MOLECULAR AND CELLULAR BIOLOGY, Oct. 1990, p. 5324–5332
0270-7306/90/105324-09$02.00/0

Vol. 10, No. 10

Effect of a Dominant Inhibitory Ha-*ras* Mutation on Neuronal Differentiation of PC12 Cells

JÓZSEF SZEBERÉNYI,† HONG CAI, AND GEOFFREY M. COOPER*

Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

Received 30 March 1990/Accepted 13 July 1990

A dominant inhibitory mutation of Ha-*ras* which changes Ser-17 to Asn-17 in the gene product p21 [p21 (Asn-17)$^{Ha\text{-}ras}$] has been used to investigate the role of *ras* in neuronal differentiation of PC12 cells. The growth of PC12 cells, in contrast to NIH 3T3 cells, was not inhibited by p21(Asn-17)$^{Ha\text{-}ras}$ expression. However, PC12 cells expressing the mutant Ha-*ras* protein showed a marked inhibition of morphological differentiation induced by nerve growth factor (NGF) or fibroblast growth factor (FGF). These cells, however, were still able to respond with neurite outgrowth to dibutyryl cyclic AMP and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Induction of early-response genes (*fos*, *jun*, and *zif268*) by NGF and FGF but not by TPA was also inhibited by high levels of p21(Asn-17)$^{Ha\text{-}ras}$. However, lower levels of p21(Asn-17) expression were sufficient to block neuronal differentiation without inhibiting induction of these early-response genes. Induction of the secondary-response genes SCG10 and transin by NGF, like morphological differentiation, was inhibited by low levels of p21(Asn-17) whether or not induction of early-response genes was blocked. Therefore, although inhibition of *ras* function can inhibit early-response gene induction, this is not required to block morphological differentiation or secondary-response gene expression. These results suggest that *ras* proteins are involved in at least two different pathways of signal transduction from the NGF receptor, which can be distinguished by differential sensitivity to p21(Asn-17)$^{Ha\text{-}ras}$. In addition, *ras* and protein kinase C can apparently induce early-response gene expression by independent pathways in PC12 cells.

Members of the mammalian *ras* gene family (Ha-*ras*, Ki-*ras*, and N-*ras*) encode small proteins (p21s) which are localized to the inner surface of the plasma membrane. The *ras* proteins bind guanine nucleotides, have intrinsic GTPase activity, and alternate between active (GTP bound) and inactive (GDP bound) forms. These features make p21s analogous to the G proteins, which are involved in signal transduction from a variety of cell surface receptors to intracellular target molecules.

In *Saccharomyces cerevisiae*, *RAS* proteins act to stimulate adenylate cyclase (41), but the targets for p21 action in vertebrate cells have not yet been identified. Nonetheless, it is apparent that *ras* proteins are involved in the transduction of mitogenic signals in mammalian cells in culture. In addition to the transforming activity of mutated *ras* genes, it has been demonstrated that introduction of active p21 into quiescent fibroblasts induces cell proliferation in the absence of growth factor stimulation (11, 38). Conversely, inhibition of endogenous proto-oncogene *ras* function by microinjection of anti-Ras monoclonal antibody (30) or by transfection with a dominant inhibitory Ha-*ras* mutant (10) inhibits the proliferation of NIH 3T3 cells.

In addition to their role in cell proliferation, *ras* proteins may also be involved in the signal transduction pathway(s) that leads to nerve growth factor (NGF)-induced neuronal differentiation. The NGF-responsive rat pheochromocytoma cell line PC12 has been widely used as a model system to study this signal transduction process (19). Cultured PC12 cells display a chromaffin cell-like morphology but undergo rapid changes following treatment with NGF, acquiring a number of features characteristic of sympathetic neurons, including neurite outgrowth (for a review, see reference 17). Expression of oncogenic *ras* proteins in PC12 cells leads to neurite outgrowth and acquisition of other neuronlike characteristics similar to those observed after NGF treatment (2, 31). In addition, it has been reported that microinjection of PC12 cells with anti-p21 monoclonal antibody blocks neuronal differentiation induced by NGF, suggesting that *ras* proto-oncogene proteins may normally be involved in the NGF signal transduction pathway (21).

In the present study, we used a dominant inhibitory Ha-*ras* mutant (10) to investigate endogenous *ras* proto-oncogene function in the neuronal differentiation process. The mutant gene Ha-*ras* Asn-17 has a point mutation in codon 17 resulting in the substitution of serine by asparagine in p21. The Ha-*ras* Asn-17-encoded p21 preferentially binds GDP versus GTP and strongly inhibits proliferation of NIH 3T3 cells, apparently by acting as a dominant inhibitor of normal *ras* function (10). Yeast *RAS* genes with mutations corresponding to positions 15, 16, and 18 of mammalian p21 display similar dominant inhibitory activities (33, 36).

In order to study the role of *ras* proto-oncogene proteins in neuronal differentiation, we introduced the Ha-*ras* Asn-17 mutant into PC12 cells. In this article we report that Ha-*ras* Asn-17 does not affect PC12 cell proliferation but strongly inhibits neuronal differentiation induced by either NGF or fibroblast growth factor (FGF). Analyses of the characteristic alterations in gene expression induced by NGF further indicate that *ras* proteins function in at least two distinct NGF signal transducing pathways.

MATERIALS AND METHODS

Cell culture. PC12 cells were grown in Temin modified Eagle medium supplemented with 10% fetal bovine serum and 5% horse serum. NGF and FGF were purchased from Collaborative Research, Inc. Dibutyryl cyclic AMP (dbcAMP),

* Corresponding author.
† On leave from the Department of Biology, University Medical School of Pécs, Pécs, Hungary.

12-*O*-tetradecanoylphorbol-13-acetate (TPA), and dexamethasone were obtained from Sigma Chemical Co.

Ha-*ras* expression plasmids. The Ha-*ras* Asn-17 gene was subcloned into three different mammalian expression vectors.

(i) pZIPrasH(Asn-17) was constructed by inserting a *Bgl*II-*Bam*HI Ha-*ras*-containing fragment of pXCR(Asn-17) into the *Bam*HI site of pZIPneoSV(X) (10). In this plasmid, the mutant gene is coexpressed with a Neo^r gene from a Moloney murine leukemia virus (M-MuLV) long terminal repeat (LTR).

(ii) pMTrasH(Asn-17) was constructed by ligating an *Xba*I-*Pst*I Ha-*ras*-containing fragment of pXCR(Asn-17) (10) into the polylinker region of pMT64AA (C. Dionne and L. Gudas, unpublished). In this construct, the mutant gene is transcribed from the promoter of the mouse metallothionein-I gene (15).

(iii) The plasmid pMMTVrasH(Asn-17) was constructed by replacing the thymidine kinase (*tk*) gene in pLTL3 (46) with the mutant Ha-*ras* Asn-17 gene and by replacing pBR322 sequences with linearized pSV2neo (37). In this plasmid, the Ha-*ras* Asn-17 gene is expressed from a mouse mammary tumor virus (MMTV) LTR and the Neo^r gene is expressed from the simian virus 40 promoter.

Transfection of PC12 cells. Transfection of PC12 cells with plasmid DNAs was performed with the calcium phosphate precipitation technique as described previously (8). From 0.5 to 10 μg of plasmid DNA was used together with 20 μg of carrier NIH 3T3 DNA. Since pMTrasH(Asn-17) does not carry a selectable marker, this plasmid was cotransfected with 1 μg of pSV2neo (37) to confer G418 resistance to transformed cells. Three days after transfection, cells were transferred to medium containing G418 (0.4 mg/ml). Drug-resistant colonies were isolated after 4 to 6 weeks of G418 treatment and were propagated in the presence of G418. However, G418 was not included in the medium during growth or differentiation experiments.

Western immunoblot analysis. Cells were harvested at 50% confluence by scraping with a rubber policeman and extracted with buffer containing 1% Triton X-100, 20 mM Tris hydrochloride (pH 7.4), 1 mM $MgCl_2$, 125 mM NaCl, and 1% aprotinin. Protein samples (100 μg) were electrophoresed in 12% sodium dodecyl sulfate–polyacrylamide gels. Proteins were electroblotted to nitrocellulose filters and analyzed with anti-p21 monoclonal antibody ras-10 (6). Blots were developed with goat anti-mouse immunoglobulin G horseradish peroxidase conjugate (Bio-Rad Laboratories).

Northern (RNA) blot analysis. Total cytoplasmic RNA was isolated as described previously (10) except that cells were scraped with a rubber policeman rather than trypsinized and RNasin was replaced by vanadyl ribonucleoside complex (Bethesda Research Laboratories) in the extraction buffer. For blot hybridization, 5 to 15 μg of RNA was electrophoresed in 1.25% agarose–formaldehyde gels and transferred to Gene Screen Plus membranes (NEN Research Products). Filters were prehybridized, hybridized, and washed according to the manufacturer's instructions. Autoradiography was performed at −70°C with intensifying screens. The following DNA fragments were ^{32}P-labeled with a nick translation kit (Amersham) for use as probes: Ha-*ras*, a 0.7-kilobase (kb) *Pst*I-*Xba*I fragment from pXCR (10); *fos*, a 1-kb *Pst*I fragment from pfos-1 (9); *jun*, a 0.9-kb *Pst*I fragment containing human *jun* (4); *zif268*, a 2.2-kb *Eco*RI-*Hin*dIII fragment from a pUC19-*zif268* construct (25); transin, a 1.6-kb *Eco*RI fragment from pTR1 (28); and SCG10, a 1.9-kb *Eco*RI fragment from pSCG10-3 (39).

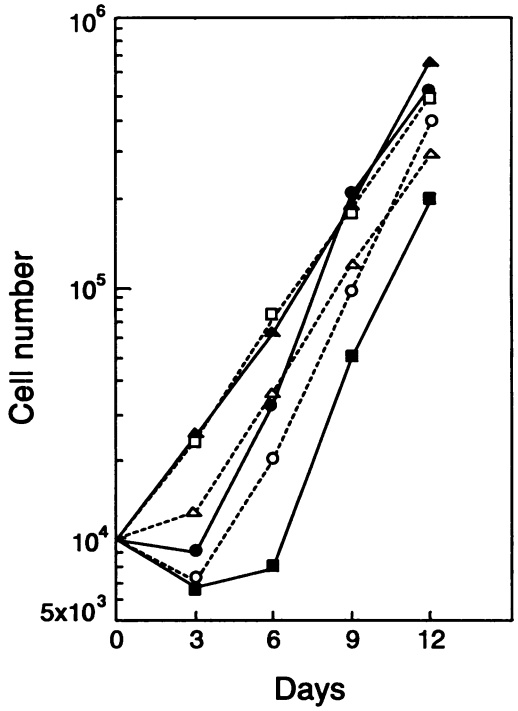

FIG. 1. Effect of Ha-*ras* Asn-17 on PC12 cell growth. PC12 (△), Z-1 (□), M-7 (○), Z-M17-5 (■), M-M17-2 (▲), and M-M17-26 (●) cells were plated in 60-mm dishes in medium containing 10% fetal bovine serum plus 5% horse serum at a density of 10^4 cells per plate. Cell numbers were determined every 3 days by counting cells in duplicate plates. Z-1 and M-7 cells are control subclones, whereas Z-M17-5, M-M17-2, and M-M17-26 express the Ha-*ras* Asn-17 gene.

RESULTS

Effect of Ha-*ras* Asn-17 on growth and differentiation of PC12 cells. The biological activity of the dominant inhibitory Ha-*ras* Asn-17 gene was investigated by transfection of PC12 cells with the mutant gene inserted into three different mammalian expression vectors, in which Ha-*ras* Asn-17 was expressed from either the M-MuLV LTR [pZIPrasH(Asn-17)], the metallothionein promoter [pMTrasH(Asn-17)], or the MMTV LTR [pMMTVrasH(Asn-17)]. In each case transformants were selected for G418 resistance conferred by a Neo^r gene contained either within the Ha-*ras* expression plasmids (pZIPrasH and pMMTVrasH constructs) or introduced by cotransfection with pSV2neo (for the pMTrasH construct). Control cultures were transfected with pZIPneoSV(X) or pMT64AA plus pSV2neo.

In contrast to the results of comparable experiments with NIH 3T3 cells as recipients (10), the number of G418-resistant PC12 cell colonies was not reduced by transfection with the Ha-*ras* Asn-17 mutant. In addition, we did not observe significant differences in the growth rate of control and Ha-*ras* Asn-17-expressing PC12 transformants (Fig. 1). Thus, in contrast to its effect in NIH 3T3 cells, expression of Ha-*ras* Asn-17 did not inhibit proliferation of PC12 cells.

A number of G418-resistant PC12 subclones were isolated after transfection with plasmids expressing the Ha-*ras* Asn-17 gene (M17 subclones) and screened for resistance to NGF-induced morphological differentiation (Table 1). Under the conditions used, PC12 cells displayed 85 to 90% NGF-induced neurite outgrowth, and 8 of 10 G418-resistant PC12

TABLE 1. NGF-induced morphological differentiation of M17 subclones

Subclone[a]	No. tested	No. showing indicated % differentiation[b]		
		>50%	20–50%	<20%
Z-M17	6	5	0	1
M-M17	8	1	1	6
MMTV-M17[c]				
Without Dex	13	6	4	3
With Dex	13	2	6	5
Controls[d]	10	8	2	0

[a] G418-resistant PC12 subclones were isolated after transfection with pZIPrasH(Asn-17) (Z-M17 subclones), pMTrasH(Asn-17) (M-M17 subclones), and pMMTVrasH(Asn-17) (MMTV-M17 subclones).

[b] The fraction of cells bearing processes longer than the cell diameter was determined for each subclone after 10 days of treatment with NGF (10 ng/ml).

[c] Dexamethasone (Dex, 0.5 μM) was added 24 h before NGF where indicated.

[d] Control subclones were isolated after transfection with pZIPneoSV(X) (five subclones), pZIPrasH (one subclone), pSV2neo (one subclone), or pSV2neo plus pMT64AA (three subclones).

subclones isolated after transfection with control plasmids responded comparably. In contrast, 19 of 27 M17 subclones showed significant resistance to NGF-induced neurite outgrowth. Notably, among those subclones in which the Ha-*ras* Asn-17 gene was expressed from the MMTV LTR, induction by dexamethasone reduced the extent of morphological differentiation in response to NGF (Table 1). These results indicate that, rather than affecting PC12 cell proliferation, the mutant Ha-*ras* gene inhibited NGF-induced neuronal differentiation.

Characterization of PC12 subclones transfected with Ha-*ras* Asn-17. Four M17 subclones showing less than 10% neurite outgrowth after NGF treatment were selected for further analysis. Z-M17-5 and MMTV-M17-21 were isolated after transfection of PC12 cells with pZIPrasH(Asn-17) and pMMTVrasH(Asn-17), respectively. M-M17-2 and M-M17-26 were obtained by cotransfection with pMTrasH(Asn-17) plus pSV2neo. Subclones Z-M17-5, M-M17-2, and M-M17-26 contained approximately 10 copies of the exogenous Ha-*ras* gene, whereas the MMTV-M17-21 subclone contained only one to two copies of the transfected gene, as judged by Southern blot hybridization (data not shown). Control subclones Z-1 and M-7 were isolated after transfection of PC12 cells with pZIPneoSV(X) and pMT64AA plus pSV2neo, respectively.

The expression of Ha-*ras* Asn-17 in the M17 subclones was studied by both Northern blot analysis of total cytoplasmic RNA (Fig. 2A and C) and Western blot analysis of protein extracts (Fig. 2B and D). Control subclones of PC12 cells transfected with vector plasmids alone (Z-1 and M-7) expressed levels of Ha-*ras* RNA comparable to those in PC12 cells (Fig. 2A). In contrast, increased levels of Ha-*ras* mRNA were detected in M17 subclones, in which the Ha-*ras* Asn-17 gene was under the control of either the M-MuLV LTR (Z-M17-5) or the metallothionein promoter (M-M17-2 and M-M17-26) (Fig. 2A). In addition, the levels of p21 expressed in these M17 subclones, as judged by Western blot analysis, were proportional to the levels of Ha-*ras* mRNA. The level of p21 in Z-M17-5 was only slightly higher than in control cells, while subclones M-M17-2 and especially M-M17-26 showed significantly increased p21 amounts (Fig. 2B).

The levels of mutant Ha-*ras* Asn-17 RNA and protein

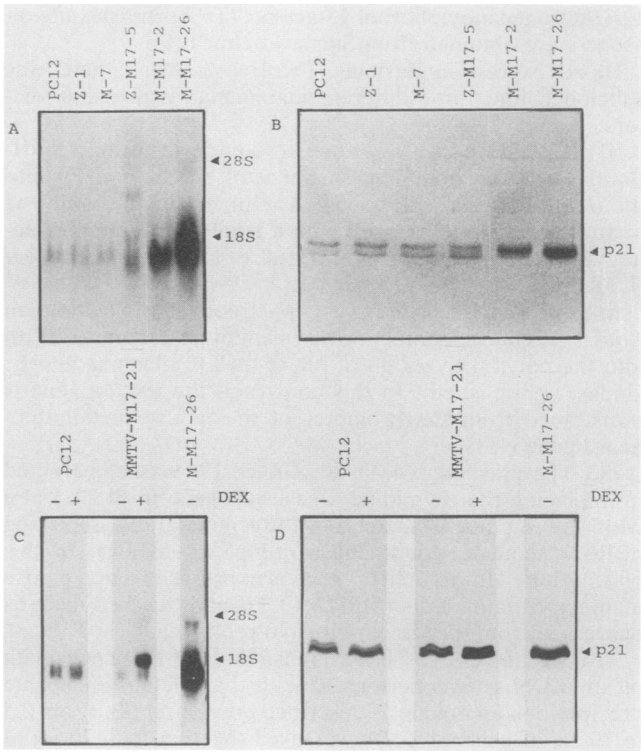

FIG. 2. Expression of Ha-*ras* Asn-17 in PC12 subclones. Control (PC12, Z-1, M-7) and Ha-*ras* Asn-17-transfected (Z-M17-5, M-M17-2, M-M17-26, MMTV-M17-21) cells were cultured and processed for Northern (A and C) and Western (B and D) blot analyses as described in Materials and Methods. For Northern blot analysis, lanes contained either 15 μg (A) or 8 μg (C) of total cytoplasmic RNA, and filters were probed with nick-translated Ha-*ras*. For Western blot analysis, lanes contained 100 μg of protein and blots were analyzed with anti-p21 monoclonal antibody ras-10. Where indicated, 0.5 μM dexamethasone (DEX) was added 24 h prior to preparation of samples. Ethidium bromide staining revealed comparable amounts of 28S and 18S rRNAs in each lane, except that the PC12 lanes in panel C contained about twice the amount of RNA as the MMTV-M17-21 and M-M17-26 lanes.

expressed from the metallothionein promoter in the M-M17-2 and M-M17-26 subclones (Fig. 2A and B) represented high levels of basal expression, which could not be further induced by exposure to zinc (data not shown). In contrast, expression of the Ha-*ras* Asn-17 gene was inducible by dexamethasone in MMTV-M17 subclones in which the gene was transcribed from the MMTV LTR. In these cells, expression of Ha-*ras* Asn-17 was induced by dexamethasone at the level of both RNA (Fig. 2C) and protein (Fig. 2D).

Ha-*ras* Asn-17 inhibits NGF- and FGF-induced neuronal differentiation of PC12 cells. The M17 subclones described above were selected for their reduced ability to grow neurites after 10 days of NGF treatment. In Fig. 3 we show a more detailed analysis of the morphological response of control (PC12, Z-1, and M-7) and M17 subclones (Z-M17-5, M-M17-2, and M-M17-26) to treatment with NGF and other agents known to induce neurite development in PC12 cells. Cells were seeded at a low cell density and treated for 10 days. Light micrographs were taken after 10 days of treatment (Fig. 3A), and the fraction of cells bearing processes was determined every other day (Fig. 3B). As expected, control cell lines extended long neurites in response to NGF

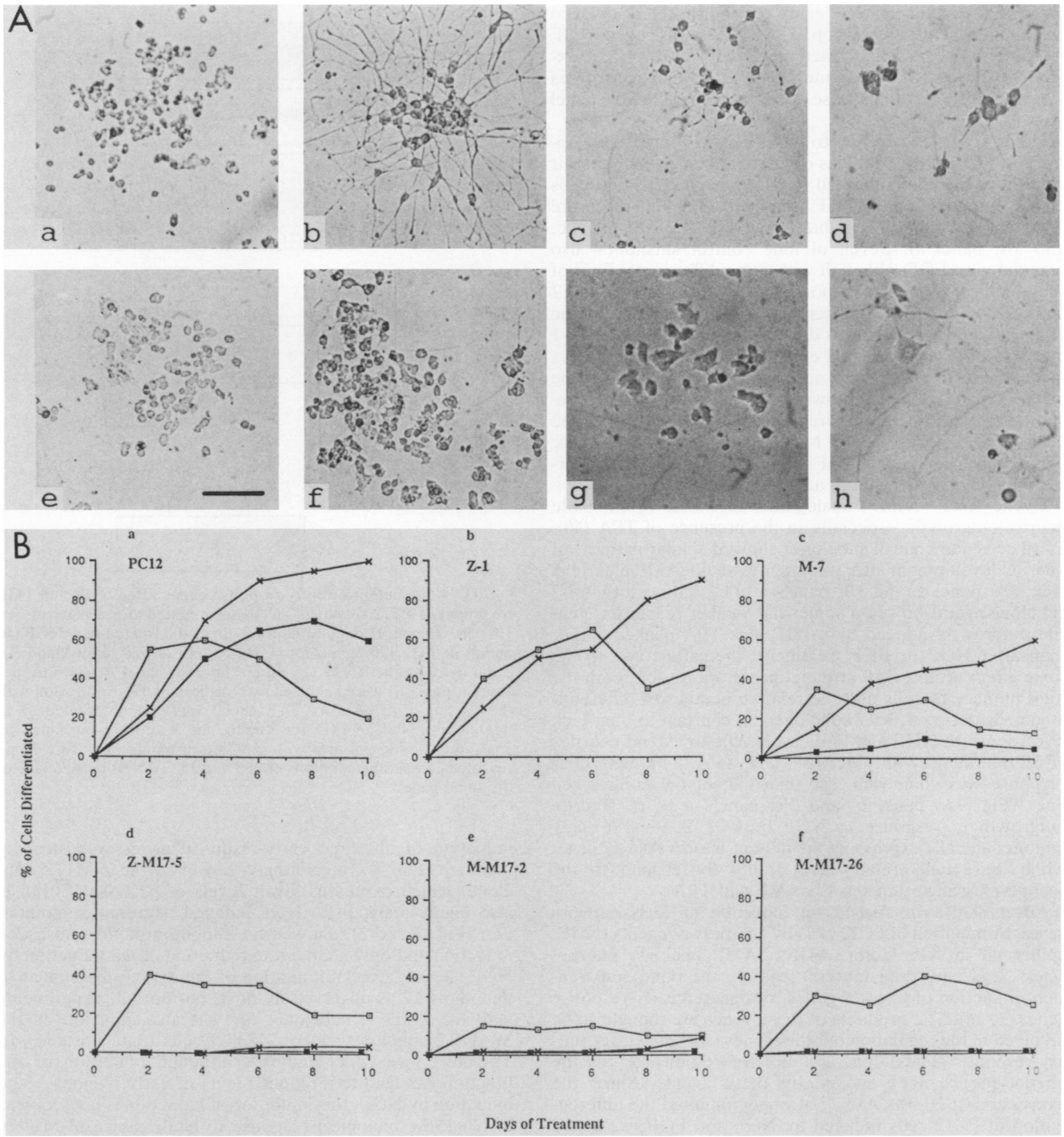

FIG. 3. Differentiation of control and Ha-*ras* Asn-17-expressing PC12 subclones. Cells were seeded in 24-well dishes at a density of 10^4 cells per well and treated with NGF (10 ng/ml), FGF (50 ng/ml), or dbcAMP (0.5 mM) plus TPA (20 nM) for 10 days. (A) PC12 (a to d) and M-M17-26 (e to h) cells were photographed after 10 days without treatment (a and e) or treatment with NGF (b and f), FGF (c and g), or dbcAMP plus TPA (d and h). Bar, 100 μm. (B) Approximately 200 cells were counted in each well every other day during treatment with NGF (×), FGF (■), or dbcAMP plus TPA (□). Cells were scored as differentiated if they carried processes longer than the diameter of the cells. Untreated cells displayed <1% differentiation for all subclones at any time point. The extent of differentiation of Z-1 cells in response to FGF is not shown, since these cells formed clusters after FGF treatment, making the extent of differentiation difficult to quantitate. However, after 10 days of FGF treatment, most (>80%) Z-1 cells had extended neurites.

treatment (Fig. 3A, panel b). After 8 to 10 days of treatment, >80% of PC12 and Z-1 cells and ~60% of M-7 cells had extended processes (Fig. 3B, panels a to c). In contrast, all three M17 subclones were highly resistant to NGF-induced morphological differentiation. Even after prolonged NGF treatment, only a small fraction of cells (<10%) had extended neurites (Fig. 3B, panels d to f), and these processes were much shorter than those of control cells (Fig. 3A, panel f).

Other agents in addition to NGF induce neurite development in PC12 cells, so it was of interest to see whether their activities were also inhibited by Ha-*ras* Asn-17. PC12 cells display processes after FGF treatment (42), but these are much shorter than those induced by NGF (Fig. 3A, panel c, and 3B, panel a). Seven of nine control subclones also responded to FGF, although with some differences. Four of these control subclones responded to FGF similarly to PC12 cells. Z-1 cells formed clusters after FGF treatment, so the extent of differentiation was difficult to quantitate. Most Z-1 cells (>80%), however, had extended neurites after 10 days of exposure to FGF (data not shown). M-7 cells and one other control subclone showed a significantly reduced response to FGF (~10% differentiation) compared with PC12 cells (Fig. 3B, panel c). All M17 subclones, however, were completely resistant to FGF-induced morphological differentiation (Fig. 3A, panel g, and 3B, panels d to f).

Cyclic AMP (cAMP) analogs are also able to stimulate neurite outgrowth, especially in the presence of TPA (40). PC12 cells and control subclones showed similar patterns of neurite development after treatment with dbcAMP plus TPA (Fig. 3A, panel d, and 3B, panels a to c), although the M-7 subclone again showed a somewhat weaker response. Neurite outgrowth induced by cAMP plus TPA peaked earlier than after NGF or FGF treatment; thereafter, due to the toxic effect of dbcAMP at the concentration used, both the total number of cells and the relative number of differentiated cells declined. M17 subclones, in contrast to their lack of response to NGF and FGF, were able to extend neurites after treatment with dbcAMP and TPA, although their response was somewhat less than that of the control cell lines (Fig. 3A, panel h, and 3B, panels d to f). Neurite outgrowth in response to NGF or FGF is thus strongly inhibited in cells expressing the mutant Ha-*ras* Asn-17 gene, while these cells retain their ability to respond to the second-messenger analogs dbcAMP and TPA.

Effect of Ha-*ras* Asn-17 on induction of early-response genes. Stimulation of PC12 cells by a variety of agents (NGF, epidermal growth factor, FGF, cAMP analogs, phorbol esters, and neurotransmitters) leads to the rapid and transient induction of a set of genes, designated early-response genes (3, 16). The products of these genes are thought to be involved in the regulation of other genes (designated secondary-response genes) that are directly responsible for the phenotypic changes evoked by these agents. Since the expression of Ha-*ras* Asn-17 strongly inhibited the differentiation of PC12 cells induced by NGF and FGF, we asked whether the induction of early- and secondary-response genes was similarly affected.

The early-response genes selected for these studies included the nuclear proto-oncogenes *fos* and *jun* as well as *zif268*, which encodes a protein with zinc finger DNA-binding domains (7). As expected (3), Northern blot analysis showed that all three genes were rapidly induced by NGF in PC12 cells and in the control Z-1 and M-7 subclones, peaking after 30 min of NGF treatment (Fig. 4). In contrast, in the highly expressing M17 subclone M-M17-26 (Fig. 2), the

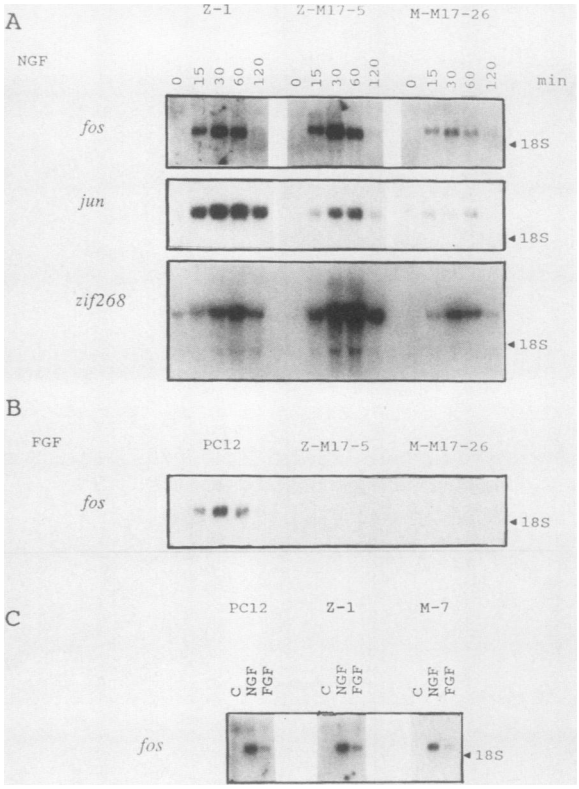

FIG. 4. Induction of early-response genes after NGF and FGF treatment of PC12 subclones. Cells were plated at a density of 3×10^6 per 100-mm plate, cultured for 3 days, and treated with NGF (50 ng/ml) or FGF (50 ng/ml) for 0, 15, 30, 60, or 120 min (A and B). Total cytoplasmic RNAs (15 μg per lane for panel A and 6 μg per lane for panel B) were analyzed by Northern blot hybridization with the *fos*, *jun*, or *zif268* probe. In panel C, cells were not treated (C) or treated with NGF or FGF for 30 min, and 5 μg RNA per lane was analyzed. Staining of gels with ethidium bromide prior to blotting revealed comparable amounts of 28S and 18S rRNAs in each lane of the same panel.

induction of all three early-response genes was strongly inhibited (Fig. 4). Interestingly, however, the Z-M17-5 subclone, which expresses lower levels of p21(Asn-17) but is also highly resistant to NGF-induced neuronal differentiation (Fig. 2 and 3), showed no inhibition of *fos* and *zif268* induction and only a moderate reduction of *jun* induction by NGF (Fig. 4). Partial inhibition of *jun* expression similar to that shown was observed in most, but not all, experiments with the Z-M17-5 subclone. *fos* was also inducible in the M-M17-2 subclone, as in Z-M17-5 cells (data not shown). Therefore, we concluded that although inhibition of *ras* function can lead to a reduced level of early-response gene induction by NGF, this inhibition of induction is not required to block the biological response to NGF. Since M-M17-26 cells expressed higher levels of p21(Asn-17) than Z-M17-5 or M-M17-2 cells (Fig. 2), it appears that a higher level of expression of the mutant protein is required to block NGF induction of these early-response genes than to block morphological differentiation.

FGF is also able to induce early-response genes (16) and induced *fos* in PC12 cells and control subclones (Fig. 4). However, induction of *fos* by FGF was virtually completely blocked in both Z-M17-5 and M-M17-26 subclones (Fig. 4). It therefore appeared that induction of *fos* by FGF was more

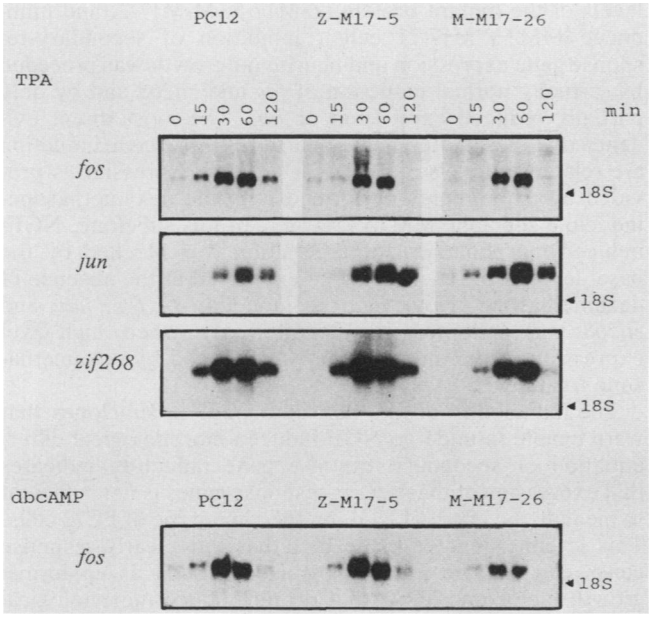

FIG. 5. Induction of early-response genes by TPA and dbcAMP. Cells were treated with TPA (200 nM) or dbcAMP (0.5 mM) and analyzed by Northern blot hybridization as described in the legend to Fig. 4. Lanes contained 6 μg of RNA from dbcAMP-treated cells and 7 μg of RNA from TPA-treated cells.

sensitive to inhibition by the mutant *ras* gene than induction by NGF.

Of the second messengers known to activate early-response genes, we tested the effect of cAMP and TPA in M17 subclones (Fig. 5). The cAMP derivative dbcAMP induced *fos* in all cell lines tested, although the level of induction in M-M17-26 was somewhat reduced compared with that in PC12 and Z-M17-5 (Fig. 5). Likewise, *fos*, *jun*, and *zif268* were readily induced by TPA, reaching their maximum levels after 30 to 60 min in PC12 cells as well as in both M17 subclones (Fig. 5). Even in the highly expressing M17 subclone M-M17-26, the levels of induction of all three early-response genes by TPA were comparable to those in control PC12 cells. This observation suggests that *ras* function is not required for the activation of early-response gene expression by protein kinase C. Furthermore, induction of early-response genes by TPA and dbcAMP is consistent with the biological response of PC12 cells to these agents: neither of these events was significantly affected by the expression of p21(Asn-17).

In order to rule out the possibility that the observed differences in the induction of early-response genes between different M17 subclones were due to clonal variation rather than to differences in levels of Ha-*ras* Asn-17 expression, we performed experiments with the MMTV-M17-21 cell line, in which the expression of the mutant gene is under control of the dexamethasone-inducible MMTV LTR (Fig. 2C and D). In control PC12 cells, dexamethasone did not alter the expression of the three early-response genes investigated (Fig. 6). In MMTV-M17-21, a subclone resistant to NGF- and FGF-induced neurite outgrowth in both the presence and absence of dexamethasone, *fos*, *jun*, and *zif268* were inducible by NGF, FGF, and TPA in the absence of dexamethasone. Pretreatment with dexamethasone, however, inhibited the induction of all three genes by NGF and FGF but did not affect induction by TPA. These results confirm that inhibition of NGF-induced early-response gene expression is dependent on the level of p21(Asn-17) and that TPA does not require *ras* function to induce these genes.

Effect of Ha-*ras* Asn-17 on induction of secondary-response genes. Unlike early-response genes, which can be induced by a wide array of agents in PC12 cells, the activation of secondary-response genes is more restricted; it is related to the neuronal phenotype, is specific for NGF or FGF treatment, and requires new protein synthesis (26, 27, 39). To analyze the possible involvement of *ras* proteins in the events leading to secondary-response gene induction, we studied the effect of Ha-*ras* Asn-17 on the NGF-induced activation of SCG10 and transin. SCG10, a gene coding for a small membrane-associated protein, is expressed in untreated PC12 cells, and the level of its mRNA is increased during the first 4 days of NGF treatment (39) (Fig. 7). SCG10 mRNA was also expressed in the M17 subclones; however, induction of this mRNA by NGF was inhibited in both Z-M17-5 and M-M17-26 cells (Fig. 7). Transin, a secreted metalloprotease, is synthesized from a 1.9-kb mRNA which is undetectable in untreated PC12 cells and is strongly induced by NGF treatment (27) (Fig. 7). In contrast, the induction of transin by NGF was completely blocked in both Z-M17-5 and M-M17-26 cells (Fig. 7). Similar results were obtained with five other M17 subclones tested, whereas Z-1

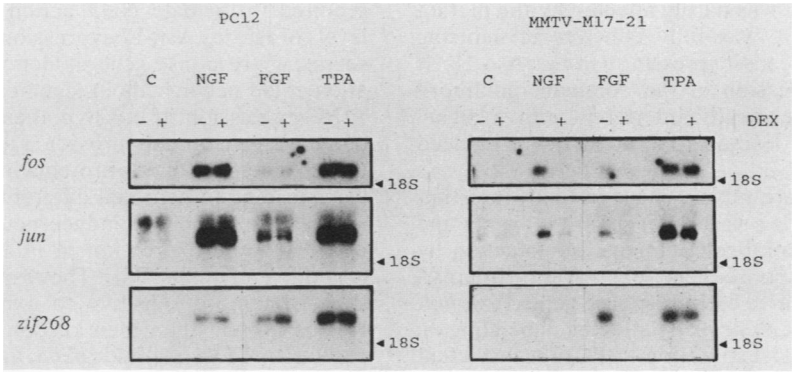

FIG. 6. Effect of inducible Ha-*ras* Asn-17 expression on early-response gene induction. PC12 and MMTV-M17-21 cells were cultured as described in the legend to Fig. 4 except that, where indicated, dexamethasone (DEX, 0.5 μM) was added to the medium 24 h before treatment with growth factors or TPA. Cells were untreated (C) or treated with NGF (50 ng/ml), FGF (50 ng/ml), or TPA (200 nM) for 30 min. RNAs (6 μg per lane) were analyzed by Northern blot hybridization as in Fig. 4.

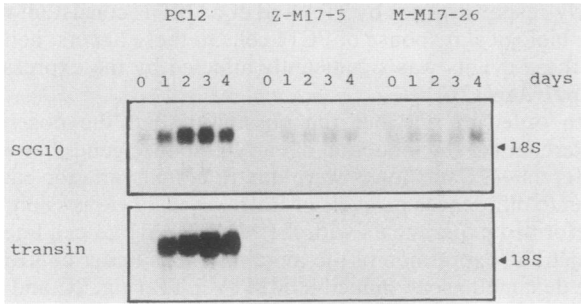

FIG. 7. Expression of secondary-response genes after NGF treatment. Cells were plated at a density of 2×10^6 per 100-mm dish. The following day, the medium was replaced by low-serum medium (0.5% horse serum in Temin modified Eagle medium). Cells were exposed to NGF (50 ng/ml) for 0, 1, 2, 3, or 4 days, and RNAs (12 μg per lane) were analyzed by Northern blot hybridization with the SCG10 and transin probes. Ethidium bromide staining revealed similar amounts of 28S and 18S rRNAs in each lane.

control cells were indistinguishable from PC12 cells (data not shown). The effect of Ha-*ras* Asn-17 on induction of secondary-response genes therefore seems to be related more to its effect on morphological differentiation than to induction of the early-response genes *fos*, *jun*, and *zif268*.

DISCUSSION

Previous studies have demonstrated that oncogenic *ras* proteins induce morphological and biochemical changes in PC12 cells that mimic the events of NGF-induced neuronal differentiation (2, 31, 35, 40). The possible involvement of normal *ras* proteins in NGF-induced differentiation of PC12 cells has also been suggested by experiments in which microinjection of an anti-p21 monoclonal antibody was found to block NGF-induced neurite outgrowth (21). In the present study, we used a dominant inhibitory *ras* mutant, Ha-*ras* Asn-17 (10), to further investigate the role of the *ras* proto-oncogene in this neuronal differentiation model.

In contrast to NIH 3T3 cells (10), we found that proliferation of PC12 cells was not inhibited by the expression of p21(Asn-17). Instead, stable transfectants of PC12 cells expressing Ha-*ras* Asn-17 were resistant to NGF- or FGF-induced morphological differentiation. Induction of early- and secondary-response genes by growth factors was also inhibited by the expression of Ha-*ras* Asn-17. In contrast, the effect of dbcAMP on neurite outgrowth and early-response gene expression was hardly affected by the mutant Ha-*ras* protein, and TPA was fully effective in inducing early-response genes in cells expressing Ha-*ras* Asn-17. It thus appeared that expression of the dominant inhibitory Ha-*ras* Asn-17 gene specifically interfered with a signal transduction pathway(s) leading to growth factor-induced differentiation.

Early-response genes are rapidly induced following stimulation of PC12 cells with a variety of different agents and are thought to play a central role in signal transduction by regulating secondary-response gene expression. Interestingly, the inducibility of three early-response genes (*fos*, *jun*, and *zif268*) by NGF was differentially affected depending on the level of Ha-*ras* Asn-17 expression in PC12 transfectant subclones. In subclones expressing high levels of p21(Asn-17)$^{Ha\text{-}ras}$ (M-M17-26 and dexamethasone-induced MMTV-M17-21 cells), NGF induction of *fos*, *jun*, and *zif268* was strongly inhibited. However, in subclones expressing lower levels of the mutant protein (Z-M17-5, M-M17-2, and uninduced MMTV-M17-21 cells), inhibition of secondary-response gene expression and neurite outgrowth was preceded by virtually normal induction of *fos* and *zif268* and by only partially reduced expression of *jun*. The most direct evidence that these differences in early-response gene induction are related to levels of p21(Asn-17)$^{Ha\text{-}ras}$ expression is provided by experiments performed with the dexamethasone-inducible subclone MMTV-M17-21. In this subclone, NGF-induced morphological differentiation was blocked by the basal level of p21(Asn-17)$^{Ha\text{-}ras}$ expressed in the absence of dexamethasone. However, the induction of *fos*, *jun*, and *zif268* was inhibited significantly only when high-level expression of the mutant gene was induced by dexamethasone treatment.

The inducibility of *fos*, *jun*, and *zif268* in subclones that were unable to undergo NGF-induced morphological differentiation or secondary-response gene induction indicates that expression of these early-response genes is not sufficient to mediate the effect of NGF on the phenotype of PC12 cells. This is consistent with the fact that these early-response genes can also be induced by agents, such as epidermal growth factor and TPA, that do not induce morphological differentiation (3, 16) and with experiments suggesting that induction of morphological differentiation by *ras* does not require induction of *fos* (20). Moreover, the inhibition of NGF-induced differentiation by low levels of p21(Asn-17)$^{Ha\text{-}ras}$ implies that *ras* proteins are involved in at least two different pathways of signal transduction from the NGF receptor. One pathway, which is blocked only by high levels of p21(Asn-17)$^{Ha\text{-}ras}$, is sufficient to lead to *fos*, *jun*, and *zif268* induction. A second signaling pathway, however, which is sensitive to inhibition by lower levels of the mutant protein, also appears to be required to elicit secondary-response gene expression and morphological differentiation. Similar results have been obtained in recent studies of the effect of Ha-*ras* Asn-17 expression on mitogenic stimulation of NIH 3T3 cells (5). In this case, a low level of Ha-*ras* Asn-17 expression was sufficient to block the mitogenic effect of epidermal growth factor without affecting induction of *fos* or *myc*, although *fos* induction was blocked by higher levels of the mutant protein.

The targets of this second Ha-*ras* Asn-17-sensitive signaling pathway in PC12 cells remain to be identified. However, it should be noted that the early-response gene family is estimated to include 50 to 100 genes (1), only three of which were analyzed in the present study. It is therefore possible that induction of a subset of early-response genes, which are required to mediate NGF action, may be blocked by low levels of Ha-*ras* Asn-17 expression. In this case, inhibition of secondary-response gene induction and neuronal differentiation could occur without significant changes in *fos*, *jun*, or *zif268* expression. If this hypothesis is correct, identification of such a gene(s) may provide a new approach to studies of the involvement of *ras* proteins in signal transduction pathways leading to neuronal differentiation.

FGF is also able to induce neurite outgrowth and early-response gene expression in PC12 cells (16, 42), although less efficiently than NGF. The response of PC12 cells to FGF was even more sensitive to expression of the inhibitory Ha-*ras* protein than their response to NGF. In particular, induction of *fos*, *jun*, and *zif268*, as well as neurite extension, were strongly inhibited even in subclones expressing relatively low levels of p21(Asn-17)$^{Ha\text{-}ras}$. Comparison with the NGF inducibility of early-response genes in these subclones suggests that the effects of FGF may be mediated primarily,

or perhaps solely, by a signal transduction pathway that is sensitive to inhibition by low levels of the mutant protein.

The second-messenger analog dbcAMP effectively induced *fos* in PC12 subclones with either low or high levels of p21(Asn-17)$^{Ha\text{-}ras}$ expression, though *fos* induction by dbcAMP was somewhat reduced in M-M17-26 cells. Similarly, dbcAMP plus TPA induced morphological differentiation of all Ha-*ras* Asn-17-expressing subclones, although this response was also somewhat reduced compared with PC12 cells. It therefore appears that *ras* function is not required for signal transduction mediated by cAMP, although there may be some inhibitory effect of high-level expression of the Ha-*ras* Asn-17 gene.

In several systems, *ras* proteins appear to affect the metabolism of phosphatidylinositides and/or diacylglycerol (12, 22, 24, 32, 34, 43, 44). The interaction between *ras* proteins and protein kinase C, however, is a subject of controversy. On the one hand, inhibition of *ras* function by a monoclonal antibody (45) or by Ha-*ras* Asn-17 expression (5) has been found to abolish the mitogenic effect of TPA in NIH 3T3 cells, indicating that *ras* function is required to mediate stimulation of DNA synthesis by protein kinase C. In other studies, however, downregulation or inhibition of protein kinase C has been found to block the mitogenic effect of *ras* (14, 23, 29). The present experiments indicate that the inhibitory effect of Ha-*ras* Asn-17 on *fos*, *jun*, and *zif268* induction in PC12 cells can be bypassed by treatment with TPA. All three genes were induced by TPA in all subclones tested at a level comparable to control cells. This observation indicates that *ras* function is not required for gene activation by protein kinase C in PC12 cells, suggesting that *ras* either acts upstream from protein kinase C or can induce early-response genes via an independent pathway. Conversely, recent studies with PC12 cells in which protein kinase C had been downregulated indicate that functional protein kinase C is not required for induction of *fos* by either NGF or oncogenic *ras* protein (13). Together, these observations clearly favor the notion that both *ras* and protein kinase C can independently induce *fos* expression.

ACKNOWLEDGMENTS

We are grateful to J. Wagner for PC12 cells and to D. J. Anderson, C. Dionne, M. Greenberg, L. Gudas, L. Matrisian, and K. Yamamoto for various plasmids used in these experiments.

This research was supported by Public Health Service grant RO1 CA18689 from the National Institutes of Health.

LITERATURE CITED

1. **Almendral, J. M., D. Sommer, H. Macdonald-Bravo, J. Burckhardt, J. Perera, and R. Bravo.** 1988. Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol. Cell. Biol. **8**:2140–2148.
2. **Bar-Sagi, D., and J. R. Feramisco.** 1985. Microinjection of the *ras* oncogene protein into PC12 cells induces morphological differentiation. Cell **42**:841–848.
3. **Bartel, D. P., M. Sheng, L. F. Lau, and M. E. Greenberg.** 1989. Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of *fos* and *jun* induction. Genes Dev. **3**:304–313.
4. **Bohmann, D., T. J. Bos, A. Admon, T. Nishimura, P. K. Vogt, and R. Tjian.** 1987. Human proto-oncogene c-*jun* encodes a DNA-binding protein with structural and functional properties of transcription factor AP-1. Science **238**:1386–1392.
5. **Cai, H., J. Szeberényi, and G. M. Cooper.** 1990. Effect of a dominant inhibitory Ha-*ras* mutation on mitogenic signal transduction in NIH 3T3 cells. Mol. Cell. Biol. **10**:5314–5323.
6. **Carney, W. P., P. J. Hamer, J. LaVecchio, S. Ng, D. Petit, T. G. Pullano, and K. L. Trimpe.** 1989. Monoclonal antibodies for detection of normal and oncogenic *ras* p21, p. 53–62. *In* R. S. Metzgar and M. S. Mitchell (ed.), Human tumor antigens and specific tumor therapy. Alan R. Liss, Inc., New York.
7. **Christy, B. A., L. F. Lau, and D. Nathans.** 1988. A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc. Natl. Acad. Sci. USA **85**:7857–7861.
8. **Copeland, N. G., and G. M. Cooper.** 1979. Transfection by exogenous and endogenous murine retrovirus DNAs. Cell **16**: 347–356.
9. **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.
10. **Feig, L. A., and G. M. Cooper.** 1988. Inhibition of NIH 3T3 cell proliferation by a mutant *ras* protein with preferential affinity for GDP. Mol. Cell. Biol. **8**:3235–3243.
11. **Feramisco, J. R., M. Gross, T. Kamata, M. Rosenberg, and R. W. Sweet.** 1984. Microinjection of the oncogene form of the human H-*ras* (T-24) protein results in rapid proliferation of quiescent cells. Cell **38**:109–117.
12. **Fleischman, L. F., S. B. Chahwala, and L. Cantley.** 1986. *ras*-Transformed cells: altered levels of phosphatidylinositol 4,5-bisphosphate and catabolites. Science **232**:407–410.
13. **Fukumoto, Y., K. Kaibuchi, N. Oku, Y. Hori, and Y. Takai.** 1990. Activation of the c-*fos* serum-response element by the activated c-Ha-*ras* protein in a manner independent of protein kinase C and cAMP-dependent protein kinase. J. Biol. Chem. **265**:774–780.
14. **Gauthier-Rouviere, C., A. Fernandez, and N. J. C. Lamb.** 1990. *ras*-Induced c-*fos* expression and proliferation in living rat fibroblasts involves c-kinase activation and the serum response element pathway. EMBO J. **9**:171–180.
15. **Glanville, N., D. M. Durnam, and R. D. Palmiter.** 1981. Structure of mouse metallothionein I gene and its mRNA. Nature (London) **292**:267–269.
16. **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.
17. **Greene, L. A.** 1984. The importance of both early and delayed responses in the biological actions of nerve growth factor. Trends Neurosci. **7**:91–94.
18. **Greene, L. A., and J. C. McGuire.** 1978. Induction of ornithine decarboxylase by nerve growth factor dissociated from effects on survival and neurite outgrowth. Nature (London) **276**:191–194.
19. **Greene, L. A., and A. S. Tischler.** 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA **73**:2424–2428.
20. **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.
21. **Hagag, N., S. Halegoua, and M. Viola.** 1986. Inhibition of growth factor-induced differentiation of PC12 cells by microinjection of antibody to *ras* p21. Nature (London) **319**:680–682.
22. **Lacal, J. C., P. De La Pena, J. Moscat, P. Garcia-Barreno, P. S. Anderson, and S. A. Aaronson.** 1987. Rapid stimulation of diacylglycerol production in *Xenopus* oocytes by microinjection of H-*ras* p21. Science **238**:533–536.
23. **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.
24. **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**:269–272.
25. **Lau, L. F., and D. Nathans.** 1985. Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. EMBO J. **4**:3145–3151.
26. **Lindenbaum, M. H., S. Carbonetto, F. Grosveld, D. Flavell, and**

W. E. Mushynski. 1988. Transcriptional and post-transcriptional effects of nerve growth factor on expression of the three neurofilament subunits in PC12 cells. J. Biol. Chem. **263**:5662–5667.

27. **Machida, C. M., K. D. Rodland, L. Matrisian, B. E. Magun, and G. Ciment.** 1989. NGF induction of the gene encoding the protease transin accompanies neuronal differentiation in PC12 cells. Neuron **2**:1587–1596.
28. **Matrisian, L. M., N. Glaichenhaus, H. C. Gesnel, and R. Breathnach.** 1985. Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. EMBO J. **4**:1435–1440.
29. **Morris, J. D. H., B. Price, A. C. Lloyd, A. J. Self, C. J. Marshall, and A. Hall.** 1989. Scrape-loading of Swiss 3T3 cells with *ras* protein rapidly activates protein kinase C in the absence of phosphoinositide hydrolysis. Oncogene **4**:27–31.
30. **Mulcahy, L. S., M. R. Smith, and D. W. Stacey.** 1985. Requirement for *ras* proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature (London) **313**:241–243.
31. **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.
32. **Pan, B.-T., and G. M. Cooper.** 1990. Role of phosphatidylinositol metabolism in *ras*-induced *Xenopus* oocyte maturation. Mol. Cell. Biol. **10**:923–929.
33. **Powers, S., K. O'Neill, and M. Wigler.** 1989. Dominant yeast and mammalian *RAS* mutants that interfere with the *CDC25*-dependent activation of wild-type *RAS* in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9**:390–395.
34. **Preiss, J., C. R. Loumis, W. R. Bishop, R. Stein, J. E. Niedel, and R. M. Bell.** 1986. Quantitative measurement of *sn*-1,2-diacylglycerols present in platelets, hepatocytes, and *ras*- and *sis*-transformed normal rat kidney cells. J. Biol. Chem. **261**: 8597–8600.
35. **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.
36. **Sigal, I. S., J. B. Gibbs, J. S. D'Alonzo, G. L. Temeles, B. S. Wolanski, S. H. Socher, and E. M. Scolnick.** 1986. Mutant *ras*-encoded proteins with altered nucleotide binding exert dominant biological effects. Proc. Natl. Acad. Sci. USA **83**:952–956.
37. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
38. **Stacey, D. W., and H. F. Kung.** 1984. Transformation of NIH 3T3 cells by microinjection of Ha-*ras* p21 protein. Nature (London) **310**:508–511.
39. **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.
40. **Sugimoto, Y., M. Noda, H. Kitayama, and Y. Ikawa.** 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.
41. **Toda, T., I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broek, S. Cameron, J. Broach, K. Matsumoto, and M. Wigler.** 1985. In yeast, *ras* proteins are controlling elements of adenylate cyclase. Cell **40**:27–36.
42. **Togari, A., G. Dickens, H. Kuzuya, and G. Guroff.** 1985. The effect of fibroblast growth factor on PC12 cells. J. Neurosci. **5**:307–316.
43. **Wakelam, M. J. O., S. A. Davies, M. D. Houslay, I. McKay, C. J. Marshall, and A. Hall.** 1986. Normal $p21^{N\text{-}ras}$ couples bombesin and other growth factor receptors to inositol phosphate production. Nature (London) **323**:173–176.
44. **Wolfman, A., and I. G. Macara.** 1987. Elevated levels of diacylglycerol and decreased phorbol ester sensitivity in *ras*-transformed fibroblasts. Nature (London) **325**:359–361.
45. **Yu, C.-L., M.-H. Tsai, and D. W. Stacey.** 1988. Cellular *ras* activity and phospholipid metabolism. Cell **52**:63–71.
46. **Zaret, K. S., and K. R. Yamamoto.** 1984. Reversible and persistent changes in chromatin structure accompany activation of a glucocorticoid-dependent enhancer element. Cell **38**:29–38.