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# Activated Transcription of the Human Neuropeptide Y Gene in Differentiating SH-SY5Y Neuroblastoma Cells Is Dependent on Transcription Factors AP-1, AP-2α, and NGFI

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**Abstract:** Activated transcription of the human neuropeptide Y gene (*NPY*) was investigated in SH-SY5Y neuroblastoma cells at the onset of sympathetic neuronal differentiation induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and serum or by nerve growth factor (NGF). As determined by transient expression, two NGF response elements (REs) were required for transcription induced by NGF in SH-SY5Y cells with stable expression of an exogenous NGF receptor *TRK-A* gene (SH-SY5Y/trk). TPA treatment in the presence of serum induced *NPY* transcription in both wild-type SH-SY5Y (SH-SY5Y/wt) and SH-SY5Y/trk cells. A TPA RE (TRE), overlapping the proximal NGF RE, was identified by expression of the v-Jun oncoprotein that enhanced *NPY* transcription. Suppression of TPA-induced *NPY* transcription was obtained by expression of a dominant negative Jun protein, selective protein kinase C inhibition, or introduction of a mutated TRE, whereas NGF-induced *NPY* transcription was inhibited to a lesser degree. The transcription factor AP-2α was shown to bind cooperatively to the *NPY* promoter with either AP-1 or NGFI-A to the shared TRE and NGF RE and to the distal NGF RE, respectively. These results show that transcription factors AP-1, AP-2α, and NGFI-A are involved in activated *NPY* transcription during the onset of neuronal differentiation. **Key Words:** Neuropeptide Y—Transcription—SH-SY5Y neuroblastoma—AP-1—AP-2α—NGFI.
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Neuropeptide Y gene (*NPY*) expression in response to treatment with phorbol ester, nerve growth factor (NGF), and forskolin has been extensively studied in both neuroblastoma and PC12 pheochromocytoma cells (Minth et al., 1986; Allen et al., 1987; Higuchi et al., 1988; Minth and Dixon, 1990; Sabol and Higuchi, 1990; Andersson et al., 1994; Balbi and Allen, 1994; Jalava and Mai, 1994; Minth-Worby, 1994). Recently, the rat *NPY* gene was shown to be actively transcribed in response to membrane depolarization in PC12 cells (Higuchi et al., 1996). Both transcriptional and posttranscriptional mechanisms appear to be operative for maximal *NPY* gene expression in PC12 cells in response to combined treatment with phorbol ester and forskolin. However, mainly posttranscriptional effects are activated in response to this treatment in LA-N-5 neuroblastoma cells (Lerchen et al., 1995). In fact, an NGF responsive element (RE) in the human *NPY* promoter, located between positions −87 and −36 relative to the start site for transcription, was previously identified (Minth-Worby, 1994).

Treatment of human SH-SY5Y wild-type (SH-SY5Y/wt) neuroblastoma cells with nanomolar concentrations of the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in serum-containing medium activates sustained protein kinase C (PKC)-dependent signal transduction events, leading to differentiation into a functional neuronal sympathetic phenotype (Påhlman et al., 1981, 1984, 1991, 1992, 1995; Parrow et al., 1992). The novel PKC isoform nPKC-ε was recently implicated in neurite outgrowth in these cells (Fagerström et al., 1996). *NPY* gene transcription in SH-SY5Y/wt neuroblastoma cells is induced following activated PKC signaling (Påhlman et al., 1991; Andersson et al., 1994; Jalava and Mai, 1994). A rapid synchronous and bipha-

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*Abbreviations used:* AP, activator protein; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; fpu, footprint unit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NGF, nerve growth factor; NPY, neuropeptide Y; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; RE, responsive element; RSV, Rous sarcoma virus; TK, thymidine kinase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TRE, TPA RE.

sic induction of c-*fos* and c-*jun* mRNA expression is obtained by treatment of SH-SY5Y/wt cells with TPA (Hammerling et al., 1987; Andersson et al., 1994). The second peak of c-*fos* and c-*jun* gene transcription correlates with maximal *NPY* gene transcription as well as with the onset of functional neuronal sympathetic differentiation. TPA-induced interactions of the activator protein 1 (AP-1) family of transcription factors and of AP-2$\alpha$ to a TPA RE (TRE) in the *NPY* promoter were identified in SH-SY5Y/wt nuclear extracts. Unlike the TPA-induced c-*jun* mRNA expression, *AP-2*$\alpha$ mRNA is constitutively expressed in SH-SY5Y/wt cells (Andersson et al., 1994). *NPY* transcription in SH-SY5Y/wt cells in response to PKC signaling is likely to be activated by cooperation between the transcription factors AP-1 and AP-2$\alpha$ (Andersson et al., 1994). Thus, transcriptional as well as posttranscriptional mechanisms activated by PKC appear to be important for the induced *NPY* gene transcription in differentiating neuroblastoma cells.

SH-SY5Y/wt cells express both the high-affinity ($p140^{c\text{-}trk\text{-}A}$) as well as the low-affinity (p75) NGF receptor (Azar et al., 1990; Lavenius et al., 1995). Even so, SH-SY5Y/wt cells, like other cultured neuroblastoma cells, do not show NGF responsiveness, indicating a defective *TRK-A*-dependent signal transduction or low receptor levels (Lavenius et al., 1994, 1995). Stable expression of an exogenous functional *TRK-A* gene in the SH-SY5Y cell line (SH-SY5Y/trk) allows these cells to undergo neuronal sympathetic differentiation and to express *NPY* after exposure to NGF (Lavenius et al., 1995). Therefore, SH-SY5Y/trk cells offer an advantageous biological system to study distinct effects of NGF and TPA on *NPY* gene transcription.

To compare the mechanisms responsible for activated *NPY* gene expression in SH-SY5Y/wt and SH-SY5Y/trk cells in response to TPA and NGF treatments, we have performed transient transfection analyses with *NPY* promoter target plasmids. Transcriptional activation was studied under conditions where PKC and AP-1 activities were either positively or negatively modulated. Furthermore, DNA-protein binding analyses identified transcription factors of importance for the TPA- and NGF-dependent regulation of *NPY* gene expression in these two cell types.

## EXPERIMENTAL PROCEDURES

### Cell cultures

The human neuroblastoma cell line SH-SY5Y/wt, a subclone of the cell line SK-N-SH (Biedler et al., 1978), was kindly provided by Dr. J. Biedler (Sloan Kettering Institute, New York, NY, U.S.A.). The establishment of the stable *TRK-A*-transfected SH-SY5Y/trk cell clone 6:2 has been described (Lavenius et al., 1995). The cells were cultured in Eagle's minimum essential medium in the presence of 10% fetal calf serum (GIBCO, Gaithersburg, MD, U.S.A.), penicillin (100 IU/ml), and streptomycin (50 $\mu$g/ml). Conditions for induction of differentiation of these cell lines have been described (Påhlman et al., 1981, 1984; Lavenius et al., 1995). In brief, SH-SY5Y/wt and SH-SY5Y/trk cells, at low density ($1-2 \times 10^6$ cells/8.5-cm Petri dish), were induced with 16 n*M* TPA in fetal calf serum-containing medium. Alternatively, SH-SY5Y/trk cells were induced with 100 ng/ml of mouse NGF 2.5S (Promega, Madison, WI, U.S.A.). Prior to TPA or NGF treatment, cells were grown in serum-containing Eagle's medium for 24 h. Stock solutions of TPA (Sigma Chemical Co., St. Louis, MO, U.S.A.) were prepared in ethanol. The final ethanol concentration was kept below 0.1% in all experiments. The selective PKC inhibitor GF 109203X (Toullec et al., 1991) was used at a concentration of 2 $\mu$*M* when indicated and added 30 min prior to TPA or NGF (Fagerström et al., 1996).

### Northern hybridization

RNA was isolated essentially as previously described (Auffray and Rougeon, 1980) with minor modifications (Hammerling et al., 1987). Purification of poly(A)$^+$ mRNA, electrophoresis, and northern analysis were performed as outlined (Hammerling et al., 1987) except that Hybond-C membranes (Amersham Int., Amersham, Buckinghamshire, U.K.) were used. mRNA was quantified by absorbance at 260 nm. Human *NPY*, *NGFI-A*, and *NGFI-C* (gift from Dr. J. Milbrandt, Washington University, St. Louis, MO, U.S.A.) cDNA probes (Minth et al., 1986; Crosby et al., 1991) were used in these experiments. A cDNA probe encoding the human glyceraldehyde-3-phosphate dehydrogenase (pHcGAP3; a gift from Dr. R. Wu, Cornell University, Ithaca, NY, U.S.A.) served as quantitative standard for the amount of mRNA loaded measured by phosphorimage analyses using Fuji BAS 2000 equipment (Fuji, Japan). cDNA probes were labeled according to the manufacturer (Amersham).

### Transient expression assays

Transfections shown in Fig. 4B were performed with the calcium phosphate technique as described (Chen and Okayama, 1987). All other transfections were performed with the DEAE-dextran chloroquine bisphosphate method as previously described (Sompayrac and Danna, 1981; Andersson et al., 1994). The *NPY* promoter target plasmids used in this study have already been described (Minth-Worby, 1994). In brief, 15 $\mu$g of each plasmid target DNA was used in the experiments. In co-transfection experiments, 2 and 5 $\mu$g of a dominant negative v-*jun* plasmid denoted $\Delta$9 (Lloyd et al., 1991) or 2 $\mu$g of a v-*jun* activator plasmid (Baichwal and Tjian, 1990) was used as indicated. Five million untreated SH-SY5Y/wt or SH-SY5Y/trk cells were transfected on each plate. Before addition of TPA or NGF, the cells were allowed to recover for 24 h. After treatment with additives for an additional 24 h, the cells were harvested and lysates were prepared for chloramphenicol acetyltransferase (CAT) assays. Fifty micrograms of protein was used in each CAT assay at 37°C for 16 h. The CAT values were quantified by phosphorimage analyses (Fuji BAS 2000) and normalized to the activity obtained by positive and negative control target plasmids. Each transfection was performed in either duplicate or triplicate more than three times using different plasmid preparations. Error bars indicate the standard deviations from the means. Results were standardized, where values for uninduced resting cells were set to 1. In parallel transfection assays, p*RSVCAT* was used as the positive (Gorman et al., 1982) and p*TKCAT* (Tsang et al., 1988; Minth-

Worby, 1994) served as the negative control plasmids. Transfection efficiency was evaluated by comparison of the CAT activities with these plasmids in multiple independent transfections.

### Preparation of nuclear protein extracts

Nuclear extracts were prepared from SH-SY5Y/wt and SH-SY5Y/trk cells as described (Andrews and Faller, 1991). Cells were harvested on ice and washed with phosphate-buffered saline, and cell pellets were resuspended in 400 μl of isotonic buffer A [10 m*M* HEPES-KOH (pH 7.9), 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol (DTT), 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF)]. After incubation on ice for 10 min, cells were vortexed and centrifuged. Nuclei were collected, resuspended in cold buffer C [20 m*M* HEPES-KOH (pH 7.9), 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.2 m*M* EDTA, 0.2 m*M* PMSF, 25% glycerol, 0.5 m*M* DTT], and incubated on ice for 20 min. Debris was removed by a short centrifugation. Protein concentration was determined using the Bio-Rad system (Bio-Rad Scandinavia, Sweden). The nuclear extracts were aliquoted, frozen in liquid nitrogen, and stored at −70°C.

### DNA-protein binding assays

Electrophoretic mobility shift assay (EMSA) (Fried and Crothers, 1981) was performed using SH-SY5Y/wt and SH-SY5Y/trk nuclear extracts. The extracts were first quality tested using an AP-1 oligonucleotide, and comparable protein-DNA complexes to those obtained previously were observed. The conditions for protein-DNA binding were according to Minth-Worby (1994) to allow comparison with complexes obtained in PC12 nuclear extracts. In brief, 20,000 cpm of $^{32}P$ radioactively labeled double-stranded oligonucleotides was incubated with 4 μg of SH-SY5Y/wt or SH-SY5Y/trk nuclear extracts prepared from control or stimulated cells in a binding buffer [10 m*M* HEPES (pH 7.9), 50 m*M* KCl, 1 m*M* EDTA, 5 m*M* DTT, 5 m*M* $MgCl_2$, 10% glycerol]. Two micrograms of polydeoxyinosinic–deoxycytidylic acid (Pharmacia & Upjohn, Uppsala, Sweden) was included as competitor for nonspecific DNA-protein interaction in each reaction. Specific competition was monitored using 100- to 200-fold molar excess of nonlabeled double-stranded oligonucleotides as indicated. The oligonucleotides used are listed in Table 1. For antibody interference and supershift experiments, 1 μl of undiluted or 1 μl of 0.1× diluted antiserum against AP-2α (sc-184 X), c-Fos (sc-52 X), c-Jun (sc-45 X), JunD (sc-74 X), and c-Jun/AP-1 (D) [α-Jun (sc-44 X); Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.], as indicated in the legends to Figs. 5 and 6, either was added to the nuclear extracts 1 h before addition of labeled oligonucleotide or was added after incubation with labeled oligonucleotide and incubated 1 h on ice. Binding reactions were incubated at room temperature for 30 min and then separated on 5% nondenaturing polyacrylamide gels (29:1 acrylamide/bisacrylamide) in either a TGE buffer (50 m*M* Tris, 0.38 m*M* glycine, 2 m*M* EDTA) (Fig. 5A) or a TBE buffer [22 m*M* Tris-borate (pH 8.3), 0.05 m*M* EDTA] (Figs. 5B and 6). After electrophoresis at 4°C, the gels were dried and visualized by phosphorimage analysis or by standard autoradiography at −70°C using Kodak X-Omat film (Kodak AB, Järfälla, Sweden). The plasmid pJDM1502 (provided by Dr. J. Milbrandt), containing the rat *NGFI-A* gene inserted in the pCITE1 mammalian expression vector (Novagen, Madison, WI, U.S.A.), was digested with *Sal*I to generate a linearized DNA template. Coupled in vitro transcription and translation in TNT-T7-coupled rabbit reticulocyte lysates (Promega) according to the manufacturer's instructions were performed using this DNA template. One microliter of the total 50-μl reaction mixture of in vitro translated *NGFI-A* was used in EMSA with an oligonucleotide containing the GSG-1 and GSG-2 boxes (oligonucleotide no. 5 in Table 1).

## RESULTS

### Enhanced *NPY*, *NGFI-A*, and *NGFI-C* mRNA expression in SH-SY5Y/trk cells in response to NGF treatment

Northern hybridization experiments were performed to determine *NPY* mRNA expression profiles in SH-SY5Y/wt and SH-SY5Y/trk cells. mRNA was prepared from untreated SH-SY5Y/trk cells and after treatment with 100 ng/ml of NGF for indicated times (Fig. 1A). Exposure of SH-SY5Y/trk cells to NGF leads to an induction pattern of *NPY* mRNA comparable with that obtained in SH-SY5Y/wt cells after TPA treatment (Fig. 1A) (Andersson et al., 1994). Maximal *NPY* mRNA expression was detected 4 days after NGF treatment in this analysis (Fig. 1A).

Defective NGF responsiveness in SH-SY5Y/wt cells has previously been monitored by examination of c-*fos* mRNA expression following NGF treatment. Both c-*fos* and c-*jun* mRNA levels in SH-SY5Y/wt cells were unaffected by NGF treatment, whereas enhanced expression was shown in SH-SY5Y/trk cells (Lavenius et al., 1995; data not shown).

To determine the mRNA expression pattern of the NGF-inducible immediate early *NGFI-A* and *NGFI-C* genes (Swirnoff and Milbrandt, 1995), mRNA was prepared from untreated SH-SY5Y/trk cells and, after treatment with either 100 ng/ml of NGF or 16 n*M* TPA for the indicated times, hybridized with *NGFI-A* and *NGFI-C* cDNA probes. In resting SH-SY5Y/trk cells, mRNA expression of these genes was undetectable (Fig. 1B). A synchronous and transient induction of these immediate early genes was obtained with both NGF and TPA treatments. Maximal expression was seen after 1-h treatment. Further, TPA treatment led to stronger and more persistent induction in comparison with NGF treatment (Fig. 1B). A second minor peak of *NGFI-A* mRNA expression was reproducibly seen after 12 h of TPA and NGF treatment (Fig. 1B).

### Transcription of *NPY* gene is activated in response to NGF in SH-SY5Y/trk cells

To determine whether the observed *NPY* mRNA induction reflects a transcriptional activation, transient transfection analyses were performed in SH-SY5Y/wt and SH-SY5Y/trk cells. Cells were transfected with a target plasmid containing *NPY* promoter sequences between positions −87 and −47 cloned in front of a heterologous thymidine kinase (*TK*) promoter and the bacterial *CAT* gene. This region of the rat and human *NPY* promoters spans an NGF RE as previously defined in PC12 cells (Higuchi et al., 1992; Minth-

**TABLE 1.** *Nucleotide sequences of oligonucleotides used in EMSA*

| No. | Sequence |
|---|---|
| 1 | −73/−45: 5′-GCGTGACTGCCCGAGGCCCCTCCTGCCGC-3′ |
| 2 | −87/−56: 5′-GGGAGTCACCCAAGCGTGACTGCCCGAGGC-3′ |
| 3 | Amut: 5′-GGGAGTCACCCAATATGTCATGCCCGAGGC-3′ |
| 4 | AP-1: 5′-AGATGCGTCATCTCAAAA |
| 5 | GSG: 5′-AGTGCCTGGGGCGGGAGGGTTGG-3′ |
| 6 | mGSG: 5′-AGTTAATGTTTAGGTATGGTTGG-3′ |
| 7 | AP-2: 5′-CAACTGACCGCCCGCGGCCAACTGACCGCCCGCGGCCG-3′ |
| 8 | Sp1: 5′-CCGGCCCCGCCCATCCCCGGCCCCGCCCATCC-3′ |

1–3: oligonucleotides corresponding to the human *NPY* promoter. 1, positions −73 to −45; 2, positions −87 to −56. Oligonucleotides 1 and 2 contain partially overlapping sequences. 3, positions as in 2 where the AP-1 site is mutated. Mutated positions are underlined. 4, oligonucleotide containing an AP-1 site from the human *DRA* gene promoter corresponding to positions −100 to −87 (Andersson and Peterlin, 1990). 5, oligonucleotide containing two GSG elements corresponding to positions −195 to −173 in the *NPY* promoter. 6, oligonucleotide where the GSG elements are mutated. Mutated positions are underlined. 7, oligonucleotide containing two AP-2 binding sites from the human metallothionein IIA promoter (Imagawa et al., 1987). 8, oligonucleotide with two 16-bp tandem copies containing Sp1 consensus binding sites from the herpes simplex virus *IE-3* gene promoter (Westin and Schaffner, 1988). All oligonucleotides were synthesized with *Bam*HI/*Bgl*II sticky ends for the purpose of radioactive labeling. Only the sense strands of the double-stranded oligonucleotides are shown.

Worby, 1994). This NGF RE contains several *cis* elements with implications for phorbol ester- and NGF-dependent transcription (Fig. 2). In particular, two AP-1-like sites (5′-GGAGTCA-3′, 5′-TGACTGC-3′) are present in the *NPY* promoter. Moreover, an AP-2 site (5′-GCCCGAGGC-3′) is located 5′ of the CT box that binds Sp1 family proteins (Andersson et al., 1994; Minth-Worby, 1994) (Fig. 2). Following a recovery period after transfections, SH-SY5Y/wt and SH-SY5Y/trk cells were stimulated with NGF for 24 h, or, as a control for basal activity, cells were not exposed to any reagent (see Experimental Procedures). In SH-SY5Y/wt cells, NGF treatment did not significantly increase the CAT activity above the control level, whereas a pronounced (ninefold) induction was obtained in response to NGF in SH-SY5Y/trk cells (Fig. 3A). As expected, TPA treatment induced *NPY* promoter activity in both SH-SY5Y/wt and SH-SY5Y/

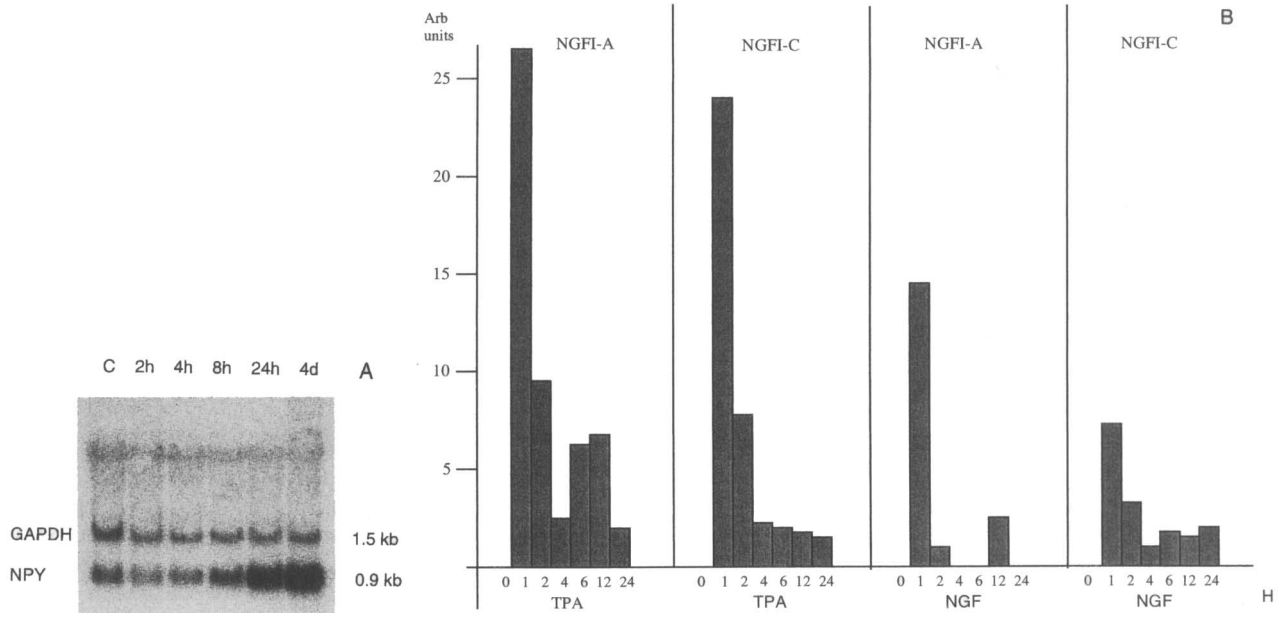


**FIG. 1.** Northern blotting analysis of *NPY*, *NGFI-A*, and *NGFI-C* mRNA expression in SH-SY5Y/trk cells. **A:** mRNA was prepared from nontreated cells and from cells treated with 100 ng/ml NGF for indicated times. Northern filters were probed for *NPY* and *GAPDH*, and hybridization signals were visualized by phosphorimage analysis. **B:** mRNA was prepared from resting cells (0) and from cells treated with either 16 n*M* TPA or 100 ng/ml of NGF for indicated times. Northern filters were probed for *NGFI-A* and *NGFI-C*, and hybridization signals were visualized by phosphorimage analysis and presented in a diagram with arbitrary units (arb units).

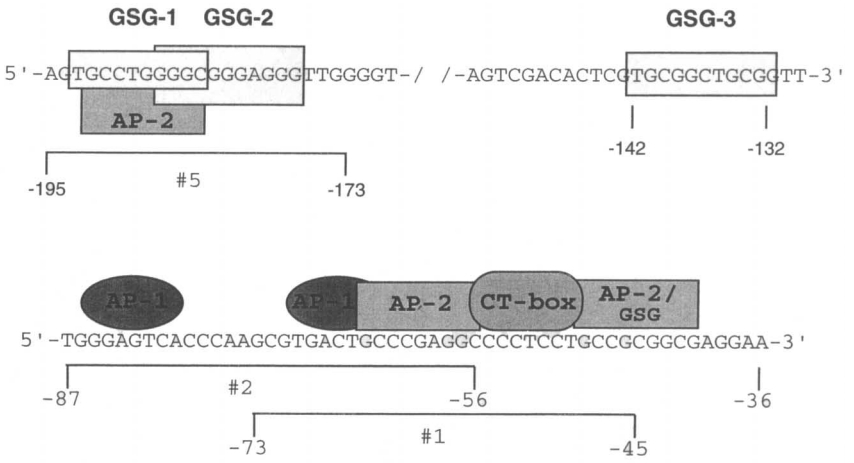


**FIG. 2.** Schematic diagram of *cis* elements and DNA binding activities to the NGF REs of the human *NPY* promoter. DNA binding activities located within the NGF REs are indicated. The 5′ and 3′ borders of the oligonucleotides used for EMSA (no. 1, −73/−45; no. 2, −87/−56; no. 5, −195/−173) are indicated below the sequence. The borders of the GSG-3 element are also indicated.

trk cells (Fig. 3A). The relatively higher induction observed in SH-SY5Y/wt cells than in SH-SY5Y/trk cells reflects a higher basal *NPY* transcription in SH-SY5Y/trk cells. Note that in resting SH-SY5Y/trk cells, *NPY* mRNA expression is higher than in resting SH-SY5Y/wt cells (Fig. 1A) (Andersson et al., 1994). Thus, this part of the *NPY* promoter is essential for TPA activation in both cell types and activated transcription of the *NPY* gene induced by NGF in SH-SY5Y/trk cells.

### NGF-dependent transcriptional activation of *NPY* gene in differentiating SH-SY5Y/trk cells

To investigate the *NPY* promoter sequences responsible for the activation by NGF in differentiating SH-SY5Y/trk cells, transient transfections were performed with *NPY* targets carrying successive deletions of the *NPY* promoter. The following *NPY* promoter deletion constructs were used: positions −246, −118, −83, −63, and −51. In addition, all plasmids contained 51 nucleotides of the *NPY* 5′-UT exon (see Minth-Worby, 1994; also Experimental Procedures). Following a recovery period after the transfections, cells were subjected to stimulation with NGF for 24 h, or, as a control, cells were not exposed to any reagents. Treatment of SH-SY5Y/wt cells with NGF did not induce CAT activities of any of the *NPY* promoter targets (data not shown). Maximal NGF responsiveness in SH-SY5Y/trk cells was obtained with the −246 *NPYCAT* target plasmid (fourfold). Successive deletions of the *NPY* promoter led to decreased activities. A weak NGF responsiveness was obtained with the −83 *NPYCAT* target plasmid. Deletion to position −63 abolished the NGF induction (Fig. 3B). At positions −193 to −176 in the distal NGF RE, two GSG-like-elements were identified (Fig. 2). GSG sequences have been reported to bind NGFI proteins (Swirnoff and Milbrandt, 1995). Thus, we conclude that NGF-inducible transcription of the *NPY* gene involves these GSG promoter elements.

### TPA-dependent transcriptional activation of *NPY* gene is abolished by mutations of proximal AP-1-like site or distal AP-2 site

To determine the effects of the TPA treatment on the expression from the *NPY* promoter, transient expression assays with the wild-type −246 *NPYCAT* target plasmid as well as two target plasmids bearing clustered point mutations in the context of the −246 *NPYCAT* target were performed. The mutant *NPY* targets either abolish the AP-1-like site (5′-TGACTGC-3′) or destroy the AP-2 site (5′-GCCCGAGGC-3′) (see Fig. 2). The basal activities of the mutated target plasmids were lower than those of the wild-type target, and TPA-mediated induction could not be obtained when either of the mutated target plasmids was analyzed (not shown). Thus, both the AP-1-like and the distal AP-2 site are essential for activated transcription of the *NPY* gene induced by TPA in SH-SY5Y cells.

### Overexpression of v-Jun enhances transcriptional activation of *NPY* gene in SH-SY5Y/wt cells

To directly address Jun-dependent *NPY* transcription activation in SH-SY5Y/wt cells, the −87/−47 *NPY/TKCAT* target plasmid, which contains the two AP-1-like sites (5′-GGAGTCA-3′, 5′-TGACTGC-3′), was co-transfected with a v-*jun* activator (Baichwal and Tjian, 1990) in resting SH-SY5Y/wt cells. Overexpression of v-Jun led to fivefold relative induction of CAT activities in these SH-SY5Y/wt cells (Fig. 4A). Similar results were obtained with SH-SY5Y/trk cells (not shown).

### A dominant negative Jun protein suppresses PKC-dependent *NPY* expression, whereas NGF-induced activation is inhibited to lesser degree

To further analyze the effects of Jun on *NPY* expression, the −87/−47 *NPY/TKCAT* target plasmid was co-transfected with increasing amounts of Δ9, which directs the synthesis of a dominant negative Jun protein (Lloyd et al., 1991), in either TPA-stimulated or NGF-stimulated SH-SY5Y/trk cells. This mutated form of

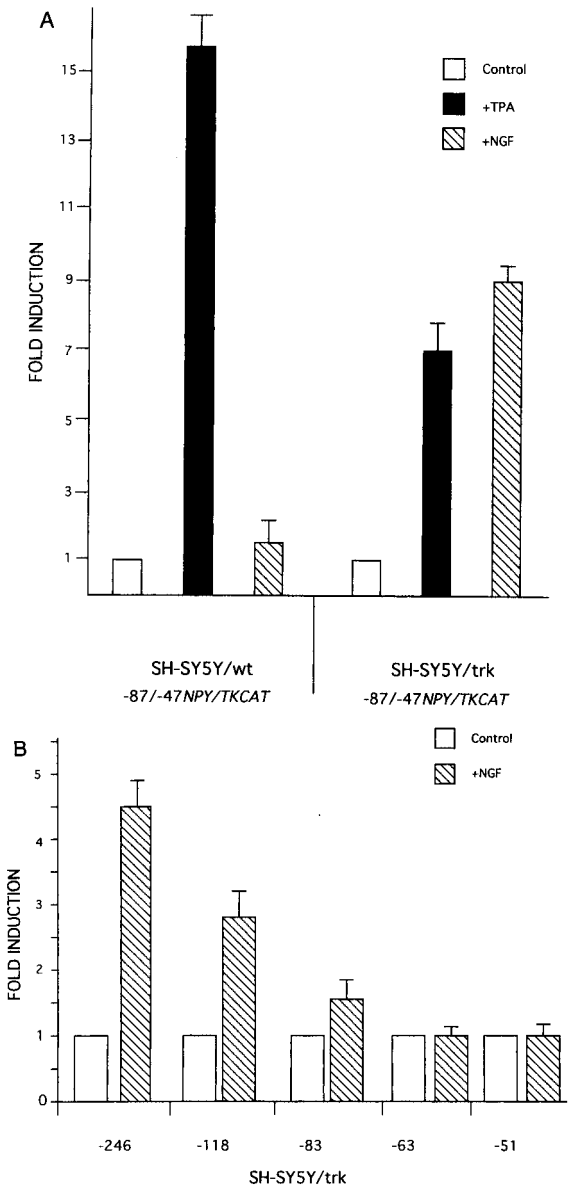


**FIG. 3. A:** Transient transfection analyses of the −87/−47 *NPY/TKCAT* reporter plasmid. Open columns represent the normalized CAT activities obtained from transient transfections of untreated SH-SY5Y/wt and SH-SY5Y/trk cells with the −87/−47 *NPY/TKCAT* reporter plasmid. Filled columns represent the relative CAT induction obtained following TPA treatment. Hatched columns represent the relative CAT induction obtained in response to NGF treatment. **B:** Transient transfection analyses of *NPY/CAT* promoter deletion plasmids. Open columns represent the normalized CAT activities obtained from transient transfections of untreated SH-SY5Y/trk cells with *NPY* deletion plasmids with deletions ranging from −246 to −51. Hatched columns represent the relative induction in response to treatment with 100 ng/ml of NGF 24 h prior to harvest. Data represent an average of three transfections for each plasmid. The error bars represent the standard deviation from the mean.

Jun lacks the transcriptional activation domain and blocks Jun-dependent transcription and transformation activities (Lloyd et al., 1991). The Δ9 plasmid has previously also been shown to effectively inhibit c-Jun-dependent collagenase transcription in normal and *ras*-transformed fibroblasts (Shoshan and Linder, 1994). The overexpression of Δ9 in SH-SY5Y/trk cells resulted in ~85 and 70% decreased *NPY* promoter activities of the TPA- and NGF-dependent activities, respectively, when the highest amounts of the Δ9 plasmid were co-transfected (Fig. 4B). Specificity of the Δ9 plasmid was controlled by co-transfection with a p*RSVCAT* target plasmid. Transcription driven by the *RSV* promoter was not inhibited by the Δ9 plasmid in either control or TPA-treated cells (not shown). We conclude from these experiments that proteins of the Jun family of transcription factors are necessary for the increased transcription of the *NPY* gene in activated SH-SY5Y cells and that Jun binding is mediated by the AP-1-like sites.

### Selective PKC inhibition suppresses *NPY* expression in SH-SY5Y/trk cells in response to TPA but only partially in response to NGF

Treatment of SH-SY5Y/trk cells with the selective PKC inhibitor GF 109203X (Toullec et al., 1991) should reveal PKC-dependent transcriptional activities of the *NPY* promoter. Therefore, SH-SY5Y/trk cells were transfected with the −87/−47 *NPY/TKCAT* target plasmid, and 30 min before addition of either TPA or NGF, the cells were treated with 2 μ*M* GF 109203X, which revealed that the basal *NPY* promoter activity was unaffected by the PKC inhibitor (Fig. 4C). In contrast, GF 109203X treatment resulted in 87 and 40% suppression of the transcriptional activation of *NPY* in response to TPA and NGF, respectively (Fig. 4C). Thus, activated PKC signaling appears to be required for *NPY* transcription. Activation of this pathway was less important during NGF-evoked *NPY* transcription.

### Analyses of proteins that bind to −87/−56 region of *NPY* promoter in SH-SY5Y nuclear extracts

Previously, EMSA was used to identify TPA-enhanced AP-1 and AP-2 DNA binding activities in SH-SY5Y/wt nuclear extracts using an oligonucleotide corresponding to the evolutionarily conserved region at position −73/−45 (oligonucleotide no. 1 in Table 1) of the human *NPY* promoter (Andersson et al., 1994). In the present study, an oligonucleotide corresponding to the proximal NGF RE at position −87/−56 (no. 2 in Table 1 and Fig. 2) containing the two AP-1-like sites and the distal AP-2 binding site was analyzed by EMSA and compared with the complexes (CI to CIV) (Andersson et al., 1994) obtained with the −73/−45 oligonucleotide (no. 1 in Table 1). Nuclear extracts from resting, NGF-treated, and TPA-treated SH-SY5Y/wt and SH-SY5Y/trk cells were analyzed. No major qualitative differences were seen in the different nuclear extracts (not shown). Three DNA binding activities denoted C-A to C-C were obtained in SH-SY5Y/trk nuclear extracts using the −87/−56 oligonucleotide (Fig. 5A, lane D). The complexes ob-

tained with the −73/−45 and the −87/−56 oligonucleotides in SH-SY5Y/trk nuclear extracts were compared. This revealed that neither C-B nor C-A could be formed with the −73/−45 oligonucleotide (Fig. 5A, lane A). A co-migration between CII and C-C was observed (Fig. 5A, lanes C and D). Moreover, the −87/−56 oligonucleotide (no. 2 in Table 1) competed only for CI and CII complexes formed with the −73/−45 oligonucleotide (Fig. 5A, lane B). The abundance of C-B and C-A was similar, whereas the abundance of C-C differed between nuclear extract preparations. Compare Fig. 5A and B. The DNA binding activities of these complexes in response to TPA and NGF treatment of SH-SY5Y/wt and SH-SY5Y/trk cells, respectively, were, in principle, unaffected (not shown). Competition of the C-B and C-C complexes was obtained with both oligonucleotide no. 1 (Table 1) and the oligonucleotide containing a mutation at position −73/−67 (no. 3 in Table 1), whereas C-A formation was unaffected by these oligonucleotides (Fig. 5A, lanes E and F). Competition using the −73/−45 oligonucleotide for −87/−56 binding resulted in the disappearance of C-B and C-C (Fig. 5A, lane E).

### AP-2α and JunB participate in complex formation in SH-SY5Y/wt nuclear extracts

To determine whether AP-1 and AP-2 proteins interact with the −87/−56 oligonucleotide, EMSAs were performed with a combination of nuclear extracts prepared 24 h after TPA treatment and recombinant AP-2α protein as well as in the presence of AP-1 antisera. Addition of recombinant AP-2α led to an induction of complexes migrating similarly to C-C and possibly

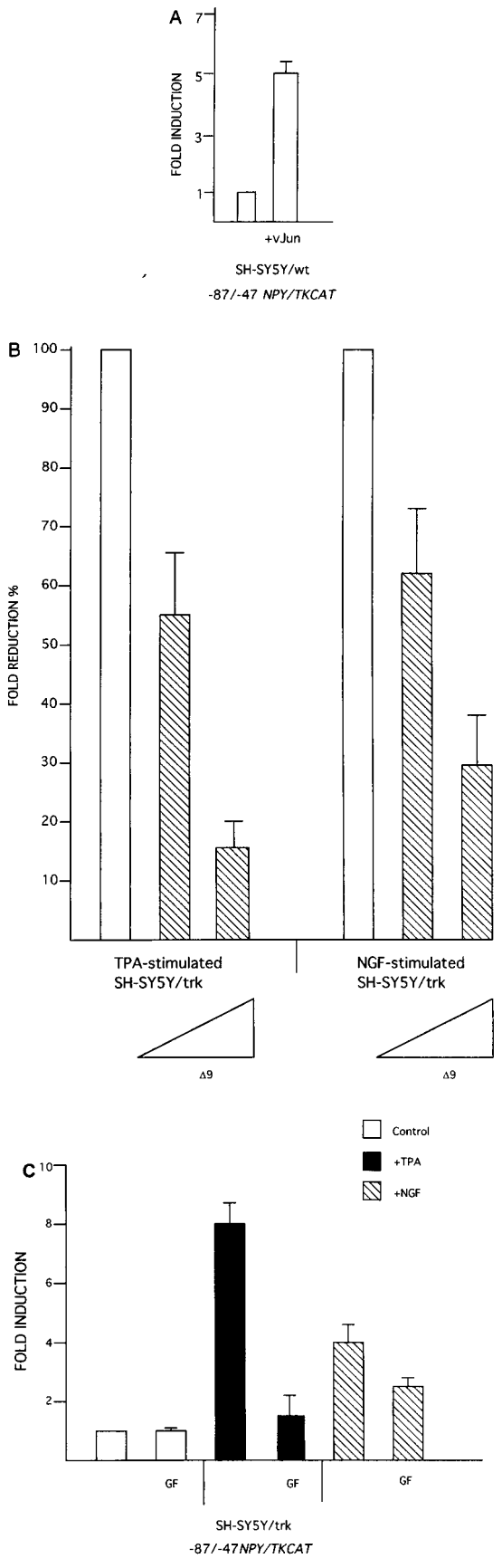


**FIG. 4. A:** *NPY* promoter activation by overexpression of v-Jun. Open columns represent the CAT activities obtained from transient transfections of untreated SH-SY5Y/wt cells with the −87/−47 *NPY/TKCAT* reporter plasmid. Co-transfections with the v-Jun activator plasmid are indicated below the columns. **B:** Suppression of *NPY* promoter activity by a dominant negative Jun plasmid. Co-transfections of TPA- and NGF-stimulated SH-SY5Y/trk cells with the −87/−47 *NPY/TKCAT* plasmid and increasing amounts (2 and 5 μg) of a dominant negative Jun plasmid (Δ9). Open columns represent the relative CAT activities obtained from TPA- and NGF-treated cells, respectively, in the absence of the Δ9 plasmid. Hatched columns represent the fold reduction of CAT activities obtained in co-transfections with Δ9 plasmid. SH-SY5Y/trk cells were treated with 16 n*M* TPA or with 100 ng/ml of NGF 24 h prior to harvest. Specificity of the Δ9 plasmid was monitored using the positive *pRSVCAT* plasmid in separate co-transfections (not shown). Data represent an average of two transfections for each plasmid. The error bars represent the standard deviation from the mean. **C:** Inhibition of *NPY* promoter activity by the selective PKC inhibitor GF 109203X. Open columns represent the CAT activities obtained from transient transfections of untreated SH-SY5Y/trk cells with the −87/−47 *NPY/TKCAT* reporter plasmid. Filled and hatched columns represent activities obtained following TPA and NGF treatment, respectively, 24 h prior to harvest. In these cultures, GF 109203X was added 30 min prior to NGF or TPA stimulation (indicated below the bars) and was present during the following 24 h until harvest of the transfected cells.

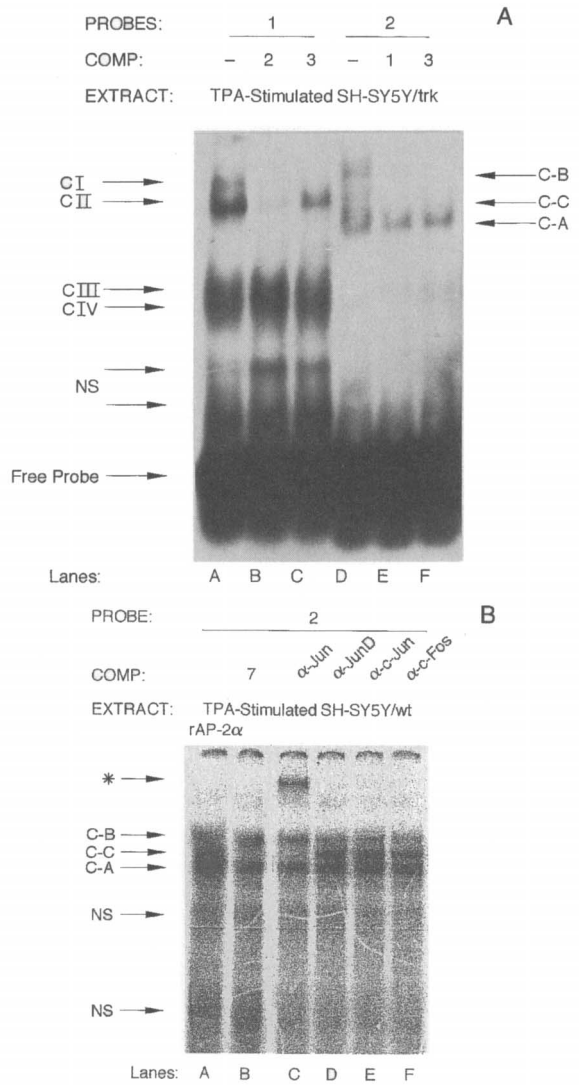


**FIG. 5.** EMSA of proteins that bind to the *NPY* promoter. **A:** Nuclear extracts prepared from TPA-treated SH-SY5Y/trk cells were incubated with radioactively labeled double-stranded *NPY* promoter oligonucleotides, −73/−45 (no. 1, Table 1; lanes A–C) or −87/−56 (no. 2, Table 1; lanes D–F). Binding reactions in the presence of 100-fold molar excess amounts of nonradioactive competition oligonucleotides are indicated above the lanes. Specific DNA-protein complexes CI to CIV and C-A to C-C are indicated to the left and right, respectively. NS, nonspecific complex. For nucleotide sequences of the oligonucleotides used, see Table 1. **B:** EMSA in the presence of recombinant AP-2α (rAP-2α) and AP-1 antisera. Nuclear extracts prepared from TPA-treated SH-SY5Y/wt cells were incubated with the −87/−56 *NPY* promoter oligonucleotide (no. 2, Table 1). In lane A, 0.01 fpu of rAP-2α was included in the binding reaction. Lane B, competition with an AP-2 oligonucleotide (no. 7 in Table 1). In lanes C–F, binding was monitored in the presence of AP-1 antiserum added after the radioactive −87/−56 oligonucleotide and incubated on ice for 1 h. Lane C, α-c-Jun/AP-1 (D)/α-Jun (sc-44 X); lane D, α-JunD (sc-74 X); lane E, α-c-Jun (sc-45 X); lane F, α-c-Fos (sc-52 X). The asterisk points to an anti-Jun supershifted complex.

also to C-A (Fig. 5B, lane A). Competition with an AP-2 oligonucleotide (no. 7 in Table. 1) in the absence of recombinant AP-2α in the binding reaction resulted in specific competition of C-C (Fig. 5B, lane B). Thus, AP-2α is clearly involved in the in vitro formation of the C-C complex. Postincubation with an antiserum broadly reactive with c-Jun, JunB, and JunD resulted in a supershifted complex and concomitant disappearance of C-C (Fig. 5B, lane C), whereas antisera specific for c-Jun, JunD, and c-Fos, respectively, did not reproducibly affect the C-C complex (Fig. 5B, lanes D–F). We conclude that both AP-2α and JunB or an unknown Jun family member participates in the formation of the C-C complex. The specificities of the different antisera were analyzed using a consensus AP-1 oligonucleotide (no. 4 in Table 1) as well as unrelated oligonucleotides and nuclear extracts prepared from SH-SY5Y/wt cells (data not shown).

### Analyses of proteins that bind to GSG elements in *NPY* promoter

To investigate DNA binding activities to the GSG-1 and GSG-2 elements located in the distal NGF RE in the *NPY* promoter at positions −193 to −176 (Fig. 2), EMSA was performed using a GSG oligonucleotide containing these two GSG boxes as well as an AP-2 site overlapping GSG-1 (no. 5 in Table 1 and Fig. 2). Three distinct complexes denoted GSG-I, -II, and -III were obtained (Fig. 6A, lanes A and H). The latter complex (GSG-III) was only weakly detectable. The specificity of these complexes was revealed by competition with an excess amount (200-fold molar excess) of unlabeled GSG oligonucleotide (Fig. 6A, lane F). Preincubation of the binding reaction with 200-fold molar excess of an oligonucleotide bearing clustered mutations in the GSG boxes (no. 6 in Table 1) resulted in competition for GSG-I and -III, whereas GSG-II binding activity was clearly detectable (Fig. 6A, lane G). We further analyzed whether recombinant AP-2α could interact with this oligonucleotide, as shown above for the −87/−56 oligonucleotide (Fig. 5B). Incubation with recombinant AP-2α alone failed to generate a complex under these binding conditions (Fig. 6A, lane B). However, incubation with nuclear extracts prepared from NGF-treated SH-SY5Y/trk cells together with different amounts of recombinant AP-2α resulted in a strong complex formation (Fig. 6A, lanes C and I). Also, GSG-II and -III complexes competed with an AP-2 oligonucleotide (no. 7 in Table 1; Fig. 6A, lane D). When 0.03 fpu of recombinant AP-2α was added, a co-migration of the enhanced complexes with the GSG-II and -III complexes was evident (Fig. 6A, lane I). Furthermore, the presence of transcription factor AP-2α in the GSG-II and -III complexes was revealed by incubation with an AP-2α-specific antiserum that resulted in a supershift of these complexes (Fig. 6A, lane J). Further, addition of an SP1 oligonucleotide (no. 8 in Table 1) in the binding reaction led to competition of GSG-I (Fig. 6A, lane E). EMSA using an oligonucleotide corresponding to the proximal GSG-3 element (see Fig. 2) failed

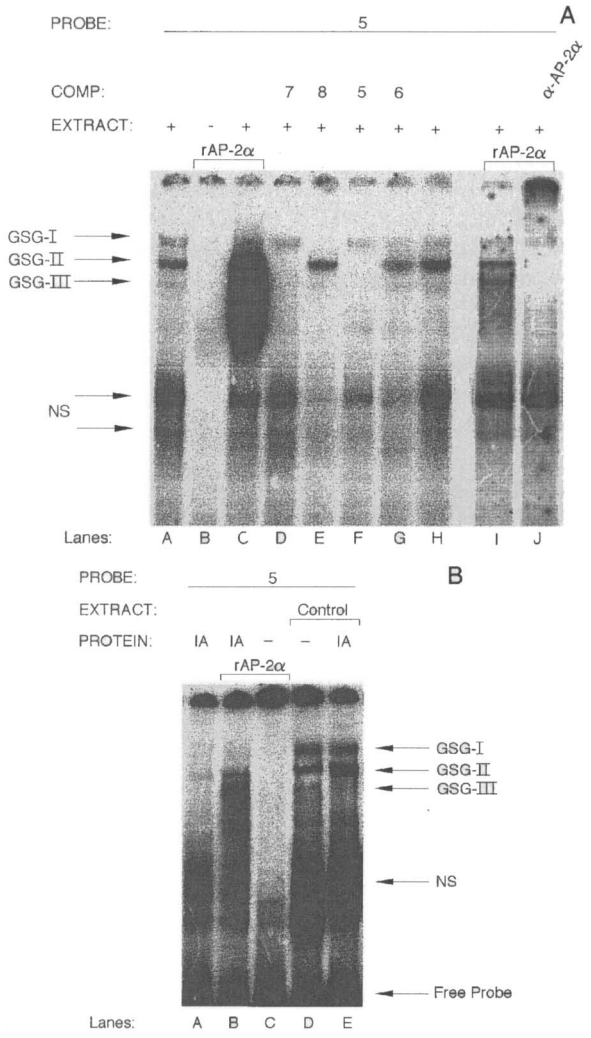


**FIG. 6.** DNA-protein interactions at the GSG elements in the *NPY* promoter. **A:** SH-SY5Y/trk nuclear extracts from NGF-treated cells were incubated with the −195/−173 *NPY* promoter oligonucleotide (no. 5, Table 1). In lanes A–C and H–J, binding reactions were without competitors. In lane B, 0.1 fpu of recombinant AP-2α (rAP-2α) was added alone; in lane C, it was combined with nuclear extracts. In lanes D–G, GSG binding competed with oligonucleotides as indicated. In a separate experiment, 0.03 fpu of rAP-2α was added to the nuclear extract alone (lane I). In lane J, an AP-2α-specific antiserum was included after the binding reaction. **B:** In vitro translated NGFI-A (lane A) and rAP-2α (lane C) were incubated alone with the GSG oligonucleotide (no. 5, Table 1). In lane B, NGFI-A and rAP-2α were co-incubated. Lanes D and E, SH-SY5Y/trk nuclear extracts were incubated alone or co-incubated with in vitro translated NGFI-A.

to generate specific DNA binding activities (not shown).

### Cooperative interactions between AP-2α and NGFI-A at distal GSG element in *NPY* promoter

To determine whether AP-2α binds to the GSG box together with previously identified GSG box binding proteins, recombinant AP-2α and in vitro translated NGFI-A (Crosby et al., 1991) were incubated together with the GSG oligonucleotide (no. 5 in Table 1). Prominent DNA binding activities were obtained upon co-incubation of these two proteins with the GSG oligonucleotide apparently generating the GSG-II and -III complexes seen with the nuclear extracts (Fig. 6B, lane B). NGFI-A alone in this binding reaction resulted only in a weak protein-DNA interaction with a size similar to the GSG-II complex (Fig. 6B, lane A). When increasing amounts of NGFI-A were incubated alone with the oligonucleotide, the formation of a distinct complex became evident (not shown). The cooperation between NGFI-A and other proteins binding to this element (e.g., AP-2α) was revealed by addition of in vitro translated NGFI-A to nuclear extracts prepared from resting SH-SY5Y/trk cells. Compare lanes D and E, where the latter lane contains in vitro translated NGFI-A. Furthermore, a luciferase vector was used in a parallel binding reaction to examine the specificity of NGFI-A. Binding of in vitro translated luciferase to the GSG oligonucleotide could not be obtained (not shown). From these experiments we conclude that transcription factors NGFI-A and AP-2α bind cooperatively to the GSG element in the *NPY* promoter.

## DISCUSSION

In this report, two NGF REs located at positions −246 to −118 and −87 to −47 were demonstrated to increase *NPY* transcription in activated SH-SY5Y neuroblastoma cells. The proximal NGF RE overlaps with a TRE. Following NGF treatment, human SH-SY5Y/trk neuroblastoma cells differentiate to a functional sympathetic neuronal phenotype accompanied by enhanced *NPY* expression levels (Lavenius et al., 1995). Here we show that activation of *NPY* transcription in response to 24-h NGF treatment occurs in SH-SY5Y/trk cells, whereas in SH-SY5Y/wt cells, *NPY* transcription was unaffected. Increased *NPY* transcription was obtained by phorbol ester treatment of both SH-SY5Y/wt and SH-SY5Y/trk cells. Thus, the mechanisms involved in NGF-evoked *NPY* transcription are defective in SH-SY5Y/wt cells. Clustered point mutations of the AP-1 and AP-2 sites in the proximal NGF RE/TRE abrogated the activation of *NPY* transcription. In the distal NGF RE, two GSG elements including a consensus AP-2 site were identified. GSG elements have been reported to be recognized by the NGFI/Egr family of immediate early proteins including NGFI-A/Egr1, Egr3, NGFI-C/Egr4, and Egr2/Krox20 (Crosby et al., 1991; Swirnoff and Milbrandt, 1995). Deletion of the GSG elements in the distal NGF RE of the *NPY* promoter resulted in decreased NGF responsiveness. These results revealed that increases in rates of transcription from the *NPY* promoter in response to NGF signaling are dependent on both the proximal and the distal NGF REs. Induction of *NGFI-A* and *NGFI-C* mRNAs following brain injury in regions where also AP-1 mRNA expression

was enhanced has recently been reported (Honkaniemi et al., 1995). In SH-SY5Y/trk cells, *NGFI-A* and *NGFI-C* mRNA expression was induced in response to both TPA and NGF treatment. Further, cooperative DNA binding between AP-2$\alpha$ and NGFI-A proteins to the GSG element located at positions $-193$ to $-176$ was revealed.

We noted that *NPY* transcription is activated by partly distinct mechanisms in response to phorbol ester- and NGF-induced signaling. The selective PKC inhibitor GF 109203X was administered in transfections of SH-SY5Y/trk cells with the $-87/-47$ *NPY/TKCAT* target plasmid. Almost a complete abrogation of the TPA induction but only a partial suppression of the transcriptional activation in response to NGF treatment were obtained. Co-transfections with a v-Jun activator plasmid with the $-87/-47$ *NPY/TKCAT* target revealed that transcription factor AP-1 activates *NPY* gene transcription in these cells. Furthermore, the dependence of transcription factor AP-1 for *NPY* gene expression in response to TPA and NGF was compared by co-transfecting the $-87/-47$ *NPY/TKCAT* target with the dominant negative $\Delta$9 Jun plasmid. In SH-SY5Y/trk cells, $\Delta$9 strongly suppressed the TPA-induced transcription from the *NPY* promoter. In contrast, NGF-evoked responses in SH-SY5Y/trk cells were affected to a lesser degree by the $\Delta$9 Jun plasmid. These results clearly establish that AP-1 is essential for activated *NPY* transcription in response to PKC signaling and that partly distinct pathways are operating to achieve transcriptional activation of the *NPY* gene in response to NGF and TPA in SH-SY5Y cells.

Furthermore, binding of the differentiation-associated transcription factor AP-2$\alpha$ was shown to be essential for efficient binding of both AP-1 and NGFI-A to the *NPY* promoter. The two NGF REs in the *NPY* promoter contain in total two perfect AP-2 sites (see Fig. 2). In addition, an imperfect AP-2/GSG-like box is located 3′ of the CT box (Fig. 2). Thus, interaction of both AP-1 and AP-2$\alpha$ with the *NPY* promoter appears to be crucial for *NPY* expression as clustered mutations of these promoter elements result in abolished *NPY* promoter activity. Moreover, protein binding to the *NPY* promoter was monitored in the presence of antisera specific for AP-1 proteins and revealed that JunB or a related member of the AP-1 family of transcription factors binds to the AP-1-like sites in the *NPY* promoter (Figs. 2 and 5B).

In conclusion, several lines of evidence underscore the importance of the NGFI-A, AP-1, and AP-2$\alpha$ proteins for NGF- and TPA-dependent *NPY* gene expression (Andersson et al., 1994; Minth-Worby, 1994; this study). Furthermore, two NGF REs were shown to be required for optimal NGF-evoked transcription of the *NPY* gene. In both these NGF REs, transcription factor AP-2$\alpha$ was shown to be involved in cooperative interactions with NGFI-A and AP-1 proteins, respectively. Transcription factor AP-2$\alpha$ has recently been implicated in inducing terminal differentiation and in inhibiting cancer cell growth by activation of $p21^{WAF/CIP1}$ expression (Zeng et al., 1997). Thus, the identification of induced AP-2$\alpha$ activities during the onset of neuronal differentiation further corroborates a crucial function for this transcription factor in cell growth control and differentiation. Activation of transcription factor AP-2$\alpha$ leads to a broad neuronal differentiation as demonstrated by activated transcription of the *NPY* gene in these neuroblastoma cells. We have shown that AP-2$\alpha$, NGFI-A, and AP-1 cooperate to activate *NPY* transcription in differentiating SH-SY5Y neuroblastoma cells. Further characterization of the mechanisms by which these transcription factors become activated should reveal the functional consequences for neuronal differentiation and growth control.

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