Transcriptional Activation of the Inducible Nuclear Receptor Gene nur77 by Nerve Growth Factor and Membrane Depolarization in PC12 Cells*

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nur77 is an immediate-early gene inducible by nerve growth factor or membrane depolarization in the rat pheochromocytoma cell line PC12 and by serum growth factors in fibroblasts. The nur77-encoded protein is a member of the steroid/thyroid hormone receptor superfamily and can act as a potent transcription activator. The induction of nur77 in PC12 cells is rapid and transient, with kinetics similar to those of the c-fos protooncogene. Induction does not require de novo protein synthesis. Whereas transcriptional activation of c-fos by nerve growth factor in PC12 cells requires a 20-base pair serum response element in its promoter, there is no such sequence in the nur77 promoter. To understand the mechanism for the activation of nur77, we have analyzed the inducibility of a series of transfected nur77 minigenes using an S1 nuclease protection assay. We identified the sequence 22–86 nucleotides upstream of the transcription start site as necessary and sufficient for nur77 induction by nerve growth factor and membrane depolarization in PC12 cells. Sequences farther upstream enhance the induction. Analysis of base substitution mutations allowed us to identify three sequence elements within this region that are essential for induction. These sequence elements include two copies of an AP1-like element and a GC-rich sequence. Unlike transcriptional activation of c-fos, the sequence requirements for the activation of nur77 by nerve growth factor and membrane depolarization cannot be readily separated. Taken together, our data suggest that activation of nur77 and c-fos by nerve growth factor occurs through different mechanisms in PC12 cells.

A variety of extracellular signaling agents, including mitogenic growth factors, differentiation agents, neurotransmitters, steroid hormones, and other environmental changes, exert their effects, at least in part, by eliciting specific changes in gene expression (1–3). The primary genomic response to these extracellular signals is the rapid transcriptional activation of a set of immediate-early response genes. Transcription of these genes does not require de novo protein synthesis. Many immediate-early genes encode transcription factors, including members of the Fos and Jun families, Myc, Rel, and several zinc finger proteins (1, 2). These transcription factors may act to interpret the extracellular signals into long-term cellular responses by regulating the expression of specific genes. Interestingly, many of the same immediate-early genes, particularly those encoding transcription factors, are induced in different cell types by various stimulating agents that cause divergent long-term biological effects. This observation suggests that the target genes of these immediate-early transcription factors may be cell type- and/or stimulus-specific.

A particularly instructive cell system for studying immediate-early gene activation is the rat pheochromocytoma-derived cell line PC12, which can be stimulated by various agents to undergo disparate cellular responses (4, 5). NGF stimulates these cells to differentiate into sympathetic neuron-like cells after completing one round of cell division. In contrast, epidermal growth factor stimulates proliferation without causing differentiation. In addition, PC12 cells have excitable membranes that can be depolarized by specific neurotransmitters or elevated levels of extracellular KCl (6).

The transcriptional regulation of the c-fos protooncogene has been extensively studied in fibroblasts and in PC12 cells and serves as a paradigm for the regulation of immediate-early genes. Numerous studies have identified the SRE, a 20-bp sequence of imperfect dyad symmetry, as being critical for the transcriptional activation of c-fos by serum growth factors in fibroblasts and by NGF in PC12 cells (7–9). A complex of at least six proteins is known to bind the SRE and its flanking sequences (10–12), although the precise mechanistic roles of these proteins in gene activation have not yet been clearly defined. Among these proteins, the serum response factor plays a critical role (13).

In contrast, transcriptional activation of c-fos by membrane depolarization, Ca2+ influx, or cAMP is mediated through a sequence resembling the CRE and binds to CREB in vitro (14–16). Phosphorylation of CREB by cAMP-dependent protein kinases, which increases the transcriptional efficacy of CREB, correlates with the transcriptional activation of c-fos by membrane depolarization (14, 17–19). These observations suggest that membrane depolarization, acting through cAMP-dependent protein kinase or Ca2+-dependent kinases, regulates CREB, which in turn activates c-fos.

To what extent can these regulatory mechanisms be generalized for the transcriptional control of other immediate-early genes? Although a simple hypothesis might be that all immediate-early genes activated by a particular stimulatory agent are regulated through the same mechanism, there is

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1 The abbreviations used are: NGF, nerve growth factor; SRE, serum response element; CRE, cAMP response element; CREB, CRE-binding protein; CAT, chloromethylphenyl acetyltransferase; nt, nucleotide(s); bp, base pair(s); PIPES, 1,4-piperazinediethanesulfonic acid.
currently little information on the mechanism of immediate-early gene activation in PC12 cells other than what is known about c-fos. To address this question, we sought to analyze the transcriptional regulation of another immediate-early gene, nur77 (also known as NGFI-B, N10, TIS1, and NAK-1) (20–23), which, like fos, is also activated rapidly and transiently by NGF, epidermal growth factor, or KCl treatment in PC12 cells (21, 24).

The nur77-encoded protein is a member of the steroid/thyroid hormone receptor superfamily, suggesting that it may act as a ligand-dependent transcription regulator (25, 26). Although a specific ligand for Nur77 has not been identified, a DNA sequence with which Nur77 interacts has been defined (26). Thus, Nur77 may act to mediate cellular responses to extracellular signals by activating specific genes. Furthermore, distinct Nur77 phosphopeptides specific to NGF- or KCl-treated PC12 cells have been identified, suggesting that differential phosphorylation of Nur77 upon induction by different agents might modulate its activity (27).

To understand the transcriptional regulation of nur77 by a differentiation agent versus a membrane-depolymerizing agent, we have analyzed the sequence requirements in its promoter for activation by NGF and KCl in PC12 cells. In contrast to that of c-fos, activation of nur77 by NGF is not mediated through an CRE. Rather, the required sequence contains two copies of an AP1-like element and a GC-rich sequence that includes the binding sites for SP1 (28) and a family of zinc finger proteins (29, 30). Mutation of any of these sequence elements abolishes nur77 inducibility by either NGF or KCl. The differences in promoter elements required for activation of c-fos and nur77 by NGF and KCl suggest that the same stimuli induce these immediate-early genes by alternate mechanisms.

MATERIALS AND METHODS

Cell Culture—PC12 cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated horse serum (Sigma), 5% fetal bovine serum (Biologics, N.erville, IL), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C with 10% CO2. Cells were stimulated by addition of NGF (2.5 S) (Bioproducts for Science, Indianapolis, IN) to 50 ng/ml for various durations as indicated. Membrane depolymerization was achieved by adding KCl to the culture medium to a final concentration of 60 mM.

Calcium Phosphate Precipitation—PC12 cells were transfected either by calcium phosphate precipitation or by electroporation as indicated. Calcium phosphate precipitation was carried out as described (15) using 20 µg of the test plasmid and 10 µg of the pSV2 neo reference plasmid pK300, which contains sequences corresponding to nt -1148 to +122 of nur77 followed by a polylinker. Into the polylinker we inserted the 2193-bp Nur77-XbaI cDNA fragment corresponding to nt 123–2315 of the nur77 cDNA. Downstream of the cDNA we inserted a 276-bp XhoI-ScaI nur77 genomic DNA fragment that contains the polyadenylation signal and sequences farther downstream. Sequence analysis was carried out after each step to ensure that the correct product was obtained.

5'–Deletion mutants (see Fig. 2) were derived from the 1148/77TR plasmid using a polymerase chain reaction procedure. The 5'–primers (see below), which defined the deletion end points, were used with the 3'–primer (5'-CCAGGTCTCACTGTTAGCTGTTGT), to generate polymerase chain reaction products. These products were digested with XbaI and NcoI, purified via agarose gel electrophoresis, and substituted for the XbaI-NcoI fragment of 1148/77TR (nt -1148 to +428). The 5'–primers used are as follows: mutant 226/77TR, 5'-TGCTCTAGAGGCGCCAGACATTTCCGAC; mutant 126/77TR, 5'-TGCTCTAGAGCCCCCGCGAGACCTTCCGAC; mutant 106/77TR, 5'-TGCTCTAGAGCGCCCGAGGTCGCGGACCCGAGCGACGCCGAGACCTTCCGAC; mutant 77TR, 5'-TGCTCTAGAGGCCA; mutant 68/77TR, 5'-TGCTCTAGAGCGCCCGAGGTCGCGGACCCGAGCGACGCCGAGACCTTCCGAC; mutant 60/77TR, 5'-TGCTCTAGAGGCC; mutant 55/77TR, 5'-TGCTCTAGAGGAACCCGGCGACCTTCCGAC; and mutant 33/77TR, 5'-TGCTCTAGAGGACCGTCCTAAGATGGTTG.

To test the function of nur77 promoter elements in a heterologous promoter, portions of the nur77 promoter were placed upstream of the herpes simplex virus thymidine kinase promoter linked to the CAT gene (see Fig. 4A). These constructs were made by modifying the 17MX2 TKCAT plasmid (a gift from P. Chambon) (34), which contains two copies of the transcription factor GAL4-binding site upstream of the thymidine kinase promoter. The GAL4-binding sites were removed by HindIII and XbaI digests, and in their place we inserted a DNA fragment containing nt -278 to -22 of the nur77 promoter adapted with HindIII and XbaI linkers, creating the plasmid 278/22 TKCAT. To obtain the 126/22 TKCAT plasmid, the polymerase chain reaction product amplified using 278/22 TKCAT as template for the 5'–primer, 126Tdx (5'--278 to -22 of the nur77 promoter), the CAT PEI (5'-CCATTGGGATATATCAACGGTGG), and 3'–primer, CATPEI (5'-CCATTTGGGATATATCAACGGTGG), was restricted with HindIII and BglII and inserted into HindIII-BglII-digested 278/22 TKCAT. 126/91 TKCAT and 80/22 TKCAT were made by crimping the BglII-XbaI linker sequence to the thymidine kinase promoter, respectively, of 126/22 TKCAT followed by end repair with Klenow DNA polymerase and religation. The sequence of each construct was confirmed by direct sequence analysis. Plasmid DNA for transfection was prepared by the alkaline lysis procedure followed by Calf density gradient centrifugation (35).

In Vitro Mutagenesis—Mutagenesis of the nur77 promoter was carried out by the procedure described by Kunkel et al. (5). Briefly, the DNA fragment spanning nt -126 to +122 of the nur77 genomic sequence was inserted into M13mp19 replicative form DNA. Recombinant plasmids were obtained by transfection into Escherichia coli (36). The M13mp19 host cells and used to transformed E. coli C3266 cells to prepare uracil-containing single-stranded M13 DNA. Single-stranded plasmid DNA was annealed to oligonucleotides containing specific mutations (see Fig. 5A). Mutated double-stranded DNAs were obtained by synthesis of the complementary strand with T, DNA polymerase in the presence of dT, gene protein (Bio-Rad) and T, DNA ligase. Mutant plasmids were identified by DNA sequencing analysis. Altered nur77 sequences were isolated from the M13 replicative form DNA and inserted into the 126/77TR plasmid restricted with XbaI and BamHI.

Nucleotide Protection Assay—After transfection, total RNA samples were prepared as described (36) and analyzed using an S1 nucleotide protection assay (37). To prepare the 447-nt nur77 S1 probe (see Fig. 1A), the 1148/77TR plasmid was restricted with NcoI, dephosphorylated, and then phosphorylated with γ-[32P]ATP by T, DNA ligase (New England BioLabs, Inc., Beverly, MA). The resulting DNA was digested with BssHII, and the 447-bp nucleotide fragment was purified on 1.2% agarose gel.

To obtain the 847-bp double-stranded neo S1 probe, pSV2 neo was...
digested with BglII, dephosphorylated, and labeled with \([\gamma-\text{P}]\)ATP. After digestion with AccI, the probe was purified on 1.2% agarose gel. The single-stranded S1 probe for the CAT gene was generated by the method described previously (37) using the CATPE1 primer and the 278/22 TKCAT plasmid as template. The oligonucleotide primer was Klenow-labeled and hybridized to the template, followed by synthesis of the probe DNA using E. coli polynucleotide Klenow fragment. The synthesized DNA was restricted with XbaI, and the labeled single-stranded probe was purified from denaturing alkaline agarose gels.

For S1 nuclease protection assays, total cellular RNA (30 \(\mu\)g) was precipitated and resuspended in 30 \(\mu\)l of S1 hybridization buffer (400 mM NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA in 80% deionized formamide) containing 1 \(\times\) 10\(^{-5}\) cpm of S1 probe. Hybridization was carried out at 55 °C for \(nur77\) mRNA and at 50 °C for neo and CAT mRNAs overnight. RNA was digested with 400 units of S1 nuclease (Boehringer Mannheim) in buffer containing 0.28 M NaCl, 50 mM sodium acetate, pH 4.5, and 4.5 mM ZnSO\(_4\), for 1 h at 37 °C for \(nur77\) mRNA or at 30 °C for neo and CAT mRNAs. The digestion was stopped with 80 \(\mu\)l of 4 M ammonium acetate, 20 mM EDTA, pH 8.0, and 5 \(\mu\)g of tRNA. The reaction products were precipitated with ethanol at -80 °C for 1 h. The protected products were fractionated on 8 M urea, 6% polyacrylamide gel followed by autoradiography.

**RESULTS**

**Inducible Expression of nur77 Minigene upon NGF and KCl Treatment in PC12 Cells**—It was previously found that \(nur77\) transcript is induced rapidly and transiently by treatment with NGF or KCl in PC12 cells (21, 24). \(nur77\) mRNA is detectable within 15-30 min of NGF addition, accumulates to peak levels by 1-2 h, and declines to an undetectable level by 4 h. Membrane depolarization induces greater and more prolonged \(nur77\) expression than NGF; thus, the mRNA accumulates beyond 4 h upon KCl stimulation (24). Treatment with either NGF or KCl in the presence of the protein synthesis inhibitor cycloheximide results in superinduction (21, 24). These results place the 5'-boundary of the minimal promoter region sequences that mediate induction by NGF and membrane depolarization in PC12 cells.

**Promoter—** Although the inducible expression of immediate-early genes including \(nur77\) does not require de novo protein synthesis, we tested whether the promoter sequence identified exhibited this property. The 126/77TR and 86/77TR minigenes were transfected into PC12 cells, which were stimulated with NGF or KCl for 30 min or 3 h in the presence or absence of cycloheximide. After 30 min of NGF stimulation, the levels of expression were similar in the presence or absence of cycloheximide (Fig. 3A). By 3 h after NGF addition, \(nur77\) mRNA declined to low levels in the absence of cycloheximide, whereas in its presence, the mRNA accumulated to a high level. This was observed for both the endogenous \(nur77\) gene and the transfected minigenes. Similar results were also obtained when cells were stimulated with KCl (Fig. 3B). These results show that inducible expression directed by the first 86 nt of the \(nur77\) promoter closely mimics that of the endogenous gene and does not require de novo protein synthesis.
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**Fig. 1.** Expression of transiently transfected nur77 minigene. A, structure of the intronless nur77 minigene. Various sequence elements are indicated schematically. The nur77 promoter, including 1148 bp of 5'-flanking sequence, was fused to the nur77 cDNA interrupted by an 11-bp linker. The 447-nt S1 probe corresponds to a segment of the minigene including the linker and was labeled at the NcoI site marked with an asterisk. Transcripts correctly initiated from the transfected minigene yield a 242-nt S1 nuclease-protected product, whereas the endogenous nur77 mRNA yields a 109-nt protected product. The numbers indicated on the promoter refer to 5'-deletion end points (see Fig. 2). B, induction of the transiently transfected nur77 minigene by NGF. The 1148/77TR minigene was transfected into PC12 cells by calcium precipitation; cells were then stimulated with NGF (50 ng/ml) in the absence or presence of cycloheximide (CHX) (10 μg/ml) for the indicated periods, and total cellular RNA was analyzed by S1 nuclease protection. Protected products corresponding to transcripts from the transfected minigene, the endogenous nur77, and the reference plasmid pSV,NEO are indicated. Control samples include S1 protection assays carried out using tRNA or untransfected cell RNA (-). C, induction of nur77 minigene by KCl. PC12 cells transiently transfected with the 1148/77TR minigene were stimulated with KCl (60 mM) in the absence or presence of cycloheximide for the indicated periods.

Therefore, we tested whether the nur77 5'-flanking sequence can faithfully confer inducibility on a heterologous gene. Various nur77 promoter fragments were inserted upstream of the herpes simplex virus thymidine kinase gene basal promoter linked to the bacterial CAT gene coding sequence (Fig. 4A). The numeric designation of each test plasmid indicates the position of the 5'- and 3'-end points of the nur77 promoter fragment inserted. Following cotransfection of the test plasmid and the reference plasmid pSV,NEO, cells were treated with NGF or KCl for 1 h followed by RNA isolation. Using a 264-nt probe in an S1 nuclease protection assay, the CAT message correctly initiated from the thymidine kinase promoter should yield a 138-nt protected product (Fig. 4A).

As expected, the parental TKCAT plasmid containing only the thymidine kinase basal promoter failed to show inducible expression upon NGF or KCl treatment (Fig. 4B). By contrast, the 278/22 TKCAT construct was strongly inducible by NGF and KCl. The 126/22 TKCAT and 90/22 TKCAT plasmids were also efficiently induced by NGF or KCl (Fig. 4B). The 126/91 TKCAT construct was also inducible, although the magnitude of induction was lower than that of the other inducible constructs. The 90/61 TKCAT construct, however, was virtually uninducible by either NGF or KCl. These results indicate that the functional elements sufficient for induction by either NGF or KCl are located between nt -90 and -22, whereas sequences between nt -126 and -91 can also mediate a low level of induction. Furthermore, induction of 90/22 TKCAT is also cycloheximide-resistant (data not shown). These results are consistent with those from the 5'-deletion analysis (Fig. 2), which placed the 5'-boundary of the minimal inducible promoter between nt -86 and -60. Taken together, these data indicate that the sequence between nt -86 and -22 contains the minimal elements sufficient to confer induction by both NGF and membrane depolarization. In addition, sequences between nt -126 and -91 may also participate in mediating induction.

Requirements of AP1-like and GC-rich Sequences for Inducibility—To gain further insight into the sequence requirements for the induction of nur77, we examined the promoter region more closely (Fig. 5A). Between nt -126 and -22 there
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are several potential binding sites for known transcription factors, including c-Ets (39), SP1 (28), ZIF268 (29), and CREB (40) or AP1 (41, 42). We tested the importance of these sequences for nur77 induction by assessing the effects of mutations that alter them (Fig. 5A). All such mutations were derived from the 126/77TR minigene and are identical to it except for the specified base substitutions.

Among the mutants tested, three severely affected inducibility by NGF and KCl (Fig. 5). First, mutant M5, which destroys the AP1-like sequence centered at nt -67, lost virtually all inducibility by NGF or KCl. Second, mutant M6, which alters the GC-rich/SP1 sequence centered at nt -51, was uninducible. Third, alterations in the AP1-like sequence centered at nt -38 in mutant M7 also abolished inducibility. These results show that the GC-rich/SP1 sequence at nt -54 and the AP1-like sequences at nt -38 and -67 are essential for induction by both NGF and KCl. Mutation of any of these elements abolishes inducibility.

Mutants M5–M7 alter sequences within the 69-bp fragment (nt -90 to -22) defined by chimeric promoter analysis to be required for conferring inducibility. The M4 mutation, which alters the GC-rich element centered at nt -77, had no discernible effect on inducibility. Since deletion analysis placed the 5' boundary of the essential sequence for induction between nt -86 and -60 and since mutations in nt -73 to -75 had no effect, it is likely that the true 5' boundary for the minimal inducible promoter is downstream of nt -72.

The induction of c-fos by NGF is mediated through the SRE, which contains a CArG box as its central core (15). The CArG box is defined by the consensus sequence CC(A/T)GG (43) and can by itself mediate serum induction in fibroblasts (44). There is a CArG-like sequence in the nur77 promoter centered at nt -95 (Fig. 5A). This sequence (CTTGTATGG) deviates from the consensus sequence by having a GC pair within the A/T core. To determine whether this sequence plays a role in the activation of nur77, we created mutant M3, in which the CArG-like sequence was changed to completely destroy any ability to interact with the serum response factor (14, 45). Since mutant M3 is still inducible by NGF or KCl (Fig. 5), we conclude that this CArG-like sequence is not required for the activation of nur77 by NGF or KCl in PC12 cells. The only effect we observed with the M3 mutation is an increased basal level of expression in unstimulated cells (Fig. 5B).

In examining the sequences farther upstream, we found that the M2 mutation, which changes the GC-rich/SP1 sequence centered at nt -110, causes somewhat reduced induction by NGF and KCl. This sequence may help to enhance the level of induction. Altering the c-Ets binding site (M1 mutation) has no discernible effect on induction by NGF or KCl.

Taken together, these data indicate that induction of nur77 by both NGF and KCl requires sequences between nt -86 and -22, most likely between nt -72 and -22. Within this sequence, mutations at any of the three recognizable protein-binding sites (two potential AP1-binding sites and a GC-rich sequence) completely abolish inducibility. Furthermore, none of the deletion or base substitution mutations analyzed were able to dissociate inducibility by NGF from that by membrane depolarization.

DISCUSSION

Recent studies have identified a large number of immediate-early response genes that are coordinately regulated by extracellular signals, including growth or differentiation factors (1–3). Although a wealth of information has been garnered on the regulation of the c-fos protooncogene (9, 10), relatively little is known about the regulation of other immediate-early genes. While there are indications that multiple regulatory mechanisms control immediate-early gene expression upon mitogenic stimulation in fibroblasts (46), information on the complexity of regulatory mechanisms mediating gene activa-

\(^{1}\) G. T. Williams and L. F. Lau, manuscript in preparation.
**Fig. 4.** *nur77* promoter fragments confer inducibility on heterologous promoter. *A*, structure of *nur77*/TKCAT fusion gene constructs. Various *nur77* promoter fragments were inserted upstream of the herpes simplex virus thymidine kinase (HSV TK) promoter linked to the CAT gene coding sequence. The numeric designation of each TKCAT construct denotes the nucleotide positions of the 5' and 3' boundaries of the promoter fragment inserted. An *asterisk* indicates the labeled end of the 264-nt S1 probe. A 138-nt protected product represents the correctly initiated CAT transcript derived from the transfected fusion gene. *B*, analysis of promoter fusion constructs. *nur77*/TKCAT fusion genes were transiently transfected into PC12 cells; cells were harvested either without stimulation (UN) or after stimulation with NGF (N1) or KCl (K1) for 1 h. Total cellular RNA was analyzed by S1 nuclease protection analysis. The positions of the protected products corresponding to the neo and CAT transcripts are indicated, as are those of the undigested neo and TKCAT probes. A nonspecific S1 nuclease digestion product, which appears in the control digestion using only tRNA, is marked by an *asterisk.*

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**Fig. 5.** Essential sequence elements for activation of *nur77* by NGF and KCl. *A*, nucleotide sequence of *nur77* promoter from nt -126 to +4. Potential binding sites for known transcription factors are *underlined* and indicated. The sequences of base substitution mutations are shown. *B*-D, S1 nuclease protection analysis of base substitution mutants. The mutant or parental 126/77TR plasmid (WT) was transiently transfected into PC12 cells by electroporation. The cells were harvested either without stimulation (*B*) or after stimulation with NGF (*C*) or KCl (*D*) for 1 h. S1 nuclease protection products corresponding to the transfected *nur77* mutants, the endogenous gene, and the neo control are indicated.
tion by other agents in other cell types is scant. To understand how growth factors coordinately control the expression of immediate-early genes, it is important to determine how broadly the mechanism regulating c-fos expression can be generalized.

In this study, we have focused on identifying the sequences required for the activation of the immediate-early gene nur77 by NGF and membrane depolarization in PC12 cells. Our study utilizes an S1 nuclease protection assay to examine the expression of transfected nur77 minigenes designed to determine the sequence requirements for induction. Two lines of evidence indicate that sequences between nt -22 and -86 are sufficient to mediate induction by NGF and membrane depolarization. First, deletion analysis placed the 5'-boundary for the minimal essential element between nt -86 and -60 upstream of the transcription start site (Fig. 2). Second, the sequence between nt -22 and -90 is sufficient to confer correct immediate-early induction on a heterologous promoter (Fig. 4).

Within the sequence (nt -22 to -86) defined by deletion and chimeric fusion gene analyses, there are several potential binding sites for known transcription factors, including two AP1-like sequences and two GC-rich sequences (Fig. 5A). Analysis of base substitution mutations showed that alterations in the GC-rich sequence farthest upstream (nt -75 to -82) had no effect on activation by NGF or membrane depolarization. In contrast, mutations in any of the other three sequence elements, which lie between approximately nt -30 to -70 upstream of the major transcription start site, completely abolish inducibility. Taken together, our results argue that these three sequence elements, including two AP1-like sites and a GC-rich sequence, compose the minimal essential sequence for nur77 induction by NGF and membrane depolarization in PC12 cells. However, we have not yet identified the cellular proteins that actually bind to these sequences and activate transcription.

The two AP1-like elements contain an identical 7-bp sequence (TGCGTCA). This sequence can bind to members of the Fos and Jun families of proteins (data not shown) (48), suggesting that members of the AP1 family might participate in nur77 induction. However, most Fos and Jun family members appear to be absent or present at very low levels in the unstimulated PC12 cells (24). The exception is JunD, which is constitutively expressed. Interestingly, the same sequence has been shown to mediate transactivation of the proenkephalin gene promoter by JunD (49). Thus, the possibility that JunD might participate in nur77 induction is an attractive hypothesis.

In addition to members of the Fos/Jun families, the nur77 promoter AP1-like sequences might also function as CREs and interact with CREB. These AP1-like sequences are very similar to the FAP element (CTGCCTCA), which acts as a functional CRE in PC12 cells (50). They also contain the core sequence CGTCA found in most CREs (40, 51). The c-fos gene is known to be activated by membrane depolarization through the interaction of CREB with the c-fos CRE in PC12 cells (7, 15). Thus, the AP1-like sequences might function as CREs and mediate activation through interaction with CREB.

The GC-rich/SP1 element contains two overlapping copies of the SP1-binding site (28) and a binding site for a family of zinc finger proteins including ZIF268, Krox20, NGFI-C, and the Wilms tumor gene product (WT1), which recognize the sequence GCGGGGCGG (29, 30). Since the M6 mutation disrupted both SP1 sites and the zinc finger protein-binding site (Fig. 5A), we cannot pinpoint which site(s) is essential for activation. The zinc finger proteins mentioned above, except WT1, are encoded by immediate-early genes and may not be present in unstimulated PC12 cells. Since the activation of nur77 does not require de novo protein synthesis, it is likely that constitutively expressed transcription factors such as SP1 may act upon this site. Two other GC-rich/SP1 sequences centered at nt -110 and -170 have been identified. It is possible that these sequences enhance the magnitude of inducible expression, as 5'-deletion analysis suggests that sequences upstream of the minimal essential promoter are important for maximal expression (Fig. 2).

In this study, we failed to distinguish any difference in the sequence requirements for activation of nur77 by NGF and KCl. An interesting possibility is that NGF and membrane depolarization might mobilize different signal transduction pathways that ultimately converge on the same sequences on the promoter. These alternate pathways might involve JunD and CREB, acting through the identical AP1-like sequences. Alternatively, there may be distinct sequence requirements for NGF and KCl induction of nur77 that can be revealed only upon further mutagenesis. Future studies aimed at identifying the protein complexes that form on the promoter following NGF or KCl stimulation will help to address the mechanisms of activation mediated by these two agents.

For the c-fos protooncogene, it is known that NGF and serum growth factors activate transcription through the SRE, whereas KCl and cAMP activate the gene through the interaction of CREB with the CRE sequence in the promoter (9, 10). These two sequences are distinct and separable. Interestingly, at nt -95 in the nur77 promoter there is a sequence that resembles the CArG box, which composes the core of the SRE. However, several lines of evidence indicate that this CArG box-like sequence is not the key element in NGF-mediated induction. First, this sequence deviates from the CArG consensus sequence by having a G/C bp within the central A/T region, and it binds poorly to the serum response factor translated in vitro (data not shown). Second, 5'-deletion analysis places the minimal essential sequence downstream of this CArG box-like sequence. Third, a promoter fragment (nt -22 to -90) identified as able to confer NGF inducibility excludes this sequence. Finally, mutations of this sequence in the context of a nur77 minigene do not affect NGF induction. These data suggest that the activation of nur77 and c-fos by NGF occurs through different mechanisms. Since a promoter fragment (nt -91 to -126) that includes this sequence can confer a small level of inducibility on a heterologous promoter (Fig. 4B), the possibility that it might play an auxiliary role in induction cannot be ruled out.

Recently, Freter et al. (47) reported that a 7-nt motif located in the 3'-untranslated region of the JE gene is able to mediate immediate-early expression in fibroblasts. Although this 7-nt motif is present in the 3'-untranslated region of nur77, it is not able to mediate nur77 induction in PC12 cells by NGF or membrane depolarization. Two lines of evidence support this conclusion. 1) nur77 promoter sequences alone can confer NGF inducibility on a heterologous promoter; and 2) specific mutations within the nur77 promoter sequence not affecting this 7-nt motif can abolish inducibility in the context of a nur77 minigene. These observations further support the diversity of mechanisms for immediate-early gene activation in different cell types induced by different stimulatory agents.

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