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Nerve Growth Factor Employs Multiple Pathways to Induce Primary Response Genes in PC12 Cells

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Nerve growth factor (NGF) leads to neuronal differentiation of PC12 cells and promotes their survival in serum-free medium. Past studies have shown that purine analogues block some of the effects of NGF but not others and thus that they can be used to dissect the mechanistic pathways of its action. In the present work we used 2-aminopurine (2-AP) and 6-thioguanine (6-TG) to examine whether NGF causes activation of primary response genes through a single signaling pathway or via multiple pathways. Northern blot analysis and nuclear run-off transcription assays were used to assess the activation of c-*fos*, c-*jun*, *TIS1*, *TIS8*, and *TIS11* after exposure of PC12 cells to NGF in the presence or absence of 2-AP and 6-TG. Our findings indicate that NGF appears to employ at least three distinct pathways to induce early genes in PC12 cells. This suggests that the NGF signaling mechanism diverges at an early point after interaction of NGF with its receptor.

INTRODUCTION

Nerve growth factor (NGF) promotes survival, growth, and differentiation of neuronal populations in the peripheral as well as in the central nervous system (Levi-Montalcini and Angeletti, 1968; Levi-Montalcini, 1987), but the mechanism of these actions is only partially understood (Levi and Alemá, 1991). Rat PC12 pheochromocytoma cells have been very useful for the study of the signaling pathways used by NGF because they can be maintained in a "naive" state without prior exposure to this factor (Greene and Tischler, 1976; Greene and Tischler, 1982; Levi and Alemá, 1991). NGF leads to neuronal differentiation of PC12 cells and promotes their survival in serum-free medium (Greene, 1978).

Past studies have demonstrated that purine analogues, such as 2-aminopurine (2-AP) and 6-thioguanine (6-TG), can be used to dissect the pathways employed by NGF to elicit its actions (Volonté *et al.*, 1989; Greene *et al.*, 1990; Volonté and Greene, 1990). It has been shown that these compounds suppress some but not all of the effects of NGF on PC12 cells and neurons (Volonté *et al.*, 1989; Greene *et al.*, 1990). For example, they inhibit the NGF-promoted neurite regeneration by PC12 cells and neurite outgrowth by cultured sympathetic and sensory neurons (Volonté *et al.*, 1989; Greene *et al.*, 1990). In addition, in PC12 cultures, both analogues inhibit the NGF-stimulated increase of ornithine decarboxylase mRNA levels and activity (Volonté and Greene, 1990). In contrast, they do not affect the NGF-promoted survival of neuronal target cells (Greene *et al.*, 1990) and the rapid NGF-induced phosphorylation of tyrosine hydroxylase and of microtubule-associated protein 1.2 (MAP1.2) in PC12 cells (Volonté *et al.*, 1989). These findings have suggested the presence of at least two separate pathways in the NGF mechanism of action. The presence of at least a third pathway was indicated by the observation that some actions can be blocked by 2-AP but not by 6-TG. These responses include the induction of c-*fos* mRNA and the activation of protein kinase N (PKN), an NGF-regulated serine protein kinase (Rowland *et al.*, 1987; Volonté *et al.*, 1989).

Although the mechanisms by which purine analogues differentially block NGF actions have not been entirely established, recent findings suggested inhibition of protein kinase activities. 2-AP has been reported to inhibit at least several different protein kinase activities, including PKN and an NGF-activated MAP kinase (Farrell *et al.*, 1977; Volonté *et al.*, 1989; Mahadevan *et al.*, 1990; Tsao *et al.*, 1990; Volonté and Greene, 1992). 6-TG, in contrast, appears thus far to be a quite specific inhibitor, blocking only the activity of PKN but not the activity of a variety of other protein kinases (Volonté and Greene, 1992).

Part of the initial response of PC12 cells to NGF is the rapid and transient activation of a number of pri-

mary response genes (Greenberg *et al.*, 1985; Kujubu *et al.*, 1987; Wu *et al.*, 1989). Many of these genes encode possible transcription factors, and thus they may play roles in the initiation and regulation of subsequent responses to NGF (for review, Herschman, 1991). The aim of this study has been to use purine analogues to further dissect the NGF mechanism of action, in particular the pathways by which NGF induces early genes in PC12 cells. Specifically, we have studied the effects of 2-AP and 6-TG on the induction of five genes that are rapidly and transiently activated by NGF; these include c-*fos*, c-*jun*, *TIS1* (also designated *NGFI-B* or *nur77*), *TIS8* (also designated *NGFI-A*, *egr-1*, *zif-268*, or *krox24*), and *TIS11*. Our findings suggest that NGF regulates primary response genes by at least three distinct pathways.

MATERIALS AND METHODS

Materials

Mouse NGF was prepared from adult male submaxillary glands as previously described (Mobley *et al.*, 1976) and was used at a concentration of 50 ng/ml. Purine analogues were purchased from Sigma Chemical (St. Louis, MO). Stock solutions were prepared in RPMI 1640 (pH 7.4). Plasmids containing cDNA inserts for *TIS1*, *TIS8*, and *TIS11* (Lim *et al.*, 1987) were kindly provided by Dr. H.R. Herschman, University of California, Los Angeles.

Cell Culture

PC12 cells were cultured as previously described (Greene and Tischler, 1976, 1982) on collagen-coated dishes in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum.

Northern Blot Analysis

PC12 cells were cultured in 100-mm dishes and pretreated for 1 h, where applicable, with 2-AP (10 mM final concentration) or 6-TG (100 μM final concentration). The compounds were added directly to the medium from 10 to 50 times stock solutions. The concentrations of the purine analogues used are comparable with those found to be effective in previous studies (Volonté *et al.*, 1989; Volonté and Greene, 1990). NGF (from a 5000 times stock solution) was added to the cultures for different times, the cells were then washed with cold phosphate-buffered saline (PBS), and immediately used for isolation of total RNA by a single step acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Fifteen micrograms of RNA per sample were separated on 1% agarose-formaldehyde gels and transferred to nitrocellulose membranes (Maniatis *et al.*, 1982). ^{32}P-labeled probes for *TIS1*, *TIS8*, *TIS11* (Lim *et al.*, 1987), c-*fos* (Curran *et al.*, 1982), or c-*jun* (Bohmann *et al.*, 1987) were prepared by random priming reactions (Maniatis *et al.*, 1982). The blots were hybridized with radiolabeled probes in the presence of 3× SSC at 37°C overnight, washed in 0.1× SSC and 0.5% sodium dodecyl sulfate (SDS) at 60°C for 2 h, and subjected to autoradiography. Subsequently, blots were stripped and rehybridized with a ^{32}P-labeled probe to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Piechaczyk *et al.*, 1984), a gene whose message is not rapidly regulated by NGF. Quantitative densitometry was performed on autoradiograms using a MicroScan 1000 Gel Analyzer (Technology Resources, Nashville, TN). Levels of mRNA expression were normalized against levels of GAPDH mRNA. Effects of the purine analogues were assayed in two to three independent experiments for each gene.

Nuclear Run-Off Transcription Assay

PC12 cells were grown in 100-mm dishes and pretreated where applicable with 2-AP (10 mM final concentration) or 6-TG (100 or 500 μM final concentration). NGF was added for different times, the cultures were washed with cold PBS, and run-off assays were carried out on isolated nuclei using a previously described protocol (Ausubel *et al.*, 1988). After hybridization the filters were washed in 2× SSC and 0.1% SDS at 65°C for 2 h and subjected to autoradiography.

RESULTS

The basic protocol used in these experiments was as follows. PC12 cells were pretreated, where applicable, for 1 h with 10 mM 2-AP or 100–500 μM 6-TG. NGF was then added to the cultures for various times, and the cells were harvested and used for isolation of total cellular RNA or of intact nuclei. Subsequently, Northern blot analysis and run-off assays were carried out using probes for five different NGF-induced immediate early genes.

Among the early effects of NGF is the induction of *TIS8* (also designated *NGFI-A*, *egr-1*, *zif-268*, or *krox24*) mRNA, which encodes a DNA-binding protein with "zinc-finger" motifs (Kujubu *et al.*, 1987; Milbrandt, 1987; Herschman, 1991). As shown in Figure 1 (A and B), neither 2-AP nor 6-TG inhibit the NGF-induced increase of *TIS8* mRNA. Although 2-AP and 6-TG alone do not induce *TIS8* mRNA, they do cause a degree of superinduction in the presence of NGF. In two independent experiments, the peaks of the mRNA increase in the presence of 2-AP and NGF were about two- and fivefold that induced by NGF alone. The peaks in the presence of 6-TG and NGF were (for 2 independent experiments) about 1.5- and 3-fold greater than with NGF alone. This effect is transient, because by 8 h of NGF treatment (in the presence or absence of the purine analogues) *TIS8* mRNA again reaches basal levels. Nuclear run-off analysis on PC12 cells harvested at 15, 30, and 60 min after treatment with NGF showed that purine analogues also do not block *TIS8* transcription (Figure 2). Consistent with the mRNA levels, in the presence of the compounds the NGF-dependent activation of *TIS8* transcription is equal or higher than that in control cells treated only with NGF. Run-off assays of cells cultured in the absence of NGF with or without the compounds did not reveal detectable signal.

c-*fos* and c-*jun* encode transcription-regulatory proteins that are also rapidly induced by NGF (Greenberg *et al.*, 1985; Milbrandt, 1986; Wu *et al.*, 1989; Herschman, 1991). Past studies have shown that 2-AP inhibits c-*fos* mRNA induction caused by a variety of stimuli in several human cell lines (Zinn *et al.*, 1988) and by epidermal growth factor (EGF) and phorbol ester in C3H 10T½ fibroblasts (Mahadevan *et al.*, 1990). In PC12 cells it has been shown that the increase of c-*fos* mRNA after 30 or 60 min of treatment with NGF is inhibited by 2-AP but not by 6-TG (Volonté *et al.*, 1989; Machida *et*

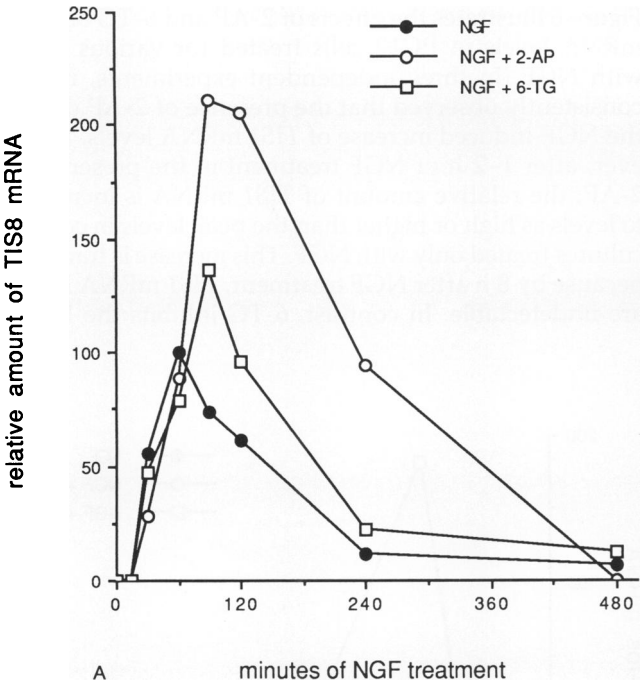
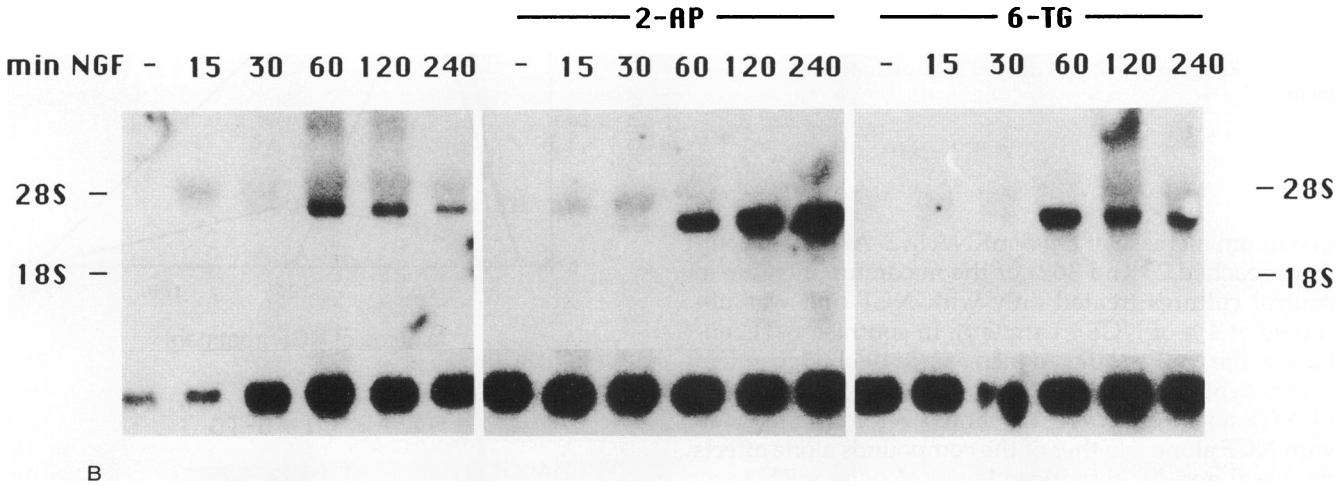


Figure 1. Effects of purine analogues on the NGF-dependent increase of *TIS8* mRNA. PC12 cells were pretreated where applicable with 10 mM 2-AP or 100 μM 6-TG for 1 h and then treated with NGF (50 ng/ml) for different times. Total cellular RNA was prepared as described under MATERIALS AND METHODS. Fifteen micrograms of total RNA were separated on 1% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized with ^{32}P-labeled *TIS8* probe. Subsequently, the blots were stripped and hybridized with ^{32}P-labeled GAPDH probe. (A) Autoradiograms of Northern blots were analyzed by densitometric scanning. Levels of *TIS8* mRNA were normalized against levels of GAPDH mRNA. Relative levels of mRNA are presented as percentage of the maximum increase with NGF. (B) Autoradiograms of Northern blots from a representative experiment. Upper autoradiogram shows results obtained with a radiolabeled *TIS8* probe. Lower autoradiogram shows results after reprobing the same blot with a radiolabeled probe for GAPDH.



al., 1991). The time course of c-*fos* mRNA induction in the presence or absence of the purine analogues is presented in Figure 3A. Consistent with previous reports, 2-AP suppresses c-*fos* mRNA induction, allowing only a small increase after several hours (in 2 independent experiments, the maximum levels of c-*fos* mRNA in the presence of 2-AP and NGF reached 7 and 21% of the peak reached with NGF alone). 2-AP alone does not affect c-*fos* mRNA. 6-TG alone also does not induce c-*fos* mRNA, but in contrast to 2-AP, it enhances the NGF-induced increase (Figure 3A). Nuclear run-off analysis of c-*fos* performed on PC12 cells treated for 15 min with NGF showed that the rapid NGF-activated transcription of c-*fos* was inhibited by 92% in the presence of 2-AP but was in contrast slightly enhanced by 6-TG (the levels of c-*fos* transcription in the presence of 6-TG and NGF are 30% higher than in the presence of NGF alone; Figure 3B).

The data in Figure 4 show that the effects of purine analogues on induction of c-*jun* mRNA are similar to their effects on the induction of c-*fos* mRNA. The NGF-induced increase of c-*jun* mRNA is inhibited by 2-AP, whose presence allows only a small delayed NGF-regulated increase (in 2 independent experiments, the

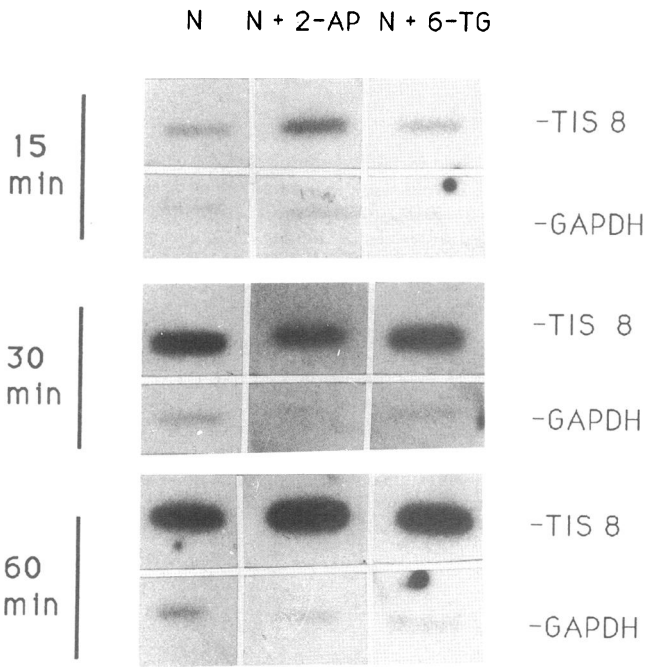


Figure 2. Effects of purine analogues on the transcriptional activation of *TIS8* by NGF. PC12 cells were pretreated where applicable with 10 mM 2-AP or 100 μM 6-TG for 1 h. Nuclei were isolated at 15, 30, or 60 min after NGF addition. Labeled RNA was hybridized to 20 μg of linearized *TIS8* or GAPDH DNA that was immobilized on nitrocellulose. Each time point was derived from an independent experiment.

maximum increase of c-*jun* mRNA in 2-AP-treated cultures reached 23 and 30% of the maximum increase in control cultures treated only with NGF and was observed at 4 h of NGF treatment). In contrast, 6-TG enhances the NGF-regulated increase. In two independent experiments, the peaks of c-*jun* mRNA in the presence of NGF and 6-TG were 1.6- and 1.7-fold higher than with NGF alone. Neither of the compounds alone affects the basal non-NGF-induced levels of c-*jun* mRNA.

TIS11 is yet another NGF-inducible gene (Kujubu *et al.*, 1987; Varnum *et al.*, 1989; Herschman, 1991). The function of the *TIS11* protein is unknown. As shown in Figure 5, 2-AP inhibits the NGF-induced increase of *TIS11* mRNA. In three independent experiments, the levels of *TIS11* mRNA in the presence of 2-AP and NGF reached 6–15% of the peak levels with NGF alone. 6-TG does not inhibit the NGF-induced increase. Thus, *TIS11* induction shows a profile of sensitivity to the purine analogues that is similar to those shown by c-*fos* and c-*jun*.

The *TIS1* (also designated *NGFI-B* or *nur77*) gene is a member of a family of ligand-binding transcription factors and it likewise shows rapid induction by NGF (Kujubu *et al.*, 1987; Milbrandt, 1988; Herschman, 1991).

Figure 6 illustrates the effects of 2-AP and 6-TG on *TIS1* mRNA levels in PC12 cells treated for various times with NGF. In three independent experiments, it was consistently observed that the presence of 2-AP delays the NGF-induced increase of *TIS1* mRNA levels. However, after 1–2 h of NGF treatment in the presence of 2-AP, the relative amount of *TIS1* mRNA is increased to levels as high or higher than the peak levels in control cultures treated only with NGF. This increase is transient because by 8 h after NGF treatment, *TIS1* mRNA levels are undetectable. In contrast, 6-TG inhibits the NGF-

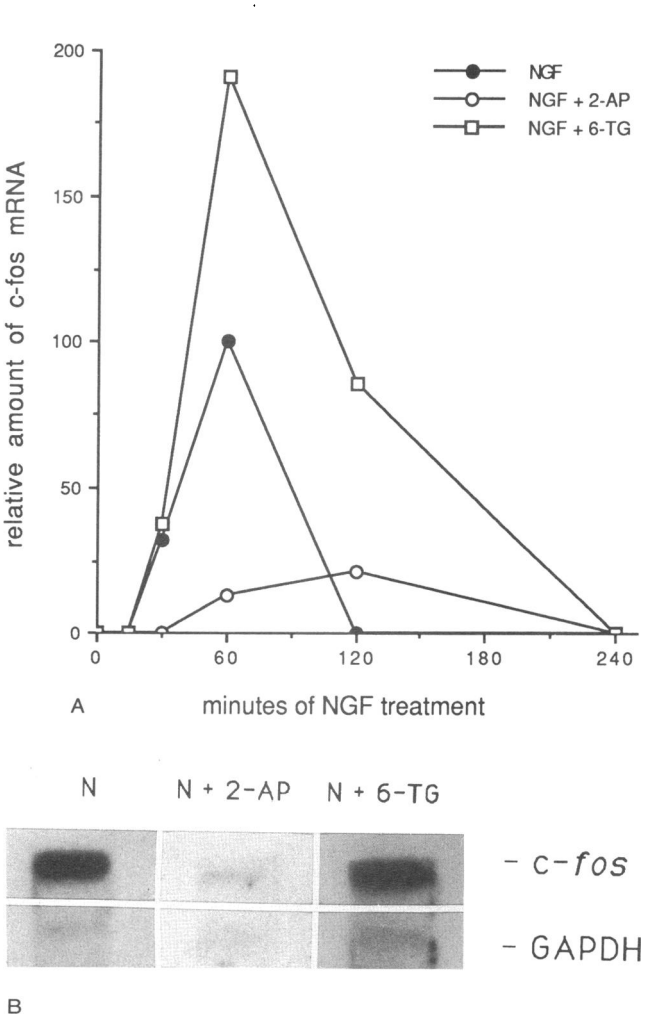


Figure 3. Effects of purine analogues on the NGF-dependent activation of c-*fos*. (A) Northern blots from PC12 cells performed as described in MATERIALS AND METHODS were hybridized with ^{32}P-labeled c-*fos* probe and subsequently with ^{32}P-labeled GAPDH probe. Autoradiograms of Northern blots were analyzed by densitometric scanning. Levels of c-*fos* mRNA were normalized against levels of GAPDH mRNA. Relative levels of mRNA are presented as percentage of the maximum NGF increase. (B) Nuclear run-off analysis of c-*fos* and GAPDH performed as described in MATERIALS AND METHODS. PC12 cells were pretreated with 10 mM 2-AP or 500 μM 6-TG for 1 h where applicable and then treated with NGF for 15 min.

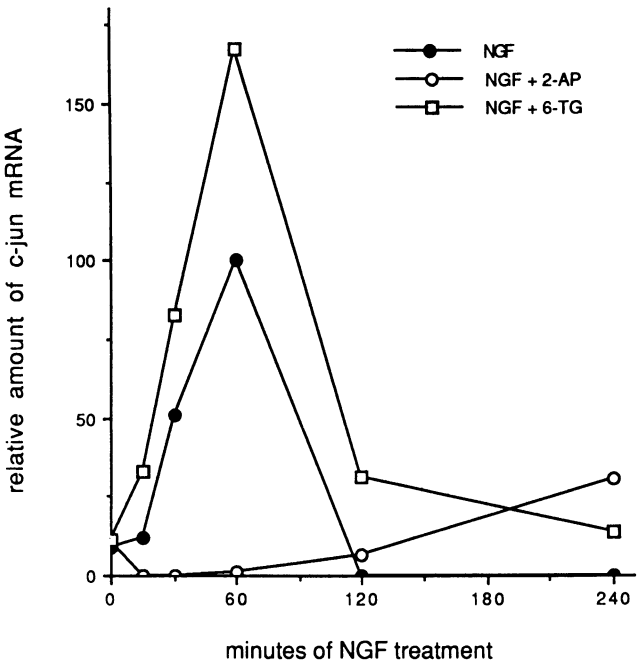


Figure 4. Effects of purine analogues on the NGF-dependent increase of c-*jun* mRNA. Blots of PC12 cell RNA were performed as described in MATERIALS AND METHODS and were hybridized with ^{32}P-labeled c-*jun* probe and subsequently with ^{32}P-labeled GAPDH probe. Autoradiograms of Northern blots were analyzed by densitometric scanning. Levels of c-*jun* mRNA were normalized against levels of GAPDH mRNA. Relative levels of mRNA are presented as percentage of the maximum NGF increase.

induced increase of *TIS1* mRNA, allowing only a small increase (13–20% of peak levels) that occurs at a time later than the peak expression of the gene in control cells treated only with NGF.

DISCUSSION

It has been well established that different agents can lead to induction of the same primary response genes (Herschman, 1991). For example, with PC12 cells, not only NGF but also fibroblast growth factor, EGF, c-AMP analogues, tumor promoters, and membrane-depolarizing agents can lead to induction of a similar set of immediate early genes (Greenberg *et al.*, 1985; Milbrandt, 1986; Kujubu *et al.*, 1987; Cho *et al.*, 1989; Herschman, 1991). The induction of these genes can occur in the absence of protein synthesis and thus appears to require only modification of pre-existing factors (for review, Herschman, 1991). Studies on the kinetics and levels of induction of these genes in wild-type and mutant PC12 cells (Altin *et al.*, 1991; Herschman, 1991) suggest that the different agents utilize independent signaling pathways to lead to induction of the same gene.

The aim of the present study has been to determine whether a single agent, NGF, activates different primary response genes by means of a single pathway or by means of multiple signaling pathways. We chose five primary response genes that have different properties and are rapidly activated by NGF with different kinetics: c-*fos*, c-*jun*, *TIS1*, *TIS8*, and *TIS11*. The c-*fos* and c-*jun* genes encode proteins that form heterodimers and cause transcriptional activation (Halazonetis *et al.*, 1988). The product of *TIS1* is a member of the superfamily of ligand-binding transcription factors (Kujubu *et al.*, 1987; Milbrandt, 1988; Herschman, 1991) and the product of *TIS8* is a zinc-finger containing DNA-binding protein (Kujubu *et al.*, 1987; Milbrandt, 1987; Herschman, 1991). The function of the *TIS11* product is presently unknown; however, it is suggested to be a transcriptional regulator (Kujubu *et al.*, 1987; Varnum *et al.*, 1989; Herschman, 1991).

To dissect the possible pathways by which NGF causes activation of immediate early genes, we used the purine analogues 2-AP and 6-TG. The use of these compounds in past studies indicated that there are several distinct pathways by which NGF exerts its effects (Volonté *et al.*, 1989; Greene *et al.*, 1990; Volonté and Greene, 1990). There are actions not inhibited by either 2-AP or 6-TG, such as NGF-promoted neuronal survival, actions inhibited by both compounds, such as NGF-dependent neurite outgrowth and induction of ornithine decarboxylase activity and message, and actions inhibited by 2-AP but not 6-TG, such as the NGF-induced activation of PKN and increase of c-*fos* mRNA.

We found here that purine analogues have differential effects on the induction of specific genes by NGF. The activation of *TIS8* transcription and the induction of *TIS8* mRNA are not inhibited by either 2-AP or 6-TG, whereas the inductions of c-*fos* transcription and mRNA and of c-*jun* and *TIS11* mRNAs are inhibited by 2-AP but not by 6-TG. Finally, induction of *TIS1* mRNA was inhibited by 6-TG and significantly delayed by 2-AP. The observation that certain genes are blocked and others are not rules out nonspecific actions on cellular metabolism. Thus, it appears that at least three distinct pathways are involved in the induction of primary response genes by NGF.

In several of the above cases, gene induction is elevated in the presence of the analogues. One possibility is that the purine analogues nonspecifically inhibit the expression of gene products that are involved in message degradation. Arguing against this is the observation that at least in the cases of c-*fos* and *TIS8* the mRNA levels correlate well with the rates of transcription. Another possibility is that the purine analogues nonspecifically interfere with synthesis of the gene products in question, thus abolishing possible feed-back transcriptional repression of these genes. If this is true, purine analogues should nonselectively block protein synthesis. However, this does not seem to be the case because in previous studies it has been shown that 6-TG does not inhibit the NGF-induced increase of ornithine decarboxylase

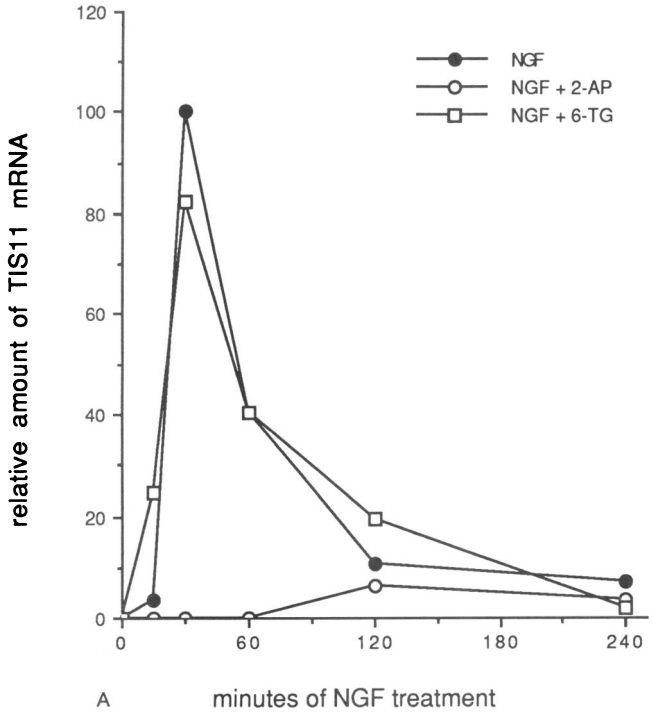
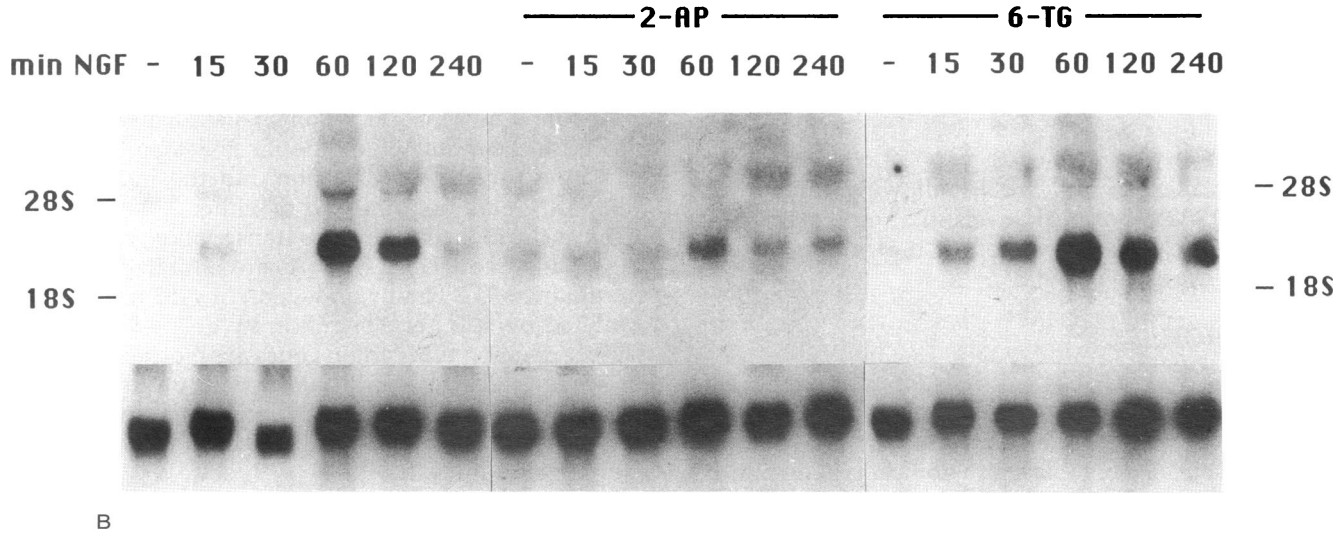


Figure 5. Effects of purine analogues on the induction of *TIS11* mRNA by NGF. (A) Autoradiograms of Northern blots hybridized with ^{32}P-labeled *TIS11* probe and subsequently with ^{32}P-labeled GAPDH probe were analyzed by densitometric scanning. Levels of *TIS11* mRNA were normalized against levels of GAPDH mRNA. Relative levels of mRNA are presented as percentage of the maximum NGF increase. (B) Autoradiograms of Northern blots from a representative experiment. Upper autoradiogram shows results obtained with a radiolabeled *TIS11* probe. Lower autoradiogram shows results after reprobing the same blot with a radiolabeled probe for GAPDH.



activity when it is added to PC12 cell cultures as early as 3 min after the addition of NGF (Volonté and Greene, 1990). An attractive possibility is that the purine analogues selectively derepress one or more alternative pathways for NGF-induced transcription, thus leading to an additional increase in transcription that is nonetheless transient.

The consistent delay of *TIS1* induction by NGF in the presence of 2-AP suggests that the initial preferred pathway that leads to the induction of this gene is inhibited. It is possible that the 2-AP-dependent transient superinduction of *TIS1* mRNA observed later is due to the derepression or permission of expression of a slower secondary pathway that does not otherwise lead to gene induction. This is consistent with the concept that a single growth factor, such as NGF, can stimulate induction of a given gene by multiple pathways (Altin *et al.*, 1991). 6-TG also inhibits the initial NGF-dependent pathway that induces *TIS1* mRNA but in contrast to 2-AP does not appear to release an alternative pathway for induction of this gene.

The means by which purine analogues differentially

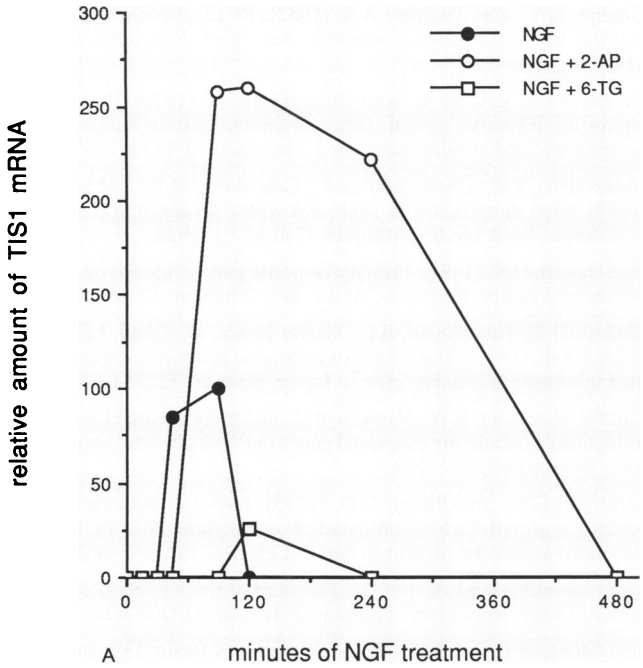
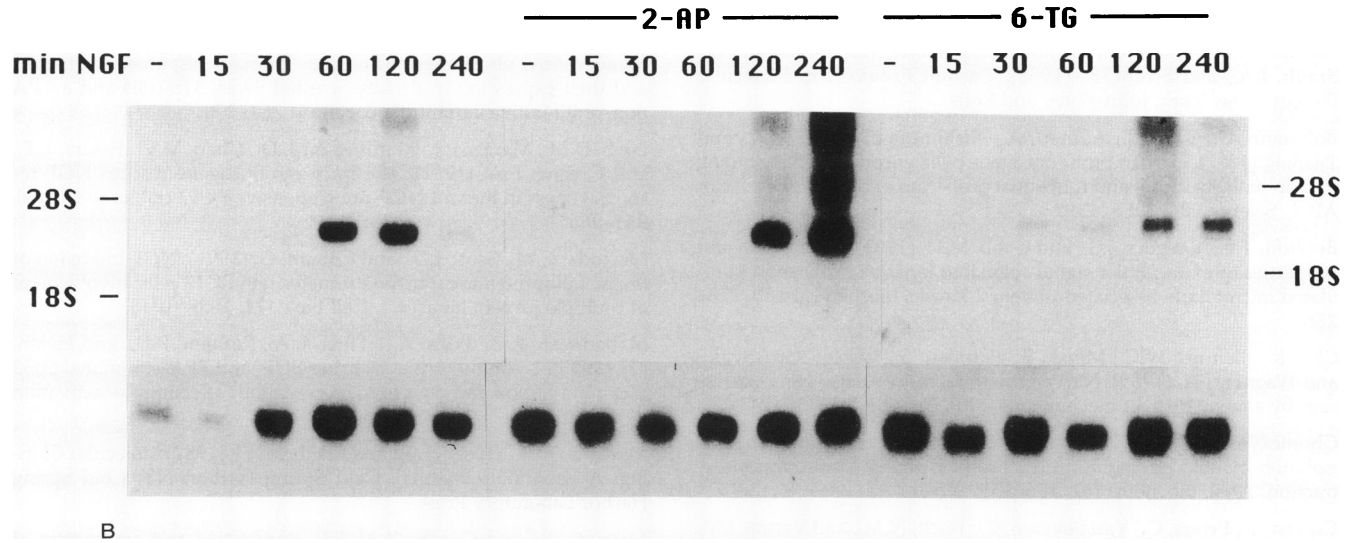


Figure 6. Effect of purine analogues on the NGF-dependent increase of *TIS1* mRNA. (A) Autoradiograms of Northern blots hybridized with ^{32}P-labeled *TIS1* probe and subsequently with ^{32}P-labeled GAPDH probe were analyzed by densitometric scanning. Levels of *TIS1* mRNA were normalized against levels of GAPDH mRNA. Relative levels of mRNA are presented as percentage of the maximum NGF increase. (B) Autoradiograms of Northern blots from a representative experiment. Upper autoradiogram shows results obtained with a radiolabeled *TIS1* probe. Lower autoradiogram shows results after reprobing the same blot with a radiolabeled probe for GAPDH.



affect the induction of various primary response genes are presently unknown. Activation of these genes is thought to require only modification of pre-existing transcriptional factors. Regulation of protein kinases leads to early gene induction in a variety of cell types (Herschman, 1991; Sheng *et al.*, 1991) and NGF rapidly stimulates several different protein kinase activities in PC12 cells (Landreth and Rieser, 1985; Matsuda and Guroff, 1987; Rowland *et al.*, 1987; Tsao *et al.*, 1990; Levi and Alemá, 1991). Previous studies have shown that 2-AP inhibits several, but not all, protein kinase activities. For example, it inhibits RNA-dependent eIF-2a protein kinase (Farrell *et al.*, 1977), calcium/calmodulin-dependent protein kinase type I (Volonté and Greene, 1992), MAP kinases (Tsao *et al.*, 1990; Boulton *et al.*, 1991), and PKN (Volonté *et al.*, 1989) but does not block protein kinase C or casein kinase II (Volonté and Greene, 1992). 6-TG, in contrast, appears to be a much more specific inhibitor. PKN is the only protein kinase, among the many thus far tested, to be inhibited by 6-TG (Volonté and Greene, 1992). It is therefore possible that 2-AP and 6-TG affect the pathways of

early gene induction by selectively inhibiting protein kinases, at least one of which is PKN.

Recent findings have indicated that NGF binds and rapidly activates the tyrosine kinase gp140^{prototrk} that leads to at least several NGF responses in PC12 cells (Kaplan *et al.*, 1991; Loeb *et al.*, 1991). If activation of gp140^{prototrk} is the single initial step in induction of the genes studied here, then our findings indicate that the NGF mechanism must thereafter diverge into at least three separable pathways, each of which regulates early gene transcription.

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