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Nerve Growth Factor Regulates Gene Expression by Several Distinct Mechanisms

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To help elucidate the mechanisms by which nerve growth factor (NGF) regulates gene expression, we have identified and studied four genes (*a-2*, *d-2*, *d-4*, and *d-5*) that are positively regulated by NGF in PC12 cells, including one (*d-2*) which has previously been identified as a putative transcription factor (NGF I-A). Three of these genes, including *d-2*, were induced very rapidly at the transcriptional level, but the relative time courses of transcription and mRNA accumulation of each of these three genes were distinct. The fourth gene (*d-4*) displayed no apparent increase in transcription that corresponded to the increase in its mRNA, suggesting that NGF may regulate its expression at a posttranscriptional level. Thus, NGF positively regulates gene expression by more than one mechanism. These genes could also be distinguished on the basis of their response to cyclic AMP. The expression of *d-2* and *a-2* was increased by cholera toxin and further augmented by NGF; however, cholera toxin not only failed to increase the levels of *d-5* and *d-4* mRNA but also actually inhibited the NGF-dependent increase. The expression of each of these genes, including *d-2* (NGF I-A), was also increased by fibroblast growth factor, epidermal growth factor (EGF), phorbol myristate acetate, and in some cases insulin, showing that the regulation of these genes is not unique to NGF. Because each of these genes was expressed in response to phorbol myristate acetate and EGF, their expression may be necessary but is certainly not sufficient for neurite formation. The protein kinase inhibitor K-252a prevented the NGF-associated, but not the acidic FGF-associated, induction of *d-2* and *d-5* gene expression, suggesting that these two growth factors may regulate gene expression via different cellular pathways. The study of the regulation of the expression of these and other NGF-inducible genes should provide valuable new information concerning how NGF and other growth factors cause neural differentiation.

Nerve growth factor (NGF) is a peptide hormone that is essential for the development and the survival of sympathetic nerves, sensory nerves, and certain populations of nerves in the central nervous system (32, 52). It also influences many aspects of cell metabolism, including RNA synthesis, protein synthesis, protein phosphorylation, and ion flux (1, 11, 22, 46, 54). However, studies of NGF action on neural differentiation have been hampered because NGF influences both cell survival and cell differentiation.

PC12 cells, a cloned line derived from a rat pheochromocytoma, has been a useful model system for studying NGF-dependent differentiation (18). Although NGF is not required as a survival factor for these cells, it retains the ability to stimulate differentiation and process formation. The heparin-binding peptide growth factors, acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF), also stimulate neurite outgrowth and differentiation in PC12 cells in a pattern that is not distinguishable from that of NGF (47, 51). In the presence of NGF, aFGF, or bFGF, PC12 cells take on many of the properties of a mature sympathetic nerve. In contrast, both epidermal growth factor (EGF) and insulin can bind to PC12 and elicit biochemical changes, but neither can induce differentiation (18, 19, 24).

The exact role of second messengers in mediating the effects of these peptide hormones is not yet well understood, but there has been reasonable evidence that cyclic AMP (cAMP) and phosphatidylinositol-derived second messengers play a role in mediating some of the effects of NGF on PC12 cells. cAMP can induce neurite formation and mimic many of the effects of NGF. However, accumulating evidence suggests that cAMP is not the key second messenger utilized by NGF to stimulate differentiation. Firstly, the cAMP-induced neurites are much shorter than NGF-induced neurites and are found at a much earlier time (21). Secondly, cAMP is known to antagonize the effect of NGF on neurite extension or protein synthesis in certain cases (12, 17, 40). Lastly, mutant PC12 cells that are deficient in the cAMP-dependent protein kinases are not responsive to the effects of adenosine and cAMP analogs but retain their sensitivity to NGF (50). However, the pathways are not completely separate because at least some NGF-dependent phosphorylations are mediated by the cAMP-dependent protein kinases (8). Both NGF and aFGF are known to stimulate phosphatidylinositol turnover and activate protein kinase C, and at least some NGF-dependent phosphorylations are mediated by protein kinase C (7, 8, 23, 48, 49). However, diacylglycerol is also not the key second messenger for NGF because phorbol myristate acetate (PMA) can neither stimulate the formation of neurites nor potentiate the action of NGF (3). Thus, there must exist additional second messenger systems that mediate the actions of NGF, aFGF, and bFGF.

One of the essential features of NGF action in these cells is the induction of a specific set of genes that are required for neurite formation to occur (4). A number of NGF-dependent transcription events have been identified (30, 31, 35, 37, 43), and at least one NGF-induced gene may encode a transcrip-

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tion factor (36). We have isolated several genes whose expression is regulated by NGF, and studied their regulation by NGF, fibroblast growth factor (FGF), EGF, insulin, dibutyryl cAMP (dbcAMP), cholera toxin, and PMA in order to better understand the role of second messenger systems in the action of the peptide growth factors. Here we present the preliminary characterization of four independently isolated clones and show that each of them has a distinct pattern of temporal expression and response to various agents.

MATERIALS AND METHODS

Cell culture. PC12 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and 5% horse serum, and the medium was changed every 2 days. Cells were plated in 150-mm-diameter dishes (50 to 70% confluency) and were treated with the following agents: NGF (100 ng/ml), EGF (10 ng/ml), aFGF (10 U/ml) plus heparin (100 μg/ml), bFGF (5 U/ml), cholera toxin (3×10^{-11} M), dbcAMP (1 mM), insulin (200 ng/ml), PMA (100 ng/ml), cycloheximide (10 μg/ml), or K-252a (250 nM). One unit of FGF is defined as the amount of FGF that is sufficient to give half maximal stimulation of mitogenic activity in NIH 3T3 fibroblast cells (10). FGFs were used at concentrations greater than that required to induce maximal neurite outgrowth. β-NGF was prepared in this laboratory by the method of Mobley et al. (38), and it was over 99% pure. EGF, PMA, and dbcAMP were purchased from Sigma Chemical Co. Insulin was purchased from Collaborative Research, Inc. and cholera toxin was purchased from Schwarz/Mann. K-252a was purchased from Kyowa Medex Company. FGFs were kindly provided by P. D'Amore. For either run-on assay or Northern (RNA) analysis, approximately 10^8 cells were used.

Construction of cDNA library. Subconfluent PC12 cells (~50 to 70% confluency) were treated with NGF for 2 h in the presence of fresh medium. At the end of the treatment, cells were washed with ice-cold phosphate-buffered saline two times and harvested. poly(A)$^+$ RNA from approximately 10^8 treated cells was prepared by using guanidium isothiocyanate (5, 34) and oligo(dT) chromatography as described previously (34). cDNA was prepared from 10 μg of poly(A)$^+$ RNA, and a fraction of this cDNA was used for the construction of cDNA library by the method of Gubler and Hoffman (20). λgt10 (26) and λ packaging extract (purchased from Stratagene) were used to construct the library. Approximately 8×10^6 independent bacteriophages were obtained.

Isolation of NGF-induced clones from the library. Approximately 20,000 phages of the 2-h library were plated onto 150-mm-diameter plates (2,000 phages per plate) and duplicated filters were prepared as described previously (2). Radioactive probe was made by using cDNA prepared from poly(A)$^+$ RNA of either untreated cells (0-h probe) or cells treated with NGF for 2 h (2-h probe) and labeled using random hexamer as a primer (14). Each set of filters was hybridized with either the 0- or 2-h probe. The plaques that hybridized only to the latter probe, not to the former, were picked and rescreened after plaque purification. To characterize these NGF-induced clones, phage DNA was prepared from clones following secondary screening, restricted with *Eco*RI, and subcloned into pUC19 (53). The sizes of the cDNA inserts were determined by measuring their mobility on an agarose gel relative to λ DNA that had been digested with *Hin*dIII. cDNA inserts of *a-2*, *d-2*, *d-5*, and *d-4* were 2.2, 2.7, 1.9, and 1.9 kilobases (kb), respectively. The plasmids, pmca-1 (unpublished), which was used to detect α-tubulin mRNAs, and pvfos-1 (9), which was used to detect c-*fos* mRNAs, were kindly provided by C. Chiu and C. Stiles, respectively.

Nuclear run-on assay and Northern analysis. Nuclei and cytoplasmic RNA were prepared as described previously (15). Nuclear run-on assays were performed as described previously (15) with two modifications. First, labeled nuclear RNA was precipitated by ethanol and collected by centrifugation, rather than by filtering trichloroacetic acid-precipitated nuclear RNA. Second, DNA applied to the nitrocellulose was heat denatured at 100°C for 5 to 10 min, rather than by alkaline denaturation. For Northern analysis, 20 μg of cytoplasmic RNA was size fractionated on 1.2% agarose-formaldehyde gels and transferred to either a nitrocellulose filter or gene screen as described by Maniatis et al. (34). pUC19 DNA containing each cDNA insert was labeled with [α-^{32}P]dCTP using random hexamer as a primer. Hybridization and washing conditions were used as described previously (34). For run-on assays, the intensity of radioactive signals was quantitated by measuring the radioactivity of cut filters. Northern blots were quantitated by measuring the signal level of autoradiogram with a densitometer or by the method of Suissa (45).

RESULTS

Isolation of NGF-induced cDNA clones. We constructed a cDNA library in λgt10 using cDNA that had been synthesized from poly(A)$^+$ RNA from PC12 cells which were exposed to NGF for 2 h. This library was differentially screened with radioactively labeled cDNA probe made from either untreated PC12 cells or cells that had been treated with NGF for 2 h. This time was chosen to maximize the chance of isolating a cDNA induced directly by the NGF-dependent second messenger system(s). A total of 45 clones were picked, rescreened, and plaque purified. Of these clones, 11 were tested for temporal expression after the addition of NGF to cells. This report describes four NGF-inducible clones that fall into distinct classes on the basis of their temporal regulation by NGF and other agents. Some of the major conclusions about these genes are summarized in Table 1.

Rapid transcriptional induction by NGF. We performed both nuclear run-on transcription assays (Fig. 1) and Northern analyses (Fig. 2) to examine, respectively, the changes in transcription rates of these genes and the increases in the levels of their cytoplasmic mRNAs. α-Tubulin served as a gene whose transcription should not be significantly influenced by NGF for short times (15). These data show that each of these genes was regulated in a different kinetic pattern by NGF.

Transcription of *d-2* mRNA (~3.3 kb) was detected within 5 min after NGF addition, peaked within 15 min to a level that was greater than 36-fold over uninduced levels, and declined to near-basal levels within 3 h (Fig. 1). This rapid transcriptional induction of *d-2* was reflected by the cytoplasmic level of *d-2* mRNA (Fig. 2). An increase in *d-2* cytoplasmic mRNA was seen within 5 min after NGF addition, and peak levels, which were at least 50-fold over the basal levels, were reached by 15 to 30 min and were maintained until 1 h. Subsequently, the level of *d-2* mRNA decreased substantially, but there was a residual level of *d-2* mRNA that was above the basal level even after 7 days of NGF treatment. On the basis of time course of expression, restriction map, and comparison of several hundred bases of *d-2* with the published sequence of NGF I-A, the *d-2* gene

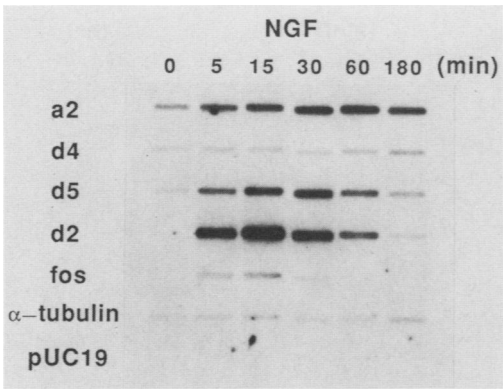

FIG. 1. Transcription rates of NGF-induced mRNAs. Nuclei were isolated from NGF-treated PC12 cells and nascent RNA was extended in vitro as described in Materials and Methods. Labeled RNAs were hybridized to 5 μg of linearized DNA that had been immobilized on nitrocellulose. Each DNA contains an insert representing a cDNA, *a-2*, *d-2*, *d-4*, or *d-5*, in the vector, pUC19. Background was indicated by the level of hybridization to pUC19. α-Tubulin RNA, which is not significantly affected by NGF at early times, was used as an internal control (13, 15). Transcriptional induction of *fos* mRNA by NGF was detected as expected (15, 29).

was identical to NGF I-A, which is homologous to *Xenopus* transcription factor TFIIIA (36).

Transcription of *d-5* mRNA (~5.7 kb) was rapidly and transiently induced about sevenfold over uninduced levels by 30 min of NGF treatment in a pattern reminiscent of that of *d-2* (Fig. 1). In contrast to *d-2*, however, appearance of *d-5* mRNA in cytoplasm was delayed; measurable levels of *d-5* cytoplasmic mRNA were not achieved until about 2 h, and peak levels (13-fold induction) were not achieved until 5 h after NGF addition (Fig. 2).

In contrast to *d-2* and *d-5*, which were clearly induced at the transcriptional level, there was little, if any, increase in the observed transcription of *d-4* (Fig. 1; also see Fig. 5); however, the increase in the level of *d-4* mRNA was clearly seen with Northern analysis. Therefore, *d-4* mRNA may be induced at a posttranscriptional level, although it is impossible to rule out a transcriptional effect of NGF on this gene from our data. The level of *d-4* mRNA (~2.9 kb) began increasing at about 1 h after NGF addition, peaked in 3 h (15-fold induction), and declined slowly (Fig. 2).

The transcription of *a-2* began to show an increase as early as 5 min after NGF addition (Fig. 1). Unlike *d-2* and *d-5*, the maximal level of transcription of *a-2* mRNA (~2.3 kb) was not achieved until 1 h after NGF addition. Also, its rate of transcription after 3 h of NGF induction was still quite substantial (Fig. 1). The cytoplasmic level of *a-2* mRNA was maximal at about 7 h (20-fold induction) and subsequently declined (Fig. 2). The delay between transcription and accumulation in cytoplasm of *a-2* mRNA was similar to that seen with *d-5*, although its degree was smaller. It is clearly different from the very rapid change in mRNA seen with *d-2*.

Protein synthesis-independent transcriptional induction by NGF. The rapid induction of *d-2*, *d-5*, and *a-2* suggests that they are among the first genes induced by NGF (Fig. 1 and 2). To determine if the increase in transcription of these genes was independent of protein synthesis, we measured the transcription rates of these genes in the presence and absence of cycloheximide (10 μg/ml of medium). Transcription rates were measured at both 30 and 180 min after NGF addition. The increase in the transcription of *d-2*, *d-5*, or *a-2* was not prevented by exposing the cells to cycloheximide for 2.5 h before NGF was added (Fig. 3); thus, new protein synthesis does not appear to be absolutely required for the induction of these genes. A slight reduction in the transcription rate of *a-2* and *d-5* by 30 min of cycloheximide treatment was detected in this experiment; however, this reduction was small and it was not observed in all experiments. It remains possible that continued protein synthesis may marginally enhance the expression of these genes by NGF.

The most striking effect of cycloheximide was that *d-2* continued to be transcribed at 3 h of NGF treatment, a time when its expression was normally greatly reduced (Fig. 1 and 3). This suggests that, although the induction of this gene was independent of protein synthesis, the termination of its transcription required protein synthesis. Thus, the protein(s) responsible for the decrease in the transcription of *d-2* were either activated by NGF or were short-lived or both. The increase in the transcription of *d-2* and *d-5* by cycloheximide in the absence of NGF is also consistent with the existence of a short-lived protein that represses the transcription of these genes. For *d-5*, protein synthesis may also be required to turn off NGF-dependent transcription. However, the high level of transcription at 3 h in the presence of NGF may be due only to an independent action of cycloheximide, since cycloheximide can independently increase the expression of this gene (Fig. 3).

Differential effects of cAMP on gene expression. cAMP mimicked some, but not all, of the effects of NGF, and it may play a role as a second messenger for some NGF-dependent effects. We therefore determined the effects of dbcAMP and cholera toxin on the regulation of NGF-induced genes. These genes fall into two distinct classes on the basis of their responses to elevated levels of cAMP. While the *d-4* and *d-5* genes were not obviously induced by cAMP, the *a-2* and *d-2* genes were induced at the transcriptional level (Fig. 4A). This effect of cAMP on the expression of these genes was clearly substantiated by the levels of the mRNAs induced by cholera toxin (Fig. 4B).

For *a-2* and *d-2*, the combined treatment of cAMP and NGF produced a level of induction higher than that of either agent alone (Fig. 4), suggesting that cAMP and NGF can stimulate the expression of *a-2* and *d-2* by independent pathways. On the other hand, cAMP strongly compromised the induction of *d-4* and *d-5* by NGF (Fig. 4B). For the *d-5* gene, this antagonistic effect occurred at the transcriptional level (Fig. 4A). Thus, cAMP plays a significantly different role in the expression of different NGF-inducible genes.

Effects of other agents on gene expression. Genes that are induced by either NGF or FGF may be important for neurite outgrowth and differentiation, but the expression of these genes may also be elicited by other growth factors. It is clear that none of these genes we have identified is induced by platelet-derived growth factor or other factors present in the serum in PC12 cells. Under our experimental conditions, growth factors were added to the cells in fresh serum-containing media, and the expression of these genes was absolutely dependent on the presence of an exogenously added growth factor (data not shown). To further examine whether the induction of these genes was NGF specific, we examined the effects of FGF, EGF, insulin, and PMA on the transcription of the NGF-induced genes. *d-2* was induced by all the agents tested, with the possible exception of insulin; however, the level of induction among different agents was somewhat variable (Fig. 5). The *d-5* gene was efficiently induced by FGF, EGF, and PMA, and to a lesser extent by insulin (Fig. 5). The *a-2* gene was also induced by all the

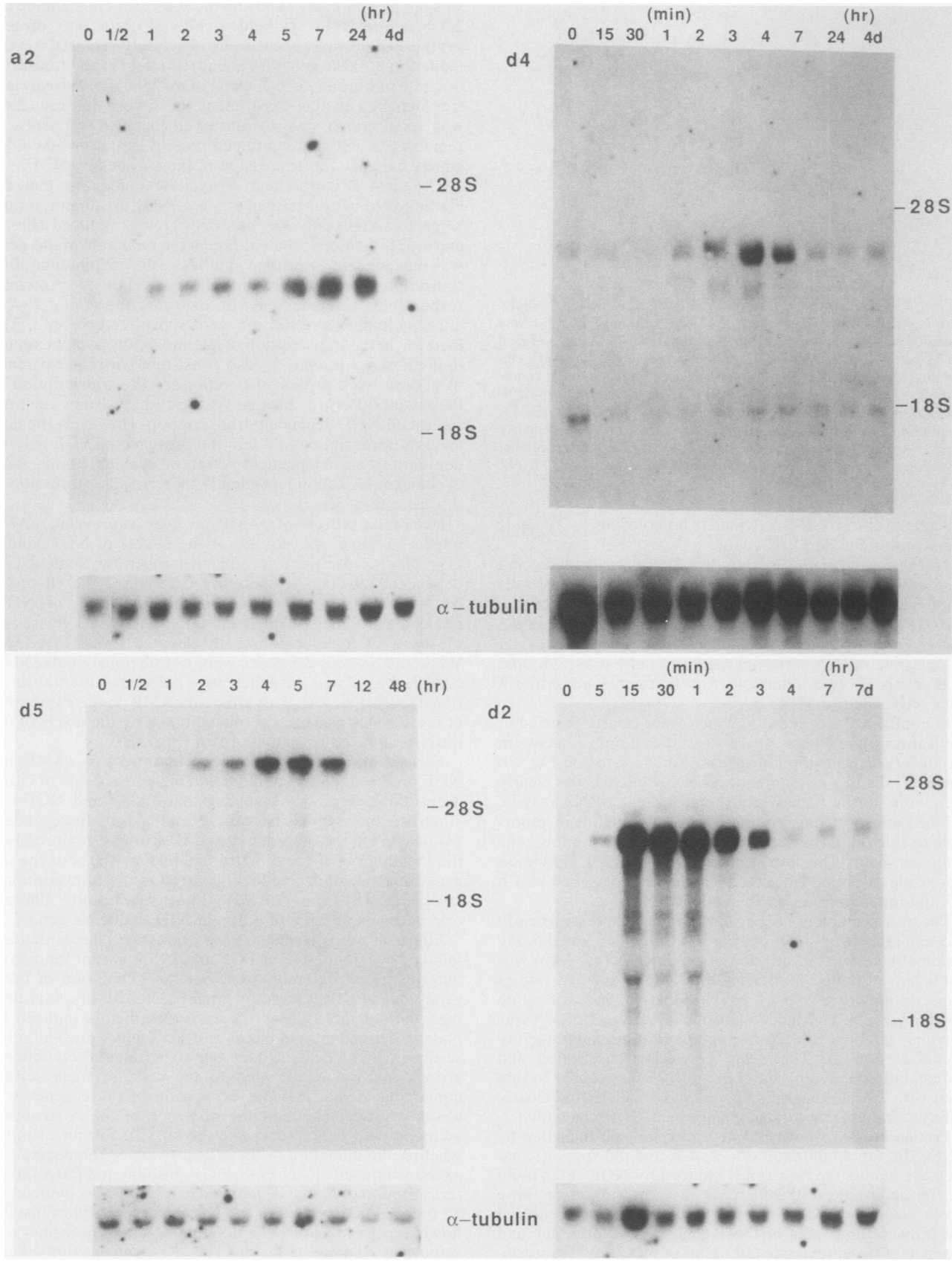

FIG. 2. Northern blot analysis of NGF-induced mRNAs. RNA isolated from PC12 cells treated with NGF for various lengths of time was fractionated on an agarose gel, transferred to a nitrocellulose membrane, and hybridized with a ^{32}P-labeled cDNA probe. The signal near 18S rRNA in *d-4* Northern analysis is residual α-tubulin mRNA from a previous hybridization.

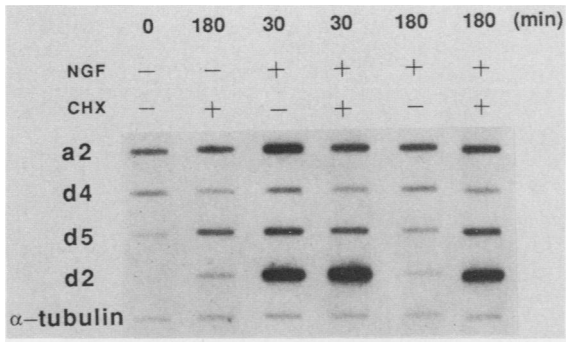

FIG. 3. Effect of cycloheximide on NGF-induced transcription rate. Nuclei were isolated from cells that had been treated with cycloheximide (CHX) or NGF for various times as indicated above the gels. In the fourth lane (30, +, +), cells were pretreated with cycloheximide for 2.5 h and then treated with both cycloheximide and NGF for 1/2 h. RNA was extended in vitro, isolated, and hybridized to immobilized DNA as indicated in the legend to Fig. 1.

agents tested, although the effect of insulin was small. As expected from our transcription studies (Fig. 1), the *d-4* gene did not show any consistent induction at the transcriptional level with any agent tested (Fig. 5A), so we measured the influence of these agents on the level of *d-4* mRNA. We found that all the agents were capable of increasing the expression of *d-4* mRNA (Fig. 5B). As might be expected, aFGF behaved identically to NGF and bFGF in every case tested (Fig. 5 and unpublished data).

Inhibition of NGF induction by K-252a. K-252a, an inhibitor of both protein kinase C and the cAMP-dependent protein kinases, has been reported to specifically interfere with the actions of NGF while not interfering with several events under the control of FGF, including FGF-dependent neurite outgrowth of PC12 cells (28). Because the induction of *d-2* and *d-5* are extremely rapid events that may be directly dependent on the second messenger systems under the control of NGF or aFGF, we decided to determine if the expression of these genes could be inhibited by K-252a.

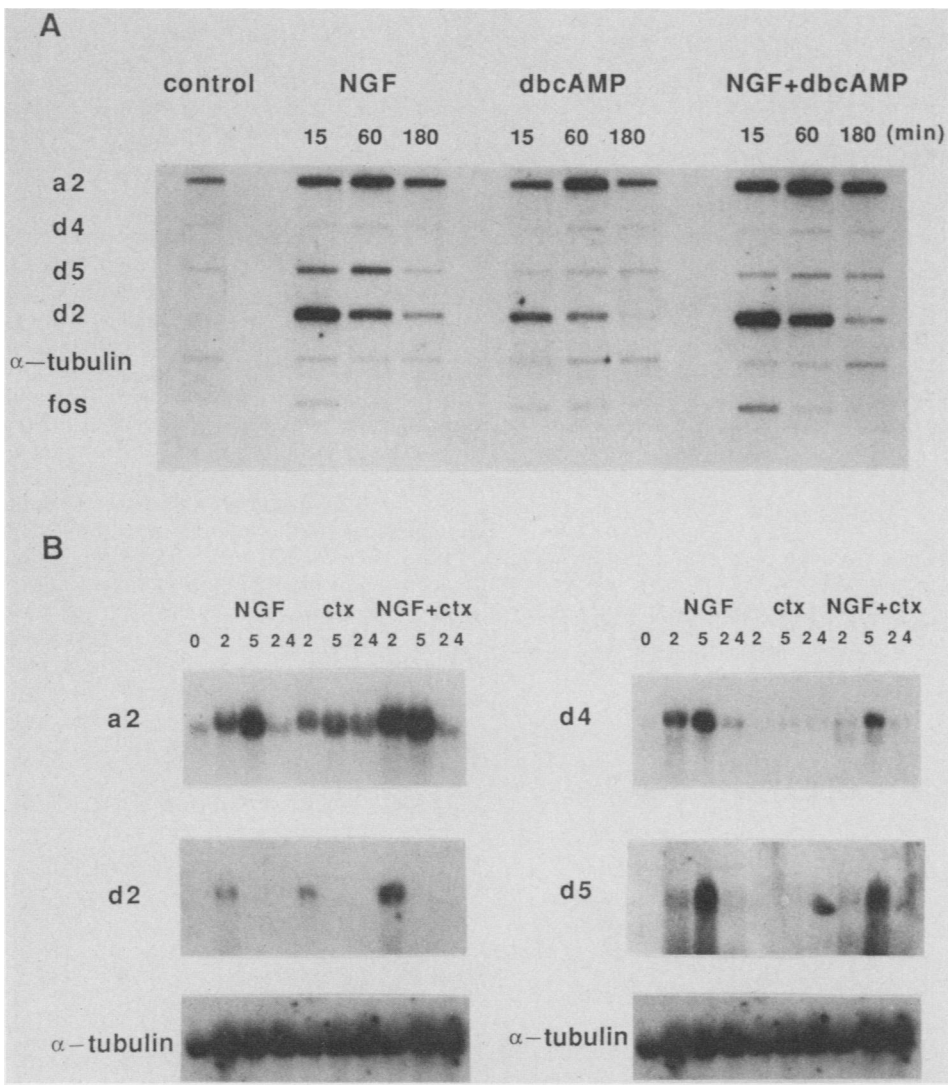

FIG. 4. Effect of dbcAMP or cholera toxin on the induction of *a-2*, *d-4*, *d-5*, and *d-2* mRNAs. (A) The effect of NGF, dbcAMP, or NGF plus dbcAMP on transcription rate at 15, 60, or 180 min was measured for the indicated genes as described in the legend to Fig. 1. (B) Northern analysis of mRNAs obtained from cells treated with NGF, cholera toxin (ctx), or NGF plus cholera toxin (NGF+ctx) for 2, 5, and 24 h.

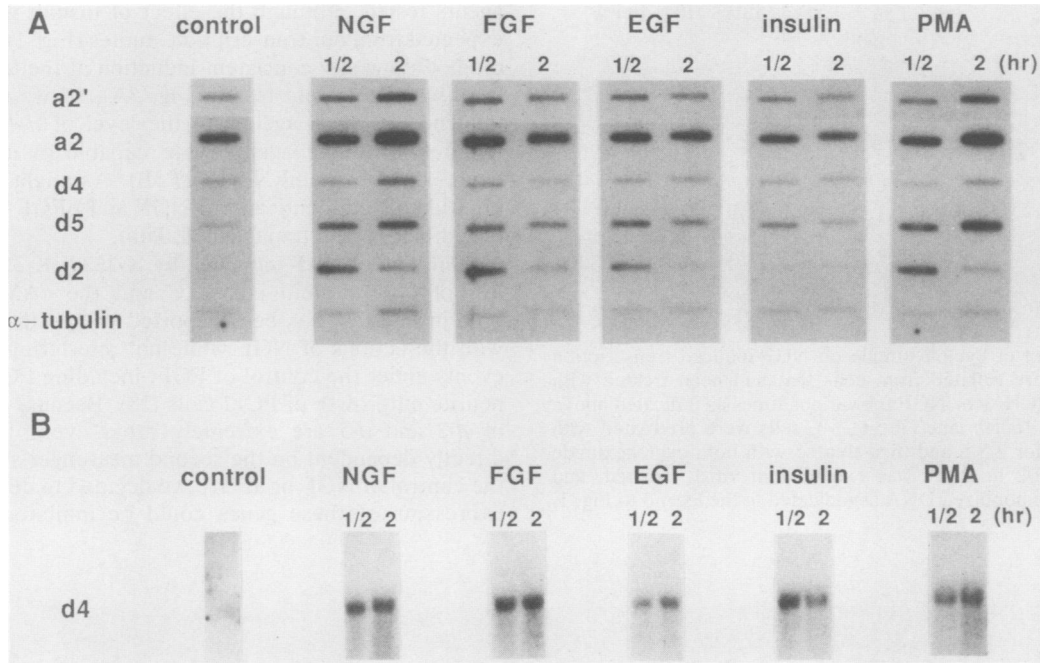

FIG. 5. Effect of FGF, EGF, insulin, and PMA on the expression of *a-2*, *d-4*, *d-5*, and *d-2* mRNAs. (A) The rate of transcription in cells treated with NGF, FGF, EGF, insulin, or PMA for 1/2 or 2 h was measured as described in the legend to Fig. 1. The row *a-2'* was exposed for 1/4 the time of the other rows to show the induction more clearly. (B) Increases in *d-4* mRNA level by various agents at 1/2 or 2 h was measured by Northern analysis.

These genes were induced by both NGF and aFGF; in the presence of K-252a, however, the induction by NGF was strongly inhibited, although there was little or no effect on the induction by aFGF (Fig. 6). Thus, K-252a is a potentially useful reagent with specific effects on the regulation of gene expression by NGF. Although K-252a may inhibit an NGF-dependent protein kinase, it is also possible that it may interfere with some other signaling step that is unique to NGF.

DISCUSSION

Each of the genes that we have isolated showed a characteristic pattern of regulation by NGF and other growth factors (Table 1). The different patterns can be explicitly defined by considering the following: the transcription time course, the rate of mRNA accumulation, the requirement of these processes for protein synthesis, the role of second messengers in regulating expression, and the ability of these genes to be induced by other growth factors (EGF, insulin, and FGF).

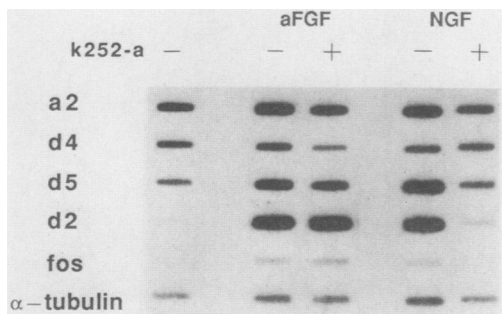

FIG. 6. Effect of K-252a on the induction of *a-2*, *d-4*, *d-5*, and *d-2* by either NGF or aFGF. Cells were preincubated with K-252a (250 nM) for 10 min and further incubated for 30 min in the presence of either NGF or aFGF. Nuclei were isolated from cells, and nuclear run-on assays were performed. Cells used for the first column were not treated with any agents.

The *d-2* and *d-5* genes were similar in two respects. (i) Both genes were rapidly induced at the transcriptional level, and this induction was independent of new protein synthesis, suggesting that the transcription of these genes is directly activated by a second messenger system whose activity is dependent on the interaction of NGF with its receptor. (ii) Both *d-2* and *d-5* were transiently induced by NGF; the decrease in transcription of these genes was dependent on continued protein synthesis. This suggests that the expression of these genes may be repressed by a protein with a short half-life. This repressor may itself be regulated by NGF. Alternatively, these proteins may autoregulate by a mechanism that is yet to be defined. Both the existence of a short-lived repressor or an autoregulatory mechanism are

TABLE 1. Summary of NGF-inducible genes[a]

NGF-induced gene	Size (kb) of mRNA	Transcriptional induction (fold)	Increase (fold) in mRNA level	Effect of cAMP on NGF induction[b]
a-2	~2.3	~5	~20	++
d-2	~3.3	~36	~50	++
d-4	~2.9	ND[c]	~15	−−
d-5	~5.7	~11	~13	−−

[a] All four genes were also induced by PMA, EGF, FGF, and insulin.
[b] ++, cAMP agonists induced the expression of this gene and the gene could be further induced by NGF; −−, cAMP agonists did not induce the expression of this gene and they inhibited the expression of this gene by NGF.
[c] ND, Not detectable.

supported by the partial induction of the transcription of *d-2* and *d-5* by cycloheximide in the absence of NGF.

Although the *d-2* and *d-5* genes were both rapidly and transiently induced at the transcriptional level, these genes were very different in the following three respects. (i) The appearance of *d-2* mRNA in the cytoplasm was extremely rapid (as early as 5 min after NGF addition), while the appearance of *d-5* mRNA was delayed for 1 h. Thus, for *d-5*, mRNA accumulation was delayed until after the transcription of the gene was beginning to subside. This delay in the appearance of *d-5* mRNA may reflect either a slow step in processing or a delay in transport and may serve as a mechanism regulating the amount of this message. (ii) *d-2* and *d-5* also showed differences in the induction pattern by cAMP. cAMP could induce *d-2* and acted additively with NGF to further induce the gene; in contrast, cAMP did not induce *d-5* and actually inhibited the induction of this gene by NGF. (iii) Insulin seemed to be more effective in inducing *d-5* than in inducing *d-2*.

The *a-2* gene was rapidly induced by NGF, but it could be distinguished from *d-2* and *d-5* in three ways. (i) The basal level of transcription was higher. (ii) Although there was a rapid induction of the gene, full expression did not occur until 1 h after NGF addition. (iii) NGF produced a long-term induction of *a-2* that lasted for at least 7 h after the treatment. (iv) Finally, cAMP alone can induce *a-2* or act in combination with NGF to further induce *a-2* expression.

The accumulation of *d-4* mRNA was induced by NGF, EGF, FGF, insulin, and PMA; however, in contrast to *d-2* and *d-5*, a transcriptional induction was not obvious. In some experiments, there was a small induction of *d-4* (two- to threefold), but this observation was not reproduced in all experiments. The background level of transcription of *d-4* in the run-on assay was high relative to the maximum level of transcriptional induction we observed. Thus, the induction of this gene may be explained by a posttranscriptional effect, as shown with neurofilament mRNA in PC12 cells (33), although a transcriptional effect cannot be ruled out. Further studies are needed to address this issue. As with *d-5*, cAMP did not induce the expression of *d-4* but inhibited its induction by NGF.

cAMP mimics many of the effects of NGF and has been suggested as a second messenger for NGF (42). However, cAMP does not mimic some of the most striking effects of NGF. For example, Greene et al. (17) reported that both cholera toxin and forskolin suppressed regeneration of neurites by NGF, although they significantly increase the initial formation of short cytoplasmic extensions that may be a precursor of the long neurite (21, 25). We observed that cAMP mimicked the effects of NGF in the induction of some genes (*d-2* and *a-2*) but not others (*d-5* and *d-4*). Interestingly, cholera toxin acted additively with NGF to further induce the *d-2* and *a-2* genes, while it inhibited the induction of the *d-4* and *d-5* genes by NGF. There are at least two possible explanations for these observations. First, these genes may have a positive or a negative regulatory element that is sensitive to cAMP. Occupation of this regulatory element by unidentified regulatory protein may increase or decrease the effect of NGF on the expression of these genes. Second, the regulatory protein(s) involved in NGF regulation of these genes could also be affected by cAMP-dependent protein kinases, leading to an enhancement or a diminution of their biological activity. Identification and characterization of the regulatory region of these genes will be essential to distinguish these two possibilities.

It is striking that these NGF-induced genes were also induced by EGF, PMA, and, in certain cases, by insulin, agents which do not stimulate differentiation of these cells. One cannot conclude that the increase in the expression of these genes is not a key step in neural differentiation, since the induction of these genes might be necessary, but not sufficient, for neural differentiation. On the other hand, these genes could be involved in general steps that prepare the cell to respond to many growth factors and, thus, not be unique to any one pathway. It is possible that the signal to differentiate requires the induction of a set of genes, none of which is unique to any particular pathway. Two mechanisms are possible to explain how these genes are expressed by different growth factors and effectors. First, there may be one regulatory element and a unique transcription factor may be modified by several pathways (6, 16, 39, 44); alternatively, there may be an array of regulatory elements, each of which responds to a different effector.

By using a differential screening technique, it is more likely to isolate cDNAs representing abundant mRNAs than cDNAs representing rare mRNAs. Indeed we observed that the three of the isolated cDNA clones, *a-2*, *d-2*, and *d-5*, were repeatedly isolated, reflecting the greater abundance of these mRNAs in NGF-treated cells. The fact that these cDNA clones were repeatedly isolated suggests that the differential screening performed here may be approaching saturation. Since no NGF-specific cDNAs were isolated, it is possible that mRNAs expressed specifically by NGF may be quite rare and that the differential screening method that we used does not allow the corresponding cDNAs to be identified. Alternatively, NGF-specific mRNAs may be induced at later times than we chose for our screen.

NGF-induced cDNA clones have been isolated by various groups using differential screening (30, 31, 35–37, 43). NGF-induced genes such as *fos*, NILE, and GAP-43, were also cloned and studied (27, 29, 41). We have obtained sequence information of several hundred bases of each of our cDNAs. These sequences were compared with those in the data base of the National Biology Research Foundation, and no significant homology was found. However, a manual comparison of the partial DNA sequence of *d-2* revealed that a 300-base-pair sequence of this gene is identical to NGF I-A, which contains a zinc finger domain present in *Xenopus* transcription factor TFIIIA and other DNA-binding proteins (36). Thus, we conclude that we have independently isolated and further studied the NGF I-A gene. We have extended the results of Milbrandt (36) by showing that *d-2* (NGF I-A) is transcriptionally induced and that induction, rather than being unique to NGF, is mimicked by several other agents, some of which do not cause neurite formation. It is possible that other NGF-induced clones could also be involved in the regulation of subsequent transcription by NGF and other growth factors. The study of the NGF-inducible genes isolated in this lab and by others may help establish how NGF regulates gene expression and how the induction of these genes promotes neural differentiation.

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