Transcriptional regulation of early growth response genes in FOS-expressing PC-12 cells

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Deregulated *c-fos* expression in the rat pheochromocytoma cell line, PC-12, causes pronounced downregulation of nerve growth factor (NGF)-induced *c-fos* and *c-jun* activation, accompanied by a block in NGF-induced differentiation of PC-12 cells. The FOS-expressing PC-12 cells were exposed to diverse agents such as NGF, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), dibutyl cyclic adenosine 3',5' monophosphate (db cAMP), and Ca-ionophore; and the expression of *egr-1*, *c-fos*, *c-jun*, *jun-B*, and *jun-D* was analyzed. Pronounced downregulation of *c-fos*, *c-jun*, and—to a lesser extent—*jun-B* was observed on treatment with NGF, bFGF, db cAMP, and Ca-ionophore, whereas EGF-induced activation of these early response genes was not inhibited in FOS-expressing PC-12 cells. Ca-ionophore- and db cAMP-induced *egr-1* activation in PC-12 *fos* cells was completely inhibited. Both parent and PC-12 *fos* cells expressed similar high basal levels of *jun-D*, whose expression was the least regulatable by all of these agents. Transfection of *fos* promoter–chloramphenicol acetyltransferase (promoter-CAT) plasmid into these stable FOS-expressing PC-12 cells revealed that these effects are exerted at the *fos* promoter level.

Introduction

Tissue development and maintenance of integrity in an organism require carefully controlled regulation of both cell proliferation and differentiation. Growth factors and perhaps protooncogenes play a role in both of these processes (Bishop, 1985). Transcription of a distinct set of genes, variously termed early growth response (Sukhatme *et al.*, 1987), primary response, or

cellular immediate early genes (Curran and Morgan, 1987; Lau and Nathans, 1987), is transiently activated when growth factors stimulate quiescent fibroblasts to reenter the cell cycle (Cochran *et al.*, 1983; Greenberg and Ziff, 1984; Lau and Nathans, 1987; Lim *et al.*, 1987; Sukhatme *et al.*, 1987; Almendral *et al.*, 1988). Accumulating evidence indicates that various members of the early response family of genes encode putative transcriptional factors that may mediate cells' response to growth factors (Chavrier *et al.*, 1988; Chiu *et al.*, 1988; Christy *et al.*, 1988; Hazal *et al.*, 1988; LeMaire *et al.*, 1988). Among the early response genes, *c-fos* and *c-jun* protooncogenes are the most extensively studied. Products of both the *c-fos* and *c-jun* genes are induced within minutes of being treated with a variety of stimuli, mediated by ligand binding (Greenberg and Ziff, 1984; Curran and Morgan, 1985; Kruijer *et al.*, 1984; Morgan and Curran, 1986; Quantin and Breathnach, 1988; Rauscher *et al.*, 1988a). The c-JUN protein forms a complex with the product c-FOS (Curran and Franza, 1988; Rauscher *et al.*, 1988b; Sassone-Corsi *et al.*, 1988a,b; Gentz *et al.*, 1989), and the FOS/JUN heterodimeric complex binds to the AP-1 site with high affinity, regulating transcription of genes that contain this DNA element (Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988; Rauscher *et al.*, 1988a). Two other members of the family, *jun-B* and *jun-D*, which share sequence homology with *c-jun*, can also form transcription complexes with c-FOS (Nakabeppu *et al.*, 1988; Ryder *et al.*, 1988); and two *fos*-related genes, *fra-1* (Cohen and Curran, 1988; Cohen *et al.*, 1989) and *fos-B* (Zerial *et al.*, 1989), can similarly form complexes with JUN.

The rat pheochromocytoma cell line PC-12 (Greene and Tischler, 1976) offers a powerful in vitro system to study the mechanism of growth factor-induced proliferation and differentiation. Agents such as nerve growth factor (NGF), basic fibroblast growth factor (bFGF), and interleukin-6 (IL-6) cause neuronal differentiation in PC-12 cells (Greene and Tischler, 1976; Greenberg *et al.*, 1985; Rydel and Greene, 1987; Satoh *et al.*, 1988). Within minutes after addition, these diverse differentiation-inducing agents also cause

transient activation of *c-fos* and *c-jun* (Curran and Morgan, 1985; Greenberg *et al.*, 1985; Kruijer *et al.*, 1985; Bartel *et al.*, 1989). Although initially mitogenic, on prolonged treatment NGF causes growth arrest and differentiation into sympathetic neuron-like cells (Greene and Tischler, 1976, 1982; Boonstra *et al.*, 1983). Epidermal growth factor (EGF), on the other hand, is only mitogenic, and causes transient activation of *c-fos* and *c-jun* in PC-12 cells (Boonstra *et al.*, 1985; Sukhatme *et al.*, 1987). Diverse agents such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or depolarization with either K⁺, Ca-ionophore A23187, or barium also induce *c-fos* in PC-12 cells (Greenberg *et al.*, 1985; Kruijer *et al.*, 1985; Morgan and Curran, 1986; Milbrandt, 1987).

We have recently demonstrated that constitutive expression of *c-fos* in PC-12 cells causes a block in NGF- and bFGF-induced differentiation as well as a pronounced downregulation of *c-fos* and *c-jun* (Ito *et al.*, 1989). To understand the role of FOS in the regulation of other early growth response genes, we utilized a PC-12 cell line that expresses FOS. We demonstrate a pronounced downregulation of *c-fos*, *c-jun*, and—to a lesser extent—*jun-B* in FOS-expressing PC-12 cells, not only in response to NGF and bFGF but also to dibutyl cyclic adenosine 3',5' monophosphate (db cAMP), IL-6, and Ca-ionophore. Conversely, the mitogenic growth factor EGF-induced activation of these early growth response genes was not inhibited in FOS-expressing PC-12 cells.

Results

Downregulation of *c-fos* in FOS-expressing PC-12 cells

Two clones of FOS-expressing PC-12 cells (FOS-76 #1 and #4) were chosen for the study (Ito *et al.*, 1989). Diverse agents—including NGF, EGF, bFGF, cAMP analogues, neurotransmitters, IL-6, adenosine, TPA, Ca-ionophore, and barium—caused stimulation of PC-12 cells (Greene and Tischler, 1982; Greenberg *et al.*, 1985; Curran and Morgan, 1986; Rydel and Greene, 1987; Satoh *et al.*, 1988). Induction of *c-fos* and *c-jun* seems to be one of the earliest responses of PC-12 cells to these diverse stimuli (Curran and Morgan, 1985; Greenberg *et al.*, 1985, 1986; Kruijer *et al.*, 1985; Morgan and Curran, 1986; Bartel *et al.*, 1989). Recently it has been demonstrated that the FOS protein directly modulates JUN function by means of a heterodimeric FOS/JUN complex (Sassone-Corsi *et al.*, 1988a,b). Our ability to generate a FOS-expressing PC-12 cell line enabled us to

investigate the complex interaction of FOS with various members of the JUN family. Among various agents that have been implicated in PC-12 cells, we chose NGF, bFGF, EGF, db cAMP, Ca-ionophore, interleukin-1 β (IL-1 β), and IL-6 to compare their effects on PC-12 cells and PC-12 FOS clones. Although PC-12 cells cannot be cultured for a prolonged time (>4 d) in the absence of serum, our preliminary results revealed that they can be maintained serum free for 24–48 h without loss of viability; accordingly, all the agents were tested in serum-free conditions. Similar results were obtained when serum-containing medium was used (Ito *et al.*, 1989). *c-fos* expression in the parent PC-12 cells was markedly stimulated within 30 min of treatment with NGF, bFGF, EGF, db cAMP, and Ca-ionophore (Figure 1). IL-6 was marginally effective, whereas IL-1 β did not stimulate *c-fos* expression. In the PC-12 *fos* clones, the exogenous *c-fos* transcript (2.8 kb) can be easily distinguished from the endogenous 2.2 kb *c-fos* transcript (Ito *et al.*, 1989). In two different PC-12 *fos* clones, the activation of *c-fos* by NGF and db cAMP was significantly downregulated (Figure 1). bFGF-induced *c-fos* activation was less susceptible to downregulation (2.5-fold) in the *fos* clones than NGF (6-fold). Ca-ionophore-induced *c-fos* activation was more sensitive to downregulation in the presence of deregulated FOS than was db cAMP. IL-6-induced *fos* activation was also inhibited in PC-12 *fos* clones. The mitogenic growth factor EGF caused pronounced stimulation of *c-fos* in both parent PC-12 and PC-12 *fos* clones (Figure 1). In fact, EGF was the only agent showing a relative resistance to downregulating endogenous *c-fos* in FOS-expressing PC-12 cells. Recently Bartel *et al.*, (1989) have observed no significant differences in *c-fos*, *c-jun*, and *jun-B* expression on treatment of PC-12 cells with NGF and EGF. These authors have suggested that the different effects of NGF and EGF on PC-12 cell function may not be a consequence of differential induction of the constituents of FOS/JUN. However, PC-12 *fos* cells' apparent resistance to showing downregulation of *c-fos* when treated with EGF led us to analyze these clones for expression of the *jun* family of genes in response to EGF.

Downregulation of *c-jun* in the presence of deregulated *c-fos* in response to diverse agents

Similar to the *c-fos* results, NGF, EGF, and bFGF caused pronounced stimulation of *c-jun* expression in PC-12 cells (Figure 2). db cAMP, IL-6, and Ca-ionophore were marginally effective,

Figure 1. Downregulation of C-fos in PC-12 fos cells in response to diverse agents. Total cellular RNA (20 μ g) from PC-12 cells and PC-12 fos cells (clones #1 and #4) was treated for 30 min with Ca-ionophore (lane 1), 10 μ g/ml; db cAMP (lane 2), 1 mM; bFGF (lane 3), 5 ng/ml; EGF (lane 4), 10 ng/ml; IL-1 β (lane 5), 200 U/ml; IL-6 (lane 6), 20 ng/ml; and NGF (lane 7), 50 ng/ml. Untreated control (lane 8) was analyzed by Northern blot for c-fos expression as described in Materials and Methods. Endo, endogenous c-fos; exo, exogenous c-fos.

while IL-1 β was not effective. Although c-jun induction was similar to c-fos, a low constitutive expression of c-jun was seen in parent PC-12 cells. Two species of c-jun mRNAs (2.7 kb and 3.4 kb) were detected in PC-12 cells treated with various agents, with two- to threefold higher levels of the smaller transcript. Treatment of PC-12 fos cells with various agents caused a downregulation of c-jun analogous to c-fos (Figure 2). EGF was the only agent that showed a relative resistance to downregulation of c-jun in PC-12 fos cells. Thus both c-fos (Figure 1) and c-jun were subject to a similar type of transcriptional control in response to the mitogenic growth factor EGF in constitutively FOS-expressing PC-12 cells.

Constitutive expression of c-fos in PC-12 cells and jun-B expression in response to various stimuli

Another member of the jun family of genes is jun-B, which is homologous but not identical to

c-jun (Ryder *et al.*, 1988). The induction of jun-B in response to various stimuli follows the same pattern as c-fos and c-jun. The protein encoded by jun-B shares two regions of homology with c-jun, including the DNA binding domain and the activator domain (Ryder and Nathans, 1988). Because it has been suggested that the complex regulation of the jun family of genes may play an important role in the response of cells to growth factors (Ryder and Nathans, 1988), it was of interest to investigate the regulation of jun-B in PC-12 fos cells. The induction of jun-B in NGF-treated PC-12 cells followed the same pattern as c-fos and c-jun, peaking at 30 min (Figure 3a), although jun-B induction followed a slower kinetics than c-fos and c-jun. Within 30 min, PC-12 cells treated with EGF, bFGF, and db cAMP showed pronounced stimulation of jun-B expression (Figure 3b). Ca-ionophore, IL-6, and IL-1 β were not effective. Of interest is the stimulation of jun-B by db cAMP, in contrast

Figure 2. Downregulation of C-jun in FOS expressing PC-12 cells. Total cellular RNA from untreated and treated (30 min, as in Figure 1) PC-12 and PC-12 *fos* cells was analyzed for *c-jun* expression.

to the relatively low levels of *c-jun* induction by db cAMP (Figure 2 and Bartel *et al.*, 1989). In PC-12 *fos* cells, the activation of *jun-B* by NGF, bFGF, and db cAMP was downregulated to a lesser extent than *c-jun* (Figure 3b). EGF-induced activation of *jun-B* was the least inhibited in the presence of deregulated FOS.

Expression of *jun-D* was least regulatable in response to various stimuli in PC-12 cells

A third member of the *jun* family of genes, *jun-D*, has recently been reported (Ryder *et al.*, 1989). *jun-D* is more closely related to *c-jun* than to *jun-B*. *jun-D* has been detected at high basal levels in quiescent 3T3 cells, and is only marginally stimulated by serum, platelet-derived

growth factor (PDGF), or bFGF (Ryder *et al.*, 1989). Furthermore, *jun-D* mRNA is found to be stable on serum stimulation (Ryder *et al.*, 1989). These results seem to suggest that *jun-D* expression is regulated by a different mechanism(s) than *c-jun* and *jun-B*. Hence it was of interest to investigate the expression of *jun-D* in PC-12 and PC-12 *fos* cells. The initial time course with NGF in PC-12 cells revealed a high basal level of *jun-D* expression (Figure 4a). In contrast to other early growth response genes, *jun-D* was only marginally stimulated with NGF (1.5-fold, Figure 4a). Because the PC-12 *fos* cells showed a differential responsiveness to EGF, we then analyzed the response of PC-12 cells to EGF (Figure 4a). *jun-D* expression was the least affected in EGF-treated PC-12 cells, similar

Figure 3. Effect of diverse agents on *jun-B* expression in PC-12 cells and PC-12 *fos* cells. (a) NGF time course: parent PC-12 cells were treated with NGF (0–24 h), and the total cellular RNA was analyzed by Northern blot for *jun-B* expression. (b) Both the PC-12 cells and PC-12 *fos* cells, untreated and treated (30 min) with diverse agents as in Figure 1., were analyzed by Northern blot for *jun-B* expression.

to the results for NGF-treated PC-12 cells. Diverse agents used in the study stimulated *jun-D* expression only marginally in parent PC-12 cells (Figure 4b). Both the parent PC-12 cells and PC-12 *fos* cells showed a high basal level of *jun-D* (Figure 4c) that was not regulatable on treatment with the diverse agents used in the study (not shown). These results clearly demonstrate that, in contrast to other early growth response genes, the high basal level of *jun-D* in PC-12 cells is not regulatable either by polypeptide growth factors or db cAMP, and that deregulated FOS expression in PC-12 cells does not interfere with *jun-D* expression.

Differential responsiveness of *egr-1* expression in PC-12 *fos* cells

We have recently demonstrated that in PC-12 *fos* cells the NGF-induced activation of *egr-1* was not affected (Ito *et al.*, 1989). Because other agents such as high K⁺ or EGF also activate *egr-1* in PC-12 cells (Kujubu *et al.*, 1987), it was

of interest to investigate the effect of diverse agents used in the study on *egr-1* expression. Figure 5 demonstrates that in both the PC-12 cells and PC-12 *fos* cells, NGF, EGF, and bFGF stimulated *egr-1* expression. Ca-ionophore was marginally effective, whereas IL-1 β and IL-6 were not effective. *egr-1* induction by db cAMP, on the other hand, showed a differential responsiveness in PC-12 *fos* cells. The pronounced stimulation of *egr-1* by db cAMP in the parent PC-12 cells was completely inhibited in FOS-expressing PC-12 cells. Ca-ionophore-induced *egr-1* activation was also inhibited in PC-12 *fos* cells.

Expression of a neural-specific gene is not affected in PC-12 *fos* cells

In an earlier work (Ito *et al.*, 1989) we showed that PC-12 *fos* cells exhibited a pronounced inhibition of NGF-induced morphological differentiation (Ito *et al.*, 1989). Furthermore, the expression of intermediate markers such as or-

Figure 4. Expression of *jun-D* in PC-12 and PC-12 *fos* cells. (a) Total RNA from the parent PC-12 cells treated with EGF 20 ng/ml or NGF 50 ng/ml for 0–24 h was analyzed by Northern blot for *jun-D* expression. (b) Total RNA from parent PC-12 cells untreated or treated (30 min) as in Figure 1 was analyzed for *jun-D* expression. (c) Total RNA from the PC-12 cells and PC-12 *fos* clones (untreated) was analyzed from *jun-D* expression.

nithine decarboxylase (ODC), an enzyme involved in polyamine synthesis, was completely inhibited in NGF-treated PC-12 *fos* cells (Ito *et al.*, 1989). The downregulation of the early growth response genes in PC-12 *fos* cells raised the possibility that terminal markers of PC-12 differentiation might be inhibited in the presence of deregulated FOS. We chose IF-clone 73 (Leonard *et al.*, 1987), a cDNA clone obtained from prolonged treatment of PC-12 cells with NGF, for this purpose. This gene is NGF inducible on prolonged treatment in PC-12 cells. As shown in Figure 6, both the PC-12 cells and PC-12 *fos* cells expressed basal levels of this late marker of differentiation. Prolonged treatment with NGF resulted in similar induction levels of this gene in both the parent PC-12 cells and PC-12 *fos* cells.

Downregulation of *c-fos* expression by diverse agents involves the *fos* promoter

As demonstrated in Figure 1, *c-fos* is induced in PC-12 cells in response to various agents. The constitutive expression of *c-fos* in PC-12 *fos* cells enabled us to investigate the *fos* promoter's function and its interaction with various agents. A plasmid containing upstream sequences of human *c-fos* gene was linked to the bacterial chloramphenicol acetyltransferase (CAT) gene and was transfected into both PC-12 cells and PC-12 *fos* cells. The construct F-

700 comprises both the serum-responsive element (SRE) and the cyclic AMP-like responding element (CRE). As shown in Figure 7, the parent PC-12 cells responded to NGF, EGF, bFGF, and db cAMP in serum-free conditions as indicated by a four- to sixfold increase in CAT activity. Similar results were obtained in serum-containing conditions (not shown). However, in the presence of constitutive expression of FOS in PC-12 cells, this induction was significantly inhibited, as demonstrated by very low CAT activity. Transfection of Rous sarcoma virus (RSV) CAT plasmid into both PC-12 cells and PC-12 *fos* cells showed comparable levels of CAT activity (Figure 7). These results strongly suggest that the constitutive *c-fos* expression in PC-12 *fos* cells transrepresses *c-fos* promoter activity when the promoter is introduced into PC-12 *fos* cells. Treatment of PC-12 *fos* cells with EGF after F-700 transfection did not reverse the repression of the *fos* promoter, in contrast to the Northern blot results (Figure 1), raising the possibility that the repression mechanism might involve posttranslational pathways.

Discussion

Our results demonstrate that in the presence of constitutive expression of *c-fos*, the transient activation of *c-fos*, *c-jun* and, to a lesser extent, *jun-B*, is significantly downregulated when induced with the differentiation-inducing agents

NGF, bFGF, and IL-6. Of interest are our observations that db cAMP-induced activation of *c-fos* and of *jun-B* was significantly downregulated in the PC-12 *fos* cells. Induction of *c-jun*, however, was only marginally affected in db cAMP-treated PC-12 and PC-12 *fos* cells. It is still possible that the low basal levels of *c-jun* seen in PC-12 cells might be sufficient to participate in the response to various agents. Thus, in the presence of deregulated *c-fos*, the relative availability of the *jun* and *fos* family of genes in the PC-12 *fos* cells is significantly reduced in response to a stimulus; and this reduction interferes with the normal signal transduction mechanisms that are involved in the differentiation pathway, thereby resulting in a block.

Recently, Bartel *et al.* (1989) have demonstrated that the opposing effects of NGF and EGF in PC-12 cells cannot be explained by differential induction of any early growth response gene. What then causes the PC-12 cells to respond differentially to mitogenic signals from EGF compared with differentiation signals from

NGF? Are there other growth response genes that are NGF/EGF specific that are yet to be identified? NGF has both mitogenic and anti-proliferative activity (Burstein and Greene, 1982). Does this imply that both NGF and EGF induce similar genes but are modified differently? In fact, it has been demonstrated that in PC-12 cells treated with EGF, NGF, or membrane depolarization, FOS and FOS-associated proteins are modified differently (Kruijer *et al.*, 1985; Curran and Morgan, 1986; Morgan and Curran, 1986).

FOS-expressing PC-12 cells showed pronounced downregulation of *egr-1* expression only in response to db cAMP or Ca-ionophore but not to the polypeptide growth factors, NGF, EGF, or bFGF. The promoter region of the rat homologue of *egr-1*, viz. NGFI-A, has recently been identified (Changelian *et al.*, 1989). This region contains both SRE- and CRE-like elements. The SRE region, however, does not contain the extended dyad symmetry of the *c-fos* promoter (Changelian *et al.*, 1989). Using the

Figure 5. Expression of *egr-1* in PC-12 cells and PC-12 *fos* cells. Both PC-12 cells and PC-12 *fos* clones, untreated and treated (30 min) with diverse agents as in Figure 1, were analyzed by Northern blot for *egr-1* expression.

Figure 6. Expression of a neural-specific gene in PC-12 *fos* cells. Parent PC-12 cells and PC-12 *fos* clones were untreated and treated with NGF (50 ng/ml) for 12 d and total RNA was analyzed by Northern blot hybridization with the IF-clone 73 cDNA probe. (Lane 1) PC-12 *fos* #4 treated, (lane 2) untreated; (lane 3) PC-12 *fos* #1 treated, (lane 4) untreated; (lane 5) control PC-12 cells treated, and (lane 6) untreated with NGF.

CAT gene, these authors have demonstrated that the NGFI-A promoter is NGF inducible. It is not clear whether the promoters of NGFI-A and *egr-1* are identical. However, our observation that only db cAMP- and Ca-ionophore-induced *egr-1* expression was inhibited in the presence of constitutive expression of FOS in PC-12 cells indicates that the CRE of *egr-1* was repressed by FOS.

Our results also demonstrate that the expression of *jun-D* was the least affected by the presence of deregulated FOS in PC-12 cells as well as the least regulatable by any of the agents tested; this implies that the relative levels of other early growth response genes (viz.,

c-fos, *c-jun*, and *jun-B*) have no effect on *jun-D* expression. Furthermore, a marker of neuronal differentiation was not downregulated in response to NGF in FOS-expressing PC-12 cells, suggesting that the inhibition of differentiation is probably exerted at an early stage of the signal transduction process. The overall reduction in expression of the early growth response gene(s) in response to differentiation-inducing agents perhaps interferes with the differentiation signals, causing the cells to proliferate. This would account for the fact that the PC-12 *fos* cells can be made to proliferate over a prolonged period of time in the presence of NGF (Ito *et al.*, 1989).

The c-FOS protein product has been shown to specifically repress the serum-induced promoter activity of the *c-fos* gene (Sassone-Corsi *et al.*, 1988c). The negative feedback mechanism (transrepression) occurs only when *c-fos* transcription is induced by serum (Sassone-Corsi *et al.*, 1988c). These observations suggest that transcription of *c-fos* is repressed by its own product. Our observation, that, in PC-12 *fos* cells, the expression of the *fos*-CAT fusion gene was significantly inhibited when exposed to both polypeptide growth factors and db cAMP, supports the repression hypothesis. The steady state levels of mRNAs for *c-fos*, *c-jun*, and to a lesser extent, *jun-B* showed a relative resistance to downregulation in response to the mitogenic growth factor EGF in FOS-expressing PC-12 cells, as monitored by Northern blot analyses. Hence the mechanism of repression of the *fos* promoter by deregulated FOS might involve interference at the posttranscriptional and/or posttranslational levels. Nonetheless, the immediate early growth response genes'

Figure 7. Analysis of *fos* promoter function by transient CAT assay. Both the PC-12 cells and PC-12 *fos* clone (#1) were transfected with RSV CAT or F700 CAT by electroporation and the cells were cultured and treated as described in Materials and Methods and analyzed for CAT expression. (Lane 1) untreated; (lane 2) NGF, 50 ng/ml; (lane 3) EGF, 10 ng/ml; (lane 4) db cAMP, 1 mM; and (lane 5) RSV CAT-electroporated cells. S, substrate; A and B, acetylated products of CAT.

apparent resistance to downregulation by EGF in PC-12 *fos* cells in contrast to the differentiation-inducing agents gives us an approach to separate signals of mitogenesis from those of differentiation. Studies involving PC-12 cells expressing various early growth response genes are underway, which should enable us to delineate the role of these genes in the complex signal transduction processes involved in differentiation versus proliferation.

Materials and methods

Cell culture and stimulation

PC-12 cells (Greene and Tischler, 1976) were grown in RPMI containing 5% calf serum and 10% horse serum in plastic dishes without collagen coating. For subculture, cells were mechanically dislodged from dishes by trituration. PC-12 *fos* transfectants were grown as described elsewhere (Ito *et al.*, 1989). NGF, bFGF, and EGF were obtained from collaborative research. Ca-ionophore A23187 and db cAMP were from Sigma. IL-1 β was purified in Hoffmann-La Roche, Inc. and IL-6 was from R & D Systems, Minneapolis, MN.

For stimulation by various agents, cells were cultured in complete medium; and 12 h before stimulation, the medium was changed to RPMI 1640 without serum. The final concentration of each reagent was as follows: NGF, 50 ng/ml; EGF, 10 ng/ml; bFGF, 5 ng/ml; IL-1 β , 200 U/ml; IL-6, 20 ng/ml; db cAMP, 1 mM; and Ca-ionophore A23187, 10 μ g/ml. The concentrations of bFGF and IL-6 were chosen after an initial dose response by morphological differentiation (>90% of PC-12 cells differentiated within 7 d). Cells were treated with various agents for indicated time and harvested for RNA extraction.

RNA extraction and analysis

Total RNA was extracted by RNeasy (Cinna-Biotex, TX) and fractionated by 1% agarose/formaldehyde gel, stained with ethidium bromide, and transferred to Nitroplus 2000 (Separation Sciences, Inc.). Filters were hybridized using ³²P-labeled nick-translated probes. The probes used included the 2.2-Kb *Eco*RI fragment of rat *c-fos* cDNA (Curran *et al.*, 1987); the 1.8-kb *Eco*RI fragment of *c-jun* (Rauscher *et al.*, 1988b); the 3.1-kb *Eco*RI fragment of *egr-1* (Sukhatme *et al.*, 1988); the 1.8-kb *Eco*RI fragment of human *jun-B* cDNA (Nomura *et al.*, in preparation); the 1.6-kb *Eco*RI fragment of human *jun-D* cDNA (Nomura *et al.*, in preparation); the 0.7-kb *Pst* I-*Eco*RI fragment of IF-clone 73 cDNA (Leonard *et al.*, 1987); and the 1.3-kb *Pst* I fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort *et al.*, 1985). Prehybridization and hybridization conditions were as recommended by the manufacturer (high stringency hybridization). Washing conditions for individual probes after room temperature wash were as follows: *c-fos*, 0.2 $\times$ SSC/0.1% SDS, 65°C; *c-jun* and *egr-1*, 2 $\times$ SSC/0.1% SDS, 55°C; *jun-B* and *jun-D*, 0.2 $\times$ SSC/0.1% SDS, 65°C; IF-clone 73, 0.5 $\times$ SSC/0.1% SDS, 65°C; and GAPDH, 0.1 $\times$ SSC/0.1% SDS, 65°C for 3 $\times$ 25 min. RNA concentrations were quantitated by laser densitometric scanning of the autoradiograms (E-C Apparatus Corp.). When the RNA blot was used repeatedly for hybridization, the previous probe was removed by boiling in H₂O for 2 $\times$ 15 min. GAPDH was used to quantitate the amount of RNA loaded in different lanes (not shown). Each blot was repeated with two or more independent preparations of RNA, and a similar pattern of hybridization was observed with various probes.

Plasmid construction (F700 CAT)

A 5.4-Kb *Bam*HI fragment of pc-*fos*-1 (human, Curran *et al.*, 1983) encompassing the entire *c-fos*(h) gene was first cloned into the *Bam*HI site of PDP-1 vector (USB, Cleveland, OH) to generate PDP-*fos*. The PDP-*fos* was cleaved with *Nae* I (which cleaves at position +41 of *c-fos* gene and in the vector), the coding sequence of *c-fos* was removed, and the ends were modified into *Bgl*II with linkers. SV₀ CAT plasmid was digested with *Hind*III, and the *Hind*III site was converted into a *Bgl*II site by linkers. The *Bgl*II-*Bam*HI fragment comprising the CAT coding sequence and the SV40 polyA sequences was cloned into the *Bgl*II site of PDP-*fos* to generate F-700-*fos*.

Transient expression assay

Supercoiled plasmid DNAs were transfected onto PC-12 and PC-12 *fos* cells as described (Ito *et al.*, 1989). After electroporation, cells were incubated in complete medium for 12 h in 100 mm collagen-coated culture dishes (Costar), the medium was replaced with RPMI without serum for 24 h followed by addition of indicated agents, and the cells were further incubated for an additional 12 h before harvesting for CAT assay (Gorman *et al.*, 1982). CAT activity was quantitated by densitometric scanning of the autoradiograms.

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