NERVE growth factor (NGF)\(^1\) is a well-characterized trophic factor for sympathetic neurons, neural crest-derived sensory neurons, and forebrain cholinergic neurons (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980; Johnson et al., 1986). However, the mechanism of signal transduction and consequent gene activation underlying NGF action are still poorly understood. Most studies on genetic responses to NGF have been performed on PC12 cells, which are chromaffin-like cells (Greene and Tischler, 1976) that undergo a morphological switch when treated with NGF. The appearance of a neuronal phenotype is preceded by activation of a set of immediate early genes (Kujubu et al., 1987). One of these is the proto-oncogene c-fos (Kruijver et al., 1985; Greenberg et al., 1985) which, by dimerization with the Jun proteins, forms the transcription factor API (for review see Morgan and Curran, 1991). The API complex in turn activates the expression of intermediate and late genes characteristic of the neuronal cells (Gizang-Ginsberg and Ziff, 1990).

The c-fos promoter includes a serum response element (SRE), also termed dyad symmetry element (DSE), located 300-bp upstream of the transcriptional start site (Treisman, 1985; Gilman et al., 1988; Greenberg et al., 1987), which mediates activation by protein kinase C and growth factors that bind to receptors with tyrosine kinase activity, including NGF (Maher, 1988; Bothwell, 1991). The SRE also represents the major target for autoregulation of c-fos transcription in HeLa cells (Shaw et al., 1989). An API binding site adjacent to the SRE participates in the basal promoter activity in NIH-3T3 fibroblasts (Koening et al., 1989; Schoenthal et al., 1989). C-fos induction by cyclic AMP is independent of SRE (Gilman, 1988), and is mediated by the cyclic AMP response element (CRE) located 65-bp upstream of the start of transcription (Montminy et al., 1986; Sassone-Corsi et al., 1988). In PC12 cells this latter promoter element is also involved in c-fos induction by Ca\(^{2+}\) (Sheng et al., 1990). NGF induction of the c-fos gene is cooperatively mediated by SRE and an adjacent sequence of the c-fos promoter, termed SRE2 (Visvander et al., 1988). Induction of c-fos by NGF has been previously studied by measuring the mRNA levels in adult rat DRG neurons (Lindsay et al., 1990), and by immunocytochemistry of Fos protein in neonatal rat sympathetic neurons (Buckmaster et al., 1991).

In this paper we describe for the first time transfection of chicken DRG neurons (E8–E12) with plasmids bearing c-fos promoter sequences linked to the CAT gene by means of the calcium phosphate co-precipitation method, and subsequent investigation of the mechanism of c-fos transcriptional control by NGF in these neurons. Utilization of this method allowed for assessment of the intracellular messenger systems linked to NGF receptor via identification of the c-fos promoter regulatory elements that mediate the biological response to this growth factor.

**Materials and Methods**

**Preparation of Neuronal Cultures**

Chicken dorsal root ganglia taken at embryonic day 8 (E8) and E12 were cultured as previously described (Skaper et al., 1990). Briefly, after dissociation the cell suspensions were plated into tissue culture dishes (Falcon Plastics, Cockeysville, MD) to obtain high neuronal purity (80–90%). Purified DRG neurons were cultured on 0.01 mg/ml poly-l-lysine (P 1274, 2 ml for each 35 mm dish; Sigma Chemical Co., St. Louis, MO) using DME supplemented with 33.3 mM D-glucose, 2 mM l-glutamine, 26.4 mM NaHCO\(_3\), 100 U/ml penicillin, 10% (vol/vol) heat-inactivated FCS (Scromed) and 1 ng/ml of murine NGF.

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\(^1\) Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; CRE, cyclic AMP responsive element; DRG, dorsal root ganglion; NGF, nerve growth factor; SRE, serum responsive element.
DNA Transfection

Cultured E8/E12 DRG neurons were seeded at 5-6 x10^3 cells per 35-mm dish. Shortly before transfection, neurons were washed twice with DME and fed with DME containing 0.5% FCS. Transfection was performed by the DNA-calcium phosphate co-precipitation procedure (Graham and van der Eb, 1973; Chen and Okayama, 1987). Plasmid DNA was prepared and purified through Qiagen columns (Diagen, Düsseldorf, Germany). DNA precipitates at 1-10 μg/ml were prepared by mixing the DNA solution with 250 mM CaCl₂ followed by dropwise addition of the same volume of 2 x HBPS buffer containing 50 mM Hepes, pH 7.0, 1.5 mM Na₂HPO₄, 280 mM NaCl, 10 mM KCl, and 12 mM glucose. This latter solution was carefully distributed over the cultures using one-tenth of the growth medium volume. 7 h after DNA addition, the cell monolayers were washed twice and refed with medium containing 0.5% FCS. NGF was then added at 1 ng/ml, depending on the experiment. The cells were incubated for a further 16–24 h to allow DNA expression.

Plasmids

Mouse c-fos promoter CAT constructs were prepared as previously described (Gabellini et al., 1991). pDE3tkCAT including three repeats of the mouse DSE sequence: GATCTCCGAGCTCCCATTTGAGCATCC-CONTGG, linked to the minimal herpes simplex virus minimal promoter (tk) of pBluescript (Luckow and Schutz, 1987); and the construct pATF3tkCAT, bearing three copies of ATF oligonucleotides: AGCTCCGTACCTACCC-CCGG, including the CRE sequence (Lin and Green, 1988) in the same vector, were constructed in the laboratory of Dr. W. Kruijer (Utrecht). Plasmids pSVCAT and pSVbeta-galactosidase were purchased from Stratagene (La Jolla, CA).

CAT Assay

Transfected neurons were washed with ice-cold PBS and collected by scraping into 1 ml of cold TEN buffer (40 mM Tris/Cl, pH 7.5, 10 mM EDTA, 150 mM NaCl). CAT activity was determined as described (Gorman et al., 1982), using 1.3 nmol (75 nCi) of [³⁵Cl]chloramphenicol (Amersham Corp., Arlington Heights, IL), 0.35 mM acetyl CoA (Sigma Chemical Co.), 20–80 μl of cell lysate and 250 mM Tris/Cl pH 8 to a final volume of 250 μl. Acetylated [³⁵Cl]chloramphenicol was quantified by Bioscan (Packard, Canberra). CAT activity was normalized for protein content using the Coomassie protein assay reagent (Pierce Chemical Co., Rockford, IL).

Beta-Galactosidase Immunostaining

E8 and E12 DRG neurons plated at the standard density and grown in the presence of NGF (1 ng/ml) were transfected with pSV-beta-galactosidase at 1 μg/ml of DNA. Cells were fixed in 4% paraformaldehyde and immunostained with anti-beta-galactosidase mAb (Promega Biotec) using a peroxidase-conjugated secondary antibody (Histostain-SP; Zimed, South San Francisco, CA).

Results

DNA Transfection of Sensory Neurons by Calcium–Phosphate Coprecipitation

DRG neurons were initially transfected with a bluescript plasmid derivative termed BSFC-579, that comprises the whole mouse c-fos promoter (Curran et al., 1983; Renz et al., 1985) linked to the CAT reporter gene (Fig. 1) by the DNA calcium phosphate co-precipitation method. Transfection was performed with dissociated chicken embryonic dorsal root ganglion neurons (E8/E12 DRG) that were plated in the presence of NGF at 1 ng/ml and 10% FCS to ensure maximal survival. Transfection was most efficient when carried out 3–18 h after plating. Maximal CAT activity was already detected at a DNA concentration of 0.5 μg/ml, while 50% activity was obtained using 0.2 μg/ml DNA. Before DNA addition, the cultures were washed with fresh medium containing 0.5% FCS, with or without NGF. Incubation with the plasmid DNA (1 μg/ml) for 6–8 h was sufficient for maximal CAT expression. Omission of NGF during this short transfection period did not influence the DNA uptake capabilities of these neurons. Incubation with DNA up to 18 h, in the presence of NGF, did not modify cell survival and neurite outgrowth. Both morphological appearance and metabolic activity of DRG neurons, the latter determined by a colorimetric test with the MTT dye (Mosmann, 1983), were similar to non-transfected cells.

When DRG neurons (E8 and E12) were grown and transfected in the continuous presence of NGF (1 ng/ml), CAT activity 30 h posttransfection was in the range of 1 μMol of modified chloramphenicol/h/mg cell protein. This high CAT expression in DRG neurons is a function of c-fos promoter strength, which is about 3.5-fold higher than that of pSVβCAT in both E8 and E12 DRG (Table 1). Furthermore, when cultured DRG neurons were transfected with plasmid pSV-beta-galactosidase and the expressed recombinant protein visualized by immunocytochemistry, ~90% of the neurons showed strong beta-galactosidase immunoreactivity (Fig. 2), whereas only very few (<10%) non-neuronal cells were stained. In long-term, NGF-treated cultures expression of CAT activity reached maximal levels 24 h from transfection and remained constant for at least one week, in both E8 or E12 DRG neurons. Hence, DNA calcium-phosphate co-precipitation appears to be a very convenient and simple method for introduction of DNA in developing sensory neurons. The transfection procedure was equally efficient with DRG cultured on polyornithine or laminin substrata. Because the latter may potentiate NGF effects on these neurons (Ernsberg and Rohrer, 1988; Millaruello et al., 1988), subsequent experiments were performed on polylysine to minimize substrate interference with NGF effects.

SRE Is a Strong Transcriptional Enhancer in Developing Sensory Neurons

To identify the c-fos promoter regions that confer high transcriptional activity, cultured DRG neurons (E8 and E12) were transfected with bluescript plasmid derivatives bearing different length fragments of the mouse c-fos promoter region linked to the CAT gene (Gabellini et al., 1991). Fig. 1 shows the results of a representative experiment, in which E8 DRG neurons were grown for 48 h in the presence of NGF. The DNA region of the c-fos promoter from base −579 to base −412 was not relevant for high transcriptional activity. A dramatic decrease in CAT expression (90%) was obtained with a deletion up to base −307 lacking 6 bp of the 5' SRE sequence. This element is an important mediator of NGF responses in PC12 cells (Visvander et al., 1988). These results indicate that the DNA region from nucleotides −412 to −307 includes a major transcriptional regulatory element in developing DRG neurons. The remaining portion from base −307 to −49, which bears both an intact API binding site downstream of the SRE and a CRE centered at position −60, is responsible for only 5% of the overall c-fos promoter activity.

To evaluate the relative contributions of SRE and CRE in transcription from c-fos promoter as a function of developmental age, E8 and E12 DRG neurons, continuously maintained in the presence of NGF, were transfected with plasmids pDE3tkCAT and pATF3tkCAT, containing three...
Figure 1. Mapping of enhancer elements active in DRG neurons. The DNA region comprising the mouse c-fos promoter in a HindIII-Accl fragment is shown at the top, together with positions of the main transcriptional enhancers SRE, CRE, and SCM. Arrows indicate the extent of 5' deletions linked to the CAT gene in the BSFC plasmids used for transfection. The lower panel (left) shows a representative experiment for CAT expression in E8 DRG neurons grown in the continuous presence of NGF for 30 h from transfection (DNA: 1 µg/ml). Positions of the origin (o), chloramphenicol (c), and acetylated chloramphenicol (ac) on the chromatogram are marked. The CAT assay was performed using 2 µg of protein and 1-h incubation. The percentage of total activity (mean ± SD) was calculated from the results of three independent transfections performed in duplicate (lower panel, right).

<table>
<thead>
<tr>
<th>DNA</th>
<th>E8 DRG</th>
<th>E12 DRG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSFC-579</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>pDSE3tkCAT</td>
<td>58 ± 5</td>
<td>545 ± 110</td>
</tr>
<tr>
<td>pATF3tkCAT</td>
<td>20 ± 10</td>
<td>45 ± 5</td>
</tr>
<tr>
<td>pSVCAT</td>
<td>33 ± 1</td>
<td>36 ± 8</td>
</tr>
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Values are the mean ± SD (n = 6) and are normalized for protein content. All cultures were in the presence of NGF. Absolute values of BSFC-579 were similar in E8 and E12 DRG neurons.

Table I. Relative Promoter Strength in Sensory Neurons

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<th>DNA</th>
<th>E8 DRG</th>
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Copies of DSE/SRE or ATF/CRE oligonucleotides, respectively, linked to the thymidine kinase minimal promoter of the plasmid pBLCAT2 (see Materials and Methods for details). As shown in Table I, although both DRG populations expressed similar CAT levels from c-fos promoter, expression from the pDSE3tkCAT in E8 DRG neurons was 50% lower than the c-fos promoter but was ~10-fold more active in E12 DRG neurons. In comparison, pATF3tkCAT showed lower activity (in the range of 20 to 40% of the c-fos promoter) in both neuronal populations. No CAT expression could be detected with pBLCAT2 or with a derivative containing only one ATF/CRE element. These results are in agreement with the expression of the deletion mutants of c-fos promoter, and suggest that SRE is a strong transcriptional enhancer in DRG neurons. Furthermore, the strong increase of activity detected from this element is developmentally regulated, and may be functionally related to the content of trk proto-oncogene product, as reported for the early development of mouse sensory ganglia (Martin-Zanca et al., 1991).

Control of c-fos Transcription by NGF

Chicken E8 DRG neurons depend on NGF for survival, whereas most E12 DRG neurons survive without NGF but remain sensitive in terms of neurite outgrowth (Skaper and Varon, 1983). Thus to correlate c-fos expression with NGF...
effects, we first evaluated c-fos promoter activity in cultured E8 DRG neurons transfected with BSFC-579 and grown in the presence of different NGF concentrations (from 4 pM to 1 nM) up to 48 h. Consistent with the presence of a high-affinity receptor-NGF interaction, the EC50 for CAT activity was 20 pM NGF (data not shown). The c-fos transcription correlated fairly well with the neurofilament protein content of equally treated DRG cultures measured by immunoreaction with mAb RT97 (Doherty et al., 1984), which showed a EC50 of 50 pM NGF.

To define a regulatory role of NGF on c-fos promoter activity, we next developed a NGF-deprivation paradigm. In particular to allow for optimal culture and transfection conditions, E8 DRG neurons were plated and cultured for the first 16 hours in the presence of 1 ng/ml of NGF and 10% FCS. 1 h before transfection with BSFC-579 or other plasmid constructs, the cultures were washed and subsequently maintained in medium containing 0.5% FCS or 10% FCS with or without NGF, for an additional 24 h. In these culture conditions, the DRG neurons showed no evident signs of cell death. As shown in Fig. 3, cells exposed to only 0.5% FCS expressed a CAT basal activity of only 30% of that measured in cultures having both NGF and 10% FCS. With 10% FCS or NGF + 0.5% FCS, CAT activity was about twice that obtained with cells grown in 0.5% FCS only. In control transfections with pSVCAT containing the NGF non-responsive SV-40 enhancer, CAT expression was not modified by NGF deprivation (Fig. 3). Hence, both NGF and FCS have a stimulatory effect on c-fos transcription. The combination of these two agents produced an additive effect on CAT expression, suggesting distinct pathways of c-fos activation.

In view of the above results, the ability of NGF to stimulate transcription was then evaluated following its readdition after a deprivation period. In particular E8 DRG neurons were deprived of NGF and serum shortly before transfection for 8 h with BSFC-579, and maintained in 0.5% FCS together with or without NGF for other 16 h. As shown in Fig. 4, NGF readdition to DRG cultures (E8) induced a significant increase in CAT expression in the presence of NGF alone or in combination with 0.5% FCS. These results suggest that NGF can stimulate c-fos transcription in a manner that is additive to the effects of FCS, and that the combination of these two agents can activate distinct pathways of c-fos expression.

**Figure 2.** Phase contrast light micrograph of dissociated E8 DRG neurons transfected for 8 h with pSV-beta-galactosidase and immunostained with peroxidase-conjugated specific mAb. The arrow indicates a non-transfected neuron.

**Figure 3.** Effect of NGF and FCS deprivation on c-fos transcription. E8 DRG neurons were transfected with BSFC-579 and with pSVCAT (1 μg/ml) and incubated for an additional 24 h in medium containing either 10 or 0.5% FCS with or without NGF (1 ng/ml) as indicated. Values are the mean ± SD (n = 3) of a representative experiment.
increase in transcriptional activity that was already detectable 2 h later. The transcription rate in neurons re-exposed to NGF increased linearly, reaching a twofold stimulation over control after 12 h. During this period control (NGF-deprived) cells were still metabolically active, as shown by the slow but constant increase of CAT activity. Furthermore, when the DNA concentration was lowered to a minimum of 0.1 μg/ml to counteract possible DNA excess over transcription factors and repressors, the basal activity was correspondingly reduced and c-fos inducibility by NGF was still about twofold. As Fig. 5 shows, similar results were obtained when using El2 DRG neurons in which NGF starvation was prolonged for 30 h, a time sufficient for repression and subsequent re-induction of the c-fos promoter in other cell types (Shaw et al., 1989). Furthermore, the linear accumulation of CAT activity in DRG neurons under these conditions (Fig.

**Figure 4.** NGF induction of c-fos transcription. E8 DRG neurons in 0.5% FCS were transfected with BSFC-579 (1 μg/ml) and deprived of NGF for 16 h. Cells were collected at the indicated times after NGF readdition. Controls were kept in 0.5% FCS only. Each experimental point is the mean ± SD of triplicate samples run in parallel.

**Figure 5.** Opposite effects of NGF on transcription from DSE and CRE. El2 DRG neurons were transfected with BSFC-579, DSE3tkCAT (DSE 3×) and ATF3tkCAT (ATF 3×) at 0.1 μg/ml. After 30 h of NGF and serum starvation, promoter induction was performed by addition of NGF (1 ng/ml) or forskolin (FSK) at 10 μM for 8 h. Control cells (CON) were kept in medium with 0.5% FCS. The values of CAT activity shown next to each treatment were determined using 2 μg protein and 2-h incubation. Average of induction (mean ± SD) refers to two independent experiments run in duplicate. Position of the origin (o), chloramphenicol (c), and acetylated chloramphenicol (ac) are as indicated.
NGF induction of c-fos transcription requires SRE. E8 DRG neurons were transfected with BSFC-412, -307, and -49 using 0.1 μg/ml of each DNA. After 16 h of NGF and serum starvation, NGF (1 ng/ml) was added (+) or not (−) as indicated. CAT activity was determined 8 h from NGF addition. The positions of the origin (o), chloramphenicol (c), and acetylated chloramphenicol (ac) on the thin layer chromatogram are as indicated.

4), instead of the transient induction pattern typical of the endogenous c-fos, probably reflects a loss of c-fos promoter repression by the fos protein itself (Lucibello et al., 1989).

**Transcriptional Control by NGF on DSE and CRE**

To identify the c-fos promoter sequences responsive to NGF, transfections with the BSFC plasmids described in Fig. 1 were performed, using the above described transient NGF-deprivation paradigm. E8 DRG neurons in 0.5% FCS were deprived of NGF for 16 h followed by 8 h of incubation with NGF. The CAT activity determined at this time showed a 1.5–1.8-fold increase in c-fos promoter activity, as compared to cells deprived of NGF for 24 h. Promoter deletion up to base −407 retained maximal inducibility, whereas deletions up to base −307 and −49 were no longer inducible by NGF (Fig. 6). This indicates that the DNA region between bases −412 and −307, including the DSE, mediates NGF-induced stimulation of transcriptional activity. Thus, the CRE element present in construct BSFC-307 (see Fig. 1) is not appreciably involved in c-fos induction by NGF. Furthermore, the SRE2 sequence that partially mediates c-fos induction by NGF and serum in PC12 cells (Visvander et al., 1988), which is also present in BSFC-307, is inactive in DRG neurons.

The results obtained with c-fos promoter deletions showed that a region including the SRE sequence is relevant for NGF-induced expression of c-fos. To evaluate the contribution of the SRE for induction of c-fos transcription by NGF, the plasmid pDSE3tkCAT was tested for NGF inducibility. As Fig. 5 shows, the SRE is a main target for NGF stimulation (2.1-fold induction). The elements SCM or SIF (Fig. 1), which have been shown to mediate genetic responses to PDGF (Wagner et al., 1990), do not seem to be relevant in this system.

cAMP is known to activate transcription of c-fos and other genes via CRE. Neurons transfected with pATF3TKCAT showed an increase in CAT activity (1.5-fold) after a 12-h stimulation with forskolin (Fig. 5). Interestingly, transcription from CRE was down-regulated ~50% by NGF in both E8 and E12 DRG neurons. These results suggest that NGF exerts a positive effect on the transcription complex (SRF) binding to DSE/SRE, producing an inhibition of CRE binding factor activity.

**Discussion**

**Transfection of Primary Neuronal Cultures**

High efficiency non-cytotoxic DNA transfection of cultured avian embryonic sensory neurons was obtained by DNA calcium phosphate co-precipitation. This method was optimized for maximal CAT expression in DRG neurons, in terms of DNA concentration, time of incubation with co-precipitated DNA, age of the neurons in culture and cell density. The most efficient DNA transfer was obtained within a few hours, after plating in the presence of NGF. One possible explanation for this is that the dissociation process may increase the membrane permeability to macromolecules due to mechanical lesion (Borasio et al., 1989) and/or trypsin proteolysis. Furthermore the strong trophic activity that NGF plays on these neurons, may increase the active transport of macromolecules, which could positively influence DNA uptake. Thus, this experimental procedure together with the high purity of the neuronal preparations used and very low transfection efficiency of the non-neuronal cells allowed for assessment of nuclear responses to NGF, following its removal and readdition, in developing dorsal root neurons.

This procedure is of greater simplicity and efficiency, when compared with an alternative method based on DNA lipid complexes (Behr et al., 1989) that was used for transfection of cerebellar granule cells (Loeffler et al., 1990). The transfection method herein outlined can also be used for gene transfer of primary central nervous system neurons, as hippocampal neurons although with lower efficiency (Gabellini and Skaper, unpublished data).

**Positive Control of c-fos Transcription by NGF**

NGF addition consistently increased the strong expression from the c-fos promoter obtained in developing sensory neurons. The similarity between E8 and E12 DRG neurons in the induction of c-fos transcription by NGF suggests that in-
increased c-fos expression is not simply a reflection of enhanced cell survival. This is compatible with the results obtained with PC12 cells (Krujier et al., 1985, Greenberg et al., 1985) and adult rat DRG neurons (Lindsay et al., 1989), in which induction of c-fos expression by NGF occurs independently of any effect on survival. Thus the NGF induced high transcriptional activity from c-fos promoter is likely more relevant for the neurotogenic effect of this trophic factor on developing DRG neurons rather than its effect on survival.

**DSE Mediates NGF Induction of c-fos Transcription**

In chicken embryonic DRG neurons in the presence of NGF, very high levels of CAT expression driven by the c-fos promoter were obtained. In 3T3 fibroblasts and primary astrocytes, transfection with the same construct yielded 1,000 and 50-fold lower levels of CAT expression, respectively (Gabellini et al., 1991; Koenig et al., 1989). In comparison, in cell lines such as NIH-3T3 fibroblasts c-fos promoter activity was only 50% of that obtained with the SV-40 enhancer (Deschamps et al., 1985) whereas in DRG neurons expression from c-fos promoter was threefold stronger than that from pSVCAT. Such high expression is determined by the DSE, which contributes 90% of the total promoter activity in sensory neurons. In commonly used cell lines, e.g., fibroblasts and HeLa cells (Deschamps et al., 1985) or primary cultured astrocytes (Gabellini et al., 1991), the contribution of DSE in the c-fos promoter was only 50% of the total activity. The transcriptional activity from this element is strongly regulated during development, as it increases about 10-fold during embryonic cell maturation (E8 vs E12). This could indicate that additional or different control mechanisms of gene transcription are involved in sensory neurons when they become independent from NGF for survival. In contrast, the total c-fos promoter activity did not differ significantly in the two cell populations (E8 vs E12), suggesting that an as yet undefined additional complexity is operative in the control of the intact promoter.

Maximal induction of c-fos transcription measured after an NGF starvation period up to 30 h was about twofold over the basal levels determined in NGF deprived controls. In contrast, the NGF-induced phenotypic switch in PC12 cells is preceded by a 12-fold induction of c-fos which is mediated by SRE/DSE and by an adjacent downstream SRE2 sequence, the latter being apparently irrelevant in DRG neurons. This discrepancy probably reflects different cellular responses elicited by NGF in these two cell types (maturation in DRG neurons versus phenotypic switch and differentiation in PC12 cells). The lower c-fos induction by NGF detected in developing sensory neurons, as compared to naïve PC12 cells, may be related to the high basal c-fos expression in DRG neurons due to pre-exposure to NGF and may involve an insufficient repression of the c-fos promoter by its own or other gene products. The absence of such a negative regulatory system has been shown to prevent induction of the c-fos gene expression (Shaw et al., 1989; Koenig et al., 1989).

**Negative Regulation by NGF on CRE**

It is well established that increased cytoplasmic cyclic AMP levels can induce c-fos expression via the CRE. This result has been also confirmed in our model, as shown by the increase in CAT activity under control of the c-fos or ATF3tk promoter after forskolin addition to the culture medium. In this context, it is interesting that NGF seems to negatively regulate the protein kinase A–mediated transcription pathway. This result is supported by the observation that forskolin inhibits the expression of low-affinity NGF receptors in adult rat DRG (Lindsay et al., 1990) and decreases neurofilament synthesis in PC12 cells (Doherty et al., 1987). Both of these genes are known to be positively regulated by NGF, presumably through a signalling pathway involving tyrosine phosphorylation of the high-affinity receptor complex (see Bothwell, 1991). Tyrosine kinase activity might be inhibitory for the protein kinase A pathway in DRG neurons, by modification of the phosphorylation state of CRE binding protein, CREB (Gonzales and Montminy, 1989; Lamph et al., 1990). The observation that increased levels of cyclic AMP mimic the effect of NGF on some genes, while inhibiting others (Cho et al., 1989), may reside on the promoter structure of the gene under consideration.

In summary we show that the calcium phosphate DNA co-precipitation method allows for efficient transfection of primary cultured neurons and that in avian embryonic DRG neurons NGF controls the high CAT expression from the transfected c-fos promoter, that is mediated by DSE. Further experimentation using this method for gene transfer in other neuronal cell types, will further our understanding of the regulatory mechanisms of gene expression controlled by growth factors and other extracellular stimuli in the nervous system.

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