Transcriptional Regulation of the Human Neuropeptide Y Gene by Nerve Growth Factor*

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(Received for publication, March 7, 1994)

Neuropeptide Y (NPY) is the most ubiquitously expressed peptide in the mammalian nervous system. Transcription of the NPY gene in PC12 cells is regulated by a number of agents, including the neurotrophic peptide nerve growth factor (NGF). In this paper, we define the cis-acting promoter elements which respond to NGF and characterize the trans-acting factors which interact with these sequences. The NGF-responsive elements of the NPY gene lie between nucleotides -87 and -36. At least four proteins interact with this promoter region. One of these proteins interacts with a Ct-rich sequence centered at position -51, which closely abuts a binding site for transcription factor AP-2 centered at position -63. Two newly characterized factors bind between positions -87 and -58. These proteins are expressed in a tissue-specific manner and, together with the other binding activities, modulate the transcriptional activity of the NPY gene. These results suggest that the concerted interplay of these proteins, in response to NGF, increases the transcriptional activity of the NPY gene.

Nerve growth factor (NGF)* is the best characterized member of a family of neurotrophins. These molecules promote the growth and differentiation of distinct neuronal populations. NGF is required for the survival of sympathetic neurons of the peripheral nervous system and cholinergic neurons in the central nervous system (1). The actions of NGF have been extensively studied in rat pheochromocytoma cells (PC12), a population of cells derived from neoplastic adrenal chromaffin cells which display a bipotent developmental potential (2). These cells closely resemble their non-tumor counterparts and, when treated with glucocorticoids, continue to develop into adrenal chromaffin-like cells. However, a divergent developmental fate occurs upon exposure to NGF. Over a period of a few days, the NGF-treated cells stop dividing and mature into cells that exhibit characteristics reminiscent of sympathetic neurons, including neurite outgrowth and electrical excitability (3).

Part of the biochemical basis underlying the differentiation into these neuronal-like cells has been elucidated and shown to involve a cascade of events initiated by NGF. Characterization of the NGF receptor has shown that it is composed of two disparate proteins, one of which is the product of the trk protooncogene (4, 5). When NGF binds to its receptor, trk’s intrinsic tyrosine kinase activity leads to the activation of several signal transducing proteins. These proteins ultimately stimulate the transcription of immediate early genes including c-fos, c-myc, NGF1-A, NGF1-B, and NGF1-C (6-9). These proteins, in turn, function as transcription factors influencing the expression of genes expressed during the intermediate (2-6 h) and late (>6 h) phases of the NGF cascade.

Neuropeptide Y is one of the genes whose transcription is increased in the intermediate wave of NGF-stimulated gene expression (10). This peptide hormone/neurotransmitter is expressed in a wide variety of neuronal tissues, including sympathetic neurons and cells of the adrenal medulla (11). PC12 cells produce very low levels of NPY mRNA prior to treatment with NGF. Previous studies have shown that treatment of these cells with NGF causes a maximal 60-fold increase in the steady state levels of NPY mRNA within 24 h. Increased transcriptional activity accounts for only 2-7-fold of this increase, occurring within 6-8 h of treatment (12). The remainder of this response is presumably due to increased mRNA stability. Dual control of NPY mRNA levels is also manifested by LA-N-5 and LA-N-1 neuroblastoma cells upon treatment with 12-O-tetradecanoylphorbol-12-acetate and forskolin. Increasing the stability of the mRNA allows for rapid increases in NPY production. This may be particularly important under conditions of intense neuronal stimulation. However, since many different stimuli can impinge upon a single neuron, it is likely that different signaling pathways may selectively regulate transcription while others regulate mRNA stability.

NPYs predominantly neuronal expression pattern and NGF responsiveness make it a good candidate for elucidating the biochemical mechanisms responsible for the development of a neuronal phenotype. We have undertaken a study to determine the mechanisms responsible for the NGF elicited increase in the transcription of the NPY gene in PC12 cells. We report the characterization of cis-acting sequences and their corresponding trans-acting factors which participate in this developmental response. These results are a first step toward a better understanding of how differential gene expression occurs during neuronal development.

MATERIALS AND METHODS

Construction of Recombinant Plasmids—NPY/CAT chimeras were made by fusing varying amounts of the NPY gene 5′-flanking sequence to the coding sequences for chloramphenical acetyltransferase as described previously (13). The number following the Δ indicates the end of the 5′-flanking sequence with respect to the transcriptional start start (designated +1) which was determined by in vitro transcription analyses and primer extension analyses (13, 14). In addition to the specified amounts of 5′-flanking sequence, these constructs contained the start of transcription and 51 base pairs of 5′-untranslated leader sequence.

Plasmids for analysis of individual NPY gene elements were constructed with the minimal Herpes simplex virus thymidine kinase (TK) promoter (-105 to -51) directing expression of the CAT gene (TRCat, Ref. 15). NPY elements (-87/-47) were created by annealing comple-

* This work was supported by National Institutes of Health NINDS Grant NS234496. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The abbreviations used are: NGF, nerve growth factor; CAT, chloramphenical acetyltransferase; TK, thymidine kinase; NPY, neuropeptide Y; DEMEM, Dulbecco’s modified Eagle’s medium; TRH, thyrotropin-releasing hormone; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding; NGF-RE, NGF-responsive element.

‡ C. Minth-Worby, manuscript in preparation.
The blackened bars represent the relative CAT activity obtained from transient co-transfections of PC12 cells with the deletions ranging from -796 to -30 and the internal control, pRSVLacZ. The hatched bars represent identically transfected plates except that the cells were transiently co-transfected with deletions ranging from -796 to -30 and the internal control, pRSVLacZ. The hatched bars represent data from untransfected cells.

Data represent an average of 8-11 transfections for each construct. The error bars represent the standard deviation from the mean.

Nuclear Extract Preparation and Gel Retardation Analysis—PC12 nuclear extracts were prepared as described by Dignam et al. (19). Protein concentrations were determined using the BCA protein assay kit (Pierce Chemical Co.).

DNA-protein binding reactions contained 15,000 cpm (~0.3 ng) of γ-32P-labeled oligonucleotide, 1 μg of poly(dI-dC) (Pharmacia LKB Technology Inc.) and 5–15 μg of PC12 nuclear extract in 1 x Hepes binding buffer (1 x = 10 mM Hepes, pH 7.9, 50 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 5 mM MgCl2, 10% glycerol). Binding reactions (20 μl) were incubated for 30 min at room temperature, the DNA-protein complexes were then resolved on 4% polyacrylamide gels (40:0.5, acrylamide:bisacrylamide, 50:1). Methylation interference footprinting was performed using the T4 polynucleotide kinase. After electrophoresis in nondenaturing 10% polyacrylamide gels (acrylamide:bisacrylamide, 50:1) and exposure to autoradiographic film, the gel was scanned using a phosphorimager.

Methylation Interference Footprinting—Methylation interference footprinting was performed with a double-stranded oligonucleotide spanning the -87 to -58 region of the human NPY gene. Oligonucleotides for each strand were separately labeled with γ-32P/ATP using T4 polynucleotide kinase. Following inactivation of the kinase by heating for 10 min at 90 °C, the labeled oligonucleotides were annealed to their unlabeled counterparts, and double-stranded complexes were purified after electrophoresis in nondenaturing 10% polyacrylamide gels (acrylamide:bisacrylamide, 50:1). Methylation interference footprinting was performed as described by Andrisani et al. (20). Cleavage fragments resulting from piperidine treatment of the methylated DNA were loaded onto denaturing 20% polyacrylamide sequencing gels. The sequencing reactions were performed using a standard sequencing reaction, with the exception that the sequencing reaction was loaded onto a 10% polyacrylamide sequencing gel. Methylation interference footprinting was performed as described by Andrisani et al. (20). Cleavage fragments resulting from piperidine treatment of the methylated DNA were loaded onto denaturing 20% polyacrylamide sequencing gels. The sequencing reactions were performed using a standard sequencing reaction, with the exception that the sequencing reaction was loaded onto a 10% polyacrylamide sequencing gel. The sequencing reactions were performed using a standard sequencing reaction, with the exception that the sequencing reaction was loaded onto a 10% polyacrylamide sequencing gel.

Cell Culture and Transient Transfection Analyses—PC12 cells (kindly donated by Dr. Gordon Guroff, National Institutes of Health) were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% horse serum and 5% fetal calf serum in a humidified 8% CO2 atmosphere. The cells were routinely divided 1:4 onto collagen-coated dishes prior to transfection. Only cell passages 6 through 16 were used in these experiments because older cells appeared to have an altered phenotype and a diminished response to NGF (generously provided by Dr. Gordon Guroff, National Institutes of Health) with the following modifications. Fifteen micrograms of NPY/CAT fusion constructions plus 5 pg of pRSVLacZ, containing the Rous sarcoma virus promoter, were precipitated per 100-mm dish at 4 °C. Aliquots of the extracts used for CAT analyses were heated for 15 min at 65 °C to remove endogenous deacetylating activities (16). o-nitrophenyl-P-D-galactopyranoside is the substrate for the β-galactosidase assay and has an absorbance of A405 nm. CAT activities for the NGF-treated samples were determined using the BCA protein assay kit (Pierce Chemical Co.).

 Following electrophoresis in nondenaturing 10% polyacrylamide gels (acrylamide:bisacrylamide, 50:1), the gel was scanned using a phosphorimager. Methylation interference footprinting was performed as described by Andrisani et al. (20). Cleavage fragments resulting from piperidine treatment of the methylated DNA were loaded onto denaturing 20% polyacrylamide sequencing gels. The sequencing reactions were performed using a standard sequencing reaction, with the exception that the sequencing reaction was loaded onto a 10% polyacrylamide sequencing gel. The sequencing reactions were performed using a standard sequencing reaction, with the exception that the sequencing reaction was loaded onto a 10% polyacrylamide sequencing gel. The sequencing reactions were performed using a standard sequencing reaction, with the exception that the sequencing reaction was loaded onto a 10% polyacrylamide sequencing gel.
NGF-induced NPY Gene Transcription

A. The sequence of the NPY promoter spanning -75 to -46. The CT box Sp1-like binding site is boxed, whereas the putative AP-2 binding site is circled. The nucleotide changes in the mutant oligonucleotides listed to the right are indicated above. B, lane 1 represents the gel retardation pattern obtained using PC12 extract in the absence of any competitor oligonucleotides. Lanes 2-9 contain 0.1 pg of the indicated competitor DNAs. The specific complexes shifted using PC12 cell extract are designated C1-C3. C, lanes 1-3 represent the sequence -75/-46 shifted with 1 footprint unit of the indicated purified proteins.

RESULTS

Determination of the Human NPY Gene's 5'-Flanking Sequences Important for the Transcriptional Increase Elicited by NGF—To define the elements important for NGF regulation, plasmid constructions containing varying amounts of the human NPY gene's 5'-flanking sequences fused to the bacterial reporter gene CAT were transiently introduced into the PC12 cell line. Analysis of the 5'-flanking sequences spanning -796 to -30 indicated that sequences between -246 and -30 were sufficient to increase CAT activity 5-fold in response to NGF.
treatment (Fig. 1). These results are consistent with the NGF-induced transcriptional increase (2–7-fold) measured for the endogenous NPY gene by nuclear runoff analyses (12). Finer dissection of the region between -246 and -30 demonstrated that as little as 63 base pairs 5’ to the transcriptional start site were capable of increasing CAT activity when the cells were exposed to NGF (Fig. 1). The addition of sequences between -83 and -63 increased the basal activity of the promoter as well as maximizing the NGF response. The increases for the Δ83 and the Δ63 constructs were consistently 3- and 2-fold, respectively. The Δ30 construct which was previously shown to be incapable of correctly initiating transcription (13) did not consistently respond to NGF. This experiment defined the NGF response element to be contained within the sequence spanning -83 to -30.

Gel Retardation Analysis—The sequence between -87 and -36 was subjected to gel retardation analyses to determine the number and nature of interacting proteins present in PC12 cell crude nuclear extracts. Due to the complexity of these interactions, this region was divided into three overlapping double-stranded oligonucleotides, -65 to -36, -75 to -46, and -87 to -58 (Figs. 2A, 3A, and 4A, respectively). Computer analysis (MacVector 3.5, International Biotechnologies, Inc., New Haven, CT) of these sequences for potential DNA-protein interactions revealed two putative AP-2 binding sites centered around positions -61 and -45 (Fig. 2A). Previous analysis of the region between -65 and -36 carried out in a human neuroblastoma cell line, LA-N-5, demonstrated that the CT-rich sequence designated the CT box in Fig. 2A was necessary for basal level expression. The associated protein binding activity had a specificity similar to that of transcription factor Spl (13). It is noteworthy in this regard that transcription factor AP-2 also binds to GC-rich sequences. Gel retardation analysis of this region using PC12 cell nuclear extracts is shown in Fig. 2B. Three major complexes were observed with no apparent NGF-dependent differences (Fig. 2B, compare lanes 1 and 10 with 2). Analogous to the LA-N-5 cell experiments, the slowest mobility bands, S1 and S2 are efficiently competed by addition of an excess of unlabeled oligonucleotide -65/-36 (Fig. 2B, lane 3) and an oligonucleotide containing two high affinity Spl binding sites (Fig. 2B, lane 4). A GC-rich AP-2 binding oligonucleotide is also able to partially compete these complexes (Fig. 2B, lane 5). Competitors which do not contain the CT sequence or have mutations in this binding site are not able to compete for S1 and S2 binding (Fig. 2B, lanes 6, 7, and 9). The -75/-46 oligonucleotide, containing the CT binding site near the 3’ end (Fig. 4A), displays somewhat reduced competition (Fig. 2B, lane 8). This sequence contains not only the CT box but also a high affinity AP-2 binding site centered at position -61. Therefore, its reduced ability to compete the S1 complex could be due either to the noncentral location of the CT binding site or to blockage of CT box protein binding to -75/-46 by an AP-2-like
factor which is bound. However, the inability of -75/-46M1 to compete suggests that the CT box is the sequence responsible for competition by this oligonucleotide (Fig. 2B, lane 5). Since the oligonucleotide representing the AP-2 consensus binding site is able to partially compete the S1 and S2 complexes (Fig. 2B, lane 5), we sought to determine whether AP-2 as well as Spl-like molecules are able to interact with this sequence. When purified Spl and AP-2 are used in the gel retardation analysis along with the -65 to -36 oligonucleotide, AP-2 is not able to specifically interact with this oligonucleotide (compare Fig. 2B, lane 11 with Fig. 3C, lane 4) and Spl1 is able to interact only marginally (Fig. 2B, lane 12). Furthermore, the Spl1 complex runs at a slower mobility than complex S1, suggesting that complex S1 is not formed by Spl1. Complex S3 probably represents a nonspecific binding reaction, because it is inefficiently competed by all competitors tested.

The sequences of the oligonucleotide -75/-46 and selected mutants are shown in Fig. 3A. Both the CT box binding element and an AP-2 consensus binding site are located in this sequence. Three major complexes, designated C1-C3, are formed when this labeled oligonucleotide is incubated in the presence of PC12 nuclear extracts (Fig. 3B, lane 1). Although not shown, extracts prepared from cells treated with NGF display no discernible differences in complex formation. Competition with an excess of unlabeled -75/-46 reduces the intensity of C1-C3 (Fig. 3B, lane 2) as does competition with consensus binding sites for AP-2, Spl1, and the CT box containing oligonucleotide -65/-36 (Fig. 3B, lanes 3, 4, and 6). All oligonucleotides compete C3 to a similar degree, suggesting that it is generated by a nonspecific interaction. The Spl consensus oligonucleotide and -65/-36 compete the most efficiently for complex C1 and C2 formation. Oligonucleotides which do not contain a CT sequence (~87/-58) or have a mutated version of this sequence (~65/-36M, -75/-46M1) are no longer able to efficiently compete (Fig. 3B, lanes 5, 7, and 8). Oligonucleotide -75/-46M2, which contains mutations in the AP-2 binding site, retains partial competitive ability (Fig. 3B, lane 9). Therefore, similar to the overlapping sequence (~65 to -36), -75/-46 is capable of forming a DNA-protein complex involving nucleotides in the CT box. Additionally, it is capable of interacting with AP-2. The ability of this sequence to interact with purified AP-2 and Spl1 was determined as shown in Fig. 3C. The AP-2 binding site located in the center of the sequence binds purified AP-2 strongly while interacting only marginally with Spl1. (Fig. 3C, lanes 1 and 2). The strength of the AP-2 binding to the NPY sequence in comparison with the consensus oligonucleotide, defined by its interaction with the hMT-IIA gene, can be seen by comparing lanes 1 and 2. When added in concert these proteins
that come into direct contact with the proteins forming complexes A-C. A pattern of reduced contact with methylated G residues was apparent only for complex A (Fig. 5A). Three G residues on the coding strand at positions -73, -71, and -69 appeared to be involved in complex formation. On the noncoding strand the intensity of 2 G residues were reduced at positions -72 and -67 (summarized in Fig. 5B). No G residues were observed to be critical for complex B or C binding. It is possible that the binding sites for these complexes are too close to the ends of the oligonucleotide for this type of analysis or that they do not contact the DNA through analyzable G residues.

To determine the importance of the identified G residues in complex formation, specific bases were mutated within the -87 to -58 sequence (Fig. 4A) and subjected to gel retardation analysis (Fig. 5C). The gel retardation pattern produced by the oligonucleotide -87/-58 without the addition of competitors is shown in lane 7 preceded by the addition of increasing amounts of unlabeled oligonucleotide (Fig. 5C, lanes 6 and 5), respectively. Competitors -87/-58M3 and -87/-58M4, which contain mutations in the binding site defined by the methylation interference assay, are unable to effectively compete complex A (Fig. 5C, lanes 3 and 4). However, complex B is differentially competed by these two sequences. The M3 but not the M4 oligonucleotide is able to partially compete this complex (Fig. 5C, lane 3 versus 4). This evidence suggests that these complexes are generated by different proteins. Furthermore, M2, which contains mutations in the CAAT-like sequence, very efficiently competes only complex A. Conversely, M1, which contains mutations within complex A's binding site, efficiently competes only complex B.

These complexes also display tissue specificity. The gel retardation pattern in lane 8 (Fig. 5C) was generated by incubation of labeled -87/-58 with HeLa cell nuclear extracts. Neither complex A nor B is formed to any appreciable extent.

Southwestern Analysis—In order to further characterize the factors binding to the sequences between -87 and -58, a Southwestern analysis was performed. The ability of this sequence to specifically interact with proteins was tested in HeLa, LA-N-5, and PC12 extracts. LA-N-5 cells are human neuroblastoma
cells which highly express NPY (13). Using this technique, three bands were visualized (Fig. 6). The highest molecular weight band (A) was nonspecific. All labeled oligonucleotides produced this signal (data not shown). Band B, however, appears to be specific in that it is completely competed by cold self but not by NPY mutant oligonucleotides -87/-58M1 and -87/-58M2 nor oligonucleotides representing the consensus sequences for AP-1, Sp1, or NF1-CAAT. The apparent molecular mass of band B is approximately 40 kDa. It is also present in LA-N-5 cell extract but not in HeLa extract, consistent with the inability of the -87/-58 oligonucleotide to produce the characteristic pattern in the gel retardation analysis when HeLa extract is used. The fastest mobility band, C, is not consistently produced and is not characterized further in this report.

DNase I Footprint Analysis—The complexity of the DNA binding activities defined by the gel retardation analyses suggested the possibility of multiple DNA-protein interactions occurring within the sequence between -87 and -46. In order to further define the combinatorial nature of these interactions, the fragment spanning -143 to +51 was labeled on the coding strand, incubated with PC12 cell nuclear extracts, and treated with DNase I (Fig. 7A). The DNA ladder obtained by treating this labeled fragment with DNase I in the absence of any extract is represented in lanes 1, 7, and 14. Consistent with the gel retardation analyses, treatment of the cells with NGF 8 h prior to extract preparation did not alter the footprinting pattern (compare lanes 2 and 3). Footprinted regions, labeled FP1–FP3, define the approximate boundaries of the NPY oligonucleotides used in the previous experiments. The first footprinted region (FP1), spanning -65 to -35, encompasses the CT box binding site and the juxtaposed AP-2-like binding site. The next protected region (FP2) spans -93 to -66 and represents the binding sites contained in the -87/-58 oligonucleotide. The final footprinted region (FP3) extends from -132 to -106 and includes another CT-rich sequence.

FP1 is efficiently competed by addition of 1 μg of unlabeled oligonucleotide -65/-36 as well as oligonucleotide -75/-46 (Fig. 7A, lanes 4 and 6). Mutation of the CT box in -65/-36M abolishes this competition (Fig. 7A, lane 5). GC-rich oligonucleotides containing consensus binding sites for AP-2, Sp1, and oligonucleotides NPY -98/-71 and NPY -132/-106 also partially compete this footprint (Fig. 7A, lanes 9 and 11–13). FP2 is only marginally competed by the aforementioned oligonucleotides and is not competed by unlabeled oligonucleotide -87/-58 (Fig. 7A, lane 8). The inability of oligonucleotides -87/-58 and -98/-71 to compete FP2 is perplexing. It is possible that more unlabeled oligonucleotide may be necessary to compete
Binding sites designated mutations generated were shown previously to disrupt protein in the context of the NPY/TKCat fusion construct (Fig. 8). The capable of responding to NGF to the level of the wild type were transiently introduced into PC12 cells, the basal level of binding in the gel retardation analyses. When these constructs expression decreased to approximately that of TKCat (Fig. 8). When these sequences were placed 5' to the NPY promoter between -87 and -36 contains the binding sites for at least four proteins, we sought to ascertain which of these sequences in front of a heterologous promoter to deter-

The region between -87 and -36 designated TK tract, indicating a more complex interaction than AP-2 binding alone. Purified Sp1 is not able to footprint this fragment providing further evidence that the protein which interacts with the CT box is not Sp1 (Fig. 7B, lane 2). Furthermore, when added in concert with AP-2, Sp1 does not significantly alter the footprint of this region (Fig. 7B, lane 5). AP-1 is also not able to interact with these sequences and does not change the footprint generated by AP-2 (Fig. 7B, lanes 4 and 6).

Mutational Analysis of the NGF Response Element—Due to the low magnitude of the response produced by NPY fusion constructs Δ53 and Δ63, coupled with the variability inherent in transient transfection analyses, it was desirable to place these sequences in front of a heterologous promoter to determine if they were capable of eliciting a transcriptional increase in a different context. When these sequences were placed 5' to the minimal TK promoter, they enabled this promoter to be more efficiently expressed in PC12 cells and conferred NGF inducibility. The region between -87 and -36 designated TK-87/-36 produced around a 2.5-fold increase in CAT activity (Fig. 8). This is consistent with the fold increases observed for the Δ53 and Δ63 constructs. Since the aforementioned experiments have shown that the sequence of the NPY promoter between -87 and -36 contains the binding sites for at least four proteins, we sought to ascertain which of these interactions were important for the NGF response. The binding sites designated A/B, CT box, and AP-2 were mutated in the context of the NPY/TKCat fusion construct (Fig. 8). The mutations generated were shown previously to disrupt protein binding in the gel retardation analyses. When these constructs were transiently introduced into PC12 cells, the basal level of expression decreased to approximately that of TKCat (Fig. 8). Moreover, none of the individually mutated constructs were capable of responding to NGF to the level of the wild type construct. This suggests that the NGF response may be generated by the interaction of all these proteins on the NPY promoter.

**DISCUSSION**

The binding of NGF to its receptor on PC12 cells activates a cellular program leading to the differentiation of these cells into sympathetic-like neurons. Early events in the NGF cascade increase the transcription of proto-oncogenes such as c-fos and c-myc as well as that of other putative transcriptional regulatory factors (9). These proteins function in the control of other genes which ultimately serve to initiate the differentiation program. We have determined the sequences involved and partially characterized the DNA-binding proteins responsible for increasing NPY mRNA at the transcriptional level during this developmental process.

Transient transfection analyses determined that the NPY gene sequences between positions -87 and -36 were capable of increasing the activity of NPY/CAT reporter when transfected PC12 cells were exposed to NGF. These same sequences were also able to confer approximately a 2.5-fold increase in the ability of the TK promoter to respond to NGF. We have therefore designated this sequence as the NGF-responsive element (NGF-RE) of the NPY gene.

Several proteins are capable of interacting with the NPY gene's NGF-RE (Fig. 9). The CT box-binding protein displays the same type of binding specificity as the transcriptional activator, Sp1, and potentially represents a member of this rapidly growing family. Sp1 is a transcription factor whose consensus binding site is defined as the GC box and whose DNA binding domain consists of structures known as zinc fingers (24). Additionally, other proteins such as Sp2 and Sp3, which bind to CT box consensus sequences, have been characterized recently (26). These proteins were isolated based on the premise that their DNA binding domains would contain sequence homology to the Sp1 DNA binding domain. Additional GC sequence binding proteins include NGFI-A, NGFI-C, Krox-20, and the Wilm's tumor gene product (6, 27). The proteins NGFI-A (A–C) are of interest as they were isolated as early response genes of the NGF cascade. Since purified Sp1 is not able to footprint the CT box, it is likely that this binding activity is either another one of these family members or represents a novel addition to this family.

Immediately upstream of the CT-rich sequence (NPY 5'-flanking sequences -65 to -57) is a consensus AP-2 binding site (Fig. 9). The AP-2 protein binds GC-rich recognition sequences present in the cis-regulatory regions of the SV40 enhancer, HTLV-1 enhancer, as well as the human metallothionein IIA, proenkephalin, and mouse major histocompatibility complex h-2K genes (25, 28, 29). It is also promiscuous in its binding to GC-rich consensus binding sites for Sp1 and NF1 (30). AP-2 is developmentally regulated and displays selected tissue specificity (31, 32). It has also been demonstrated to mediate induction of the hMT-IIA enhancer by combination of the protein kinase C and cAMP signal-transduction pathways (25). The AP-2 protein binds as a dimer to sequences with the consensus 5'-GCCCGAGGC-3'. This sequence differs by only one nucleotide from the NPY sequence between -65 and -57. The effects of NGF on the expression of AP-2 in differentiating PC12 cells is not known, and we were unable to demonstrate any differences in binding intensities of the AP-2 complex as measured by the gel retardation analysis in extracts prepared from NGF treated cells versus nontreated cells. Since, the pattern of AP-2 expression in neural crest cells and the developing nervous system indicates that it may play a major role in establishing the peripheral nervous system and its connection with the central nervous system, it would be consistent for AP-2 to play a
role in the NGF-elicited differentiation of neural crest derived PC12 cells. Whether it acts in conjunction with the CT box-binding protein or by excluding its interaction remains to be elucidated, but previous examples of regulation by AP-2 involving the displacement of other DNA-binding proteins have been reported (33).

The DNA-protein interactions occurring between -87 and -58, as illustrated by complexes B and A (Fig. 9), are not known to be represented by previously characterized DNA binding activities. Addition of these sequences to NPYICAT constructs increases the transcription observed in PC12 cells whether or not the cells have been exposed to NGF. This suggests that this sequence binds a positive regulator(s) whose activity is further increased via the NGF cascade. The purification and characterization of these proteins will provide important information concerning their role in modulating the promoter activity of the NPY gene and the ability of the NGF cascade to further increase this activity. The availability and potential modifications of these proteins as modulated by the NGF cascade are likely to determine the overall activity of the NPY gene during the differentiation process occurring in PC12 cells. However, no differences in gel retardation patterns nor footprinting patterns were apparent between extracts prepared from NGF-treated cells versus non-NGF-treated cells. It is possible that protein modifications may not be apparent under these circumstances and just as likely that any quantitative differences would be muted by the artificial concentration of proteins during nuclear extract preparation. It will, therefore, be of interest to determine the identity of these proteins and to study not only their interactions with the NPY promoter but also their potential ability to interact with one another and the other proteins which bind to proximal NPY promoter sequences.

Acknowledgments—We thank Jim Brinegar for help with the computer and Drs. Randy Haun, Seung Kwak, James Clements, and Randy Stone for critically reading this manuscript. The skilled technical assistance by R. Krajcik (methylation interference analysis) and R. Lerchen was much appreciated. Many thanks to Dr. Gordon Gurdon for purified CREB, and Dr. Steve Sabol for purified NGF.

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Fig. 9. Schematic diagram of the NPY promoter region containing the NGF-RE. DNA binding activities defined in this study include the NGF-RE (bracket) detected by transient transfections, the regions protected by DNase I (indicated by bars), G residues which participate in the binding of complex A as determined by methylation interference (indicated by asterisks), and binding activities of B, A, AP-2, and the CT box-binding proteins identified by gel retardation analyses.