MOLECULAR AND CELLULAR BIOLOGY, Apr. 1990, p. 1556–1563
0270-7306/90/041556-08$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 10, No. 4

Oncogene N-*ras* Mediates Selective Inhibition of c-*fos* Induction by Nerve Growth Factor and Basic Fibroblast Growth Factor in a PC12 Cell Line

TIMOTHY M. THOMSON,[1†] STEVEN H. GREEN,[2] ROBERT J. TROTTA,[1] DAVID E. BURSTEIN,[1] AND ANGEL PELLICER[1*]

Department of Pathology and Kaplan Cancer Center, New York University Medical Center, New York, New York 10016,[1] and Department of Biology, University of Iowa, Iowa City, Iowa 52242[2]

Received 28 August 1989/Accepted 9 December 1989

A cell line was generated from U7 cells (a subline of PC12 rat pheochromocytoma cells) that contains a stably integrated transforming mouse N-*ras* (Lys-61) gene under the control of the long terminal repeat from mouse mammary tumor virus. Such cells, designated UR61, undergo neuronal differentiation upon exposure to nanomolar concentrations of dexamethasone, as a consequence of expression of the activated N-*ras* gene (I. Guerrero, A. Pellicer, and D. E. Burstein, Biochem. Biophys. Res. Commun. 150:1185–1192, 1988). Exposure of UR61 cells to either nerve growth factor (NGF) or basic fibroblast growth factor (bFGF) results in a marked induction of c-*fos* RNA, with kinetics paralleling those of NGF- or bFGF-induced expression of c-*fos* RNA in PC12 cells. Dexamethasone-induced expression of activated N-*ras* p21 results in blocking of c-*fos* RNA induction by NGF or bFGF in a time-dependent manner. Activated N-*ras* p21-mediated inhibition of c-*fos* RNA induction in UR61 cells is selective for NGF and bFGF and is not due to selective degradation of c-*fos* RNA. Normal and transforming N-*ras* can *trans* activate the chloramphenicol acetyltransferase gene linked to mouse c-*fos* regulatory sequences when transient expression assays are performed. Our observations suggest that N-*ras* p21 selectively interacts with pathways involved in induction of c-*fos* expression which initiate at the receptors for NGF and bFGF.

The *ras* genes were initially isolated as the transforming principle of certain oncogenic retroviruses (reviewed in reference 3). Cellular counterparts for these genes were subsequently found. A number of tumors contained *ras* alleles with transforming capacity in NIH 3T3 focus-forming assays, a capacity conferred by point mutations that lead to the expression of products with single amino acid substitutions (3). More recently, *ras* genes with transforming capacity for mouse fibroblasts have been shown to induce terminal differentiation in a number of cellular systems, including neuronal (4, 28, 49), endocrine (47), and lymphoid (54) cells.

Cellular *ras* genes code for a protein, p21, whose physiological function is still elusive. There is growing evidence, however, that *ras* p21 is involved in the process of signal transduction for certain growth factor receptors (31). Thus, *ras*-transformed fibroblasts show increased membrane phospholipid turnover, leading to accumulation of inositol triphosphate and diacylglycerol (1, 16), in a fashion similar to activation of phosphatidylinositol metabolism by several growth factors (7). Similarly, *ras* p21 stimulates the metabolism of membrane phosphatidylcholine, with accumulation of phosphocholine (38). Furthermore, p21 binds guanine nucleotides and contains regions of high homology to the guanine-binding site of other guanine nucleotide-binding proteins (32, 41, 45, 48) and, like other members of this class of proteins, hydrolyzes GTP (17, 60). These characteristics, together with its subcellular localization on the inner aspect of plasma membranes, have prompted the inclusion of this molecule within the category of G proteins involved in signal transduction for a number of transmembrane receptors (18). On the basis of observation of ligand-mimicking effects and blocking by specific antibodies, several receptors for peptide growth factors have been proposed as candidates for interaction with *ras* p21, including the receptors for nerve growth factor (NGF) (4, 28, 29, 49), transforming growth factor β (50), and insulin (14, 35).

PC12 rat pheochromocytoma cells undergo neuronal differentiation and growth arrest when exposed to NGF (26). Interaction between this peptide and its surface receptor on PC12 cells is followed by transcriptional activation of specific sets of genes which are responsible for the resulting differentiated phenotype (reviewed in reference 26). NGF-induced transcriptional activation occurs according to distinct temporal patterns (39). One of the first genes whose expression is induced by NGF in PC12 cells is the nuclear proto-oncogene c-*fos* (12, 24, 36). In the present study, we have used transient changes in the level of c-*fos* RNA as a marker for signal transduction (12, 24, 35) to analyze possible functional interactions between the product of oncogene N-*ras* and the receptor for NGF. Our observations suggest that N-*ras* p21 selectively interacts with pathways involved in induction of c-*fos* expression which initiate at the receptors for NGF and basic fibroblast growth factor (bFGF).

MATERIALS AND METHODS

Cell cultures. UR61 (27) cells were grown in 85% Dulbecco modified Eagle medium–10% donor horse serum–5% fetal bovine serum (complete DMEM), supplemented with penicillin, streptomycin, and G418 (200 μg/ml). U7 cells (9) and PC12 cells were grown in 85% RPMI 1640 medium–10% donor horse serum–5% fetal bovine serum. Factors were added directly to the cells from concentrated stocks. The factors used were NGF (SUNY Technology Transfer, Albany, N.Y.), epidermal growth factor (Collaborative Re-

* Corresponding author.
† Present address: Departamento de Inmunología, Instituto Municipal de Investigaciones Médicas, 08003 Barcelona, Spain.

search, Inc., Bedford, Mass.), bFGF (Collaborative Research), and the following products from Sigma Chemical Co., St. Louis, Mo.): dexamethasone, dibutyryl cyclic AMP, phorbol-12-myristate-13-acetate, and forskolin.

RNA extraction and analysis. Total cellular RNA was isolated by the guanidine thiocyanate-cesium chloride method (10), electrophoresed on 1% agarose–formaldehyde gels (15 μg per lane), and transferred to nitrocellulose filters by standard procedures (43). Samples were normalized by hybridization with a probe for glucose-6-phosphate dehydrogenase. Filters were hybridized with random primer-labeled *Pst*I fragment of pfos-1 (13).

Transfection. For assays of transient expression of the chloramphenicol acetyltransferase (*CAT*) gene, we performed transfection experiments as described previously (27). Briefly, cells were plated on collagen- and poly-L-lysine-coated petri dishes 24 h prior to transfection. The cells were then washed with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline (137 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM Na_2HPO_4, 21 mM HEPES), and 1 ml of DNA-calcium phosphate precipitate was added per 10-cm petri dish. After 6 h of incubation in complete DMEM containing 100 μM chloroquine, cells were washed and subjected to glycerol shock. For cotransfection experiments, equimolar ratios of test plasmids were maintained. The plasmids used were c-*fos-CAT*, which contains mouse c-*fos* regulatory sequences (from −356 to +109) linked to the *CAT* gene (20), and ptl1-N-*ras* (Lys-61) or ptl1-N-*ras* (normal), which contain the corresponding mouse N-*ras* genes placed after the long terminal repeat LTR of Moloney murine leukemia virus (28). As a control for transfection efficiency, 4 μg of plasmid pCH110 (*lacZ*) was included in all transient-expression experiments (30). At 24 h after transfection, cells were harvested and CAT activity was assayed as described previously (21). The amount of cell extracts used for these assays was normalized according to their β-galactosidase activity (30). For generation of the T5H clone, U7 cells were cotransfected with pMMTV-N-*ras* (27) and pIBW3neo (8), by using the same transfection procedure as above, and colonies were isolated after 2 weeks of incubation in complete medium plus 400 μM G418. Thereafter, clones were grown in complete medium plus 200 μg of G418 per ml.

NGF binding. NGF binding was assayed as described by Green et al. (23). Briefly, cells were plated on poly-L-lysine-coated dishes in complete RMPI 1640 medium, incubated for 60 min with 200 pM ^{125}I-NGF in phosphate-buffered saline–0.2% bovine serum albumin–0.1% glucose, and washed with phosphate-buffered saline. Surface-bound NGF, not internalized, was removed by treatment with 0.5 M NaCl–0.2 M acetic acid and quantitated in a gamma counter. The remaining cell-associated counts represent the internalized NGF. Specific binding was determined by incubating labeled NGF (22) with 200 nM unlabeled NGF to determine nonspecific binding and subtracting this value from the total binding. The internal-to-external ratio represents the internal NGF normalized to the amount of NGF bound to the surface of the cells at steady state (23) and is thus a measure of the internalization rate.

RESULTS

NGF, but not stably integrated N-*ras*, induces endogenous c-*fos* RNA in UR61 cells. Interaction of NGF with its surface receptor on PC12 cells induces rapid transient transcriptional activation of the nuclear proto-oncogene c-*fos*, with increased steady-state RNA levels that peak at 45 to 60 min (12, 24, 36). U7 cells are a clone derived from PC12 cells which express high-affinity receptors for NGF (23) and possess partial responses to its differentiating effects (9). These cells were used as recipients for transfection of constructs carrying activated mouse N-*ras* (Lys-61) under the transcriptional control of the LTR from mouse mammary tumor virus (28), owing to their favorable characteristics as recipients of foreign DNA and faster growth than PC12 cells (9). One of the clones derived from U7 cells that carries this dexamethasone-inducible construct was designated UR61 (28). Dexamethasone at nanomolar concentrations induces N-*ras* expression and neuronal differentiation of UR61 cells (28).

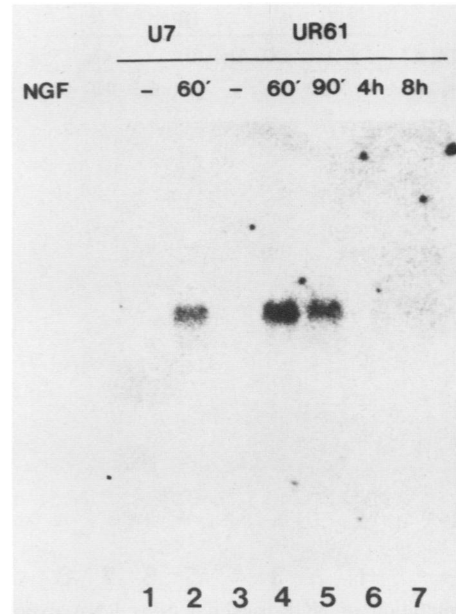

FIG. 1. c-*fos* RNA induction by NGF in U7 and UR61 cells. U7 cells (lanes 1 and 2) and UR61 cells (lanes 3 to 7) were incubated for the indicated periods with medium containing 50 ng of NGF per ml and washed, and RNA was extracted for Northern blot analysis of c-*fos* expression. Lanes 1 and 3 are control lanes (no NGF added).

Both U7 and UR61 cells showed transcriptional activation of c-*fos* in response to NGF, with kinetics similar to those observed for PC12 cells (Fig. 1). Induction of expression of N-*ras* by incubation with dexamethasone, however, did not induce c-*fos* expression, measured as steady-state RNA levels at time points ranging from 15 min to 12 h after the addition of dexamethasone. Time points corresponding to 60 min, 2 h and 4 h are shown in Fig. 2; no c-*fos* RNA was detected at any of the following points after addition of dexamethasone to the culture: 15-min intervals from 15 min through 60 min, 30-min intervals from 1 h through 6 h, and 1-h intervals from 6 h through 12 h (Fig. 2) (27; data not shown).

Reports from other laboratories have shown induction of c-*fos* by *ras* genes or *ras* products when analyzed in transient-expression assays (52, 57). We therefore performed transient-expression assays to evaluate *trans* activation of the reporter gene *CAT* linked to mouse c-*fos* 5' regulatory sequences (20) by either cotransfected or dexamethasone-induced integrated N-*ras*. The c-*fos–CAT* construct used in these experiments includes sequences −356 to +109 from the mouse c-*fos* transcription start site (20). This region

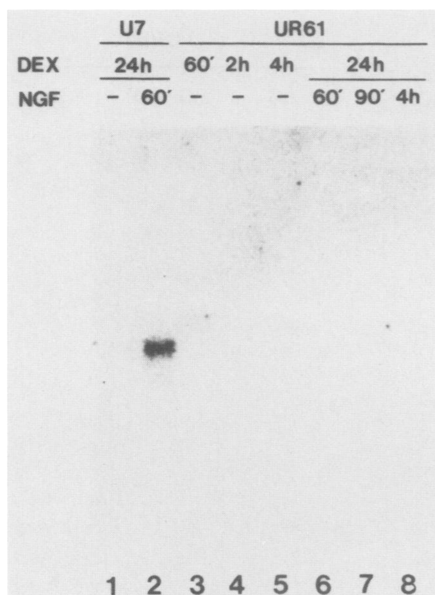

FIG. 2. Inhibition of NGF-triggered c-*fos* RNA expression in UR61 cells by dexamethasone-induced N-*ras*. U7 cells (lanes 1 and 2) and UR61 cells (lanes 3 to 8) were incubated for the indicated periods in medium containing 1 μM dexamethasone (DEX). In lanes 1, 2 and 6 to 8, NGF (50 ng/ml) was added for the indicated periods at the end of a 24-h incubation with dexamethasone. After the incubations, cells were washed and RNA was extracted for Northern blot analysis of c-*fos* expression.

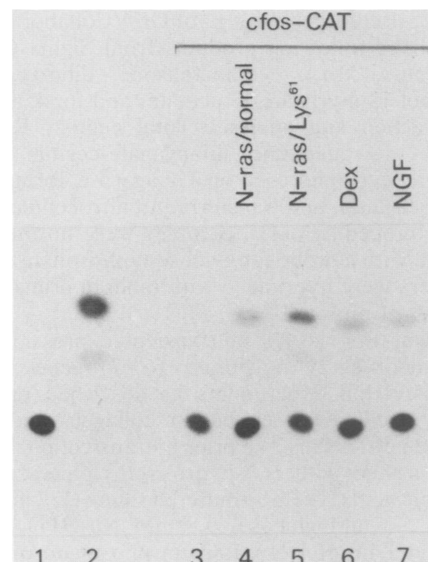

FIG. 3. *trans* activation of c-*fos–CAT* by N-*ras*. Lanes 1 and 2 correspond to *CAT* reporter gene activities of transfected pSV0*CAT* (lane 1) and pSV2*CAT* (lane 2) (21). Lanes 4 and 5 correspond to cotransfection experiments with the indicated plasmids. Basal-level expression of *fos-CAT* is shown in lane 3. At the time of transfection with c-*fos–CAT*, dexamethasone (DEX) (1 μM) was added to the cells, until extracts were prepared (lane 6). At 8 h prior to preparation of extracts, NGF (50 ng/ml) was added to cells transfected with c-*fos–CAT* (lane 7). Transfection of UR61 cells and CAT assays were performed as described in Materials and Methods.

contains the serum-responsive element and the AP-1-binding site, which are essential for transcriptional activation of c-*fos* by serum, 12-*O*-tetradecanonylphorbol-13-acetate (TPA), and other factors (20, 53, 62, 63), as well as for autoregulation mediated by *fos* and *jun* products (34, 56). Both activated N-*ras* and, to a lesser extent, normal N-*ras* induce CAT expression when cotransfected with c-*fos–CAT* (Fig. 3). Similarly, when UR61 cells transfected with c-*fos–CAT* were incubated with dexamethasone or NGF, CAT activity was induced (Fig. 3; Table 1). Therefore, N-*ras* can induce c-*fos* expression in transient-expression assays, confirming previously reported observations.

Lack of endogenous c-*fos* induction in UR61 cells by dexamethasone-induced, integrated N-*ras* p21 could be related to basal-level expression of the mutated p21, leading to continuous stimulation of low levels of c-*fos* transcription that would hamper rapid transient increases of expression in response to the same kind of stimulus (19, 46). Under basal conditions, however, steady-state levels of c-*fos* RNA in UR61 cells were always essentially undetectable (Fig. 1). Results from the transient-expression assays described above would suggest that the low levels of c-*fos* protein expressed under basal conditions would be sufficient to cause negative autoregulation of expression of the endogenous gene (56), but not repression of expression from c-*fos–CAT* plasmids, multiple copies of which are introduced into the cells (see Discussion).

N-*ras* p21 inhibits NGF-induced expression of c-*fos*. Exposure of U7 cells to 1 μM dexamethasone for 24 h did not affect the NGF-induced transcriptional activation of c-*fos* (Fig. 3). Dexamethasone treatment of UR61 cells, however, inhibited NGF-induced c-*fos* expression at least 20-fold (Fig. 3). Since these cell lines differ by the integration of the mouse oncogene N-*ras* in UR61 cells, the inhibitory effect observed must be due to the expression of activated p21. This is a reflection of lower levels of NGF-induced c-*fos* RNA in dexamethasone-treated UR61 cells, and is not related to a temporal shift in peak levels of steady-state c-*fos* RNA, since the low levels of transcription induced by NGF under conditions that allowed oncogene N-*ras* expression in UR61 cells followed similar kinetics in both dexamethasone-treated and untreated samples (Fig. 3).

Inhibition of NGF-induced expression of c-*fos* by N-*ras* p21 was time dependent, and it was observed after 6 h of exposure of UR61 cells to dexamethasone (Fig. 4). Activated p21 is expressed after 2 to 3 h of exposure to dexamethasone, reaching peak levels after 6 to 9 h (27; data not shown). Maximal inhibition of NGF-induced c-*fos* activation was seen only after 24 h of incubation. This was partially reversed at 48 h of exposure to dexamethasone, concomitant with a decrease in activity of the glucocorticoid-dependent mouse mammary tumor virus LTR and lower levels of the

TABLE 1. Relative CAT activities in transient-expression assays with c-*fos–CAT*

Plasmid	Treatment[a]	c-*fos* activation[b]
c-*fos–CAT*	–	1.0
c-*fos–CAT* + N-*ras*	–	2.8
c-*fos–CAT* + N-*ras* (Lys-61)	–	5.7
c-*fos–CAT*	Dex	3.1
c-*fos–CAT*	NGF	2.8

[a] Transfected UR61 cells were grown in medium without factors (–), with 1 μM dexamethasone for 24 h (Dex), or with 50 ng NGF per ml for the last 8 h prior to extraction for the CAT assay (NGF).

[b] Fold induction of CAT activity over the activity of transfected c-*fos–CAT* alone. Values are averages of two independent experiments.

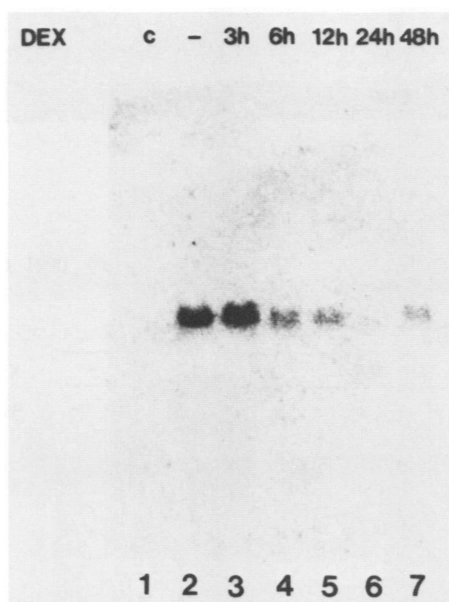

FIG. 4. Time dependence of *ras*-mediated inhibition of c-*fos* transcriptional activation. UR61 cells were incubated for the indicated periods in medium containing 1 μM dexamethasone (DEX). NGF (50 ng/ml) was added for the last 60 min of incubation. Subsequently, cells were washed and RNA was extracted for Northern blot analysis of c-*fos* expression. Lane 1 was the control lane (no factors added).

mutated N-*ras* p21 (J. C. Lacal, A. Cuadrado, J. E. Jones, R. J. Trotta, D. E. Burstein, T. M. Thomson, and A. Pellicer, submitted for publication). The latter phenomenon can be explained by the negative transcriptional effects of *ras* p21 on the mouse mammary tumor virus LTR transcription (32). Thus, inhibition of NGF-induced transcriptional activation of c-*fos* in UR61 cells correlates with the level of activated N-*ras* p21.

Binding of NGF to UR61 cells is not altered by *ras* induction. Exposure of PC12, U7, or UR61 cells to 1 μM dexamethasone for 24 h did not alter NGF binding levels in any of the cell lines (Table 2). Therefore, the observed inhibitory effect on NGF-induced c-*fos* transcription is not due to down regulation of surface NGF receptors on UR61 cells. Although levels of NGF binding remained constant, our data indicated a small change in the rate of NGF internalization in UR61 cells following a 24-h exposure to dexamethasone.

TABLE 2. ^{125}I-NGF-binding assay on UR61 cells exposed to dexamethasone

Cell line	No. of expts	Incubation time (h) with dexamethasone[a]	Amt of NGF bound[b] (fmol/mg of protein)		I/E[c]
			External	Internal	
UR61	9	0	60.4 ± 1.9	29.2 ± 1.9	0.48
UR61	9	24	60.6 ± 4.5	38.0 ± 1.7	0.63
U7	9	0	75.1 ± 3.7	61.7 ± 3.3	0.82
U7	9	24	78.0 ± 6.4	59.4 ± 3.8	0.76
PC12	3	0	30.6 ± 2.0	105.3 ± 7.1	3.45
PC12	3	24	34.5 ± 2.7	129.0 ± 5.4	3.74

[a] Incubation in medium containing 1 μM dexamethasone.
[b] Values shown are the means ± standard errors.
[c] Ratio between internal and external counts (see Materials and Methods).

This effect may be due to metabolic changes in the cells resulting from *ras* expression, since it was not seen in dexamethasone-treated U7 cells. However, since our observations showed an increase rather than a decrease in internalization rate, it is unlikely to be a cause of decreased NGF responsiveness.

N-*ras* p21-mediated inhibition of c-*fos* induction is selective for NGF and bFGF. Many other factors that exert their actions through diverse mechanisms are known to induce transcriptional activation of c-*fos* (24, 25). Thus, we wished to test whether c-*fos* activation by other factors would be affected in UR61 cells by expression of the oncogene N-*ras*. Of several factors tested, only bFGF-induced expression of c-*fos* RNA was inhibited by incubation of UR61 cells with 1 μM dexamethasone for 24 h (Fig. 5). In contrast, N-*ras* expression did not affect c-*fos* activation by epidermal growth factor, phorbol-12-myristate-13-acetate, dibutyryl cyclic AMP, forskolin, and 50 mM K^+ (which depolarizes PC12 membranes). Thus, activated p21-dependent inhibition of c-*fos* RNA induction in UR61 cells is selective for NGF and bFGF.

Inhibition of NGF induction of c-*fos* RNA in other sublines. Other cell lines carrying oncogenic N-*ras* were analyzed for NGF-induced transcriptional activation of c-*fos*. T5H and UR17 cells are, like UR61 cells, clones of U7 cells that contain integrated copies of mouse N-*ras* (Lys-61) under the control of mouse mammary tumor virus LTR. T5H cells show relatively high levels of basal expression of this gene and, as a consequence, show a partially differentiated phenotype in normal culture medium (T. M. Thomson and A. Pellicer, unpublished observations). UR17 cells show a limited phenotypic response to incubation with dexamethasone (27). The NGF-induced increase in c-*fos* RNA was not affected by dexamethasone in control U7 cells, whereas it was inhibited in UR17 cells (Fig. 6). T5H cells did not show a response even in the absence of dexamethasone induction, consistent with the high levels of basal expression of mutated p21.

DISCUSSION

Our results show that expression of the integrated, dexamethasone-inducible activated p21 in PC12 sublines results in inhibition of NGF- and bFGF-induced c-*fos* transcriptional activation. This N-*ras* p21-mediated effect correlated with levels of p21. Thus, the inhibitory effect was seen only after 6 h of incubation of UR61 cells with dexamethasone, and it was maximal after 24 h. This correlated well with the kinetics of expression of p21 in UR61 cells (27). The observed reversal of inhibition after 48 h of exposure to dexamethasone also coincided with a decrease in the expression of mouse mammary tumor virus LTR-driven N-*ras*, probably as a consequence of the negative transcriptional effect of activated p21 on the mouse mammary tumor virus LTR (33). The p21 dependence of the inhibitory effect on c-*fos* activation is further confirmed by the observation that NGF failed to induce c-*fos* expression in a clone which expressed relatively high basal levels of transforming p21 even in the absence of added dexamethasone (Fig. 6).

Dexamethasone induction of integrated activated N-*ras* does not result in a detectable increase in c-*fos* RNA. It is possible that integrated N-*ras*, under the transcriptional control of the mouse mammary tumor virus LTR, is expressed at low levels in UR61 cells under the usual (basal) conditions of culture. This would induce sufficient levels of c-*fos* protein to achieve a transcription-repressive effect on

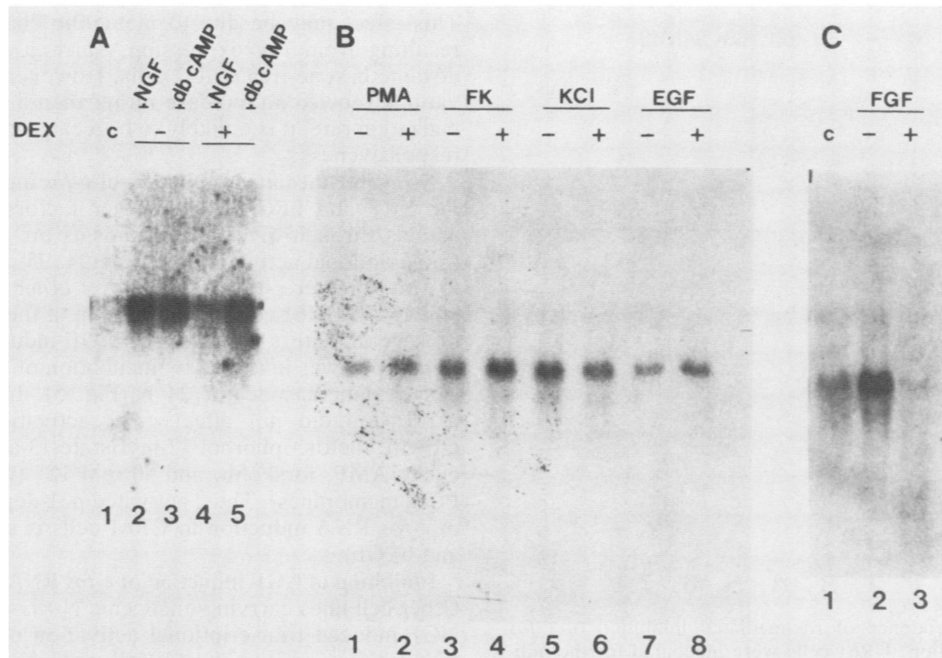

FIG. 5. Factor specificity of *ras*-mediated inhibition of c-*fos* transcriptional activation. UR61 cells were incubated for 24 h in complete medium without or with 1 μM dexamethasone (DEX). Subsequently, medium containing one of the following factors was added: NGF (50 ng/ml), dibutyryl cyclic AMP (db cAMP) (1 mM), phorbol-12-myristate-13-acetate (PMA) (50 nM), forskolin (FK) (1 μM), 50 mM KCl, epidermal growth factor (EGF) (10 ng/ml), or bFGF (10 ng/ml), as indicated. After 1 h of incubation, cells were washed and RNA was extracted for Northern blot analysis of c-*fos* expression. Lanes 1 in panels A and C were control lanes (no factors added).

its own gene (34, 53, 56) and to produce a refractory state (46) to further induction in response to increased levels of mouse mammary tumor virus LTR-driven N-*ras*. These basal levels of c-*fos* would be below the detection limits in our Northern (RNA) blots. That N-*ras*, both normal and transforming, can activate c-*fos*-directed expression, is shown by transient-expression assays (Fig. 3). Under the conditions used in these experiments, basal levels of c-*fos* protein already present in UR61 cells, which are sufficient for autoregulation of the endogenous c-*fos* gene, would not be sufficient for negative regulation of c-*fos* transcription from the multiple copies of the c-*fos–CAT* plasmid.

In contrast to the lack of detectable c-*fos* activation by dexamethasone-induced expression of N-*ras*, NGF induced high levels of c-*fos* RNA in UR61 cells. Thus, NGF and other stimuli seem to induce factors that activate the transcription of c-*fos* through interaction with regulatory elements of the c-*fos* promoter. These stimuli would exert their positive activity at different sites from those blocked by negative regulators induced by *ras*.

Increased expression of the mouse mammary tumor virus LTR-driven N-*ras*, while failing to induce detectable c-*fos* RNA, is able to block NGF- and bFGF-induced activation of c-*fos* transcription. The selectivity of this effect and its close correlation to levels of p21 suggest that it is due to more direct interactions between N-*ras* p21 and the receptors or other elements directly involved in signal transduction rather than through factors that affect c-*fos* transcription. Thus, it is possible that normal *ras* p21 can undergo reversible dissociation-reassociation cycles with other elements in the transduction pathway; these cycles would be activated upon ligand interaction in a fashion similar to G protein subunit activation and dissociation-reassociation (18). Transforming p21, being in active conformation in a constitutive manner (3), would bind putative substrate molecules (44) in competition with normal *ras* p21. The resulting effect would be the sequestering of substrate by constitutively activated *ras* p21 from physiologically activated pathways. This model could also explain *ras* p21-mediated uncoupling of ligand-receptor interactions observed in other systems, in which the molecular markers used represent some of the first events involved in such interactions, namely, products of membrane phospholipid metabolism (1, 15, 55), prostaglandines (5, 6), and cytosolic Ca^{2+} concentration (42).

The fact that N-*ras*-mediated inhibition of c-*fos* activation was selective for NGF and bFGF may be a reflection of the use of common mediators of actions triggered by both factors in PC12 cells. Both NGF and bFGF are capable of inducing neuronal differentiation in PC12 cells (61) and can promote the survival of certain neurons in vitro (66), indicating that their receptors share at least some of the pathways involved in such processes. Divergent pathways would account for effects specific for each of these factors. In this regard, it appears that NGF may use at least two signaling pathways (59). Other studies have shown that activated p21, in addition to inhibiting growth factor receptor induction of c-*fos* expression, affects transcriptional activation of c-*fos* by phorbol-12-myristate-13-acetate, dibutyryl cyclic AMP, and calcium ionophores (11, 40). In those reports, cell lines long transformed by *ras* were used, rather than transient or inducible systems. Thus, constitutive expression of high levels of the activated *ras* product could lead to long-term metabolic alterations by *ras* p21 that could result in alterations in other second-messenger pathways, thus making it more difficult to interpret effects that are directly due to activated p21.

Previous work had suggested that activation of protein kinase C in PC12 cells uncouples signal transduction for muscarinic receptors (65). Since there is evidence that *ras* p21 uses protein kinase C-mediated pathways in certain cell

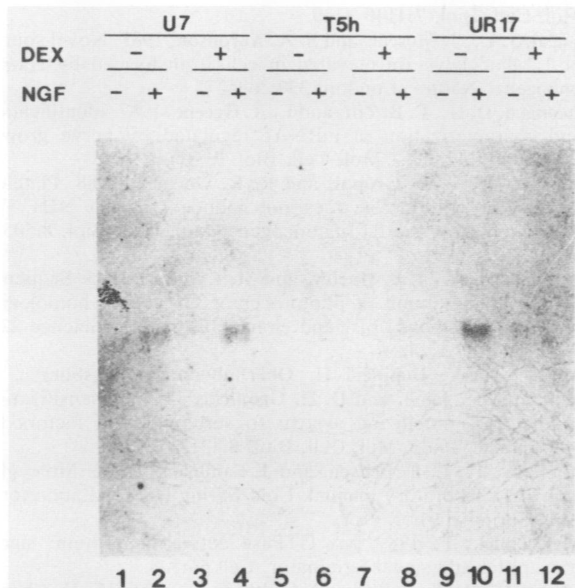

FIG. 6. N-*ras*-mediated inhibition of c-*fos* transcriptional activation in clones other than UR61. U7 cells (lanes 1 to 4), T5H cells (lanes 5 to 8), and UR17 cells (lanes 9 to 12) were incubated for 24 h in medium without or with 1 μM dexamethasone (DEX), as indicated. NGF (50 ng/ml) was added (even lanes) or not added (odd lanes) during the last 60 min of incubation. Subsequently, cells were washed and RNA was extracted for Northern blot analysis of c-*fos* expression.

types (37), it is conceivable that such an interaction in PC12 cells can regulate signal transduction. In this regard, cell type specificity must be taken into account to interpret our observations, since p21 can modulate protein kinase C expression and activity in PC12 cells (R. J. Trotta, T. M. Thomson, J. C. Lacal, A. Pellicer, and D. E. Burnstein, J. Cell. Physiol., in press; Lacal et al., submitted).

It could be argued that the use of dexamethasone as the inducing agent for conditional expression of N-*ras* represents a potential problem of our system. Glucocorticoids drive PC12 cells toward differentiation to chromaffin cells (2, 64) and can repress transcription of certain genes (51), including genes induced by NGF and bFGF (58). In our experiments, however, NGF induction of c-*fos* in parental U7 cells (which do not contain dexamethasone-inducible *ras* oncogenes) was not impaired by prolonged exposure to dexamethasone (Fig. 2) (data not shown). Moreover, inhibition of c-*fos* RNA induction by NGF was observed in a clone expressing high constitutive levels of activated N-*ras* in the absence of added dexamethasone. Therefore, the dexamethasone-induced effects of UR61 cells reported here are a consequence of the conditional expression of activated N-*ras* and are not a direct effect of dexamethasone.

Evidence for involvement of p21, the product of protooncogene *ras*, in the process of signal transduction in the NGF receptor system is based on NGF mimicking phenotypic effects of the transforming variants of this molecule (4, 27, 28, 49) and on blocking by antibody microinjection (29). Our observations lend further support to involvement of *ras* p21 in the process of signal transduction and specific gene activation by NGF and bFGF in PC12 cells. Our results, together with evidence for involvement of p21 in the early events that follow ligand-receptor interaction (1, 5, 6, 15, 54), indicate a close interaction between N-*ras* p21 and the receptors for NGF and bFGF and/or molecules critical for signal transduction and gene activation.

ACKNOWLEDGMENTS

We are grateful to R. Paciucci for help and advice with the CAT assays, J. M. Kahn for assistance with plasmids, and M. Z. Gilman for providing the *fos-CAT* plasmid.

This work was supported by Council for Tobacco Research grant 2466 (D.E.B.), Public Health Service grants NS21648 (D.E.B.), CA 01025 (D.E.B.), CA36327 (A.P.), and CA16239 (A.P.) from the National Institutes of Health, and a grant from the Familial Dysautonomia Foundation (D.E.B.). T.M.T. was supported by Basic Pathobiology Training Program grant GM07552. R.J.T. is an American Cancer Society fellow. D.E.B. is an awardee of the Irma T. Hirsch/Monique Weill-Caulier Trust. A.P. is a Leukemia Society Scholar.

LITERATURE CITED

1. **Alonso, T., R. O. Morgan, J. C. Mervizon, H. Zarbl, and E. Santos.** 1988. Malignant transformation by *ras* and other oncogenes produces common alterations in inositol phospholipid signalling pathways. Proc. Natl. Acad. Sci. USA **85:**4271–4275.
2. **Anderson, D. J., and R. Axel.** 1986. A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoids. Cell **47:**1079–1090.
3. **Barbacid, M.** 1987. *ras* genes. Annu. Rev. Biochem. **56:**779–827.
4. **Bar-Sagi, D., and J. R. Feramisco.** 1985. Microinjection of the *ras* oncogene protein into PC12 cells induces morphological differentiation. Cell **42:**841–848.
5. **Benjamin, C. W., W. G. Tarpley, and R. R. Gorman.** 1987. Loss of platelet-derived growth factor-stimulated phospholipase activity in NIH 3T3 cells expressing the EJ-*ras* oncogene. Proc. Natl. Acad. Sci. USA **84:**546–550.
6. **Benjamin, C. W., W. G. Tarpley, and R. R. Gorman.** 1987. The lack of PDGF-stimulated PGE_2 release from *ras*-transformed NIH 3T3 cells results from reduced phopholipase C but not phopholipase A_2 activity. Biochem. Biophys. Res. Commun. **145:**1254–1259.
7. **Berridge, M. J.** 1987. Inositol triphosphate and diacylglycerol: two interacting second messengers. Annu. Rev. Biochem. **56:** 159–193.
8. **Bond, V. C., and B. Wold.** 1987. Poly-L-ornithine-mediated transformation of mammalian cells. Mol. Cell. Biol. **7:**2286–2293.
9. **Burstein, D. E., and L. A. Greene.** 1982. Nerve growth factor has both mitogenic and antimitogenic activity. Dev. Biol. **94:** 477–482.
10. **Chirgwin, J. M., A. B. Przybyla, R. J. McDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
11. **Coletta, G., A. M. Cirafici, E. Consiglio, and G. Vecchio.** 1987. Forskolin and a tumor promoter are able to induce c-*fos* and c-*myc* expression in normal, but not in a v-*ras*-transformed rat thyroid cell line. Oncogene Res. **1:**459–466.
12. **Curran, T., and J. I. Morgan.** 1985. Superinduction of c-*fos* by nerve growth factor in the presence of peripherally active benzodiazepines. Science **229:**1265–1268.
13. **Curran, T., G. Peters, C. Van Beveren, N. M. Teich, and I. M. Verma.** 1982. FBJ murine osteosarcoma virus: identification and molecular cloning of biologically active proviral DNA. J. Virol. **44:**674–682.
14. **Deshpande, A. K., and H. F. Kung.** 1987. Insulin induction of *Xenopus laevis* oocyte maturation is inhibited by monoclonal antibody against p21 *ras* proteins. Mol. Cell. Biol. **7:**1285–1288.
15. **Downward, J., J. de Gunzburg, R. Riehl, and R. A. Weinberg.** 1988. p21 *ras*-induced responsiveness of phophatidylinositol

turnover to bradykinin is a receptor number effect. Proc. Natl. Acad. Sci. USA **85:**5774–5778.

16. **Fleischman, L. F., S. B. Chahwala, and L. Cantley.** 1986. *ras*-transformed cells: altered levels of phosphatidylinositol-4,5-biphosphate and catabolites. Science **231:**407–410.
17. **Gibbs, J. B., I. S. Sigal, M. Poe, and E. M. Scolnick.** 1984. Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. Proc. Natl. Acad. Sci. USA **81:**5704–5708.
18. **Gilman, A. G.** 1987. G proteins: transducers of receptor-generated signals. Annu. Rev. Biochem. **56:**615–649.
19. **Gilman, M. Z.** 1988. The c-*fos* serum response element responds to protein kinase C-dependent and -independent signals but not to cyclic AMP. Genes Dev. **2:**394–402.
20. **Gilman, M. Z., R. N. Wilson, and R. A. Weinberg.** 1986. Multiple protein-binding sites in the 5′-flanking region regulate c-*fos* expression. Mol. Cell. Biol. **6:**4305–4316.
21. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
22. **Green, S. H., and L. A. Greene.** 1986. A single $Mr \approx 103{,}000$ ^{125}I-nerve growth factor-affinity-labeled species represents both the low and high affinity forms of the nerve growth factor receptor. J. Biol. Chem. **261:**15316–15329.
23. **Green, S. H., R. E. Rydel, J. L. Connolly, and L. A. Greene.** 1986. PC12 cell mutants that possess low- but not high-affinity nerve growth factor receptors neither respond to nor internalize nerve growth factor. J. Cell. Biol. **102:**830–843.
24. **Greenberg, M. E., L. A. Greene, and E. B. Ziff.** 1985. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. J. Biol. Chem. **260:**14101–14110.
25. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. Nature (London) **311:**433–438.
26. **Greene, L. A., and E. M. Shooter.** 1980. Nerve growth factor: biochemistry, synthesis, and mechanism of action. Annu. Rev. Neurosci. **3:**353–402.
27. **Guerrero, I., A. Pellicer, and D. E. Burstein.** 1988. Dissociation of c-*fos* from ODC expression and neuronal differentiation in a PC12 subline stably transfected with an inducible N-*ras* oncogene. Biochem. Biophys. Res. Commun. **150:**1185–1192.
28. **Guerrero, I., H. Wong, A. Pellicer, and D. E. Burstein.** 1986. Activated N-*ras* gene induces neuronal differentiation of PC12 rat pheochromocytoma cells. J. Cell. Physiol. **129:**71–76.
29. **Hagag, N., S. Halegoua, and M. V. Viola.** 1986. Microinjection of antibody to *ras* p21 inhibits nerve growth factor induced differentiation of PC12 cells. Nature (London) **319:**680–682.
30. **Hall, C. V., P. E. Jacob, G. M. Ringold, and F. Lee.** 1983. Expression and regulation of *Escherichia coli lacZ* gene fusions in mammalian cells. J. Mol. Appl. Genet. **2:**101–109.
31. **Hanley, M. R., and T. Jackson.** 1987. The *ras* gene. Transformer and transducer. Nature (London) **328:**668–669.
32. **Hurley, J. B., M. E. Simon, D. B. Teplow, J. D. Robinshaw, and A. D. Gilman.** 1984. Homologies between signal transducing G proteins and *ras* gene products. Science **226:**860–862.
33. **Jaggi, R., B. Salmons, D. Muellener, and B. Groner.** 1986. The v-*mos* and H-*ras* oncogene expression represses glucocorticoid hormone-dependent transcription from the mouse mammary tumor virus LTR. EMBO J. **5:**2609–2616.
34. **König, H., H. Pontal, U. Rahmsdordf, M. Büscher, A. Schöntal, H. J. Rahmsdorf, and P. Herrlich.** 1989. Autoregulation of *fos*: the dyad symmetry element as the major target of repression. EMBO J. **8:**2559–2566.
35. **Korn, L. J., C. W. Siebel, F. McCormick, and R. A. Roth.** 1987. *Ras* p21 as a potential mediator of insulin action in *Xenopus* oocytes. Science **236:**840–843.
36. **Kruijer, W., D. Shubert, and I. M. Verma.** 1985. Induction of the proto-oncogene *fos* by nerve growth factor. Proc. Natl. Acad. Sci. USA **82:**7330–7334.
37. **Lacal, J. C., T. P. Fleming, B. S. Warren, P. M. Blumberg, and S. A. Aaronson.** 1987. Involvement of functional protein kinase C in the mitogenic response to the H-*ras* oncogene product. Mol. Cell. Biol. **7:**4146–4149.
38. **Lacal, J. C., J. Moscat, and S. A. Aaronson.** 1987. Novel source of 1,2-diacylglycerol elevated in cells transformed by Ha-*ras* oncogene. Nature (London) **330:**269–271.
39. **Leonard, D. G., E. B. Ziff, and L. A. Greene.** 1987. Identification and characterization of mRNAs regulated by nerve growth factor in PC12 cells. Mol. Cell. Biol. **7:**3156–3167.
40. **Lin, A. H., V. E. Groppi, and R. R. Gorman.** 1988. Platelet-derived growth factor does not induce c-*fos* in NIH 3T3 cells expressing the EJ-*ras* oncogene. Mol. Cell. Biol. **8:**5052–5055.
41. **Lochrie, M. A., J. B. Hurley, and M. I. Simon.** 1985. Sequence of the alpha subunit of photoreceptor G protein: homologies between transducin, *ras*, and elongation factors. Science **228:**96–99.
42. **Maly, K., W. Doppler, H. Oberhuber, H. Meusburger, J. Hofmann, R. Jaggi, and H. H. Grunicke.** 1988. Desensitization of the Ca^{2+}-mobilizing system to serum growth factors by Ha-*ras* and v-*mos*. Mol. Cell. Biol. **8:**4212–4216.
43. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **McCormick, F.** 1989. *ras* GTPase activating protein: signal transmitter and signal terminator. Cell **56:**5–8.
45. **Medynski, D., K. Sullivan, D. Smith, C. Van Dop, F.-H. Chang, B. K.-K. Fung, P. H. Seeburg, and H. R. Bourne.** 1985. Amino acid sequence of the alpha-subunit of transducin deduced from the cDNA sequence. Proc. Natl. Acad. Sci. USA **82:**4311–4315.
46. **Morgan, J. I., D. R. Cohen, J. L. Hempstead, and T. Curran.** 1987. Mapping patterns of c-*fos* expression in the central nervous system after seizure. Science **237:**192–197.
47. **Nakagawa, T., M. Mabry, A. de Bustros, J. H. Ihle, B. D. Nelkin, and S. B. Baylin.** 1987. Introduction of v-Ha-*ras* oncogene induces differentiation of cultured human medullary thyroid carcinoma cells. Proc. Natl. Acad. Sci. USA **84:**5923–5927.
48. **Noda, M., T. Haga, A. Ichiyama, K. Kangawa, N. Minamino, H. Matsuio, and S. Numa.** 1985. Primary structure of the alpha-subunit of transducin and its relationship to *ras* proteins. Nature (London) **315:**242–245.
49. **Noda, M., M. Ko, A. Ogura, D. Liu, T. Amano, T. Takano, and Y. Ikawa.** 1985. Sarcoma viruses carrying *ras* oncogenes induce differentiation-associated properties in a neuronal cell line. Nature (London) **318:**73–75.
50. **Olson, E. N., G. Spizz, and M. A. Tainsky.** 1987. The oncogenic forms of N-*ras* or H-*ras* prevent skeletal myoblast differentiation. Mol. Cell. Biol. **7:**2104–2111.
51. **Sakai, D. D., S. Helms, J. Carlstedt-Duke, J. A. Gustafsson, F. M. Rottman, and K. R. Yamamoto.** 1988. Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Genes Dev. **2:**1144–1154.
52. **Sassone-Corsi, P., C. J. Der, and I. M. Verma.** 1989. *ras* induced neuronal differentiation of PC12 cells: possible involvement of *fos* and *jun*. Mol. Cell. Biol. **9:**3174–3183.
53. **Sassone-Corsi, P., J. C. Sisson, and I. M. Verma.** 1988. Transcriptional autoregulation of the proto-oncogene *fos*. Nature (London) **334:**314–319.
54. **Seremetis, S., G. Inghirami, D. Ferrero, E. W. Newcomb, D. M. Knowles, G.-P. Dotto, and R. Dalla-Favera.** 1989. Transformation and plasmacytoid differentiation of EBV-infected human B lymphoblasts by *ras* oncogenes. Science **243:**660–663.
55. **Sewkien, D., A. Lagarde, and J. Pouyssegur.** 1988. Deregulation of hamster fibroblast proliferation by mutated *ras* oncogenes is not mediated by constitutive activation of phosphoinositide-specific phospholipase C. EMBO J. **7:**161–168.
56. **Shaw, P. E., S. Frasch, and A. Nordheim.** 1989. Repression of c-*fos* transcription is mediated through $p67^{SRF}$ bound to the SRE. EMBO J. **8:**2567–2574.
57. **Stacey, D. W., T. Watson, H. F. Kung, and T. Curran.** 1987. Microinjection of transforming *ras* protein induces c-*fos* expression. Mol. Cell. Biol. **7:**523–527.
58. **Stein, R., S. Orit, and D. J. Anderson.** 1988. The induction of a

neural-specific gene, SCG10, by nerve growth factor in PC12 cells is transcriptional, protein synthesis dependent, and glucocorticoid inhibitable. Dev. Biol. **127**:316–325.

59. **Sugimoto, Y., M. Noda, H. Kitayama, and Y. Ytakawa.** 1988. Possible involvement of two signaling pathways in induction of neuron-associated properties by v-Ha-*ras* gene in PC12 cells. J. Biol. Chem. **263**:12102–12108.
60. **Sweet, R. W., S. Yokoyama, T. Kamata, J. R. Feramisco, M. Rosenberg, and M. Gross.** 1984. The product of *ras* is a GTPase and the T24 oncogenic mutant is deficient in this activity. Nature (London) **311**:273–275.
61. **Togari, A., G. Dickens, H. Kuzuya, and G. Guroff.** 1985. The effect of fibroblast growth factor on PC12 cells. J. Neurosci. **5**:307–316.
62. **Treisman, R.** 1985. Transient accumulation of c-*fos* RNA following serum stimulation requires a conserved 5′ element and c-*fos* 3′ sequences. Cell **42**:889–902.
63. **Treisman, R.** 1986. Identification of a protein-binding site that mediates transcriptional response of the c-*fos* gene to serum factors. Cell **46**:567–574.
64. **Unsicker, K., B. Krisch, J. Otten, and H. Thoenen.** 1978. Nerve growth factor-induced fiber outgrowth from isolated rate adrenal chromaffin cells: impairment by glucocorticoids. Proc. Natl. Acad. Sci. USA **75**:3498–3502.
65. **Vicentini, L. M., F. DiVirgilio, A. Ambrosini, T. Pozzan, and J. Meldolesi.** 1985. Tumor promoter phorbol 12-myristate, 13-acetate inhibits phosphoinositide hydrolysis and cytosolic Ca^{2+} rise induced by the activation of muscarinic receptors in PC12 cells. Biochem. Biophys. Res. Commun. **127**:310–317.
66. **Walicke, P. W. M., N. Cowan, N. Ueno, A. Baird, and R. Guillemin.** 1986. Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. Proc. Natl. Acad. Sci. USA **83**:3012–3016.