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Mutually Dependent Elements in the Neurotensin/Neuromedin N Gene Promoter Integrate Multiple Environmental Stimuli in PC12 Cells: a Thesis

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MUTUALLY DEPENDENT ELEMENTS IN THE NEUROTENSIN/NEUROMEDIN N GENE PROMOTER INTEGRATE MULTIPLE ENVIRONMENTAL STIMULI IN PC12 CELLS

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June 14, 1990

This thesis is dedicated to my spouse

Cynthia and a future together

blessed with love, children and prosperity.

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ABSTRACT

This thesis examines the structure and regulated expression of the gene encoding the neuroendocrine peptides neurotensin and neuromedin N (NT/N gene). Previous studies have shown that expression of NT/N mRNA and NT peptide in PC12 cells are strictly dependent on simultaneous exposure to combinations of nerve growth factor (NGF), glucocorticoids, activators of adenylate cyclase, and lithium ion. My objective was to characterize the cis-regulatory DNA sequences involved in regulated expression of this gene.

The initial focus of this study was an analysis of the structure, tissue-specific expression, and exon evolution of the rat NT/N gene. Nucleotide sequence comparisons between the rat gene and the canine and bovine cDNA sequences indicated that the predicted structure of a 170 amino acid precursor protein is highly conserved. Furthermore, the close similarity between the two cDNAs suggested that identical precursor proteins are expressed in neural and endocrine tissues. RNA analysis revealed that the gene is transcribed to yield two distinct mRNAs, 1.0 kb and 1.5 kb in size. The two mRNA species differ only in the size of their 3' untranslated regions. Interestingly, the smaller mRNA is predominant in the gastrointestinal tract, while both mRNAs are equally abundant in all neural tissues examined, except the cerebellum, where no expression was observed.

Transient transfection assays were used to delineate the rat NT/N gene cis-regulatory DNA sequences. Progressive deletion of the NT/N 5' flanking region revealed that sequences between -216 and +56 of the NT/N gene are sufficient to confer the full spectrum of responses of the endogenous gene to either of two reporter genes. A detailed mutational analysis of the NT/N control region

indicated that it is composed of an array of inducible cis-regulatory elements, including an AP-1 site, two cAMP-responsive elements (CREs), and a glucocorticoid-responsive element (GRE). Specific mutations to the AP-1 site and either CRE suggested that these elements are functionally interdependent. I propose that this array of cis-regulatory sequences in the NT/N transcriptional control region serves to integrate multiple environmental stimuli into a unified transcriptional response.

To further examine the role of the AP-1 site and CREs in the NT/N promoter, reporter genes containing either a single or multiple AP-1 or CRE sites were expressed in PC12 cells and protein kinase A-deficient PC12 cells treated with forskolin, NGF, and lithium, either individually, or in combination. The results indicated that lithium and NGF markedly activate promoters containing multiple AP-1 sites, but not a single site, and that these effects were additive. Both agents potentiated forskolin-induced activation of promoters containing a single or multiple CREs, but had no effect, individually. Also, in contrast to the activation of multiple AP-1 sites by lithium and NGF, activation of the NT/N promoter and promoters containing CREs is absolutely dependent on protein kinase A activity. These results suggested that promoters containing multiple AP-1 sites, or a single AP-1 site in the context of nearby active CREs, are selectively activated by lithium and NGF in PC12 cells.

Based on the results of this thesis I have proposed a model to account for the complex transcriptional regulation of the NT/N gene in PC12 cells. I have also addressed the relevance of these findings to the mechanisms of phenotypic plasticity of embryonic neural crest cells, NGF-induced neuronal differentiation, and the pharmacological actions of lithium.

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COLLABORATORS

CHAPTER II:

Bryant Bullock isolated and plaque purified the phage containing the rat NT/N gene.

Sandra McNeil subcloned exon three and four of the rat gene, and was involved in the initial characterization and sequencing of those exons. Sandy also isolated a phage containing at least 10 kb of additional NT/N gene 5' flanking sequences.

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I

INTRODUCTION

Overview. The development and function of the nervous system in higher animals is largely influenced by extracellular stimuli (Black et al., 1987). The effects of extracellular stimuli on neuronal cells range from gross morphological changes to subtle modulation in the synthesis of neurotransmitters. Such phenotypic plasticity often occurs at a fundamental level of neuronal function, altered gene expression (Goelet et al., 1986; Morgan and Curran, 1989). To understand how the extracellular environment regulates specific changes in neuronal gene expression, I have examined the regulated expression of neurotensin, a neuroendocrine peptide, in the rat PC12 pheochromocytoma cell line.

In the nervous system, extracellular regulators of phenotypic plasticity are often collectively referred to as "environmental stimuli" (Black et al., 1987; Goodman, 1990). They include: (1) growth factors, (2) hormones, (3) neuropeptides and neurotransmitters, and (4) pharmacological agents. A variety of growth factors such as nerve growth factor (NGF), brain-derived growth factor (BDGF), ciliary neurotrophic factor (CNTF), and the fibroblast growth factors (aFGF and bFGF) influence the survival and differentiation of neuronal cells (Barde, 1989). NGF, for instance, initiates a cascade of events which culminates in neuronal differentiation (Greenberg et al., 1985). Steroid hormones influence the growth and metabolism of most cell types. In the brain, steroids may coordinately modulate neural physiology (Evans and Arriza, 1989). Glucocorticoids, in particular, appear to be important during the differentiation of the embryonic neural crest (Doupe et al., 1985). Neuropeptides and the classical neurotransmitters are prominent modulators of neural activity (Lynch and Synder, 1986). For example, recent evidence suggests that neurotensin

either directly or indirectly mediates the stimulatory effects of ovarian steroids on gonadotropin releasing hormone (GNRH) secretion in the preoptic nucleus of the brain (Alexander et al., 1989). Pharmacological agents, such as anti-psychotic drugs, represent a class of non-physiological stimuli often characterized for their behavioral effects. In total, both physiological and non-physiological stimuli modulate neuronal development and function. Combinatorial mechanisms, involving a small number of environmental stimuli, may provide an efficient means of generating greater phenotypic diversity and specificity in response to these stimuli.

Two classes of receptors receive and transduce the effects of environmental stimuli, membrane-associated and intracellular receptors (Morgan and Curran, 1989). Membrane-associated receptors are typically coupled to signal tranduction pathways which generate second messengers such as cAMP and diacylglycerol, and these second messengers appear to activate phosphorylation events involved in gene expression. Intracellular receptors, on the other hand, exemplified by the steroid/thyroid hormone receptor superfamily, modulate gene expression by a different mechanism. They couple extracellular signals directly to specific gene expression in the nucleus. Detailed analysis of specific genes targeted by environmental stimuli in neuronal cells could expand our understanding of the molecular mechanisms underlying stimulus/response coupling and neuronal plasticity.

Neural crest: a model for phenotypic plasticity. The complex process of neural crest cell development provides an interesting example of how environmental stimuli can generate phenotypic diversity through changes in

specific gene expression. The neural crest is a transient structure originating on the apical surface of the neural tube. During embryogenesis, neural crest progenitor cells migrate to form the peripheral nervous system and several non-neural cell types, including adrenal chromaffin cells (Bronner-Fraser and Fraser, 1989). Grafting experiments in which quail neural crest cells were transplanted to different axial positions in a chicken embryo have determined that the developmental potential of a neural crest progenitor cell at a given axial position is greater than its actual developmental fate (LeDouarin, 1980). More recently, fluorescent dextran microinjection and immunohistochemistry techniques have been used successfully to verify the multipotent nature of emigrating and pre-migratory trunk neural crest cells (Bronner-Fraser and Fraser, 1989). These types of experiments suggest that the various derivatives of the neural crest arise from a series of transitions, including migration, proliferation, differentiation, survival, and commitment, each influenced by environmental stimuli (Anderson, 1989). Two such environmental determinants, nerve growth factor (NGF) and glucocorticoids, are proposed to play key antagonistic roles in the development of the major catecholamine-containing neural crest-derivatives (sympathetic neurons, adrenal chromaffin cells and small intensely-fluorescent cells) which together form the sympathoadrenal sublineage (Unsicker et al., 1978). Glucocorticoids, synthesized and present in high concentrations in the adrenal gland, are necessary for adrenal chromaffin differentiation, in vitro (Doupe et al., 1985a; 1985b; Unsicker et al., 1978). NGF, normally expressed from target tissues innervated by neurons, promotes phenotypic conversion to sympathetic neurons (Greene and Shooter, 1980). The developmental fate and survival of

sympathoadrenal progenitors may reflect the relative concentrations of NGF and glucocorticoids that these cells become exposed to during or following their migration (Anderson and Axel, 1986).

The antagonism between NGF and glucocorticoids during sympathoadrenal differentiation is also evident at the level of specific gene expression. Expression of several neural specific genes is extinguished during chromaffin cell differentiation and enhanced upon NGF-induced phenotypic conversion (Anderson and Axel, 1985). A number of NGF-inducible mRNAs have been identified, many of which are regulated in a reciprocal manner by glucocorticoids (Halazonetis et al., 1988; Leonard et al., 1987; Levi et al., 1985,; Sabol and Higuchi, 1990). Frequently, simultaneous treatment with both agents leads to an intermediate level of expression (Federoff et al., 1988; Stein et al., 1988). Thus, antagonisms between environmental determinants, like NGF and glucocorticoids, illustrate a regulatory mechanism for the generation of phenotypic plasticity.

Cellular responses to environmental stimuli. A model proposed by Curran and Morgan (1987) to distinguish between short-term and long-term cellular responses to extracellular stimuli can be applied to neuronal plasticity (Curran, 1987). These authors propose that short-term responses are typically rapid and transcription-independent. They occur immediately following stimulation, take from milliseconds to minutes, and likely involve post-translational, covalent modification of existing substrates. However, long-term responses occur within hours or days, are transcription-dependent, and involve changes in gene expression. Both types of responses to extracellular stimuli involve altered second messenger activity. These second messengers either act directly to elicit

short-term responses or through transcription regulatory proteins, which modulate the expression of other genes, to elicit long-term responses. Thus, short-term responses may account for the the instantaneous release of the contents of secretory vesicles from a synapse while long-term responses may occur following the sequential activation of sets of genes.

The cellular "immediate-early" genes are excellent candidates for modulators of long-term responses (Sheng, 1990). This class of genes is rapidly transcribed in response to different environmental stimuli in the absence of protein synthesis (Curran and Morgan, 1985; Greenberg et al., 1985; Kruijer et al., 1984). Several protooncogenes, including c-fos and c-jun, are classified as "immediate-early" genes. Both c-fos and c-jun are members of a family of functionally related nuclear phosphoproteins that form heterodimeric and homodimeric complexes capable of binding a common DNA sequence, the AP-1 site (Kouzarides and Ziff, 1988, Sassone-Corsi et al., 1988). The presence of functional AP-1 binding sites within many cellular and viral promoters, including certain members of the "immediate-early" family, adds support to the hypothesis that phenotypic plasticity results from sequential gene activation (Angel et al., 1988; Angel et al., 1987; Lee et al., 1987). Furthermore, evidence suggests that AP-1 binding proteins may be involved in the regulation of growth and development of the nervous system. Messenger RNAs encoding putative transcription factors, including c-fos, c-jun, jun-B, and erg-1, are transiently induced in the rat brain following chemically-induced seizure (Morgan and Curran, 1989). Subsequent to this intense stimulation and the appearance of c-fos and c-jun transcripts, a transient burst in enkephalin gene expression occurs (Sonnenberg et al., 1989). In total, the temporal and cell-

specific expression of several members of the AP-1 family suggest that differential combinatorial mechanisms may be involved in a programmed genetic response to environmental stimuli (Bartel et al., 1989). This mechanism may underlie the diversity and specificity of cellular responses to these signals.

As mentioned above, in response to certain stimuli "immediate-early" genes are induced rapidly and transiently, independent of transcription. Later, genes which require protein synthesis are induced, including c-myc, ornithine decarboxylase, GAP-43, the neural-specific gene SCG10, and several neurofilament subunit genes (Sheng, 1990). These genes, termed the late response genes, may represent targets of the transcription factors encoded by certain "immediate-early" genes. In the nervous system, important downstream targets of these putative transcription factors might include genes involved in the synthesis of neurotransmitters (Gizang-Ginsberg and Ziff, 1990), and neuropeptides, such as enkephalin (Comb et al., 1989; Sonnenberg et al., 1989) and neurotensin.

Neurotensin. The thirteen amino acid peptide neurotensin (NT) was originally isolated from extracts of the bovine hypothalamus based on its acute hypotensive effects (vasodilation and decreased blood pressure) in anesthetized rats (Carraway and Leeman, 1973; 1975). NT-immunoreactivity is distributed throughout both neural and endocrine tissues (Reinecke, 1985). In the mammalian gut, NT is most prominent throughout the distal portion of the small intestine. In the central nervous system NT is widely distributed in the forebrain and limbic system. The biological importance of NT is underscored by the number of related peptides isolated from genetically distinct organisms

(Reinecke, 1985). This family of NT-related peptides possess common carboxyl-terminal sequences essential for biological activity and receptor binding.

Although the function of NT is unclear, evidence supports its role in several different activities. In the digestive system, NT may perform a paracrine or endocrine role in fat metabolism (Ferris et al., 1985). In the mammalian central nervous system, NT may function as a neurotransmitter or neuromodulator (Elliot and Nemeroff, 1986; Leeman and Carraway, 1982). The observation that NT/N mRNA levels are induced in response to estrogen treatment in ovariectomized rats lends support to the role of neurotensin in modulating the pre-ovulatory surge in luteinizing hormone (Alexander et al., 1989). Also, considerable evidence supports a close functional association between NT and the dopamine (DA) systems, probably through synaptic interactions (Reinecke, 1985). The distribution of these two candidate neurotransmitters overlap considerably. Both NT and DA have been co-localized to subsets of catecholamine-containing neurons and adrenal medullary cells (Hokfelt et al., 1984; Terenghi et al., 1983). Interestingly, in the clonal PC12 cell line, DA synthesis is constituitive and NT synthesis is inducible (Tischler et al., 1986; Tischler et al., 1982). The isolation of cloned cDNAs encoding a putative precursor for both NT and a related six amino acid neuroendocrine peptide, neuromedin N (N), has made possible molecular approaches to understanding the biosynthesis of the peptide and the regulated expression of the gene which encodes them (Dobner et al., 1987).

Neurotensin Regulation in PC12 Cells. The clonal pheochromocytoma PC12 cell line provides an excellent model system to study neuroendocrine gene regulation in response to environmental stimuli since it can be stimulated to synthesize and secrete a number of neuroendocrine peptides, including neurotensin (Greene and Tischler, 1982). Previous work has shown that NT/N gene expression is strictly dependent on simultaneous exposure to multiple inducers in PC12 cells. Combinations of nerve growth factor (NGF), dexamethasone (a synthetic glucocorticoid), and activators of adenylate cyclase act synergistically to increase NT content and production in PC12 cells (Tischler et al., 1986). Individual inducers, or a combination of dexamethasone and forskolin, had virtually no effect on NT content. Interestingly, after prolonged exposure (24-48 hrs) NGF in combination with dexamethasone or forskolin resulted in detectable NT peptide levels, suggesting that NGF facilitated the actions of both dexamethasone and forskolin. When lithium ion, an agent wihich inhibits NGF-inducible neurite outgrowth in PC12 cells, was included in subsequent experiments an unanticipated observation was made: lithium ion substituted for, and strongly potentiated, responses to combinations of the other three inducers on both NT peptide and NT/N mRNA levels (Dobner et al., 1988). However, similar to each of the other three inducers, lithium alone did not induce NT/N gene expression.

Several observations suggest that the observed regulation of NT/N gene expression in vitro reflects regulation in vivo. First, PC12 cells exhibit similar phenotypic plasticity in response to NGF, glucocorticoids, and activators of intracellular cAMP as their normal cellular counterpart, the adrenal chromaffin cell (Tischler et al., 1986). PC12 cells convert from an endocrine

phenotype resembling an adrenal chromaffin cell to that of a post-mitotic sympathetic neuron in response to NGF (Greene and Tischler, 1976). The phenotypic changes include the extension of long neurite-like processes, increased electrical excitibility, and altered neurotransmitter synthesis (Greene and Tischler, 1982). Glucocorticoids antagonize NGF-induced phenotypic conversion in primary cultures of chromaffin cells (Doupe et al., 1985a). This antagonism is evident at the level of specific gene expression, in PC12 cells (Stein et al., 1988). Secondly, NT-immunoreactivity has been detected in the adrenal medulla of a variety of mammals, including rats (Goedert et al., 1983; Rokaus et al., 1984), and in sympathetic ganglia (Terenghi, et al., 1983). Lastly, all three regulators can be accounted for within the region of the adrenal gland: glucocorticoids are synthesized in high concentrations in the adrenal gland; hormone-mediated signal tranduction or innervation could increase intracellular cAMP in both adrenal chromaffin cells or sympathetic neurons, alike (Walicke and Patterson, 1981); and evidence suggests that the adrenal gland expresses NGF (Harper et al., 1976).

A review the mechanisms of action of each of the four inducers, NGF, cAMP, dexamethasone, and lithium, on gene expression will provide the necessary framework for discussion of the complex regulation of NT/N gene expression in PC12 cells. Although the focus of the following section is on transcription, this does not imply that the effects of each inducer are solely transcriptional. However, experiments presented in this thesis argue that each of the four environmental stimuli modulate NT/N gene expression at the transcriptional level in PC12 cells.

Nerve growth factor. A continuous flow of information from the periphery to the nervous system is apparent from the effects of NGF on the peripheral nervous system. The development, maintenance, and survival of sympathetic and neural crest-derived sensory neurons is absolutely dependent on the continuous supply of NGF expressed from tissues targetted by the peripheral nervous system (Greene and Shooter, 1980; Levi-Montalcini and Angeletti, 1968; Theonen and Barde, 1980).

In spite of the progress in our understanding of these biological effects of NGF, its mechanism of action remains unclear. Cloning the NGF receptor has not provided further clues (Johnson et al., 1986; Radeke et al., 1987). No conspicious ATP binding site or homology with receptors possessing protein kinase activity or GTP-binding properties were found in the cytoplasmic domain. However, recent evidence has suggested a role for the ras oncogene proteins in NGF signal transduction. The capacity of activated ras p21 to mimic NGF-induced phenotypic differentiation (Bar-Sagi and Feramisco, 1985; Barbacid, 1987), and its resemblance to GTP-binding proteins, suggests that GTP-binding proteins might couple NGF receptor to second messenger pathways. NGF receptor-activated signal transduction may involve phosphorylation events mediated by multiple second messenger pathways. Evidence suggests that the cAMP and protein kinase C second messenger pathways are activated in response to NGF (Contreras and Guroff, 1987; Cremins et al., 1986; Koizumi et al., 1988). Furthermore, the partial purification of NGF-specific kinases suggested that NGF action may involve distinct phosphorylation mechanisms (Rowland-Gagne and Greene, 1990). However, no clear connection has been established between any NGF-activated second messengers and the biological actions of NGF.

The rat PC12 cell line has been extensively characterized as a model of NGF-induced neuronal differentiation (Greene and Tischler, 1982). Much of our current knowledge of stimulus-transcription coupling has been achieved with PC12 cells; most notably, the NGF-induced expression of a class of "immediate early" genes, which include members of the AP-1 family transcription factors (Sheng, 1990). The rapid transcription of genes encoding AP-1 proteins by NGF has led to the speculation that AP-1 complexes may be involved in NGF-induced neuronal differentiation, specifically the activation of genes regulated by promoters containing AP-1 binding sites. Two candidate genes regulated by AP-1 binding sites are the neuropeptides proenkephalin (Sonnenberg et al., 1989) and neuropeptide Y (Gizang-Ginsberg and Ziff, 1990). Evidence presented in this thesis suggests that the NT/N gene might also constitute a target of AP-1 mediated transcription.

Cyclic AMP. Cyclic AMP mediates the the intracellular response to a wide variety of hormones and neurotransmitters (Sunderland, 1972). The cascade of cAMP-induced phosphorylation events, following activation of adenylate cyclase, modulate multiple cellular process, including transcription (Roesler et al., 1988). Among the variety of viral and cellular genes which are regulated by elevated levels of intracellular cAMP are several neuropeptide genes, including somatostatin (Montminy et al., 1986), vasoactive intestinal polypeptide (Tsukada et al., 1987), proenkephalin (Comb et al., 1986), and the alpha subunit of choriogonadotropin (Deutsch et al., 1987).

Most cAMP-regulated genes contain a conserved sequence in the 5' flanking region related to the palindromic octamer 5'-TGACGTCA-3' (cAMP-responsive

element; CRE) . The 17 bp vasoactive intestinal peptide (VIP) gene CREs, containing two inverted copies of the 5'-CGTCA-3' motif, are also active (Fink et al., 1988). However, the mere presence of an 8-bp CRE consensus or 5'-CGTCA-3' motif within a promoter may not be sufficient for function. Both the bovine parathyroid hormone (PTH) gene, and rat glucogon gene contain a CRE consensus, but are unresponsive to cAMP. Montminy et al. (1986) have shown that a 10 bp oligonucleotide encompassing the somatostain CRE was unable to confer cAMP-regulation to the SV40 minimal promoter linked to chloramphenicol acetyltransferase (CAT) reporter gene while a 32 bp sequence was cAMP-responsive . Relevant to these observations, Deutsch et al. (1988) have demonstrated that sequences flanking the related consensus sequences of CREs and AP-1 binding sites markedly influence their respective responses to cAMP and phorbol esters when these sites are fused to the human a gonadotropin minimal promoter; although, no clear flanking sequence consensus was derived. Interconversion of CRE and AP-1 sites by the insertion or deletion of a single base can dissociate the functional properties of these two elements. The non-responsive PTH gene CRE was converted to a phorbol ester-inducible AP-1 consensus binding site by deleting a single base. In certain contexts AP-1 consensus sequences mediate responses to both cAMP and phorbol esters. The dual and sometimes synergistic response to combinations of cAMP and phorbol esters mediated by AP-1 binding sites illustrates the potentially complex interactions between two parallel signal transduction mechanisms at the level of transcription (Deutsch et al., 1988; Hoeffler et al., 1989).

A prominent 43 kd nuclear CRE-binding protein, CREB, has been characterized and cloned from PC12 cells and brain tissue, (Hoeffler et al.,

1988; Montminy and Bilezikjian, 1987). Phosphorylation of CREB at a specific serine residue (SER-133) by cAMP-dependent protein kinase A is proposed to activate CREB-mediated transcription, but does not increase its affinity for DNA (Bokar et al., 1988; Delegeane et al., 1987; Gonzalez and Montminy, 1989), suggesting that the CREB protein may be constituitively bound to the CRE (Roesler et al., 1988). A cluster of consensus phosphorylation sites for protein kinase C, protein kinase A, and casein kinase II between postions 130 and 133 in the predicted CREB protein sequence has implicated multiple phosphorylation pathways in the regulation of CREB activity. Indeed, phosphorylation of CREB by protein kinase C stimulates dimerization, but does not activate transcription, in vitro (Yamamoto et al., 1988). These phosphorylation sites could coordinately regulate CREB activity. Synergy between activators of protein kinase A and protein kinase C support such a mechanism (Deutsch et al., 1988; Hoeffler et al., 1989). However, the existence of other structurally-related CRE-binding proteins offers an additional means of generating diversity in response to cAMP (Hai et al., 1988; Hai et al., 1989). Interestingly, only certain members of this family can form heterodimers. Furthermore, those heterodimers which od form, differ in their precise interactions with a CRE consensus sequence.

Lithium ion. Lithium has profound effects on human behavior and early embryogensis (Berridge et al., 1989). The mechanism responsible for these actions remains unknown, although intensively investigated. Common to many hypotheses concerning lithium's actions are alterations in receptor-mediated signal transduction (Avissar et al., 1988, Berridge et al., 1982; Newman and Belmaker, 1987). The inositol depletion hypothesis, in particular, appears to

best address the neural and developmental specificity of lithium's actions (Berridge et al., 1989). It argues that lithium inhibits particular phosphatases involved in the dephosphorylation of inositol mono- and polyphosphates which are necessary for the synthesis and recycling of inositol lipids. Low-permeability of the blood-brain barrier to dietary inositol and the absence of a circulatory system (in the developing embryo) presumably accounts for its neural specificity. Thus, cells sequestered from plasma inositol are particularly sensitive to lithium action. This model also accounts for the time-dependent and stimulus-dependent physiological actions of lithium. As an uncompetitive inhibitor binding to the enzyme-substrate complex, lithium would exert a lower effect on less-active cells than hyper-stimulated cells. Therefore, in the central nervous system lithium could drive hyperactive receptors into a normal operating range, while during embryogenesis lithium could respecify the normal gradient of phoshoinositol turnover in the dorsal-ventral axis. Support for the phosphoinositol (PI) signalling pathway in neural signalling and development stems from the ability to rescue lithium-induced dorsalized embryos by injections of myo-inositol into the ventral pole (Busa and Gimlich, 1989).

The logical extension of these alterations in signal transduction are changes in gene expression. Oddly, little attention has been directed toward understanding lithium's effects on gene expression. As a consequence of perturbed phosphoinositol- and cAMP-mediated signal transduction, lithium could modulate environmentally-induced changes in gene expression negatively. However, Dobner et al. (1988) have shown that lithium potentiates NT/N gene expression markedly. One possible explanation might be was that lithium both dampens and enhances responses dependent on the PI turnover. For example, the

phosphoinositol-mediated contractile response to the muscarinic receptor agonist, carbachol, is potentiated by lithium treatment (Menkes et al., 1986). Furthermore, lithium treatment results in the accumulation of diacylglycerol (DAG) in a rat pituitary tumor cell line (Drummond and Raeburn, 1984) which could activate protein kinase C (Nishizuka, 1984) and enhance adenylate cyclase activity (Otte et al., 1989). Evidence strongly suggests that protein kinase C-dependent mechanisms include the AP-1-mediated activation of transcription (Lee et al., 1987) and the potential modification of CREB (Yamamoto et al., 1988). Experimental evidence presented in this thesis indicates that lithium activates specific gene transcription through promoters containing AP-1 binding sites and potentiates forskolin-induced CRE-mediated transcription.

Glucocorticoids. The effects of glucocorticoids on the differentiation of neural crest progenitors are well characterized (Doupe et al., 1985a; 1985b). However, the modulating role of steroid hormones in the central nervous system, while aggressively studied, is far from understood. The recent observation that estrogen induces NT/N mRNA levels in the preoptic nucleus in brains of ovariectomized rats lends support to the specific regional effects of steroids in the brain (Alexander et al., 1989). A combination of structural, biochemical, and genetic analyses of the steroid receptors and hormone-responsive genes has led to a more detailed understanding of the molecular mechanisms of the steroid response and a unifying hypothesis for receptor structure and hormone action (Beato, 1989; Evans and Arriza, 1989).

Structural similarities between the steroid and thryroid hormone receptors support the existence of a steroid/thyroid hormone receptor superfamily of

transcriptional regulators (Evans, 1988). Based on structural and functional characteristics, these receptors can be divided into two groups (Beato, 1989). One group contains the glucocorticoid, progesterone, androgen, and mineralcorticoid receptors. The other group contains the estrogen, thyroid hormone, retinoic acid , and vitamin D3 receptors. The typical receptor is composed of a variable N-terminal domain, a short highly-conserved and cysteine-rich central domain, and a moderately-conserved C-terminal domain. Extensive mutational analyses of receptor cDNAs suggest that: (1) the C-terminal domain is functionally complex, mediating hormone binding, nuclear translocation, dimerization, and the activation of transcription (trans-activation); (2) the central domain mediates DNA binding; and (3) the N-terminal domain co-modulates transactivation with the C-terminal domain. Based on those and numerous other studies, steroid-regulated gene expression has been divided into several loosely defined steps. Basically, upon binding of steroid to its cytoplasmic receptor, an allosteric activation (termed 'transformation') of the receptor is proposed to occur, possibly involving the dissociation of attendant heat shock proteins from the receptor, thereby enabling hormone-receptor complexes to localize to the nucleus and interact specifically with DNA (O'Malley, 1990). In this manner, the steroid receptor functions as a hormone-dependent transcription factor.

Sequence-specific interactions between the steroid-hormone receptor complex and DNA have been proposed to account for both positive and negative effects on transcription (Ackerblom et al., 1988; Sakai et al., 1989; Yamamoto, 1985). Steroid-responsive elements (SREs) are often found clustered with binding sites for other transcription factors in several promoters (Schule et al.,

1988). In general, synergy between these elements appears to be inversely related to the individual strength of each SRE. For example, the proximal glucocorticoid-responsive element (GRE) of the tryptophan oxygenase gene is weakly active in the absence of a CACCC-box, a common transcription factor binding site (Schule et al., 1988). In addition, mutation of the NF I site within the MMTV enhancer results in severely diminished glucocorticoid responsiveness (Strahle et al., 1988). Thus, certain steroid-responsive elements are functionally dependent on other cis-regulatory elements. The NT/N promoter, characterized in this thesis, is an example of a complex cis-regulatory region composed of functionally interdependent elements.

Experimental approach. To begin elucidating the molecular mechanisms of synergy between environmental stimuli which induce NT/N gene expression in PC12 cells, I have characterized the rat NT/N gene. Chapter II of this thesis descibes the NT/N gene, its structure, tissue-specific regulation, and sequence evolution. In Appendix A, a considerable amount of unpublished sequence information is provided, including the sequencing strategy for the bovine hypothalamic cDNA, intron sequences, and 1.4 kb of 5' flanking sequence. Appendix B reviews the experimental approach to establishing a transient transfection methodology for analyzing NT/N gene cis-regulatory sequences in PC12 cells. Chapter III reports the analysis of mutually dependent elements in the NT/N gene cis-regulatory region which are involved in the synergistic responses to cAMP, dexamethasone, NGF, and lithium. The experiments of Chapter IV investigate the mechanism of synergy between three inducers (NGF, forskolin, and lithium) on two types of elements present in the NT/N

transcriptional control region, AP-1 and cAMP-responsive elements. Chapter V summarizes the results of the previous chapters and proposes a model for the complex transcriptional regulation of the NT/N gene in PC12 cells.

COMPENDIUM OF MANUSCRIPTS

Sections II, III, and IV are taken, verbatim, from the following manuscripts:

Section II:

Kislauskis, E., Bullock, B., McNeil, S. and Dobner, P. R. (1988) The rat gene encoding neurotensin and neuromedin N: structure, tissue-specific expression, and evolution of exon sequences. J. Biol. Chem. *263*, 4963-4968.

Section III:

Kislauskis, E. and Dobner, P. R. (1990) Mutually dependent response elements in the cis-regulatory region of the neurotensin/neuromedin N gene integrate environmental stimuli in PC12 cells. Neuron *4*, 783-795.

Section IV:

Kislauskis, E., Wagner, J. A. and Dobner, P. R. (submitted for publication) Nerve growth factor and lithium selectively activate promoters containing multiple AP-1 sites in PC12 cells.

II

THE RAT GENE ENCODING NEUROTENSIN AND NEUROMEDIN N: STRUCTURE, TISSUE-SPECIFIC EXPRESSION, AND EVOLUTION OF EXON SEQUENCES

Kislauskis, E., Bullock, B., McNeil, S. and Dobner, P.R. (1988) J. Biol. Chem. 263, 4963-4968.

ABSTRACT

Recombinant DNA clones encoding the neurotensin/neuromedin N precursor protein have been isolated from both bovine hypothalamus cDNA and rat genomic libraries using a heterologous canine cDNA probe. Nucleotide sequence analysis of these clones and comparison with the previously determined canine sequence has revealed that 76% of the amino acid residues are conserved in all three species. The protein precursor sequences predicted from bovine hypothalamus and canine intestine cDNA clones vary at only nine of 170 amino acid residues suggesting that within a species identical precursors are synthesized in both the central nervous system and intestine. The rat gene spans approximately 10.2 kb and is divided into four exons by three introns. The neurotensin and neuromedin N coding domains are tandemly positioned on exon four. RNA blot analysis has revealed that the rat gene is transcribed to yield two distinct mRNAs, 1.0 and 1.5 kb in size, in all gastrointestinal and all neural tissues examined except the cerebellum. There is a striking variation in the relative levels of these two mRNAs between brain and intestine. The smaller 1.0 kb mRNA greatly predominates in intestine while both mRNA species are nearly equally abundant in hypothalamus, brain stem, and cortex. Sequence comparisons and RNA blot analysis indicate that these two mRNAs result from the differential utilization of two consensus poly(A) addition signals and differ in the extent of their 3' untranslated regions. The relative combined levels of the two mRNAs in various brain and intestine regions correspond roughly with the relative levels of immunologically detectable neurotensin except in the cerebral cortex where mRNA levels are 6 times higher than anticipated.

INTRODUCTION

Neurotensin (NT) is a member of a family of structurally related peptides which cause contraction of smooth muscle and, when injected into the periphery of anesthetized rats, an acute hypotensive response. The carboxy-terminal portion of NT is the major determinant of biological acitivity (1) and is the most conserved region of the different family members. The frog skin peptide, xenopsin (2), shares four of five carboxy-terminal amino acid residues with NT. Similarly, the four carboxy-terminal amino acids of NT and a six amino acid peptide isolated from porcine spinal cord, neuromedin N, are identical (3). Recently, we have determined the primary structure of a 170 amino acid precursor protein which encodes both NT and neuromedin N by sequencing cDNA clones derived from the canine intestine (4).

NT was first isolated from bovine hypothalamus (5) and is likely to serve as a neurotransmitter or neuromodulator in the central nervous system (6). The development of a radioimmunoassay for NT led to the discovery of NT in the gastrointestinal tract (7) where it may be involved in the regulation of fat metabolism (8). Discrete NT-containing endocrine cells are found dispersed throughout the intestinal mucosa, most prominently in the distal small intestine (9). NT has also been detected in the adrenal medulla of a variety of mammals (10). In cats, a specific subpopulation of noradrenaline-containing chromaffin cells also contain NT (11). Interestingly, the rat pheochromocytoma PC12 cell line, which displays some of the phenotypic characteristics of adrenal chromaffin cells, can be induced to produce high levels of NT immunoreactive material by combinations of hormones (12, 13). NT content and production are increased up

to 600-fold by the combined action of nerve growth factor (NGF), dexamethasone, and activators of adenyl cyclase. The induction is highly cooperative with NGF exerting primarily a permissive effect. This is exciting in view of the importance of NGF as a survival and differentiation factor affecting cells which migrate from the neural crest during development (14). PC12 cells are thought to be representative of pleuripotent neural crest cells and differentiate along a sympathetic neuronal pathway in response to NGF (15).

The isolation of cDNA clones encoding NT has made possible molecular approaches to understanding the biosynthesis of the peptide and the regulation of the gene which encodes it. To examine these questions in tractable experimental models, we have isolated and characterized the rat gene encoding the NT/neuromedin N (NT/N) precursor and examined its tissue-specific expression. We also report that it is likely that an identical NT/N precursor is synthesized in the gut and central nervous system.

MATERIALS AND METHODS

Materials. Avian myeloblastosis virus reverse transcriptase, Life Sciences (St. Petersburg, FL); restriction and other enzymes, Boehringer Mannheim and New England Biolabs; 32P-labeled nucleotides, Amersham; deoxy and dideoxy nucleotides, PL Biochemical.

Construction of recombinant libraries. DNA was isolated from rat testes, partially digested with Sau 3a, and size-fractionated on sucrose gradients. Digestion products 15-20 kb in size were ligated with Bam HI digested lEMBL4 phage arms, and packaged in vitro as described (16). A cDNA library was constructed from poly(A)+ RNA isolated from bovine hypothalamus in lgt11 as described (4).

Screening procedures. Escherichia coli (K802 or Y1088) were infected with recombinant phage and plated onto 15 cm bacterial plates at a density of 20,000-30,000 placques per plate. The Eco RI insert of a full-length canine neurotensin precursor cDNA (4) was 32P-labeled by nick translation and used to screen recombinants as described (16). Hybridization was performed in 5 X NaCl/sodium citrate (1 X NaCl/sodium citrate = 0.15 M NaCl/0.015 M sodium citrate, pH 7), 10 X Denhardt's solution (1 X Denhardt's solution = bovine serum albumin, polyvinylpyrrolidone, and Ficoll all at 0.2 mg/ml), 50 mM sodium phosphate buffer (pH 7), 0.5% sodium dodecyl sulfate at 60°C and the filters were washed in several changes of 2 X NaCl/sodium citrate, 0.1% sodium dodecyl sulfate at room temperature followed by a wash in the same solution at

50°C. Positively hybridizing bacteriophage were placque purified and I DNA was prepared as described (16).

DNA sequence analysis. Restriction fragments of rat genomic clones encompassing exons were identified by Southern blotting using a 32P-labeled canine neurotensin precursor cDNA probe and subcloned into either pGEM4 (Promega) or pUC12 (17). Various defined fragments were subcloned into M13mp10 or mp11 (17) and sequenced by the dideoxy method (18).

RNA analysis. RNA was extracted from various tissues by the guanidine thiocyanate procedure (19) and poly(A)+ RNA was selected by passage over oligo dT cellulose (20). RNA blot analysis, and nuclease protection experiments were performed as described (21, 22). For primer extension, 32P-labeled DNA primer (100,000 cpm) was hybridized to RNA in 0.1 M NaCl, 20 mM Tris-HCl (pH7.9), 0.1 mM EDTA for 10 hr at 60°C. An equal volume of a solution containing 80 mM Tris-HCl (pH 7), 10 mM MgCl2, 4 mM each of dGTP, dCTP, dATP, TTP and 10 units of reverse transcriptase were added on ice. The reactions were incubated at 37°C for 30 min and the products were analyzed as described (22). Uniformly labeled single-stranded probes were synthesized using an Eco RI/Hinf I fragment of rNT19 subcloned in M13mp10. The probes used for primer extension and nuclease protection were generated by cleavage with Sst I and Eco RI, respectively, followed by isolation of the labeled strand on 6% acrylamide, 7 M urea gels.

RESULTS

Screening of Libraries. Recently, we have demonstrated using a canine NT/N cDNA probe that different sized NT/N mRNAs are expressed in bovine hypothalamus and canine intestine (4). A 1.5 kb poly(A)+ RNA is detected in bovine hypothalamus while a 1.0 kb poly(A)+ RNA is detected in the canine intestine. These results opened the possibility that these mRNAs encoded different precursor proteins specific to either neural or gastrointestinal tissues. They also suggested that the canine cDNA probe could be used to isolate corresponding sequences from other mammalian species. Southern blot analysis of dog, rat, and human genomic DNA revealed that an unique gene was identified by the canine NT/N probe in each case (data not shown). To examine the structure of the larger 1.5 kb RNA detected in bovine hypothalamus and to characterize the rat NT/N gene, we screened a lgt11 cDNA library derived from bovine hypothalamic poly(A)+ RNA and a rat genomic library constructed using lEMBL4 with a 32P-labeled canine NT/N cDNA probe.

Screening of approximately 240,000 plaques from the bovine hypothalamus cDNA library resulted in the identification of fourteen hybridization-positive clones. Based on preliminary restriction analysis, two of these (bhNT3 and bhNT12) were selected for detailed analysis. Approximately 360,000 independent rat genomic recombinants were screened resulting in the isolation of three hybridization-positive clones. Two of these were nearly identical (rNT18 and rNT19) while the third (rNT23) overlapped throughout half of the cloned sequences. Southern blot analysis using the canine cDNA probe indicated that

rNT18 and rNT19 contained the entire rat NT/N precursor gene and rNT19 was chosen for detailed analysis.

Equivalent Protein Precursors in Hypothalamus and Intestine. The composite structure of the bovine hypothalamus cDNA clones is depicted in Fig. 1A along with the structures of the rat gene and previously described canine cDNA clones. The complete cDNA sequences and comparable exon sequences from the rat gene are presented in Fig. 2. The NT/N protein precursor predicted from the bovine hypothalamus cDNA sequence is nearly identical to that determined previously from canine intestine cDNAs. The general features of the precursor are the same with the neuromedin N and neurotensin coding domains located in tandem near the carboxy terminus of a 170 amino acid protein, bounded and separated by Lys-Arg basic amino acid pairs. There is also considerable similarity in the untranslated regions; however, the 3' untranslated region of the bovine sequence extends 402 bases beyond the comparable region of the canine sequence. In addition to a consensus poly(A) signal common to the two cDNA sequences, the bovine sequence contains a consensus signal near the end of the extended region.

Structure of the Neurotensin/Neuromedin N Gene. Restriction fragments of rNT19 harboring exons were identified by Southern blot analysis and subcloned for sequence analysis. Nucleotide sequences were determined by the dideoxy method using the strategy shown in Fig. 1B. The gene spans approximately 10.2 kb of DNA and comparison with heterologous canine and bovine cDNAs has revealed the positions of three intervening sequences which divide the mRNA sequence into four exons. The sequences at the junctions between exons and

introns conform to consensus splice donor and acceptor sequences (23). Exon one encodes the putative signal peptide; however, the remaining divisions of the gene do not clearly demarcate functional domains. The neurotensin and neuromedin N coding domains are both located in tandem on exon four.

Evolution of Exon Sequences. The predicted precusor protein sequences from dog, cow, and rat are compared in Fig. 3. The canine and bovine sequences are closely conserved displaying 95% amino acid identities. The rat sequence is more divergent as judged by comparison with either the dog or cow sequence with 77 and 78% amino acid identities, respectively. However, most of the substitutions between species are conservative (as defined by the Dayhoff PAM250 matrix [24]).

At the nucleotide level, the sequence conservation in the coding region is roughly the same as that at the amino acid level. In addition, there is substantial homology in the untranslated regions. The 5' and 3' untranslated regions of the cow and dog are closely related with 92 and 93% of the comparable positions conserved, respectively. The rat gene untranslated sequences are more divergent and substantial gaps must be introduced to obtain optimal alignment. However, sequences strikingly similar to both the proximal portion of the 3' untranslated region shared between the dog and cow cDNAs and the extended portion contained only in the cow cDNA are contiguous on exon four of the rat gene. Two consensus poly(A) addition signals are present in both the rat gene and bovine cDNA sequences.

Expression of the Rat Neurotensin/Neuromedin N Gene. To define the start point of transcription, primer extension and nuclease protection experiments were performed using RNA isolated from both gastrointestinal and neuronal tissues (Fig. 4). Hybridization of a single-stranded probe extending from exon one through to the 5' end of the sequences contained in rNT19 with poly(A)+ RNA from either tissue source and subsequent digestion with mung bean nuclease resulted in a group of closely spaced protected fragments (Fig. 4A); the two most prominent bands map to positions 152 and 154 of the rat NT/N gene. Hybridization of a single stranded primer (see Fig. 4C) with RNA from either tissue source and subsequent extension with reverse transcriptase resulted in the formation of two prominent extension products which map to positions 154 and 157 (Fig. 4B). Thus, the cap site is either at or near position 154. This assignment is supported by the occurrence of a "TATA" homology (positions 125-132) 29 base pairs upstream of the assigned cap site. Also noteworthy, is a sequence beginning at position 99 which bears a striking resemblence to the cAMP response element previously defined for the rat somatostatin (25) and human preproenkephalin (26) genes. In addition, two 21 base pair imperfect direct repeats are encountered upstream of the "TATA" homology beginning at positions 27 and 59.

To examine the tissue specificity of rat NT/N gene expression, poly(A)+ RNA was prepared from various tissues and examined by RNA blot analysis (Fig. 5). Using a 32P-labeled single-stranded probe corresponding to the coding region and part of the 3' untranslated region of exon four (rNTB61, Fig. 5C), two prominent bands of approximately 1.0 and 1.5 kb were observed in all neural tissues except the cerebellum (Fig. 5A). In gastrointestinal tissues, the 1.0 kb

RNA is clearly identified but the 1.5 kb RNA is only faintly visible (Fig. 5A, most evident in lane 7) suggesting that the ratio of these two mRNAs may vary in a tissue-specific manner (see Discussion).

Comparison of the rat gene sequence with the bovine and canine cDNA sequences suggested that the two mRNAs might arise as a result of the utilization of both of the poly(A) addition signals encountered in exon four of the rat gene. To test this possibility, a 32P-labeled single-stranded probe (3'UT-1, Fig. 5C) corresponding to the distal portion of the 3' untranslated region (as defined by the bovine cDNA sequence) was used to probe the same RNA blot (Fig. 5B). This probe identifies only one mRNA species corresponding to the upper band identified by the coding region probe. This band is present in all gastrointestinal and neuronal tissues with the exception of the cerebellum. As with the previous probe, there is no detectable NT/N mRNA expressed in testis, liver, or kidney. These results strongly support the contention that the two NT/N mRNAs are the result of the utilization of two different poly(A) addition signals.

DISCUSSION

We have previously characterized cDNA clones derived from the canine intestinal mucosa which encode neurotensin and neuromedin N (4). By screening rat genomic and bovine hypothalamic cDNA libraries with the canine probe, we have isolated the rat NT/N gene and bovine hypothalamic NT/N cDNA clones. Nucleotide sequence analysis has revealed that the general features of the predicted NT/N precursor are the same in all three species. The cow and dog sequences are closely conserved differing at only nine of 170 amino acid residues. The rat sequence is missing one of the two tandem methionine codons which begin the cow and dog sequences and is more divergent. The near identity of the precursor predicted from either canine intestine or bovine hypothalamus cDNA clones indicates that both NT and neuromedin N are processed from an equivalent precursor in the gut and the nervous system.

The rat NT/N gene coding region is divided into four exons by three introns as revealed by comparison with the two heterologous cDNA sequences. Although exon one encodes only the putative signal peptide (amino acids 1-23) with the aspartate codon split by intron one probably comprising part of the cleavage signal (27), the positions of the remaining two introns do not divide the gene into obvious functional domains. The remaining exons encode amino acids 24-44 (exon two), 45-119 (exon three), 120-169 (exon four). The NT and neuromedin N coding domains are located in tandem near the carboxy terminus of the predicted precursor on exon four. This feature of the gene in addition to the fact that we have thus far isolated only cDNAs which encode both NT and

neuromedin N indicates that distinct mRNAs encoding only NT or neuromedin N cannot be generated by alternative splicing mechanisms.

Comparison of the complete NT/N precursor protein sequence from dog, cow, and rat has revealed a tight evolutionary conservation. Of 169 comparable positions, 76% are identical in all three species and most of the substitutions are conservative. Based on our data and previously reported sequences for bovine neurotensin (28) and porcine neuromedin N (3), the sequences of these two peptides appears to be invariant among mammals. The high degree of conservation of the entire precursor could reflect structural requirements for appropriate precursor processing to yield the biologically active peptides. Alternatively, the precursor might be processed to yield biologically acitve peptides other than neurotensin and neuromedin N which are subject to high selective constraints. The precursor sequence is much more highly conserved between dog and cow (95%) than between either of these species and rat (77 and 78%, respectively). This could be due to the higher rate of nucleotide substitution which has been postulated for rodents (29, 30).

In addition to the close conservation of the coding region between the three species, there is also substantial similarity in the untranslated regions (see Fig. 2). Comparison of the 3' untranslated regions of the canine and bovine sequences has revealed that, in addition to a common proximal region spanning approximately 200 nucleotides which is >90% identical, the bovine sequence extends approximately 400 nucleotides further where a second consensus poly(A) addition signal is encountered. This extended 3' untranslated region accounts nicely for the difference in the sizes of canine intestine and bovine hypothalamus NT/N mRNAs we have observed previously (4). The composite

bovine cDNA sequence is 1166 nucleotides in length and, allowing for a 150-200 base poly(A) tail, the mRNA size of 1316-1366 is similar to our previous estimate of 1.5 kb based on RNA blot analysis. Comparison of the bovine cDNA with the rat gene sequence reveals sequences strikingly similar to both the proximal part of the 3' untranslated region (as defined by dog and cow cDNAs) and the distal part (contained only in the bovine cDNA). These sequences are contiguous on exon four of the rat gene.

We have examined the tissue specificity of rat NT/N gene expression by RNA blot analysis using probes derived from exon sequences of the rat gene. Using a probe containing the coding and proximal part of the 3' untranslated region of exon four, two poly(A)+ RNAs were detected in all gastrointestinal and neuronal tissues examined with the exception of cerebellum (Fig. 5A). Quantitation of the two bands by scanning densitometry (data not shown) revealed that the ratio of the level of the 1.5 kb to the 1.0 kb species ranges from 1.1 in the cerebral cortex to 0.8 in hypothalamus and brainstem. This ratio is drastically altered in gastrointestinal tissues where the 1.0 kb mRNA is at least 10 times more abundant than the 1.5 kb mRNA. The sizes of these two mRNAs (1.0 and 1.5 kb) correspond roughly to the sizes of the canine intestine (1.0 kb; previously reported smaller RNA species have not been observed in subsequent experiments; Diane L. Barber and P.R.D., unpublished observations) and bovine hypothalamus (1.5 kb) NT/N mRNAs (4). Reprobing the same blot with a probe corresponding to only the distal portion of the 3' untranslated region (Fig. 5B) resulted in the identification of only the 1.5 kb mRNA species, strongly indicating that the difference between the two mRNAs is in the extent of their 3' untranslated regions. Thus, in the rat, both of the mRNA types defined by heterologous cDNAs

are produced apparently by the utilization of different poly(A) addition signals. Furthermore, the proximal site is highly favored in intestinal tissues. Whether or not the inclusion or exclusion of the distal 3' untranslated region has any functional consequence remains to be determined.

The relative levels of NT/N mRNA (quantitated by scanning several different exposures of the blot shown in Fig. 5A and summing the values obtained for both the 1.0 and 1.5 kb bands, data not shown) are roughly equivalent to the relative levels of NT in these tissues as determined by radioimmunoassay (7). For instance, the level of NT/N mRNA in the hypothalamus is 5.7 times greater than in brainstem which is similar to the 4.7-fold difference in NT content between these two tissues. However, although the NT content of the hypothalamus is 30 times that of the cortex, the NT/N mRNA levels in cortex are only 5-fold lower than in hypothalamus. This striking difference opens the possibility that the NT/N protein precursor is differentially processed in cortex and hypothalamus resulting in the disparity between the levels of immunologically detected NT and the relative NT/N mRNA levels. The best example of such tissue-specific processing is the differential processing of the proopiomelanocorticotropin precursor in the anterior and intermediate lobes of the pituitary (31).

We have mapped the rat NT/N gene cap site to position 154 by both primer extension and nuclease protection experiments using RNA isolated from both intestine and brain. As is the case for many eukaryotic genes transcribed by RNA polymerase II (32), a "TATA" homology is encountered starting 29 base pairs upstream of the cap site. In addition, a sequence matching the previously defined cAMP response element (25, 26) at seven of eight positions is located between positions 103 and 110 of the rat gene and is immediately preceded by another

half repeat of this palindromic sequence (99-102). This cAMP response element could be involved in the control of NT levels in the rat PC12 cell line by combinations of nerve growth factor, dexamethasone, and activators of adenylate cyclase (13). The isolation of the rat NT/N gene will enable investigations of the molecular mechanisms underlying this complex synergistic control.

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REFERENCES

1. Carraway, R. and Leeman, S.E. (1975) in Peptides: Chemistry, Structure, and Biology (Walter, R. and Meienhofer, J. eds.) pp. 679-685, Science Publishers, Ann Arbor, MI

2. Araki, K., Tachibana, S., Uchiyama, M., Nakajima, T. and Yasuhara, T. (1973) Chem. Pharm. Bull. 21, 2801-2804

3. Minamino, N., Kangawa, K. and Matsuo, H. (1984) Biochem. Biophys. Res. Commun. 122, 542-549

4. Dobner, P.R., Barber, D.L., Villa-Komaroff, L. and McKiernan, C. (1987) Proc. Natl. Acad. Sci. USA 84, 3516-3520

5. Carraway, R. and Leeman, S.E. (1973) J. Biol. Chem. 248, 6854-6861

6. Leeman, S.E., Aronin, N. and Ferris, C. (1982) Recent Prog. Horm. Res. 38, 93-132

7. Carraway, R. and Leeman, S. (1976) J. Biol. Chem. 251, 7045-7052

8. Peterson, B.J., Christiansen, J., Rokaeus, A. and Rosell, S. (1984) Scand. J. Gastroenterol. 19, 48-51

9. Sundler, F., Hakanson, R., Hammer, R.A., Alumets, J., Carraway, R., Leeman, S.E. and Zimmerman, E.A. (1977) Cell Tiss. Res. 178, 313-321

10. Goedert, M., Reynolds, G.P. and Emson, P.C. (1983) Neuroscience Letters 35, 155-160

11. Terenghi, G., Polak, J.M., Varndell, I.M., Lee, Y.C., Wharton, J., and Bloom, S.R. (1983) Endocrinology 112, 226-233

12. Tischler, A.S., Lee, Y.C., Slayton, V.W. and Bloom, S.R. (1982) Regulatory Peptides 3, 415-421.

13. Tischler, A.S., Lee, Y.C., Costopoulos, D., Slayton, V.W., Jason, W.J. and Bloom, S.R. (1986) J. Neuroscience 6, 1719-1725

14. Levi-Montalcini, R. (1987) Science 237, 1154-1162

15. Greene, L.A. and Tischler, A.S. (1976) Proc. Natl. Acad. Sci. USA 73, 2424-2428

16. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual,Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

17. Messing, J. (1983) Methods Enzymol. 10, 20-78

18. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467

19. Chirgwin, J.M., Przybyla, A.E., MacDonald, R. and Rutter, W.J. (1979) Biochemistry 18, 5294-5299

20. Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408-1412

21. Dobner, P.R., Kawasaki, E.S., Yu, L.-Y. and Bancroft, F.C. (1981) Proc. Natl. Acad. Sci. USA 78, 2230-2234

22. Dobner, P.R., Kislauskis, E., Wentworth, B.M. and Villa-Komaroff, L. (1987) Nucl. Acids Res. 15, 199-218

23. Padgett, R.A., Grabowski, P.J., Konarska, M.M., Seiler, S., and Sharp, P.A. (1986) Ann. Rev. Biochem. 55, 1119-1150

24. Dayhoff, M. (1978) Atlas of Protein Sequence and Structure Vol. 5, supplement 3, NationalBiomedical Research Foundation, Silver Spring, MD

25. Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G. and Goodman, R.H. (1986) Proc. Natl. Acad. Sci. USA 83, 6682-6686

26. Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E. and Goodman, H.M. (1986) Nature 323, 353-356

27. Von Heijne, G. (1983) Eur. J. Biochem. 133, 17-21

28. Carraway, R. and Leeman, S.E. (1975) J. Biol. Chem. 251, 1907-1911

29. Britten, R.J. (1986) Science 231, 1393-1398

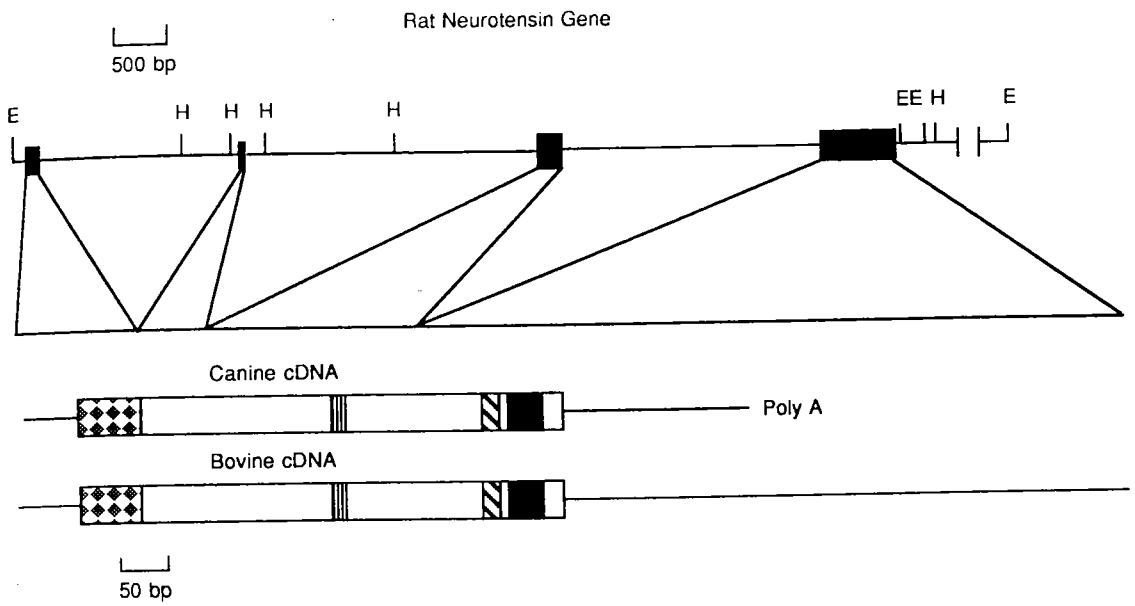
30. Wu, C.-I. and Li, W.-H. (1985) Proc. Natl. Acad. Sci. USA 82, 1741-1745

31. Douglass, J., Civelli, O., and Herbert, E. (1984) Ann. Rev. Biochem. 53, 665-715

32. Breathnach, R. and Chambon, P. (1981) Ann. Rev. Biochem. 50, 349-383

Figure 1. Structure of rat neurotensin/neuromedin N gene and sequencing strategy. (A) The structure of rNT19 isolated from a rat genomic recombinant library. In the rat gene, black boxes denote exons and thin lines denote either introns or flanking sequences. Eco RI (E) and Hind III (H) restriction sites are indicated. The structure of the gene is projected onto schematic representations of cow and dog NT/N cDNA clones. Boxes represent coding sequences and known functional domains are indicated by diamonds, signal sequence; vertical lines, neuromedin N-like; diagonal lines, neuromedin N; black, neurotensin; open, regions of unknown function. (B) Regions of rNT19 corresponding to exons were identified by Southern blot analysis and subcloned into plasmid vectors. Exon sequences were determined by the dideoxymethod using the strategy depicted. Exon sequences are indicated by either black boxes (coding) or open (untranslated) boxes; other sequences are indicated by a thin line.

A


Rat Neurotensin Gene
500 bp
E
H
H
H
H
EE H
E
Canine cDNA
Poly A
Bovine cDNA
50 bp

B

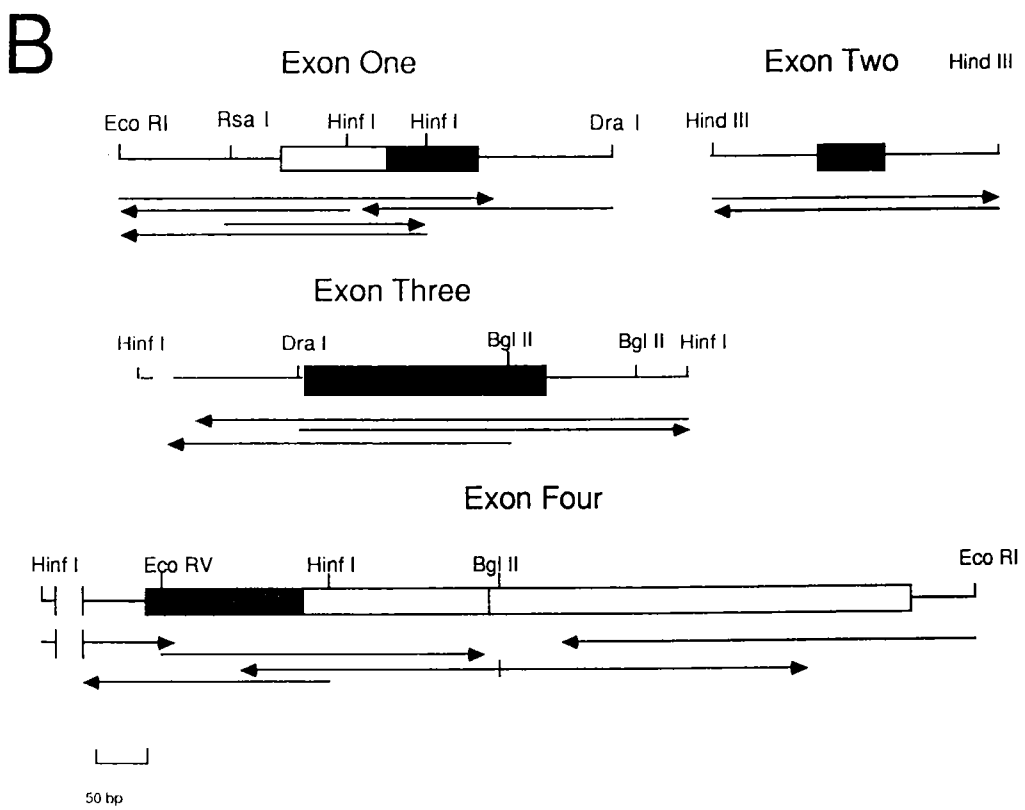

Exon One
Exon Two
Hind III
Eco RI
Rsa I
Hinf I
Hinf I
Dra I
Hind III
Exon Three
Hinf I
Dra I
Bgl II
Bgl II
Hinf I
Exon Four
Hinf I
Eco RV
Hinf I
Bgl II
Eco RI
50 bp

Figure 2. Sequence of the rat neurotensin/neuromedin N precursor gene and corresponding sequences of bovine hypothalamus and canine intestine cDNAs. Rat gene sequences were determined by the dideoxy method using the strategy depicted in Fig. 1B. Bovine hypothalamus cDNA sequences were compiled from two independent recombinants (bhNT3 and bhNT12) by strategies similar to those used previously to determine the canine intestine cDNA sequence (4). The canine sequence has been extended using data compiled from two recombinants not previously reported (ciNT11E and ciNT5B). The numbering of the rat sequence begins at the first nucleotide of the Sau 3A cloning site and does not include intervening sequences which have not been completely determined. The bovine (middle) and canine (lower) cDNA sequences starting at positions 200 and 207, respectively, are presented below the rat sequence. The positions of the three intervening sequences are indicated by arrows and the conserved consensus splice donor and acceptor sequences are shown. The cap site (arrowhead), "TATA" homology (bold underline), and a sequence resembling a cAMP response element (bold lines above and below) are indicated. The first and last nucleotides of the cDNA sequences are indicated by an asterick and a plus sign, respectively. The positions of two direct 21 base pair repeats and of two poly(A) addition signals are indicated by thin underlines. The predicted amino acid sequence of the rat NT/N precursor protein is shown above the rat DNA sequence. Differences between the rat and either cow, or dog cDNA sequences are indicated with gaps necessary to obtain maximum alignment indicated by dashes. Sequences were aligned using DNAstar (Madison, WI.) software. The lengths of the composite cow and dog cDNA sequences are 1,166 and 759 nucleotides, respectively.

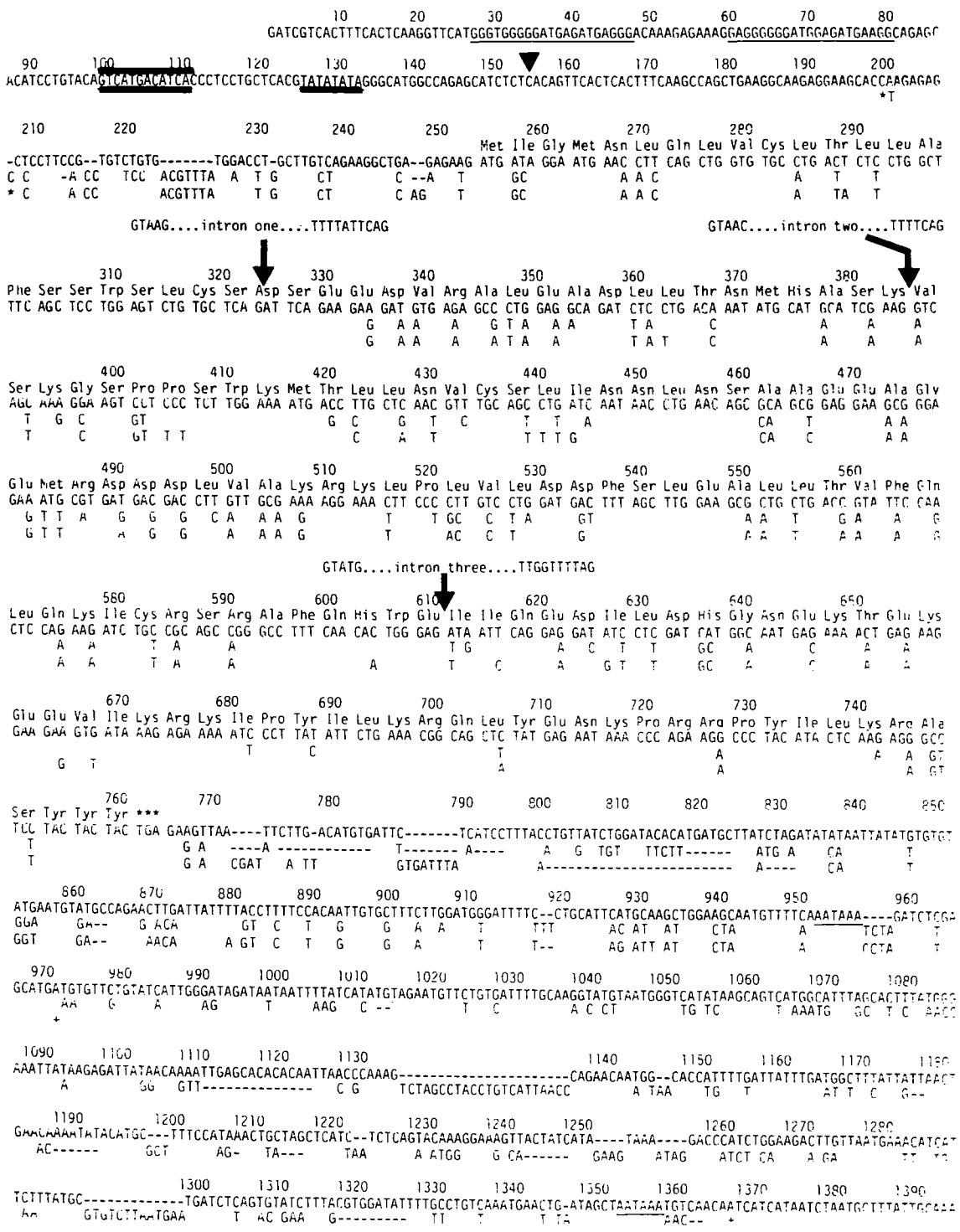

GTAAG....intron one....TTTTATTCAG
GTAAC....intron two....TTTTCAG
GTATG....intron three....TTGGTTTTAG

Figure 3. Comparison of the predicted neurotensin/neuromedin N precursor sequences from rat, cow and dog. The complete precursor sequences are depicted using the single letter amino acid code. Amino acids which are identical in all three species are boxed.

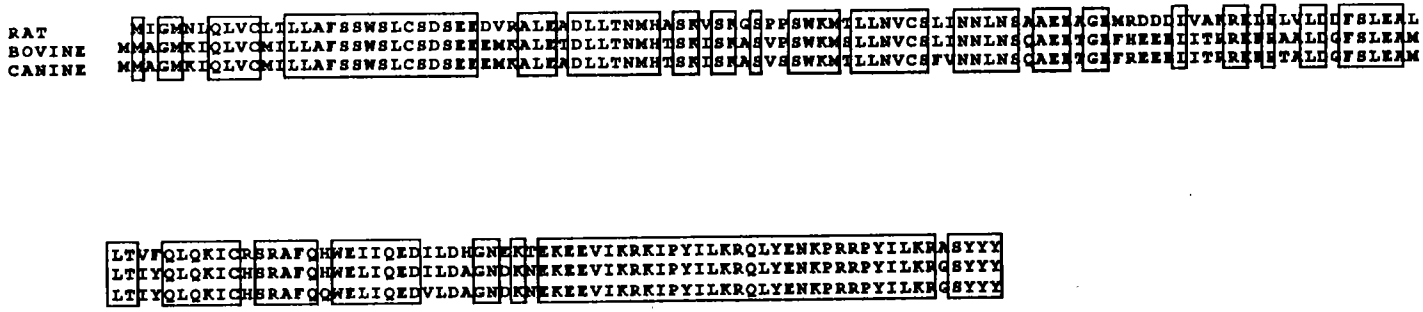

RAT
BOVINE
CANINE

Figure 4. Determination of transcription initiation site. (A) Nuclease protection. A 32P-labeled single-stranded probe was hybridized to either 10 mg of rat hypothalamus (lanes 3 and 4), or jejunum-ileum (lanes 5 and 6) poly(A)+ RNA, or total yeast RNA (lane 2) followed by digestion with either 50 (lanes 3 and 5) or 100 (lanes 2, 4 and 6) units of mung bean nuclease. The products of the reaction were analyzed on a 6% polyacrylamide/7M urea gel. Two arrows denote the major protection products. Undigested probe, lane 1; dideoxy sequencing reactions using the template used to synthesize the probe (GATC). (B) Primer extension. 32P-labeled single-stranded primer was hybridized to 10 mg of either jejunum-ileum (lane 3), cerebrum (lane 4), or testis (lane 5) poly(A)+ RNA, or total yeast RNA (lane 2) and extended with reverse trancriptase. Reaction products were analyzed as described above. Unreacted primer, lane 1; dideoxy sequencing sequencing reations (GATC). The extension products can be directly positioned on the gene sequence and are indicated by an arrow. (C) The positions of the probe used in (A) and the primer used in (B) are depicted schematically. 5' flanking (thick line), 5' untranslated (open bar), coding region (diagonal lined bar), and vector (thin line) sequences are indicated. The extent of the probe, primer, protected fragment, and the extent to which the primer was extended (zig-zag line) are also indicated. To make the template, an Eco RI/ Hinf I fragment of rNT19 was subcloned into M13mp11.

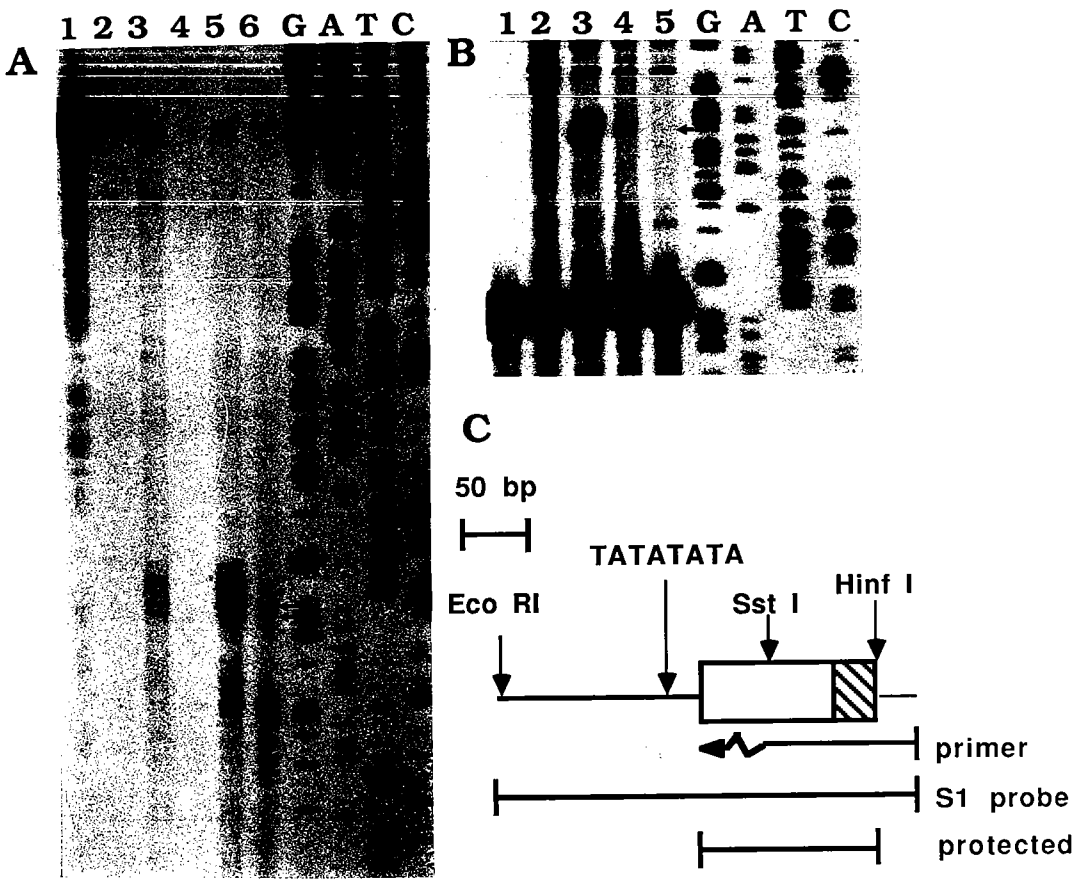
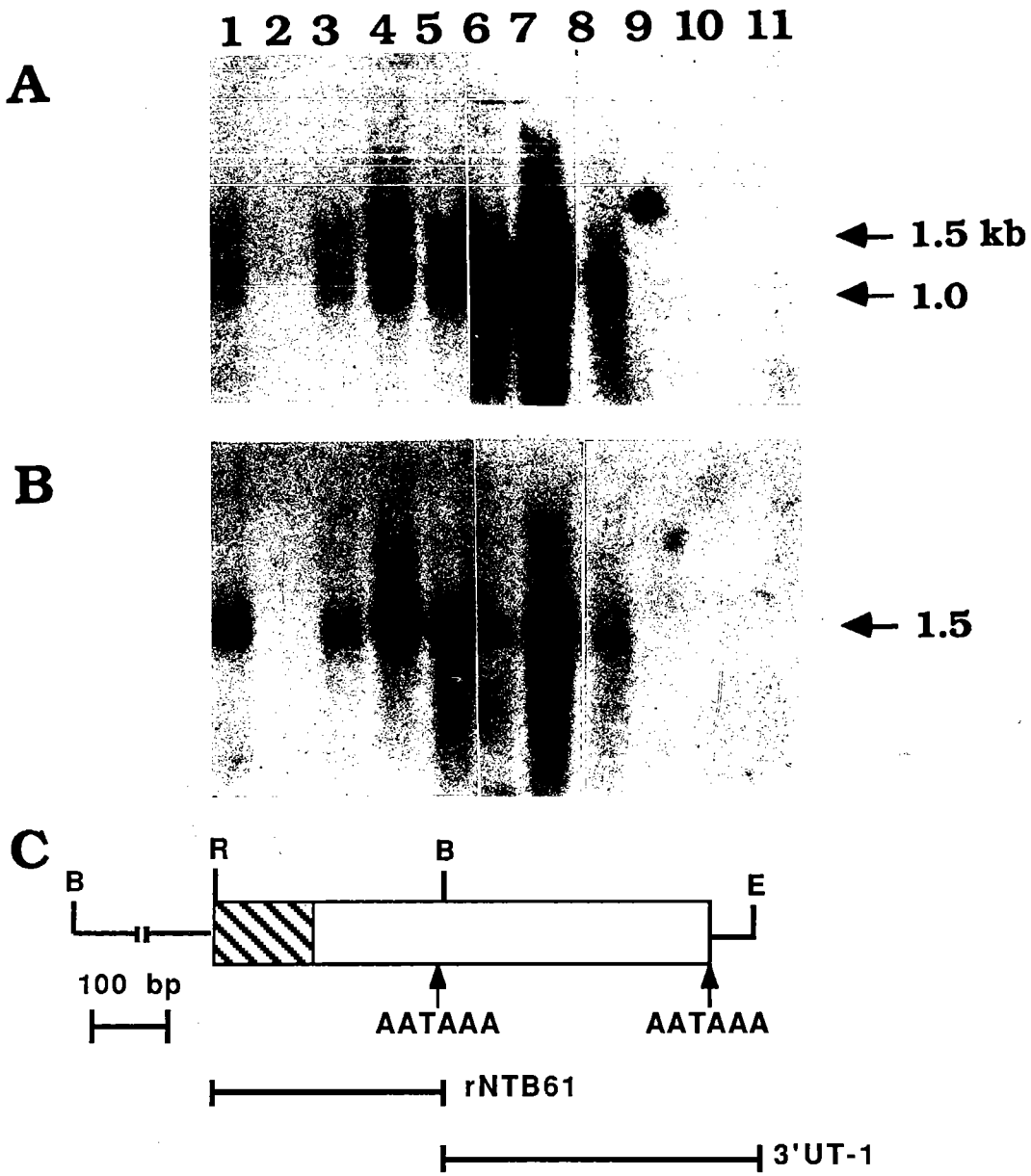

A
1 2 3 4 5 6 G A T C
B
1 2 3 4 5 G A T C
C
50 bp
TATATATA
Eco RI
Sst I
Hinf I
primer
S1 probe
protected

Figure 5. RNA blot analysis. Poly(A)+ RNA was isolated from various adult rat tissues, size-fractionated on a 1% formaldehyde-agarose gel , transfered to a Zetabind filter (Cuno, Meriden, Ct.), and hybridized with either of two 32P-labeled single-stranded probes (panel C). Hybridizing bands were visualized by autoradiography with an intensifying screen for 70 hours. 5 mg poly(A)+ RNA from brainstem, lane 1; cerebellum, lane 2; cerebrum, lane 3; hypothalamus, lane 4; total brain, lane 5; duodenum-jejunum, lane 6; jejunum-ileum, lane 7; large intestine, lane 8; testis, lane 9; kidney, lane 10; and liver, lane 11. The blot was hybridized with a probe (rNTB61) spanning coding region and proximal 3' untranslated sequences (panel A), subsequently stripped, and re-hybridized with a probe (3'UT-1) spanning only the distal 3' untranslated region (panel B). The probes are depicted schematically in panel C. Intron (thin line), coding (diagonal lined bar), 3' untranslated (open bar), and 3' flanking (thin line) sequences are indicated. The positions of two consensus poly(A) addition signals and the extent of the anti-sense probes, rNTB61 and 3'UT-1, are also indicated.


1 2 3 4 5 6 7 8 9 10 11
A
1.5 kb
1.0
B
1.5
C
B
R
B
E
100 bp
AATAAA
AATAAA
rNTB61
3'UT-1

III

MUTUALLY DEPENDENT RESPONSE ELEMENTS IN THE CIS-REGULATORY REGION OF THE NEUROTENSIN/NEUROMEDIN N GENE INTEGRATE ENVIRONMENTAL STIMULI IN PC12 CELLS

Kislauskis, E. and Dobner, P. R. (1990) Neuron 4, 783-795.

ABSTRACT

The expression of the gene encoding the neuroendocrine peptides neurotensin and neuromedin N is strictly dependent on simultaneous exposure to multiple inducers in PC12 pheochromocytoma cells. NT peptide and NT/N mRNA levels are synergistically induced by combinations of nerve growth factor, dexamethasone, activators of adenylate cyclase, and lithium ion. We have used transient transfection assays to delineate the rat neurotensin/neuromedin N gene sequences necessary for this complex regulation. Progressive deletions of the 5' flanking region revealed that sequences between -216 and +56 are sufficient to confer the full spectrum of responses exhibited by the endogenous gene to a reporter gene. Detailed mutational analysis of this region indicates that it is composed of an array of inducible cis-regulatory sequences, including AP-1, cyclic AMP response (CRE), and glucocorticoid response elements. Specific mutation of either the AP-1 site or each of two CREs indicates that they are functionally interdependent. This array of response elements serves to integrate multiple environmental stimuli into a unified transcriptional response.

INTRODUCTION

Neurotensin (NT), a tridecapeptide, is widely expressed in both the central nervous system and gastrointestinal tract (Reinecke, 1985). Sequence analysis of cDNA and genomic clones has revealed the structure of a putative 169-170 amino acid precursor protein encoding NT, and a structurally related six amino acid peptide, neuromedin N (Dobner et al., 1987; Kislauskis et al., 1988). NT has been implicated in the regulation of fat metabolism in the gut, and in diverse functions in the central nervous system, where it is likely to serve as a neurotransmitter or neuromodulator (Reinecke, 1985). Several lines of evidence support a functional relationship between NT and catecholamines, particularly dopamine (Nemeroff, 1980). Immunohistochemical localization studies have provided suggestive evidence that NT is synthesized along with catecholamines in subpopulations of catecholamine-containing neurons and adrenal medullary cells (Terenghi et al., 1983; Hokfelt et al., 1984). Interestingly, the PC12 adrenal medullary tumor cell line produces the catecholamines dopamine and norepinephrine, and can be induced to synthesize and secrete NT (Tischler et al., 1982; Tischler et al., 1986; Dobner et al., 1988).

PC12 cells, like sympathetic neurons in primary culture, elaborate long neurites in response to nerve growth factor (NGF) (Greene and Tischler, 1976). Similar phenotypic plasticity is exhibited by adrenal chromaffin cells both in tissue culture and in vivo (Aloe and Levi-Montalcini, 1979; Anderson and Axel, 1985; Doupe et al., 1985a; Doupe et al., 1985b; Anderson and Axel, 1986). Common precursor neural crest cells give rise to sympathetic neurons, adrenal

chromaffin cells, and a diverse collection of other adult cell types (LeDouarin, 1980). A variety of transplantation and tissue culture experiments indicate that environmental determinants, like NGF, dictate the final adult phenotypes adopted by crest cell precursors (Landis and Patterson, 1981). Glucocorticoids and NGF play key antagonistic roles in the decision between the adrenal medullary and sympathetic neuronal phenotypes (Unsicker et al., 1978; Doupe et al., 1985a; Doupe et al., 1985b; Anderson and Axel, 1986). Additional factors are likely to be involved in the subspecialization of these cell types. For instance, sympathetic neurons acquire cholinergic properties in response to a factor produced by heart myocytes and fibroblasts (Patterson, 1978). Thus, the existence of multiple environmental determinants could help to account for the numerous adult phenotypes adopted by crest cells. Additional diversity could result from the combined influence of multiple environmental factors on single cells.

The elaboration of different crest cell phenotypes is likely to require distinct patterns of gene expression specific to each cell type (Anderson and Axel, 1985; Anderson and Axel, 1986). The expression of several neural-specific mRNAs and antigens is extinguished during chromaffin cell differentiation. In contrast, NGF-induced phenotypic conversion of primary adrenal chromaffin cells is accompanied by the increased expression of several neural-specific mRNAs. Similarly, NGF treatment of PC12 cells results in changes in the expression of numerous genes, including neural-specific genes (Levi et al., 1985; Anderson and Axel, 1985; Leonard et al., 1987; Milbrandt, 1987; Stein et al., 1988). Many of these genes are regulated in a reciprocal fashion by glucocorticoids, and simultaneous treatment with both agents frequently results in an intermediate

level of expression (Leonard et al., 1987; Stein et al., 1988). Thus, the antagonism exhibited by NGF and glucocorticoids in the choice between the adrenal medullary and sympathetic neuronal phenotypes is reflected at the level of specific gene expression in PC12 cells, even though NGF-induced neurite outgrowth is not inhibited by glucocorticoids in these cells. These observations provide preliminary evidence that the transcriptional responses evoked by multiple environmental signals in crest cells may be more complex than the separate responses to individual signals. The exposure of crest cells to multiple environmental signals could indeed constitute a prerequisite for the expression of certain genes.

NT/N gene expression in PC12 cells is strictly dependent on simultaneous exposure to multiple inducers (Dobner et al., 1988). NT peptide and NT/N mRNA levels are synergistically increased in PC12 cells by combinations of NGF, dexamethasone, and activators of adenylate cyclase. Lithium ion, which inhibits NGF-induced neurite outgrowth by PC12 cells (Burstein et al., 1985), strongly potentiates these responses. The gene is not detectably expressed in control cells, and little or no detectable increase in expression is observed upon treatment with individual inducers. High level expression requires simultaneous treatment with three, or all four inducers. At maximum, intracellular levels of NT are increased 1,000-fold over control values, accompanied by an increase in NT/N mRNA levels of at least equal magnitude.

To understand the complex control of NT/N gene expression in PC12 cells, we have delineated the rat NT/N gene cis-regulatory sequences necessary for appropriate regulated expression in PC12 cells. Sequences between -216 and -28 are sufficient to confer the full spectrum of responses exhibited by the

endogenous rat NT/N gene to a reporter gene driven by a heterologous minimal promoter element. Detailed mutational analysis of this region indicates that it is composed of an array of inducible cis-regulatory motifs. This control element is capable of integrating inputs from multiple environmental stimuli at the level of specific gene expression. We discuss the possible implications of such integrative properties on the generation of diverse adult cell phenotypes from common equipotent precursor cells.

RESULTS

Rat NT/N gene proximal 5' flanking sequences mediate responses to inducers. To identify cis-regulatory sequences necessary for the regulated expression of the rat NT/N gene, a series of plasmids containing 5' flanking sequences fused to the bacterial chloramphenicol acetyl transferase (CAT) gene were co-transfected with a luciferase standardization plasmid into PC12 cells by electroporation (Chu et al., 1987). Transfected cells were plated in six well tissue culture dishes, and after a 48 hr recovery period either treated with lithium alone or the indicated combinations of NGF (100 ng/ml), dexamethasone (1 mM), forskolin (1 mM), and lithium (20 mM) for 12 hr. The expression of a CAT fusion gene containing 1.4 kb of 5' flanking sequence was induced to high levels in cells treated with combinations of inducers, but not in lithium treated and control cells (Figure 1A). This pattern of expression was virtually unchanged by further deletion of approximately 1.2 kb of sequences to -216. Similar results were obtained with plasmids containing either 5.0 kb more of 5' flanking sequences, or various intragenic sequences cloned upstream of the 1.4 kb promoter construct (data not shown). These results indicate that most if not all of the cis-regulatory sequences required for induction are located within the -216/+56 fragment.

Further 5' deletions revealed the approximate 5' end of the control region (Figure 1A). Deletion to -173 resulted in dramatically reduced responses (>75%) to all combinations of inducers tested as compared to the -216 construct. A further decline in activity resulted upon deletion to -102, and plasmids with deletion endpoints at -62 or -55 were nearly silenced. The -43

plasmid, which contains only 12 base pairs upstream of the "TATA" box, was silent. These results locate the 5' boundry of the control region necessary for full activity between -216 and -173. Interestingly, a perfect consensus AP-1 sequence (Angel et al., 1987) is located in this region (Figure 1B). AP-1 sites were first defined in a region of the human metallothionein gene necessary for basal level expression, and in the SV40 enhancer (Lee et al., 1987). This same site was subsequently shown to mediate transcriptional responses to phorbol esters (Angel et al., 1987). The region between -216 and the "TATA" box contains several other near-consensus cis-regulatory motifs (Figure 1B). These include elements which in other genes mediate responses to either cyclic AMP (CRE), or glucocorticoids (GRE). Two 21 base pair imperfect direct repeats (IDR) are also located in this region.

To determine if the induction of CAT enzyme activity was the result of increases in correctly initiated fusion gene transcripts, the -216/+56 CAT fusion gene was transfected into PC12 cells. Following a 48 hr recovery period, the indicated inducers were added for 12 hr, total RNA was prepared, and transcripts were quantitated using a S1 nuclease protection assay (Figure 2A). Fusion gene transcripts were detected using a 415 nucleotide 32P-labeled probe spanning sequences from -43 to +56 of the rat NT/N gene and the first 253 nucleotides of the CAT gene. Hybridization with 40 mg of total RNA isolated from cells treated with either all four inducers, or the combination NGF, dexamethasone, and forskolin resulted in a 309 nucleotide protection product, the size expected for correctly initiated transcripts (Figure 2A, lanes 3 and 4). No fusion gene transcripts were detected in either lithium treated, or control cells (Figure 2A, lanes 1 and 2). Similar results were obtained using a 32P-

labeled probe derived from exon four of the rat NT/N gene (Dobner et al., 1988) to detect endogenous transcripts (Figure 2A, lanes 9-12) and by measuring CAT activity in extracts prepared from the same cells used for preparation of RNA (Figure 2B). The relative increases in levels of fusion gene transcripts, endogenous gene transcripts, and CAT enzyme activity upon treatment with either three or four inducers were remarkably similar (Figure 2C). Thus, induction of CAT activity is the result of correctly initiated transcription of the transiently expressed fusion gene, and these increases closely parallel the responses of the endogenous gene.

The expression patterns of transfected CAT and luciferase fusion genes driven by the rat NT/N -216/+56 promoter fragment were strikingly similar to that of the endogenous NT/N gene in PC12 cells treated with all possible combinations of NGF, dexamethasone, forskolin, and lithium (Figure 3). High level expression of the endogenous rat NT/N gene (Figure 3, inset) and both reporter genes required simultaneous treatment with combinations of three or all four inducers. At maximum, reporter gene function was induced 600 to 1,200-fold over control values, a response similar in magnitude to that of the endogenous gene. Little or no increases were observed in cells treated with individual inducers. The small responses of the reporter genes to treatment with either forskolin or lithium alone in this experiment are not reproducibly observed. Certain pairwise combinations of inducers (dexamethasone and forskolin, forskolin and lithium) more effectively stimulated expression of the transfected fusion genes than is typical for the resident NT/N gene. Except for these minor differences, the sequences between -216 and +56 of the rat NT/N gene appear sufficient to

confer the full spectrum of responses to inducers exhibited by the endogenous gene.

To test whether rat NT/N 5' flanking sequences upstream of the rat NT/N "TATA" box could confer regulation to a heterologous promoter, sequences between -216 and -28 were cloned in either orientation upstream of the rat prolactin (PRL) "TATA" box and 5' untranslated sequences (-39 to +38) linked to CAT (Figure 4). PC12 cells were transfected with these plasmids, allowed to recover, and treated with the indicated inducers for 12 hr. The induction profile observed for both plasmids was similar to that obtained with the CAT fusion gene driven by the wild type -216/+56 promoter fragment. Thus, the NT/N "TATA" box and downstream sequences can be substituted by similar sequences derived from the rat prolactin gene indicating that the cis-regulatory sequences necessary for responses to inducers are located 5' to the "TATA" box. The ability of the regulatory region to function in either orientation is suggestive of enhancer function. However, a PRL promoter construct in which the -216/-28 fragment was cloned downstream of the CAT gene was essentially silent even in maximally induced cells suggesting that these sequences do not function in the position-independent manner characteristic of most enhancers (data not shown).

Multiple response elements are necessary for full NT/N promoter activity. A series of linker scanner and internal deletion mutant constructs were analyzed to more precisely map regulatory sequences in the 5' flanking region (Figure 5). These mutants were created by joining appropriate 5' and 3' deletion clones via a 10 bp Bam HI linker. Mutant plasmids were transfected into PC12 cells, and following a 48 hr recovery period the cells were treated with either the indicated

combinations of inducers or no additives. The activity of each mutant relative to wild type (-216/+56) for each induction condition is shown (Figure 5). The activity of the wild type promoter in uninduced cells was very close to background and this low level activity was not increased by any of the mutations analyzed (data not shown). The mutations define both distal and proximal domains of the regulatory region which are separated by two imperfect direct repeats.

Two distal elements were identified. The first is a perfect consensus AP-1 site located between the 5' deletion endpoints -216 and -173 (see Figure 1). Two linker mutations (-189/-182, -182/-173) which alter the AP-1 site were analyzed and provide direct evidence for its function (Figure 5A). Extensive alteration of the AP-1 sequence (-189/-182) resulted in dramatically reduced responses (79-93%) to all inducer combinations tested. Similar reductions (>90% in most cases) were observed with linker mutant -182/-173 in which the terminal A nucleotide of the AP-1 consensus sequence is changed to a C, although the response to combined treatment with dexamethasone, NGF, and lithium was less severely attenuated (67%). These results strongly imply that the consensus AP-1 site is an important component of the NT/N promoter and a focal point of synergistic interactions. The second distal element is defined by linker mutation -145/-136 which changes four nucleotides of a CGTCA motif. The human vasoactive intestinal peptide (VIP) promoter contains two inverted repeats of this sequence both of which are required for full transcriptional responses to cAMP (Tsukada et al., 1987). The consensus CRE (TGACGTCA) also includes this motif (Montminy et al., 1986). Fusion genes driven by the -145/-136 mutant promoter displayed dramatically reduced

responses (73-89%) to combinations of inducers which included the adenylate cyclase activator, forskolin (Figure 5A). However, the response to combined treatment with NGF, dexamethasone, and lithium was reduced only ~two-fold. Thus, the distal CGTCA (CRE) motif is required for maximal responses to inducers, but mutation of this sequence has a more severe impact on inducer combinations which include forskolin. The importance of this site is further substantiated by the internal deletion mutant -145/-125 in which the CGTCA motif is partially deleted (Figure 5A). Interestingly, the analysis of 5' deletion constructs (Figure 1A, compare deletion plasmids -150 and -120) did not reveal the importance of this site indicating that its activity could depend on upstream sequences, perhaps the AP-1 site.

The proximal domain is defined most precisely by the linker mutations -52/-43, and -58/-48 which alter CRE and GRE consensus sequences (Figure 5B). The CRE-like sequences in the proximal domain (-52 to -41) consist of an octamer (TGACATCA) which differs by one nucleotide from the consensus CRE palindrome, and a CRE half repeat (GTCA) immediately upstream of the octamer. This CRE-like octamer sequence is identical to an autoregulatory element located upstream of the c-jun proto-oncogene which binds AP-1 complexes in vitro (Angel et al., 1988). Extensive alteration of the CRE-like sequences in linker mutant -52/-43 resulted in dramatically reduced (>85%) responses to inducer combinations which include forskolin, but the response to combined treatment with NGF, dexamethasone, and lithium was reduced only ~2-fold. This biased effect was more pronounced, but similar to that observed when the distal CRE-like sequence was mutated (-145/-136, Figure 5A). The -58/-48 linker mutation affects both the CRE-like sequences, and a GRE-like sequence which

differs by one nucleotide from a functional tyrosine amino transferase gene GRE (Jantzen et al., 1987). This mutation markedly curtailed responses (86-92%) to all inducer combinations tested, and was the most severe mutation analyzed (Figure 5B). Removal of portions of the GRE consensus sequence in two internal deletion mutants (-129/-64, -129/-56) selectively compromised responses to inducer combinations which included dexamethasone (Figure 5B). This biased effect was abolished by further deletion into the adjacent CRE consensus sequence (-129/-43). These results taken together strongly suggest that a functional GRE is located within the sequences deleted in the -129/-64 and -129/-56 internal deletion mutants. Surprisingly, extensive substitution of the distal arm of the GRE in linker scanner mutant -73/-63 resulted in relatively mild decreases in responses to all inducer combinations tested. One possible explanation for this result is that the proximal half of the GRE is functional, but only in the context of upstream sequences not present in the internal deletion mutants. A functional estrogen response element half site has been characterized in the chicken ovalbumin gene promoter (Tora et al., 1988). The discrepancy between the substitution and deletion mutants could also be due to effects of the linker sequence or the context of the sequences in different mutants.

The sequences between the proximal and distal elements described above are largely composed of two imperfect direct repeats (IDRs). The analysis of 5' deletion constructs (Figure 1A) suggested that the IDRs may be required for full NT/N promoter function (compare deletion endpoints -120 and -102). Two linker mutations in the distal IDR resulted in mild reductions in responses to inducers (-129/-119, -112/-103) while a third (-116/-106) resulted in increased responsiveness (Figure 5C). However, a consensus SP1 binding site

(Mitchell and Tjian, 1989) was created at the junction of the Bam HI linker in the -116/-106 mutant. Since the SP1 transcription factor is widely, if not ubiquitously expressed, the SP1 site could account for the increased responsiveness of the -116/-106 mutant. Two small deletions in the proximal IDR (-100/-87, -90/-77) also had a relatively mild impact decreasing induced activity ~2-fold for all conditions tested. Deletion of both IDRs (-115/-74) had very little effect on overall activity, however, a SP1 binding site was adventitiously created by the Bam HI linker in this mutant and could have replaced a lost function. These results taken together suggest that the IDRs may be required for maximum responses to inducers, but are not essential components of the regulatory region.

Expression in other cell lines. To determine whether the NT/N promoter could function in other cell types, rat GH3 pituitary and Rat2 embryo fibroblast cells were transfected with CAT fusion genes. Following a 48 hr recovery period, cells were induced with various combinations of forskolin, dexamethasone, and lithium for an additional 12 hr. NGF was not used since neither GH3 nor Rat2 cells have been reported to possess NGF receptors, and in preliminary experiments NGF did not influence expression of transfected fusion genes in Rat2 cells (data not shown). The CAT fusion gene driven by the NT/N -216/+56 promoter fragment was active in both cell types, but displayed surprisingly different patterns of expression (Figure 6). The fusion gene was expressed at relatively high levels in uninduced Rat2 cells, and expression was increased only a modest 3-fold upon simultaneous treatment with all three inducers (Figure 6A). The high basal level expression was dependent on sequences between -216

and -43 since deletion of these sequences nearly silenced the promoter (Figure 6A). In contrast, the expression of the CAT fusion gene driven by the -216/+56 promoter fragment was highly dependent on treatment with inducers in GH3 cells (Figure 6B), and is reminiscent of the expression pattern in PC12 cells. A notable difference is the induction of fusion gene expression in response to treatment of GH3 cells with just forskolin. These results indicate that the -216/+56 NT/N promoter fragment is active in diverse cell types, but displays strikingly different patterns of expression in fibroblasts, and endocrine and neuroendocrine cell types.

DISCUSSION

Multiple response elements. We have used transient transfection assays to examine the sequence requirements for appropriate synergistic regulation of the rat NT/N gene in PC12 cells. Sequences between -216 and +56 are sufficient to confer the full spectrum of responses exhibited by the endogenous gene to two different reporter genes. At maximum, reporter gene function is induced 600- to 1,200-fold over control values, a response similar in magnitude to that of the endogenous gene (Dobner et al., 1988). Sequences between -216 and and -28 function in a relatively orientation-independent manner, suggestive of enhancer function. Although we have not examined position-dependence in detail, this fragment did not function when cloned downstream of a CAT gene driven by a minimal promoter.

The analysis of linker scanner and deletion mutants which span sequences between -216 and the "TATA" box indicates that the control region can be functionally divided into two domains, proximal and distal, separated by two imperfect direct repeats. The distal domain contains consensus AP-1 and CRE sequences while the proximal domain includes near consensus GRE and CRE sequences. Specific mutation of these elements revealed, in most cases, that they play key roles in the overall function of the control region. Mutations in the AP-1 and CGTCA (CRE) sequence motifs in the distal domain, and the TGACATCA (CRE) sequence motif in the proximal domain sharply diminish responses to most inducer combinations. Interestingly, most mutations in the two CRE and GRE consensus sequences had biased effects on inducer combinations which included

forskolin or dexamethasone, respectively (see below). In contrast, the AP-1 site appears to participate in responses to all productive inducer combinations.

The effect of inducers on NT/N gene expression is clearly synergistic. Individual inducers have virtually no effect on the expression of the gene, while treatment with three or all four inducers results in high level expression. Synergy could be generated at the level of the gene, at the level of signal transduction, or at both levels. The severe impact of mutating the AP-1 site or either of two CREs within the NT/N promoter on the responses to different combinations of inducers suggests that these sites may be functionally interdependent. Synergy between different classes of sites which bind distinct transcription factors and between multiple copies of single sites is a common feature which has emerged from the examination of a variety of enhancers and complex promoters (Dynan, 1989). Inducible response elements are frequently found tightly associated with other transcription factor binding sequences, and in some cases are dependent on these sites for high level activity. AP-1 sites within a basal enhancer element of the metallothionein promoter and the SV40 enhancer display a marked dependence on adjacent SP1 and AP-4 sites, respectively, for transcriptional activation in vitro (Lee et al., 1987; Mermod et al., 1988). Similarly, a sequence which confers responses to cAMP and phorbol esters in the upstream region of the human proenkephalin gene is composed of multiple interdependent sequence elements, including an essential CGTCA (CRE) motif (Comb et al., 1989; Hyman et al., 1989). Multiple sites of a single functional class are also frequently observed to act cooperatively (Tsukada et al., 1987; Schule et al., 1988; Strahle et al., 1988).

Functional synergy between the AP-1 and CRE sites could account, at least in part, for the requirement for multiple inducers to obtain high level NT/N gene expression. Each of these types of sites could be bound by distinct transcription factors which are separately regulated by different inducers, and since each site functions only poorly by itself, high level expression would result only when both factors are present in an active form. However, the consensus AP-1 and CRE sequences are closely related, and, although they appear to bind different proteins or protein complexes in many circumstances, recent evidence suggests that there may be at least some overlap in the proteins which bind these related sequences (see below). Thus, an additional possibility is that synergy could be generated through the combined effects of inducers, for instance multiple phosphorylations, on a single protein or protein complex which binds to both the AP-1 and CRE sequences. These mechanisms are not necessarily related, but are also not mutually exclusive. For each of these possibilities, the apparent interdependence of the AP-1 site and the CREs within the NT/N promoter region could account, at least in part, for the high degree of synergy observed in the induction of NT/N gene expression in PC12 cells.

Whether or not the GRE may also function in an interdependent manner is less clear. Several internal deletion mutants indicate that the GRE consensus sequence actually mediates transcriptional responses to glucocorticoids within the context of the NT/N promoter, although a linker mutant which altered the distal half of the GRE had a comparatively mild effect on activity. The remaining half palindrome of the GRE in this mutant could retain function, since hormone responsive half sites have been observed in other genes (Tora et al., 1988). The GRE appears to be only weakly active since the addition of dexamethasone with

different combinations of inducers typically increases the expression of fusion genes driven by the wild type NT/N promoter only 2 to 5-fold (see Figures 1 and 3). Weakly active GREs have been demonstrated to act synergistically with a variety of DNA binding sites (Schule et al., 1988; Strahle et al., 1988). The GRE within the NT/N promoter could depend on either the AP-1 site, the CREs, or both classes of sequences for activity. However, a two-fold effect would be particularly difficult to discern in the context of a mutated AP-1 site or CRE where the activity of the NT/N promoter has been reduced to 10% of the wild type level. Thus, the GRE could function independently of the other sites.

The NT/N gene as a possible downstream target of NGF action. NGF treatment of PC12 cells results in the rapid transient activation of a class of cellular genes, termed early response or immediate early genes, typified by the c-fos and c-jun proto-oncogenes (Curran and Morgan, 1985; Greenberg et al., 1985; Kruijer et al., 1985). The protein products of these two proto-oncogenes form heterodimeric complexes which bind the AP-1 site with high affinity in vitro (Kouzarides and Ziff, 1988; Sassone-Corsi et al., 1988), and are present in purified preparations of AP-1 (Bohmann et al., 1988). However, purified AP-1 complexes are heterogeneous and contain additional fos-related, and jun-related proteins (Bohmann et al., 1988; Curran and Franza, 1988). At least two fos-related (Fos B, fra 1) and two jun-related (jun B, jun D) cDNAs have been cloned, and the proteins encoded by these cDNAs are capable of forming heterodimers which bind the AP-1 site with high affinity in vitro (Nakabeppu et al., 1988; Halazonetis et al., 1988; Cohen et al., 1989; Hirai et al., 1989; Rauscher et al., 1989; Zerial et al., 1989). The protein products of the c-jun,

Jun-B, and Jun-D genes are also capable of binding to the AP-1 sequence as homodimers, but with lower affinity than when complexed with Fos (Nakabeppu et al., 1988; Hirai et al., 1989). They also bind the related CRE with around 10-fold lower affinity than the AP-1 sequence and this activity is not enhanced by Fos (Nakabeppu et al., 1988). These different complexes may differentially regulate specific promoters. For instance, c-jun efficiently activates promoters containing either single or multiple AP-1 sites while Jun B inhibits expression of promoters containing single AP-1 sites and activates expression from promoters containing multiple sites (Chiu et al., 1989). The transcriptional regulation of the fos-related and jun-related genes appears to be a major level at which AP-1 activity is controlled in the cell, although both Fos and Jun are subject to post-translational modification which could also regulate their activity (Franza et al., 1987; Angel et al., 1988). NGF induces both c-jun and jun B transcription, and the appearance of a set of fos-related proteins in PC12 cells (Franza et al., 1987; Bartel et al., 1989). At least some fos-related proteins are induced more slowly and persist longer than c-fos (Franza et al., 1987). The rapid activation of genes encoding transcription factors (like c-fos and c-jun) by NGF has led to the proposal that these proteins in turn activate genes involved in neuronal differentiation, although the downstream targets of this initial cascade have not been identified to our knowledge.

The presence of a functional AP-1 site in the NT/N promoter regulatory region is suggestive that the NT/N gene constitutes such a downstream target, specifically of AP-1 complexes, and that NGF may exert its influence, at least in part, through this site by increasing the levels of these complexes. The transient induction of AP-1 complex genes and proteins in response to NGF leads to the

prediction that the expression of downstream genes might also be transiently induced. Consistent with this prediction, we observe a transient burst in the accumulation of unspliced nuclear NT/N pre-mRNAs which reaches a peak 3-4 hr after combined treatment of PC12 cells with NGF, dexamethasone, forskolin, and LiCl (B.P. Bullock and P.R.D., unpublished results). These kinetics are similar to the more delayed transient appearance of fos-related antigens in PC12 cells (Franza et al., 1987). Specific mutation of the AP-1 site in the NT/N promoter decreased responses to all inducer combinations by about an order of magnitude, perhaps indicating that multiple inducers act through the AP-1 site. Indeed, AP-1 sites cloned upstream of the human gonadotropin a-subunit gene mediate transcriptional responses to both cAMP and phorbol esters, and the two agents together result in synergistic responses in HeLa cells and the JEG-3 choriocarcinoma cell line (Deutsch et al., 1988; Hoeffler et al., 1989). However, this dual regulation was not observed in HeLa cells with a b-globin gene construct driven by multimerized AP-1 sites (Imagawa et al., 1987). In PC12 cells, forskolin does not increase expression of a CAT fusion gene driven by a promoter containing multimerized AP-1 sites, however, this construct is activated by either NGF, or lithium treatment (E.K. and P.R.D., manuscript in preparation). Thus, the AP-1 site within the NT/N promoter is likely to be a focus of both NGF and lithium action in PC12 cells.

Recently, seizure activity has been shown to induce the expression of c-fos, c-jun, and related genes in specific regions of the brain, particularly the hippocampus (Curran and Morgan, 1987; Morgan et al., 1987; Saffen et al., 1988; Sagar et al., 1988; Sonnenberg et al., 1989a). These increases result in a protracted increase in AP-1 activity which appears to depend on the sequential

appearance of Fos and several fos-related antigens (Sonnenberg et al., 1989a). Seizure activity also results in increased preproenkephalin gene expression in the hippocampus, and the expression of a CAT fusion gene driven by the preproenkephalin promoter is induced by cotransfection with c-fos and c-jun expression plasmids suggesting that AP-1 complexes regulate neuropeptide gene expression in the brain (Sonnenberg et al., 1989b). The NT/N gene is expressed widely in the rat forebrain including the CA1 region of the hippocampus (Alexander et al., 1989). The AP-1 site identified by transfection into PC12 cells could also be required for NT/N gene expression in the central nervous system.

Biased effect of mutations in the CREs and GRE. Specific mutations in either of two CRE-like sequences and deletion mutations which remove part of the GRE consensus sequence had biased effects on responses to inducer combinations. Mutations in either the distal or the proximal CRE-like sequences selectively attenuated responses to inducer combinations which included the adenylate cyclase activator forskolin, supporting their function as CREs. This selective effect is particularly apparent when the proximal CRE-like sequence is mutated. Similarly, responses to inducer combinations which included dexamethasone were selectively compromised by removal of portions of the GRE consensus sequence in two internal deletion mutants (-129/-64 and -129/-56). When the internal deletion extended further into the proximal CRE-like sequences (-129/-43), the selective effect was abolished and all responses to inducers were severely compromised. These results taken together suggest that there is a functional GRE within the region deleted in the -129/-64 and -129/-56

mutants, most likely the GRE consensus sequence. Likewise, the CRE consensus sequences appear to be primarily required for responses to forskolin. However, none of these sequences were capable of mediating a simple response in PC12 cells to any of the inducers tested when they were used individually.

The selective effects of mutations in the CRE and GRE sequences suggest that synergy may be generated at a step distal to signal transduction, perhaps through binding of transcription factors, or transcriptional activation by these factors. The close match of the CRE and GRE sequences to consensus suggests that the glucocorticoid receptor, and the major CRE-binding protein in PC12 cells (CREB) may be involved. The glucocorticoid receptor is a ligand-dependent transcriptional activator (reviewed by Evans, 1988) while CREB activity is modulated by phosphorylation by protein kinase A (Gonzalez and Montminy, 1989). The dimerization of CREB is increased in vitro by phosphorylation with protein kinase C indicating that CREB activity may be modulated by more than one kinase (Yamamoto et al., 1988). The recent demonstration that a CRE-driven reporter plasmid is activated when co-transfected with a c-jun expression plasmid suggests that Jun could also be involved in regulation through the CRE (Chiu et al., 1989). In fact, the proximal CRE in the NT/N promoter is identical to an autoregulatory site upstream of the c-jun gene which binds AP-1 complexes in vitro (Angel et al., 1988). Thus, although the biased effect of mutating either CRE is suggestive that these sequences actually function as CREs, most likely by binding CREB, we cannot rule out the possibility of other interactions. In fact, CREB is only one member of a family of CRE-binding proteins (Hai et al., 1989; Maekawa et al., 1989) and at least one other member of the family is expressed in PC12 cells (Maekawa et al., 1989). The

availability of cloned genes for these proteins, and various AP-1 complex proteins should enable us to examine the mechanisms underlying the highly synergistic operation of the NT/N promoter.

Generating phenotypic diversity in neural crest progenitor cells. Immunohistochemical co-localization studies have demonstrated that NT is produced in a subpopulation of catecholamine-containing adrenal medullary cells in cats (Terenghi et al., 1983) and subpopulations of catecholamine-containing neurons in the rat central nervous system (Hokfelt et al., 1984). NT may also be produced by principal sympathetic neurons, although this has not been clearly established (Reinecke, 1985). These in vivo correlates suggest that the regulated expression of NT in the catecholamine-producing PC12 cell line may indeed be representative of expression in the intact animal. Two of the inducers we have used, NGF and the synthetic glucocorticoid, dexamethasone, represent important environmental determinants which influence the differentiation of neural crest cells (Landis and Patterson, 1981). NGF is essential for the development and survival of sympathetic and some sensory neurons, and perhaps adrenal medullary cells (Aloe and Levi-Montalcini, 1979; Levi-Montalcini, 1987). In addition, intracellular levels of cAMP might be influenced in crest-derived cells by circulating hormones, or neurotransmitters (Walicke and Patterson, 1981). Lithium is not a physiological regulator, but is used extensively for the treatment of manic-depressive illness (reviewed by Berridge et al., 1989). The effects of lithium on NT/N gene expression are paradoxical in view of its known negative influence on both the cyclic AMP and phosphatidylinositol signal transduction pathways. The results presented here

clearly indicate that lithium can influence gene transcription, possibly by stimulating a cellular pathway that normally responds to one or more environmental determinants.

The evidence presented here indicates that the NT/N gene promoter serves to integrate multiple environmental signals in PC12 cells into a unified transcriptional response. This property may have important implications for the generation of phenotypic diversity in neural crest progenitor cells. Such promoters could restrict the expression of particular sets of genes to limited populations of adult crest derivatives which have migrated to an environment which provides the necessary multiple cues. Although NGF and glucocorticoids appear to be the major determinants involved in the decision between the sympathetic neuronal and adrenal medullary phenotypes, other agents could influence the further diversification of these basic cell types. The acquisition of cholinergic properties by sympathetic neurons provides an example of such sub-specialization, and requires a factor produced by fibroblasts and heart myocytes (Patterson, 1978). As has been suggested elsewhere, pleuripotent neural crest cells could maintain batteries of genes, required in various specialized cell types, in a chromatin conformation that permits transcription, but the actual genes expressed would be determined by environmental signals (Anderson and Axel, 1985). By making the expression of certain genes or sets of genes dependent on multiple stimuli, a relatively small number of environmental cues could specify a large number of adult cell phenotypes.

EXPERIMENTAL PROCEDURES

Plasmid Constructions. To construct pUCAT, the Hind III site of pSV2CAT (Gorman et al., 1982) was converted to a Sac I site, the resulting plasmid was digested with Sac I and Bgl II, the 1.6 kb fragment containing the chloramphenicol acetyl transferase (CAT) gene and SV40 splice and polyadenylation signals was gel-isolated, and ligated into a Sac I/Bgl II digested pUC19 derivative in which the Eco RI site had been converted to a Bgl II site. Plasmids containing various amounts of rat gene 5' flanking sequences were constructed by ligating various promoter fragments into pUCAT. Initial promoter fragments were prepared from a subcloned 3.5 kb Hind III fragment containing exon one, part of intron one, and approximately 1.4 kb of 5' flanking sequences of the rat NT/N gene. Fragments were prepared by digestion with Sac I which cuts at nucleotide +55 within the 5' untranslated region, and either Hind III (-1414), Bam HI (-1056), Bgl II (-570), or Pst I (-216), followed by ligation into pUCAT digested with the appropriate enzymes. Additional plasmids containing intragenic sequences were constructed by ligation of several Hind III fragments derived from subclones of the NT/N gene into the -1414 plasmid at the Hind III site. A plasmid containing additional 5' flanking sequences was constructed by ligating a 5.0 kb Bam HI fragment into the -1056 plasmid at the Bam HI site. The resultant plasmid contains approximately 6 kb of contiguous 5' flanking sequence.

5' and 3' deletions were created from a plasmid containing a Pst I/Sac I fragment of the rat NT/N gene encompassing sequences from -216 to +56. For 5' deletions, the plasmid was linearized at the polylinker Hind III site followed by

either Bal 31, or exo III/mung bean nuclease digestion. Bam HI linkers (CGGGATCCCG) were added, DNA was digested with Bam HI, and recircularized. Selected deleted plasmids were digested with Bam HI and Sac I, the liberated insert was isolated and ligated into Bam HI/Sac I digested pUCAT. 3' deletions were constructed similarly except that the initial digestion was with Sac I. Linker scanner mutants were created by combining appropriate 5' and 3' deletions as described (McKnight and Kingsbury, 1982).

Rat NT/N gene sequences between -216 and -28 were cloned in either orientation upstream of the rat prolactin gene "TATA" homology and cap site in a pUC9 based vector (pPRLCAT) containing the rat prolactin minimal promoter (Lufkin and Bancroft, 1987). The insert fragment was obtained from a 3' deletion mutant of the -216 (Pst I site) to +56 (Sac I site) fragment in which sequences between -28 and +56 were deleted and a Bam HI linker was attached. The Pst I site was converted to a Bam HI site and the insert was removed by digestion with Bam HI and ligated into a Bgl II digested pPRLCAT plasmid derivative in which the Sal I site had been converted to a Bgl II site. To obtain inserts downstream of the CAT gene, pPRLCAT was digested with Bam HI.

The two luciferase control plasmids used were pRSV/L (de Wet et al., 1987), and pPSLUC which was created by cloning a Pst I/Sac I rat NT/N promoter fragment encompassing sequences between -216 and +56 into Pst I/Sac I digested pXP2 which contains the firefly luciferase gene (Nordeen, 1988).

Luciferase and CAT assays. Following induction, cells were pelleted in a microcentrifuge for 2 sec., washed with ice-cold phosphate buffered saline, washed with extraction buffer, resuspended in 200 ml of extraction buffer (100

mM potassium phosphate [pH 7.8], 1 mM dithiotreitol), and disrupted by three cycles of freezing and thawing. Cell debris was pelleted in a microcentrifuge for 5 min. at 4°C, and protein concentrations were determined by Coomassie brilliant blue G250 binding (Bradford, 1976). Luciferase assays were performed with 20 mg of protein as described (de Wet et al., 1987) using a model 2010 luminometer (Analytical Luminescence Laboratories, San Diego, CA). CAT assays were performed as described (Gorman et al., 1982) using 0.1 mCi 14C-chloramphenicol (Amersham) and 20 mg protein in a 1 hr reaction, except for Rat2 cell extracts where 5 mg was used.

To control for variations in transfection efficiency, CAT activity was standardized to luciferase activity as follows: Luciferase activity was determined for each sample (at least in duplicate) and corrected for background by subtracting the activity determined for an extract prepared from mock-transfected cells. These values were then normalized by dividing each one by the average luciferase activity obtained in all extracts under the same induction condition. The normalized luciferase activities were then used to standardize the CAT activities measured in the corresponding extracts. Corrected CAT activity is the measured activity (%14C-chloramphenicol converted to acetylated forms) divided by the normalized luciferase activity. For the analysis of linker and internal deletion mutants, relative CAT activity was calculated by dividing the corrected CAT activity for a given mutant by the corrected CAT activity obtained with the parental wild-type plasmid (-216/+56). We note that expression from RSV/L (de Wet et al., 1987) was reproducibly induced around 10-fold by lithium either alone or in combination with other inducers in PC12 cells. This was not due to an enhancement of DNA uptake or retention since a CAT fusion gene

driven by the Herpes Simplex virus thymidine kinase promoter was not induced by lithium.

Cell culture and electroporation. PC12 cells (Greene and Tischler, 1976) were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 50 units/ml penicillin, 50 mg/ml streptomycin (all from GIBCO) on plastic tissue culture dishes. GH3 cells were grown as described (Lufkin and Bancroft, 1987). Rat2 cells were grown in DMEM supplemented with 5% calf serum (Hazleton, Lenexa, KS). Electroporation of DNA was performed as described (Chu et al., 1987) using a single pulse from a 960 mF capacitor (Gene pulser, Biorad) charged to either 200 V (PC12 cells), or 300 V (GH3 and Rat2 cells). Typically PC12 and GH3 cells were transfected with 25 mg of CAT plasmid and 2.5 mg of luciferase plasmid. The amounts of each plasmid were doubled for Rat2 cells. Following electroporation, cells were plated onto either six well (~2 x 106 cells/well) or 100 mm (5 x 106 cells) plastic tissue culture dishes and allowed to recover for 48 hr. Cells were induced with appropriate combinations of NGF (100ng/ml), dexamethasone (1mM), forskolin (1 mM) and, LiCl (20 mM) for 12 hr. NGF was either prepared as described (Mobley et al., 1976), or purchased from Bioproducts for Science, Inc. (Indianapolis, IN). Dexamethasone was a gift from Merck Sharp and Dohme, LiCl was from Fluka, and forskolin was from Sigma.

RNA analysis. RNA was extracted from PC12 cells by the LiCl/urea extraction procedure, and S1 nuclease protection assays were performed using 32P-labeled single-stranded DNA probes as described (Dobner et al., 1988).

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REFERENCES

Alexander, M. J., Miller, M. A., Dorsa, D. M., Bullock, B. P., Melloni, R. H., Jr., Dobner, P. R., and Leeman, S. E. (1989). Distribution of neurotensin/neuromedin N mRNA in rat forebrain: Unexpected abundance in hippocampus and subiculum. Proc. Natl. Acad. Sci. USA 86, 5202-5206.

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

Anderson, D. J. and Axel, R. (1985). Molecular probes for the development and plasticity of neural crest derivatives. Cell 42, 649-662.

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

Angel, P., Hattori, K., Smeal, T., and Karin, M. (1988). The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell 55, 875-885.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P., and Karin, M. (1987). Phorbol ester-inducible genes contain a

common cis element recognized by a TPA-modulated trans-acting factor. Cell 49, 729-739.

Bartel, D. P., Sheng, M., Lau, L. F., and Greenberg, M. E. (1989). Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of fos and jun induction. Genes Dev. 3, 304-313.

Berridge, M. J., Downes, C. P., and Hanley, M. R. (1989). Neural and developmental actions of lithium: a unifying hypothesis. Cell 59, 411-419.

Bohmann, D., Admon, A., Turner, D. R., and Tjian, R. (1988). Transcriptional regulation by the AP-1 family of enhancer-binding proteins: a nuclear target for signal transduction. Cold Spring Harb. Symp. Quant. Biol. 53, 695-700.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Burstein, D. E., Seeley, P. J., and Greene, L. A. (1985). Lithium ion inhibits nerve growth factor-induced neurite outgrowth and phosphorylation of nerve growth factor-modulated microtubule-associated proteins. J. Cell Biol. 101, 862-870.

Chiu, R., Angel, P., and Karin, M. (1989). Jun-B differs in its biological properties from, and is a negative regulator of, c-jun. Cell 59, 979-986.

Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the efficient transfection of mammalian cells with DNA. Nucl. Acids Res. 15, 1311-1325.

Cohen, D. R., Ferreira, P. C. P., Gentz, R., Franza, R. F., Jr., and Curran, T. (1989). The product of a fos-related gene, fra-1, binds cooperatively to the AP-1 site with Jun: transcription factor AP-1 is comprised of multiple protein complexes. Genes Dev. 3, 173-184.

Comb, M., Mermod, N., Hyman, S. E., Pearlberg, J., Ross, M. E., and Goodman, H. M. (1989). Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible transcription. EMBO J. 7, 3793-3805.

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

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

Curran, T. and Morgan, J. I. (1987). Memories of fos. BioEssays 7, 255-258.

de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., and Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. 7, 725-737.

Deutsch, P. J., Hoeffler, J. P., Jameson, J. L., and Habener, J. F. (1988). Cyclic AMP and phorbol ester-stimulated transcription mediated by similar DNA elements that bind distinct proteins. Proc. Natl. Acad. Sci. USA 85, 7922-7926.

Dobner, P. R., Barber, D. L., Villa-Komaroff, L., and McKiernan, C. (1987). Cloning and sequence analysis of cDNA for the canine neurotensin/neuromedin N precursor. Proc. Natl. Acad. Sci. USA 84, 3516-3520.

Dobner, P. R., Tischler, A. S., Lee, Y. C., Bloom, S. R., and Donahue, S. R. (1988). Lithium dramatically potentiates neurotensin/neuromedin N gene expression. J. Biol. Chem. 263, 13983-13986.

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

Doupe, A. J., Patterson, P. H., and Landis, S. C. (1985b). Small intensely fluorescent cells in culture: role of glucocorticoids and growth factors in their development and interconversions with other neural crest derivatives. J. Neurosci. 5, 2143-2160.

Dynan, W. S. (1989). Modularity in promoters and enhancers. Cell 58, 1-4.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. Science 240, 889-895.

Franza, B. R., Jr., Smabucetti, L. C., Cohen, D. R., and Curran, T. (1987). Analysis of Fos protein complexes and Fos-related antigens by high-resolution two-dimensional electrophoresis. Oncogene 1, 213-221.

Gonzalez, G. A. and Montminy, M. R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59, 675-680.

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

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

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

Hai, T., Liu, F., Coukos, W. J., and Green, M. R. (1989). Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev. 3, 2083-2090.

Halazonetis, T. D., Georgopoulos, K., Greenberg, M. E., and Leder, P. (1988). c-Jun dimerizes with itself and with c-fos, forming complexes of different DNA binding affinities. Cell 55, 917-924.

Hirai, S.-I., Ryseck, R.-P., Mechta, F., Bravo, R., and Yaniv, M. (1989). Characterization of junD: a new member of the jun proto-oncogene family. EMBO J. 8, 1433-1439.

Hoeffler, J. P., Deutsch, P. J., Lin, J., and Habener, J. F. (1989). Distinct adenosine 3',5'-monophosphate and phorbol ester-responsive signal transduction pathways converge at the level of transcriptional activation by the interactions of DNA-binding proteins. Mol. Endo. 3, 868-880.

Hokfelt, T., Everitt, B. J., Theodorsson-Norheim, E., and Goldstein, M. (1984). Occurrence of neurotensinlike immunoreactivity in subpopulations of hypothalamic, mesencephalic, and medullary catecholamine neurons. J. Comp. Neurol. 222, 543-559.

Hyman, S. E., Comb, M., Pearlberg, J., and Goodman, H. M. (1989). An AP-2 element acts synergistically with the cyclic AMP- and phorbol ester-inducible enhancer of the human proenkephalin gene. Mol. Cell. Biol. 9, 321-324.

Imagawa, M., Chiu, R., and Karin, M. (1987). Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell 51, 251-260.

Jantzen, H.-M., Strahle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksicek, R., and Schutz, G. (1987). Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. Cell 49, 29-38.

Kislauskis, E., Bullock, B., McNeil, S., and Dobner, P. R. (1988). The rat gene encoding neurotensin and neuromedin N: structure, tissue-specific expression, and evolution of exon sequences. J. Biol. Chem. 263, 4963-4968.

Kouzarides, T. and Ziff, E. (1988). The role of the leucine zipper in the fos-jun interaction. Nature 336, 646-651.

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

Landis, S. C. and Patterson, P. H. (1981). Neural crest cell lineages. Trends Neurosci. 4, 172-175.

LeDouarin, N. M. (1980). The ontogeny of the neural crest in avian embryo chimeras. Nature 286, 663-669.

Lee, W., Haslinger, A., Karin, M., and Tjian, R. (1987). Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. Nature 325, 368-372.

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

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

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. Science 237, 1154-1162.

Lufkin, T. and Bancroft, C. (1987). Identification by cell fusion of gene sequences that interact with positive trans-acting factors. Science 237, 283-286.

Maekawa, T., Sakura, H., Kanei-Ishii, C., Sudo, T., Yoshimura, T., Fujisawa, J., Yoshida, M., and Ishii, S. (1989). Leucine zipper structure of the protein CRE-BP1 binding to the cyclic AMP response element in brain. EMBOJ 8, 2023-2028.

McKnight, S. L. and Kingsbury, R. (1982). Transcriptional control signals of a eukaryotic protein-coding gene. Science 217, 316-324.

Mermod, N., Williams, T. J., and Tjian, R. (1988). Enhancer binding factors AP-4 and AP-1 act in concert to activate SV40 late transcription in vitro. Nature 332, 557-561.

Milbrandt, J. (1987). A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science 238, 797-799.

Mitchell, P. J. and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245, 371-378.

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

Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986). Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. USA 83, 6682-6686.

Morgan, J. I., Cohen, D. R., Hempstead, J. L., and Curran, T. (1987). Mapping patterns of c-fos expression in the central nervous system after seizure. Science 237, 192-197.

Nakabeppu, Y., Ryder, K., and Nathans, D. (1988). DNA binding activities of three murine Jun proteins: stimulation by Fos. Cell 55, 907-915.

Nemeroff, C. B. (1980). Neurotensin: perchance an endogenous neuroleptic? Biol. Psychiatry 15, 283-302.

Nordeen, S. K. (1988). Luciferase reporter gene vectors for analysis of promoters and enhancers. Biotechniques 6, 454-458.

Patterson, P. H. (1978). Environmental determination of autonomic neurotransmitter functions. Annu. Rev. Neurosci. 1, 1-17.

Rauscher, F. J., III, Voulalas, P. J., Franza, B. R., Jr., and Curran, T. (1989). Fos and Jun bind cooperatively to the AP-1 site: reconstitution in vitro. Genes Dev. 2, 1687-1699.

Reinecke, M. (1985). Neurotensin: immunohistochemical localization in central and peripheral nervous system and in endocrine cells and its functional role as neurotransmitter and endocrine hormone. Progr. Histochem. Cytochem. 16, 1-175.

Saffen, D. W., Cole, A. J., Worley, P. F., Christy, B. A., Ryder, K., and Baraban, J. M. (1988). Convulsant-induced increase in transcription factor messenger RNAs in rat brain. Proc. Natl. Acad. Sci. USA 85, 7795-7799.

Sagar, S. M., Sharp, F. R., and Curran, T. (1988). Expression of c-fos protein in brain: metabolic mapping at the cellular level. Science 240, 1328-1331.

Sassone-Corsi, P., Ransone, L. J., Lamph, W. W., and Verma, I. M. (1988). Direct interaction between fos and jun nuclear oncoproteins: role of the "leucine zipper" domain. Nature 336, 692-695.

Schule, R., Muller, M., Kaltschmidt, C., and Renkawitz, R. (1988). Many transcription factors interact synergistically with steroid receptors. Science 242, 1418-1420.

Sonnenberg, J. L., Macgregor-Leon, P. F., Curran, T., and Morgan, J. I. (1989a). Dynamic alterations occur in the levels and composition of transcription factor AP-1 complexes after seizure. Neuron 3, 359-365.

Sonnenberg, J. L., Rauscher, F. J., III, Morgan, J. I., and Curran, T. (1989b). Regulation of proenkephalin by Fos and Jun. Science 246, 1622-1625.

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

Strahle, U., Schmid, W., and Schutz, G. (1988). Synergistic action of the glucocorticoid receptor with transcription factors. EMBO J. 7, 3389-3395.

Terenghi, G., Polak, J. M., Varndell, I. M., Lee, Y. C., Wharton, J., and Bloom, S. R. (1983). Neurotensin-like immunoreactivity in a subpopulation of noradrenaline-containing cells of the cat adrenal gland. Endocrinology 112, 226-233.

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

Tischler, A. S., Lee, Y. C., Slayton, V. W., and Bloom, S. R. (1982). Content and release of neurotensin in PC12 pheochromocytoma cultures: modulation by dexamethasone and nerve growth factor. Peptides 3, 415-421.

Tora, L., Gaub, M. -., Mader, S., Dierich, A., Bellard, M., and Chambon, P. (1988). Cell-specific activity of a GGTCA half-palindromic oestrogen-responsive element in the chicken ovalbumin gene promoter. EMBO J. 7, 3771-3778.

Tsukada, T., Fink, J. S., Mandel, G., and Goodman, R. H. (1987). Identification of a region in the human vasoactive intestinal polypeptide gene responsible for regulation by cyclic AMP. J. Biol. Chem. 262, 8743-8747.

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

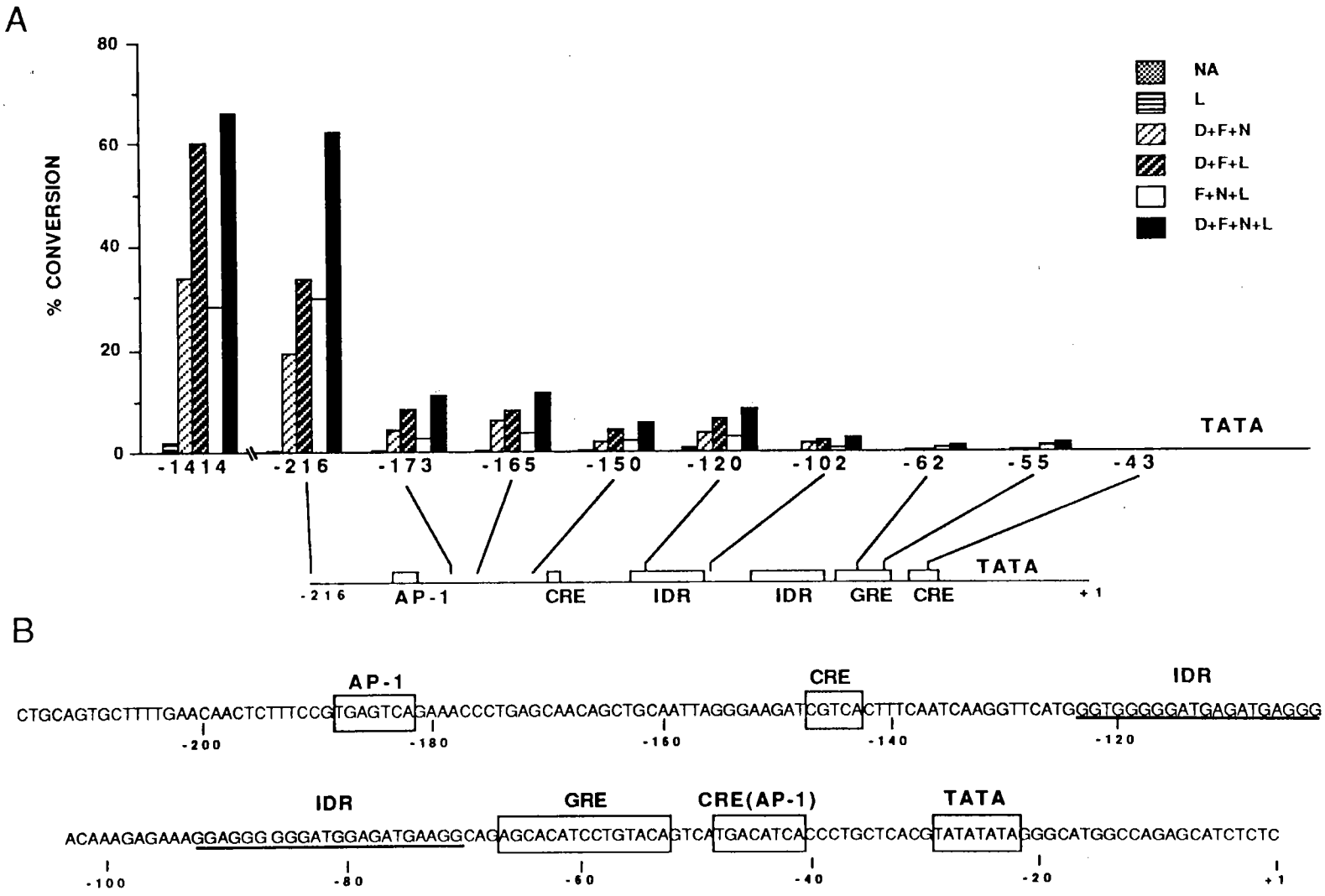
Walicke, P. A. and Patterson, P. H. (1981). On the role of cyclic nucleotides in the transmitter choice made by cultured sympathetic neurons. J. Neurosci. 1, 333-342.

Yamamoto, K. K., Gonzalez, G. A., Biggs, W. H., III, and Montminy, M. R. (1988). Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature 334, 494-498.

Zerial, M., Toschi, L., Ryseck, R.-P., Schuermann, M., Muller, R., and Bravo, R. (1989). The product of a novel growth factor activated gene, fos B, interacts with JUN proteins enhancing their DNA binding activity. EMBO J. 8, 805-813.

Figure 1. Transient transfection analysis of 5' deletion clones

(A) PC12 cells were transfected with plasmids (25 mg) containing the indicated rat NT/N gene 5' flanking sequences cloned upstream of the bacterial CAT gene in pUCAT. To control for variations in transfection efficiency, 2.5 mg of a control plasmid in which the firefly luciferase gene is driven by the rat NT/N -216 to +56 promoter fragment (pPSLUC) was co-transfected in each case. Following a 48 hr recovery period, cells were treated with the indicated combinations of inducers for 12 hr, harvested, and assayed for CAT and luciferase enzyme activity using 20 mg protein for each assay. CAT activity (% conversion of 14C-chloramphenicol to acetylated forms per 20 mg protein) for each induction condition is plotted (corrected for variations in luciferase activity as described in Materials and Methods). The deletion endpoints are depicted schematically below the abscissa. The positions of sequences similar to consensus AP-1, cyclic AMP (CRE) and glucocorticoid response (GRE) elements, and two imperfect direct repeats (IDR) are indicated. The induction conditions are indicated and the inducers are abbreviated as follows: NA, no additives; L, 20 mM LiCl; D, 1μM dexamethasone; F, 1μM forskolin; N, 100ng/ml NGF. (B) The nucleotide sequence of the 5' flanking region of the rat NT/N gene between -216 and +1. Sequences similar or identical to consensus sequences for AP-1, CRE, GRE, and the TATA box are boxed. The two IDRs are underlined.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80
60
40
20
0
% CONVERSION
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L
D+F+N
D+F+L
F+N+L
D+F+N+L
-1414
-216
-173
-165
-150
-120
-102
-62
-55
-43
TATA
-216
AP-1
CRE
IDR
IDR
GRE
CRE
TATA
+1
B
AP-1
CRE
IDR
CTGCAGTGCTTTTGAACAACTCTTTCCGTGAGTCAGAAACCCTGAGCAACAGCTGCAATTAGGGAAGATCGTCACTTTCAATCAAGGTTCATGGGTGGGGGATGAGATGAGGG
-200
-180
-160
-140
-120
IDR
GRE
CRE(AP-1)
TATA
ACAAAGAGAAAGGAGGG GGGATGGAGATGAAGGCAGAGCACATCCTGTACAGTCATGACATCACCCTGCTCACGTATATATAGGGCATGGCCAGAGCATCTCTC
-100
-80
-60
-40
-20
+1

Figure 2. Increases in CAT activity are accompanied by similar increases in the levels of accurately initiated fusion gene transcripts

(A) PC12 cells were transfected with 25 mg of a CAT fusion gene plasmid driven by the rat NT/N -216/+56 promoter, plated onto 100 mM dishes, allowed to recover, and treated with combinations of inducers as in Figure 1. Total RNA was prepared from ~1 x 107 cells for each condition tested. Fusion and endogenous gene transcripts were quantitated by hybridizing 32P-labeled single-stranded DNA probes with either 40 mg or 5 mg of RNA, respectively, followed by S1 nuclease treatment. The reaction products were analyzed on sequencing gels and visualized by autoradiography for either 12.5 hr (lanes 1-6) or 3.5 hr (lanes 7-13) with an intensifying screen. The probes and expected protection products are depicted schematically. The exon four probe spans 336 nucleotides of rat NT/N gene sequences (thick bar) including the neuromedin N (diagonal fill) and neurotensin (black fill) coding domains. The fusion gene probe contains rat NT/N sequences between -43 and +56 (thick open bar), and the first 253 nucleotides of the CAT gene (thick stippled bar). Vector sequences are represented by thin lines. Fusion gene probe, lanes 1-4, and 6; rat NT/N gene exon 4 probe, lanes 7-12. RNA from transfected PC12 cells treated with no additives, lanes 1 and 9; LiCl, lanes 2 and 10; NGF, dexamethasone, and forskolin, lanes 3 and 11; NGF, dexamethasone, forskolin, and LiCl, lanes 4 and 12. The concentrations of additives were as in Figure 1. Lanes 5 and 13, radiolabeled DNA size standards (upper, 396 bases; lower, 350 bases); lanes 6 and 8, control reactions with either 40 mg or 5 mg yeast RNA, respectively; lane 7, undigested exon 4 probe. The expected protection products are indicated by arrows. (B) CAT assays were performed using protein extracts prepared

from the same cells used in (A). The induction conditions are indicated using the single letter abbreviations used in Figure 1. (C) Quantitation of fusion gene transcripts (diagonal fill), endogenous gene transcripts (black bars), and CAT enzyme activity (horizontal fill). Transcripts levels were quantitated either by direct counting on a Betascope analyzer for the exon 4 probe (CPM x 10^{-2}) or by densitometric scanning of an autoradiograph for the fusion gene probe (arbitrary scanner units). CAT enzyme activity is expressed as in Figure 1. Induction conditions are indicated as in (B).

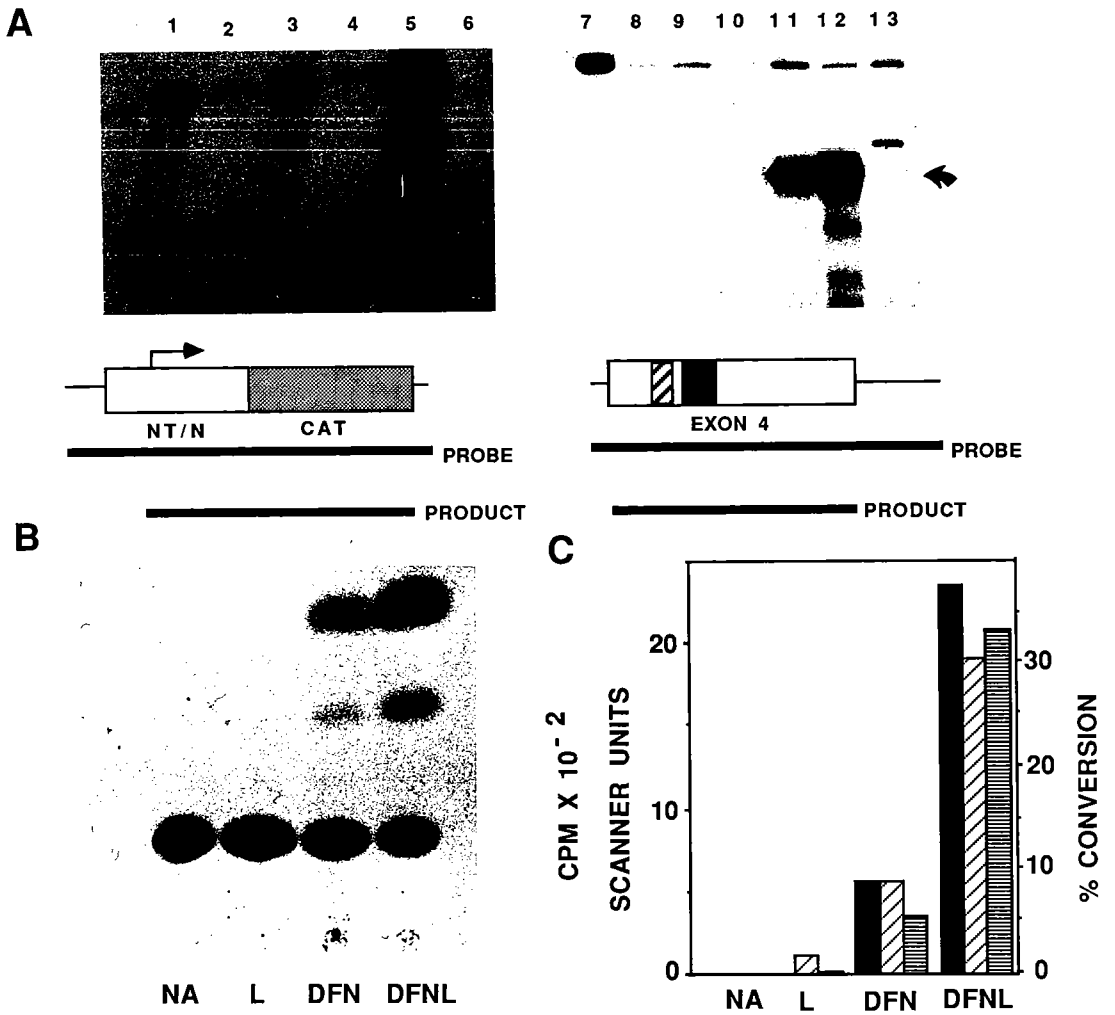
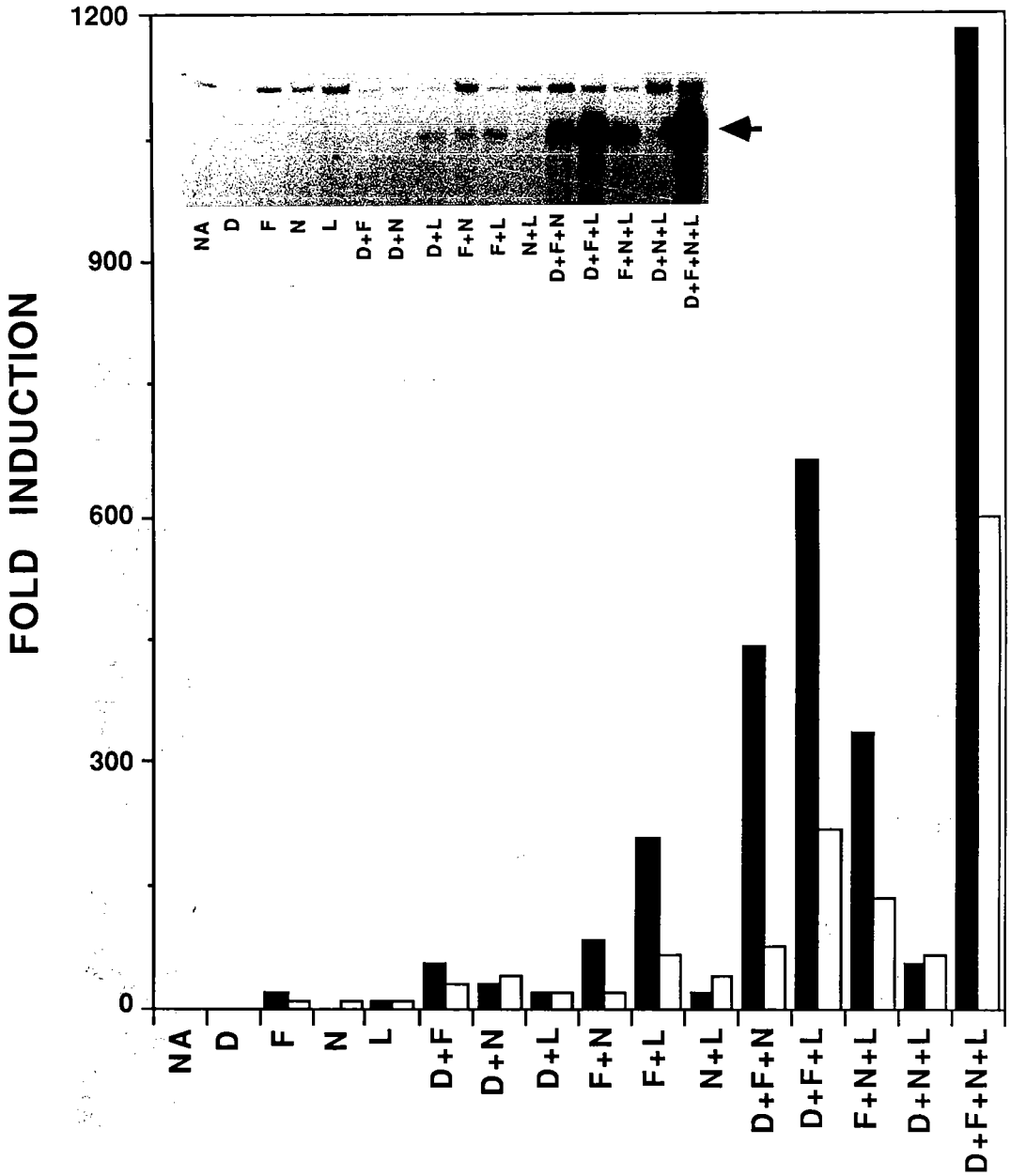

A
1 2 3 4 5 6
7 8 9 10 11 12 13
NT/N
CAT
PROBE
PRODUCT
EXON 4
PROBE
PRODUCT
B
NA L DFN DFNL
C
CPM X 10 - 2
SCANNER UNITS
% CONVERSION
0
10
20
30
NA L DFN DFNL

Figure 3. Synergistic induction of transiently expressed fusion gene constructs PC12 cells were co-transfected with CAT and luciferase fusion gene plasmids driven by the -216 to +56 rat NT/N gene promoter fragment and induced. The induction conditions, abbreviations, and concentrations of inducers are as in Figure 1. CAT (open bars) and luciferase (black bars) enzyme activities were determined, and used to calculate the fold-induction over control values (no additives). Background values for the two assays were determined using an extract prepared from mock transfected PC12 cells, and subtracted. In a separate experiment, endogenous NT/N gene transcripts were analyzed by S1 nuclease protection using a 32P-labeled exon four probe as in Figure 2A. The S1 nuclease protection products were analyzed on a sequencing gel and visualized by autoradiography (inset). The position of the expected protection product is indicated by an arrow. The induction conditions were the same as for the transfection experiment except cells were induced for 40 hr.


FOLD INDUCTION
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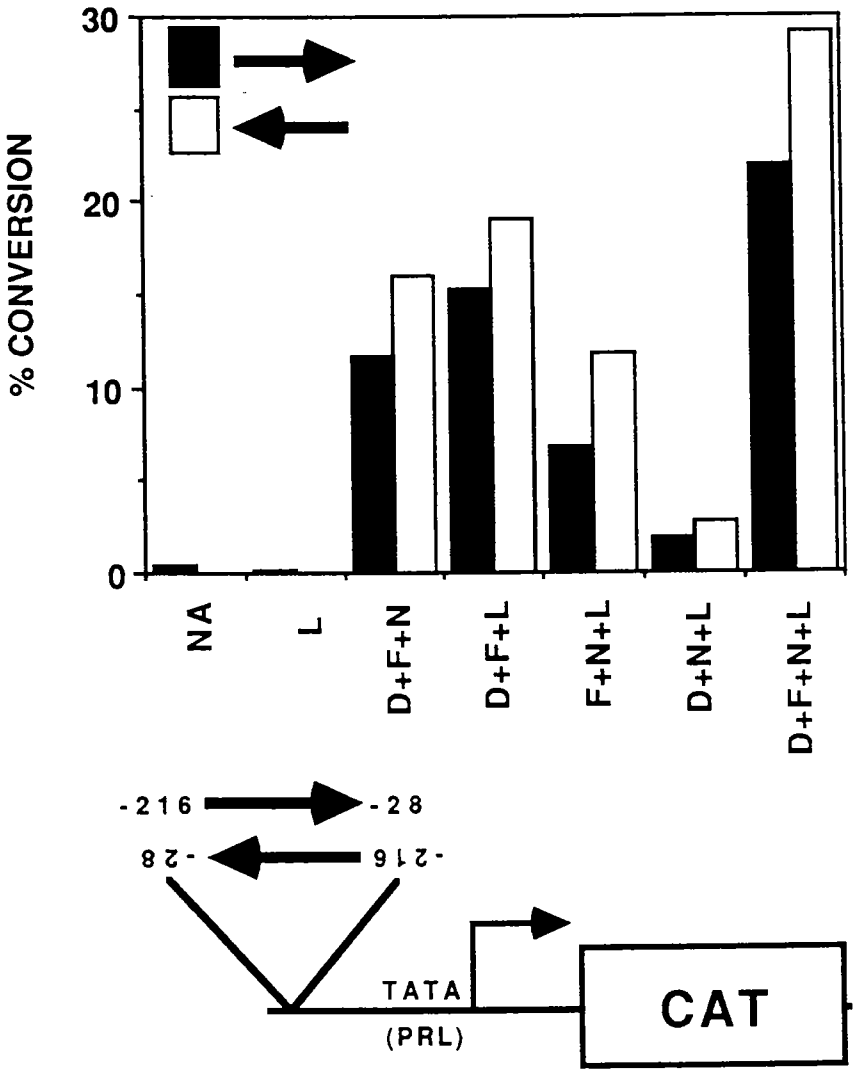
900
600
300
0
NA
D
F
N
L
D+F
D+N
D+L
F+N
F+L
N+L
D+F+N
D+F+L
F+N+L
D+N+L
D+F+N+L

Figure 4. The NT/N gene control region functions in either orientation upstream of a heterologous promoter

NT/N gene sequences between -216 and -28 were cloned in either orientation upstream of a minimal prolactin promoter fused to the CAT gene, and the resulting plasmids were transfected into PC12 cells. The cells were treated with the indicated combinations of inducers and CAT and luciferase enzyme activities were determined. The fusion gene constructs are depicted schematically.


30
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0
% CONVERSION
NA
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D+F+N
D+F+L
F+N+L
D+N+L
D+F+N+L
-216
-28
-28
-216
TATA
(PRL)
CAT

Figure 5. Mutational analysis of the NT/N promoter

A series of linker scanner and internal deletion mutants were constructed from the -216/+56 wild type rat NT/N promoter fragment and cloned upstream of the CAT gene in pUCAT. The activity of each plasmid was determined by transient transfection into PC12 cells and compared to the activity of the parent wild type plasmid. Several separate transfection experiments were performed to evaluate all the constructs and each set included the parent wild type plasmid for comparison. To control for variations in transfection efficiency within an experiment, a control luciferase plasmid was co-transfected, either RSV/L (de Wet, Wood et al., 1987) , or pPSLUC (see Fig. 1), and luciferase activity was used to correct CAT activity as described in Materials and Methods. All constructs were evaluated at least twice except for those in panel (C). The activity of the mutants is expressed relative to wild type activity (% conversion mutant/% conversion wild type). The location and extent of linker scanner (open box) and internal deletion mutations are depicted schematically. The numbers refer to the 5' and 3' endpoints of each mutation. The induction conditions tested are indicated alongside the histogram for the -189/-182 mutation. The single letter abbreviations and concentrations of inducers used are as in Figure 1. Conditions not tested are indicated by an asterisk. Mutations in the distal (A) and proximal (B) domains, and the region between these domains (C) were analyzed.

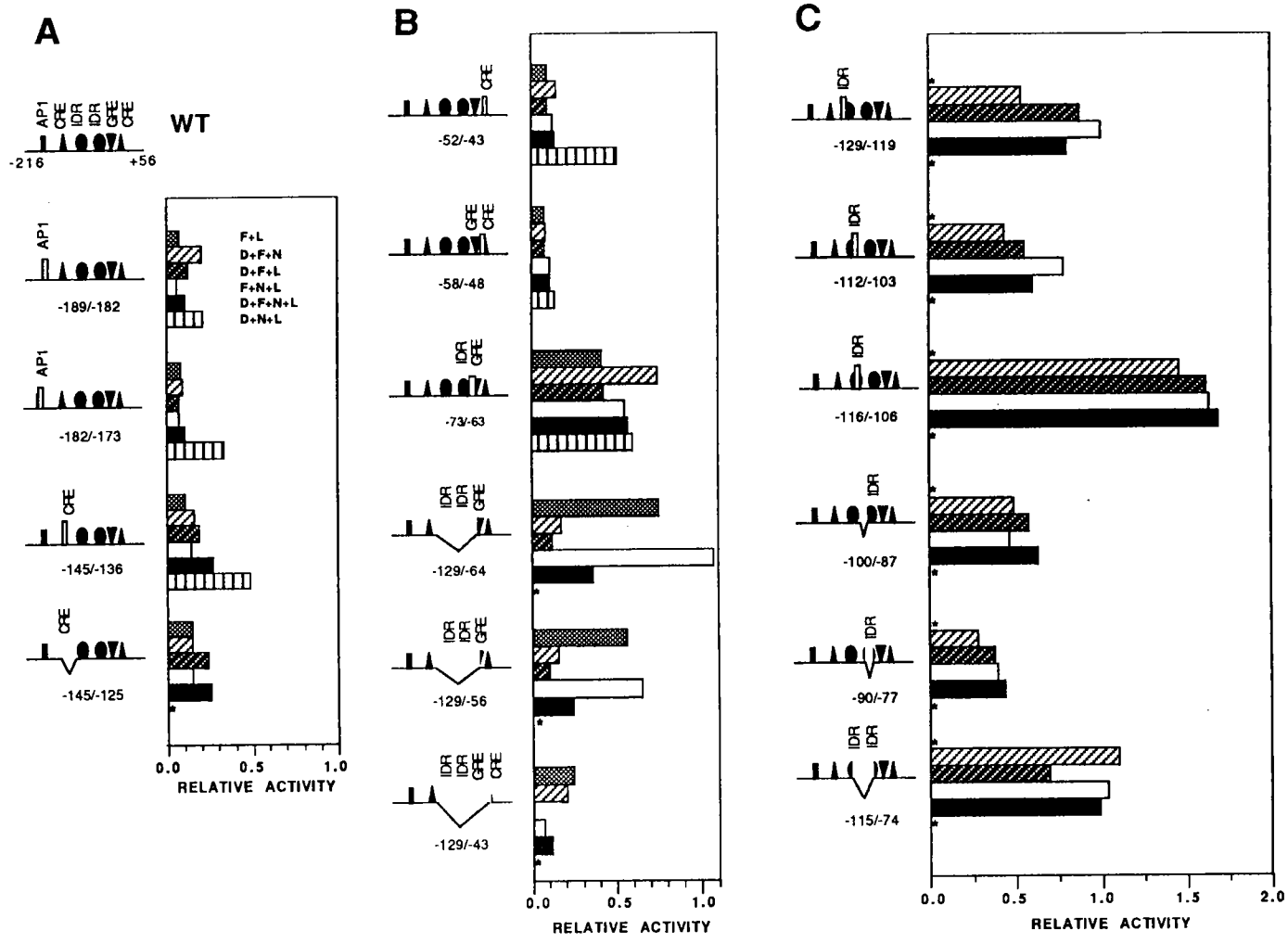
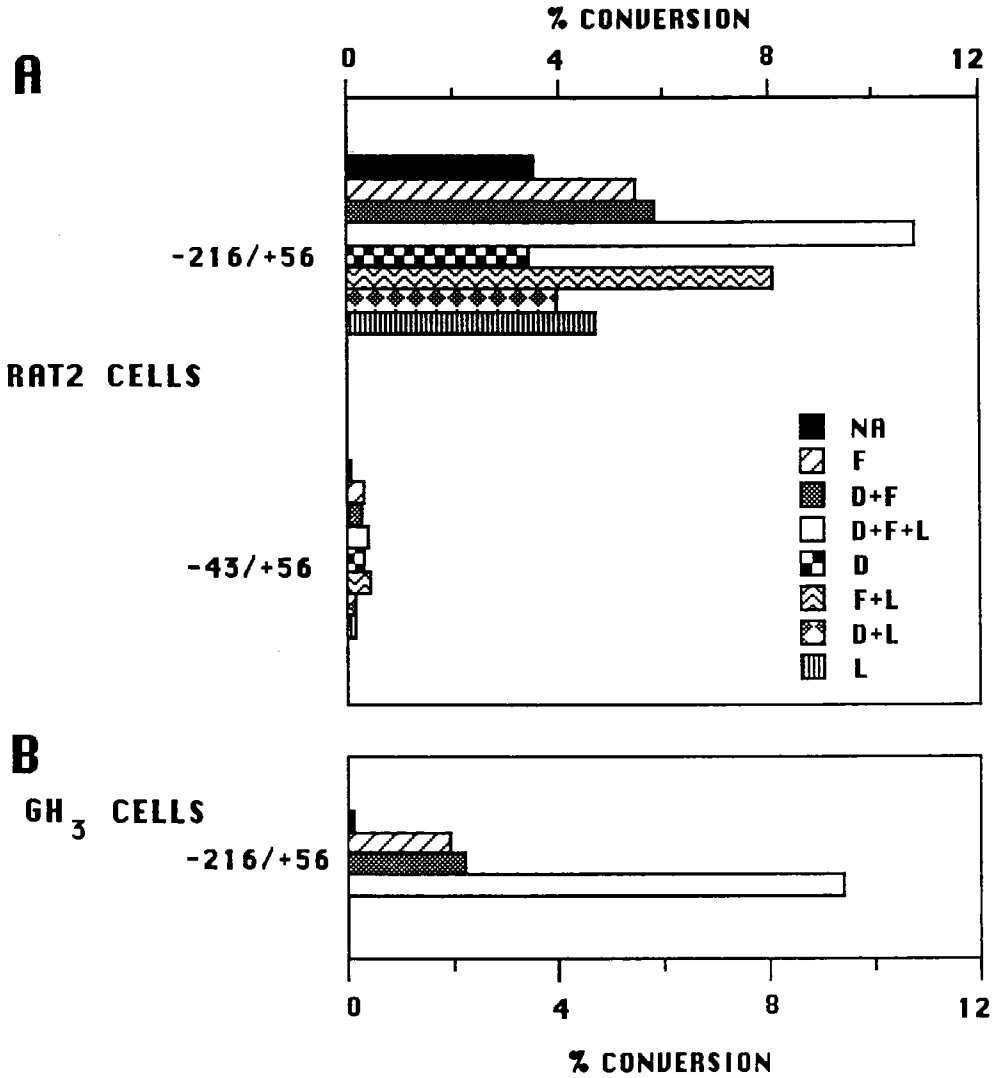

A
AP1
CRE
IDR
IDR
GRE
CRE
WT
-216
+56
AP1
-189/-182
AP1
-182/-173
CRE
-145/-136
CRE
-145/-125
F+L
D+F+N
D+F+L
F+N+L
D+F+N+L
D+N+L
0.0
0.5
1.0
RELATIVE ACTIVITY
B
CRE
-52/-43
GRE
CRE
-58/-48
IDR
GRE
-73/-63
IDR
IDR
GRE
-129/-64
IDR
IDR
GRE
-129/-56
IDR
IDR
GRE
CRE
-129/-43
0.0
0.5
1.0
RELATIVE ACTIVITY
C
IDR
-129/-119
IDR
-112/-103
IDR
-116/-106
IDR
-100/-87
IDR
-90/-77
IDR
IDR
-115/-74
0.0
0.5
1.0
1.5
2.0
RELATIVE ACTIVITY

Figure 6. Expression in Rat2 fibroblasts and GH3 pituitary cells

(A) 50 mg of either the wild type -216/+56 CAT plasmid (as evaluated in PC12 cells), or the -43/+56 5' deletion plasmid, which retains only the rat NT/N TATA box and 5' untranslated region (see Figure 1), were transfected into Rat2 cells as described in Materials and Methods. Following a 48 hr recovery period, cells were treated with the indicated combinations of inducers for 12 hrs, then harvested. The concentrations and single letter abbreviations of the inducers are as in Figure 1. 5 mg of the RSV/L luciferase control plasmid was co-transfected in each case. CAT activity was determined using 5 mg of protein and is plotted as in Figure 1.

(B) The -216/+56 CAT plasmid was assayed in GH3 rat pituitary cells as described for Rat2 cells in (A) except that 25 mg of the plasmid was transfected and CAT activity was determined using 20 mg of protein.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8
12
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-216/+56
RAT2 CELLS
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NA
F
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D+F+L
D
F+L
D+L
L
B
GH3 CELLS
-216/+56
0
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8
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% CONVERSION

IV

NERVE GROWTH FACTOR AND LITHIUM SELECTIVELY ACTIVATE PROMOTERS CONTAINING MULTIPLE AP-1 SITES IN PC12 CELLS

Kislauskis, E., Wagner, J. A., and Dobner, P.D. (submitted for publication).

ABSTRACT

Treatment of PC12 cells with nerve growth factor (NGF) results in the rapid transcriptional activation of several genes encoding AP-1 transcription factors, suggesting that AP-1 may be involved in NGF-induced neuronal differentiation [1, 2, 3, 4, 5]. NGF also acts permissively in the synergistic regulation of neurotensin/neuromedin N gene expression in PC12 cells by NGF, glucocorticoids, and activators of adenylate cyclase [6, 7]. Surprisingly, lithium can substitute for NGF in this permissive role. These transcriptional effects require mutually dependent AP-1 and cAMP-response elements (CREs) located within 200 bp of the NT/N gene transcriptional start site [7]. To further examine the role of these sites in NT/N promoter function, we examined the effects of inducers on the expression of CAT fusion genes driven by promoters containing either single or multiple sites of each class in PC12 cells. Both NGF and lithium treatment resulted in the striking activation of promoters containing multiple, but not single AP-1 sites. Both agents also potentiated forskolin-induced activation of promoters containing CREs. These results suggest that promoters containing multiple AP-1 sites, or a single AP-1 site in the context of nearby active CREs, are selectively activated by either NGF or lithium in PC12 cells.

RESULTS AND DISCUSSION

To examine the effects of inducers on promoters containing AP-1 sites, chloramphenicol acetyltransferase (CAT) fusion genes controlled by the herpes simplex virus thymidine kinase promoter (pBLCat4) and single (1xAP-1), or multiple (5xAP-1) AP-1 sites 8 were transiently expressed in PC12 cells treated with inducers for 12 hr (Fig. 1). Lithium (20 mM) treatment resulted in a striking 58-fold induction of 5xAP-1 CAT expression, but had little effect on either 1xAP-1 or pBLCat4 plasmids. NGF and phorbol 12-myristate 13-acetate (PMA) also selectively activated 5xAP-1 expression, although less effectively than lithium. Treatment with the adenylate cyclase activator, forskolin, resulted in a small, but reproducible, 5-fold stimulation of 5xAP-1 CAT activity. Combinations of inducers acted additively to selectively increase 5xAP-1 expression by over 100-fold at maximum in sharp contrast to their synergistic effect on NT/N gene expression [6, 7].

The expression of CAT fusion genes driven by promoters containing CREs markedly differed from those controlled by AP-1 sites. Neither NGF nor lithium alone activated promoters containing single (1xCRE) or multiple (4xCRE) CREs, although both types of promoters were activated (3- and 9-fold, respectively) in response to forskolin treatment of PC12 cells (Fig. 2). However, both lithium and NGF acted synergistically with forskolin to activate promoters containing either single or multiple CREs. Lithium cooperated more effectively with forskolin to activate 1xCRE and 4xCRE CAT expression by 18- and 57-fold, respectively. Both forskolin induction and potentiation by lithium and NGF were dependent on protein kinase A activity, since the 4xCRE CAT fusion gene was not

regulated in mutant A126-1B2 cells 9 deficient in protein kinase A activity (Fig. 2c). Forskolin activation of promoters containing multiple AP-1 sites was similarly compromised in the kinase A deficient mutant, while the selective activation of such promoters by lithium or NGF was relatively unimpaired (Fig. 1c).

Mutational analysis of the NT/N promoter indicates that the AP-1 site and CREs are functionally interdependent suggesting that these elements serve to integrate responses to environmental stimuli at the level of promoter function [7]. To examine the relative importance of protein kinase A in NT/N promoter function, a CAT fusion gene driven by the wild type NT/N promoter (-216/+56) was transiently expressed in both A126-1B2, and PC12 cells treated with the indicated inducers (Fig 3). In striking contrast to the induction of CAT expression in response to combinations of inducers inPC12 cells, the promoter was effectively silenced in A126-1B2 cells.

Lithium has previously been shown to substitute for NGF in the cooperative regulation of NT/N gene expression in PC12 cells [6, 7]. The results presented here demonstrate that both lithium and NGF influence the expression CAT fusion genes driven by promoters containing either AP-1 sites or CREs. Most striking is the ability of either NGF, or lithium to selectively activate promoters containing multiple AP-1 sites. AP-1 is comprised of a collection of related proteins typified by the products of the c-fos and c-jun proto-oncogenes [10, 11]. Recently, Jun-B has been shown to activate promoters containing multiple, but not single AP-1 sites when transfected into F9 embryonal carcinoma cells [12]. In contrast, c-jun activated both types of promoters, but c-jun activation of promoters containing single AP-1 sites was inhibited by co-expression of Jun-

B. NGF simultaneously and rapidly activates the transcription of the c-fos, c-jun, and Jun-B genes in PC12 cells [1, 2, 3, 4, 5]. Thus, the selective activation of genes controlled by multimerized AP-1 sites in response to NGF may be due to the coordinate induction of c-jun and Jun-B gene expression by NGF in these cells.

The expression pattern of the NT/N gene in PC12 cells suggests that additional promoter configurations may confer specificity in responses to particular AP-1 complexes. Mutational analysis of the NT/N promoter indicates that a single consensus AP-1 site and two CREs function interdependently to integrate the effects of multiple inducers [7]. The mutually dependent functioning of these elements, coupled with the differential effects of NGF and lithium on promoters containing AP-1 sites and CREs, suggests strongly that the single AP-1 site within the NT/N promoter functions within the context of the NT/N promoter by cooperating with adjacent CREs. The cAMP pathway is indispensable for NT/N promoter function since the promoter was inactive in a mutant PC12 cell line deficient in protein kinase A activity. The major CRE-binding protein in PC12 cells, CREB, is structurally related to the c-fos and c-jun proteins [13, 14] and could specifically interact with one or more AP-1 protein.

The striking effect of lithium on promoters containing multiple AP-1 site suggests that lithium treatment may also induce genes encoding AP-1 transcription factors. AP-1 sites mediate responses to phorbol esters in certain cell types [15] suggesting that lithium may act through its well established effects on the phosphatidylinositol (PI) signal transduction pathway [16]. Lithium amplifies agonist-stimulated inositol phosphate accumulation in PC12 cells resulting in the accumulation of inositol mono- and polyphosphates, although not

inositol-1,4,5-trisphosphate which mobilizes intracellular calcium [17]. In GH3 pituitary cells, lithium enhances the agonist-stimulated accumulation of diacylglycerol, the intracellular messenger required for the activation of protein kinase C [18]. NGF also stimulates PI hydrolysis and a transient increase in protein kinase C activity in PC12 cells [19, 20]. Thus, both agents could exert their effects, at least in part, through the PI pathway.

The effects of lithium on gene expression may be particularly relevant to its dorsalizing effects on developing amphibian embryos [16]. Lithium potentiates growth factor induction of mesoderm in isolated animal cap tissue at an estimated intracellular concentration of 6.4 mM [21]. Similar concentrations of lithium (5 mM) potentiate NT/N gene expression in PC12 cells, although maximal effects are observed between 10 and 20 mM [6]. The cooperative effects of lithium on NT/N gene expression and the forskolin-induced activation of promoters containing CREs suggest that lithium could also potentiate the growth factor-induced expression of specific genes involved in mesodermal differentiation.

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REFERENCES

1. Curran, T. & Morgan, J.I. Science 229, 1265-1268 (1985).

2. Greenberg, M.E., Greene, L.A. & Ziff, E.B. J. Biol. Chem. 260, 14101-14110 (1985).

3. Kruijer, W., Schubert, D. & Verma, I. Proc. Natl. Acad. Sci. USA 82, 7330-7334 (1985).

4. Curran, T. & Morgan, J.I. BioEssays 7, 255-258 (1987).

5. Bartel, D.P., Sheng, M., Lau, L.F. & Greenberg, M.E. Genes Dev. 3, 304-313 (1989).

6. Dobner, P.R., Tischler, A.S., Lee, Y.C., Bloom, S.R. & Donahue, S.R. J. Biol. Chem. 263, 13983-13986 (1988).

7. Kislauskis, E. & Dobner, P.R. Neuron 4,783-795 (1990).

8. Angel, P., et al. Cell 49, 729-739 (1987).

9. Van Buskirk, R., Corcoran, T. & Wagenr, J.A. Mol. Cell. Biol. 5, 1984-1992 (1985).

10. Curran, T. & Franza, R.B., Jr. Cell 55, 395-397 (1988).

11. Bohmann, D., Admon, A., Turner, D.R. & Tjian, R. Cold Spring Harb. Symp. Quant. Biol. 53, 695-700 (1988).

12. Chiu, R., Angel, P. & Karin, M. Cell 59, 979-986 (1989).

13. Hoeffler, J.P., Meyer, T.E., Yun, Y., Jameson, J.L. & Habener, J.F. Science 242, 1430-1433 (1988).

14. Gonzalez, G.A., et al. Nature 337, 749-752 (1989).

15. Angel, P., et al. Mol. Cell. Biol. 7, 2256-2266 (1987).

16. Berridge, M.J., Downes, C.P. & Hanley, M.R. Cell 59, 411-419 (1989).

17. van Calker, D., Assmann, K. & Greil, W. J. Neurochem. 49, 1379-1385 (1987).

18. Drummond, A.H. & Raeburn, C.A. Biochem. J. 224, 129-136 (1984).

19. Hama, T., Huang, K.-. & Guroff, G. Proc. Natl. Acad. Sci. USA 83, 2353-2357 (1986).

20. Contreras, M.L. & Guroff, G. J. Neurochem. 48, 1466-1472 (1987).

21. Slack, J.M.W., Isaacs, H.V. & Darlington, B.G. Development 103, 581-590 (1988).

22. de Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R. & Subramani, S. Mol. Cell. Biol. 7, 725-737 (1987).

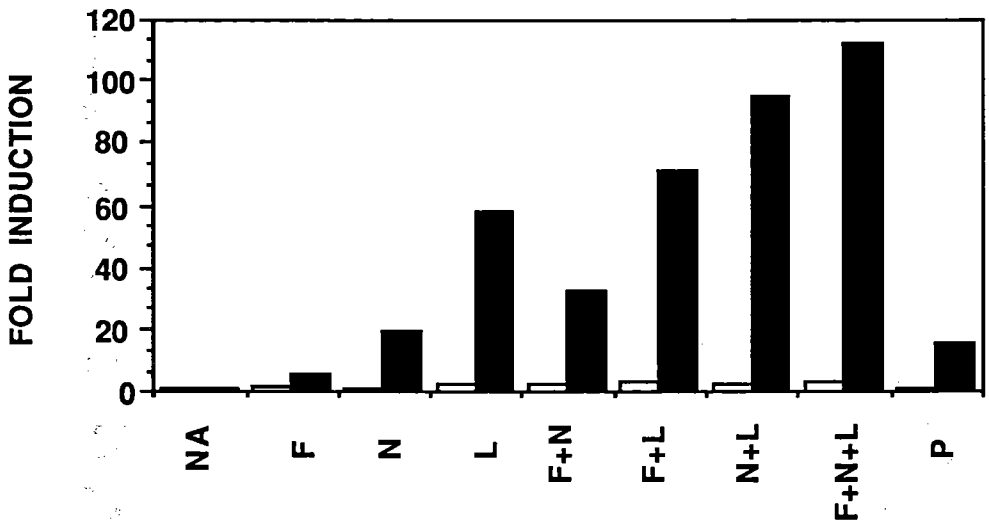
23. Fink, J.S., et al. Proc. Natl. Acad. Sci. USA 85, 6662-6666 (1988).

FIG. 1 Lithium and NGF selectively activate promoters containing multiple AP-1 sequences. a, PC12 or mutant A126-1B2 cells were co-transfected with either pBLCat4, 1xAP-1, or 5xAP-1 plasmids (depicted schematically), and a standardization plasmid in which the firefly luciferase gene is controlled by the Rous sarcoma virus long terminal repeat 22. Following a 48 hr recovery period, transfected cells were treated with the indicated combinations of inducers for 12 hr, followed by determination of CAT and luciferase enzyme activity. Representative CAT assays for each plasmid and induction condition are shown. NA, no additives; F, 10 μM forskolin; N, 100 ng/ml NGF; L, 20mM LiCl; P, 100 nM PMA. b, Fold induction of CAT gene activity (average of three separate experiments) in PC12 cells transfected with either the 1xAP-1 (open bars) or 5xAP-1 (black bars) plasmid treated with the indicated inducers (as in a). c, Same as b except mutant A126-1B2 cells deficient in protein kinase A activity 9 were transfected. The average fold induction was calculated from at least two separate experiments for each condition.

METHODS. Cell culture, transfections, and enzyme assays were all performed as described 7, 9. The A126-1B2 cells were derived from the PC12 cell line and are deficient in protein kinase A activity 9. The 1xAP-1 and 5xAP-1 plasmids were constructed as described 8, except that the vector was pBLCat4 instead of pBLCat2. They contain either one or five copies of the human collagenase gene AP-1 site inserted between vector Hind III and Bam HI sites of pBLCat4. An AP-1 site within the vector sequence of pBLCat2 has been removed from pBLCat4 (C. Jonat, personal communication). The AP-1 core sequence in the NT/N promoter (-188 to -182) is identical to the collagenase AP-1 site 7. The fold induction was calculated as the ratio of CAT activity in induced cells to the activity in

control cells. CAT activity was corrected for assay background, and variations in transfection efficiency.


PC12
A126-1B2
pBLCat4
1xAP-1
5xAP-1
NA
F
N
L
F+N
F+L
N+L
F+N+L
P


FOLD INDUCTION
120
100
80
60
40
20
0
NA
F
N
L
F+N
F+L
N+L
F+N+L
P

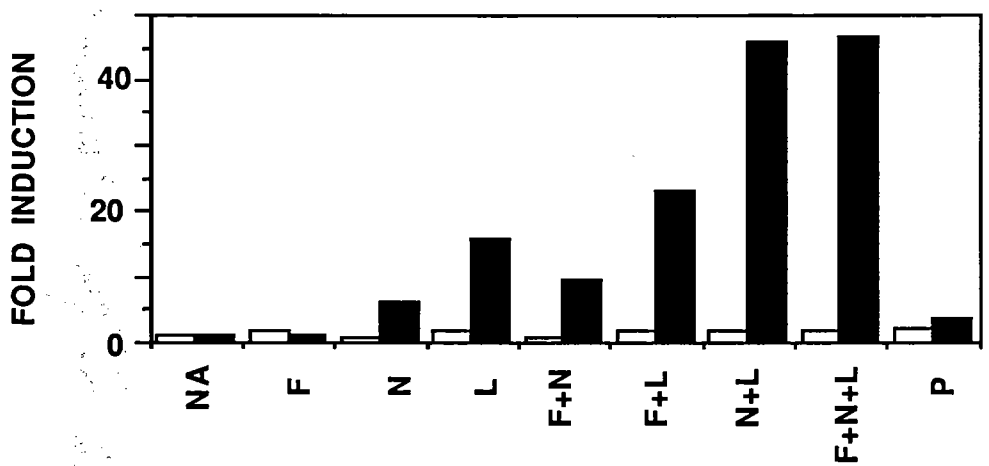

FOLD INDUCTION
40
20
0
NA
F
N
L
F+N
F+L
N+L
F+N+L
P

FIG. 2 NGF and lithium potentiate forskolin-induced activation of promoters containing single or multiple CREs: dependence on protein kinase A activity. a, PC12 or A126-1B2 cells were transfected with RSVΔE, 1xCRE, or 4xCRE CAT fusion gene plasmids (depicted schematically) and treated with the indicated inducers as described in Fig. 1. Representative CAT assays are shown. b, The average fold induction of CAT expression in PC12 cells for 1xCRE and 4xCRE was calculated as described in Fig. 1 from at least six separate experiments for 4xCRE and 3 separate experiments for 1xCRE and RSVΔE. c, Representative CAT assay from A126-1B2 cells transfected with 4xCRE and treated with the indicated inducers as in Fig. 1.

METHODS. The 1xCRE, and 4xCRE CAT fusion gene plasmids were constructed as described 23 by inserting synthetic oligonucleotides encompassing the vasoactive intestinal peptide CRE upstream of an enhancerless RSV promoter, RSVΔE. This CRE contains two inverted copies of the sequence CGTCA which is identical to the distal CRE sequence (-147 to -143) in the NT/N promoter 7. Other methods are as described in Fig. 1.

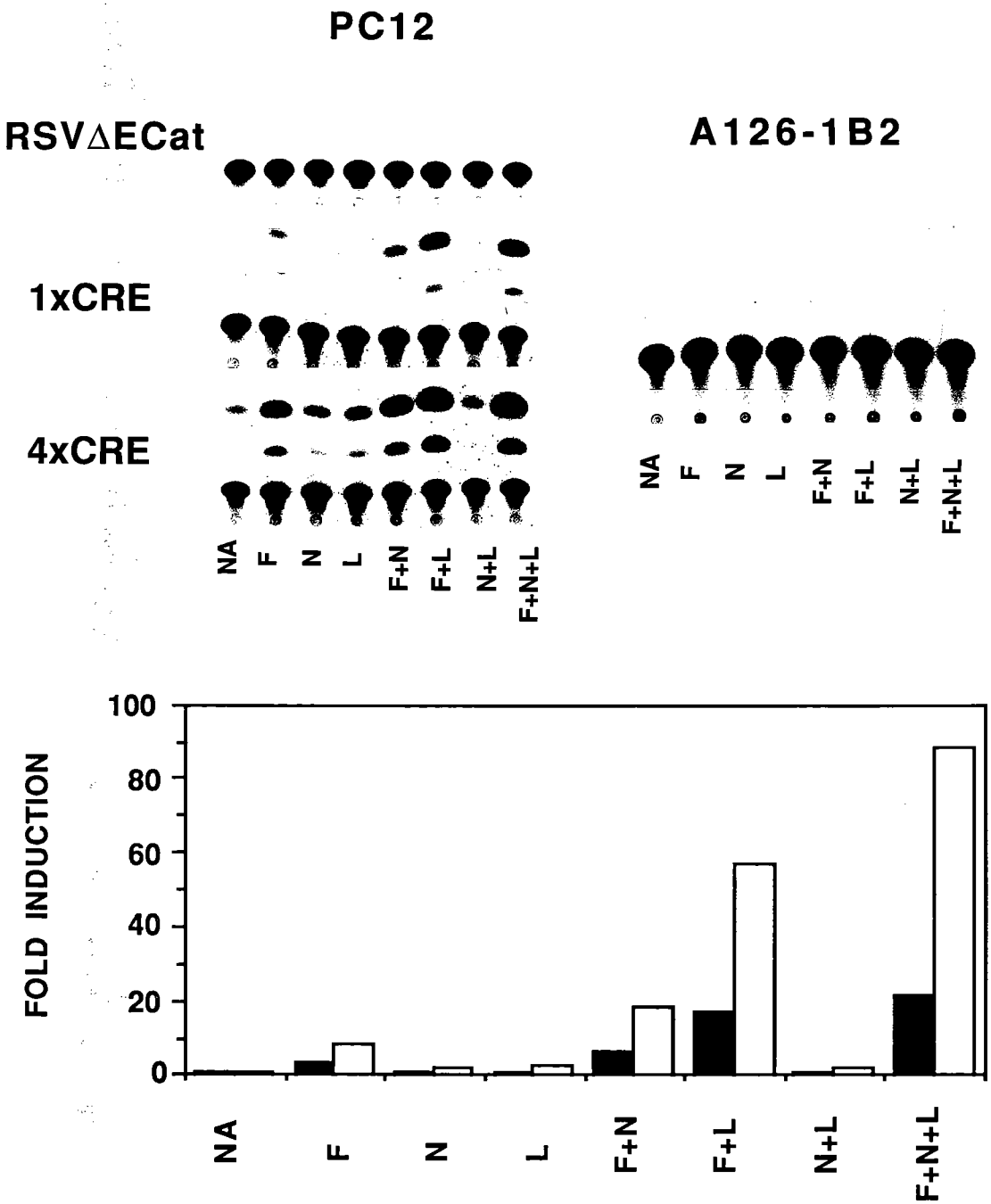
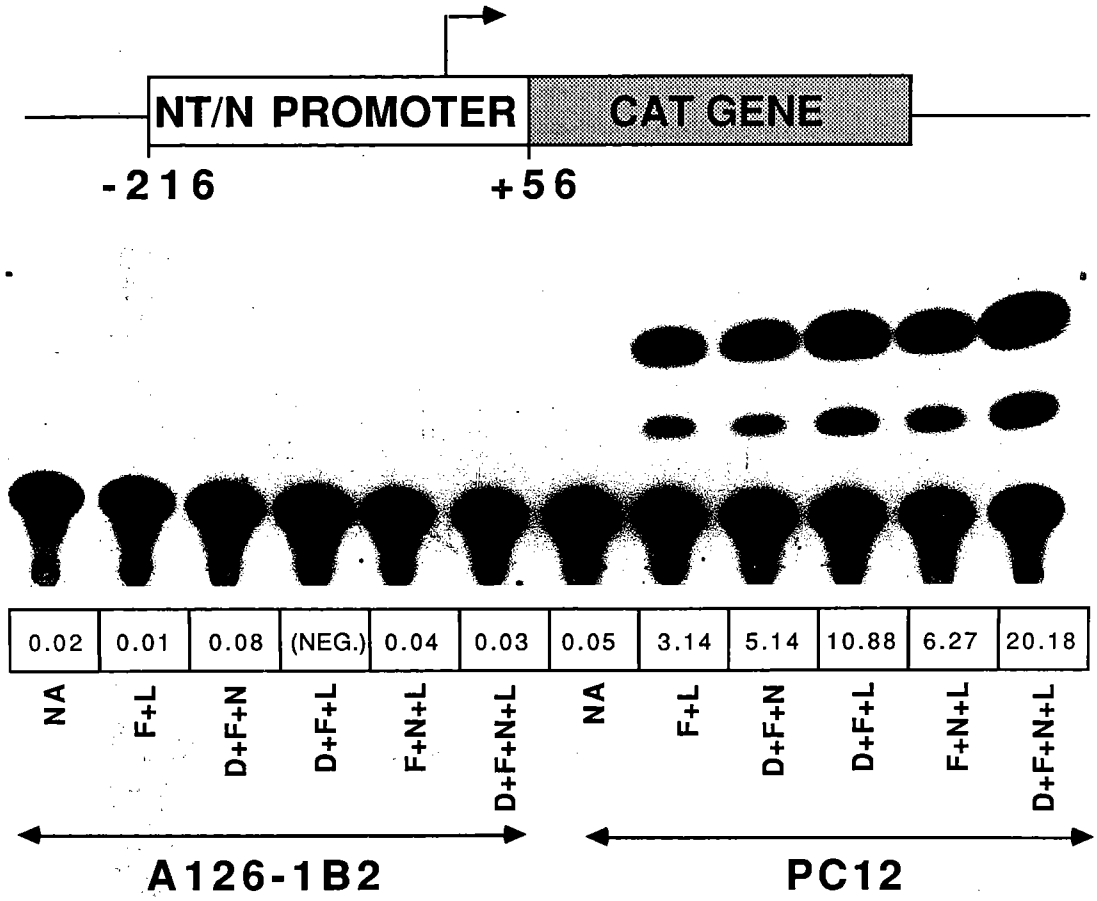

PC12
RSVΔECat
A126-1B2
1xCRE
4xCRE
NA
F
N
L
F+N
F+L
N+L
F+N+L
FOLD INDUCTION
100
80
60
40
20
0

FIG. 3 Activation of the NT/N promoter by combinations of inducers is dependent on protein kinase A activity. A CAT fusion gene controlled by the wild type NT/N promoter (-216/+56, depicted schematically) was analyzed in both A126-1B2 and PC12 cells treated with the indicated inducers (abbreviated as in Fig. 1 except; D, 1 mM dexamethasone; F, 1 mM forskolin). CAT assays, and the actual % conversion of 14C-chloramphenicol from unacetylated to acetylated forms are shown.


NT/N PROMOTER
CAT GENE
-216
+56
0.02
0.01
0.08
(NEG.)
0.04
0.03
0.05
3.14
5.14
10.88
6.27
20.18
NA
F+L
D+F+N
D+F+L
F+N+L
D+F+N+L
NA
F+L
D+F+N
D+F+L
F+N+L
D+F+N+L
A126-1B2
PC12

V

DISCUSSION

This thesis examined the structure and regulation of a neuroendocrine peptide gene encoding neurotensin (NT) and neuromedin N (N) in PC12 cells. Previous studies had shown that induction of NT peptide and NT/N mRNA levels in PC12 cells are strictly dependent on simultaneous exposure to combinations of NGF, dexamethasone, forskolin, and lithium (Dobner et al., 1988). To examine the transcriptional component of this complex regulation, the rat NT/N gene was first cloned and characterized (Chapter II). Next, the cis-regulatory sequences necessary for expression in PC12 cells were delineated using transient transfection assays, performed on plasmids containing NT/N sequences fused to reporter genes in PC12 cells (Chapter III). Finally, to address the mechanism of synergy between these inducers, the NT/N promoter and promoters containing a single functional class of cis-regulatory site were analyzed in wild-type and protein kinase A-deficient PC12 cells (Chapter IV). In this chapter, those analyses are reviewed and a model is proposed to explain the transcriptional regulation of the NT/N gene in PC12 cells. Also, the relevance of this work to three important issues in neurobiology are discussed, including (1) phenotypic plasticity of the neural crest, (2) NGF-induced neuronal differentiation, and (3) the pharmacological actions of lithium.

Progressive deletion of the NT/N 5' flanking region revealed that sequences between positions -216 and +56 function to integrate the transcriptional effects of all four inducers. These sequences, termed the NT/N promoter, are sufficient to confer the full spectrum of responses exhibited by the endogenous gene to reporter genes, including the absence of basal level expression, the negligible response to single inducers, and the extraordinary synergy between combinations

of any three and all four inducers. We conclude, therefore, that the NT/N gene is subject to complex synergistic control at the transcriptional level by all four inducers.

Cooperative interactions in the transcriptional control regions of other complex promoters have led us to consider two basic mechanisms for the synergistic control of the NT/N promoter: (1) synergy at the level of the gene, and (2) synergy at the level of signal transduction. As an extension of the model proposed by Ptashne (1988), synergy at the level of the gene could involve induced or activated transcription factors which cooperatively bind to adjacent sites in the promoter, or independently bind those sites and cooperate through a third factor (Ptashne, 1988). Alternatively, the effects of inducers may converge at the level of signal transduction to increase the synthesis and/or post-translational modification of a transcription factor which regulates one or more sites in the NT/N promoter. The functional characteristics of the NT/N promoter and its constituents suggest that synergy can occur at both levels.

Detailed mutational analysis of the NT/N promoter provided direct evidence for the functions of several inducible cis-regulatory sites, including an AP-1 binding site (AP-1), two cAMP-responsive elements (CREs), and a glucocorticoid responsive element (GRE). The severity of specific mutations in the AP-1 site or either CRE suggest that these sites function in a mutually dependent manner. The GRE may also cooperate with the other sites, but the evidence is less convincing. Furthermore, the biased effects of specific mutations in the NT/N promoter suggest that each of the three classes of sites mediates a distinct function. Mutations in either of the CREs resulted in selectively attenuated responses to combinations of inducers that include

forskolin, suggesting that these sites function as cAMP-responsive elements, while internal deletions which remove part or all of the GRE consensus resulted in selectively attenuated responses to conditions that include dexamethasone, suggesting that it functions as a glucocorticoid-responsive element. In contrast, extensive mutations in the AP-1 site did not show biased effects, suggesting that more that one inducer activates the AP-1 site. However, due to the apparent synergy between the AP-1 site and the CREs, it remained uncertain what the effects of individual inducers were on these sites.

To characterize the roles of the AP-1 site and CREs in the NT/N promoter more clearly, CAT reporter genes fused to promoters containing either a single copy or multiple copies of an AP-1 or CRE site were evaluated in parental and mutant PC12 cell lines treated with forskolin, NGF, and lithium, individually, and in combination. The results indicated that these two types of sites are regulated independently, and can be distinguished by: (1) the selective effects of single inducers; (2) the combinatorial actions of inducers; and (3) the requirement for kinase A activity. Each distinction, discussed separately below, could have significant bearing on the role of these sites in the NT/N promoter.

First, a comparison of the effects of single inducers on promoters containing either AP-1 sites or CREs suggests that each inducer preferentially activates one type of site. Promoters containing five AP-1 sites, but not a single AP-1 site, were markedly induced by lithium (58-fold) and NGF (nearly 20-fold), and modestly induced by forskolin (5-fold). In contrast, promoters containing either one or four CREs were induced by forskolin (3- and 9-fold, respectively), but were unresponsive to either lithium or NGF, individually. Therefore, lithium and NGF might act principally through the AP-1 site, and

forskolin might act principally through on the CREs, in the NT/N promoter. These results corroborate the effects of specific mutations to the AP-1 and CREs in the NT/N promoter, suggesting that multiple inducers activate the AP-1 site and forskolin principally activates the CREs. In addition, the selective activation of promoters containing multiple AP-1 sites by these single inducers, and the synergy between the AP-1 and CRE sites in the NT/N promoter, suggest that the function of a single AP-1 site is dependent on the activity of adjacent sites, in PC12 cells.

Further distinctions between the functions of AP-1 and CRE sites were revealed with combinations of inducers. Our result indicate that all three inducers act additively on promoters containing multiple AP-1 sites. In contrast, lithium and NGF potentiate the forskolin-induced activation of promoters containing four CRE (6-fold and 2-fold, respectively), and similarly affect promoters containing a single CRE. Interestingly, the potentiating effects of lithium and NGF on CRE activity also appears additive in cells treated with all three inducers. Therefore, separate mechanisms may mediate the effects of each inducer on both the AP-1 site and CREs.

The role of cAMP-mediated signal transduction in the induction of these promoters was further implicated using a mutant PC12 cell line, deficient in protein kinase A activity (van Buskirk et al., 1985). We observed that CRE activity was silenced and forskolin-induced expression from promoters containing multiple AP-1 sites was compromised. However, the effects of lithium or NGF on promoters containing multiple AP-1 sites was only marginally impaired. Therefore, forskolin's effects on these promoters are mediated by a protein kinase A-dependent mechanism, while the transcriptional

effects of lithium and NGF are mediated by both kinase A-dependent and kinase A-independent mechanisms. These results confirm the role of kinase A in the forskolin-stimulated cAMP pathway (Montminy et al., 1986; Rowland-Gagne and Greene, 1990; van Buskirk, et al., 1985), and support the role of multiple independent signal transduction mechanisms in the response to NGF (Cremins et al., 1986). Moreover, since a CAT fusion gene driven by the wild-type NT/N promoter (-216/+56) was effectively silenced in kinase A-deficient cells we conclude that the cAMP pathway is indispensible for NT/N promoter function, in PC12 cells.

Our evidence suggests that lithium and NGF influence NT/N gene expression through distinct mechanisms involving one or more AP-1 binding proteins. This conclusion was drawn from several observations. First, mutations to the AP-1 site in the NT/N promoter severely compromise NT/N promoter activity. Secondly, lithium and NGF selectively activate promoters containing multiple AP-1 sites, additively. This selective activation of multiple AP-1 sites closely resembles the properties of an NGF-inducible AP-1 protein, Jun B, recently demonstrated in F9 cells (Chiu et al., 1989), also NGF-inducible in PC12 cells. Thirdly, recent studies by Bryant Bullock have shown that the rapid and transient peak in unspliced NT/N precursor RNA in PC12 cells treated with a combinations of NGF, dexamethasone, and forskolin occurs earlier than in PC12 cells treated with a combination of lithium, dexamethasone, and forskolin. This earlier peak was protein synthesis-independent while the later peak was protein synthesis-dependent. These results suggest that NGF's actions on the NT/N promoter could involve a post-translational modification of pre-existing transcription factors while lithium's actions could involve the increased

expression of transcription factors. In PC12 cells, several members of the AP-1 family are differential regulated in response environmental stimuli, such as forskolin and NGF (Bartel et al., 1989; Chiu et al., 1989). Some are post-translationally modified, also (Curran, 1988). Combinatorial mechanisms between AP-1 proteins might play a critical role in mediating the pattern of response of the NT/N promoter to various combination of inducers. Overall, the characteristics of the NT/N promoter suggest that different AP-1 binding proteins with similar functional properties are activated by lithium and NGF through separate mechanisms involving transcription or by post-translational modification. Although, it remains possible that the protein induced at the transcriptional level by lithium could also be post-translationally modified by NGF.

The selective effects of specific mutations to cis-regulatory sites in the NT/N promoter which closely resemble GRE and CREs suggest that the glucocorticoid receptor and a major CRE-binding protein found in uninduced PC12 cells (CREB), respectively, could be involved in NT/N gene expression in PC12 cells. The glucocorticoid receptor is a ligand-dependent transcription factor which directly mediates the effects of dexamethasone through GREs (Evans, 1988). CREB, on the other hand, is activated by phosphorylation at a specific residue (Ser-133), recognized by kinase A, in response to cAMP (Gonzalez and Montminy, 1989). Phoshosphorylation of CREB at Ser-133 appears to activate an allosteric mechanism which may enable CREB to interact with other components of the transcription apparatus (Yamamoto et al., 1990). Phosphorylation at a separate site, by protein kinase C, promotes CREB dimer formation and enhances its binding affinity, but not its ability to active

transcription, in vitro (Yamamoto et al., 1988), suggesting that additional phosphorylation mechanisms might also modulate CREB functions, in vivo. However, since dephoshorylated CREB can still bind as a monomer to the CRE, and since evidence suggests that CREB monomers and dimers exist in equilibrium in uninduced PC12 cells, it has been proposed that CREs are bound constitutively by CREB (Roesler et al., 1988). If CREB is involved in the regulated transcription from the NT/N promoter, then the potentiating effects of lithium and NGF on forskolin-induced CRE activity could involve converging phosphorylation mechanisms on CREB proteins, previously bound to the NT/N promoter. An alternative possibility is that other CRE-binding proteins may mediate the effects of forskolin, NGF, and lithium on the CREs.

CREB is a member of a family of transcription factors which are structurally-related to AP-1 (Hai et al., 1989). Two other CREB-related proteins have been clonedand are present in PC12 cells: CRE-BP1 (Maekawa et al., 1989) and ΔCREB (Yamamoto et al., 1990). Eight recombinant cDNA clones encoding CRE-binding proteins have been characterized in HeLa cells, differing in their precise interactions with a consensus CRE (Hai et al., 1989). The potential for interactions between the two families is supported by recent evidence that c-Jun and Fos/Jun dimers can activate transcription through CREs, in F9 cells (Chiu et al., 1989) and JEG-3 cells (Sassone-Corsi et al., 1990). However, there is no solid evidence that specific heterodimers, which form between members of the CREB and AP-1 families, activate transcription from either site (Benbrook and Jones, 1990; Ivashkiv et al., 1990; MacGregor et al., 1990), or that CREB can bind and activate the AP-1 site (Deutsch et al., 1988; Hoeffler et al., 1989). We speculate that since promoters containing AP-1 sites

or CREs are differentially regulated in response to these inducers, and mutations to these sites in the context of the NT/N promoter have distinct effects, it is unlikely that both types of sites are mediated by the same transcription factor. It is more likely that distinct proteins mediate the effects of the CREs and AP-1 sites in PC12 cells.

The model that I propose to account for the complex regulation of NT/N gene expression in PC12 cells, depicted schematically in Figure 1, is based on the following considerations: (1) all the information necessary to confer appropriate transcriptional regulation to heterologous promoters occurs between postions -216 and +56 of the NT/N gene; (2) the NT/N promoter is composed solely of inducible cis-regulatory sites, including an AP-1 binding site, two cAMP-responsive elements, and a near-consensus glucocorticoid- responsive element; (3) the AP-1 and the CREs are mutually interdependent; (4) each inducer has a dominant effect on one type of site through a separate pathway (dexamethasone affects the GRE, forskolin affects the CRE, and both lithium and NGF affect the AP-1 site); (5) interactions occur between the cAMP pathway, activated by forskolin, and the pathways activated by lithium and NGF such that all three inducers influence both the CREs and AP-1 site; and (6) distinct transcription factors could mediate the activity of each type of site. This model accounts for the undetectable basal level of expression, the absence of an appreciable response to single inducers, and the extraordinary synergy between combinations of three and four inducers. Each of these features are addressed separately, below.

I propose that combinations of the four inducers could act synergistically to induce NT/N gene expression in PC12 cells at the level of the gene and at the level of signal transduction. At the level of signal transduction, lithium and NGF

synergistically potentiate the effects of forskolin on CREs. At the level of the gene, multiple transcription factors induced at the transcriptional level and by post-translational modification, cooperate to increase the rate of transcription from the NT/N gene. Whether these transcription factors bind each site cooperatively or bind independently and then interact cooperatively through a third protein is unknown. However, two observations suggest that the spatial organization of the NT/N promoter is important for the regulation of the NT/N promoter. First, the organization of the NT/N promoter is such that all four of these sites roughly align on one side of the DNA helix, in a position which could be favorable for interactions between different transcription factors. Secondly, the NT/N promoter sequences between -216 and -28 function in either orientation upstream of a heterologous promoter but are silent when moved downstream from the reporter gene. These results suggest that the apparent synergy between the AP-1 site, the CREs, and possibly the GRE may reflect interactions between stereospecifically aligned proteins which contact the transcription apparatus. Moreover, the severity of mutations in the AP-1 site and the two CREs indicate these sites must be occupied in order to generate high level expression. In this model, the complex pattern of responses to the various combinations of inducers reflects the particular assembly of transcription factors at the NT/N promoter at once, and their capacity to activate transcription.

I propose that a productive assembly of transcription factors does not occur on the NT/N promoter, in response to individual inducers, as a consequence of either weak binding sites in the NT/N promoter, or the properties of transcription factors which activate transcription from those site. Three sites in the NT/N promoter, the GRE and both CREs, deviate slightly from their respective

consensus sequence. In other systems, identical or nearly-identical deviations from the CRE and GRE consensus exhibit weaker activity (Bokar et al., 1988; Jantzen et al., 1987). Often, such weak sites function cooperatively with adjacent active sites in complex promoters (Comb et al., 1989; Hyman et al., 1989; Jantzen et al., 1987; Lee et al., 1987; Mermod et al., 1988). In addition, sequences flanking AP-1 and CRE consensus sites have been shown to markedly affect their capacity to respond to phorbol esters and cAMP, respectively (Deutsch et al., 1988; Hoeffler et al., 1989). Therefore, activation of the NT/N promoter in PC12 cells may involve cooperative interactions between weak binding sites. However, the alternative possibility is that each site may have high affinity for its transcription factor, but due to its specific biological properties, still not form a productive transcription complex in response to single inducers. In support of this possibility, Jun-B has been shown to bind a single AP-1 site (Nakabeppu, et al., 1988), but not activate it (Chiu, et al., 1989). Furthermore, it appears to outcompete an activator of that site, c-Jun (Chiu et al., 1989). Similarly, a CREB-related protein expressed in PC12 cells, ΔCREB, binds the CRE equally as well as CREB, but unlike CREB cannot activate it (Yamamoto et al., 1990). Both of these proteins could serve to downregulate AP-1 and CRE activity in general, or modulate specific gene expression, like the NT/N promoter.

I propose that the transcription factors which regulate the NT/N promoter are either absent or inactive in uninduced PC12 cells. The glucocorticoid receptor is cytoplasmic in the absence of hormone (Yamamoto, 1985), the levels of AP-1 protein are low in uninduced cells (Sheng, 1990), and CREB requires phosphorylation to be induced to activate transcripiton (Yamamoto, et al.,

1988). In this regard, the NT/N promoter can be considered solely composed of inducible cis-regulatory sites. However, in other cells types, these sites could be constitutively active (Lee et al., 1987; Roesler et al., 1988). The strikingly different patterns of expression of the NT/N promoter in rat GH3 pituitary cells and Rat 2 embryonic fibroblasts, particularly the response to forskolin treatment in GH3 cells and the high constitutive activity in Rat 2 cells, attest to the flexibility of this gene.

Analysis of the NT/N promoter has demonstrated that very complex regulatory properties can result from the assembly of different inducible cis-regulatory sequences near the start site of transcription. Such modular organization is common among transcriptional control regions for protein coding genes in mammalian cells. In general, promoters and enhancers are composed of multiple cis-regulatory sites activated by both constitutively active and inducible transcription factors (Dynan, 1989). However, the NT/N promoter is thus far unique in that it appears to be composed entirely of inducible elements. These inducible elements serve to integrate multiple environmental stimuli into a unified transcriptional response. During neural crest development, such a mechanism may be particularly important for the generation of phenotypic plasticity. Pluripotent neural crest cells may maintain a battery of genes, in accessible chromatin conformation, which are expressed only when cells receive specific combinations of environmental cues (Anderson and Axel, 1985). Exposure to the appropriate environmental cues would result in differential gene expression and culminate in a particular differentiated phenotype. Furthermore, synergy between several environmental cues provide a more sensitive mechanism to control the magnitude and duration of the response through small

changes in the concentration of individual inducers. Therefore, promoters composed entirely of inducible elements could engender both specificity and flexibility in the transcriptional response to combinations of environmental stimuli.

The NT/N promoter has provided insight into the mechanism by which NGF mediates neuronal differentiation in PC12 cells. One of the earliest responses to NGF in PC12 cells is the rapid, transient, and protein synthesis-independent induction of several genes encoding putative regulatory proteins, including members of the AP-1 family. This rapid transcriptional activation of AP-1 expression has led to the proposal that these proteins are involved in the cascade of transcriptional events which occur during NGF-induced neuronal differentiation (Sheng, 1990). The observed induction of AP-1 components in specific regions of the brain following chemically-induced seizure suggests that these proteins may also mediate neuronal function (Morgan and Curran, 1989). The presence of a functional AP-1 site in the proenkephalin promoter and the slightly delayed appearance of proenkephalin mRNA following seizure suggests that AP-1 proteins may be involved in neuropeptide gene expression in the brain (Sonnenberg et al., 1989). The functional AP-1 site in the NT/N promoter, and the fact that the NT/N unspliced precursor RNA is induced with slightly delayed kinetics relative to the induction of AP-1 genes in the absence of protein synthesis, implies that AP-1 proteins play a role in NT/N gene expression. Furthermore, the selective activation of multiple AP-1 sites by NGF suggests that promoters containing multiple AP-1 sites, or a single AP-1 site in the context of active CREs, may be involved in NGF-induced neuronal gene expression in PC12 cells.

The data presented in this thesis demonstrate clearly that lithium ion influences specific gene expression at the transcription level, in PC12 cells. Lithium, present in trace amounts in normal serum, is the major therapeutic agent used in the treatment of manic depressive illness in humans and a potent teratogen during early development (Berridge et al., 1989). Although investigations into its mechanism of action have focused on its inhibitory effects on signal transduction involving the cAMP and phosphoinositol pathways, the consequences of these effects on gene expression have received little attention. The ability of lithium to selectively activate promoters containing multiple AP-1 sites by a kinase A-independent mechanism, and to potentiate forskolin-induced CRE activity markedly, by a kinase A-dependent mechanism, indicate that at least two distinct signal transduction pathways mediate lithium's transcriptional effects, including the cAMP pathway. Although the concentration of lithium used in these experiments (20 mM) was greater than peak serum levels during lithium therapy (1mM), the observation that lithium can potentiate NT/N gene expression submaximally, *in vitro*, at much lower concentrations (2-5 mM) (Dobner et al., 1988) raises the possibility that lithium-induced changes in neuromodulator or neurotransmitter gene expression in the central nervous system might participate in its pharmacological effects.

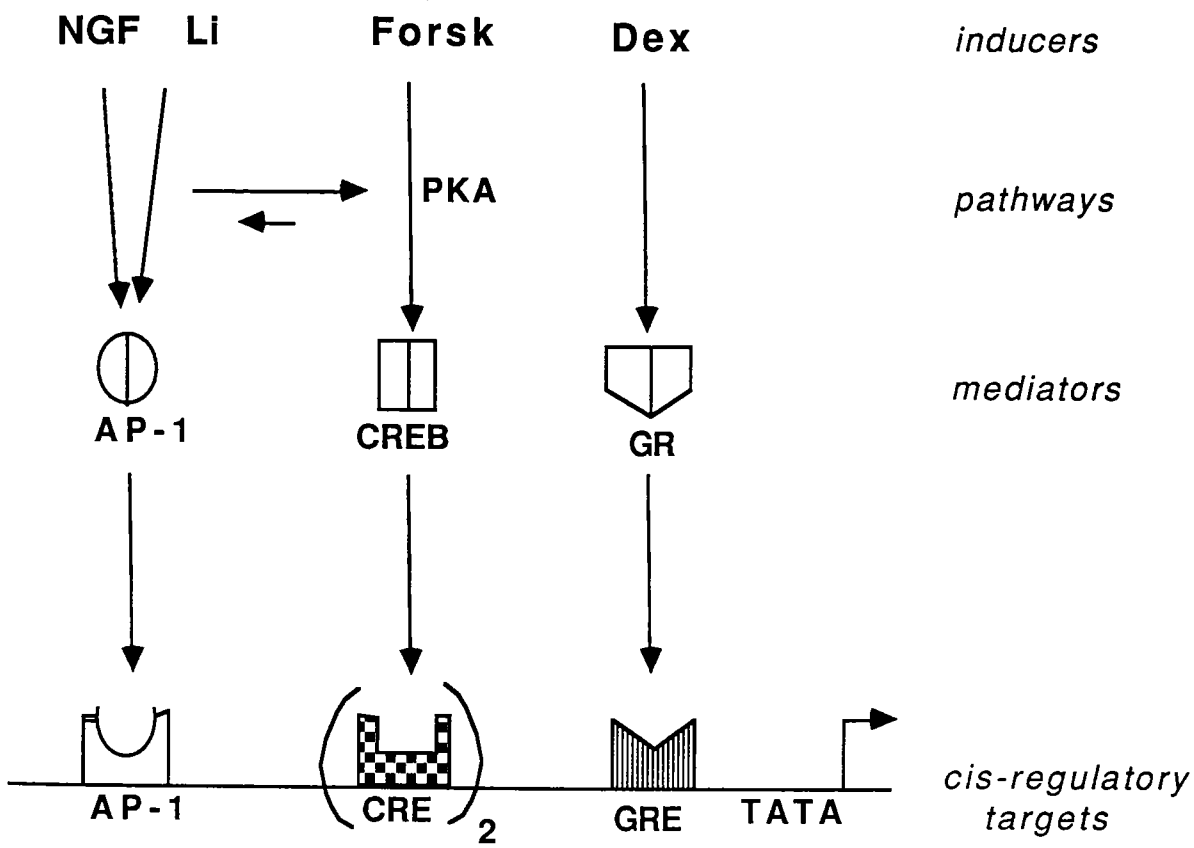


Figure 1. Model for the transcriptional regulation of the NT/N gene

VI

REFERENCES
(Sections I and V)

Ackerblom, I. E., Slater, E. P., Beato, M., Baxter, J.D. and, Mellon, P. L. (1988). Negative regulation by glucocrticoids through interference with a cAMP responsive enhancer. Science 241 , 350-353.

Alexander, M.J., Dobner, P.R., Miller, M.A., Bullock, B.P., Dorsa, D.M. and, Leeman, S.E. (1989). Estrogen induces neurotensin/neuromedin N messenger ribonucleic acid in a preoptic nucleus essential for the preovulatory surge of luteinizing hormone in the rat. Endocrinology 125 , 2111-2117.

Alexander, M. J., Mahoney, P. D., Ferris, C. F., Carraway, R. E. and, Leeman, S. E. (1989). Evidence that Neurotensin Participates in the Central Regulation of the Preovulatory Surge of Luteinizing Hormone in the Rat. Endocrinology 124 , 783-788.

Anderson, D. J. (1989). The neural crest lineage problem: neuropoiesis? Neuron 3 ,1-12.

Anderson, D.J. and, Axel, R. (1985). Molecular probes for the development and plasticity of neural crest derivatives. Cell 42 , 649-662.

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

Angel, P., Hattori, K., Smeal, T. and, Karin, M. (1988). The jun proto-oncogene Is positively autoregulated by its product, Jun/AP-1. Cell 55 , 875-885.

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

Avissar, S., Schieber, G., Danon, A. and, Belmaker, R.H. (1988). Lithium inhibits adrenergic and cholinergic increases in the GTP binding in the rat cortex. Nature 331 , 440-442.

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

Barbacid, M. (1987). Ras genes. Annu. Rev. Biochem. 56 , 779-827.

Barde, Y.-A. (1989). Trophic factors and neuronal survival. Neuron 2 , 1525-1534.

Bartel, D.P., Sheng, M., Lau, L.F. and, Greenberg, M.E. (1989). Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of fos and jun induction. Genes Dev. 3 , 304-313.

Beato, M. (1989). Gene regulation by steroid hormones. Cell 56 , 335-344.

Benbrook, D. M. and, Jones, N. C. (1990). Heterodimer formation between CREB and JUN proteins. Oncogene 5 , 295-302.

Berridge, M. J., Downes, C. P. and, Hanley, M. R. (1982). Lithium amplifies the agonist-dependent phosphatidylinositol responses in brain and salivary gland. Biochem. J. 206 , 587-599.

Berridge, M.J., Downes, C.P. and, Hanley, M.R. (1989). Neural and developmental actions of lithium: a unifying hypothesis. Cell 59 , 411-419.

Black, I.B., Adler, J.E., Dreyfus, C.F., Friedman, W.F., LaGamma, E.F. and, Roach, A.H. (1987). Biochemistry of information storage in the nervous system. Science 236 , 1263-1268.

Bokar, J. A., Roesler, W. J., Vandenbark, G. R., Kaetzel, D. M., Hanson, R. W. and, Nilson, J. H. (1988). Characterization of the cAMP-responsive elements from the genes for the α-subunit of glycoprotein hormones and phosphoenolpyruvate carboxykinase (GTP): conserved features of nuclear protein binding between tissues and species. J. Biol. Chem. 263 , 19740-19747.

Bronner-Fraser, M. and, Fraser, S. (1989). Developmental potential of avian trunk neural crest cell in situ. Neuron 3 , 755-766.

Busa, W. B. and, Gimlich, R. L. (1989). Lithium-induced teratogenesis in frog embryos prevented by polyphosphoinositide cycle intermediates or a diacylglycerol analog. Dev. Biol. 132 , 315-324.

Carraway, R. and, Leeman, S. E. (1973). Isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. J. Biol. Chem. 248 , 6854-6861.

Carraway, R. and, Leeman, S.E. (1975). Radioimmunoassay for neurotensin. J. Biol. Chem. 250 , 1912-1918.

Chiu, R., Angel, P. and, Karin, M. (1989). Jun-B differs in its biological properties from, and is a negative regulator of, c-jun. Cell 59 , 979-986.

Chu, G., Hayakawa, H., and Berg, P. (1987) Electroporation for the efficient transfection of mammalian cells with DNA. Nucl. Acids Res. 15, 1311-1325.

Comb, M., Birnberg, N. C., Seasholtz, A., Herbert, E. and, Goodman, H. M. (1986). A cyclic AMP- and phorbol ester-inducible DNA element. Nature 323 , 353-356.

Comb, M., Mermod, N., Hyman, S. E., Pearlberg, J., Ross, M. E. and, Goodman, H. M. (1989). Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible transcription. EMBO J. , 3793-3805.

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

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

Curran, T. (1988). The Fos oncogene. In The Oncogene Handbook, Reddy, G. P., Skalka, A. M., Curran, T., eds. (Amersterdam, Elsevier Science): 307-325.

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

Curran, T. and Morgan, J. I. (1987). BioEssays 7 , 255-258.

Delegeane, A. M., Ferland, L. H. and, Mellon, P. M. (1987). Tissue-specific enhancer of the human glycoprotein hormone a-subunit gene: dependence on cyclic AMP-inducible elements. Mol. Cell. Biol. 7 , 3994-4002.

Deutsch, P. J., Hoeffler, J. P., Jameson, J. L. and, Habener, J. F. (1988). Cyclic AMP and phorbol ester-stimulated transcription mediated by similar DNA elements that bind distinct proteins. Proc. Natl. Acad. Sci. USA 85 , 7922-7926.

Deutsch, P. J., Jameson, J. L. and, Habener, J. F. (1987). Cyclic AMP responsiveness of human gonadotropin-a gene transcription is directed by a repeated 18-base pair enhancer: a-promoter receptivity to the enhancer confers cell-preferential expression. J. Biol. Chem. 262 , 12169-12174.

Dobner, P. R., Barber, D. L., Villa-Komaroff, L. and, McKiernan, C. (1987). Cloning and Sequence Analysis of cDNA for the Canine Neurotensin/Neuromedin N Precursor. Proc. Natl. Acad. Sci. USA 84 , 3516-3520.

Dobner, P. R., Tischler, A. S., Lee, Y. C., Bloom, S. R. and, Donahue, S. R. (1988). Lithium dramatically potentiates neurotensin/neuromedin N gene expression. J. Biol. Chem. 263 , 13983-13986.

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

Doupe, A.J., Patterson, P.H. and, Landis, S.C. (1985b). Small intensely fluorescent cells in culture: role of glucocorticoids and growth factors in their development and interconversions with other neural crest derivatives. J. Neurosci. 5 , 2143-2160.

Drummond, A. H. and, Raeburn, C. A. (1984). The interaction of lithium with thyrotropin-releasing hormone-stimulated lipid metabolism in GH3 pituitary cells. Biochem. J. 224 , 129-136.

Dynan, W.S. (1989). Modularity in promoters and enhancers. Cell 58 , 1-4.

Elliot, P. J. and, Nemeroff, C. B. (1986). The neurobiology of neurotensin. In Neural and Endocrine peptides and receptors, Woody, T. W., ed. (New York, Plenum): 219-245.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. Science 240, 889-895.

Evans, R. M. and, Arriza, J. L. (1989). A molecular framework for the actions of glucocorticoid hormones in the nervous system. Neuron 2 , 1105-1112.

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

Ferris, C. F., Armstrong, M. J., George, J. K., Stevens, C. A., Carraway, R.E. and, Leeman, S.E. (1985). Alcohol and fatty acid stimulation of neurotensin release from the rat small intestine. Endocrinology 116 , 1133-1138.

Fink, J.S., Menno, V., Kasper, S., Tsukada, T., Mandel, G and, Goodman, R. (1988). The CGTCA sequence motif is essential for the biological activity of the vasoactive intestinal peptide gene cAMP-regulated enhancer. Proc. Natl. Acad. Sci. 85 , 6662-6666.

Gizang-Ginsberg, E. and, Ziff, E. (1990). Nerve growth factor regulates tyrosine hydroxylase gene transcription through a nucleoprotein complex that contains c-Fos. Genes and dev. 4 , 477-491.

Goedert, M., Reynolds, G. P. and, Emson, P.C. (1983). Neurosci. Letts. 35 , 155-160.

Goelet, P., Castelluci, S., S., Schacher and, Kandel, E. R. (1986). The long and short of long-term memory--a molecular framework. Nature 322 , 419-422.

Gonzalez, G. A. and, Montminy, M. R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59 , 675-680.

Goodman, R.H. (1990). Regulation of neuropeptide gene expression. Annu. Rev. Neurosci. 13 , 111-127.

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

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

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

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

Greene, L. A. and, Tischler, A. S. (1982). PC12 Pheochromocytoma cultures in neurbiological research. Adv. Cell. Neurobiol. 3 , 373-414.

Hai, T., Liu, F., Allegretto, E. A., Karin, M. and, Green, M. R. (1988). A family of immunologically related transcription factors that includes multiple forms of ATF and AP-1. Genes Dev. 2 , 1216-1226.

Hai, T., Liu, F., Coukos, W.J. and, Green, M.R. (1989). Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev. 3 , 2083-2090.

Halazonetis, T. D., Georgopoulos, K., Greenberg, M. E. and, Leder, P. (1988). c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. Cell 55 , 917-924.

Harper, G. P., Pearce, F. L. and, Vernon, C. A. (1976). Nature 261 , 251-253.

Hoeffler, J.P., Deutsch, P.J., Lin, J. and, Habener, J.F. (1989). Distinct adenosine 3',5'-monophosphate and phorbol ester-responsive signal transduction pathways converge at the level of transcriptional activation by the interactions of DNA-binding proteins. Mol. Endo. 3 , 868-880.

Hoeffler, J. P., Meyer, T. E., Yun, Y., Jameson, J. L. and, Habener, J. F. (1988). Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental cDNA. Science 242 , 1430-1433.

Hokfelt, T., Everitt, B.J., Theodorsson-Norheim, E. and, Goldstein, M. (1984). Occurrence of neurotensinlike immunoreactivity in subpopulations of hypothalamic, mesencephalic, and medullary catecholamine neurons. J. Comp. Neurol. 222 , 543-559.

Hyman, S. E., Comb, M., Pearlberg, J. and, Goodman, H. M. (1989). An AP-2 element acts synergistically with the cyclic AMP- and phorbol ester-inducible enhancer of the human proenkephalin gene. Mol. Cell. Biol. 9 , 321-324.

Ivashkiv, L. B., Liou, H-C., Kara, C. J., Lamph, W. W., Verma, I. M. and, Glimcher, L. H. (1990). mXBP/CRE-BP2 and c-Jun form a complex which binds to cyclic AMP, but not to the 12-O-Tetradecanoylphorbol-13-Acetate, response element. Mol. Cell. Biol. 10 , 1609-1621.

Jantzen, H.-M., Strahle, U., Gloss, B, Stewart, F., Schmid, W., Boshart, M., Miksicek, R. and, Schutz, G. (1987). Cooperativity of glucocorticoid response

elements located far upstream of the tyrosine aminotransferase gene. Cell 49 , 29-38.

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

Koizumi, S., Contrerass, M.L., Matsuda, Y., Hama, T., Lazarovici, P. and, Guroff, G. (1988). K-252a- A specific inhibitor of the action of NGF on PC12 cells. J. Neurosci. 8 , 715-721.

Kouzarides, T. and, Ziff, E. (1988). The role of the leucine zipper in the fos-jun interaction. Nature 336 , 646-651.

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

LeDouarin, N.M. (1980). The ontogeny of the neural crest in avian embryo chimeras. Nature 286 , 663-669.

Lee, W., Haslinger, A., Karin, M. and, Tjian, R. (1987). Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. Nature 325 , 368-372.

Leeman, S. E. and, Carraway, R. E. (1982). Neurotensin discovery, isolation, characterization, synthesis, and possible physiological roles. In Neurotensin, A Brain and Gastrointestinal Peptide, Nemeroff, C. B. and Prange, Jr., A. J., eds. (N.Y., Ann. N. Y. Med. Sci.): 1-16.

Leonard, D. G. B., Ziff, E. B. and, Greene, L. A. (1987). Identification and Characterization of mRNAs Regulated by Nerve Growth Factor in PC12 Cells. Mol. Cell. Biol. 7 , 3156-3167.

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

Levi-Montalcini, R. and, Angeletti, P.U. (1968). Nerve growth factor. Physiol. Rev. 48 , 534-569.

Lynch, D. R. and, Synder, S. H. (1986). Neuropeptides: multiple molecular forms, metabolic pathways, and receptors. Ann. Rev. Biochem. 55 , 773-799.

MacGregor, P. F., Abate, C. and, Curran, T. (1990). Direct cloning of leucine zipper proteins Jun binds cooperatively to the CRE with CRE-BP1. Oncogene 5 , 451-458.

Maekawa, T. H., Sakura, C., Kanei-Ishii, T. , Yoshimura, T. and, Ishii, S. (1989). Leucine zipper structure of th protein CRE-BP1 binding to the cyclic AMP responsive element in the brain. EMBO. J. 8 , 2023-2028.

Menkes, H. A., Baraban, J. M., Freed, A. N. and, Snyder, S. H. (1986). Lithium dampens neurotransmitter response to smooth muscle: relevance to action in affective illness. Proc. Natl. Acad. Sci. USA 83 , 5727-5730.

Mermod, N., Williams, T.J. and, Tjian, R. (1988). Enhancer binding factors AP-4 and AP-1 act in concert to activate SV40 late transcription in vitro. Nature 332 , 557-561.

Montminy, M.R. and, Bilezikjian, L.M. (1987). Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature 328 , 175-178.

Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G. and, Goodman, R. H. (1986). Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. USA 83 , 6682-6686.

Morgan, J. I. and, Curran, T. (1989). Stimulus-transcription coupling in neurons: role of cellular immediate-early genes. TINS 12 , 459-462.

Nakabeppu, Y., Ryder, K. and, Nathans, D. (1988). DNA binding activities of three murine Jun proteins: stimulation by Fos. Cell 55 , 907-915.

Newman, M. E. and, Belmaker, R. H. (1987). Effects of lithium in vitro and ex vivo on components of the adenylate cyclase system in membranes form the cerebral cortex of the rat. Neuropharmocol. 26 , 211-217.

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

O'Malley, B. (1990). The steroid receptor superfamily: more excitement predicted for the future. Mol. Endocrinol. 4 , 363-369.

Otte, A.P., van Run, P., Heideveld, M., van Driel, Roel and, Durston, A.J. (1989). Neural induction is mediated by crosstalk between the protein kinase C and cyclic AMP pathways. Cell 58 , 641-648.

Ptashne, M. (1988). How eukaryotic transcriptional activators work. Nature 335 , 683-689.

Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A. and, Shooter, E. M. (1987). Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325 , 593-597.

Reinecke, M. (1985). Neurotensin: immunohistochemical localization in central and peripheral nervous system and in endocrine cells and its functional role as neurotransmitter and endocrine hormone. Progr. Histochem. Cytochem. 16 , 1-175.

Roesler, W. J., Vandenbark, G. R. and, Hanson, R. W. (1988). Cyclic AMP and the induction of eukaryotic gene transcription. J. Biol. Chem. 263 , 9063-9066.

Rokaus, A., Fried, G. and, Lundberg, J. (1984). J. Acta Physiol. Scand. 120 , 373-380.

Rowland-Gagne, E. and, Greene, L. A. (1990). Multiple pathways of N-kinase activation in PC12 cells. J. Neurochem. 54 , 424-433.

Sabol, S. L. and, Higuchi, H. (1990). Transcriptional regulation of the neuropeptide Y gene by nerve growth factor: antagonism by glucocorticoids and potentiation by adenosine 3', 5'-monophosphate and phorbol ester. Mol. Endocrinology 4 , 384-392.

Sakai, D. D., Helms, S., Carlstedt-Duke, J., Gustafsson, J-A. and, Rottman, F. M. (1989). Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Genes and Dev. 2 , 1144-1154.

Sassone-Corsi, P., Ransone, L.J., Lamph, W.W. and, Verma, I.M. (1988). Direct interaction between fos and jun nuclear oncoproteins: role of the "leucine zipper" domain. Nature 336 , 692-695.

Sassone-Corsi, P., Ransone, L. J. and, Verma, I. M. (1990). Cross-talk in signal transduction: TPA-inducible factor jun/AP-1 activates cAMP-responsive enhancer elements. Oncogene 5 , 427-431.

Schule, R., Muller, M., Kaltschmidt, C. and, Renkawitz, R. (1988). Many transcription factors interact synergistically with steroid receptors. Science 242 , 1418-1420.

Schule, R., Muller, M., Otsuka-Murakami, H. and, Renkawitz, R. (1988). Cooperativity of the glucocorticoid receptor and the CACCC-box binding factor. Nature 332 , 87-90.

Sheng, M. and Greenberg, M. E. (1990). The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron 4 , 477-485.

Sonnenberg, J. L., Rauscher, F.J. III, Morgan, J. I. and, Curran, T. (1989). Regulation of proenkephalin by Fos and Jun. Science 246 , 1622-1625.

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

Strahle, U., Schmid, W. and, Schutz, G. (1988). Synergistic action of the glucocorticoid receptor with transcription factors. EMBO J. 7 , 3389-3395.

Sunderland, E.W. (1972). Studies on the mechanism of hormone action. Science 177 , 401.

Terenghi, G., Polak, J.M., Varndell, I.M., Lee, Y.C., Wharton, J. and, Bloom, S.R. (1983). Neurotensin-like immunoreactivity in a subpopulation of noradrenaline-containing cells of the cat adrenal gland. Endocrinol. 112 , 226-233.

Theonen, H. and, Barde, Y.-A. (1980). Physiology of nerve growth factor. Physiol. Rev. 60, 1285-1335.

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

Tischler, A.S., Lee, Y.C., Slayton, V.W. and, Bloom, S.R. (1982). Content and release of neurotensin in PC12 pheochromocytoma cultures: modulation by dexamethasone and nerve growth factor. Peptides 3 , 415-421.

Tsukada, T., Fink, J. S., Mandel, G. and, Goodman, R. H. (1987). Identification of a region in the human vasoactive intestinal polypeptide gene responsible for regulation by cyclic AMP. J. Biol. Chem. 262 , 8743-8747.

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

van Buskirk, R., Corcoran, T. and, Wagner, J.A. (1985). Clonal variants of PC12 pheochromocytoma cells with defects in cAMP-dependent protein kinases induce ornithine decarboxylase in response to nerve growth factor but not to adenosine agonists. Mol. Cell. Biol. 5 , 1884-1992.

Walicke, P.A. and, Patterson, P.H. (1981). On the role of cyclic nucleotides in the transmitter choice made by cultured sympathetic neurons. J. Neurosci. 1 , 333-342.

Yamamoto, K. K., Gonzalez, G. A., Biggs, W. H., III and, Montminy, M. R. (1988). Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature 334 , 494-498.

Yamamoto, K. K., Gonzalez, G. A., Menzel, P., Rivier, J. and, Montminy, M. R. (1990). Characterization of a bipartite activator domain in transcription factor CREB. Cell 60 , 611-617.

Yamamoto, K.R. (1985). Steroid receptor regulatoed transcription of specific genes and networks. Annu. Rev. Genet. 19 , 209-252.

APPENDIX

A

ADDITIONAL SEQUENCE DATA

Unpublished sequence information, compiled during the structural analysis of the rat NT/N gene and bovine hypothalamic cDNA, is presented below. The first schematic serves a reference of the fragments subcloned from the rat gene (Figure 1). The sequencing strategies and linear sequence of two subcloned fragments, a 1.8 kb EcoRI/Hind III fragment and a 2.2 kb HindIII fragment, are depicted schematically (Figure 2a, b). The 1.8 kb fragment contains a portion of 5' flanking sequences, Exon I, and sequences up through approximately 500 bp from Exon II. The 2.2 kb fragment contains the better part of Intron II and part of Exon III. Figure 3 depicts that analysis of 1.4 kb of NT/N 5' flanking region. Figure 4 depicts the sequence analysis of the bovine hypothalamic cDNA.

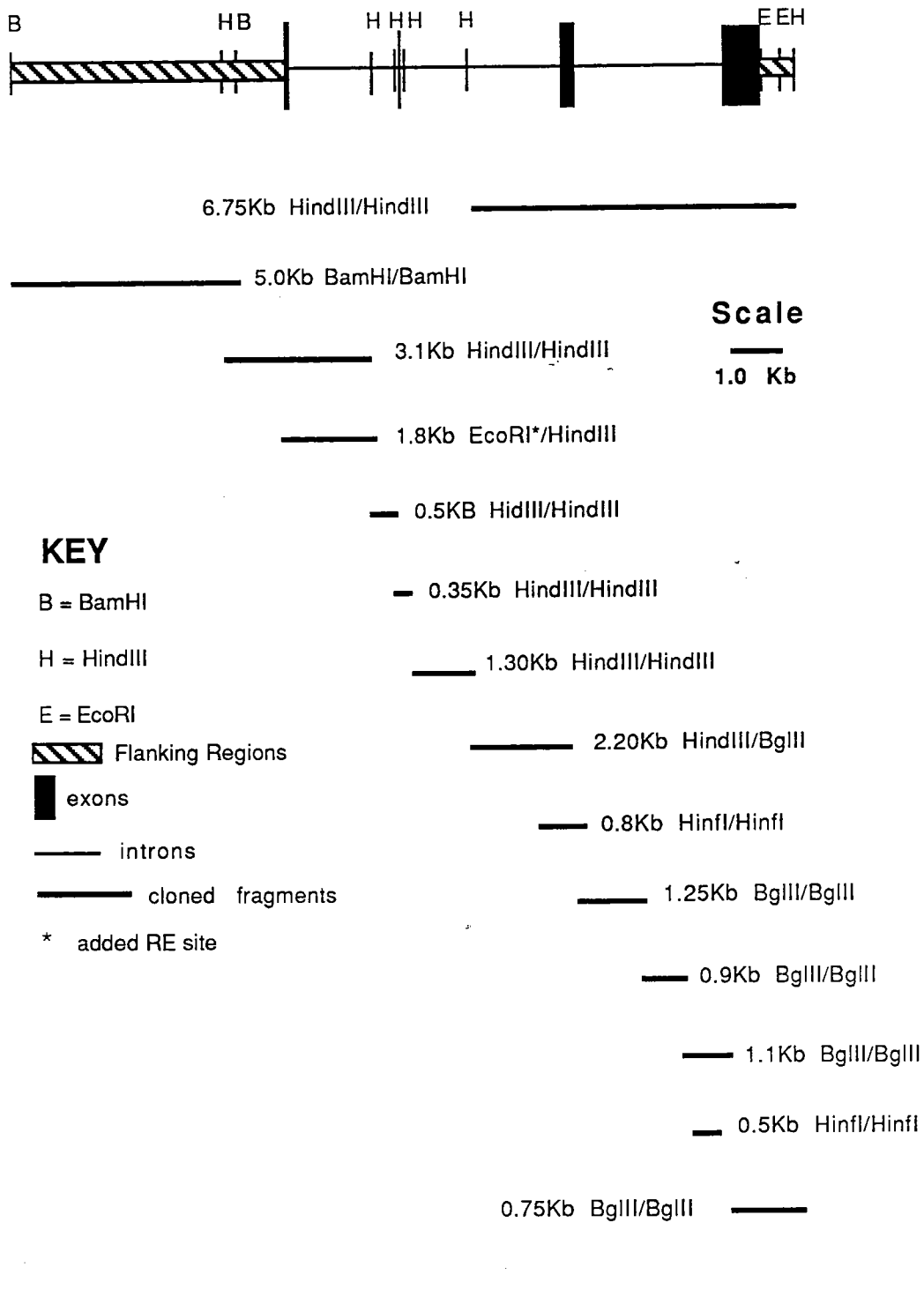
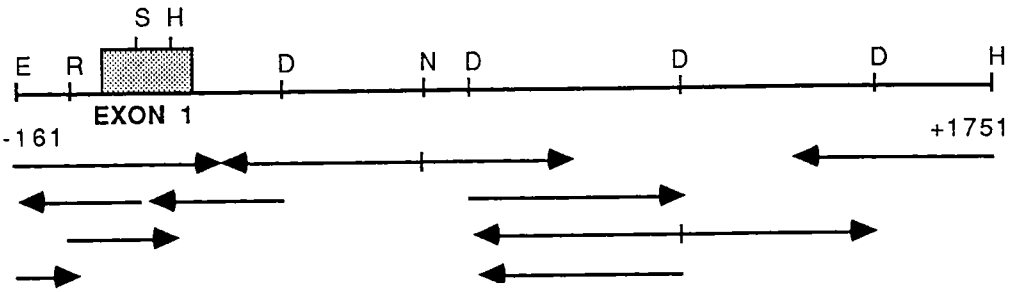


Figure 1: Structure of rat NT/N gene and subcloned fragments

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51    ATGAGGGACA AAGAGAAAGG AGGGGGGATG GAGATGAAGG CAGAGCACAT
101   CCTGTACAGT CATGACATCA CCCTCCTGCT CACGTATATA TAGGGCATGG
151   CCAGAGCATC TCTCACAGTT CACTCACTTT CAAGCCAGCT GAAGGCAAGA
201   GGAAGCACCA AGAGAGCTCC TTCCGTGTCT GTGTGGACCT GCTTGTCAGA

251   AGGCTGAGAG AAGATGATAG GAATGAACCT TCAGCTGGTG TGCCTGACTC
301   TCCTGGCTTT CAGCTCCTGG AGTCTGTGCT CAGGTAAGCT CGCACCTCTC
351   TGAAGTGAGT GATTTCTCTT ACAGTCTGCT GCTACTCAGG TAAATGAATC
401   CCAGGAGGAA TCTCTCTCTC TCTCTCTCTC TCTCTCTCTC TCTCTCTCTC
451   TCTCGTATAA GAACTGAAAC TATTATTTTT TTTTCTTTTA AAAGTCTGTA

501   AAGCAGGCAG TTCATGAGGT TTCAAGGAAA GGCAAACTAT TTTCCTTTGG
551   AATATAACTA CTAGAGGGAA AAATCAATAA GAGTAATAAA ACAAGGATGA
601   AAACTAGAAC AGAGGCAGAG CTTTTCAGCA GGCTCCCATG AAGAGATCTC
651   ATGCATGATT TTTTATTCTC ATCTTTTCTC ACTGCCTTAG CTGGTGGCTT
701   CTCTCACTTA CCTTTGTGAT TTTCCTCAAG TTATTTTCAA CTCTACATGT

751   GTGTTAGAAC ATGTGAAATA AATACCAGTA AATAAAAGAG TGTTTCTAAT
801   GCAACAAAAA GTGTCCCAAG TCCTTTGAGA ATGTATATTT TAAAGAATTT
851   CATTCTGACA GTCGTGATGC TCATATAGCT GCTGCTTTTT TGAATGTAAT
901   CTAAGTTGGC ACAACTATAA CCATAAAATT GGTATACTTC TATTTATGAT
951   AAATTTAGCT TTACAGTGTT CTGTATATAA CAGTTAAGAA GAAGAAATGT

1001  ATTGTTATCA GATGAATTAC GTCTTCTTAA AAATAAAATA AATTTAAGAC
1051  TAATATGAAA TATTTTGATG ATGGCAATGT ATTTTAATTA ATAATTGGAA
1101  ATAATTGAAC AAACACCTAA CAGTGGAAAA TCTTAAAGAC AAAATGAAGT
1151  AAAAATCACT TAACTTATAT TTGTGAATAT TGTTCTTAAT AACCAAGACT
1201  CTGAAGTGTT AGTTCCAAAC TAAGATTTTT TTAAATACAA AGATTAGCTA

1251  TCAGAAGTTT ATCAGACTGT GATATGTTTT GGATGATCTT ATTAGTATAA
1301  GAGCCTGTAA ATATTTTATA AGCCAATGGC ACTTTCCCAT AACTATAATT
1351  TTATCAAGTA GTTTACAGAA ATATAGTTCT AATTATAGCT TATTGATTAA
1401  AAATTGTATA TTAGCTCTCG AAACCCATTT CTGTGACATA AACACATTCT
1451  TAACAGAGAT TAACAAGATT AATTTTTAAC ATTCATTCTG AAAATCAAAC

1501  AGCAAACAAA TGGAGCAAAA GTGTCCCTGG AATAGCTCAG TTCTTACAGG
1551  TACTCAGGCT GGGATCTTGT GTTTTAAAGA CCACATAGTA AATAATGGGG
1601  CAATTTTAAG CTCTGCCTCC TTTATCCACA GAAGGCTAAT GGATATTAAA
1651  TGCTCTACAA TACCTTAGCG TTTCTAAGTT TTTGCTTCTC TAAACAAGAC
1701  ACATAAAAGA AATCTGGGAA AATGTGTTCT GACATTAATC AAGGATCTGT

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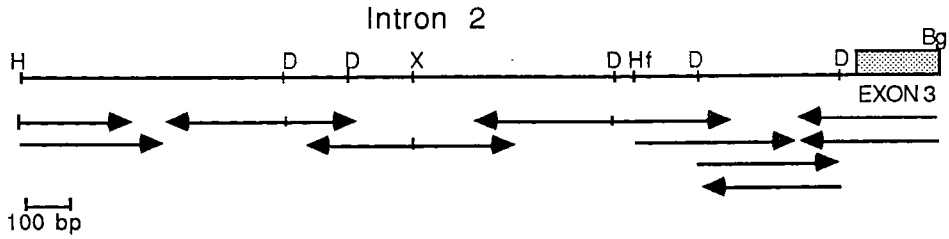
Figure 2a. NT/N gene sequences including exon 1 and a portion of intron 1.

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1     TTTATTTAGT ATTCGATCCC GGTAACAGTG ACTGCAGTCT TTTCGTACTC
51    CAAGGTAGGA AGATGGGTGT AACCCATTAA TACTATCTTA GCTTAGCTAT
101   GAAGCCCTTG GTGTCTTACA ATAGGTTTTG TTTTTGAAAT TGTAAATGTA
151   CATGTTGACA CATACATGCT CTAAATAATC CTGAAAACAT GACAAGTTTT
201   GCAAGGTCTA CCATTCTTAA ATATGGGAGA ATATGGGTTC CCTCTCTGAC

251   AGAACAATTT TGTGCCATGG TGTGATTCAA TCAGTCTAAA ACAATCCAAG
301   AGGGAAGTAC TTGCATTGCA CATTGACTCA TTTCAACTTA GTTACTAAGA
351   CATTTCAACT TAGTTACCTA AGACACTTCT TCAACAAGAA CATATGAGGT
401   AGAAAACCAC TGCAATTTGC TGCTAGGATA AAAATAAAAA GTGCATGTTT
451   TTCTATAAAA TTGTTTCTGT TATTCAACCA TAATTTTTGT AATGTTTATA

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1101  ACTTTCAGTG TTCAAATGTG TCTAGGAAGC ACATCATATT CTTCTTTTCC
1151  ATTCTCTTCT CTCCTCTTCT CCACTCTCTT CTCTTCATCT CTGCTCTTCT
1201  TTTCCTACTA ATCTACATGT ACATGCCATT GAGTATTTGC ATGTAAAGTC

1251  TCAGGATATT TAATTAAAAT TTTAAACTTC CTTCCCTACT TTTTGTAAGT
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1351  CAAACAGAGC CCAGACTCAG CAAGTTGCAT TTACCTGCGT TTGTGCACAC
1401  ATGCACACAC ATACGTGTAT GCTCCCAACA ATAATAAACA AACGAAAAGA
1451  AGGCCATTGA ACCTAAGAGT GTGGGAAGAC TGGAAGGGCT TTGAAGGAGG

1501  GAACCTCCGT GAGAATGGAG GAAGAGGAGC GAGGAAGTTG ACGTTAGTTT
1551  CCATTTCAGT TTAAAGGAAC TACGTCACTT TCCTCGCTTC CTCTTCCTCC
1601  ATTCTCACGG AGGTTTCCCT CCTTCAAAGC CCTTCCAAGT TTTCCCACAC
1651  TCTTAGGTTC ATTGGACCTC TTTTCGTTTT GTTTGTTTTA TTATTGTTGG
1701  GAGCATTACA CGTTATGTGT GTGCATGTGT GCACAACGCA GGTAAATGCA

1751  ACTTGCTTGA GTTCTGGCTC TGTTTGTGGT GTTTATAAAG TTATATATAC
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1851  TGGAAAATGA CCTTGCTCAA CGTTTGCAGC CTGATCAATA ACCTGAACAG
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2001  GTATTCCAAC TCCAGAA
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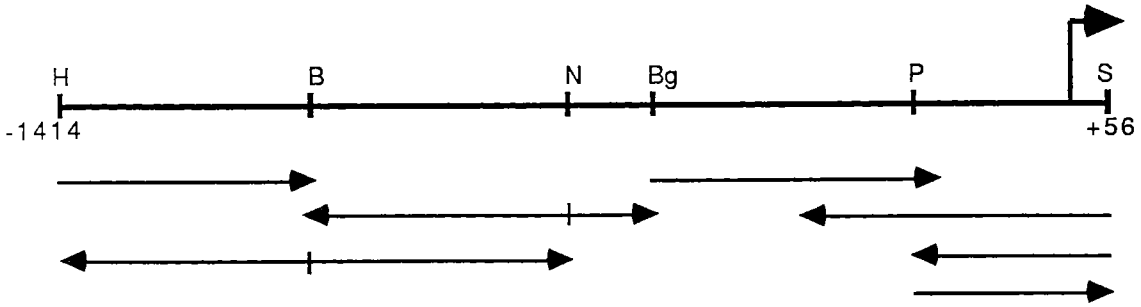
Figure 2b. NT/N gene sequences including intron 2 and part of exon 3.

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-900  TGTCTAAAGT ACAGTCACTT TTCTACAAAT AAAAAGGATT AAACTGAAGG
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-800  TCGGACAACA AATAAACAGT TAAAAATGTA GTAAAAATGT GATTAATTAT

-750  GAAATTAATT GGGGAGGTAT AATTAATACA GCATTCATAG TTACCTTTGA
-700  TGCATAACCT ATTTGTGCCT CTATTTTATA AATATTATTT AAAACACCCA
-650  TGAAAGACAG CATATGAATT TAGACTCCCC TCAGCTACAT ACATAAACTT
-600  TCAAAGAAGT AGGACATAAT ACTATTCATT AGATCTTCAA TTTCCTTCCT
-550  TTTATCTGAT ATTTTTCCTT TACCTTGGAT GTTCTGTTAC CCTATTCCAT

-500  TATTATATTC ATTTTCTTTT GTCTGTTCCC CTTCCTATTT TTAGTACATG
-450  CTATAAAAAA TTCTTTATGA GTTTTGAGAC CAGAAACACG TGCTCAAGAA
-400  GTTTGTGTTA CCAGAAAATA TACCCCAAGT CTTTGAGTCT TCCAAGCAGT
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-300  CATTTTTAAA ATCAAATGAC AAATGTCTGG AGCACGATAG GGATTAGAAA

-250  TAAAAGGTAC CGGGTGCCTC TTCCTAGCTG TACACTGCAG TGCTTTTGAA
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-150  GATCGTCACT TTCAATCAAG GTTCATGGGT GGGGGATGAG ATGAGGGACA
-100  AAGAGAAAGG AGGGGGGATG GAGATGAAGG CAGAGCACAT CCTGTACAGT
-50   CATGACATCA CCCTGCTCAC GTATATATAG GGCATGGCCA GAGCATCTCT

+1   CACAGTTCAC TCACTTTCAA AGCCAGCTGA AGGCAAGAGG AAGCACCAAG
+51  AGAGCTC
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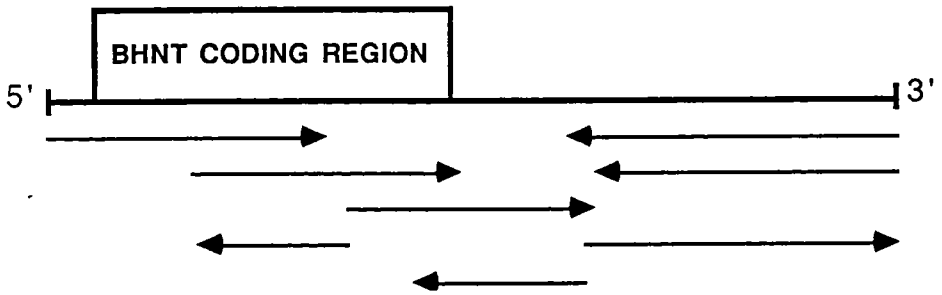
Figure 3. The NT/N gene 5' flanking region.

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151   GAAAGCGTTA GAAACAGATT TACTGACCAA TATGCATACA TCAAAGATCA
201   GTAAGGCAAG TGTTCCCTCT TGGAAAATGA GCCTGCTGAA TGTCTGCAGT
251   CTTATAAATA ACCTGAACAG CCAAGCTGAG GAAACAGGAG AGTTTCATGA

301   GGAGGAGCTC ATTACAAGAA GGAAATTTCC TGCTGCCTTA GATGGTTTTA
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401   AGGGCCTTTC AACACTGGGA GTTGATTCAG GAAGACATTC TTGATGCTGG
451   AAATGACAAA AATGAAAAGG AAGAAGTGAT AAAGAGAAAA ATTCCTTACA

501   TTCTGAAACG GCAGCTTTAT GAGAATAAAC CCAGAAGACC CTACATACTC
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751   AGATCTTGAG CATGAAATGT TGTGTATAAT TGGAGTAGAT ATTAATTAAG
801   TCACATAGAA TGTTTTGTCA TTTTGCAAAG CACTTAATGG GTTGTTCAAG
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1001  AAGACTTACT CATTAATCTC AGAAATGGGG AAGGCAATAG AAGTAAAATA
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1101  TAATGAATGA TCTTAGACTG AATTTGTTTT TCTGTTAAAT GAATTTAATA
1151  GCTAATAAAA ACACAA
```

Figure 4. Bovine hypothalamic NT/N cDNA

APPENDIX

B

TRANSIENT TRANSFECTION ASSAYS

PC12 cells have been successfully tranfected in several laboratories by a number of methods including protoplast fusion, calcium phosphate co-precipitation, and electroporation. The major advantages of the electroporation technique over these alternatives are simplicity, reproducibility, and higher efficiency [Chu et al., 1987]. Technically, electroporation involves the exposure of a cell suspension to a high-voltage, high current, exponential pulsed electric field which, presumably, causes temporary pores to form in the plasma membrane and allows DNA to enter the cell. A single exponential pulse of controlled characteristics can be delivered using the Bio-Rad Gene Pulser™ (BIO-RAD Laboratories) transfection apparatus. In this thesis, all transfection experiments involved electroporation. The following parameters were optimized to achieve maximal transfection efficiency, as measuered by reporter activity.

Electroporation voltage/capacitance. The three parameters which have major impact on transfection efficiency by electroporation are voltage, capacitance, and buffer system. As a rule, voltage-dependence for maximal efficiency changes with buffer and capacitance. In all experiments reported in this thesis, cells were electroporated in1.0 ml 80% phosphate buffered saline (Delbecco's PBS)/20 % 10 mM Tris-HCl(pH 7.6),1 mM EDTA solution containing supercoiled plasmid DNA isolated by cesium chloride gradient centrifugation.

To establish the optimal voltage in kilovolts (kV) and capacitance in microfarads (μFD) required for maximal transfection efficency (survival and expression of CAT activity) in PC12 cells, the positive control plasmid pSV2CAT was used. The

SV40 early transcription region drives the expression of chloramphenicol acetyltransferase (CAT), in SV2CAT [Gorman et al., 1982]. Capacitance affects transfection efficiency by determining the amount and duration of the current pulse passing through the cell suspension. Voltages between 0.1-0.35 kV were surveyed at the maximum capacitance of 960 μFD (Fig. 1a). The data indicate that the 0.2kV/960μFD condition yields optimal expression of CAT and survival by electroporation. Next, the optimal capacitance was determined at 0.2kV and 2.0kV (Fig 1b). The optimal survival and CAT expression occurs at 0.2kV and 960μFD. Therefore, the maximal tranfection efficiency appears to occur at 0.2 kV and 960 μFD with approximately 25% survival. The calculated field strength is 0.5 kV/cm.

CAT plasmid titration. In order not to limit cellular components involved in the expression of CAT activity in transfected cells, transfection efficiency should increase linearly as a function of DNA concentration. Because preliminary experiments had indicated that the NT/N-CAT 5' deletion plasmids with endpoints between -1414 and -216 were equally inducible in response to a combination of the four inducers (dexamethasone, forskolin, NGF, and Li), plasmid -216 was chosen to demonstrate the linearity of transfection by electroporation (Figure 2). The results indicate that transfection efficiency is linear through 50 μg.

CAT kinetics. To assure that CAT enzyme activity is linear throughout the assay, a time course was performed as in Figure 2 using 5' deletion plasmid -1414 (Fig. 3). The data indicate that CAT enzyme activity is linear through 90

min and that acetylation of the label remains linear through at least 60% conversion.

Luciferase plasmid titration. To control for variations in transfection efficiency, a luciferase plasmid was co-transfected with the test CAT plasmid. The plasmid PS-LUC was driven by the NT/N gene cis-regulatory sequences from -216 to +56. To determine the minimal concentration of this luciferase plasmid to use as a normalizing standard, increasing concentrations of PS-LUC were co-transfected with the promoterless CAT vector, pUCAT (Fig. 4). The inductions and analysis were as in Figure 3. Luciferase activity was linear through at least 10 μg. To minimize potential competition for inducible transcription factors between the CAT and luciferase plasmids, a 10:1 (CAT:LUC)DNA ratio was chosen for all subsequent experiments.

Recovery Kinetics. Preliminary experiments had suggested that recovery post-transfection by electroporation was critical for maximal response to a combination of all four inducers. To establish the optimal recovery period post-transfection for maximal responses, PC12 cells were co-transfected with the CAT and luciferase plasmids bearing NT/N seqences from -216 through +56, and allowed to recover for 12, 24, 36, and 48 hrs, prior to a 12 hr induction with all four inducers (Fig. 5). The results suggest that the optimal recovery period is 48 hrs following electroporation for response to all four inducers. However, it is not known if activity continues to increase beyond a 48 hr recovery.

Induction Kinetics. The optimal time to analyze the response to a combination of all four inducers (Fig. 6) was established in PC12 cells as in Figure 5. Induction of both CAT and luciferase activity appear to peak at or near 8 hrs. Differences in the stability of reporter gene transcripts or their products may explain the plateau in CAT activity and the rapid decay in luciferase activity. A 12 hr induction time was selected since CAT activity is optimal and since luciferase activity serves as an internal standard.

Current transfection protocol. The following protocol results in the efficient transfection and optimal expression in response of the NT/N control region fused to reporter plamids in PC12 and GH3 cells.

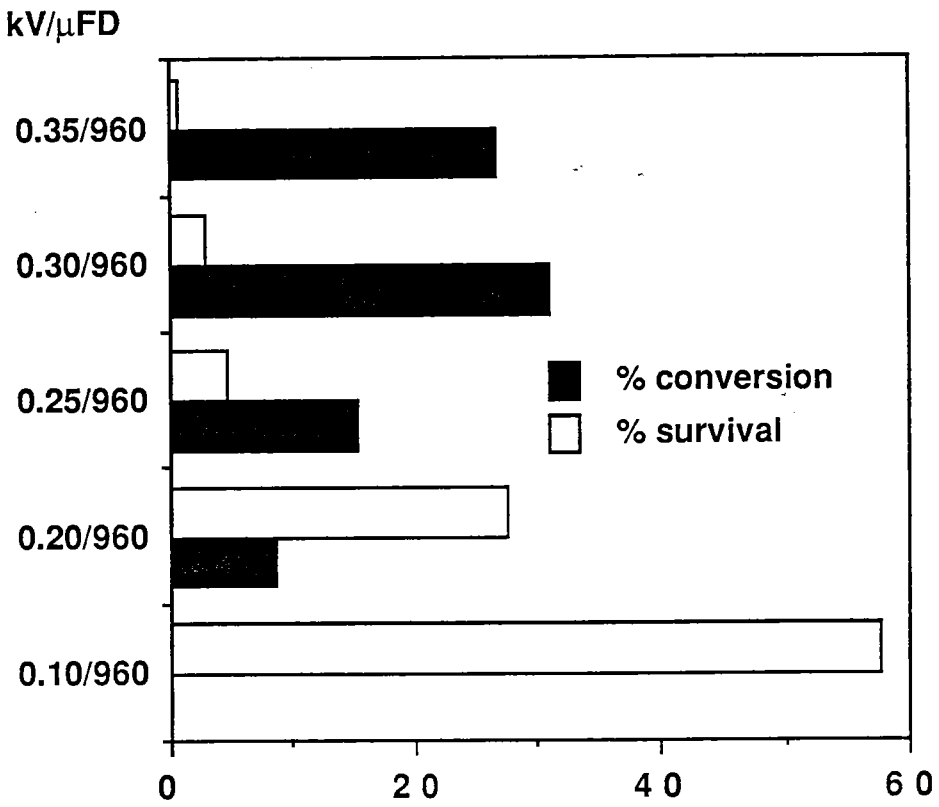
Cells are harvested at half confluence in ice cold phosphate buffered saline (Dulbecco's PBS). Twenty million cells in 800 µl are combined with 200 µl of 10 mM Tris-HCl (pH 7.6), 1 mM EDTA containing 25 µg of CAT plasmid with 2.5 µg of luciferase control plasmid for 10 min on ice, followed by a single pulse from a 960 µFD capacitor charged to 200 volts in a 0.5 cm cuvette (BioRad). After a 10 min incubation on ice, the transfected cells are resuspended in ice-cold PBS and pelleted to remove debris and DNA precipitant at 1000 rpm, 5 min at 4 °C. For analysis of more than six conditions, paired transfections are pooled and plated onto six-well plastic culture dishes (5-6 conditions/transfection cuvette). The cultures are allowed to recover for 48 hrs prior to induction in 2-3 mls of fresh medium. Following a 12 hr induction, the cells are triturated from the plate in ice-cold PBS and pelleted in an Eppendorf tube at 4 °C, for 10 sec. The pellet is rinsed with 1.5 ml of PBS, spun again, and resuspended in 100 µl of 100 mM potassium phosphate (pH 7.8), 1mM DTT. The cells are lysed with three

freeze/thaw cycles (liquid nitrogen/37 °C water bath). Cell extracts were analyzed for protein content, then CAT and luciferase activity as described above (see CHAPTER III, methods). Wagner PC12 and A126-1B2 cells were transfected identically; except, the concetration of CRE, AP-1, and luciferase plasmids was double. The transfection protocol used for the RAT2 cells was essentially the same with the following modifications. 10^7 cells were transfected in the identical solution with 25 μg of CAT plasmid and 5 or 10 μg of luciferase control plasmid. Electroporation of DNA was performed using a single pulse from a 960 μFD capacitor charged to 300 volts.

Cell culture. PC12 cells were grown in 100 mm tissue culture dishes (Corning) in Dulbecco's minimal essential medium (DMEM) supplemented with 10 % horse serum, 5 % fetal bovine serum, 50 μg/ml streptomycin, and 50 U/ml penicillin (all from GIBCO). GH3 cells were also grown as described [Lufkin and Bancroft, 1987] in the above media. Wagner PC12 and A126-1B2 cells were grown in DMEM supplemented with 10% fetal bovine serum, 5% horse serum, 50μg/ml streptomycin, and 50 U/ml penicillin. RAT2 cells were grown in DMEM supplemented with 5% newborn calf serum (Hazelton, Lenexa, KS). GH3 and RAT2 cells were cultured on poly-L-lysine (SIGMA) coated plates. Routinely, cells were split 1:3 every two to three days in fresh complete medium. The concentrations of inducers used were nerve growth factor (100 ng/ml), dexamethasone (1 μM), forskolin (1 μM), lithium chloride (20 mM), and phorbol 12-myristate 13-acetate (60 ng/ml), unless otherwise stated. NGF was either prepared as described [Mobley et al., 1976] or purchased from Bioproducts for Science, Inc. (Indianapolis, IN). Dexamethasone was a gift from

Merck, Sharpe, and Dohme. LiCl was from Fluka. Forskolin and phorbol 12-myristate 13-acetate (PMA) were from Sigma. A concentration of 10 μM forskolin was used in experiments involving the CRE and AP-1 plasmids. All cells, excluding the GH3 cells, were incubated at 37 °C and 8% CO2. The GH3 cells were incubated in 5% CO2. All cell cultures were split to approximately 50% confluence ($<10^7$/100 mm plate) in fresh media 24 hrs prior to transfection.

Figure 1. Electroporation efficiency as a function of voltage and capacitance in PC12 cells. 4 x 106 PC12 cells were transfected with 25 µg of pSV2cat by electroporation and analyzed following 64 hrs of transient expression. Cells were harvested in 0.25M Tris-HCl, resuspended at 10^7/ml, and disrupted by sonication. Percent survival was determined following transfection relative to control (untransfected) cells, prior to sonication. Cell extract from approximately 5.5 x 105 cells (55 µl) were analyzed in a 90 min CAT assay. a. Electroporation as a function of voltage at 960µFD capacitance. b. Electroporation as a function of capacitance at 0.2 kV and 2.0 kV.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0.30/960
0.25/960
0.20/960
0.10/960
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% survival
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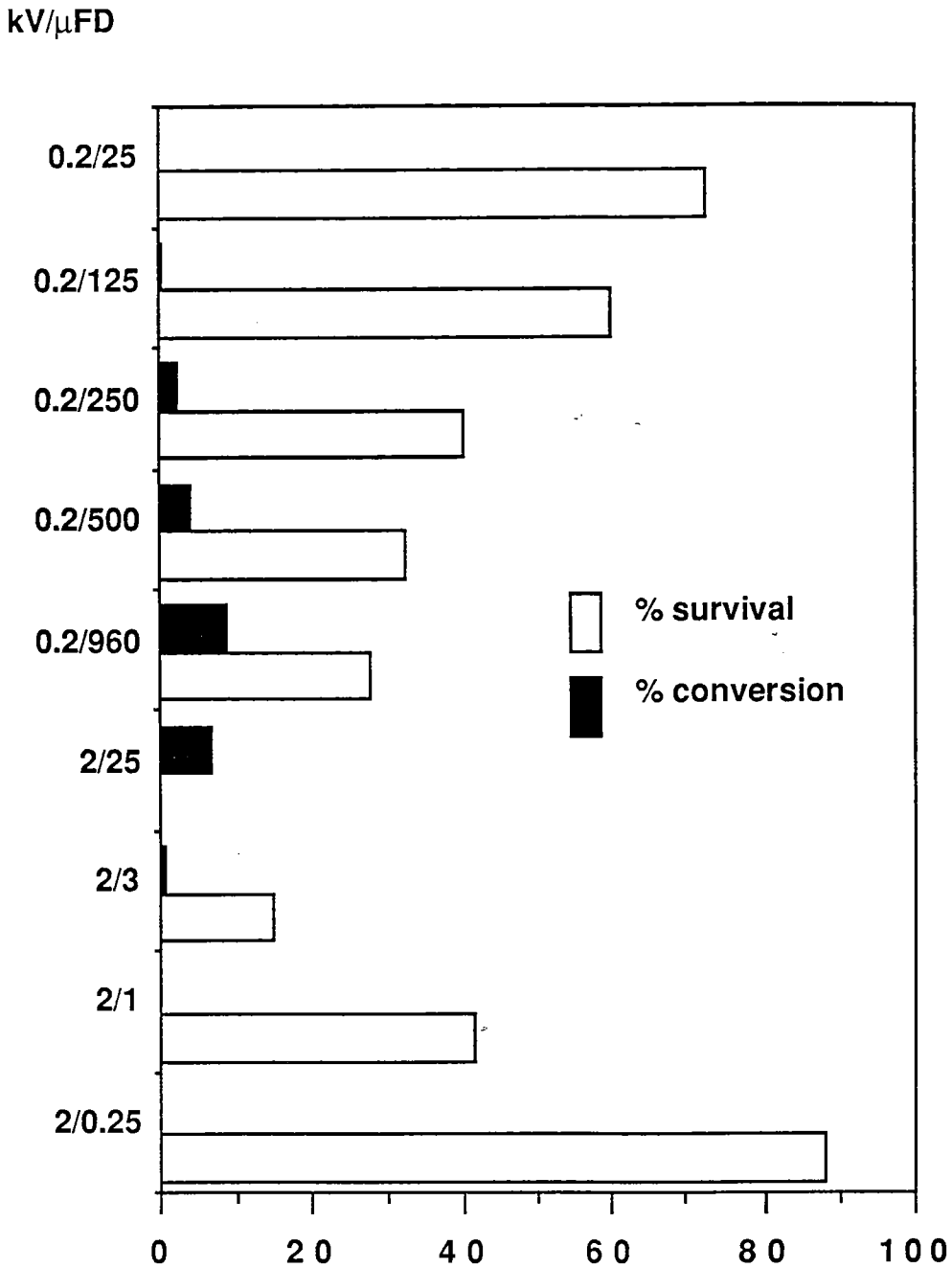
40
60


kV/μFD
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0.2/500
0.2/960
2/25
2/3
2/1
2/0.25
% survival
% conversion
0
20
40
60
80
100

Figure 2. Electroporation as a function of DNA concentration. PC12 cells were electroporated with a single pulse generated from a 960 μFD capacitor charged to 200 volts applied to 107 CAT activity was determined following transfection of between 1-50 μg of a NT/N-CAT fusion plasmid (-216). Twelve hours post-transfection, PC12 cells were induced for 48 hrs with either all four inducers (100 ng/ml NGF, 1 μM dexamethasone, 1 μM forskolin, and 20 mM LiCl) or no inducers in fresh media, and then harvested as in Fig 1. CAT assay were performed with 20 μg of cell extract. Duplicate transfections were averaged and plotted.

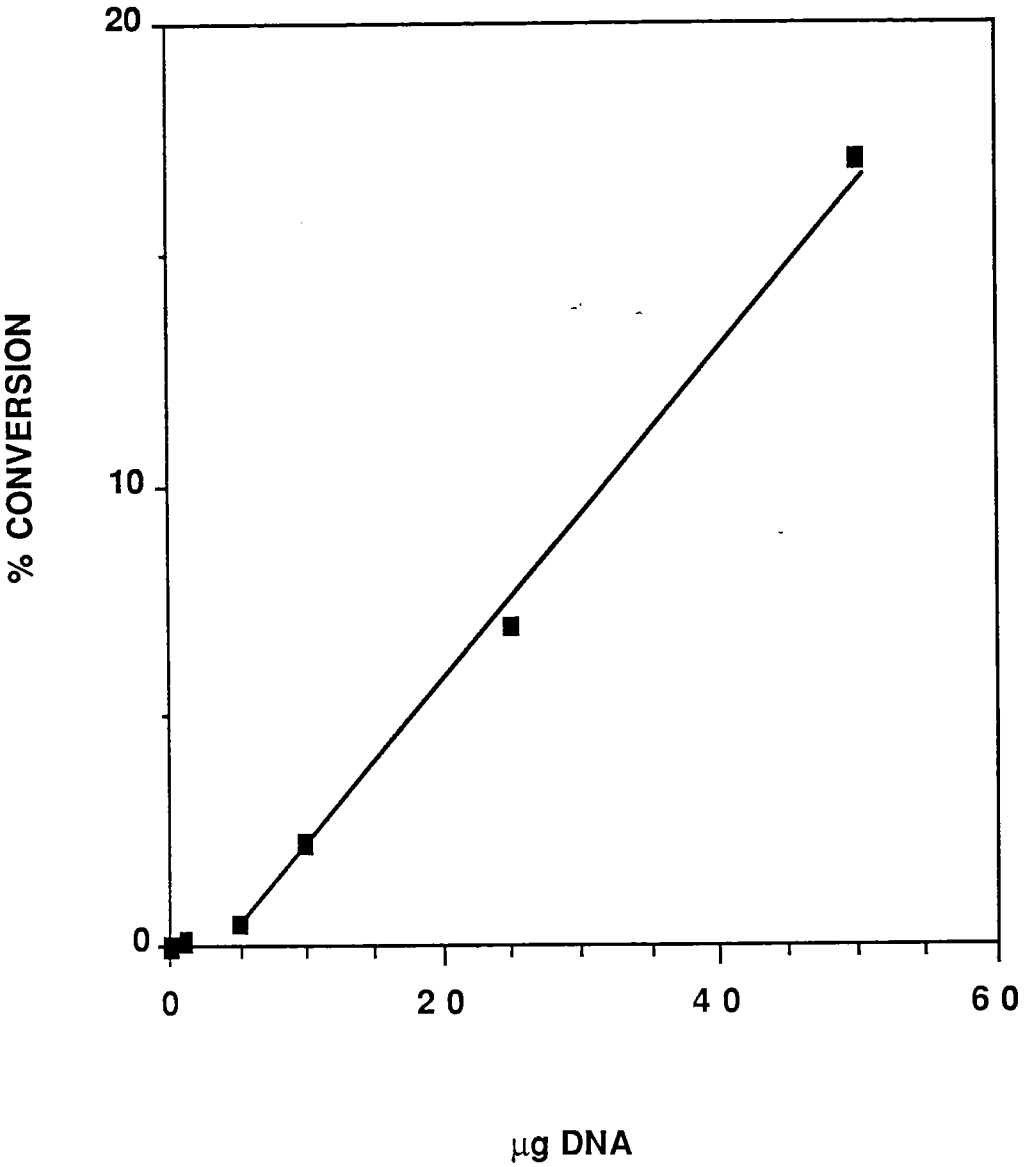

20
10
0
% CONVERSION
0
20
40
60
µg DNA

Figure 3. CAT activity as a function of time. PC12 cells transfected with a NT/N-CAT fusion plasmid containing 1.4 kb of NT/N gene 5' flanking sequences (-1414) and induced with either all four inducers or no inducers for 48 hrs (see Fig 2). CAT activity within 10 μg of cell extract was used at each time point indicated.

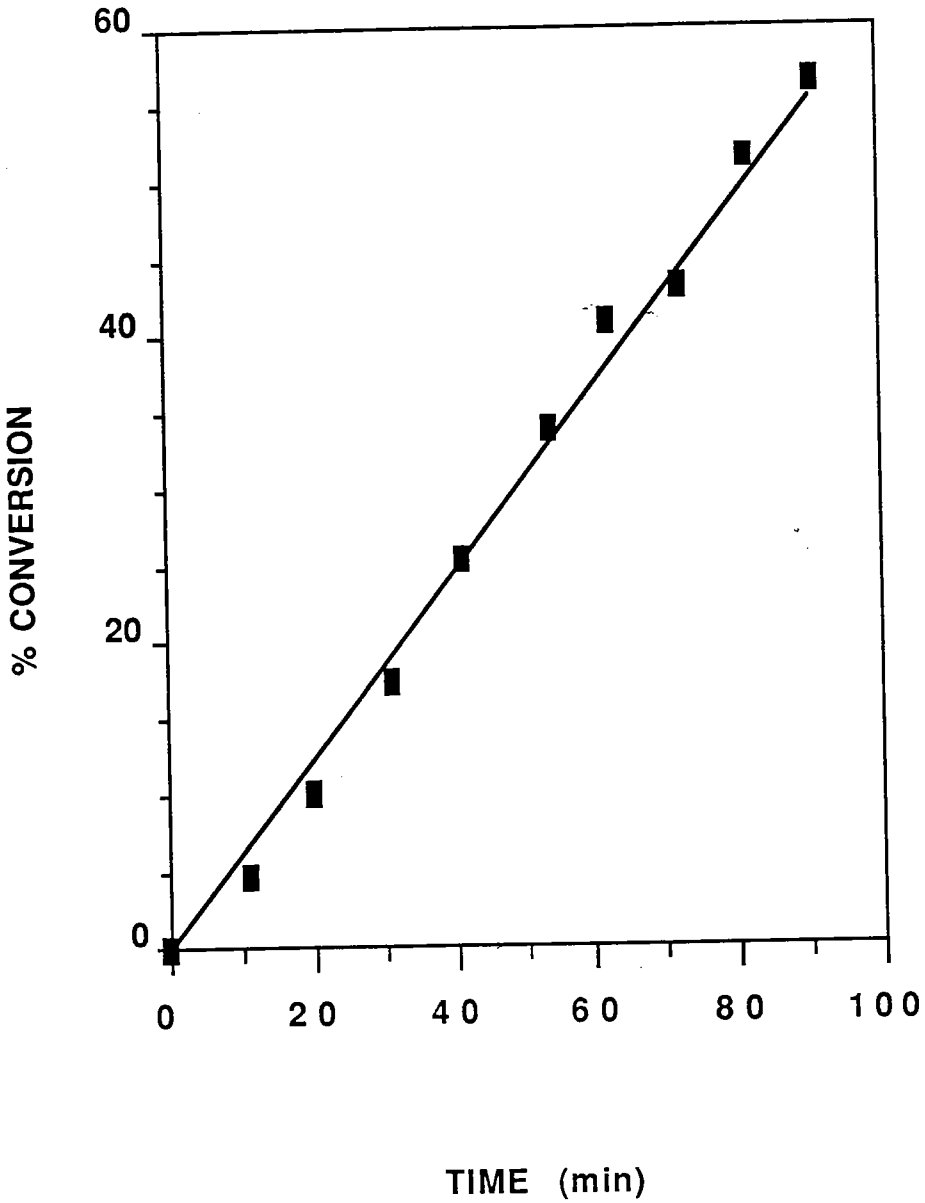
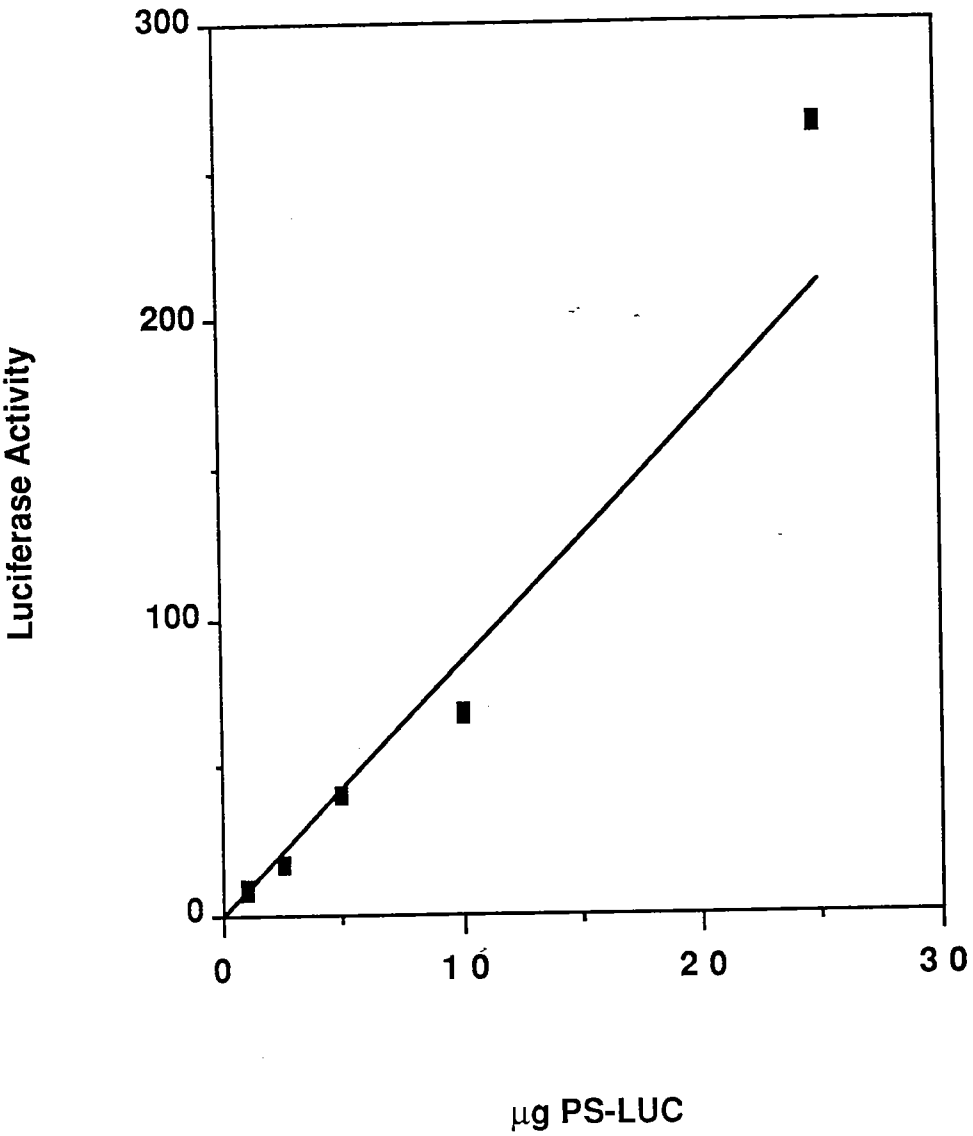

60
40
20
0
% CONVERSION
0
20
40
60
80
100
TIME (min)

Figure 4. Luciferase activity as a function of DNA concentration. PC12 cells were co-transfected with 25µg of CAT plasmid -216 and increasing amounts of PS-LUC into $20x10^7$ cells as indicated. Transfected cells were induced and analyzed (see Fig. 2). Luciferase and CAT activity was determined with samples of 20 µg of cell protein and normalized to the CAT activity.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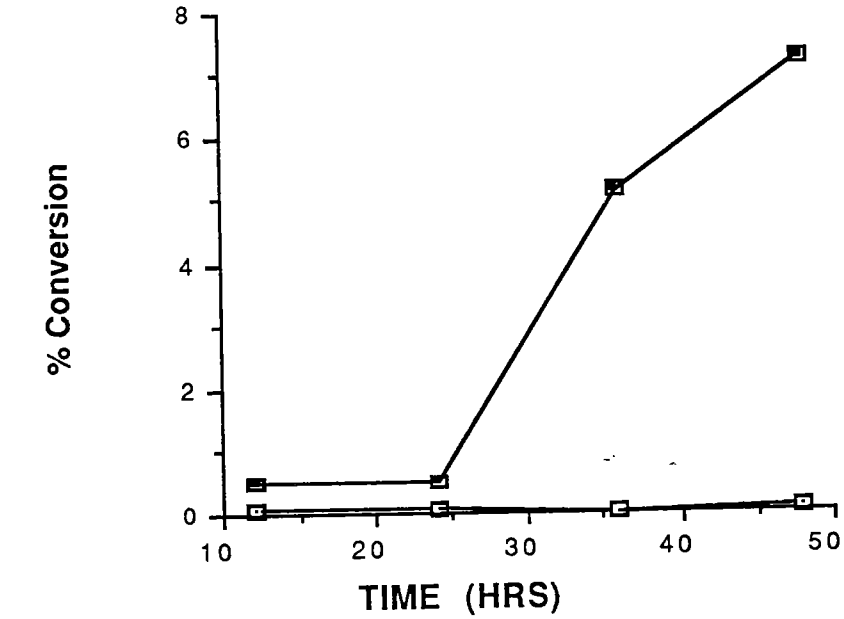
200
100
0
Luciferase Activity
0
10
20
30
µg PS-LUC

Figure 5. Induction of CAT and luciferase activity as a function of recovery time post-transfection. CAT activity in % conversion (upper panel) and luciferase activity in light units (lower panel) were determined from 20 µg of cell extract taken from triplicate inductions of PC12 cells co-transfected with 25 µg of NT/N-CAT plasmid -216 and 2.5 µg of PS-luc. PC12 cells treated with a combination of all four inducers (closed boxes), as in Fig 2, or no inducers PC12 cells (open boxes) were collected and analyzed 12, 24, 36, and 48 hrs. post-transfection. Background subtracted and averaged activity is plotted with standard error bars.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4
2
0
10
20
30
40
50
TIME (HRS)

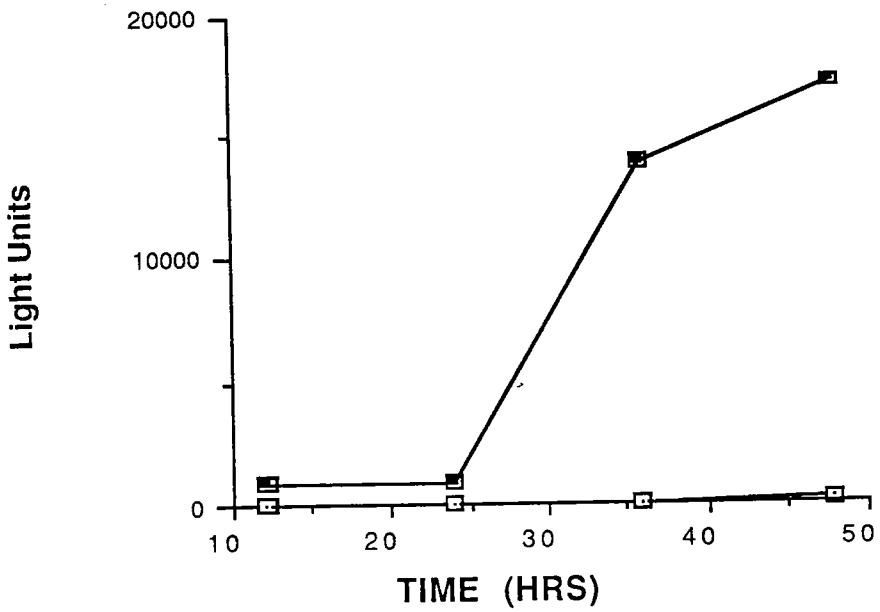
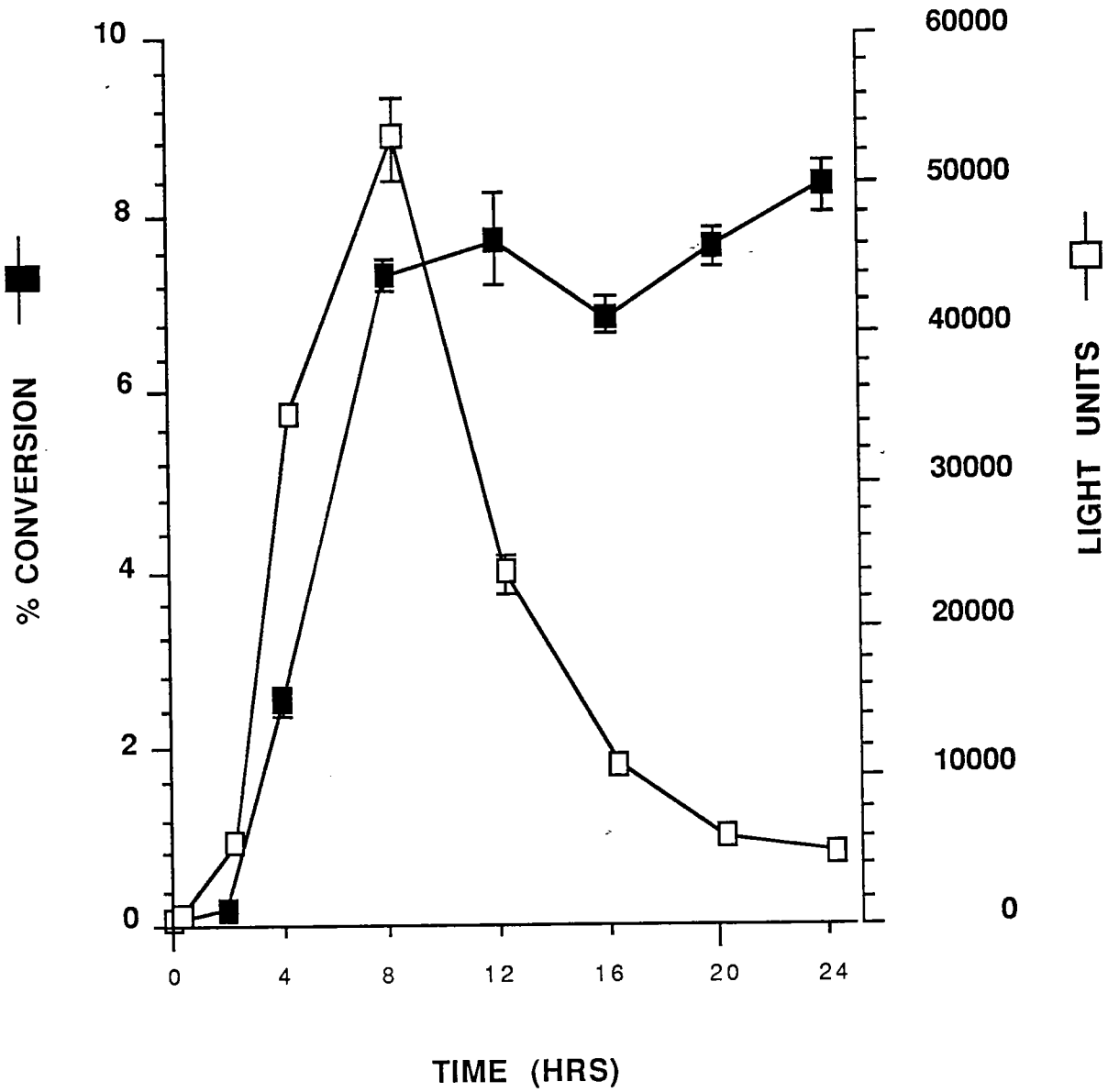

Light Units
20000
10000
0
10
20
30
40
50
TIME (HRS)

Figure 6. Induction kinetics of CAT and luciferase activity. PC12 cells were co-transfected with the -216 plasmid and PS-luc (see in Fig. 5). Forty-eight hours post-transfection fresh media including either all four inducers or no inducers was added. Following a 1, 2, 4, 8, 12 , 16, 20, and 24 hour incubation, CAT (closed boxes) and luciferase activity (open boxes) were determined. No change in uninduced CAT or luciferase activity occurred (data not shown).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