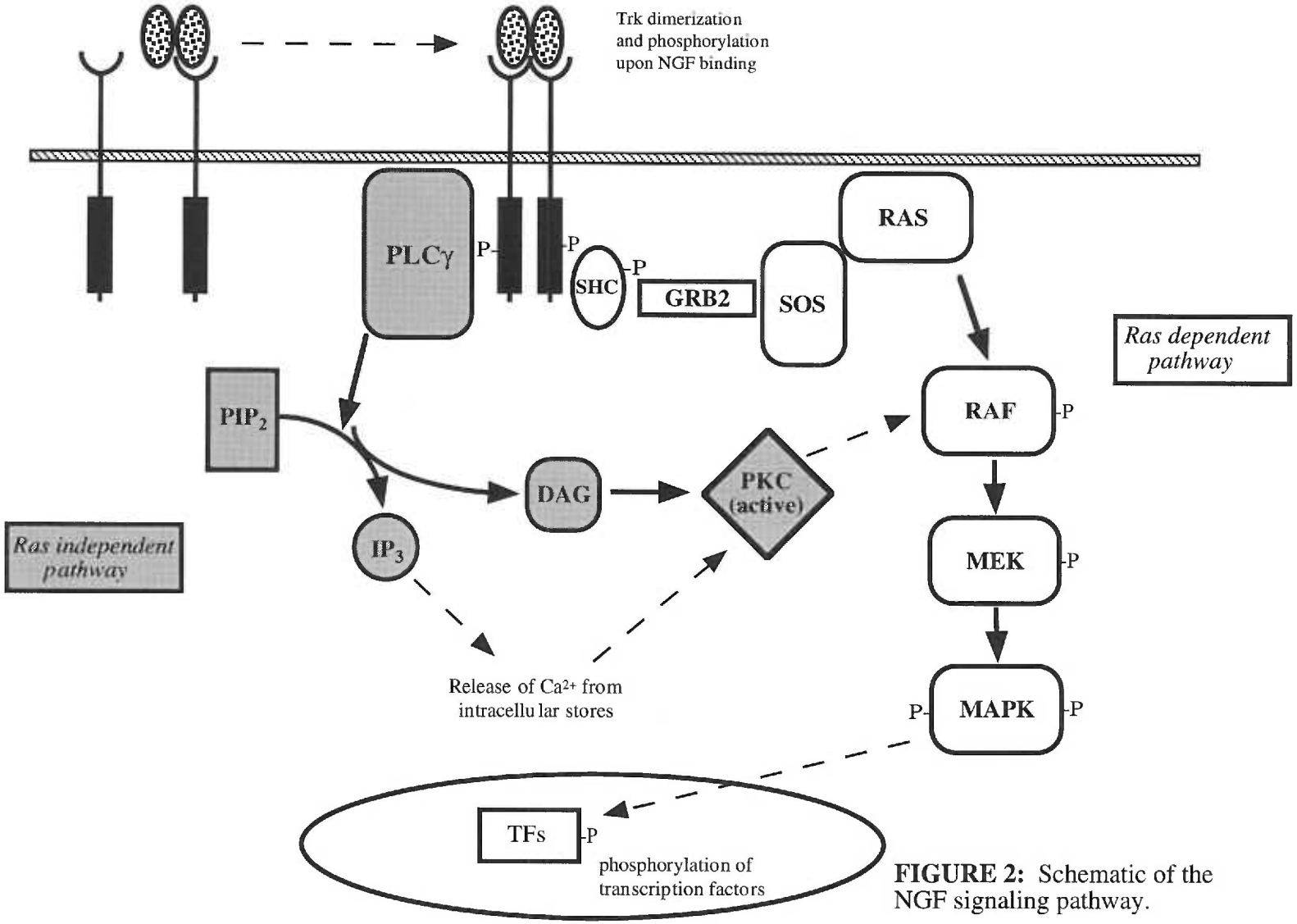

FIGURE 2: Schematic of the NGF signaling pathway.

In addition to SHC and PLCγ, *trk*A phosphorylation also activates the regulatory subunit (p85) of phophatidylinositol-3' (PI-3) kinase and ERK-1. PI-3 kinase binds directly to *trk*A at the end of the kinase catalytic domain and is activated presumably by a mechanism involving binding of the catalytic p110 subunit of PI-3 kinaseto the phosphorylated p85 adaptor (117, 118). The MAP kinase ERK1 (but not the highly related ERK2 kinase) has been found to be associated directly with *trk* receptors in immunoprecipitates derived from NGF-treated PC12 cells (119, 120). Therefore, the activation of MAP kinases by NGF may be mediated by either a direct mechanism involving ERK1 or an indirect mechanism involving the SHC/GRB-2-SOS complex.

The elucidation of the elaborate cytoplasmic events following NGF addition still does not establish how NGF affects neuronal differentiation and survival. The NGF signaling through the *trk* receptors does not necessarily lead to a differentiation process. Autocrine activation of *trk*A (as well as *trk*B and *trk*C) receptors in proliferating cells, such as NIH 3T3 fibroblasts, elicits potent mitogenic signals that result in their malignant transformation (121). This suggests the existence of neuronal-specific signaling elements that will process *trk* downstream signals into differentiation-specific pathways. One such candidate may be SNT, a 90 kDa protein that binds to a subunit of the cell-cycle regulatory complex that includes cdc2 kinase and cyclin (122). SNT is rapidly phosphorylated on tyrosine residues upon NGF stimulation in PC12 cells. More importantly, SNT is not phosphorylated in PC12 cells exposed to EGF, a mitogenic factor for both PC12 and NIH 3T3 cells (122).

The activation of MAP kinase has been shown to be not only required, but also sufficient to induce differentiation of PC12 cells (123). Since treatment of PC12 cells with EGF (which causes PC12 cells to divide rather than differentiate) through its tyrosine kinase receptor also leads to the activation of the MAP kinase cascade, the question of how these two growth factors produce such vastly different biological effects

needs to be resolved. It has been suggested that this difference could be due to differences in the intensity and/or duration of the signals elicited by the two different activated tyrosine kinase receptors rather than the receptors acting through unique signaling pathways. Recently, it has been shown that MAP kinase activation by NGF is sustained and leads to its nuclear translocation, whereas activation by EGF is transient and does not lead to pronounced nuclear translocation of MAP kinase (124). EGF, therefore, may be unable to initiate differentiation of PC12 cells because activation of MAP kinase is not maintained long enough to ensure entry of enough active kinase into the nucleus to initiate transcriptional events required for differentiation. This hypothesis of the duration of the signal as being the modulator between differentiation versus proliferation has been tested directly. PC12 cells that overexpressed an EGF desensitization-negative mutant receptor no longer proliferated in response to EGF, but instead triggered differentiation of these cells (125).

NGF regulation of gene transcription.

NGF-induced differentiation in PC12 cells is accompanied by the activation of various classes of genes whose onset of transcription occurs at different times following NGF stimulation. The "immediate early" genes, which are transiently activated within 5-10 minutes of NGF stimulation, encode several putative transcription factors whose products may mediate the growth factor response during differentiation (126-130). These include *c-fos* (128-131), *c-jun* (126, 132), NGF-IA and related zif/268 and egr-1 genes encoding products that contain a zinc-finger DNA binding domain (133-137), NGF-IB (*nur 77*), an early response gene that has sequence similarity to the glucocorticoid receptor (138, 139), and ornithine decarboxylase (140, 141). The "delayed early" genes are activated within 1-2 hours of NGF stimulation and includes genes whose products may be important for early events during the transition from a chromaffin phenotype to

the neuronal cell state. One example of such a gene is the tyrosine hydroxylase gene (67). This enzyme catalyzes the rate-limiting step in the catecholamine biosynthetic pathway to convert tyrosine to 3,4-dihydroxyphenylalanine (DOPA) which is a precursor to various neurotransmitters such as dopamine, norepinephrine and epinephrine. The last class of genes, the "late" genes, are transcriptionally active several hours post-NGF stimulation and their expression coincides with gross morphological changes occuring in the cell during differentiation, such as neurite extension. Some of these genes include several neurofilament subunits (68, 142), the peripherin gene, which encodes a neuron-specific intermediate filament protein (143, 144), voltage dependent brain type II Na^+ channels (145), GAP-43 (146, 147), SCG10 (148, 149), VGF (150), microtubule associated protein 2 (151), neuropeptide Y (152), neurotensin/neuromedin (153), and stromelysin (also known as transin) (154).

In order to understand the mechanism behind NGF-specific gene regulation, many labs, including ours, have utilized these transcriptionally regulated genes to look for NGF-specific transcription factors or DNA elements in the promoter region of these genes. Promoter elements required for induction by NGF have been analyzed for the *c-fos*, NGF-IA, tyrosine hydroxylase, VGF, neurotensin, peripherin and neuropeptide Y genes (Table 2, page 00). NGF induction of *c-fos*(155) in transfected PC12 cells is dependent upon two serum response elements (SREs) located at positions -227 and -323. A sequence similar to the cAMP response element (CRE) at -60 was later shown to mediate calcium and membrane depolarization induction of *c-fos* through the transcription factor CREB (156). Constructs containing 532 bp of upstream sequence from the NGF-IA gene (157) transfected into PC12 cells have been shown to be NGF inducible. Potential transcription factor binding sites in this region include four SREs and a CRE at -140, but the involvement of these elements in NGF induction has not been demonstrated. Both NGF inducibility and basal promoter activity of the tyrosine hydroxylase gene (158) are dependent on a fat-specific element (TH-FSE) that contains a

consensus AP1 binding site. NGF was shown to increase binding of a nucleoprotein complex that contained *c-fos* to the TH-FSE. The VGF gene is transcriptionally regulated by NGF through a 14 bp palindromic sequence that contains a CRE (159). Binding of the CRE binding protein (CREB) was found to be critical for the activation of the VGF promoter by NGF. Sequences mediating NGF induction of the neurotensin gene (153) are less well understood as synergistically acting agents are required for induction. Transcriptional induction of neurotensin by NGF in combination with other agents is decreased by mutation of a consensus AP1 site or mutations of two CRE-like sites (153). The peripherin gene is regulated via the derepression of a 11 bp unique negative regulatory element by NGF in PC12 cells. A less well-defined distal positive element contained within a fairly large 370 bp region of the promoter is also required for complete transcriptional activation of the peripherin gene (160) Finally, the neuropeptide Y promoter contained a 50 bp region within its promoter that is NGF-responsive and interacts with atleast four different proteins including AP2 and these proteins were expressed in a tissue specific manner (161). In none of these genes has a CRE, or any other known sites for inducible transcription factors, been shown to be a common site through which transcriptional induction by NGF is mediated (Table 2). Thus, it is reasonable to conclude that NGF must regulate gene expression by a variety of different mechanisms.

This thesis is an investigation of the NGF regulation of another transcriptionally induced gene, stromelysin-1 (ST-1). In keeping with the evidence presented above, it is not surprising that the transcriptional regulation of ST-1 is also different from any other NGF-induced gene and is a demonstration of yet another mechanism by which NGF may regulate gene expression.

TABLE 2: List of NGF response elements.

	PROMOTER ELEMENT/SEQUENCE		TRANSCRIPTION FACTOR	REFERENCE
Late genes				
Tyrosine hydroxylase	TH-FSE	-205 TGATTCAGAGGCA -193	*fos*	Gizang-Ginsberg and Ziff, 1990. *Genes & Dev* 4:477.
VGF	CRE	-82 TGACGTCA -75	CREB	Hawley et al., 1992. *J Neurosci* 12:2573.
Neurotensin	AP1	-188 TGAGTCA -182	AP1	Kislauskis and Dobner, 1990. *Neuron* 4:783.
	CRE/AP1	-48 TGACATCA -41	*fos/jun*	
Peripherin	NRE	-178 GGCAGGGCGCC -168	??	Thompson et al., 1992. *Mol Cell Biol* 12:2501
Neuropeptide Y	-83 AGTCACCCAAGCGTGACTGCCCGAGGCCCCTCCTG -46 AP2, CT-box		??	Minth-Worby, 1994. *J Biol Chem* 269:15460
Immediate eary genes				
c-fos	1 SRE at -308; 1 SRE-2 at -288		SRF	Visvader et al., 1988. *PNAS* 85:9474.
NGF-IA	4 SREs at -84, -106, -370, -408; 1 CRE at -140		SRF, CREB	Changelian et al., 1989. *PNAS* 86:377

B. STROMELYSIN-1

Stromelysin-1 (ST-1) is a member of the matrix metalloproteinase gene family.

ST-1 is a member of the matrix metalloproteinase (MMP) family which consists of structurally related enzymes and is so named because of its requirement for divalent cations for enzymatic activity and its involvement in degrading specific components of the extracellular matrix. There are nine members of the matrix metalloproteinase family identified so far, which are classified according to their substrate specificity. The collagenases degrade fibrillar interstitial and neutrophil collagens; the gelatinases recognize basement membrane components and denatured collagens; and the stromelysins hydrolyze the protein component of proteoglycans and extracellular matrix glycoproteins (162, 163). A murine metalloelastase has also been identified amd may represent a new subclass of elastinolytic enzymes (164).

The stromelysin subclass of MMPs include two highly related proteins of about 54 kilodaltons, ST-1 and ST-2, as well as the smaller matrilysin protein (165). Figure 3 (page 00) shows the general structure of ST-1 which, like all other MMPs, has a signal sequence and is synthesized as a pro-enzyme which is cleaved to produce the enzymatically active form of the protein (166) Although the enzyme responsible for ST-1 cleavage and activation *in vivo* is unclear, it is known that plasmin can activate it *in vitro* by cleavage at a furin-like site at position 102 (167). ST-1 also has a histidine-containing zinc-binding domain, which is critical for its catalytic activity. Site directed mutagenesis of the histidine at position 154, for example, completely destroys ST-1 enzymatic activity (168). ST-1 also contains a domain similar to hemopexin, an iron-transport protein. Although the function of this domain is not entirely clear, it has been shown to bind endogenous inhibitors of metalloproteinases, suggesting that this

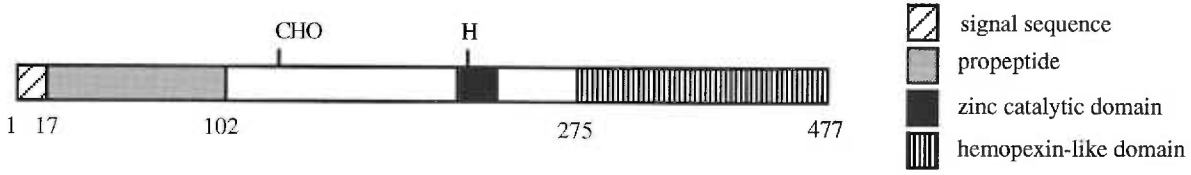

FIGURE 3: Domain structure of pre-pro-Stromelysin-1. CHO refers to a glycosylation site; H refers to the histidine at position #154 which is necessary for catalytic activity.

carboxyterminal region may play an important regulatory role for this enzyme *in vivo* (169, 170).

ST-1 function in normal and pathological processes.

ST-1 degrades various components of the extracellular matrix and basal lamina such as fibronectin, laminin, proteoglycans and collagen types III, IV, V, and IX (162, 163). During development, stromelysins and other MMPs participate in the remodelling of the extracellular environment. In the nervous system, neurites in the periphery grow within a 3-dimensional environment rich in extracellular matrix. One means of promoting and aiding axon outgrowth through this matrix would be for growing processes to release proteases capable of degrading the matrix. The local release of proteases would help create "channels" in the extracellular matrix through which growing neurites could move. This hypothesis has been tested *in vitro* using three-dimensional collagen gels where it was shown that axonal outgrowth within the gel could be inhibited using various inhibitors of MMPs (171-173). It has been shown *in vivo* that during migration and colonization of the developing head and neck, cephalic neural crest cells secrete the serine proteinase plasminogen activator (PA) (174). Plasminogen activator (both tissue plasminogen activator and urokinase) cleaves plasminogen to form the active protease, plasmin (Fig.4, page 00). Plasmin has been proposed to be the endogenous activator of MMPs by cleaving the proenzyme form of MMPs to the active peptide. Hence, production of plasminogen activator by migrating neural crest cells would increase the activity of metalloproteinases allowing a passage through the extracellular matrix through which neural crest cells can migrate. In the case of ST-1, it has been shown that cleavage at a furin-like site at position 102 by plasmin can activate it *in vitro* (167).

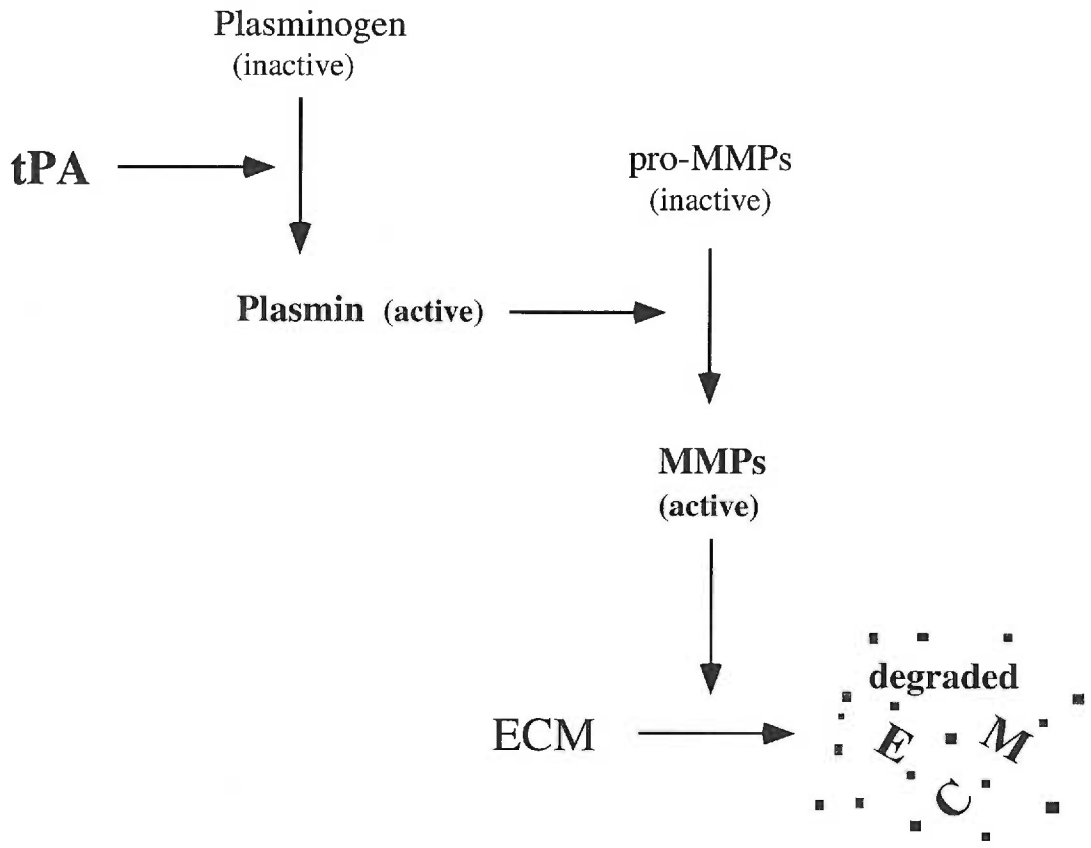

FIGURE 4: Pathway for the activation of matrix metalloproteinases (MMPs) *in vivo*. Tissue plasminogen activator (tPA) cleaves inactive plasminogen to its active form, plasmin. Plasmin cleaves the pro-peptide of several MMP family members which, in its fully activated enzymatic form, can degrade proteins in the extracellular matrix (ECM).

Recently, it has been shown that ST-1 is expressed in peripheral nerves exiting the spinal cord, doral root ganglionic neurons and sympathetic neurons (175). In PC12 cells, a neural crest derivative, it was shown that upon NGF stimulation, ST-1 protein was found to accumulate in the cell body and in growing neurites. When ST-1 protein accumulation was blocked using antisense mRNA, these cells failed to penetrate through a matrix, though normal neurite outgrowth was not compromised (175). Surprisingly, ST-1 was also found in the central nervous system in axons within the vertral white commissure in developing rat spinal cords (175). These commissural neurons never penetrate basal laminae, but instead remain within the CNS, projecting rostrally to the hind brain. However, even though the expression of ST-1 in these neurons may seem unusual, there are several possible substrates for ST-1 in the CNS, mainly proteoglycans which are found associated with the extracellular matrix of the CNS, as well as with the surfaces of some neuronal and glial cells.

In addition to nervous system development, ST-1 has been implicated in several other normal processes involving destruction and remodelling of the extracellular matrix such as wound healing (176), mammary gland involution (177-179), and blastocyst implantation (180). Stromelysins also play an important role in pathological processes such as arthritis (181) and tumor invasion and metastasis (182, 183). In mouse squamous epithelial carcinomas, ST-1 message levels were found to be much higher in malignant tumors than in benign or normal epithelial tissue (184). In breast carcinomas, ST-3 protein, which was expressed in the surrounding stromal tissue rather than the cancer cells themselves, contributed to the metastatic potential of the carcinoma (185). More recently, it was shown that the progression of mouse skin tumors from squamous carcinoma to the aggressive, highly metastatic spindle cell carcinoma is likely due to ST-1 expression (186). The role of stromelysins in the tumorigenicity of cancerous tissues has been a target of many pharmaceutical companies for the prevention of tumor progression.

ST-1 gene regulation.

Because of the medical implications and repercussions of ST-1 misexpression in normal tissues, the regulation of this gene has been an area of much focus and research. Tumor promoting agents such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and mitogenic growth factors like epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) are all able to regulate ST-1 expression in different cell types. Transin, the rat homolog of ST-1 was initially identified as a 1.9 kilobase transcript that was induced in Rat-1 fibroblasts by treatment with EGF and TPA. In addition, cells transformed with either the Rous sarcoma virus or the cellular oncogene Harvey-*ras* (H-*ras*) also showed elevated transin levels (187, 188). The induction of ST-1 (transin) by these agents was blocked by treatment with the protein translation inhibitor, cycloheximide, indicating the requirement for *de novo* protein synthesis in ST-1 induction. Nuclear run-on analysis demonstrated that the increase in the message levels of ST-1 mRNA was due to regulation at the transcriptional level (187, 189). In addition to transcription, there is also evidence that EGF regulates ST-1 by a post-translational mechanism that increases mRNA stability by 30-50 % (190).

Analysis of the ST-1 promoter reveals several known transcriptional enhancer elements (see Chapter II, Fig.2A). The ST-1 promoter has an activator protein-1 (AP1) site, which is a palindromic sequence centered at -67 (TGAGTCA) and is also known as the TPA response element (TRE) (191). This sequence is known to bind the proto-oncogenes *c-fos* and *c-jun* as heterodimers and also *c-jun* homodimers (192). There are also two sites, arranged head to head, that are similar to the polyomavirus enhancer A-binding protein-3 (PEA3) site between -208 and -191 that bind the *c-ets* oncoproteins (193, 194) and plays a role in the induction of ST-1 by TPA (195). In addition to these

enhancer elements, the ST-1 promoter has a CAAT box at -74 and a TATA box at -28, which are required for the assembly of the transcriptional machinery.

In addition to the well characterized induction of ST-1 by EGF, PDGF can also induce ST-1 in fibroblasts. Using an antisense approach, it was shown that while the induction of ST-1 by EGF did not require *c-fos,* the induction by PDGF was dependent on *c-fos* (191). The transcriptional regulation of ST-1 by PDGF in NIH 3T3 cells has been well characterized recently. A palindromic sequence present between -1218 and -1202 was shown to be necessary for the PDGF control of ST-1 gene expression (196). In addition, these researchers showed that a phorbol 12-myristate 13-acetate (PMA) insensitive protein kinase C isotype called ζPKC was critical for the ST-1 induction by PDGF. The stromelysin-1 PDGF-responsive element (SPRE) was recently shown to bind a novel transcription factor (SPBP) of 937 amino acids, which contains a putative leucine zipper region, a nuclear localization signal and a region that has homology to the DNA binding domains of *fos* and *jun* (197). This transcription factor is regulated by serum and cooperates with *c-jun* binding to the AP1 site for activation through the SPRE (196, 197). Recently, it was shown that the PDGF regulation of ST-1 occurred through two transcriptional units: the SPRE-AP1 and the PEA3-AP1 (198). Using dominant negative *ras* and *raf* proteins, they showed that induction of ST-1 by PDGF involved two independent signalling pathways downstream of *ras* -- one *raf*-dependent, operating through PEA3-AP1 and the other one *raf*-independent, operating through SPRE-AP1 (198).

Other growth factors such as insulin, fibroblast growth factor (FGF) and transforming growth factor beta-1 (TGF-β1) do not induce ST-1 transcription in fibroblasts like EGF does (187). However, TGF-β1 was also able to block ST-1 transcription induced by EGF (187). A similar transcriptional block was also produced by cyclic-AMP (cAMP) (199). The inhibitory effect of TGF-β has been shown to occur

via an element located at -709 in the ST-1 promoter (200). This TGF-β1 inhibitory element (TIE) binds a nuclear protein complex that contains the *c-fos* proto-oncogene product. These studies showed that *c-fos* expression was absolutely required for the TGF-β1 inhibition of ST-1 induced gene expression (200-202). The vitamin A derivative retinoic acid (RA) has also been found to negatively regulate ST-1 gene transcription (203). This effect was mediated through the AP1 site, though the mechanism was unclear. However, it did not appear that the RA-dependent repression was due to RA receptor binding to the AP1 site or to the AP1 protein complex (203). The AP1 site in the human ST-1 promoter was also shown to mediate the positive response of ST-1 to an inflammatory mediator, interleukin-1 (204) and to other cytokines such as tumor necrosis factor (TNF) and interferon-β (205) in human foreskin fibroblasts.

The regulation of ST-1 by these various growth factors, tumor promoters, oncogenes and cytokines are all mediated to some extent by the AP1 motif, which is present not only in the ST-1 promoters of rat, rabbit and human, but also in the promoters of other matrix metalloproteinases, such as human interstitial collagenase (206). It appears, therefore, that the AP1 site in the ST-1 promoter is critical for the transactivation of this promoter (207). In addition, the data presented in this thesis also supports this theory since the induction of ST-1 by NGF is severely compromised when the AP1 site is rendered ineffective (Chapter II) .

ST-1 regulation by NGF in PC12 cells.

In addition to fibroblasts, the ST-1 gene is also regulated by NGF in PC12 cells (154). Northern blot analysis indicated that ST-1 mRNA levels increased several hundred-fold over initially undetectable basal levels of expression, making this one of the most NGF-responsive gene products identified. The mechanism of this upregulation was

shown to be similar to that previously seen in fibroblasts induced by EGF. In both cases, ST-1 mRNA induction was blocked by treatment with cycloheximide, indicating that transcription is dependent on *de novo* protein synthesis (154, 187). Nuclear run-on experiments demonstrated that this gene was transcriptionally regulated by NGF about 1-2 hours after NGF treatment. The levels of ST-1 mRNA was at its peak about 24-72 hours after NGF treatment and then declined. The induction of ST-1 levels was shown to be largely coincident with the neuronal differentiation of PC12 cells and the extension of neurites (154). In PC12 cells, ST-1 was induced by NGF but not by other growth factors like EGF or FGF, making this gene an ideal candidate for examining NGF-specific gene regulation. In addition, most other late gene products have relatively high basal levels of expression, which limits induction by NGF to only about 2-10 fold (67, 68).

The involvement of serine kinases such as *raf,* MEK and MAP kinases in NGF signaling has already been discussed (see above). It has been hypothesized that an additional protein kinase may also be involved later in the NGF-induction of ST-1 (208). These studies involved the protein kinase inhibitor staurosporine (also known as K252a), which is known to block the autophosphorylation of the *trk* receptors (117, 209, 210). Staurosporine was found to completely block the NGF-induction of ST-1, which is not surprising since the entire NGF signal transduction pathway is blocked due to prevention of *trk* receptor phosphorylation. However, staurosporine blocked ST-1 induction even when added four hours after NGF addition (208). Four hours after NGF addition, *trk* is no longer phophorylated and *c-fos* protein is no longer present in PC12 cells (211), suggesting that this action of staurosporine is independent of its effects on *trk* and that some late kinase activity is necessary for ST-1 gene expression. This staurosporine sensitive kinase does not appear to be PKC, since down-regulation of PKC by pre-treatment with phorbol esters has no effect whatsoever on the ST-1 induction of ST-1 (208). This as yet unidentified kinase may be responsible for the phosphorylation of a cytoplasmic protein or a transcription factor involved in ST-1 gene induction via NGF.

II

RESULTS

A Novel Nerve Growth Factor-responsive Element in the Stromelysin-1 (Transin) Gene That Is Necessary and Sufficient for Gene Expression in PC12 Cells

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ABSTRACT

Stromelysin-1 (ST-1) is an extracellular matrix metalloproteinase whose expression is transcriptionally regulated by nerve growth factor (NGF) in rat pheochromocytoma (PC12) cells. In this paper, we define sequences in the proximal ST-1 promoter that contain a novel NGF-responsive element(s). We show that this *cis*-acting promoter element can bind nuclear proteins from both untreated and NGF-treated PC12 cells in a specific and saturable manner, and is sufficient to confer NGF-inducibility to a heterologous promoter. At least a portion of this NGF-responsive element lies between bases -241 and -229 of the ST-1 gene and bears no sequence homology to other known transcriptional elements. In contrast to what has been reported for fibroblasts, an AP1 site centered around position -68 does not seem to be involved in the growth factor regulation of this gene in PC12 cells. These results suggest that the interaction of *cis*-acting sequences with the *trans*-acting factors that bind to them is important for transcriptional induction of the ST-1 gene in response to NGF.

INTRODUCTION

Nerve growth factor (NGF) was the first neurotrophic factor discovered in a class of molecules responsible for the development, differentiation and growth of the nervous system. NGF supports the survival and maintenance of sympathetic and sensory neurons of the peripheral nervous system and promotes the differentiation of selected cholinergic and adrenergic neurons of the central nervous system (35, 212). NGF can also induce chromaffin cells from the rat fetal adrenal medulla, a neural crest-derived tissue, to differentiate into sympathetic neurons (64), a phenomenon recapitulated *in vitro* by the PC12 rat pheochromocytoma cell line (65). Following several days of exposure to NGF, PC12 cells undergo transcriptionally-dependent transformation into a neuronal phenotype characterized by the extension of neurites, the development of electrical excitability, and the expression of genes encoding neuronal cell-specific proteins (65, 66)

Unlike NGF, which triggers PC12 cells to cease dividing and undergo neuronal differentiation, epidermal growth factor (EGF) stimulates proliferation of PC12 cells without differentiation. Both NGF and EGF are ligands for receptor tyrosine kinases, which can activate similar intracellular signaling molecules such as PLC-γ, PI-3 kinase and Ras (109, 111, 112, 118, 213-217). Within minutes, both NGF and EGF cause the induction of several immediate early genes that encode transcriptional regulatory proteins, including c-fos, c-jun, and NGF-IA/egr-1 in PC12 cells (126, 128-130, 132, 136). Despite similarities in the signaling pathways, these growth factors have very different effects on the physiology and morphology of PC12 cells. Neuronal differentiation by NGF is accompanied by expression of a subset of genes which encode

proteins that are important for the maintenance of the differentiated phenotype. These genes, most of which are transcriptionally active hours after NGF addition, include tyrosine hydroxylase (67), neuropeptide Y (152), peripherin (143) and other neurofilament components (68), brain type II Na^+ channels (145), SCG10 (149), VGF (150), and stromelysin (also known as transin) (154). None of these late genes are activated by EGF, suggesting that some critical aspects of these two signaling pathways differ. In order to better understand the mechanisms underlying the development and expression of the neuronal phenotype in PC12 cells, several groups, including ours, have examined the transcriptional regulation of NGF-induced genes (158-161). In this study, we utilize the stromelysin gene as a model for NGF-induced regulation of gene expression.

Stromelysin-1 (ST-1) is a member of the matrix metalloproteinase gene family which includes interstitial collagenase and the gelatinases (218, 219). ST-1 itself is known to degrade various components of the extracellular matrix associated with basal laminae (220), and has been implicated in tissue remodelling events associated with embryonic development (180), tumor metastasis (184), and axonal invasiveness (175). In fibroblasts, ST-1 is transcriptionally induced by EGF, platelet derived growth factor (PDGF) and the phorbol ester TPA (187, 188, 191). This induction can be transcriptionally inhibited by transforming growth factor β-1 (TGFβ-1) through a 10 bp sequence in the ST-1 proximal promoter region known as the TGFβ-1 inhibitory element (TIE) (189, 199, 200). PDGF has also recently been shown to induce ST-1 gene expression in NIH 3T3 cells via a 6 bp palindromic sequence in the distal ST-1 promoter (196). In PC12 cells, however, we found that NGF, but not EGF nor PDGF, increased the levels of ST-1 mRNA (154). This increase in ST-1 mRNA is at least a hundred-fold over initially undetectable levels, making ST-1 one of the most highly induced NGF-responsive late gene products described (67).

In this study, we utilize the ST-1 promoter to determine the mechanisms responsible for the NGF induced increase ST-1 transcription in PC12 cells. It was previously shown that a 750 bp fragment of the proximal ST-1 promoter contained sequences which were sufficient for the NGF-induction of this gene (154). In this paper, we identify a 12 bp region of the ST-1 promoter which contains at least a portion of a novel NGF-responsive element. We show that the region of the promoter containing this element binds nuclear proteins from both untreated and NGF-treated PC12 cells and is sufficient to confer NGF-responsiveness to a heterologous promoter. This characterization of *cis*-acting sequences which participate in the developmental response is important for understanding the mechanisms which underlie differential gene expression during neuronal development.

METHODS AND MATERIALS

Oligonucleotides and Plasmids

5' promoter deletion mutants of the p750TRCAT plasmid were generated using an Erase-a-Base® kit (Promega, Madison, WI), as described (200). Deletions were analyzed by DNA sequencing with Sequenase (Version 2.0, USB, Cleveland, OH). The pCATbasicTK vectors (pCBTK) was the gift of Dr. Bruce Magun (Oregon Health Sciences University) (221). In some experiments, a modified form of the pCBTK vector was used in which an AP1 site was inserted upstream of the thymidine kinase (TK) promoter. This vector was referred to in this paper as "pAS." The NRR-ABC fragment was obtained by polymerase chain reaction (PCR)-amplification of the p715TRCAT vector using appropriate oligonucleotides with *Bgl*II and *Cla*I restriction sites placed at the termini. These oligonucleotides were: 5' - GCACAGATCTCTTCT-GGAAGTTCTTTGTAC - 3' (upstream) and 5' - GCACATCGATAAATGCTTCCTG-CCTTAG - 3' (downstream). The PCR product was then subcloned into the *Bgl*II and *Cla*I sites of the pCBTK vector. The NRR-A, NRR-B, and NRR-C fragments were obtained by restriction digestion of the NRR-ABC fragment using *Mae*III and *Hae*III, followed by gel isolation. The NRR-C fragment was treated with Klenow polymerase to fill-in the overhangs, and then blunt-end ligated into the *Sal*I site of the pAS vector.

Cell Culture and Transient transfection assays

Stock cultures of PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum and 5% horse serum at 37°C in a

95% air-5% CO_2 atmosphere. The original PC12 cell line (subclone GR-5) was obtained from Dr. Rae Nishi (Oregon Health Sciences University). For transient transfections, PC12 cells were plated at an initial density of 2 x 10^6 cells per 10 cm Primaria plate (Falcon) two days before transfections. Calcium phosphate-DNA precipitates containing 15 μg of DNA were added to the cultures for four hours (222). The cells were then "shocked" for 2.0 minutes with 15% glycerol in HEPES buffered (25 mM; pH 7.0) saline solution. The cultures were washed twice with phosphate buffered saline (PBS), allowed to recover overnight in serum-containing medium, and then cultured for 24 hours in "N2-supplemented" medium (223). The next morning, NGF (50 ng/ml) or EGF (5 ng/ml) were added directly to the cultures for an additional 24 hours prior to harvesting the cultures. Protein levels were assayed (224) and extracts containing 50 μg protein were used in a kinetic chloramphenicol acetyltransferase (CAT) assay using ^{3}H-acetyl coenzyme A, as described (225).

Gel Mobility Shift Assays

Nuclei from PC12 cells were prepared using a modified version of the method of Hagenbüchle and Wellauer (226) in which intact nuclei are used instead of nuclear extracts. PC12 cells were first dissociated by treatment with PBS, pelleted by centrifugation, washed in PBS, and then resuspended in 0.3 M sucrose in Buffer A (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM HEPES, pH 7.8, 14 mM mercaptoethanol, 0.5 mM PMSF and 8 μl/ml aprotinin). NP-40 was then added to this nuclear preparation to a final concentration of 0.1% (v/v). Cells were lysed in a Dounce homogenizer using 30 strokes with a type B pestle, and then the suspension was centrifuged atop a 0.9 M sucrose cushion in buffer A. The nuclei pellet was resuspended and treated again to Dounce homogenization in Buffer A (without NP-40) with 5 strokes with the pestle. After the second centrifugation, the pellet was resuspended in a small

volume of buffer B (75 mM NaCl, 0.5 mM EDTA, 20 mM Tris pH 7.9, 0.8 mM DTT, 0.1 mM PMSF and 50% glycerol) and the number of nuclei in each extraction was determined. Nuclei from untreated, EGF- and NGF-treated PC12 cells were then adjusted to a final concentration of 10^6/ml and then 50 µl aliquots were stored at -80°C.

DNA binding reactions involved first pre-incubating the nuclei in 10 µl containing 20 µg bovine serum albumin, 15 µg poly (dI-dC) and unlabeled competitor DNA's for 30 min. at room temperature. End-labeled probes (10,000 cpm; specific activity = 10^8/cpm/µg) was added to the nuclei in a final buffer concentration of 15 mM HEPES (pH 7.5), 60 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, and 12% glycerol. Binding reactions were allowed to incubate for 30 min. on ice. Samples were then subjected to electrophoresis in 6% SDS-polyacrylamide gels (acrylamide:bis ratio of 37:1) at 4°C in 0.5 X TBE (50 mM Tris-HCl, pH 8.3; 41 mM boric acid; 0.5 mM EDTA). Gels were dried and radiolabelled bands were visualized by autoradiography. Unlabeled competitor DNAs were present in 25-, 50-, 100-, and 200-fold molar excesses over radiolabeled probe DNA.

Site-Directed Mutagenesis of the NGF-responsive region

5 versions of the ST-1 promoter were generated using the Altered Sites™ in vitro mutagenesis system (Promega). These 5 plasmids contain contiguous 6 bp nested mutations spanning the 30 bp NRR-C' region (see Fig.6A). The *Eco*R1 fragment of p715TRCAT (containing the promoter) was subcloned into pAlter-1 in the sense orientation and used as a template for the mutagenesis reaction. The following oligonucleotides containing the required mutations were used for mutagenesis: mutant 1 (m1) 5' - CAGCTTCTGAAGGATATAGTACTTTTCCAAAGTAG - 3'; mutant 2 (m2) 5' - GAAGGATAGTTACAAGACTGAAAGTAGAAAAAAATGCC - 3'; mutant 3 (m3)

5' - GATAGTTACATTTTCCGTATCTGAAAAAAATGCCCC - 3'; mutant 4 (m4) 5' - CATTTTCCAAAGTATTACTGAATGCCCCAGTTTTC - 3'; and mutant 5 (m5) 5' - CCAAAGTAGA AAAAGCCTATGCAGTTTTCTCTTTTGC - 3'. Annealing, extension and screening of mutations were carried out using protocols supplied by the manufacturer. Mutated forms of the promoter were then reintroduced into the pCBTK vector by PCR-amplification of the mutant NRR-ABC region and subsequent subcloning of this fragment into the *Bgl*II and *Cla*I sites of pCBTK.

RESULTS

Transient transfection studies using a mutated AP1 site in the ST-1 promoter

In previous work, Kerr et al. (191) showed that the AP1 site at position -65 of the ST-1 promoter mediated the EGF-induction of ST-1 gene expression in NIH 3T3 cells. To determine whether this AP1 site was necessary for NGF-induction of ST-1, PC12 cells were transiently transfected with either the parental p750TRCAT vector containing a 753 base pair region of the ST-1 promoter, or a site-directed mutant of this vector in which two base substitutions were introduced into the AP1 site, rendering it functionally inactive (191). Fig. 1 shows that PC12 cells transfected with the wild type p750TRCAT plasmid showed a 5- to 7-fold NGF-induced increase in CAT activity as compared with untreated control cells. Basal levels of CAT activity were significantly lower in cells transfected with the plasmid containing the mutated AP1 site as compared with those transfected with the wild type AP1 site ($p < 0.001$), whereas NGF still caused a significant increase in CAT levels ($p < 0.050$). In neither case was there a statistically significant change in CAT activities following exposure to EGF. Since the ability of the mutated plasmid to respond to NGF was not lost, we infer that the AP1 site is not directly responsible for the NGF-responsiveness of the ST-1 promoter. This experiment was performed three times with qualitatively similar results.

5'-Deletion analysis of the ST-1 promoter

The observation that a mutated AP1 site failed to abolish the NGF-induction of CAT indicates that a different element must confer NGF-responsiveness to the ST-1

promoter. To localize this element, 5'-deletion mutants of the parental p750TRCAT plasmid were generated (see Fig. 2A; the arrows indicate the various deletion mutants). PC12 cells were then transiently transfected with these plasmids and treated for 24 hours with either control culture medium or medium supplemented with NGF or EGF. As a control for transfection efficiencies, all of the cultures were co-transfected with a second plasmid containing the luciferase reporter gene driven by the Rous sarcoma virus constitutive promoter. Fig. 1B shows that removal of the terminal 38 bases from the 5' end of the parental plasmid resulted in an increase in NGF-responsive CAT expression from approximately 5-fold to 11-fold above basal levels, indicating the possible presence of a negative regulatory element in this region. In contrast, there was no statistically significant change in basal or EGF-induced levels of CAT expression. Deletion of the subsequent 400 base pairs from the 5' end of the ST-1 promoter, however, produced no consistent or statistically significant changes in the levels of NGF-responsiveness. Although basal levels of CAT expression remained unchanged following deletions of this region, the levels of EGF-induced gene expression were slightly higher with the p573TRCAT and p578TRCAT plasmids. This observation, however, was not pursued further.

The largest change in NGF-responsiveness occurred following the deletion of a 68 base pair region between positions -315 and -247, in which the fold NGF-induction fell from approximately 8-fold to about 1.5-fold (averaging the results of the four experiments). In contrast, this deletion produced no significant differences in either basal levels or EGF-induced levels of CAT expression. Further deletions in the ST-1 promoter had only a slight effect on NGF-responsiveness. However, removal of the region between -247 and -228 resulted in a small but significant increase in EGF-responsive CAT expression, indicating the possible presence of a growth factor-specific silencer region. This experiment was performed three times with qualitatively similar results.

These results indicate that the region between -315 and -247 bp upstream of the ST-1 transcription start site contains sequences that are required for NGF-responsiveness.

Gel mobility shift experiments using various regions of the ST-1 promoter

DNA sequences which function as regulatory elements are likely to bind nuclear proteins (227). To determine whether the region between positions -247 and -315 would bind such a protein, gel mobility shift assays were performed. As a probe for these experiments, we first generated a polymerase chain reaction (PCR) fragment which included the region between -247 and -315 plus significant portions of 5' and 3' flanking DNA (labeled in Fig.3A "NRR-ABC" for "NGF-Responsive Region") to insure that this probe would contain the entire protein-binding site. PC12 cells were then treated for 2 hours in the presence or absence of NGF, and then nuclei were isolated and incubated with ^{32}P end-labeled probe, allowing protein/DNA complexes to form. Fig. 3B is an autoradiograph of a non-denaturing gel loaded with these binding reactions showing that, in the absence of nuclear proteins, no shifted complexes were present (lane 1). When nuclei from either control or NGF-treated PC12 cells were used, several shifted bands were visible (lanes 2 and 3). The arrow at the left indicates the most heavily labeled band, which was subsequently found to be the only one which displayed specific and saturable DNA/protein binding.

To dissect this protein binding region further, the NRR-ABC fragment was digested with *Hae*III and *Mae*III restriction enzymes, which cut the fragment into 3 similarly sized pieces (termed NRR-A, NRR-B and NRR-C; see Fig. 3A). Each of these pieces were then end-labeled and used in gel mobility shift assays, as described above. Lanes 4-9 of Fig. 3B show that neither the NRR-A nor NRR-B fragments produced shifted protein/DNA complexes, whereas the NRR-C fragment did with nuclei from

either control or NGF-treated PC12 cells (Fig.3B, lanes 10-12). This experiment was performed three times with qualitatively similar results. These results suggest, therefore, that a region within the NRR-C fragment binds DNA-binding protein(s), and that this protein(s) is present constitutively.

To determine whether these DNA/protein interactions were specific and saturable, competition gel shift assays were performed. In these experiments, molar excesses of unlabelled DNA corresponding either to the full length NRR-ABC or each of the three fragments was included in the incubation mixture along with radiolabelled probe. Figs. 4A and 4B are competition mobility shift assays using the full length NRR-ABC with nuclei isolated from untreated and NGF-treated cells, and show that in either case, only the full length NRR-ABC and the NRR-C could compete for the shifted complex, and this competition was apparent when molar excess of as little as 25X unlabelled-to-labeled DNA was used (lanes 3-6 and 15-18). Figs. 4C and 4D are the converse experiment and show that the NRR-C radiolabelled probe was effectively competed for binding with both NRR-ABC and NRR-C unlabelled DNA. And again, this competition was seen when using nuclei from either NGF-treated or control PC12 cell cultures. This experiment has been performed three times with qualitatively similar results. Several other non-specific oligonucleotides were also used as competitors and none were able to disrupt binding of nuclear protein(s) to the NRR-ABC (data not shown). These results are consistent, therefore, with the notion that a specific and saturable protein binding element can be found within region NRR-C, and that the protein recognizing this element is present in the nuclei of both NGF-treated and -untreated PC12 cells. Interestingly, the NRR-B fragment contains a 9 base pair palindromic sequence centered around position -268 (Fig. 1A, single underlined sequence). When an oligomer corresponding to this region was used in competition gel mobility shift assays, however, it was also unable to compete with the NRR-ABC probe for binding (data not shown), consistent with the notion that the NRR-B fragment does not contain this regulatory element.

Since the NRR-C contains a large segment of DNA flanking the original deletion at position -247 (see Fig. 2A), it seems likely that the protein binding element would be found near this position in the 5' half of the NRR-C fragment. To test this assumption, additional competition mobility shift assays were performed using a 30 bp synthetic oligonucleotide that corresponded to the first 30 bp of NRR-C. Fig.5A shows that this oligonucleotide (termed NRR-C') competed with radiolabeled NRR-C for binding to nuclear proteins in both control (lanes 3 - 7) and NGF-treated (lanes 9 - 13) PC12 cells. The arrow points to the shifted complex that was specifically competed by NRR-C' and the asterisk indicates another complex which is not competed with unlabelled DNA. Fig.5B shows that the NRR-C' oligonucleotide, when used as a labeled probe, could also bind nuclear proteins in both NGF-treated and untreated PC12 cells, indicating that this 30 bp region of the ST-1 promoter contains the binding site(s) for nuclear proteins from PC12 cells. These experiments were performed three times with qualitatively similar results.

To further localize the NGF-responsive element within the 30 bp region, we performed site-directed mutagenesis. For these studies, 5 mutant plasmids were first generated using the Altered Sites™ system (Promega) containing contiguous 6 bp mutations spanning the NRR-C' region (i.e., see Fig.6A). The NRR-ABC region of each of these mutant plasmids was then isolated and used in gel mobility shift assays as competitors. Fig.6B shows that m1, m2 and m3 fragments were able to compete for protein binding with radiolabelled wild type probe (lanes 5-10), whereas m4 and m5 fragments were not (lanes 11-14), suggesting that the region of the promoter necessary for protein binding is present within a 12 base pair region at the 3'-end of the NRR-C' fragment (see the bold underlined region between positions -241 and -229 of Fig.1A). Qualitatively similar results were seen in assays using nuclei from non-NGF-treated cultures (data not shown). Interestingly, gel mobility shift assays using radiolabelled probes corresponding to each of these mutated fragments showed that all five were able

to bind nuclear proteins to some extent in the absence of any competitor DNA (data not shown). This suggests that the mutations in m4 and m5 reduced, rather than abolished, protein binding affinity.

Heterologous promoter experiments using the NRR-C fragment

The mobility shift assays and 5' deletion analysis show that an element within the NRR-C region is necessary for NGF-responsiveness. To show that this element is also sufficient for NGF-responsiveness, the NRR-C was cloned into a vector containing the basal thymidine kinase promoter (TKp), with an added AP1 site, driving expression of the CAT reporter gene. The NRR-C was inserted in either the forward "AS{NRR–C(F)}" or reverse "AS{NRR-C(R)}" alignment in order to determine whether it was equally active in either orientation. PC12 cells were then transiently transfected with these plasmids or the parental AS plasmid, treated for 24 hours with culture medium containing either NGF or EGF, and then the cultures were harvested for CAT enzymatic activity. Fig. 7 shows that a single copy of the NRR-C region in the forward orientation was sufficient to induce a two-fold increase of CAT activity by NGF, but not EGF. In the reverse orientation, however, this region failed to induce a statistically significant increase in CAT activity by either NGF or EGF in the three times this experiment was performed. Similar results were obtained using the NRR-ABC fragment in either the forward or reverse orientation (data not shown). Together, these results indicate that the NRR-C region is both necessary and sufficient to function as an orientation-specific NGF-responsive element.

DISCUSSION

NGF has been shown to be critical for the normal development and maintenance of the nervous system in the embryo and for the survival of neurons in the adult, presumably via its effects on gene expression (212, 228). While many of the initial events in the NGF signaling pathway have been identified (77, 78, 94, 96, 109, 111, 112, 118-120), little is known about nuclear events that regulate growth-factor specific changes in gene expression.

In this paper, we identify a 60 bp NGF-responsive region in the ST-1 promoter that is both necessary and sufficient for NGF-responsiveness. When this region was deleted from the promoter, the remaining portions failed to display NGF-inducibility; when this region was placed next to a heterologous promoter, it was sufficient to confer NGF-inducibility. The orientation of this 60 bp region was also found to be important for heterologous gene inducibility, since experiments reversing the orientation of this region failed to display significant levels of NGF-induction. This is consistent with the fact that this 60 bp region lacks palindromic sequences of appreciable length, which are often associated with regulatory elements which have biological activities in either the forward or reverse orientations. Using various mutants of this region, we showed that the protein binding site most likely included sequences within a 12 bp region of the ST-1 promoter. Mobility shift assays confirmed, moreover, that this region bound a nuclear protein or proteins expressed by PC12 cells. Interestingly, this protein(s) seemed to be expressed in both untreated and NGF-treated cells, since the mobility shift patterns of nuclei isolated from both treatments were indistinguishable. These similar mobility shift patterns may also indicate that the binding of accessory proteins to the preformed complex is not necessary for ST-1 induction. If this were the case, one might have expected super-

shifted bands or other differences in the patterns of bands between untreated and NGF-treated cells. Alternatively, post-transcriptional modifications of this nuclear protein(s), such as phosphorylation, may be associated with the mechanism of ST-1-induction by NGF. This is consistent with observations that protein kinase A or C activators, such as forskolin and phorbol esters, augments the NGF-induction of ST-1 in PC12 cells (208). The notion that phosphorylation is involved in the NGF-induction of ST-1 is also consistent with observations that activation of the cytoplasmic kinases src, ras, and raf seem to be necessary for the NGF-induction of neuronal differentiation in PC12 cells, including the expression of ST-1 (90-93, 229). These proto-oncogenes are believed to act as upstream regulators of the MAP kinase cascade leading to the phosphorylation of critical regulatory proteins in the nucleus (230-232).

Several other NGF-responsive elements have also been described, but all differ significantly from sequences present in the ST-1 promoter region described here. These NGF-responsive elements include a fat-specific element (TH-FSE) in the tyrosine hydroxylase promoter that binds c-fos as part of the nucleoprotein complex (158) and a unique novel negative regulatory element in the peripherin gene (160). In this latter case, derepression of peripherin gene expression also involved a less well-defined "distal positive element" within a fairly large 370 bp region of the promoter. These studies did not, however, test whether these elements conferred NGF-responsiveness to a heterologous promoter. This analysis was included, however, in a recent report of a 50 bp NGF-responsive region of the neuropeptide Y promoter (161). This NGF-responsive region in the neuropeptide Y promoter also bound a transcription factor present in both untreated and NGF-treated PC12 cells and the patterns of shifted bands was similar in NGF-treated and untreated cultures. Comparison of the neuropeptide Y and the ST-1 NGF-responsive regions showed no significant sequence similarities, which may indicate that the transcription factors recognizing the two NGF-responsive elements also differ significantly. Although the 12 bp sequence in the ST1 promoter contains a

relatively high A+T content, it doesn't seem to correspond to known homeodomain binding protein consensus sequences, which are typically A+T rich (233).

We also report here that the AP1 site at position -71 regulates basal levels of ST-1 expression, at least in these transient transfection assays. Promoter constructs with point mutations in the AP1 site show, for example, large reductions in basal levels of CAT expression in transiently transfected PC12 cells, yet these cells still displayed statistically significant NGF-inducibility. Although the absolute levels of this induction were somewhat attentuated with the mutated AP1 site, the relative levels of NGF-inducibility remained unaffected in cells transfected with the mutated AP1 site. Further evidence that the AP1 site is not involved in NGF-responsiveness is that c-fos and c-jun, two transcription factors which bind to this site, are also induced by EGF in PC12 cells, yet EGF fails to elicit ST-1 expression (126, 128, 129, 132, 136). In any case, it should be noted that basal levels of endogenous ST-1 gene expression in PC12 cells are normally non-detectable (154, 208), and therefore, the notion that the AP1 site regulates basal level of expression may only apply to the truncated (i.e., 753 bp) ST-1 promoter region used in these studies.

In contrast to PC12 cells, the AP1 site in rat-1 fibroblasts seems to play a critical role in the induction of ST-1 gene expression by both EGF and PDGF (191). The AP1 site was also implicated in ST-1 gene expression in a polyomavirus-transformed rat embryonic cell line, in which the negative regulation of ST-1 mRNA levels by retinoic acid was shown to be mediated by the same AP1 site (203). The fact that NGF-induction of ST-1 gene expression in rat PC12 cells does not seem to be dependent on the AP1 site would suggest, therefore, that the tissue-specific mechanisms of regulation differ significantly. On the other hand, it is interesting to note that phorbol esters, though they cannot induce ST-1 themselves, can augment the NGF-induction of ST-1 in PC12 cells

(208). If this phorbol ester-augmentation is mediated via the AP1 site, it would suggest that this element acts to modulate, rather than activate, gene expression.

The progressive deletion mutants of the ST-1 promoter also allowed us to determine the contribution of other potential transcriptional elements to NGF-inducibility. There is, for example, an NGFI-A site at position -400 in the ST-1 promoter. This site is believed to bind the NGF-IA protein (also known as zif 268, Egr-1, Krox 24 and Tis 8), which is a zinc-finger DNA binding protein rapidly induced by either NGF or EGF in PC12 cells (128, 133, 135). Removal of this site, however, was found not to have an appreciable effect on the NGF-inducibility of CAT expression in transient transfection assays, suggesting that this NGFI-A site is not involved. This conclusion is consistent with earlier observations in which addition of the kinase inhibitor staurosporine to PC12 cells was found to have little effect on NGF-induction of the NGFI-A, yet completely blocked the NGF-induction of ST-1 mRNA expression and neurite extension (208).

In sum, we have shown that the ST-1 gene regulation by NGF is mediated by an element present in a 12 bp region of the ST-1 proximal promoter. Future studies characterizing the core element within this region, as well as studies identifying transcription factors responsible for mediating activation of the transcription apparatus, should provide important information about how the biological effects of NGF are manifested at the level of gene expression in the nervous system.

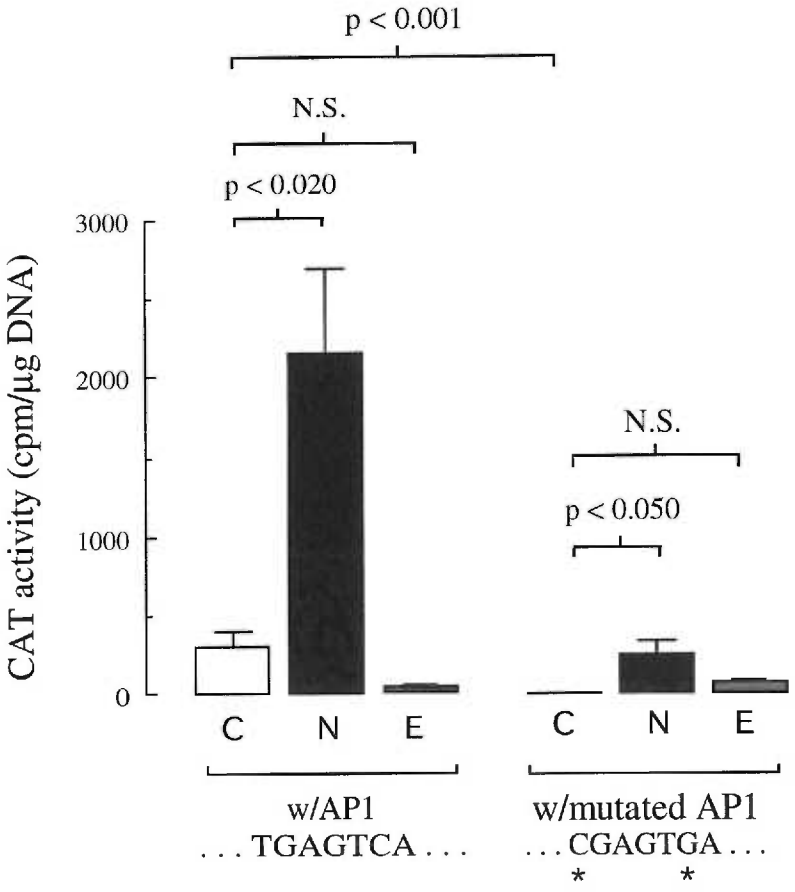

FIGURE 1: The AP1 site is not directly involved in the NGF-responsiveness of the ST-1 promoter. PC12 cells were transiently transfected with a plasmid containing 750 bp of the ST-1 promoter driving expression of the reporter gene, chloramphenicol acetyl transferase (CAT) containing the native AP1 site, or with a plasmid in which two base changes were made in the AP1 site of the ST-1 promoter (indicated by asterisks). CAT enzymatic activity was assayed for PC12 cells that were either untreated (C; open bars), treated with 50 ng/ml NGF (N; black bars), or 5 ng/ml EGF (E; stippled bars). Results from student t test analysis between bracketed samples is indicated. N.S. = not statistically significant.

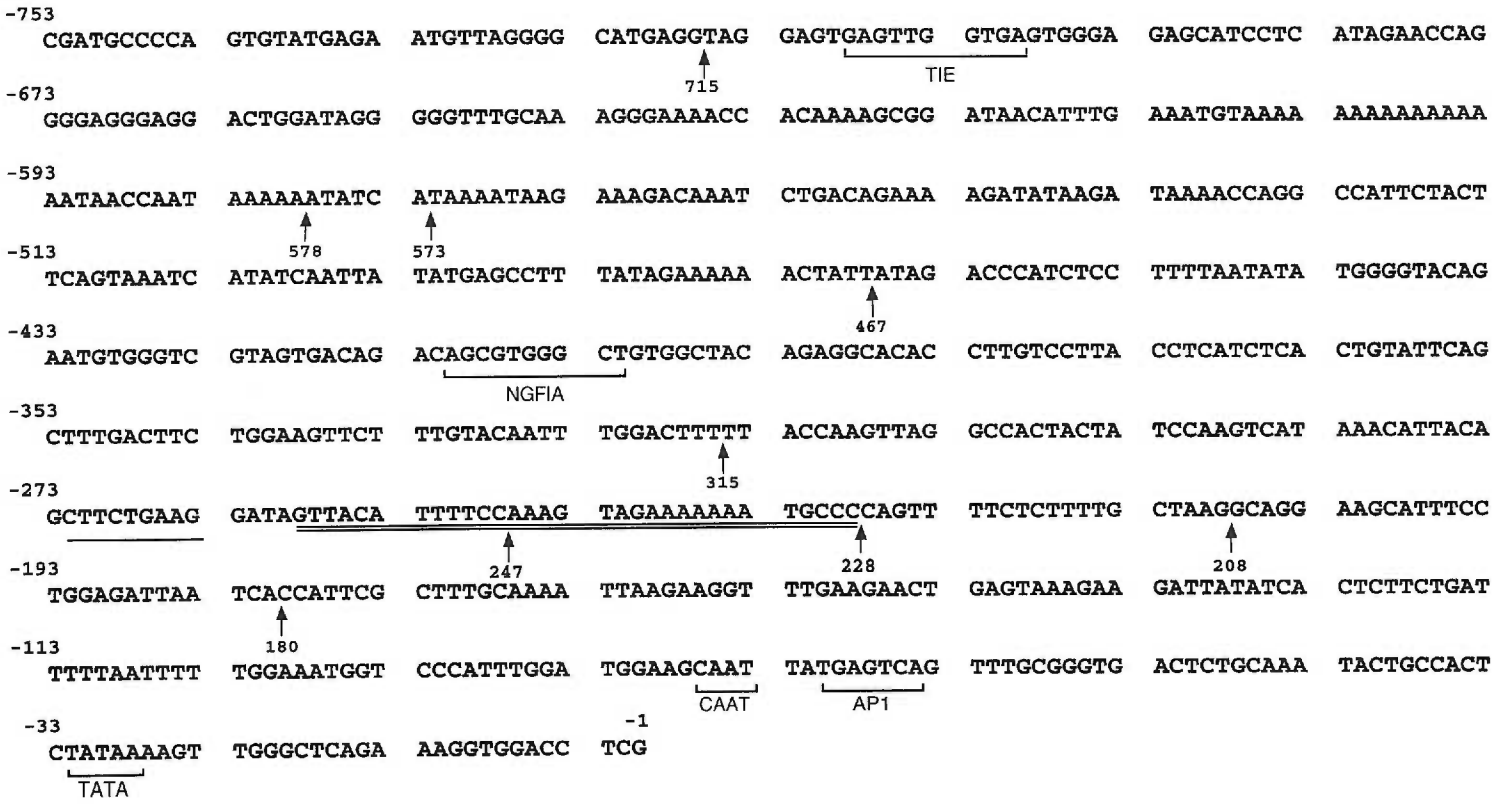

FIGURE 2A: Sequence of the ST-1 promoter numbered with respect to the transcription start site. Putative regulatory elements are bracketed and include a TIE (TGF-β inhibitory element), NGF-IA (also known as zif 268, tis 8), CAAT box, TATA box and AP1 site. The single underlined sequence corresponds to a 9bp palindromic sequence. The double underlined sequence corresponds to the 12 bp sequence that contains at least a portion of the NGF-responsive element. The arrows and numbers below the sequence refer to the various 5'-deletions analyzed for promoter activity in Fig.2B.

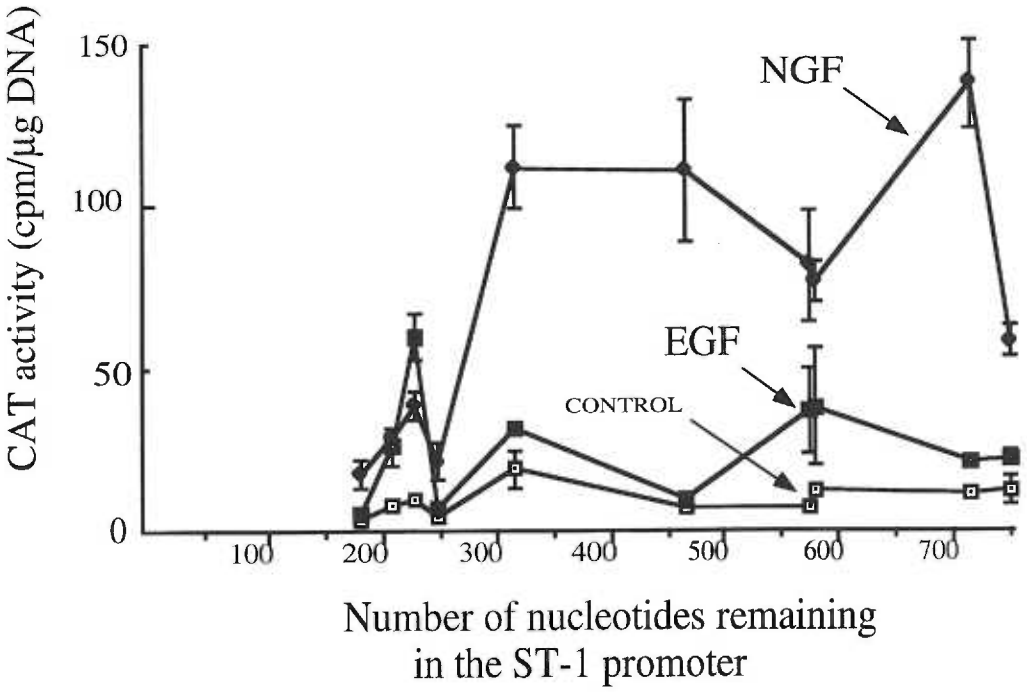

FIGURE 2B: The region between positions -247 and -315 in the ST-1 promoter is necessary for NGF-responsiveness. PC12 cells were transiently transfected with promoter deletion plasmids driving the CAT reporter gene as described in "Materials and Methods". Cultures were then incubated with medium in the absence (control; open squares) or presence of either NGF (50 ng/ml; closed circles) or EGF (5 ng/ml; closed squares) for 24 hours. All cultures were co-transfected with a plasmid containing the luciferase gene driven by the Rous sarcoma virus promoter. The results presented here have been normalized against luciferase levels. Note that there is a significant decrease in NGF-induced CAT activities between positions -315 and -247. This experiment has been repeated three times with qualitatively similar results.

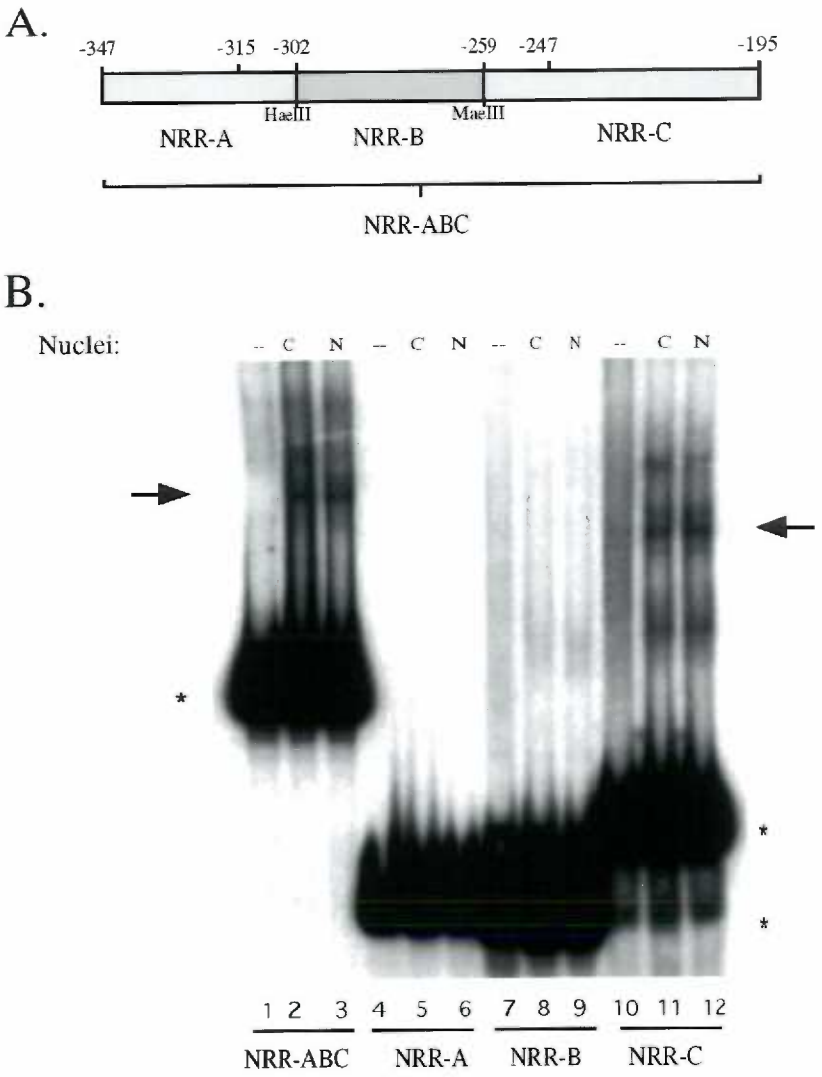

FIGURE 3: Nuclear protein(s) bind to a 60 bp NRR-C region of the ST-1 promoter. Panel A, schematic of the NRR-ABC fragment containing the region between positions -315 and -247 of the ST-1 promoter plus flanking DNA. Also diagrammed with different fill patterns are the 45 base pair NRR-A, 43 base pair NRR-B, and 60 base pair NRR-C restriction fragments. Panel B, gel mobility shift assay using different regions of the ST-1 promoter. DNA fragments were first end-labeled with ^{32}P and then incubated with nuclei from either untreated (C) or 2 hr NGF-treated (N) PC12 cells and electrophoresed in a 6% acrylamide gel under non-denaturing conditions as described in "Materials and Methods". Note that, in the absence of nuclei, all of the label is present at a single band (asterisks), but in the presence of nuclei from either untreated or NGF-treated cells, the NRR-ABC (lanes 1-3) and NRR-C fragments (lanes 10-12) produce shifted complexes containing multiple bands (arrows).

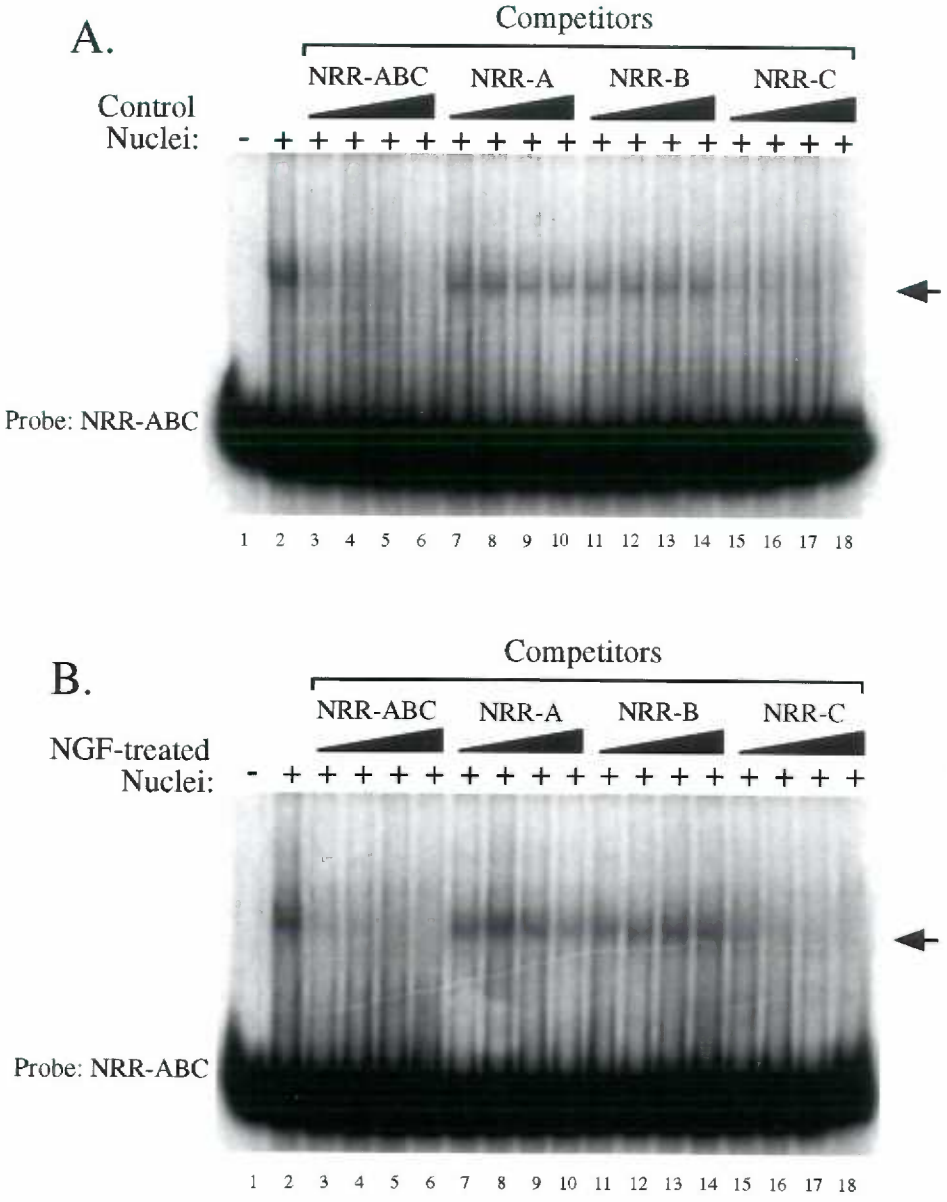

FIGURE 4A and 4B: Binding of nuclear protein(s) from both untreated and NGF-treated PC12 cells to the NRR-ABC is specific and saturable. ^{32}P-labeled NRR-ABC was incubated with nuclei from either untreated (panel A) or NGF-treated (panel B) PC12 cells and electrophoresed as described in "Materials and Methods". Competitor non-labeled DNA (NRR-ABC, NRR-A, NRR-B and NRR-C) was included in the binding reactions at increasing amounts of 25-, 50-, 100-, and 200-fold molar excess over labeled probe. Relative concentrations of competitor DNA are represented by the ramps at the top of each gel. The arrows at the right identify the shifted complexes which showed specific and saturable DNA/protein binding.

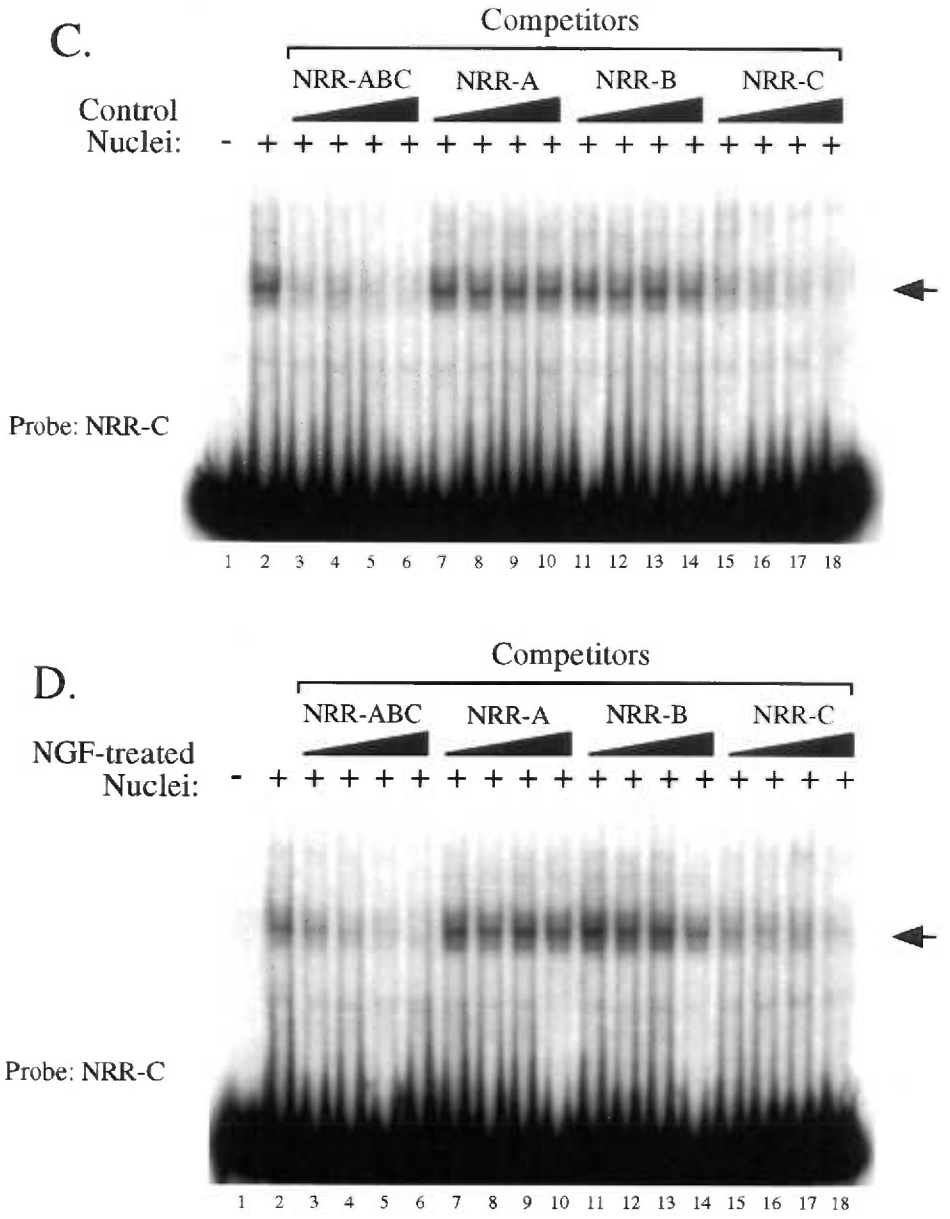

FIGURE 4C and 4D: Binding of nuclear protein(s) from both untreated and NGF-treated PC12 cells to the NRR-C is specific and saturable. ^{32}P-labeled NRR-C was incubated with nuclei from either untreated (panel C) or NGF-treated (panel D) PC12 cells and electrophoresed as described in "Materials and Methods". Competitor non-labeled DNA (NRR-ABC, NRR-A, NRR-B and NRR-C) was included in the binding reactions at increasing amounts of 25-, 50-, 100-, and 200-fold molar excess over labeled probe. Relative concentrations of competitor DNA are represented by the ramps at the top of each gel. The arrows at the right identify the shifted complexes which showed specific and saturable DNA/protein binding.

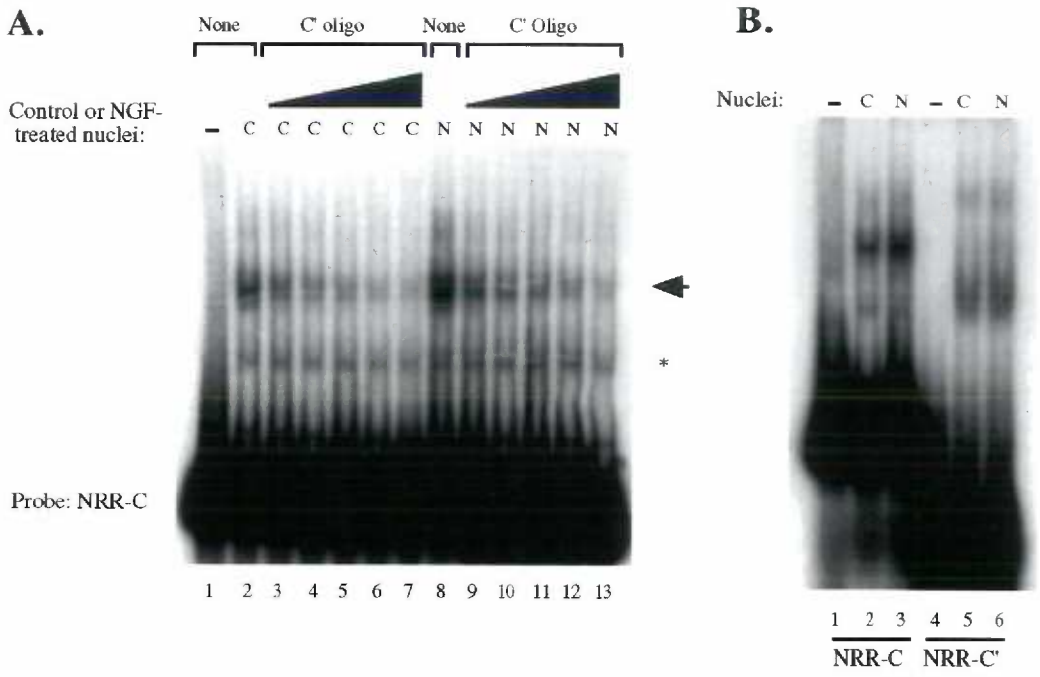

FIGURE 5: The region between -229 to -259 of the ST-1 promoter contains the nuclear protein binding site. Panel A, ^{32}P-labeled NRR-C was incubated with nuclei from either untreated (C; lanes 2-7) or NGF-treated (N; lanes 8-13) PC12 cells and electrophoresed, as described in "Methods and Materials". Competitor unlabelled synthetic oligonucleotides corresponding to the NRR-C' region of the ST-1 promoter (underlined sequence in Fig.1A) was included in the binding reactions at increasing amounts of 25-, 50-, 100-, 200-, and 400-fold molar excess over labeled probe as indicated by the ramps on the top of the gel. The arrow on the right points to the shifted protein-DNA complex that is specifically competed out by the NRR-C'. The asterisk (*) indicates a shifted complex which is not competed out by NRR-C'. Panel B, ^{32}P-labeled NRR-C (lanes 1 - 3) or radiolabeled NRR-C' (lanes 4 - 6) were mixed with nuclei from either untreated (C) or NGF-treated (N) PC12 cells and electrophoresed as described in "Methods and Materials".

A.

wild type	GTTACA	TTTTCC	AAAGTA	GAAAAA	AATGCC
m1	**TAGTAC**	TTTTCC	AAAGTA	GAAAAA	AATGCC
m2	GTTACA	**AGACTG**	AAAGTA	GAAAAA	AATGCC
m3	GTTACA	TTTTCC	**GTATCT**	GAAAAA	AATGCC
m4	GTTACA	TTTTCC	AAAGTA	**TTACTG**	AATGCC
m5	GTTACA	TTTTCC	AAAGTA	GAAAAA	**GCCTGC**

B.

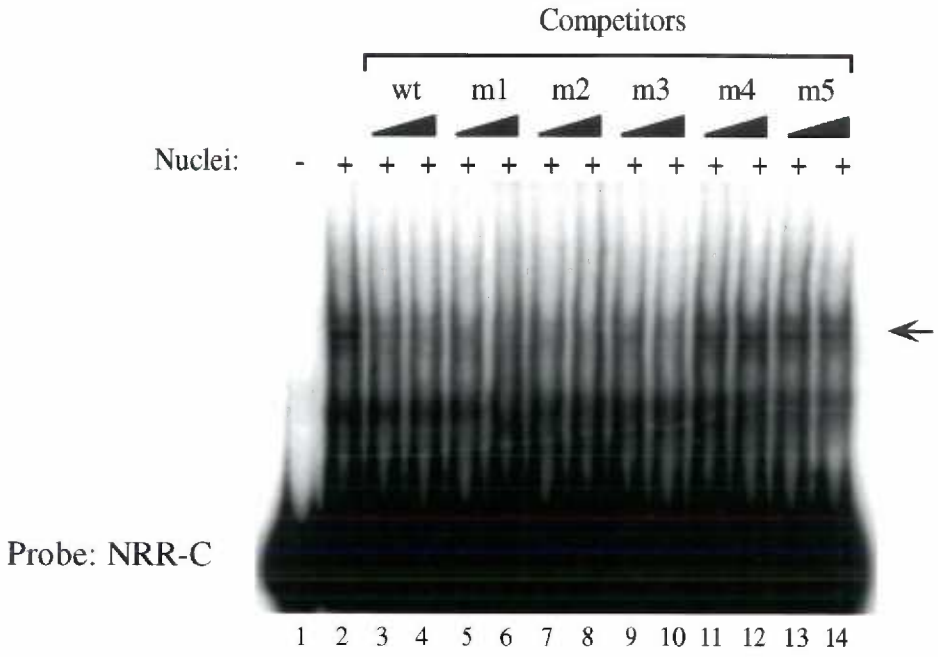

FIGURE 6: Competition gel mobility shift assay showing that the 3'-end of the 30 bp sequence is necessary for nuclear protein binding. Panel A, DNA sequences of the wild type and 5 mutated forms of the 30 bp NRR-C' region which were used as competitors in the experiment represented in panel B. Panel B, ^{32}P-labeled NRR-C was incubated with nuclei from NGF-treated PC12 cells and electrophoresed, as described in "Methods and Materials." Competitor unlabelled NRR-ABC fragments from either wild type (wt) or mutant plasmids were included in the binding reactions at 25- or 100-fold molar excesses over labelled probe as indicated by the ramps at the top of the gel. The arrow on the right points to the shifted protein-DNA complex. Note that wild type and m1, m2 and m3 were able to compete for nuclear protein binding, but that m4 and m5 were not.

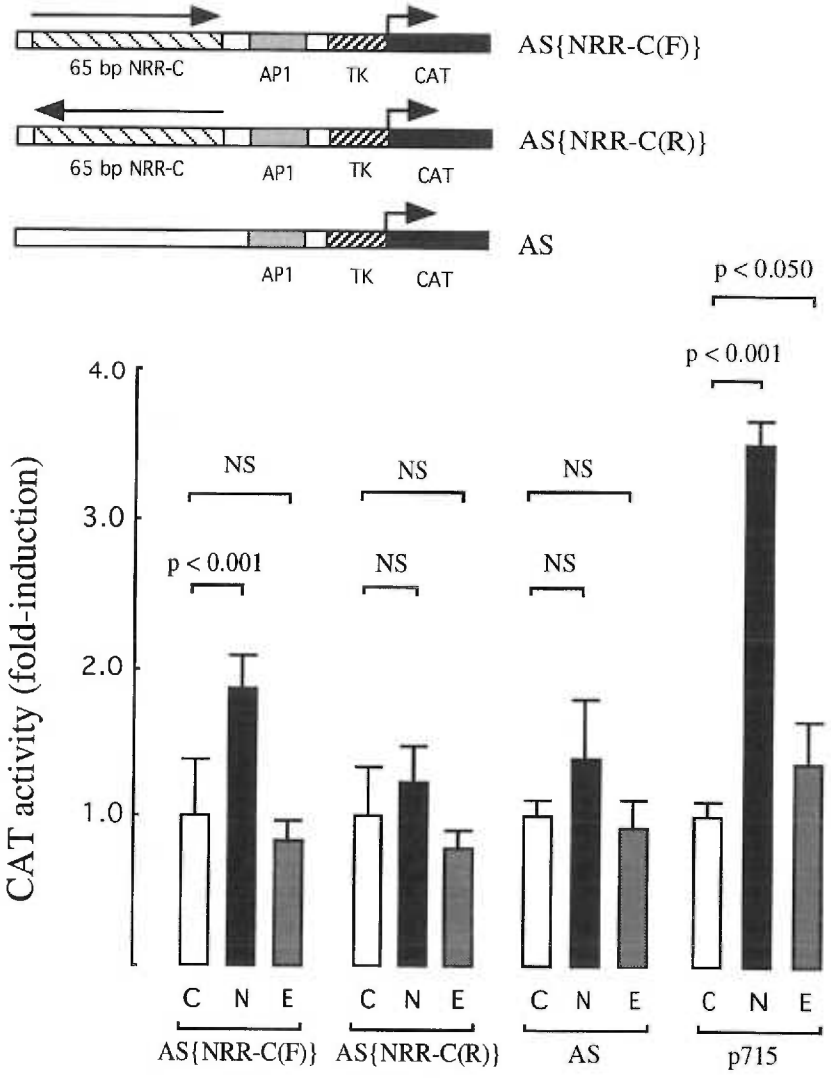

FIGURE 7: The NRR-C region of the ST-1 promoter is confers NGF-responsiveness to a heterologous promoter. PC12 cells were transiently transfected with a plasmid containing the NRR-C in either forward AS{NRR-C(F)} or the reverse AS{NRR-C(R)} orientation upstream of the basal thymidine kinase (TK) promoter driving expression of the reporter gene CAT. As a negative control, cells were transfected with the pAS vector alone. The p715 vector was used as a positive control. CAT enzymatic activity was assayed for PC12 cells that were either untreated (C; open bars), treated with 50 ng/ml NGF (N; black bars), or 5 ng/ml EGF (E; stippled bars). Results from student t test analysis between bracketed samples is indicated. N.S. = not statistically significant (i.e., $p > 0.05$).

CHAPTER III

RESULTS

Role of the bZIP Transcription Factor IREBF1 in the NGF-induction of Stromelysin-1 (Transin) Gene Expression in PC12 cells.

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in September, 1996

ABSTRACT

Stromelysin-1 (ST-1) is one of the most NGF-responsive gene products expressed in PC12 cells. In previous work, we identified a novel NGF-responsive element in the proximal promoter region of the ST-1 gene that participates in this induction, and showed that it bound a protein present in the nuclei of PC12 cells. Here, we identify a transcription factor that specifically recognizes this regulatory element -- IREBF1 -- a member of the basic leucine zipper gene family. We show that IREBF1 is constitutively expressed in PC12 cells and that over-expression of IREBF1 augments NGF-responsive ST-1 gene regulation, but does not affect basal levels of expression. On the other hand, expression of a mutated form of this transcription factor lacking the DNA binding domain attentuated NGF-responsiveness, without affecting basal levels of expression. These data suggest that IREBF1 is part of the NGF-responsive transcriptional machinery necessary for the expression of ST-1 in PC12 cells.

INTRODUCTION

Nerve growth factor (NGF) plays a critical role in the the normal development of the nervous system and supports the survival of neurons in the adult (36, 212, 228). Although some of NGF's effects on neuronal cells are direct, such as the induction of membrane ruffling at the growth cone (234) and changes in the activity of the Na^+/K^+ pump (235), most of the long-term effects of NGF are believed to result from changes in gene expression (70, 71).

One widely used model for studying NGF effects on gene expression has been the PC12 rat pheochromocytoma cell line. In response to NGF, PC12 cells acquire the properties of sympathetic neuron-like cells, including neurite outgrowth, increased electrical excitability, and changes in neurotransmitter synthesis (66). Binding of NGF to its cognate receptor, the *trk* tyrosine kinase, first activates the *ras/raf* intracellular signaling pathway in PC12 cells, which in turn, is believed to activate a MAP kinase cascade (for review see (236, 237). Expression of constitutively active forms of *ras* and *raf*-1, for example, induce ST-1 in the absence of NGF (89), and expression of dominant-negative forms of *ras* or ERK-1 block the NGF induction of ST-1 in PC12 cells (92). MAP kinase activation by NGF is believed to result in the induction of an early set of gene products which includes a number of general transcription factors, such as *c-fos*, *c-jun*, *jun* B, zif268 (126, 128, 136), as well as other proteins influencing general DNA organization, such as ornithine decarboxylase (147, 238). In some cases, it has been shown that MAP kinase-mediated phosphorylation of constitutively expressed transcription factors is directly involved in changes in immediate-early gene regulation (230, 232, 239). In any event, the mRNAs encoding these gene products appear within minutes of NGF-addition and do not require on-going protein synthesis.

After several hours of NGF-treatment, PC12 cells then begin to transcribe a second wave of gene products which require ongoing protein synthesis, and which are more specific to their neuronal phenotype. These "late" gene products include tyrosine hydroxylase (67), neuropeptide Y (152), peripherin (143), brain type-II Na^+ channels (145), and VGF (150). One continuing issue in the field concerns how expression of the immediate-early genes is linked to that of late gene expression. It seems clear that the translation of some immediate early gene product is necessary for late gene expression, since the addition of protein synthesis blockers, such as cycloheximide, block the appearance of late gene mRNAs in PC12 cells (68, 149, 208). However, the identity of these gene products is unclear. It is known, for example, that epidermal growth factor (EGF) induces the same set of immediate-early genes in PC12 cells as does NGF (126), and yet does not induce expression of the same late gene products (67, 149, 150). Although it has been suggested that the duration of the intracellular signal elicited by these two growth factors is likely to be responsible for aspects of this specificity (124), it is still unclear which immediate-early gene products are directly involved in the induction of NGF-specific late genes, and which ones function in other aspects of growth factor signaling.

In previous work, we showed that stromelysin-1 (ST-1) (also known as transin) is one of the most highly NGF-induced late gene product in PC12 cells (154). ST-1 is a member of the matrix metalloproteinase family of endopeptidases (163) whose substrates include various components of basal laminae, including fibronectin, laminin, collagen type IV and proteoglycans (169, 220, 240). ST-1 has been implicated in tissue remodelling events that occur during uterine implantation of the morula, as well as in several pathological conditions, such as arthritis (181) and tumor metastasis (184). In recent work, we have shown that ST-1 is also widely expressed in the normal developing nervous system of rat embryos and may play a role in the invasive behaviors exhibited by growth cones during axonal elongation (175).

We also recently examined the proximal promoter region of the ST-1 gene for *cis*-acting elements responsible for the effects of NGF, and found that at least two elements were involved -- an AP1 site at position -77 and a novel element further upstream (241). Transient transfection assays using the proximal ST-1 promoter driving expression of the chloramphenicol acetyltransferase (CAT) reporter gene showed that site-directed mutants of the AP1 site decreased basal levels of CAT expression, but did not seem to affect NGF-induced reporter gene activity. Using a variety of approaches, we then narrowed down this NGF-responsive region (NRR) to a 30 base pair sequence between -259 and -229, and showed that it was both necessary and sufficient for the NGF-regulation of ST-1 gene expression. Mobility shift experiments showed, moreover, that a nuclear protein from PC12 cells bound to the NRR in a specific and saturable manner (241).

In this paper, we examine the identity of this nuclear protein. We show that one candidate is a previously identified transcription factor of the basic leucine zipper gene family called interferon response element binding factor-1 (IREBF1).

METHODS AND MATERIALS

Cell culture and transient transfection assays

Stock cultures of PC12 cells were maintained as described previously (241). For transient transfection assays, 35 μg of total plasmid DNA was transfected into PC12 cells using calcium phosphate precipitates (222). Cells were treated with these DNA precipitates for 4 h and then were "shocked" for 2 min with 15% glycerol in HEPES-buffered (25mM, pH 7.0) isotonic saline solution. The cultures were rinsed twice with phosphate-buffered isotonic saline (PBS) and allowed to recover overnight in 10% serum-containing medium. Cells were then serum-deprived for 24 h in "N2-supplemented" medium (223) before NGF (100ng/ml) was added for an additional 24 h prior to harvest. A kinetic assay using [^{3}H]acetyl coenzyme A was used to measure chloramphenicol acetyltransferase (CAT) activities (225).

Recombinant protein production

To generate the wild type (wt) IREBF1 recombinant protein, the 1.0 kilobase (kb) coding region of IREBF1 from clone 25 (242) was ligated into the BglII/EcoRI site of the pTrcHisB prokaryotic expression vector, which adds 9 histidines to the amino terminus (Invitrogen, San Diego, CA). Plasmid DNA constructs were verified by automated sequencing (Applied Biosystems, Foster City, CA). Bacteria (HB101) were transformed with this plasmid and induced with isopropylthio-β-D-galactoside (IPTG) for 4 hours. Recombinant IREBF1 protein was purified from homogenates of these bacteria over a Ni^{2+} column, following the manufacturer's instructions (Invitrogen).

To generate the dominant-negative (dn) IREBF1 protein, 2 Not1 sites were introduced into the IREBF1 plasmid by PCR mutagenesis (30 cycles, 50°C annealing temperature) using T7, SP6 and the following internal primers: 5 '- GCGAAAGTTGCTTATAGTATTGCGGCCGCCCGA TTCAAACTATCATTG - 3' and 5' - CTTGGTGTGGGTGAGAAAGCGGCCGCAT CAATCAAAAAGGAATTA - 3' (the Not1 sites are underlined). Following Not1 endonuclease digestion, the plasmid was allowed to religate, resulting in an insert lacking the 38 amino acid DNA-binding domain (see Fig.1). Again, bacteria were transformed with the plasmid, induced with IPTG, and recombinant dn-IREBF1 protein was purifed over a Ni^{2+} column.

For the expression studies in PC12 cells, the coding sequences of both the wt- and dn-IREBF1 inserts (1.0 kb and 0.9 kb, respectively) were removed from the pTrcHis vectors by digestion with BamHI and EcoRI and re-ligated into the pcDNA3 expression vector (Invitrogen), which contains the cytomegalovirus strong constitutive promoter.

IREBF1 Antibodies

Anti-IREBF1 antibodies were generated in laying hens (Flock Antibodies, Seattle). The initial injection was a 1:1 mixture of 50 μg of wt-IREBF1 protein in phosphate buffered isotonic saline (PBS) with complete Freund's adjuvant. Four weeks later, the hens were boosted with a 1:1 mixture of wt-IREBF1 protein with Incomplete Freund's adjuvant. Chicken IgY antibodies were purified from the yolks of the eggs following the method of Poulson (243). Anti-IREBF1 activities were monitored by enzyme-linked immunoabsorbent assays using alkaline phosphatase-labelled goat anti-chicken IgY (Accurate Antibodies, Westbury, NY).

Western Blot Analysis of PC12 cells

Homogenates were prepared from PC12 cell nuclei as described (241, 244) and subjected to electrophoresis on a 15% sodium dodecylsulphate (SDS)-polyacrylamide gel. The proteins were then transferred electrophoretically overnight onto a nitrocellulose membrane, and non-specific binding sites on the membrane blocked with PBS containing 0.1% (v/v) Tween 20 (PBS-Tween) supplemented with 10% (v/v) nonfat dry milk for 1 h. The nitrocellulose blot was then washed with PBS-Tween and incubated for 1 h in a solution containing anti-IREBF1 antibodies (1:1000 dilution in PBS-Tween). After several washes, immunoreactive material was visualized using rabbit anti-chicken secondary antibody conjugated to alkaline phosphatase (Jackson Labs, Bar Harbor, ME) (1:5000 dilution in PBS-Tween).

Gel mobility shift and super shift assays

Gel mobility shift assays were performed as described previously (241) except that the concentration of poly(dI-dC) in the binding reaction was reduced to 5 μg and no bovine serum albumin was present. For supershift assays, varying amounts of anti-IREBF1 and preimmune antibodies were also included in the binding reaction. Both the shift and super shift binding reactions were subjected to electrophoresis in non-denaturing polyacrylamide gels at 4°C in 0.5X TBE (50mM tris-HCl, pH 8.3, 41 mM boric acid, 0.5 mM EDTA). Gels were dried, and radiolabeled bands were visualized by autoradiography.

RESULTS

Identification of IREBF1 as a transcription factor capable of binding the NRR

Approximately 3 x 10^6 plaque-forming units from a rat PC12 cell λgt11 cDNA library (Clontech, Palo Alto, CA) were screened using a ^{32}P-labelled concatemer of the 30 base pair NGF-responsive region (NRR) of the ST-1 promoter, following standard methods (245). Twelve positive clones from this screen were then purified to homogeneity through four subsequent rounds of screening. Nine out of the 12 clones showed specificity for the NRR sequence by failing to bind a ^{32}P-labelled concatemer of the cAMP responsive element. DNA sequence analysis of the inserts showed that 2 of the 9 clones (D2 and V') were overlapping regions of a single open reading frame corresponding to a previously identified mouse transcription factor called interferon response element binding factor-1 (IREBF1) (242). IREBF1 is a member of the basic/leucine zipper family of transcription factors and contains an acidic domain, three heptad repeat leucine arrays, and a region that shares similarity with the yeast transcriptional factor GAL4 DNA-binding domain (see Fig.1).

To confirm that IREBF1 protein could specifically recognize the NRR sequence, we performed gel mobility shift assays using full-length recombinant IREBF1 protein and a ^{32}P-labelled probe containing the NRR. Fig.2 shows that IREBF1 was able to bind this probe (compare lanes 1 and 2), and that this binding could be blocked by the addition of excess unlabelled probe DNA, but not an excess of an unrelated DNA sequence (i.e., "AP1," see lanes 3 and 4). This experiment was performed 3 times with qualitatively similar results. These data indicate that IREBF1 can specifically recognize and bind the NRE sequence under *in vitro* conditions.

Protein immunoblot and super shift assays

To determine whether IREBF1 protein was present in PC12 cells, protein immunoblot experiments were performed using antibodies generated against a recombinant form of IREBF1. Fig. 3 shows that these antibodies recognized a single protein of approximately 35 kilodaltons (kDa) present in nuclear extracts from NGF-treated PC12 cells (lane 2). This size corresponds to the predicted size of this protein (242). In contrast, no immunoreactive bands were detected in parallel immunoblots when pre-immune antibodies were substituted for anti-IREBF1 antibodies (lanes 3 and 4). Note that the recombinant protein runs at a slightly higher molecular weight than native IREBF1 due to the addition of the polyhistidine tag at the amino terminus used for protein purification (lane 1).

Super shift assays were then used to determine whether this antibody could also recognize IREBF1 when complexed with DNA. Fig.4 shows that higher concentrations of anti-IREBF1 antibody caused the appearance of an additional shifted band of reduced mobility (see upper arrow at the left), and that this "super shift" decreased with lower concentrations of antibody (see lanes 3-7). No detectable supershift was observed when pre-immune antibodies were substituted for the immune antibodies (lanes 8-10). Additional negative controls in these experiments included samples in which recombinant IREBF1 was not present in the binding mixture (lanes 11-14). This experiment was performed 4 times with qualitatively similar results. These data indicate, therefore, that the IREBF1 protein is present in the nuclei of NGF-treated PC12 cells and can bind the NRR sequence.

Regulation of IREBF1 in PC12 cells

In previous gel mobility shift experiments, we found that the nuclear activity in PC12 cells capable of shifting the NRR was present in both NGF-treated and control cells (241). To determine whether the IREBF1 protein was also constitutively present in PC12 cells, protein immunoblot analysis was performed. Fig.5A shows that the levels of IREBF1 protein were similar in nuclear extracts prepared from control and NGF-treated cells (lanes 2 and 3, see arrow). There was no discernible difference, moreover, in the relative mobilities of the bands isolated from NGF-treated or untreated cells. Negative controls in this experiment involved the substitution of pre-immune antibodies for the immune antibodies (Fig.5B). This experiment was performed 3 times with qualitatively similar results. Experiments using whole cell homogenates instead of nuclear protein extracts also showed similar levels of IREBF1 immunoreactivity in NGF-treated and untreated cultures (not shown). This data are consistent, therefore, with previous gel shift data (241) and indicate that IREBF1 is constitutively expressed in PC12 cells.

Overexpression of wild-type and "dominant negative" forms of IREBF1

Protein structural analysis of IREBF1 reveals a putative leucine zipper region at the carboxy terminus of the protein, suggesting that this transcription factor, like other members of the basic leucine zipper family, requires protein dimerization for function. If so, then it is theoretically possible to construct a dominant negative form of IREBF1 (dn-IREBF1) lacking the DNA binding domain. To test this possibility, we first introduced two Not1 restriction sites flanking either side of the DNA binding domain of IREBF1 (see Fig.1), removed this domain by restriction cutting with Not1, and then religated the sticky ends. Fig.6A is a protein immunoblot assay showing that IPTG-induced bacteria express a dn-IREBF1 protein that runs at a slightly lower molecular weight than wild

type IREBF1 (wt-IREBF1), corresponding to the 38 amino acid-deletion of the DNA binding domain. Bacteria that were untreated with IPTG failed to express detectable levels of IREBF1-immunoreactive material (lanes 3 and 4). To confirm that this dn-IREBF1 protein would not bind the NRR sequence, gel mobility shift assays were performed using a ^{32}P-labelled probe containing the NRR. Fig.6B shows that the dn-IREBF1 protein does not produce a detectable gel shift with this probe, as compared with the wild type protein (compare lanes 2 and 3), indicating that the DNA-binding activity of this mutant protein is greatly reduced.

We then set out to test whether expression of this dn-IREBF1 affected the NGF-induction of ST-1 in PC12 cells. These experiments involved transiently transfecting PC12 cells with a reporter plasmid containing a 715 base pair region of the ST-1 promoter driving expression of the chloramphenical acetyltransferase (CAT) gene, plus either the wt-IREBF1 or the dn-IREBF1 plasmids under the control of a constitutive promoter. These cells were then incubated for 24 hours in the absence or presence of NGF, and assayed for CAT activity. Fig.7A shows that co-transfection with the wt-IREBF1 plasmid caused an increase in the NGF-induced CAT activities as compared to cells transfected with the ST-1 promoter-CAT reporter gene alone. This increase in NGF-responsiveness was statistically significant and increased with higher concentrations of wt-IREBF1 plasmid. At these higher concentrations, wt-IREBF1 also increased basal levels of transcription slightly. In contrast, co-transfection with the plasmid containing the dn-IREBF1 was found to produce as much as a 65% decrease in the NGF-induced levels of CAT (Fig.7B). In this case, however, basal levels of transcription were unaffected, even by higher concentrations of the dn-IREBF1 plasmid. This experiment was repeated twice with qualitatively similar results.

To rule out the possibility that the inhibition of NGF-responsiveness produced by co-transfection with dn-IREBF1 was due to its effects on some early step in the NGF-

signalling pathway, parallel transient transfection assays were performed using the *c-fos* immediate early gene promoter driving expression of the CAT reporter. This promoter lacks the NRR sequence, but does respond to NGF-treatment. Therefore, if the dn-IREBF1 protein were to inhibit the NGF-signaling pathway in some non-specific fashion, one would expect that it should diminish NGF-responsiveness of the *fos* promoter. Fig.8 shows, however, that co-transfection with either the wt-IREBF1 or dn-IREBF1 plasmids had no significant effect on either basal or NGF-induced levels of CAT expression. These data indicate, therefore, that IREBF1 plays a specific role in the NGF-induction of the ST-1 gene.

DISCUSSION

In this paper, we identify and characterize a transcription factor, IREBF1, that binds to the NGF-responsive region (NRR) of the proximal ST-1 gene promoter and may regulate its activity. We show that this protein binds preferentially to the NRR *in vitro*, and that it produces a similar gel mobility shift to that seen with nuclear extracts from PC12 cells (241). Expression of the IREBF1 protein in PC12 cells was found, moreover, to augment the NGF-responsiveness of the proximal ST-1 promoter, but did not seem to affect its basal levels of expression. Conversely, expression of a mutated form of IREBF1 lacking the DNA-binding domain acted to attentuate NGF-responsiveness, but also did not affect basal levels of expression. These data would suggest, therefore, that IREBF1 contributes to the transcriptional regulation of this NGF-induced late gene product.

IREBF1 was initially isolated by Yan and Tamm (242) using a similar cloning strategy looking for gene products binding to an α/β-interferon-stimulated response element (ISRE) found in the (2'-5')-oligoadenylate synthetase gene proximal promoter region. Although little is known about the specific role of IREBF1 in interferon-regulated gene expression or in its patterns of expression *in vivo*, its amino acid sequence and predicted domain structure would suggest that it may be a member of the basic leucine zipper gene family of transcription factors. Interestingly, the DNA sequence we used to pull it out of our PC12 cell cDNA expression library was quite different from that originally used to clone it (242). Whereas the concatemer we used was 5'-GTTACATTTT CCAAAGTAGAAAAAAATGCC-3', the sequence from the OAS promoter was 5'-CGGG AAATGGAAACTG-3'. Although cursory inspection shows that there are some areas of homology between these two probes, the sequences are largely

different. This may suggest that the binding of IREBF1 to its core sequence can tolerate a relatively large number of base substitutions, as compared with other members of the basic leucine zipper family (246). It should be noted, however, that the IREBF1 protein does not bindnon-specifically to DNA, since it failed to recognize a cyclic AMP response element containing probe used as a negative control in the initial screening experiments. We have also shown that other unrelated DNA sequences fail to displace IREBF1 binding to the NRR in competition gel mobility shift assays (Fig.2). In any case, this variability in the DNA sequence being recognized by IREBF1 makes it difficult to determine *a priori* whether its recognition sequence may be present in the promoters of other NGF-induced gene products.

Although the core sequence to which IREBF1 binds is still unclear, it does not appear to correspond to either an α/β-interferon or γ-interferon response element (247). For one thing, neither of these elements are present in the OAS promoter sequence originally used to clone IREBF1 (242). Although the ST-1 proximal promoter does contain a region in which 9 out of 11 base pairs are similar to an interferon-stimulated response element (ISRE) (247), it is probably not directly involved in the binding of IREBF1. We found, for example, that the region containing this ISRE-like sequence was unable to compete for binding to the nuclear protein present in PC12 cell nuclei in competition gel mobility shift assays (data not shown). On the other hand, there are several gene products which are induced in PC12 cells by γ-interferon including OAS and the type II sodium channel (248, 249). It would be of interest to determine, therefore, whether induction of these genes by interferon also involves IREBF1. If so, this may indicate that there is some convergence in the interferon- and NGF-signaling pathways in PC12 cells.

Our studies do provide some clues as to how IREBF1 may influence the NGF-induction of ST-1 gene expression. For one thing, we found that IREBF1 was expressed

constitutively in PC12 cells. Western blot experiments showed, for example, roughly equal intensities of an IREBF1-immunoreactive band at 35 kDa in either homogenates of whole cells or nuclear protein extracts from NGF-treated and control cultures. This indicates, therefore, that IREBF1 is not an NGF-induced immediate-early gene product whose appearance is responsible for the transcriptional activation of the late gene products, such as ST-1. On the other hand, it is possible that IREBF1 may be part of a larger transcriptional complex which might include such immediate-early gene products. Although IREBF1 is capable of forming a functional homodimer *in vitro*, it is possible that IREBF1 may normally heterodimerize with some other leucine zipper family member *in vivo*, as has been shown for members of the *c-jun* family (192, 250, 251). In which case, induction of some other member of the multimeric complex may be responsible for activating gene expression.

Another, non-mutually exclusive possibility is that IREBF1 may be phosphorylated or dephosphorylated following NGF-treatment and that this modification somehow activates the basal transcriptional apparatus. The sequence of IREBF1 contains, for example, two casein kinase II consensus phosphorylation sites -- one adjacent to the DNA binding domain and another within the leucine zipper domain. This serine/threonine kinase has been shown in other cells to translocate into the nucleus following growth factor treatment, and to phosphorylate other bZIP transcription factors (252-254). In particular, phosphorylation of *c-jun* by casein kinase II decreases its ability to bind the AP1 recognition sequence in DNA (255), and probably constitutes one of the mechanisms governing *jun* function in cells (256). On the other hand, it is unlikely that either casein kinase II or IREBF1 is activated by members of the MAP kinase cascade, since both lack the serine/threonine-proline (S/TP) consensus phosphorylation sequence for members of this kinase cascade. However, the possibility that casien kinase II affects ST-1 gene regulation by regulating IREBF1 function, and the relationship between this kinase and those of the MAP kinse pathway, remains to be determined

Our working model of how IREBF1 participates in the NGF-induction of ST-1 gene expression is based, therefore, on the observations that IREBF1 is constitutively expressed by PC12 cells and binds constitutively to the NGF-responsive region of the ST-1 promoter. When NGF is presented to these cells, it seems likely that another gene product(s) is induced and either directly or indirectly initiates the formation of a larger transcriptional complex with IREBF1 which, in turn, activates ST-1 gene expression. It would be of interest to determine, moreover, whether these associated gene products are members of the *fos* and *jun* family that bind to the AP1 site located nearby on the ST-1 promoter. Alternatively, these other gene products may bind to other as-yet-unrecognized sequences in the ST-1 promoter, or may not directly bind to DNA at all, as has been shown for a number of other critical transcriptional regulators, such as CREB-binding protein (257). Studies to identify which proteins bind to IREBF1 in NGF-treated versus control cells may provide useful clues as to the underlying mechanisms involved in the NGF-regulation of ST-1 gene expression.

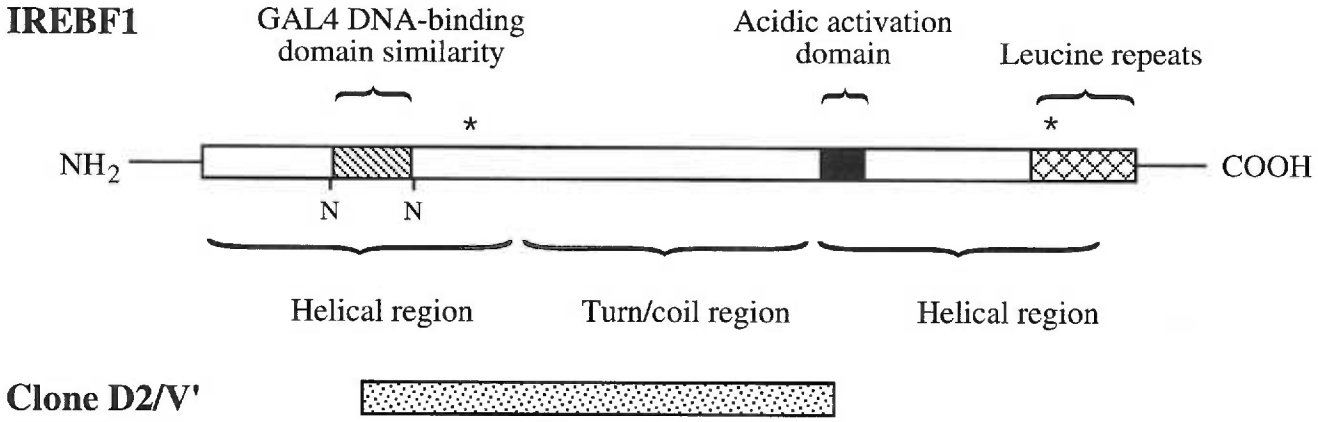

FIGURE 1: Schematic diagram of the IREBF1 protein [adapted from Yan and Tamm, 1991 *PNAS* 88:144] aligned with the region of homology with clones D2/V'. The asterisks (*) indicate putative casein kinase II phosphorylation sites, the "N's" refer to Not1 sites that were introduced which flank the GAL4 DNA-binding domain. Note that clones D2/V' contained most of the DNA-binding domain of IREBF1.

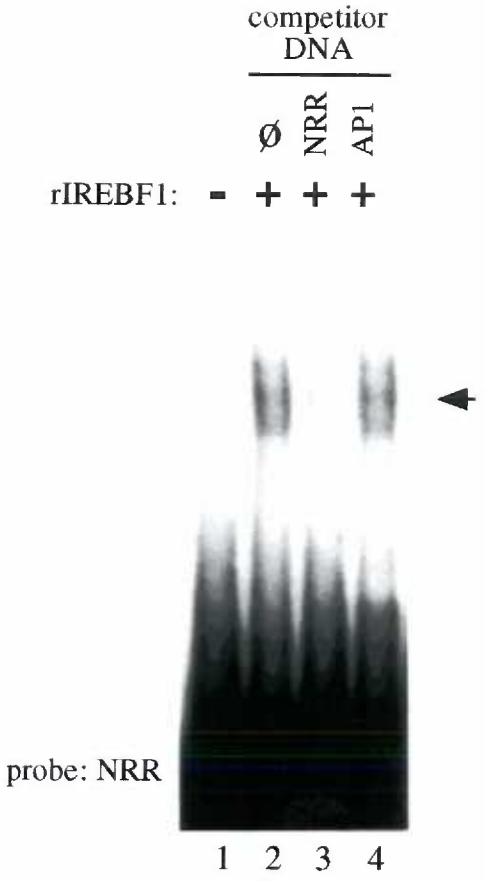

FIGURE 2: Gel mobility assay showing the specificity of IREBF1 binding to the NRR region of the ST-1 gene. Recombinant IREBF1 protein was incubated with a ^{32}P-labeled 65 bp NRR of the ST-1 promoter corresponding to bases -259 to -195, and then electrophoresed under non-denaturing conditions. Autoradiography was then performed to visualize the locations of the free probe (at the bottom) and the protein-DNA complex (at the arrow). Note that IREBF1 protein produced a gel shift (compare lanes 1 and 2), and that this shifted band was effectively competed out by excess unlabelled NRR (lane 3), but not by a non-specific competitor (lane 4).

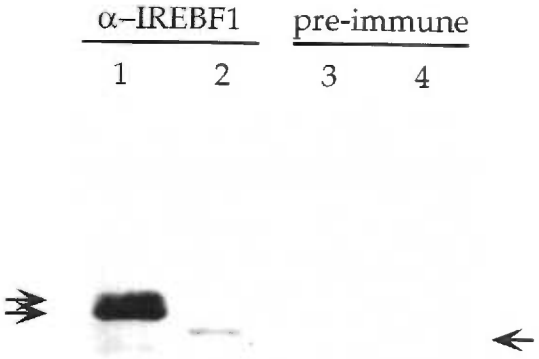

FIGURE 3: Protein immunoblot studies showing IREBF1 protein in nuclear extracts from NGF-treated PC12 cells. PC12 cells were serum-deprived for 24 hours and then treated with NGF (100ng/ml) for 2 hours prior to harvesting. Nuclear protein extracts were then subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with either anti-IREBF1 antibodies (α-IREBF1) or pre-immune antibodies. The arrow at the right indicates the position of the native 35 kD IREBF1 protein found in PC12 cells. The arrows at the left indicate the position of the recombinant IREBF1 containing a polyhistidine tag at its amino terminus, which results in its running with slightly less mobility than the native protein. Note the presence of immuno-reactive bands at the appropriate molecular weights when these blots were incubated with immune antibodies (lanes 1 and 2), but not pre-immune antibodies (lanes 3 and 4).

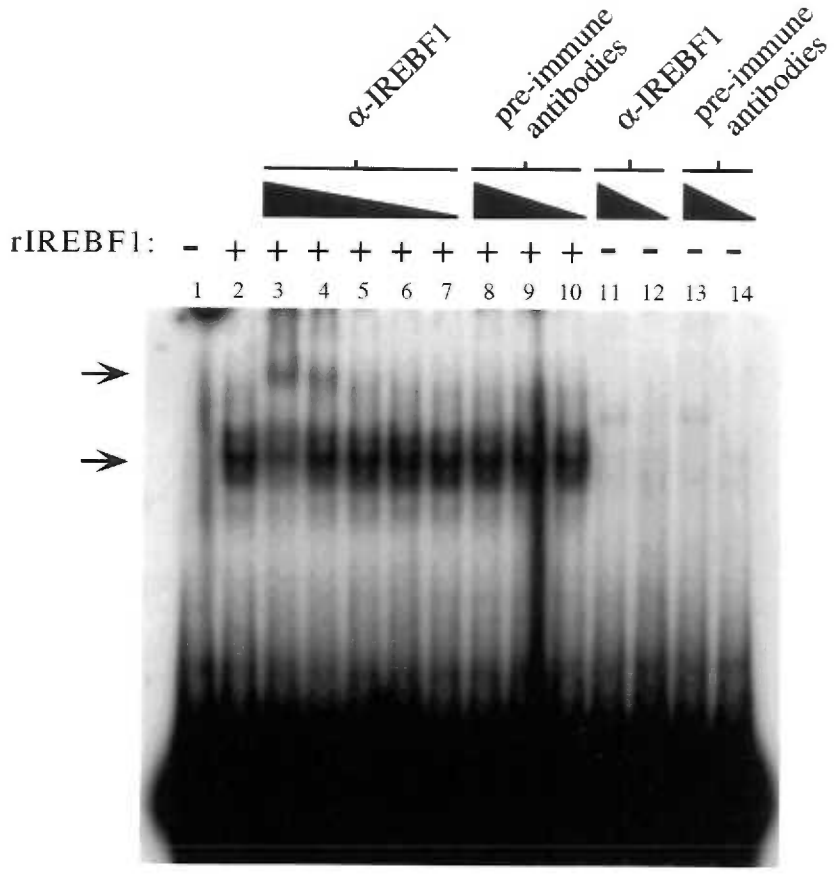

FIGURE 4: Super shift assay showing that α-IREBF1 antibodies recognize IREBF1 when complexed with DNA. The ^{32}P-labelled NRR probe was first incubated in the presence (lanes 2-10) or absence of IREBF1 protein (lanes 1 and 11 - 14). Either α-IREBF1 (lanes 3-7 and 11-12) or pre-immune antibodies (lanes 8-10 and 13-14) were added to these incubation mixtures. Finally, these binding reactions were subjected to electrophoresis in a 6% acrylamide gel under nondenaturing conditions and visualized by autoradiography. The shifted band is indicated on the left by the lower arrow and the supershifted complex is indicated by the upper arrow. Note that the higher concentrations of α-IREBF1 antibodies produced a supershift, but that pre-immune antibodies did not.

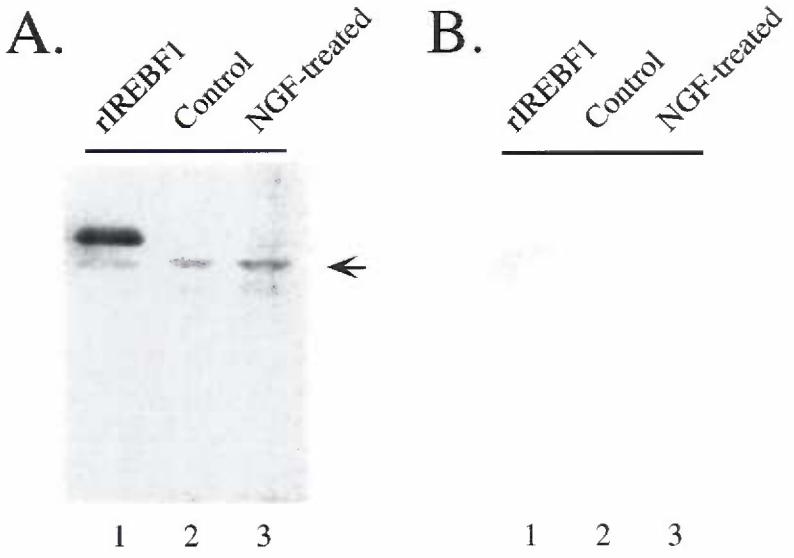

FIGURE 5: Protein immunoblot studies showing that IREBF1 protein is constitutively expressed in nuclear extracts from both control and NGF-treated PC12 cell cultures. Panel A, cells were either untreated or NGF-treated (100 ng/ml) for 2 hours, prior to harvesting. Nuclear extracts were then prepared and subjected to protein immunoblot analysis using chicken α-IREBF1 antibodies followed by alkaline phosphatase-conjugated rabbit anti-chicken IgY antibodies. Lane 1 contains 0.8 μg of recombinant IREBF1 as a positive control, which runs with slightly slower mobility due to the presence of the polyhistidine tag. Note the similar band densities in extracts of control and NGF-treated cultures (at the arrow). Panel B, similar experiment as that described in panel A, except that pre-immune antibodies were substituted for α-IREBF1 antibodies.

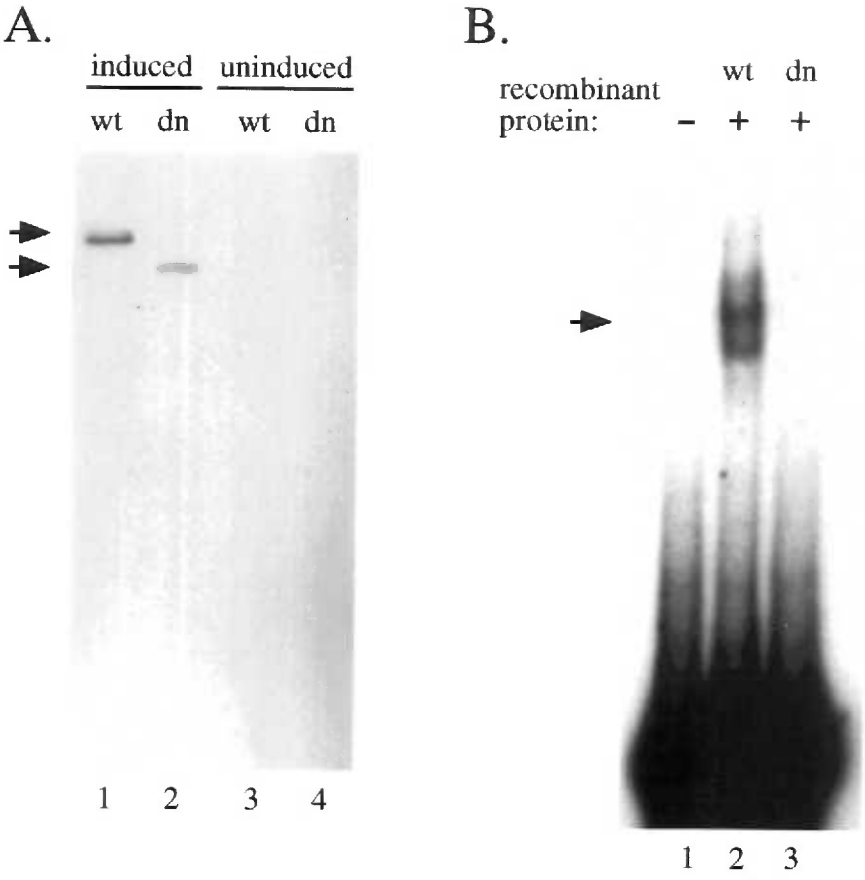

FIGURE 6: Protein immunoblot and gel mobility shift experiments showing that the dominant negative form of IREBF1 does not bind the NRR region of the ST-1 promoter. Panel A, recombinant proteins were generated in bacteria expressing either the wild type (wt) IREBF1 cDNA or a dominant negative (dn) mutant lacking the entire DNA-binding domain of IREBF1. These bacteria were either induced with IPTG for 4 h to produce protein (lanes 1 and 2) or were left uninduced (lanes 3 and 4). Finally, the bacterial proteins were isolated and a protein immunoblot assay performed using α-IREBF1 antibodies. Note that the dn-IREBF1 band runs at a slightly lower molecular weight as compared with the wild type protein due to the absence of the DNA binding domain. Panel B, gel mobility shift assay showing that dn-IREBF1 does not bind the NRR region of the ST-1 promoter. Equal amounts of either wild type (wt) or dn-IREBF1 protein (0.1 μg) were incubated with the ^{32}P-labeled NRR probe, and subjected to electrophoresis under non-denaturing conditions, followed by autoradiography. Note the presence of the shifted complex in the presence of wt-IREBF1 (lane 2), but not with dn-IREBF1 (lane 3).

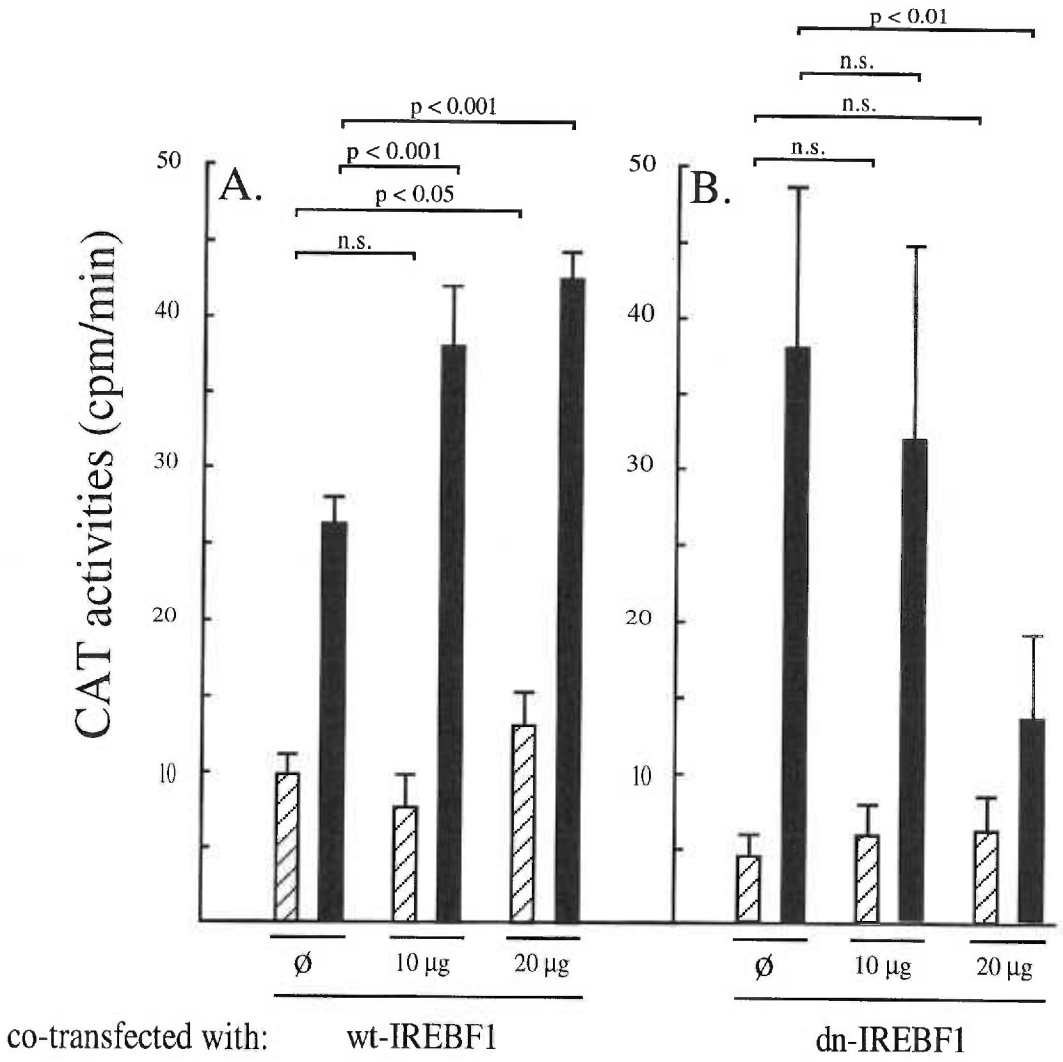

FIGURE 7: Co-transfection studies showing that expression of the wild type IREBF1 augments NGF-induced CAT activities, whereas expression of dn-IREBF1 attenuates NGF-responsiveness of the ST-1 promoter. Panel A, PC12 cells were transiently co-transfected with 15 µg of a plasmid containing 715 bp of the proximal ST-1 promoter driving CAT expression (p715TRCAT) and either 10 or 20 µg of a plasmid causing the constitutive overexpression of wt-IREBF1 protein driven by the CMV promoter. Cultures were serum-deprived for 24 h and then incubated in the presence (solid bars) or absence (striped bars) of NGF (100 ng/ml) for an additional 24 h. Cell extracts were then prepared and CAT enzymatic activity assayed. Data were analyzed by ANOVA. n.s., not statistically significant. Note that increasing concentrations of wt-IREBF1 plasmid resulted in a statistically significant increase in NGF-induced CAT activities, and only a modest increase in basal levels of CAT expression. Panel B, a similar experiment to that shown in panel A, except that the dn-IREBF1 plasmid was substituted for the wt-IREBF1 plasmid. Note that increasing concentrations of the dn-IREBF1 plasmid resulted in decreased NGF-induced CAT activities, but no significant differences in basal levels of expression.

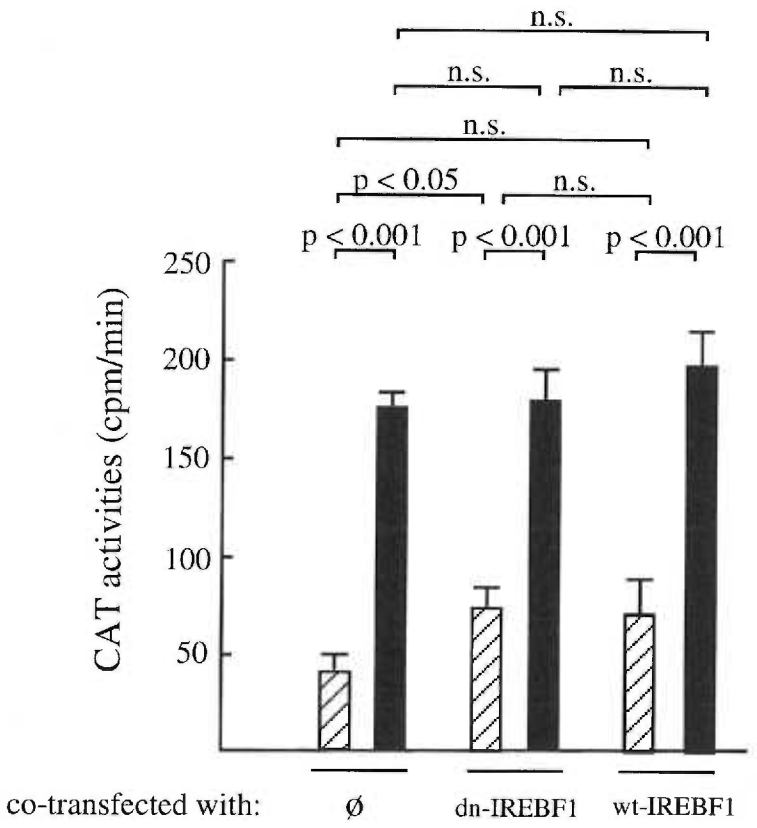

FIGURE 8: Co-transfection studies showing that expression of CAT activities driven by the *fos* promoter is unaffected by overexpression of either the wt- or dn-IREBF1. PC12 cells were transiently co-transfected with 15 μg of a plasmid containing the *c-fos* promoter driving CAT expression and 20 μg of either the dn-IREBF1 or wild type-IREBF1 plasmids. Cultures were serum-deprived for 24 h and then incubated in the presence (solid bars) or absence (striped bars) of NGF (100 ng/ml) for an additional 24 h. Cell extracts were then prepared and CAT enzymatic activity assayed. Data were analyzed by ANOVA. n.s., not statistically significant. Note that co-transfection with neither the dn-IREBF1 nor wild type-IREBF1 plasmids affected basal or NGF-induced levels of CAT activities.

IV

DISCUSSION

Involvement of the AP1 site in ST-1 regulation.

Our understanding of how NGF regulates ST-1 has increased substantially these last few years. The data presented here shows that the AP1 site present at -70 is crucial for both basal and NGF-induced transcription of this gene. Studies in which this site was mutated by two critical base substitutions lead to the complete inactivation of the ST-1 promoter (Chapter II, Fig.1), suggests the crucial role that this element plays in the regulation of this gene. The AP1 site is also present in the same location in most other matrix metalloproteinase promoters (165, 206). The AP1 complex that binds to this site contains either *c-jun* homodimers or *jun/fos* heterodimers (192, 250, 251). The *fos* and *jun* transcription factors are activated in several different cells types by a wide variety of extracellular signals (126, 128, 258); Sigmund et al., 1989; Kelly et al., 1983; Reitsma et al., 1983). It is no surprise, therefore that these immediate early genes are important for the activation of many late genes, including ST-1. The AP1 site in the ST-1 promoter appears to be a key regulator of this gene since activation by several other growth factors, cytokines and tumor promoters such as PDGF, EGF, TPA, interleukin-1, TNF and interferon-β all require this element.(191, 204, 205). In some cases, other growth factor-specific elements are also required for full activation of ST-1 (198, 241). We believe that the AP1 site is involved mainly in the basal transcription rather than in the NGF-responsiveness of ST-1. This is not the first time that the AP1 element in the ST-1

promoter has been shown to be involved in basal rather than induced gene expression (207). Buttice and co-workers showed that though the AP1 site was required for basal expression, it was not required for mediating the TPA response of ST-1 in various cell types, including fibroblasts (207).

The NRE is required and sufficient for ST-1 induction by NGF.

We have also shown that a region of the ST-1 promoter located further upstream (between -229 and -259) contains an NGF response element. Deletion of a region containing this promoter resulted in the inability of the promoter to respond to NGF in a 5' promoter deletion assay (Chapter II, Fig.2). This element was shown to bind a nuclear protein(s) that was constitutively present in the nuclei PC12 cells. The binding of this putative transcription factor was saturable and specific for the NRE sequence (Chapter II, Figs.3-6). In addition, the direct function of this sequence in the regulation of ST-1 via NGF was demonstrated. When placed upstream of the basal thymidine kinase promoter that was normally unresponsive to NGF, this sequence could confer modest NGF-reponsiveness to this promoter (Chapter II, Fig.7). In addition, the deletion of only the NRE from the native ST-1 promoter resulted in the loss of NGF responsiveness (data not shown). These experiments indicated that this 30 bp region of the ST-1 promoter contained an NRE that was required and sufficient for NGF-responsiveness.

IREBF1 binds to the NRE.

We utilized this 30 bp NRE as a probe to screen a rat PC12 λgt11 expression library. Several clones were positive and were purified to homogeniety. In order to eliminate false positives, we performed a differential screen where each purified clone

was tested in its ability to bind the NRE and another non-specific probe corresponding to the cAMP response element (CRE). Approximately a third of the clones bound both probes and were thus eliminated from further investigation. Of the remaining clones, a few were sequenced and of particular interest were two overlapping clones that had extremely high homology to a previously identified mouse transcription factor, IREBF1 (242). This transcription factor was able to bind the NRE and was present the nuclei of both untreated and NGF-treated PC12 cells (Chapter III, Fig.2-5). This was consistent with previous gel shift data in which nuclear proteins from both control and NGF-treated PC12 cell nuclei bound the NRE (Chapter II). In addition, co-transfection studies using a ST-1/CAT reporter plasmid in conjunction with plasmids overexpressing two different forms of IREBF1 implicated this protein in ST-1 gene regulation by NGF (Chapter III, Fig.7).

All these data taken together would suggests that IREBF1 plays a role in the NGF induction of ST-1 through the NRE. Though we have been able to use antibodies against IREBF1 to detect recombinant IREBF1/NRE complexes (Chapter III, Fig.4), we have not been able to detect the interaction of native IREBF1 with the NRE using a super-shift assay. From a technical standpoint, even though we know this antibody can recognize native IREBF1 (in immunoblot assays) it could be possible that this antibody cannot recognize native IREBF1 when it is complexed to DNA. Since this is a polyclonal antibody, we do not know the specific epitope that this antibody is recognizing. Upon DNA binding, this epitope could be hidden and therefore result in the loss of a recognizable epitope. This same effect could also be obtained if the native protein/DNA complex contained proteins other than IREBF1. In this case, the epitope could be lost due to masking from accessory protein binding. The data presented here would suggest, moreover, that if another, as yet identified protein is present in the native protein/DNA complex, then this protein would be constitutively present along with IREBF1 and could

also mask the recognizable epitope. This explanation would be consistent with gel shift data showing the constitutive presence of a protein complex that can bind the NRE. In addition, we did not detect any significant change in the shift pattern, so it seems unlikely that NGF stimulation caused recruitment of accesory protein(s). If this is true, then it is not surprising that we could not detect a supershift using nuclear proteins from PC12 cells since this epitope would be always be masked by protein-protein interactions. This would suggest a model in which IREBF1 is constitutively bound to the NRE along with an as yet unidentified nuclear protein(s).

The exact sequences to which IREBF1 binds is not clear. Data presented in Chapter II (Fig.6) would suggest that the protein binding sequence lies in the most proximal half of the NRE. These experiments were performed using mutated versions of the NRE as unlabelled competitiors and two mutants in particular, m4 and m5 were unable to compete for protein binding. At the time, this result led to the hypothesis that the 13 bp sequence which was mutated was the binding element for the nuclear protein. Once we isolated and purified IREBF1, we used the recombinant protein in a similar competition gel shift using mutant NRE's. To our surprise, none of these mutants were able to compete for binding to recombinant IREBF1. In addition, when each of these mutant NRE's, including m4 and m5, were used as labelled probe, all of them produced a shift when either nuclei from PC12 cells or purified IREBF1 was used (data not shown).

There are several explanations for this anomolous result. It is a possible that the DNA sequence for IREBF1 binding could be rather large and therefore the 6 bp mutations in the NRE may not have been sufficient to completely disrupt protein binding nor provide effective competition. It is also possible that IREBF1 binds the NRE in several places so that each mutant would once again be ineffective in completely disrupting DNA/protein interactions. If this is the case, then it would appear that the consensus binding sequence for IREBF1 is quite varied since there are no repeated or

palindromic sequences within the NRE. This is also supported by the fact that there was no significant homology in the NRE between the rat, human and rabbit ST-1 promoters (193). In addition, the sequence that was originally used to clone IREBF1 was an α/β interferon response element that was present in the promoter of the (2'-5')oligoadenylate synthetase ME-12 gene. Though the exact binding site of IREBF1 to this region was not analyzed by those investigators, this element, on close inspection, appeared to have no sequence homology with the 30 bp NRE.

IREBF1 is involved in the induction of ST-1 by NGF in PC12 cells.

Regardless of the exact binding site of IREBF1, it is clear that this protein does bind the NRE and is involved in the regulation of ST-1 gene induction by NGF. Structural analysis of IREBF1 showed that this protein is a member of the bZIP family of transcription factors and has a region that is similar to the GAL-4 DNA binding domain at the amino terminus and a leucine zipper region at the carboxy terminus (242). This C-terminus region, when modeled in an alpha helical structure, contains a preponderance of hydrophobic amino acids on one side of the helix and several hydrophilic amino acids on the other side of the helix (Fig.1, page 00). There are three heptad leucine arrays on the hydrophobic side of the helix that form the "leucine zipper" region. This type of structure provides a strong hydrophobic force for protein-protein interactions suggesting that IREBF1 may require protein dimerization for its function. In order to determine whether dimerization of IREBF1 was required for the trans-activation of ST-1 we made a mutant of IREBF1 that lacked the entire DNA-binding domain. The rationale behind this experiment was that should IREBF1 require dimerization for its function, then this deletion mutant would inhibit IREBF1 by heterodimerizing with wt-IREBF1 via its leucine zipper region and preventing DNA binding, thus having a "dominant negative" effect (Fig.2A, page 00). Data presented in Chapter III (Fig.7) showed that the

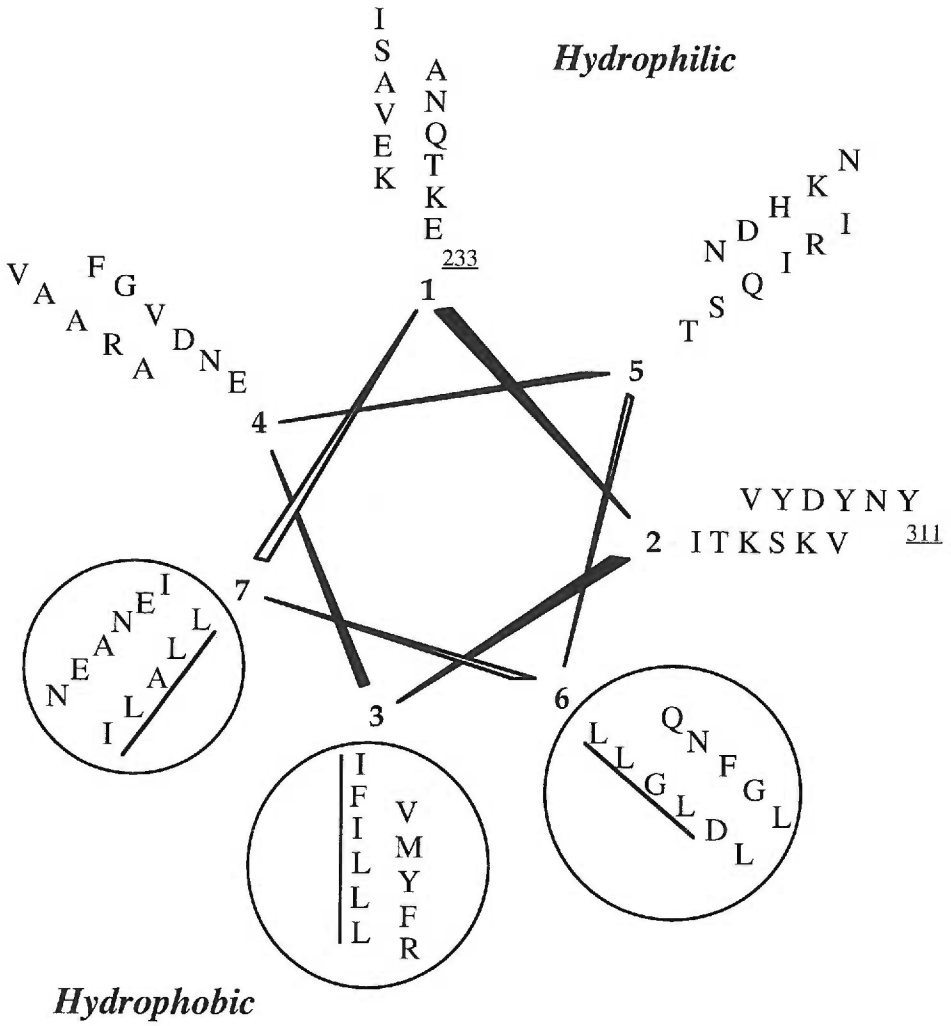

FIGURE 1: Helical wheel analysis of the C-terminus of IREBF1. Amino acids are indicated by single letters and the underlined numbers represent the starting and ending amino acids. The leucine heptads repeats are indicated by the solid line and regions of hydrophobic force is circled. Adapted from Yan and Tamm, 1991 *PNAS* 88:144.

overexpression of dn-IREBF1 caused a selective loss in the ability of the ST-1 promoter to respond to NGF but had no effect on basal promoter activity, indicating that IREBF1 was indeed involved in the NGF-responsiveness of ST-1. This data supports our original hypothesis that the mechanism of IREBF1 may involve protein dimerization. We attempted to demonstrate IREBF1 dimerization directly by performing *in vitro* protein competition experiments. These studies involved using recombinant wt-IREBF1 in the presence of increasing molar ratios of dn-IREBF1. We then used these protein mixtures in a mobility shift experiment in an *in vitro* assay to evaluate the ability of the dn-IREBF1 to compete with wt-IREBF1 for binding to the NRE. The results from this experiment was surprising as dn-IREBF1 did not effect wt-IREBF1 binding to the NRE even at a 25:1 ratio of dn- to wt-IREBF1 (data not shown). This negative result is hard to interpret due to technical reasons. We were concerned about whether we had denatured the proteins well enough to ensure that wt- and dn-IREBF1 were monomeric before they were allowed to reform as dimers. We tried several denaturing and reducing agents such as urea and beta-mercaptoethanol and unfortunately, none of them were successful. As described earlier, there are stong hydrophobic forces that favor protein dimerization and it is possible that even in the presence of these strong denaturing agents, we were unable to separate the homodimers as they get formed as soon as the protein is translated and folded. In the case of another bZIP transcription factor, cAMP response element binding factor (CREB), several mutations were made in the leucine zipper region of the protein to prevent homodimerization and selectively allow CREB to heterodimerize with other CRE binding proteins (259, 260). This approach has not been attempted with IREBF1 and could, if successful, provide us with a useful tool to investigate the mechanism of IREBF1's actions.

Though the results from the dn-IREBF1 overexpressing experiments would suggest that this protein does act as a dimer, it is possible that this mutant IREBF1 protein, in addition, could also act as a sink for other accessory factors regardless of

whether it acts as a monomer or a dimer (Fig.2A and B, page 00). If this other factor is required for gene activation, then the dn-IREBF1 could still be having a "dominant negative" effect. This could also explain our result with the wt-IREBF1 overexpressing plasmid which caused an increase in ST-1 promoter activity in response to NGF (Chapter III, Fig.7). In this case, wt-IREBF1 could be causing recruitment of this factor to the ST-1 promoter leading to the augmentation of NGF induced ST-1 promoter activity. Unfortunately, however, from the experiments presented here, we cannot distinguish between a dimer (Fig.2A) versus a monomer (Fig. 2B) model for IREBF1 activation of the ST-1 gene.

In order to rule out the possibility that overexpression of the dn- and wt-IREBF1 proteins were having a general effect on the NGF-signalling pathway, we performed similar overexpression experiments using another promoter that is also responsive to NGF, the *fos* promoter (Chapter III, Fig.8). In this experiment, we did not see any change in the NGF-responsiveness of the fos promoter when either wt- or dn-IREBF1 proteins were overexpressed in PC12 cells. Therefore, it appears that IREBF1 specifically regulates ST-1 gene expression in response to NGF.

Role of phosphorylation in the transactication of IREBF1.

The gel shift data presented in Chapter II indicates that the IREBF1 protein complex is constitutively bound to the NRE but is only activated upon NGF addition. Though it is unlikely that this activation mechanism involves recruitment of proteins in response to NGF (see above), it is possible that regulation of gene expression could occur through a post-transcriptional modification of IREBF1, such as phosphorylation. IREBF1 has two putative casien kinase II (CKII) phosphorylation sites, one adjacent to the DNA binding domain, and another within the leucine zipper region (Chapter III, Fig.1). The

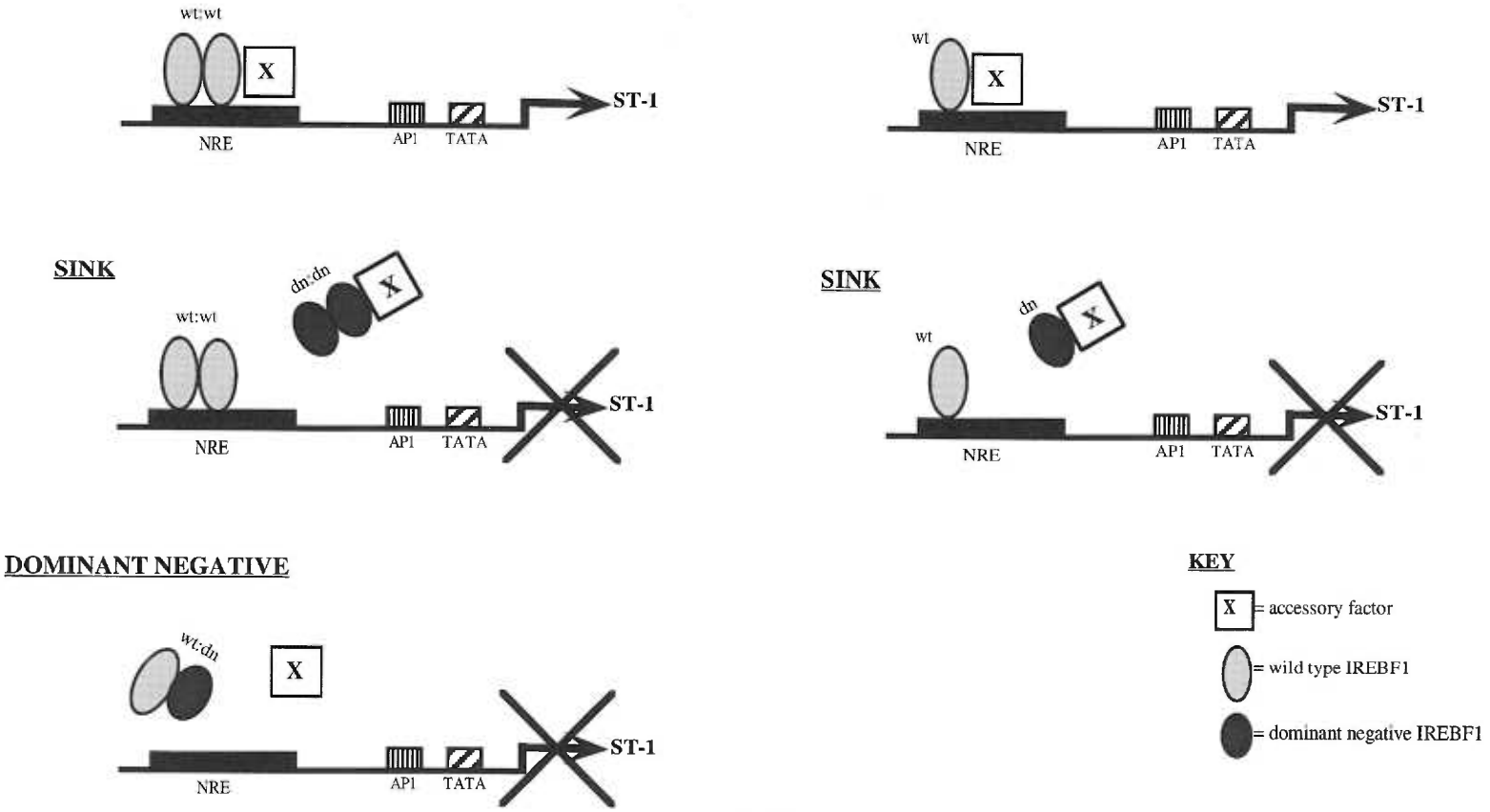

FIGURE 2: Schematic showing two possible mechanisms by which dominant negative IREBF1 could inhibit ST-1 gene transcription.

most common post-transcriptional mechanism of transactivation of transcription factors is phosphorylation (261). Phosphorylation can effect transcription factors at three different levels either by regulating the translocation to the nucleus, changing DNA binding activity or directly regulating transcription factor transactivation.

Regulation of transcription factors by compartmentilization involves either the transcription factor or its protein kinase being sequestered in the cytosol therefore being inactive since it cannot reach its target in the nucleus. In this case, activation of the kinase or phophorylation (or dephosphorylation, if constitutively phosphorylated) of the transcription factor or its cytoplasmic anchoring protein allows translocation into the nucleus. This nuclear translocation in response to phosphorylation has been observed with other transcription factor such as ISGF3 (262, 263), NF-κB(264) Schmitz et al., 1991) and the CAAT-enhancer binding protein β (C/EBPβ) (265-268). For example, in unstimulated cells, NF-κB is held in the cytoplasm in an inactive complex with the inhibitory protein IκB (269). In response to various stimuli such as TPA, the NFκB/IκB complex dissociates (270). This dissociation is probably due to phosphorylation of IκB (271-274) and causes an increase in NFκB DNA binding activity in the nucleus (269). In the case of IREBF1, it seem unlikely that this would be the mechanism of activation for a couple of different reasons. Gel mobility shift data indicates that this protein is constitutively present in the nucleus and binds DNA (Chapter II). This would not necessarily preclude the possibility of a further influx of IREBF1 into the nucleus in response to NGF. However, we tested this possibility directly by performing protein immunoblot assays using nuclei from both untreated and NGF-treated PC12 cells and saw no significant increase in IREBF1 immunoreactive material in the nucleus in response to NGF (Chapter III, Fig.5).

Transcription factor binding activity can be affected either positively or negatively by phosphorylation. The best studied transcription factor whose DNA binding is

increased upon phosphorylation is the serum response factor (SRF). SRF is phosphorylated on serine residues *in vivo* by both protein kinase A (PKA) and casein kinase II (CKII), resulting in increased DNA binding (275, 276). This increase was not due to changes in SRF dimerization but rather a conformational change in the protein which resulted in increased binding (261). Phosphorylation-induced negative regulation of transcription factor binding activity has been demonstrated for several transcription factors such as *c-myb*, *c-jun* and myogenin (255, 277-280). The proto-oncogene *c-jun* is phosphorylated *in vitro* in its C-terminal domain by several protein different protein kinases. However, only phosphorylation by GSK-3 and CKII has been shown to inhibit *c-jun* DNA binding (255) and this inhibition can be reversed by phosphatase treatment (255, 281). In negative regulation, usually the site of phosphorylation is located within or near the DNA binding domain, and it is possible that introduction of a negative charge interferes with DNA binding by creating electrostatic repulsion between phospho-groups of the protein and phosphates on the DNA. IREBF1 has a putative CKII phosphorylation site that is adjacent to the DNA binding domain (242) and it is possible that phosphorylation at this site could lead to decreased DNA binding. We have not addressed this issue as the gel mobility shift experiments performed in this study did not include phosphatase inhibitors, therefore any change in the binding affinity due to phosphorylation would not be detectable. It would be interesting to see if the inclusion of such inhibitors would result in altered DNA binding in response to NGF. In addition, we could also evaluate the strenght of the nuclear protein/NRE complex *in vitro* by performing a kinetic competition mobility shift assay using a high molar excess of unlabelled competitor for varying amounts of time and comparing the effectiveness of the competitor with untreated versus NGF-treated PC12 cell nuclear proteins.

Several transcription factors are regulated by phosphorylation-induced transactivation and the most well studied transcription factor is the cAMP responsive element binding protein CREB (282). CREB, like *c-fos* and *c-jun*, is a member of the

dimeric bZIP family of transcription factors (283). CREB is highly conserved within mammalian species and preferentially forms homodimers, although it can form heterodimers with some other members of the bZIP family (284, 285). In response to various stimuli, CREB is phosphorylated on several serine and threonine residues by PKA, CKII, PKC, GSK-3 kinase and calcium/calmodulin dependent protein kinases (CaM-kinases) (286-289). However, phosphorylation of CREB by CKII did not result in transcriptional activation whereas phosphorylation by PKA did (286, 290) Originally, the mechanism of activation by phosphorylation was believed to be a result of an allosteric change in CREB to an active conformation leading to a positive interaction with the TATA box complex of factors involved in the initiation of transcription. A CREB-binding protein (CBP) has been identified that not only binds phosphorylated CREB (257), but also interacts with the basal transcription factor TF-IIB through its C-terminal activation domain (291). Therefore, CBP could be acting as a physical bridge between transcription factors which bind to their response elements far away, like CRE, and the TATA box and basal transcription machinery. The CBP model of gene activation is a demonstration of how enhancers can stimulate promoters from a distance.

The role of phosphorylation in the activation of IREBF1 has not been determined. The location of the two putative CKII phosphorylation sites in critical regions of IREBF1 would suggest that phosphorylation (or dephosphorylation) could play a role in regulating IREBF1 function. Unfortunately, not much is known about IREBF1's regulation, function or mechanism of activation at this time. It would be interesting to determine, for instance, which of the two CKII sites are phosphorylated in response to NGF and if CKII is, indeed, the endogenous kinase. CKII is a fairly ubiquitous serine/threonine kinase which itself undergoes phosphorylation following growth factor treatment (252-254, 292). Although its name could be misinterpreted to imply that CKII's principal substrate is casein, in fact, this kinase has been shown to phosphorylate a wide variety of different intracellular proteins, including bZIP transcription factors (253, 293).

Proposed model for the mechanism of ST-1 gene induction by NGF.

We have shown that the AP1 site is absolutely crucial for transcriptional activation of the ST-1 promoter and also for basal transcription (Chapter II). We have, in addition, isolated a 30 bp region of the ST-1 promoter that is responsive to NGF (NRE) and shown that the bZIP transcription factor IREBF1 binds to the NRE and plays a role in regulating NGF-induced ST-1 gene transcription (Chapter III). Incorporating all the data presented in Chapter II and III and other experiments discussed above, I would like to present a possibile mechanism for how NGF regulates ST-1 gene expression (Fig.3, page 00).

In this model, the ST-1 promoter requires both the AP1 site and the NRE for full NGF-induction. The transcription factor IREBF1 is constituitively bound to the NRE either as a homodimer or a heterodimer with another, as yet unidentified protein "X". Upon NGF addition, either protein X is phophorylated by either MAP kinase or some other other kinase, leading to transcriptional activation of ST-1 or IREBF1 could be phophorylated by CKII and have the same effect. Based on this model, both IREBF1 and protein X have to fulfill several criteria to be in accordance with the data presented in this thesis. First of all, IREBF1 has to be present in cells in limiting quantities. If this is the case, then this could explain the observed results seen with both the dn-IREBF1 and wt-IREBF1 overexpressing plasmids on NGF induction of ST-1. If, however, in our model protein X, but not IREBF1 is rate-limiting, then though we would expect dn-IREBF1 to effect gene transcription in the same manner as was observed, this model would predict that overexpression of wt-IREBF1 would have no effect on the NGF-induced rate of ST-1 transcription since it is not rate-limiting. However, this is not what we observed as wt-IREBF1 overexpression caused an increase in NGF-responsiveness of the ST-1 promoter (Chapter III, Fig.7). Therefore, our model would predict that though protein X need not

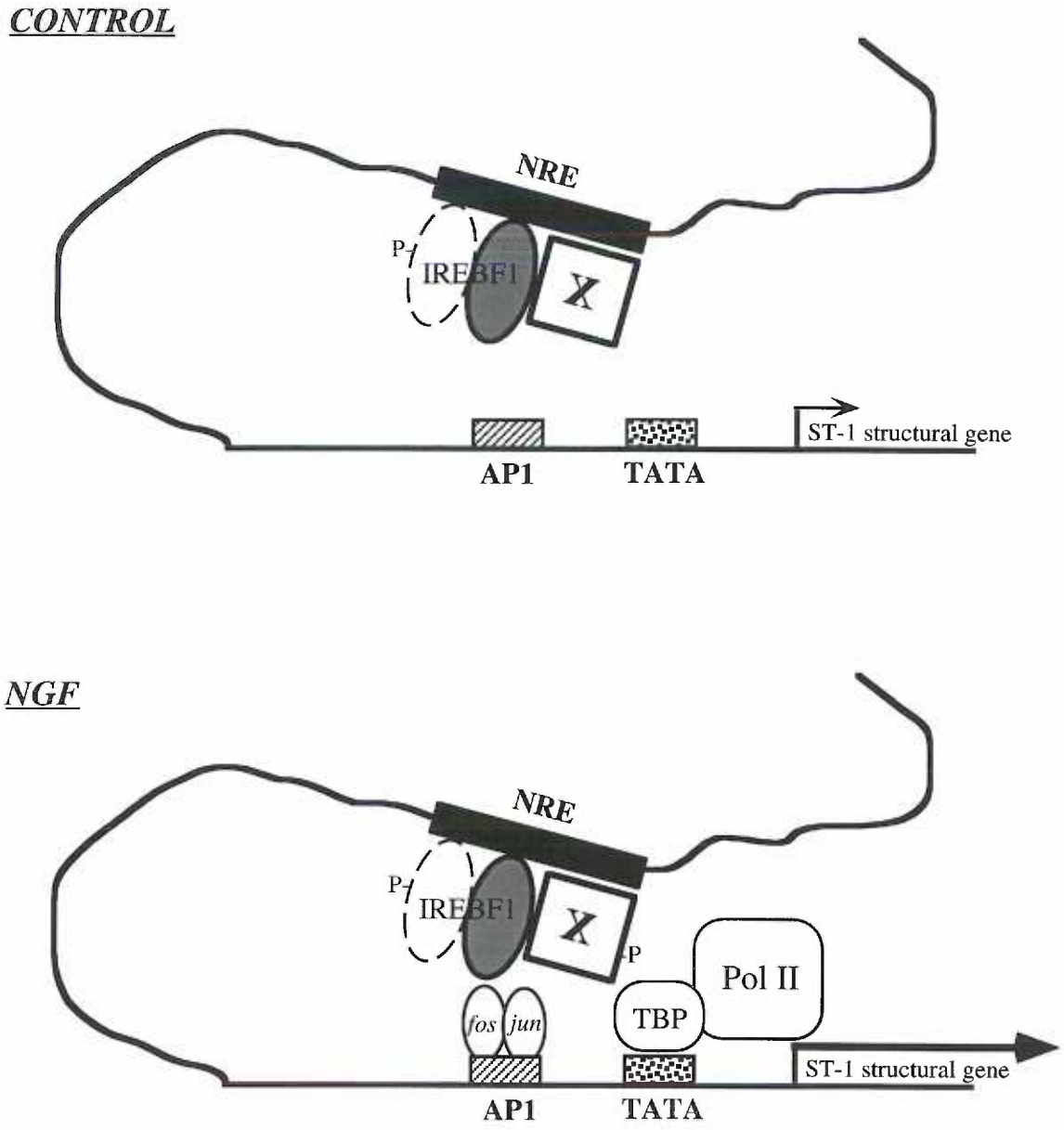

FIGURE 3: Model of the potential mechanism by which NGF induces ST-1 gene expression in PC12 cells.

be a rate-limiting transcription factor, it is able to activate transcription and bind the NRE only in the presence of IREBF1. This suggests a cooperative mechanism of DNA/protein binding between IREBF1, protein X and the NRE. This model in which protein X constitutively binds the IREBF1/NRE complex, implies that protein X is somehow transcriptionally activated, probably via phosphorylation. The exact DNA sequence to which IREBF1 binds is still unclear and perhaps some of the difficulties in elucidating the minimal binding element may be attributed to protein X association with IREBF1.

Even though much of our data is consistent with this model, there are still several functional and mechanistic questions remaining. There are several simple experiments that could be used to test the role of IREBF1 in ST-1 gene induction via NGF. For instance, we could utilize a promoter construct containing only the NRE coupled with a basal promoter in conjunction with the wt-IREBF1 overexpressing plasmid in a transient transfection assay. This would demonstrate a direct link between IREBF-1, the NRE and NGF responsiveness of the ST-1 promoter. The question of homodimerization of IREBF1 versus heterodimerization with some other transcription factor is still unresolved. Our efforts in this area were unsuccessful mainly due to technical concerns (see above). Some of these concerns may be alleviated by using an *in vitro* translation system. Different amounts of wt- and dn-IREBF1 plasmids can be translated *in vitro* simultaneously. Since the dimers would form as soon as the protein is translated, the probablility of dimer formation with either wt- or dn-IREBF1 monomers should be roughly equal. If dn-IREBF1 is able to compete for NRE binding with wt-IREBF1 in a mobility shift assay, then this would indicate that IREBF1 does act as a homodimer. If, however, IREBF1 does not appear to homodimerize, it would be interesting to see if the leucine zipper region of IREBF1 can interact with leucine zipper domains of other bZIP transcription factors. In the original λgt11 expression library screen in which we identified IREBF1, we identified a clone that was homologous to the activating transcription factor-2 (ATF-2) protein which is a member of the CREB/ATF family of

bZIP transcription factors (294) and is known to bind other bZIP proteins (295, 296). Further analysis of this interaction was disregarded primarily because this clone showed no specificity to the NRE as it also bound the CRE. However, this would suggest that the NRE can bind other members of the bZIP family of transcription factors. There are also several other clones from the library screen that have not yet been fully analyzed. It is possible that one of the remaining clones could be protein "X".

The mechanism of activation of the IREBF1/NRE transcriptional complex is currently unknown. In this model, we propose that a post-transcriptional modification of one of the DNA-binding proteins is the most likely mechanism. Based on what we know about the NGF-signaling pathway (see Chapter I) and other transcription factors, it is very likely that the mechanism of activation involves a phophorylation event. Once all the proteins in the complex are identified, this hypothesis can be tested directly by an ortho-phosphate incorporation assay. In any case, since this transcriptional unit is constitutively bound to the NRE, it is clear that some form of transcriptional activation is required for ST-1 induction, though the mechanism of this activation has not been investigated as yet at this time.

V

CONCLUSIONS

The adult nervous system arises from a sheath of cells in the ectoderm called the neural plate. The neural plate deepens into a groove that closes to form a hollow tube called the neural tube. The entire central nervous system (the brain and spinal cord) is formed from the neural tube. During closure of the neural tube, a small population of cells at the dorsal lip, called the neural crest cells, emigrate from the neuroepithelium and migrate to various locations within the embryo where they differentiate into a variety of different cell types, including most cells of the peripheral nervous system (297). Many of the neurons of the peripheral nervous system, such as those that make up the sensory, sympathetic and parasympathetic ganglia, require target-derived neurotrophins for their survival and growth. The neurotrophins are a family of molecules that have been shown to support survival and growth of a number of neurons in the central and peripheral nervous system of developing and adult animals (298). The most well characterized neurotrophin is NGF, whose role in the development of neurons in sensory and sympathetic ganglia has been well documented (35). In order to study NGF and its effects on neuronal cells, many labs, including ours, have utilized the rat PC12 cell line, a neural crest-derived cell line that responds to NGF by differentiating into a sympathetic-like neuronal phenotype (66). Along with this phenotypic differentiation, NGF stimulates the transcription of several genes that are required for the maintenance of the neuronal phenotype, such as peripherin, sodium channels, tyrosine hydroxylase and our gene of interest, the ST-1 gene (67, 143, 145, 154).

ST-1 is a matrix metalloproteinase that degrades various components of the extracellular matrix and has been implicated in several pathological conditions such as arthritis and tumor metastasis (181, 184). Because of the severe medical consequences of misexpression of ST-1 and other metalloproteases, their regulation by growth factors, cytokines and tumor promoters has been an area of intense focus for researchers (163). In PC12 cells, ST-1 mRNA is transcriptionally induced by NGF (154). We have utilized the promoter region of the ST-1 gene as a model to understand the molecular events underlying NGF's actions.

We found that the AP1 site at -70 in the ST-1 promoter was not involved in NGF-responsiveness, as mutations in this site strongly affected basal transcription but did not affect NGF-inducibility of this promoter. A study in which several bases were systematically removed from the 5' end of the promoter helped us define an NGF-responsive region of the ST-1 promoter. This region bound nuclear proteins from both untreated and NGF-treated PC12 cells and could confer NGF-responsiveness to another promoter (Chapter II).

We have identified the nuclear transcription factor that binds the NGF-responsive region of the ST-1 promoter as IREBF1, a member of the basic leucine zipper family of transcription factors. IREBF1 is constitutively present in PC12 cells and appears to be involved in the NGF-specific regulation of ST-1. Overexpression of IREBF1 caused an increase in ST-1 transcription in response to NGF, whereas a mutant form of this protein that disrupts DNA binding had the reverse effect. This effect of IREBF-1 on NGF-induced gene transcription was shown to be specific to the ST-1 promoter as overexpression of either one of these proteins had no effect on another NGF-inducible promoter, the *fos* promoter (Chapter III). All these results taken together supports our hypothesis that IREBF1 binds to the NGF-responsive region and mediates the effects of NGF on ST-1 gene transcription.

The mechanism of IREBF1 activation of gene expression, however, is still unknown. Since this transcriptional unit is constitutively bound to the NGF-responsive region of the ST-1 promoter, it is clear that some form of transcriptional activation is required for ST-1 induction. IREBF1 has two putative casein kinase II phosphorylation sites in critical regions of the protein and it is very likely that the mechanism of activation involves phosphorylation of IREBF1 and possibly, association with other members of the bZIP family of transcription factors as well (Chapter IV). Once all the proteins involved are identified, the mechanism of ST-1 induction by NGF in PC12 cells can be elucidated.

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