Involvement of JunD in Transcriptional Activation of the Orphan Receptor Gene *nur77* by Nerve Growth Factor and Membrane Depolarization in PC12 Cells

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***nur77*, an immediate-early gene that encodes an orphan nuclear receptor, is rapidly and transiently induced by nerve growth factor (NGF) stimulation or membrane depolarization in the rat pheochromocytoma-derived cell line PC12. The Nur77 protein can act as a potent transcription activator and may function to regulate the expression of downstream genes in response to extracellular stimuli. We show here that activation of *nur77* by NGF treatment and membrane depolarization is signalled through distinct pathways. These distinct signals appear to converge on the same transcription factors acting on the same promoter elements. We show that *nur77* activation by both processes requires two *cis*-acting AP1-like elements, NAP1 and NAP2, which contain the core sequence TGCCTCA centered at 67 and 38 nucleotides upstream of the transcription start site. The NAP elements can confer inducibility by NGF and membrane depolarization on an otherwise unresponsive heterologous promoter. We identified JunD as a key mediator of *nur77* activation by reason of the following observations. (i) JunD, but not CREB or other members of the Fos/Jun family, is a component of NAP binding activity in PC12 cell nuclear extracts. (ii) JunD, but not other Fos/Jun family members, specifically transactivates the *nur77* promoter through the NAP elements (iii) A dominant-negative mutant of JunD effectively abolishes the activation of *nur77* by either NGF treatment or membrane depolarization. These data draw a contrast between the regulation of *nur77* with that of *c-fos*, in which the sequence requirements for activation by NGF treatment and membrane depolarization appear separable, and CREB appears to play a role in activation by both NGF and membrane depolarization.**

The primary genetic response in mammalian cells exposed to a variety of extracellular signalling agents is the rapid and transient induction of a set of genes, called immediate-early genes (28, 36). The induction of these genes does not require *de novo* protein synthesis and is likely to be mediated by the posttranslational modification of existing transcription factors. Many immediate-early genes are known to encode transcription factors thought to regulate the expression of downstream genes, which may in turn act as effectors of the cellular responses to the extracellular stimuli.

A particularly interesting cell culture system for studying transcriptional activation of immediate-early genes is the rat pheochromocytoma-derived cell line PC12, since it can undergo disparate cellular processes depending on the stimulating agents (15, 21, 22). Nerve growth factor (NGF) stimulates PC12 cells to differentiate into sympathetic neuron-like cells, whereas epidermal growth factor is mitogenic without inducing differentiation. In addition, PC12 cells have excitable membranes that could be depolarized by specific neurotransmitters or elevated levels of extracellular KCl (13, 20, 22). Thus, PC12 cells provide a model for studying gene regulation in response to agents that induce either differentiation or proliferation or to stimuli that mimic neurotransmission.

Among immediate-early genes, the proto-oncogene *c-fos* has been the most extensively studied with regard to its transcriptional regulation (2, 16, 47, 54, 60). The activation of *c-fos* in PC12 cells by NGF, epidermal growth factor, or tetradecanoyl

phorbol acetate (TPA) is mediated primarily through the serum response element (53, 63). The *c-fos* serum response element contains a CAAT box (41), which binds to a 67-kDa serum response factor (59), and an adjacent binding site for a protein of the Ets family, most likely Elk-1 or SAP-1 (7, 29, 38). In contrast, transcriptional activation of *c-fos* by membrane depolarization, Ca²⁺ influx, and elevation of cyclic AMP (cAMP) is mediated through a cAMP response element (CRE)-like sequence that binds CREB *in vitro* (14, 53, 55). The transcription efficacy of CREB is known to be modulated by phosphorylation on serine 133, either by the cAMP-dependent protein kinase A (PKA) (19, 71) or by the Ca²⁺/calmodulin-dependent protein kinase II (CaMKII kinase) (8, 56). These results suggest that membrane depolarization might activate *c-fos* by activating CREB via signalling through cAMP- or Ca²⁺-dependent kinases. In addition, full induction of *c-fos* by NGF also requires intact CREs and involves the activation of CREB through a Ras-dependent protein kinase (17).

To expand our knowledge of the mechanisms regulating immediate-early gene activation, we have studied the transcriptional regulation of the orphan nuclear receptor gene *nur77* (72). *nur77* (also known as *N10* and *NGFI-B*) (26, 40, 51) is rapidly and transiently induced by serum growth factors in fibroblasts (26, 66) and by NGF stimulation and membrane depolarization in PC12 cells with kinetics similar to those of *c-fos* (3, 40). The *nur77*-encoded protein is a member of the steroid hormone receptor superfamily (26, 40) and has been shown to act as a potent transcriptional activator through interactions with DNA at sequence-specific sites (9, 10, 67, 68). Studies of the expression of *nur77* suggest that Nur77 may play important roles in the central and peripheral nervous systems and in steroidogenic organs, including the adrenal gland (11,

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TABLE 1. Oligonucleotides used for mutant constructions

Mutant	Oligonucleotide
M8	5'-CACACGGCGCCTTATAGCTTTGGCC
M9	5'-GCCACTGACTTGTACGGCCCCGG
M10	5'-GCGGGGGCGAAGTTGACGCACA
M11	5'-GAGGGGCGGGTTATCCACTGACGC
M12	5'-ACGCATGGAGCAATGCGGGGGCGC
M13	5'-CGTGACGCATAATCAGGGGCGGGG
M14	5'-CTCCGTGACTTGTGGAGAGGGG
M15	5'-CTTAAGCGCGAATTGACGCATG

44, 64, 65, 68), and may also play a role in activation-induced apoptosis in T lymphocytes (37, 70).

In a previous study, we have shown that the promoter region spanning nucleotides (nt) 86 to 22 upstream of the transcription start site contains the *cis*-acting sequences that are both necessary and sufficient for induction by both NGF and KCl in PC12 cells (72). This region contains two AP1-like elements (NAP-1 and NAP-2) and a GC-rich sequence that appear to be essential (72). In contrast, sequences further upstream that contain a CArG-like element and an Elk binding site are required for induction by serum growth factors in mouse fibroblasts (66).

In the present study, we have extensively analyzed the *nur77* promoter to define more precisely the elements responsible for *nur77* induction in PC12 cells. Although the signalling pathways mediating NGF and KCl are distinct, the sequence elements required for their activation are not distinguishable from each other. We present evidence that JunD, a member of the Fos/Jun family, functions as a transcriptional activator of *nur77* in PC12 cells. Furthermore, a dominant-negative JunD mutant effectively blocks the induction of *nur77* by NGF and KCl. These data point to differential regulation of *nur77* and *c-fos* by NGF and KCl in PC12 cells.

MATERIALS AND METHODS

Cell culture. PC12 cells were grown as previously described (72). Cells were stimulated with NGF (50 ng/ml), TPA (Sigma; 100 ng/ml), forskolin (Sigma; 20 μ M), or KCl (60 mM) for various durations as indicated below. The protein kinase inhibitors H-89 (4) and KN-62 (58) were dissolved in dimethyl sulfoxide according to the manufacturer's instructions (Seikagaku America Inc.) and added to culture medium to 20 and 10 μ M, respectively, 30 min before stimulation.

In vitro mutagenesis and plasmid construction. The construction of mutants M5, M6, and M7 was previously described (72). Specific mutations of the *nur77* promoter were created by site-directed in vitro mutagenesis (35). Single-stranded M13 DNA containing the *nur77* promoter spanning nt -126 to +122 was used as a template for mutagenesis as previously described (72), using the oligonucleotides listed in Table 1. 2NAP2 TKCAT (two copies of the NAP2 sequence) and 3SP1 TKCAT (three copies of the SP1 sequence) plasmids were constructed by inserting synthetic double-stranded oligonucleotides into the *Xba*I site of TKCAT plasmid. For 2NAP2 TKCAT, the oligonucleotide 5'-CTAGACTCTCCATGCGT CACGGAGCGCTCTCCATGCGTCACGGAGCGT was used with the corresponding complementary strand. For 3SP1 TKCAT, the oligonucleotide 5'-CTAGACCCGCCCTCTCCT was used with the corresponding complementary strand and three copies were inserted. *nur77* promoter-luciferase fusion plasmids were made by transferring the *nur77* promoter fragment containing nt -86 to +122 or the same region containing

mutations in the NAP elements into *Nhe*I- and *Bgl*II-digested pGL2Basic plasmid (Promega), which contains the luciferase reporter gene.

Protein expression plasmids for c-Fos, c-Jun, JunB, and JunD were made by placing the full-length mouse cDNA sequences under the control of the simian virus 40 (SV40) early promoter in plasmid pSG5 (Stratagene). A dominant-negative mutant of JunD (JunD mut) was created by an internal deletion of amino acids 271 to 283 of JunD. This mutated JunD sequence was inserted downstream of the cytomegalovirus promoter of pCMV5 (provided by Robert Costa, University of Illinois, Chicago).

The plasmids pSV_{neo} and pPGK β -gal, containing the neomycin resistance gene driven by the SV40 promoter or the β -galactosidase gene driven by the phosphoglycerate kinase promoter, respectively, were used as reference plasmids to determine transfection efficiency. Plasmid DNA for transfection was prepared by the alkaline lysis procedure followed by CsCl density gradient centrifugation as described previously (72).

Transient transfection, S1 nuclease protection, and enzymatic assays. Transient transfection was performed by electroporation as described elsewhere (72). Total RNA isolation and S1 nuclease protection were performed as previously described (72). For enzymatic assays, transfected cells were washed with phosphate-buffered saline (PBS) and harvested in TEN buffer (40 mM Tris [pH 7.5], 1 mM EDTA, and 150 mM NaCl). Cell suspensions were split into two portions for preparing cell lysates for luciferase and β -galactosidase assays. The luciferase assay was performed with the luciferase assay substrate (Promega), using the single-photon mode of a scintillation counter (Beckman). β -Galactosidase activities were measured as described elsewhere (52).

RNA blot hybridization. Samples (10 μ g) of total cellular RNA were subjected to electrophoresis on a 1.2% formaldehyde-agarose gel and then transferred to nitrocellulose filters. The filters were hybridized with ³²P-labeled *nur77* cDNA probe (26) or α -tubulin cDNA probe (6) at 45°C in a buffer containing 50% formamide, 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 4 $\times$ Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium PP_i, and 10 μ g of salmon sperm DNA per ml.

Preparation of nuclear extracts. PC12 cells were washed with ice-cold PBS twice and resuspended in buffer A (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]-KOH [pH 7.9], 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μ g of leupeptin per ml, 10 μ g of pepstatin per ml, 100 μ g of aprotinin per ml, and 1 mM sodium orthovanadate). After incubation for 15 min on ice, the cell suspension was vortexed for 10 to 15 s at medium speed. Cells were observed under the microscope to confirm plasma membrane disruption. Cell nuclei were collected by centrifugation at 1,000 $\times$ g for 2 min at 4°C. Pelleted nuclei were resuspended in buffer B (20 mM HEPES-KOH [pH 7.9], 400 mM NaCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.2 mM EDTA, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g of leupeptin per ml, 10 μ g of pepstatin per ml, 100 μ g of aprotinin per ml, 1 mM sodium orthovanadate, and 50 mM sodium fluoride), and nuclear proteins were extracted on ice for 20 min. Nuclear debris were removed by centrifugation at 10,000 $\times$ g for 5 min at 4°C after extraction; the nuclear lysates were stored at -80°C.

In vitro transcription and translation. Plasmid DNAs containing cDNA sequences of c-Fos, c-Jun, JunB, JunD, JunD mut, and CREB (kindly provided by Marc R. Montminy) were

linearized with appropriate restriction enzymes and used as templates for in vitro synthesis of RNA with either T7 or SP6 RNA polymerase. In vitro translation was performed in the rabbit reticulocyte lysates programmed with the RNAs as directed by the manufacturer (Promega) in either the absence or the presence of [35 S]methionine.

Electrophoretic mobility shift assay (EMSA). Nuclear lysate or in vitro-synthesized proteins were incubated with 0.5 ng of the appropriate 32 P-labeled oligonucleotides in 12 mM HEPES-KOH (pH 7.9)–60 mM NaCl–1 mM MgCl₂–1 mM dithiothreitol–12% glycerol in the presence of 1 μ g of poly(dI-dC) and 1 μ g of salmon sperm DNA for 30 min at room temperature. For NAP2, the oligonucleotide 5'-TCGAGC TCTCCATGCGTCACGGAGCGC and the corresponding complementary strand were used. For AP1 binding assays, the oligonucleotide 5'-GATCCATGACTCAGAGGAAAAC ATAC and the corresponding complementary strand were used. For competition, a 20- or 100-fold molar excess of cold NAP2, NAP1 (5'-CTAGACGTGTGCGTCAGTGGT), M5 (5'-GGCGCCACTTCTGCACACGGC), M7 (5'-TCTCCAT GCAGAACGGAGCGC), AP1, CRE (5'-CTAGATTGCCT GACGTCAGAGAGT), or *c-fos* CaRE (5'-CTAGAGCCCG TGACGTTTACACTCAT) oligonucleotides were added with the corresponding complementary strand to the binding reaction mixture, which was then incubated on ice for 20 min prior to the addition of the 32 P-labeled probe. Anti-JunD (kindly provided by Rodrigo Bravo, Bristol-Myers Squibb, Princeton, N.J.), anti-*c-Fos*, anti-*c-Jun*, anti-CREB (Santa Cruz Biotechnology, Santa Cruz, Calif.), or anti-Nur77 (25) antibodies were added directly to the binding reaction mixture, which was then incubated on ice for 20 min prior to the addition of probe. Protein-DNA complexes were separated on a 4% polyacrylamide gel (1:80, bisacrylamide-acrylamide) with 0.25 $\times$ Tris-borate-EDTA buffer and visualized by autoradiography.

Immunoprecipitation of Fos-JunD heterodimers. Equal amounts of 35 S-labeled, in vitro-translated Fos, JunD, or JunD mut proteins were mixed and allowed to form complexes at room temperature for 30 min. The protein mixtures were incubated with either anti-Fos antibodies (Oncogene Science) or anti-JunD antibodies for 1 h on ice and then incubated with protein A-Sepharose CL-4B (Pharmacia) for 1 h with constant shaking at 4°C. The immunoprecipitates were collected by centrifugation and washed with a buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 2 mM EDTA, and 0.2% Nonidet P-40) three times and with 10 mM Tris (pH 7.5) once. The immunoprecipitated proteins were resolved by SDS–10% polyacrylamide gel electrophoresis (PAGE) followed by fluorography.

RESULTS

Distinct signalling pathways mediate induction of *nur77* by NGF and KCl. In PC12 cells, *nur77* is rapidly induced by stimulation with NGF and by membrane depolarization (3, 40). In addition, *nur77* can be induced by other agents such as TPA or forskolin (28, 36), suggesting that *nur77* induction may be mediated through the activation of protein kinases. To gain insight into the signal transduction pathways for *nur77* activation, we have examined the possible roles of different protein kinases.

That TPA induces *nur77* suggests that protein kinase C (PKC) is able to mediate *nur77* activation. To determine whether PKC activity is obligatory for *nur77* induction, we treated PC12 cells with TPA for 24 h to down-regulate PKC activity (48). The induction of *nur77* in response to NGF or KCl in TPA-treated cells was compared with that in untreated

FIG. 1. NGF and KCl induce *nur77* through distinct signalling pathways. PC12 cells were harvested either without stimulation (UN) or after 1 h of stimulation with NGF, TPA, KCl, or forskolin (FOS). Samples (10 μ g) of total RNA from these cells were analyzed by RNA blot hybridization with a 32 P-labeled *nur77* cDNA probe; a tubulin probe was also added in the same hybridization mixture to normalize the quantity and control for the quality of RNA. (A) PKC down-regulation. Cells were stimulated without TPA treatment (-) or were pretreated with TPA for 24 h (+) prior to stimulation with the indicated agents. (B and C) Effects of the specific kinase inhibitors on *nur77* expression. Cells were stimulated in the absence (-) or in the presence (+) of the PKA inhibitor H-89 (B) or of the CaMKII kinase inhibitor KN-62 (C). H-89 and KN-62 were added to the culture medium to 20 and 10 μ M, respectively, 30 min prior to stimulation with the indicated agents.

cells (Fig. 1A). *nur77* induction by TPA was observed after 1 h in naive cells but not in TPA-pretreated cells, demonstrating that PKC activity was effectively down-regulated. Treatment with NGF and KCl, however, resulted in similar levels of *nur77* induction in TPA-pretreated cells and untreated cells (Fig. 1A), indicating that induction of *nur77* by NGF and KCl can occur through pathways that do not involve phorbol ester-sensitive PKC.

nur77 is activated by treatment with forskolin, suggesting that the cAMP-dependent PKA may be involved in its activation (Fig. 1B). To test whether PKA is required for activation of *nur77* by NGF or KCl, we treated cells with the known PKA inhibitor H-89 (4) for 30 min prior to NGF or KCl stimulation (Fig. 1B). The activation of *nur77* by NGF was partially inhibited in the presence of H-89. In contrast, *nur77* activation by either KCl or forskolin was completely blocked by the inhibitor. These results indicate that PKA may mediate one

FIG. 2. Kinetics of expression of transfected *nur77* minigene. (A) Structure of the 126/77TR minigene (72). A total of 126 bp of *nur77* 5'-flanking sequence was fused to the *nur77* cDNA, which is interrupted by an 11-bp linker. (B) Expression of the transfected minigene. PC12 cells were transfected with 26 μ g of the 126/77TR minigene plasmid and 14 μ g of pSV₂neo plasmid as a reference by electroporation. Cells were stimulated with NGF or KCl for the indicated durations in the absence or presence of cycloheximide (CHX), and total cellular RNA was analyzed by an S1 nuclease protection assay. Protected products corresponding to transcripts from the transfected minigene, the endogenous *nur77*, and the *neo* gene are indicated.

component of *nur77* activation by NGF but plays a major role in the activation of *nur77* by KCl.

Since the main effect of KCl treatment in PC12 cells is the increase of intracellular Ca^{2+} levels through the influx of extracellular Ca^{2+} (13, 20, 22), we tested whether CaMII kinase can mediate *nur77* activation by treating cells with the CaMII kinase-specific inhibitor KN-62 (58) before NGF or KCl stimulation (Fig. 1C). The activation of *nur77* by KCl was completely inhibited by KN-62, whereas activation by NGF was not significantly affected. Therefore, CaMII kinase may be involved in the activation of *nur77* by KCl but not in activation by NGF. Although pharmacological approaches in the analysis of signalling pathways have limitations with regard to the specificity of the compounds used, our results show that induction by NGF and KCl is differentially affected by the same compounds. Taken together, these results show that *nur77* activation by NGF or KCl is mediated through distinct and separable pathways and suggest that PKA and CaMII kinase may play major roles in the activation by membrane depolarization.

Accurate transcription kinetics of transfected *nur77* minigenes. The kinetics of *nur77* expression in NGF- or KCl-stimulated PC12 cells are rapid and transient (3), suggesting that transcriptional repression, or the loss of activation, occurs soon after the onset of transcriptional activation upon stimulation. We previously reported that a *nur77* minigene contain-

ing 1.1 kb of sequences upstream of the transcription start site is expressed with kinetics identical to those of the endogenous *nur77* gene (72). Here, we show that the sequence information that specifies these expression kinetics is encoded within the first 126 bp upstream of the transcription start site, contained in the 126/77TR minigene (Fig. 2). The 126/77TR minigene was transfected into PC12 cells, and the time course of expression of the transfected gene was analyzed (Fig. 2B). As described previously, the endogenous levels of *nur77* mRNA induced by NGF reached a peak by 1 h and declined to the unstimulated level by 3 h, whereas the expression induced by KCl exhibited slightly more prolonged kinetics (72). The transfected *nur77* minigene is expressed with kinetics virtually identical to those of the endogenous *nur77* and was superinduced by the protein synthesis inhibitor cycloheximide.

NAP1 and NAP2 are the major *cis*-acting elements for NGF and KCl stimulation. We previously reported that a DNA fragment containing nt -90 to -22 of the *nur77* promoter can confer inducibility by NGF or KCl on a heterologous promoter (72). Within this DNA fragment, there are two AP1-like sequences (NAP1 and NAP2), two overlapping SP1 binding sites, and a Zif268 binding site (5) (Fig. 3A). To determine more precisely the promoter sequences required for activation of *nur77*, we created a series of base substitution mutations in the context of the 126/77TR minigene (Fig. 3A). The promoter activities of these mutant minigenes were analyzed by S1

FIG. 3. Mutational analysis of the *nur77* promoter. (A) Schematic illustration of the structure of the *nur77* minigene and sequences of the *nur77* promoter and mutants analyzed. Each mutant plasmid is identical to the 126/77TR minigene except for the nucleotide changes shown. The abilities of the mutant promoters to mediate induction by NGF and KCl are summarized. (B) S1 nuclease protection analysis of the *nur77* promoter mutants. Cells were transfected with 126/77TR or mutant minigenes and analyzed as described for Fig. 2. RNA samples were isolated from transfected cells which were either unstimulated (-) or stimulated with NGF (N) or KCl (K) for 1 h. A nonspecific S1 protection product (*) is indicated. W/T, wild type.

nuclease protection analysis of the minigene-derived transcripts following transient transfection into PC12 cells. Among the mutations tested, those that disrupted either one of the NAP sequences (M9, M5, M14, and M7) completely abolished activation by NGF or KCl (Fig. 3B). Thus, both NAP1 (nt -70 to -64) and NAP2 (nt -41 to -35) appear necessary for induction by NGF and KCl. The NAP1 and NAP2 elements share a sequence, 5'-TGGCTCA, even though their 5'- and

3'-flanking sequences are different. Mutations of the 5'-flanking sequences of both NAP1 and NAP2 (M4 and M13) or the 3'-flanking sequence of NAP2 (M15) did not have any effect, whereas mutation of the 3'-flanking sequence of NAP1 (M10) abolished *nur77* induction by either NGF or KCl.

The GC-rich region between the two NAP sites contains two overlapping SP1 sites as well as a Zif268 binding site. To distinguish which of these elements are required for induction,

FIG. 4. NAP elements confer inducibility on a heterologous promoter. (A) Structure of TKCAT fusion gene constructs. Either two copies of synthetic NAP2 oligonucleotides (2NAP2 TKCAT), three copies of SP1 oligonucleotides (3SP1 TKCAT), or the *nur77* promoter fragment spanning nt -90 to -22 (90/22 TKCAT) was inserted in front of the herpes simplex virus thymidine kinase (HSV TK) basal promoter linked to the CAT gene coding sequence. (B) S1 nuclease protection analysis of TKCAT fusion genes. A 26- μ g amount of each TKCAT fusion gene plasmid and 14 μ g of pSV40neo reference plasmid were transfected into PC12 cells. Transfected cells were either not stimulated (-) or stimulated with NGF (N) or KCl (K) for 1 h, and expression of the fusion genes was analyzed by S1 nuclease protection assay of the CAT message as described elsewhere (72). The protected products corresponding to the correctly initiated CAT transcript and the *neo* reference transcript are indicated, as is a nonspecific product (*).

we created a mutation that disrupted only the Zif268 binding site without affecting the SP1 binding sites (M11) and a mutation that disrupted both the SP1 and the Zif268 binding sites (M6). The M11 construct showed strong induction by both NGF and KCl, whereas M6 exhibited no induction (Fig. 3B), indicating that the Zif268 site is not essential for activation of *nur77* by NGF and KCl. Mutations of the 3'-flanking region of the SP1 sites (M12 and M13) showed no effect. Taken together, these results indicate that the NAP1, NAP2, and SP1 elements are essential for induction of *nur77* by NGF and KCl. Thus, although we have shown that induction by NGF and KCl is mediated through distinct signalling pathways (Fig. 1), the sequence requirements for induction by these agents appear to be indistinguishable.

To further evaluate the roles of the NAP elements and SP1 sites in *nur77* activation, we examined their ability to confer inducibility on a heterologous promoter. We tested two specific constructs, one containing two copies of a region bearing the NAP2 element (nt -48 to -28) of the *nur77* promoter (2NAP2 TKCAT; Fig. 4A) and the other containing three copies of the SP1 site (3SP1 TKCAT; Fig. 4A) cloned upstream of the thymidine kinase promoter driving expression of the chloramphenicol acetyltransferase (CAT) gene. Following transient transfection, the CAT transcript was detected by S1 nuclease protection assay (Fig. 4B). The parental vector, TKCAT, which contains the thymidine kinase basal promoter, showed no induction whatever by either NGF or KCl. In

contrast, a construct that contains nt -90 to -22 of the *nur77* promoter exhibited efficient induction by both NGF and KCl, as previously reported (72). The 2NAP2 TKCAT construct showed strong induction by both NGF and KCl (Fig. 4B), indicating that the NAP elements can confer inducibility on a heterologous promoter. In addition, the induction was super-induced in the presence of cycloheximide (data not shown), showing that the activation through NAP elements is indeed an immediate-early response. These results indicate that the NAP elements mediate activation by NGF and KCl.

In contrast, the 3SP1 TKCAT construct failed to show any induction by either NGF or KCl (Fig. 4B). Since the SP1 site is required in the context of the *nur77* minigene (Fig. 3) but itself cannot function as an inducible element, we interpret these data to indicate that the SP1 element acts as an essential element for *nur77* promoter function rather than an inducible element. In the context of the thymidine kinase basal promoter, which contains SP1 sites, the NAP elements can function to confer inducibility.

JunD is a component of NAP element binding activity in PC12 cell nuclear extracts. To identify which proteins are bound to the NAP elements, an EMSA was employed to detect NAP element binding activity in PC12 cell nuclear lysates with ³²P-labeled NAP2 synthetic oligonucleotides used as probes. In nuclear extracts of cells stimulated with NGF for 20 min, we detected specific NAP binding activity (Fig. 5). This binding was abolished by the presence of excess cold NAP1 or cold

FIG. 5. NAP element binding activity in PC12 cell nuclear extracts. Nuclear extracts (20 μ g) from PC12 cells stimulated with NGF for 20 min were incubated with 32 P-labeled, double-stranded NAP2 oligonucleotides either in the absence (—) or presence of 20- or 100-fold excesses of the indicated unlabeled oligonucleotides as competitors. The DNA-protein complexes were detected as slowly migrating bands relative to the free probe in an EMSA. A nonspecific complex (*) is indicated.

NAP2 oligonucleotides, suggesting that the same binding proteins are likely to interact with both NAP1 and NAP2 elements. In contrast, the mutant oligonucleotides (M5 and M7) which contain mutations that abolished promoter activity were unable to compete with the NAP binding activity (Fig. 5). This result indicates that the NAP-binding proteins may include the transactivator of *nur77*. In addition, NAP binding activity was efficiently inhibited by oligonucleotides containing the CRE (TGACGTCA) or *c-fos* CRE/CaRE (TGACGTTT) and, to a lesser extent, by an AP1 (TGACTCA) oligonucleotide. This result suggests that members of either the CREB or the AP1 family of proteins may be components of the NAP

binding activity. Similar results were obtained with nuclear extracts from either unstimulated cells or KCl-stimulated cells (data not shown). These results are consistent with the idea that protein synthesis is not required for the activation of immediate-early genes and that preexisting factors are responsible for activation.

To further investigate the involvement of CREB or AP1 transcription factors in NAP binding, we applied antibodies specific to CREB or to Fos/Jun family members in NAP binding reaction mixtures for the EMSA (Fig. 6A). Among the antibodies tested, only anti-JunD antiserum was able to supershift the NAP element-protein complex, detected as a band with a slower electrophoretic mobility (complex II). Neither preimmune serum nor antibodies specific for CREB or any other Fos/Jun family members tested were able to supershift the NAP-protein complex. Since the same CREB or Fos/Jun antibodies are able to form supershifted complexes against their cognate *in vitro*-synthesized protein bound to DNA, this observation is not due to the inability of the antibodies to form a supershift complex in this assay (Fig. 6B). Thus, among the proteins tested, JunD can be identified as a component of the NAP binding activity in PC12 cell nuclear extracts, whereas CREB, thought to be involved in the activation of *c-fos* by both NGF and membrane depolarization (17, 54, 55), does not appear to be a component of the NAP binding complex.

To investigate the binding of JunD to the NAP elements further, *in vitro*-transcribed and -translated JunD was used in reaction mixtures for binding to the NAP2 oligonucleotide for the EMSA (Fig. 7). While JunD alone failed to interact efficiently with either NAP1 or NAP2, it could bind effectively to both NAP1 and NAP2 in the presence of *c-Fos*. This result shows that bona fide JunD synthesized *in vitro* is able to bind the NAP elements in a heterodimeric form. However, since *c-fos* is not expressed in unstimulated PC12 cells (3), JunD is not likely to act through interaction with *c-Fos*. CREB synthesized *in vitro* was unable to form a complex with NAP1 or NAP2, either alone or in the presence of JunD. From these results and the results from the antibody supershift experiment (Fig. 6), we conclude that JunD is a component of NAP

FIG. 6. JunD is a component of NAP element binding activity. (A) Antibody supershift analysis of NAP element binding activity. Nuclear extracts (20 μ g) from PC12 cells stimulated with NGF for 15 min were incubated with 2 μ l of preimmune serum (PI), 2 μ l of anti-JunD serum (JunD), or 5 μ l of antibodies specific for *c-Fos*, *c-Jun*, JunB, CREB, or Nur77 prior to the addition of the labeled NAP2 oligonucleotides. A 100-fold molar excess of unlabeled NAP2 double-stranded oligonucleotides was included in one instance (+). (B) Antibody supershift of proteins synthesized *in vitro*. 32 P-labeled AP1 or CRE oligonucleotides were incubated with *in vitro*-synthesized Fos/Jun complex or CREB as indicated. The same amounts of antibodies used in the experiment whose results are shown in panel A were added to the binding reaction mixtures.

FIG. 7. Specificity of binding of JunD to NAP elements. Proteins were obtained by in vitro translation in rabbit reticulocyte lysates programmed with either *c-fos*, *junD*, or *CREB* RNA. A 2.5- μ l sample of each reaction mixture was used in an EMSA in the presence of either the labeled NAP1 (A) or NAP2 (B) double-stranded oligonucleotides in various combinations as indicated. The total volume of reticulocyte lysates was adjusted to 5 μ l.

element binding activity and may play a role in the activation of *nur77*.

JunD transactivates *nur77*. Since JunD was recognized as being involved in NAP binding, we tested its ability to transactivate *nur77*. For this purpose, we constructed a reporter gene by fusing the *nur77* promoter region (nt -86 to +122) to the luciferase coding sequence (Fig. 8A). The *nur77* promoter activity could be detected as luciferase activity. In cotransfection experiments with vectors that express proteins of the Fos/Jun family, only expression of JunD was able to transactivate the *nur77* promoter (about 40-fold; Fig. 8B). In contrast, expression of either c-Fos, c-Jun, or JunB was unable to transactivate *nur77* (Fig. 8B). Thus, JunD protein is unique among members of the Fos/Jun family in its ability to transactivate *nur77*.

To determine whether JunD transactivates *nur77* through the NAP elements, we mutated the NAP elements in the context of the luciferase reporter construct (Fig. 8A). When either NAP1 (86LucM5) or NAP2 (86LucM7) was mutated, transactivation by JunD decreased to 30 and 10% of that of the wild-type promoter, respectively (Fig. 8C). When both NAP1 and NAP2 elements were mutated (86LucM57), induction by JunD was abolished. These results indicate that transactivation of *nur77* by JunD is mediated through the NAP elements, which we have shown to be the crucial elements for activation by NGF and KCl.

When PC12 cells were transfected with a JunD expression vector and then stimulated with either NGF or KCl, a further increase in transactivation of *nur77* was observed (Fig. 8D). Thus, while a high level of JunD protein can propel the transcription of *nur77*, stimulation of the cell with NGF and KCl can activate signalling pathways that act to enhance *nur77* expression, possibly by modification of JunD or proteins that interact with JunD.

A JunD dominant-negative mutant blocks *nur77* activation by NGF and KCl. To investigate the role of JunD in *nur77* activation more directly, we developed a dominant-negative mutant of JunD to block the activities of the wild-type JunD

protein. Since dominant-negative mutants of c-Fos and c-Jun have been successfully created by deleting their DNA binding domains (43, 45), we adopted a similar strategy (Fig. 9A). The JunD mutant, which has its DNA binding domain deleted, should retain the capacity to form homodimers or heterodimers through the leucine zipper region but should be unable to bind DNA. Thus, we reasoned that it would be possible to block the function of wild-type, endogenous JunD by overexpressing such a JunD dominant-negative mutant in cells.

We first characterized the properties of the JunD mutant protein. Wild-type and mutant JunD proteins were generated by in vitro transcription-translation and labeled with [35 S]methionine (Fig. 9B). To test their abilities to form dimers, labeled wild-type or mutant JunD was mixed with similarly labeled c-Fos to allow dimerization. The resulting proteins were subjected to immunoprecipitation with either anti-c-Fos or anti-JunD antibodies, and the precipitated proteins were fractionated by SDS-10% PAGE (Fig. 9B). Both wild-type and mutant JunD were coprecipitated by the anti-c-Fos antibodies, even though the anti-c-Fos antibodies did not recognize either form of JunD (Fig. 9B). Likewise, immunoprecipitates with anti-JunD antibodies contained c-Fos protein. These data indicate that the mutant JunD can form heterodimers with c-Fos as efficiently as the wild-type JunD.

To assess the ability of either the mutant or the wild-type JunD to bind DNA, the JunD proteins were subjected to an EMSA with 32 P-labeled AP1 oligonucleotide used as a probe in either the presence or the absence of c-Fos (Fig. 9C). While c-Fos or JunD by itself could not form a DNA-protein complex, the mixture of c-Fos and wild-type JunD formed a strong complex with the AP1 oligonucleotide. In contrast, no DNA binding activity could be detected with the mutant JunD, even in the presence of c-Fos (Fig. 9C). Moreover, when the mutant JunD protein was included in a binding reaction mixture with the wild-type JunD, the DNA binding activity of the wild-type protein was abolished (Fig. 9D). Taken together, these results indicate that the mutant JunD retains its ability to

FIG. 8. Transactivation of *nur77* by JunD. (A) Structure of *nur77*-luciferase fusion gene constructs. nt -86 to +122 of the *nur77* promoter were fused to the luciferase gene; base substitution mutations that ablated either the NAP1 (M5) or the NAP2 (M7) element or both (M57) are shown. (B) Transactivation of *nur77* by JunD. PC12 cells were cotransfected with 2.5 μ g of *nur77*-luciferase fusion gene (86LucWT), 6 μ g of pPGK β -gal plasmid, and 20 μ g of plasmid expressing either c-Fos, c-Jun, JunB, or JunD. (C) JunD transactivates through NAP elements. PC12 cells were cotransfected with 20 μ g of pSGJunD, 6 μ g of pPGK β -gal, and 2.5 μ g of either wild-type *nur77*-luciferase fusion gene (86LucWT) or mutant constructs (86LucM5, 86LucM7, or 86LucM57) as indicated. (D) Effects of NGF and KCl stimulation on transactivation by JunD. PC12 cells were cotransfected with 2.5 μ g of 86LucWT DNA, 6 μ g of pPGK β -gal plasmid, and 10 μ g of pSG5 or pSGJunD plasmid. Transfected cells were either not stimulated or stimulated with NGF or KCl. Luciferase activities were normalized with β -galactosidase activities for transfection efficiencies. Results shown are the average of four independent experiments except for panel D (three independent experiments).

form heterodimers but cannot bind DNA and interferes with the DNA binding activities of the wild-type JunD in a dominant-negative fashion.

We next questioned whether this dominant-negative mutant JunD can block the induction of *nur77* by NGF and KCl. An expression construct with the mutant JunD coding sequence driven by the cytomegalovirus promoter was transiently cotransfected with the 126/77TR *nur77* minigene into PC12 cells. As expected, transfected cells that were unstimulated did not show induction of *nur77*, whereas cells transfected with the parental vector showed strong induction of the endogenous and transfected *nur77* upon stimulation by NGF or KCl (Fig. 10). However, when the mutant JunD expression vector was titrated into the transfection mixture, activation of the transfected *nur77* by either NGF or KCl was inhibited in a dose-dependent manner (Fig. 10). Since these transfections were carried out by electroporation under conditions in which most of the cells die, a large proportion of the surviving cells were

transfected. Under these conditions, the induction of the endogenous gene was also affected by the overexpressed mutant JunD. The expression of a dominant-negative JunD protein is thus able to effectively block the induction of *nur77* by NGF or KCl. Similar results were obtained when the *nur77* promoter-luciferase construct was used as a reporter in place of the 126/77TR minigene (data not shown). From these results taken together, we conclude that JunD protein is indeed a transcription activator involved in the induction of *nur77* by NGF or KCl in PC12 cells.

DISCUSSION

NGF and KCl activate *nur77* through distinct but convergent signalling pathways. PC12 cells respond to NGF treatment and membrane depolarization with vastly different biological consequences (21, 22). Paradoxically, many of the same immediate-early genes are induced in both processes, suggest-

FIG. 9. Dominant-negative mutant of JunD. (A) Schematic diagram of wild-type and dominant-negative JunD. Amino acid residues 271 to 283 within the DNA binding domain were deleted in mutant JunD. (B) Dimerization of JunD mutant. *c-Fos*, JunD, and mutant JunD were translated in vitro in rabbit reticulocyte lysates in the presence of [³⁵S]methionine (lanes IVT). Reticulocyte lysates (10 μ l each) containing *c-Fos* were mixed with lysates containing JunD or mutant JunD, and the dimerized proteins were immunoprecipitated with either anti-*c-Fos* or anti-JunD antibodies (Ab) as indicated. Proteins were visualized by fluorography after fractionation by SDS-10% PAGE. (C) Mutant JunD is unable to bind DNA. ³²P-labeled AP1 oligonucleotides were incubated with 1 μ l of in vitro translation reaction mixtures programmed either without or with *c-fos*, *junD*, or mutant *junD* RNA and analyzed by an EMSA to detect DNA binding. Unlabeled 100-fold molar excesses of AP1 oligonucleotides were included in one reaction [AP1(100X)]. The total volume of translation reaction mixture used in each case was adjusted with reticulocyte lysate to 2 μ l. (D) Mutant JunD inhibits DNA binding of wild-type JunD. In vitro translation reaction mixtures programmed with different amounts of *c-fos*, *junD*, and/or mutant *junD* RNA (indicated in micrograms) were incubated with labeled AP1 double-stranded oligonucleotides and subjected to an EMSA. The total RNA in the translation reaction mixture was adjusted to 10 μ g with irrelevant RNA.

ing that the immediate-early proteins synthesized under these conditions might have disparate activities through different combinatorial effects or posttranslational modifications (3). In this regard, it is interesting that Nur77 synthesized upon NGF or KCl treatment is differentially phosphorylated (25).

FIG. 10. Inhibition of *nur77* induction by dominant-negative mutant of JunD. PC12 cells were cotransfected with 26 μ g of the *nur77* 126/77TR minigene, 6 μ g of pGK β -gal, and either 20 μ g of the parental pCMV plasmid or 10 or 20 μ g of pCMVJunDmut expression plasmid (adjusted to a total of 20 μ g with the parental expression plasmid pCMV5) by electroporation. After transfection, cells were either unstimulated (–) or stimulated with NGF or KCl for 1 h as indicated. Expression of the transfected and the endogenous *nur77* genes was analyzed by S1 nuclease protection assay. The RNA amount for S1 protection assay was normalized to the β -galactosidase activity of the extract, which serves as a control for transfection efficiencies.

That many of the same genes are induced by NGF and KCl treatment in PC12 cells suggests that these agents either work through different signalling pathways that act upon distinct regulatory sequences on the same genes or work through signalling pathways that converge upon the same transcription factors, which activate the identical genes. In the case of *c-fos*, the sequence elements necessary for NGF and KCl induction are separable: NGF induction requires the serum response element centered at nt –310 (53, 63) as well as two CRE-like elements (17), and membrane depolarization requires a CRE-like sequence centered at nt –60 (53, 55). These data argue that NGF and KCl work through different signalling pathways that act upon distinct regulatory sequences to activate *c-fos*. In contrast, extensive promoter analysis presented in this study shows that two similar AP1-like sequences, NAP1 and NAP2, are necessary and sufficient to mediate induction by both NGF and KCl (Fig. 3 and 4). Thus, the sequence requirements for induction of *nur77* by NGF and KCl appear indistinguishable.

Nevertheless, NGF and KCl clearly work through distinct signalling pathways to induce *nur77*, as they are differentially affected by specific kinase inhibitors (Fig. 1). Induction by NGF does not require phorbol ester-sensitive PKC- or CaMII kinase-dependent pathways (Fig. 1A and C) but is partially mediated through a PKA-dependent pathway (Fig. 1B). NGF binds to a cell surface receptor complex composed of a p140^{prototrk} and a p75^{NGFR} molecule (27, 30), resulting in the activation of an intrinsic receptor tyrosine kinase (31). Subsequent signalling events involve the activation of many kinases, including the MAP (mitogen-activated protein) kinases (24, 57, 62, 69). Thus, it is possible that MAP kinase-dependent pathways might contribute to the activation of *nur77*.

In contrast, membrane depolarization by elevated levels of extracellular KCl causes the influx of Ca²⁺ ions into the cells through voltage-dependent Ca²⁺ channels. Since the CaMII kinase inhibitor KN-62 completely blocked KCl-mediated

nur77 induction, this result supports the idea that CaMII kinase is involved in this response (Fig. 1C). In addition, whereas PKC-dependent pathways are not involved in KCl induction of *nur77* (Fig. 1A), PKA may be involved in *nur77* activation (Fig. 1B). The activation of *c-fos* and *zif268/NGFI-A* by KCl has been shown to be attenuated in PKA-deficient PC12 cell lines (18). However, as neither membrane depolarization nor Ca^{2+} affects cAMP levels in PC12 cells (56), PKA may be activated by an alternate mechanism. It has been suggested that the intermediary step of Ca^{2+} signalling may participate in the activation of PKA without affecting cAMP levels in cells (1).

Although NGF- and KCl-mediated activation of *nur77* functions through distinct signalling pathways in PC12 cells, these pathways are likely to converge upon the same transcription factors, which act on the same sequence elements in the *nur77* promoter. The NAP1 and NAP2 elements contain identical 7-bp sequences (5'-TGCGTCA) which are similar to the consensus AP1 binding site (5'-TGAGTCA) and the CRE (5'-TGACGTCA). The NAP elements can confer inducibility by both NGF and KCl on an otherwise unresponsive heterologous promoter (Fig. 4). This result suggests that the NAP element is a DNA sequence motif mediating the immediate-early response initiated by two distinct stimulatory agents, NGF and KCl, in PC12 cells. In support of this idea, a sequence similar to that of NAP has been shown to be important in mediating *zif268/NGFI-A* induction by NGF in PC12 cells (12). In addition, sequences similar to that of the NAP element have been identified in the *c-fos* and proenkephalin gene promoters as a functional CRE in PC12 cells and in C6 glioma cells, respectively (32, 61).

JunD regulates *nur77* transcription. Several lines of evidence demonstrate that JunD is one of the key transcription factors involved in the activation of *nur77* upon NGF or KCl stimulation. First, JunD, but not CREB or other members of the AP1 (Fos/Jun) family, is a component of the proteins in nuclear extracts that binds to the NAP element, which mediates transcriptional activation by NGF and KCl (Fig. 6). Second, JunD, but not other proteins of the AP1 family, can transactivate *nur77* specifically through the crucial NAP elements (Fig. 8). Third, a dominant-negative mutant of JunD effectively blocks activation of *nur77* by NGF or KCl (Fig. 10).

The above findings are significant when interpreted in the context of immediate-early gene activation. Since activation occurs in the absence of new protein synthesis, we anticipate that preexisting transcription factors are responsible. JunD, initially identified on the basis of sequence homology with c-Jun (49), is constitutively expressed in many cell types (49). JunD can bind to AP1 elements (or the TPA response element [TRE]) or related sequences as a homodimer or as heterodimers with other members of the Fos and Jun families and regulate transcription (33, 34, 42, 46, 50). In the unstimulated PC12 cell, *c-jun*, *junB*, and *c-fos* are not expressed (3). Thus, it is not surprising that the proteins encoded by these genes are not components of the NAP binding activity in nuclear extracts. It is also gratifying that JunD, but not other Fos/Jun proteins, can transactivate *nur77*. These results point to a unique specificity in the ability of JunD to transactivate *nur77*, and not all Fos/Jun proteins have this activity. Furthermore, the facts that other members of the *fos/jun* gene family are not expressed at the time of stimulation and can transactivate *nur77* lend credence to the dominant-negative experiment. Since the dominant-negative JunD retains the ability to form dimers, the absence of other Fos/Jun proteins allows us to conclude that the dominant-negative mutant blocks JunD action and not that of other members of the Fos/Jun family.

Since CREB is known to be involved in the activation of *c-fos* by both NGF and membrane depolarization in PC12 cells (17, 56), and since a CRE oligonucleotide competes with the NAP element for binding to nuclear proteins (Fig. 5), we have considered the possible involvement of CREB in the activation of *nur77*. However, CREB is not a component of the NAP element binding activity in PC12 cell nuclear extracts, and in vitro-translated CREB does not bind to the NAP element. Since JunD is unlikely to act by itself to transactivate *nur77* (Fig. 6B), and since a CRE oligonucleotide competes with the NAP element for nuclear protein binding (Fig. 5), it is possible that another member of the CREB family of proteins might participate in *nur77* activation. The ability of JunD to heterodimerize with CREB or CREM bZIP family members is consistent with this possibility (23, 39).

Distinct regulation of *nur77* transcription. We now have sufficient information concerning the transcriptional activation of *nur77* to recognize that distinct sequences regulate its induction in different cell types. In fibroblasts, the rapid and transient activation of *nur77* induced by serum growth factors is actually the superimposition of two distinct transcriptional responses: an immediate-early response which is independent of new protein synthesis and a delayed-early response which is dependent on new protein synthesis (66). The *cis*-acting sequence elements required for immediate-early activation of the gene were localized to a region of the promoter spanning nt -126 to -87 (66). This region contains a CARG-like element and the adjacent Ets binding site; both are required for significant induction (66). In contrast, delayed-early expression of *nur77* in fibroblasts is mediated through nt -86 to -26 in the promoter, which correspond to the NAP elements and SP1 and Zif268 sites identified in this study as being responsible for the immediate-early activation in PC12 cells (66). Thus, sequence elements that mediate the immediate-early response in one cell type can mediate the delayed-early response in another. In contrast to the findings for fibroblasts and PC12 cells, the promoter region responsible for the *nur77* activation by adrenocorticotrophic hormone in the adrenal cortical cell line Y-1 was localized further upstream (11). In T lymphocytes, activation of *nur77* by TPA requires sequences between nt -378 and -332, whereas activation by apoptotic signals delivered through the T-cell receptor requires sequences between nt -332 and -151 (37). Taken together, these findings point to a multiplicity of regulatory mechanisms that control the transcription of *nur77* in different cell types.

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