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Structure of the NGFI-A gene and detection of upstream sequences responsible for its transcriptional induction by nerve growth factor

(transcriptional regulator/"zinc fingers"/c-*fos*/NGFI-B protein/transfection)

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ABSTRACT **The NGFI-A gene encodes a "zinc-finger" protein that is rapidly induced by nerve growth factor (NGF) in PC12 rat pheochromocytoma cells. The complete exon/intron organization and nucleotide sequence of the rat NGFI-A gene have been determined. The gene spans 3789 nucleotides (nt) and is interrupted by a single intron at nt 588. All three zinc-finger DNA-binding domains are contiguously coded for within the 3′ exon; this is in contrast to the structure described by others for the *Xenopus laevis* transcription factor TFIIIA gene. To analyze the transcription of this gene, we have determined the transcription start site and nucleotide sequence of the 5′ flanking region. Transfection of PC12 cells with a fragment from the 5′ flanking region linked to the chloramphenicol acetyltransferase (CAT) gene revealed that it contains an element which imparts an NGF-inducible phenotype to the normally silent CAT gene. Several regions with homologies to recognizable sequence elements are present in this fragment, including a TATA box at nt −27, serum response elements at nt −84, −106, −370, and −408, a cAMP-responsive element at nt −140, and a transcription factor Sp1-binding site at nt −286. These results establish the genomic structure of this mammalian multifinger protein and demonstrate that an NGF-responsive element lies upstream of the NGFI-A gene.**

Nerve growth factor (NGF) is a polypeptide hormone required for the differentiation and survival of sympathetic and neural crest-derived sensory neurons (1). NGF is released by peripheral targets, binds to its specific receptor on the neuronal cell surface, and is retrogradely transported to the cell body. These actions initiate a series of events, including alterations in gene expression, that culminate in the differentiated phenotype. The rat pheochromocytoma cell line PC12 is an extensively characterized model of NGF-mediated neuronal differentiation (2). When treated with NGF, PC12 cells change from replicating adrenal-chromaffin-like cells to nonreplicating sympathetic-neuron-like cells that extend neurites and are electrically excitable. The expression of several genes is increased in NGF-treated PC12 cells. These include "late" genes, whose maximal induction occurs 24 hr after treatment [e.g., genes encoding intermediate filament (3), neuronal growth-associated protein GAP-43 (4), and two S-100-like proteins (5)], and "early" genes, such as those encoding c-fos (6), NGFI-A (7), and NGFI-B (8). The NGF-mediated induction of these "early" genes is very rapid: specific mRNAs are detectable within minutes after NGF stimulation and have reached maximal levels by 1 hr. These "early" genes are also characterized by the relatively short half-life of each of their corresponding mRNAs, by their activation in PC12 cells treated with phorbol 12-myristate 13-acetate or calcium ionophore A23187 (J.M., unpublished data), and by the observation that their induction is not inhibited by cycloheximide. Of greatest consequence, however, is the realization that they encode proteins which are presumptive transcriptional regulators: c-fos probably regulates transcription by virtue of its association with the transcriptional activator c-jun (9); NGFI-A contains three tandemly repeated "zinc finger" domains characteristic of many DNA-binding proteins; and NGFI-B is homologous to members of the glucocorticoid receptor family and is therefore likely to function as a ligand-dependent modulator of transcription.

The rat NGFI-A gene has been characterized with a view towards analyzing its transcriptional regulation and to explore the organization of this mammalian zinc-finger gene.* The zinc-finger DNA-binding domains are not separated by introns but are all encoded within the 2.5-kilobase (kb) 3′ exon. A fragment from the 5′ flanking region contains an element that is responsive to NGF when transfected into PC12 cells. Therefore, sequences within this fragment are at least partly responsible for the transcriptional activation of this gene in cells stimulated by NGF.

MATERIALS AND METHODS

Genomic Library Screening. A rat genomic library, constructed by cloning fragments from a *Sau*3A partial digest of rat liver DNA in λEMBL3, was obtained from R. Hynes (Massachusetts Institute of Technology). The library was screened by the method of Grunstein and Hogness (10) with ^{32}P-labeled NGFI-A cDNA.

DNA Blot Analysis. Genomic DNA was prepared from rat brain as described (11). λ phage DNA was prepared by the method of Garber (12). Both rat DNA and λ DNA were digested with restriction enzymes, electrophoresed through an agarose gel, transferred to GeneScreen*Plus* (New England Nuclear) (13), and hybridized as previously described (7). DNA probes were labeled with [^{32}P]dCTP to a specific activity of 1×10^9 dpm/μg (14).

DNA Nucleotide Sequencing. A 12-kb *Eco*RI/*Sal* I fragment from λA20 was subcloned in pBS-KS (Stratagene, La Jolla, CA). Sequencing was performed by using oligonucleotide primers situated every 200 nucleotides (nt) along the entire cDNA sequence on both strands. Additional primers were used to sequence the intron and 5′ flanking region. The primers were annealed with double-stranded plasmid template and sequencing was performed by the method of Sanger *et al.* (15) with a modified T7 DNA polymerase (Sequenase; United States Biochemical, Cleveland) as per the supplier's protocol. All nucleotide sequences were confirmed on both strands.

Primer Extension and S1-Nuclease Protection. Oligonucleotide primers were end-labeled with T4 polynucleotide kinase

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Abbreviations: NGF, nerve growth factor; CAT, chloramphenicol acetyltransferase; nt, nucleotide(s); SRE, serum-response element.
*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04154).

and [λ-^{32}P]ATP to a specific activity of 1–2 × 10^8 cpm/μg. Total RNA was prepared as described (16). Primer (1 × 10^6 cpm) and 30 μg of total RNA were precipitated with ethanol, resuspended in 5 μl of hybridization buffer (1 M NaCl/0.1 M Pipes, pH 6.4/25 mM EDTA), and annealed for 3 hr at 72°C. The extension reaction was performed at 42°C for 60 min and was initiated by adding 45 μl of conversion buffer (30 mM Tris·HCl, pH 8.3/10 mM $MgCl_2$/0.5 mM dithiothreitol/1.0 mM each dNTP) containing 40 units of RNAsin and 50 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, Saint Petersburg, FL). For S1-nuclease protection assays, a restriction fragment extending from the *Sac* II site at nt +100 to the *Sac* II site at nt −532 was dephosphorylated and end-labeled at nt +96 with 50 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP and T4 polynucleotide kinase to a specific activity of 1–2 × 10^6 cpm/μg. Then 150,000 cpm of probe and 25 μg of total RNA were precipitated with ethanol and resuspended in 15 μl of S1 hybridization cocktail [80% (vol/vol) formamide/56 mM NaCl/6 mM NaOAc, pH 4.5/0.75 mM $Zn(OAc)_2$/sonicated DNA at 3.3 μg/ml)]. The reaction mixture was treated at 85°C for 15 min and hybridized at 50°C for 16 hr. The S1-nuclease reaction was initiated by adding 300 μl of S1 cocktail (280 mM NaCl/50 mM NaOAc, pH 4.5/4.5 mM $ZnSO_4$/salmon sperm DNA at 20 μg/ml/S1 nuclease at 600 units/ml) and incubated at 37°C for 30 min. The reaction products of both assays were analyzed on urea/acrylamide DNA sequencing gels.

Transfection of PC12 Cells. PC12 cells were grown as described previously (17). Plasmids were introduced into exponentially growing cells via electroporation (18): 1 × 10^7 cells were resuspended in 400 μl of Hepes buffer (20 mM Hepes, pH 7.05/137 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4/6 mM dextrose), transferred to a plastic cuvette, and mixed with 100 μl of Hepes buffer containing 20 μg of plasmid DNA and 125 μg of herring sperm carrier DNA. The cells were electroporated with a BTX (San Diego, CA) Transfector-300 set at 225 V and a capacitance of 450 μF. Ten minutes after electroporation the transfected cells were returned to medium containing serum and incubated for 48 hr prior to analysis. The plasmids used were RSV-CAT (19), gift of T. Ley (Washington University); Sp65-CAT, gift of L. Ratner (Washington University); NGFI-A-CAT and NGFI-A-CAT(i). NGFI-A-CAT was constructed by subcloning a *Sac* II fragment (extending from nt −532 to nt +100) of the NGFI-A gene in the promoterless Sp65-CAT plasmid. NGFI-A-CAT(i) contains this *Sac* II fragment in the inverse orientation.

Analysis of Chloramphenicol Acetyltransferase (CAT) Activity. Freeze/thaw lysates of transfected cells were prepared and equivalent amounts of protein from each were assayed for CAT activity (19). The percentage of acetylated chloramphenicol was quantitated by excising areas of the TLC plate corresponding to native and acetylated choramphenicol and determining the amount of radioactivity in each sample by scintillation counting. Protein concentrations were determined by using the BCA (bicinchoninic acid) reagent (Pierce).

RESULTS

NGFI-A Is Encoded by a Single-Copy Gene. Many proteins with transcriptional regulatory activity contain DNA-binding domains known as zinc fingers (20). Recently, many mammalian zinc-finger proteins have been identified by cross-hybridization (21, 22) or differential hybridization (23) techniques, but little is known about the organization of the genes that encode these proteins. Of particular interest is the region encoding the zinc fingers; therefore we sought to determine the exon/intron structure of the rat NGFI-A gene. To obtain a clone containing the NGFI-A gene, we screened a rat genomic library constructed in λEMBL3 with the NGFI-A cDNA probe. Of several positive clones identified, λA20 contains an ≈16-kb insert that hybridizes to probes specific for both the 5′ and 3′ ends of the NGFI-A cDNA. The λA20 clone therefore spans the entire gene and was chosen for further study. To confirm that the λA20 clone represents a bona fide copy of the rat gene, a Southern blot containing *Bgl* II- and *Pst* I-digested rat brain DNA and λA20 DNA was probed at high stringency with the entire NGFI-A cDNA. In each case identically sized bands were observed (Fig. 1).

Nucleotide Sequence of the Rat NGFI-A Gene. To initiate studies of the NGF-mediated transcriptional control of NGFI-A and to determine the location of the intron/exon boundaries of this gene, we have established the nucleotide sequence of the NGFI-A gene and its 5′-flanking region (Fig. 2). The transcriptional unit is 3789 nt long and contains a single intron of 664 nt that begins at nt 588 and possesses the consensus splice signal dinucleotides. This separates the NGFI-A gene into two exons: the 5′ exon is 587 nt long and the 3′ exon is 2538 nt in length. The intron interrupts the coding sequence, but does not separate the molecule into any obvious domains; specifically, the three zinc fingers are all encoded within the 3′ exon.

The sequence of the 5′ flanking region was established to facilitate analysis of the transcriptional start site and to aid in identifying previously characterized enhancers. The sequence revealed the presence of a TATA-box-like sequence (AAATA) at nt −27, an observation consistent with the limited heterogeneity observed in the transcriptional initiation sites of this gene (Fig. 3 *Upper*) (24). Sequences similar to the serum-response elements (SREs) identified in the 5′ flanking regions of the c-fos and γ-actin genes (25) are present at nt −84, −106, −370, and −408. A GC-box, the recognition site for the transcription factor Sp1 (26), is present at nt −286. A sequence, related to the cAMP-responsive element found in the 5′ flanking region of the rat somatostatin gene (27), is located at nt −140.

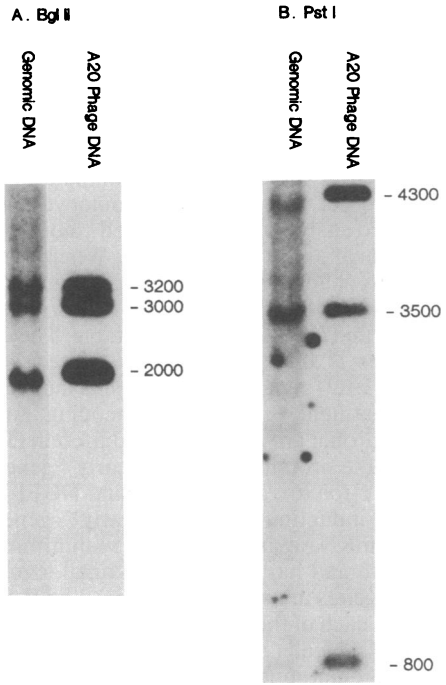

FIG. 1. DNA blot analysis of the rat NGFI-A gene. Ten micrograms of rat genomic DNA or 1 ng of λA20 DNA was digested with either *Bgl* II (*A*) or *Pst* I (*B*), electrophoresed through a 1% agarose gel, and transferred to GeneScreen*Plus*. The blot was hybridized with the NGFI-A cDNA. Molecular size markers are given in nt.

FIG. 2. Nucleotide sequence of the NGFI-A gene and 5′ flanking region. The numbers on the left refer to the nucleotide sequence using the transcription start (indicated by the arrow) as nt +1. The multiple serum response-related elements found in the 5′ flanking region are underlined, the GC-box is boxed, and the cAMP-responsive element is denoted with a broken line. The amino acids are indicated by the single-letter code, the intron is enclosed in brackets, and the zinc-finger domains are boxed. The braces denote in-frame, upstream ATGs that are alternative sites for translation initiation.

Determination of the Transcription Start Site. To define the beginning of the NGFI-A transcriptional unit, both primer extension and S1-nuclease protection analyses were performed. An oligonucleotide primer (PE118), located at nt 81–100, was annealed with RNA from uninduced PC12 cells (no NGF treatment), from PC12 cells treated with NGF (50 ng/ml) for 2 hr, or with yeast tRNA. The products of the primer extension products were analyzed by electrophoresis through a DNA sequencing gel. As shown in Fig. 3, there are three extension products (one major, two minor), 99, 100, and 101 nt in size, that are present when RNA from PC12 cells treated with NGF is analyzed (Fig. 3 *Upper*, lane 2). The major species is designated with an arrowhead and is labeled "+1" in the adjacent sequence of the transcription initiation region. There are no extended products seen when extension reactions were performed with uninduced PC12 cell RNA or yeast tRNA templates. Identical results, indicating the preferred start site to be at nt +1, were obtained with a second primer placed at nt 206 (data not shown).

To confirm the start site identified by primer extension, an S1-nuclease protection experiment was performed. A *Sac* II fragment that extends 3′ into the first exon was end-labeled at nt +96 (Fig. 3 *Lower*) and hybridized with identical aliquots of total RNA as described for the primer extension experiments. The S1 digestion products were analyzed on a DNA sequencing gel, and protected fragments were detected only in samples containing RNA from NGF-treated cells. The protected species are somewhat more heterogeneous than the primer extended products; however, the two major species, 95 and 96 nt in length, confirm the start site determined by primer

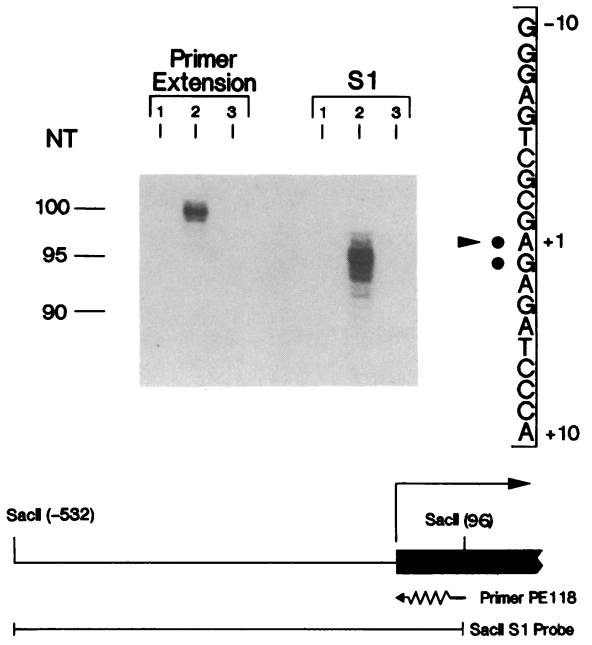

FIG. 3. (*Upper*) Analysis of the transcription initiation site. Primer extension and S1-nuclease protection were performed on total RNA isolated from uninduced PC12 cells (lanes 1), PC12 cells treated for 2 hr with NGF (lanes 2), and yeast tRNA (lanes 3). The sequence in the vicinity of the start site is shown at the right. An arrowhead indicates the initiation site determined by primer extension and the two dots represent the major sites determined by S1-nuclease protection. (*Lower*) Schematic diagram designating the relative positions of the primer and restriction fragment used in these analyses.

extension and are indicated by dots on the adjacent sequence. The transcription initiation site is 13 nt upstream from the 5′ end of the originally described NGFI-A cDNA, thus confirming that a nearly full-length clone was identified. A schematic diagram of the NGFI-A gene is shown in Fig. 4.

The NGFI-A Gene 5′ Flanking Region Contains Sequences Which Act as an NGF-Responsive Element (NRE). To understand how NGF initiates the alterations in gene expression that ultimately result in neuronal differentiation, we have concentrated on the transcriptional activation of the rapidly induced NGFI-A gene. As an initial step toward the identification of an NRE, we have attempted to identify a fragment of the NGFI-A gene that will impart an NGF-inducible phenotype on the bacterial CAT gene in a transient expression system. PC12 cells were transfected with RSV-CAT, Sp65-CAT, NGFI-A-CAT, or NGFI-A-CAT(i) plasmids by electroporation. NGFI-A-CAT consists of a *Sac* II fragment (nt −532 to +100) from the NGFI-A 5′ flanking region, which necessarily includes the transcription start site, that is positioned in the proper orientation upstream of the promoterless CAT gene of Sp65-CAT. NGFI-A-CAT(i) contains this fragment in the opposite orientation so that the promoter is inverted with respect to the CAT gene. After transfection, cells were incubated for 36 hr prior to the addition of NGF.

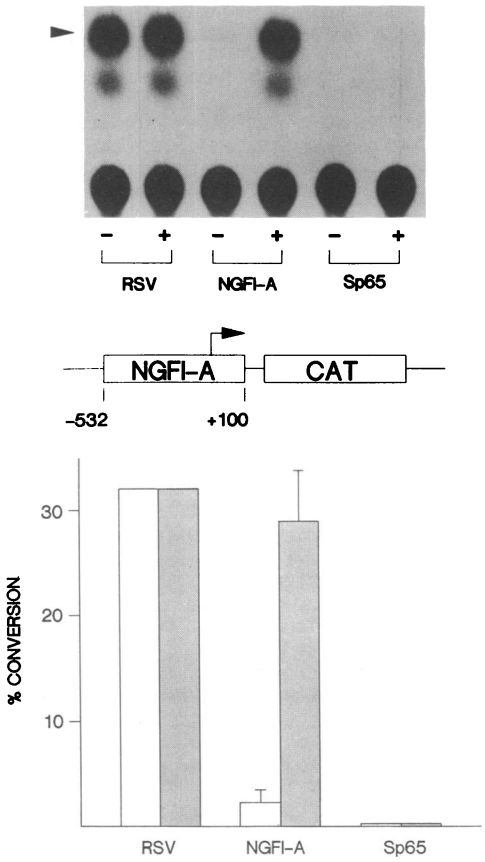

FIG. 5. Demonstration of NGF-inducible CAT activity in transfected PC12 cells. (*Upper*) PC12 cells were cultured for 36 hr after transfection in medium alone, then an additional 12 hr in the presence (+) or absence (−) of NGF (50 ng/ml). Cell lysates were assayed for CAT activity. The arrowhead indicates the position of acetylated chloramphenicol. A schematic diagram depicting the NGFI-A-CAT plasmid, with an arrow indicating the transcriptional start site, is shown at the bottom. (*Lower*) Histogram displaying the percentage of chloramphenicol converted to the acetylated form. Stippled bars, NGF-treated; open bars, control. Standard deviations are indicated for cells transfected with NGFI-A-CAT ($n = 5$). Plasmid promoters: RSV, Rous sarcoma virus long terminal repeat; NGFI-A, nt −532 to nt +100 of the NGFI-A gene; Sp65, no promoter present.

Twelve hours after NGF addition the cells were harvested and CAT activity was quantitated in the cell lysates. Results representative of this type of experiment are shown in Fig. 5 *Upper*. When PC12 cells are transfected with NGFI-A-CAT and treated with NGF, a significant amount of CAT activity is present; however, cells incubated in the absence of NGF contain little CAT activity. Cells transfected with RSV-CAT show significant CAT activity independent of the addition of NGF. Cells transfected with the promoterless Sp65-CAT contain minimal CAT activity in the presence or absence of NGF, as did cells transfected with NGFI-A-CAT(i) (data not shown). The results of numerous experiments of this type were quantitated by scintillation counting and are displayed

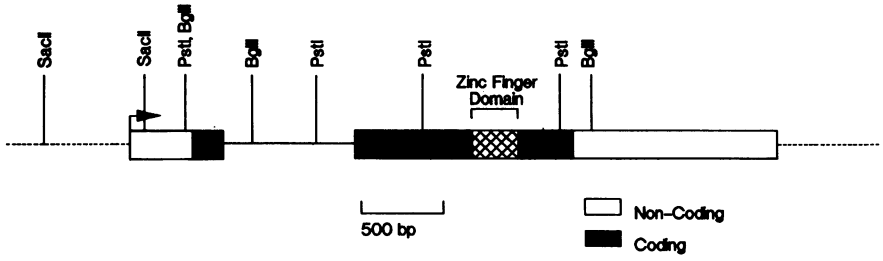

FIG. 4. Organization of the rat NGFI-A gene. The exons are designated by boxes with the coding and noncoding sequences identified. The intron is denoted by the solid line, flanking regions are denoted by the broken lines, and the transcription initiation site is marked with an arrow. The labeled *Sac* II sites mark the fragment used in transfection analysis to demonstrate NGF-mediated induction. bp, Base pairs.

as a histogram in Fig. 5 *Lower*. In the presence of NGF, cells transfected with NGFI-A-CAT convert 29% of the chloramphenicol to the acetylated form, but in the absence of NGF only 2.2% is present in the acetylated form. NGF therefore induces CAT activity approximately 15-fold in these cells, confirming that this portion of the 5′ flanking region of the NGFI-A gene contains a sequence(s) that is recognized by an NGF-activated transcription factor(s).

DISCUSSION

Using the PC12 cell model of NGF-mediated neuronal differentiation and the strategy of differential hybridization screening, we have previously isolated the NGFI-A cDNA. The NGFI-A protein contains several zinc-finger DNA-binding domains, which suggests that it is a transcriptional regulatory protein. Here we report the genomic structure and nucleotide sequence of the rat NGFI-A gene and its 5′ flanking region. To begin an analysis of the NGF-induced transcriptional activation of this gene, we have determined the transcriptional initiation site and demonstrated that sequences within the 5′ flanking region are responsive to NGF-activated transcription factors.

The zinc-finger DNA-binding motif is highly conserved. It occurs in transcription factors from yeast, *Drosophila*, *Xenopus*, rodents, and humans. Indeed, this conservation has been exploited to identify a plethora of genes encoding zinc-finger proteins by screening mouse genomic and cDNA libraries with a probe derived from the *Drosophila* Kruppel gene (21, 22). The evolution of this DNA-binding structure is unclear, but an examination of the exon/intron organization in genes that encode proteins with these domains may provide new insights. It is clear that proteins can contain widely varying numbers of zinc fingers (28). *Xenopus laevis* transcription factor TFIIIA contains nine zinc fingers, six of which are encoded by separate exons (29). In contrast, NGFI-A contains three tandemly repeated finger units and all are encoded within a single exon. Many other examples of proteins containing three zinc fingers have been identified, including transcription factors Sp1 (30) and Krox-20 (31). Both of these proteins contain zinc fingers that are very similar to those found in NGFI-A, so it will be interesting to discover whether the multiple fingers of these proteins, as well as those of other members of this multi-gene family, are also encoded within single exons. Other mammalian zinc-finger proteins, including mKr2 (21) and testis-determining factor (TDF) (32), have also been identified in which a single exon encodes multiple copies of this DNA-binding domain.

The identification of processes responsible for initiating growth and differentiation is central to developmental biology. One of the initial nuclear responses to growth factors is the rapid transcriptional activation of genes that encode putative transcriptional regulatory factors. It is thought that the expression of these factors results in a cascade of gene activation that ultimately results in the differentiated phenotype. A major site of control over this process is likely to be exerted at the initial step; therefore we have focused our attention on identifying the location within the NGFI-A gene locus of sequence elements responsible for the NGF-mediated transcriptional induction of this gene.

The NGF-mediated induction of CAT activity in PC12 cells transfected with NGFI-A-CAT is similar in character to that of the endogenous NGFI-A gene: the magnitude is high and it is rapid, occurring within 1 hr after the addition of NGF (data not shown). The nucleotide sequence of this fragment revealed that it contains several regions related to SREs, elements that mediate induction of transcription by serum components. This is of interest since the mouse homolog of NGFI-A, called Egr1, has recently been identified by virtue of its induction in serum-stimulated fibroblasts (33). The SRE-related sequences present in NGFI-A do not contain the extended dyad symmetry of SREs present in the human c-fos or *Xenopus* cytoplasmic γ-actin genes (25); instead, they are related to the central portion of these elements. Elements similar or identical to these sequences, called CArG boxes, have been identified in the 5′ flanking region of the human cardiac α-actin gene (34). Whether or not the elements mediating NGF induction are different or identical to these sequences must await further deletional analysis of this region. A determination of how NGF stimulates transcription of the NGFI-A gene, coupled with the identification of target genes regulated by NGFI-A, will increase our understanding of the role it plays in the nervous system.

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