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Effect of a Dominant Inhibitory Ha-*ras* Mutation on Mitogenic Signal Transduction in NIH 3T3 Cells

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We used a dominant inhibitory mutation of c-Ha-*ras* which changes Ser-17 to Asn-17 in the gene product p21 [p21(Asn-17)$^{Ha\text{-}ras}$] to investigate *ras* function in mitogenic signal transduction. An NIH 3T3 cell line [NIH(M17)] was isolated that displayed inducible expression of the mutant Ha-*ras* gene (Ha-*ras* Asn-17) via the mouse mammary tumor virus long terminal repeat and was growth inhibited by dexamethasone. The effect of dexamethasone induction on response of quiescent NIH(M17) cells to mitogens was then analyzed. Stimulation of DNA synthesis by epidermal growth factor (EGF) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was completely blocked by p21(Asn-17) expression, and stimulation by serum, fibroblast growth factor, and platelet-derived growth factor was partially inhibited. However, the induction of *fos*, *jun*, and *myc* by EGF and TPA was not significantly inhibited in this cell line. An effect of p21(Asn-17) on *fos* induction was, however, demonstrated in transient expression assays in which quiescent NIH 3T3 cells were cotransfected with a *fos-cat* receptor plasmid plus a Ha-*ras* Asn-17 expression vector. In this assay, p21(Asn-17) inhibited chloramphenicol acetyltransferase expression induced by EGF and other growth factors. In contrast to its effect on DNA synthesis, however, Ha-*ras* Asn-17 expression did not inhibit *fos-cat* expression induced by TPA. Conversely, downregulation of protein kinase C did not inhibit *fos-cat* induction by activated *ras* or other oncogenes. These results suggest that *ras* proteins are involved in at least two parallel mitogenic signal transduction pathways, one of which is independent of protein kinase C. Although either pathway alone appears to be sufficient to induce *fos*, both appear to be necessary to induce the full mitogenic response.

Members of the *ras* gene family are frequently activated as oncogenes in a wide array of malignancies. The proteins encoded by *ras* genes (p21s) bind guanine nucleotides, possess an intrinsic GTPase activity, and are associated with the plasma membrane. The similarity of these properties of the *ras* gene products to those of the G proteins suggests that *ras* proteins are analogously involved in intracellular signal transduction processes.

In addition to the ability of mutationally activated *ras* oncogenes to induce neoplastic transformation, the mitogenic activity of *ras* gene products has been demonstrated by the ability of microinjected *ras* oncogene proteins to induce proliferation and transient morphological transformation of quiescent fibroblasts (6, 27). Moreover, the *ras* proto-oncogene proteins appear to play a role in regulating proliferation of at least some normal cell types. Microinjection of anti-Ras antibody has been found to block the mitogenic response of NIH 3T3 cells to serum stimulation (19). Likewise, interference with *ras* proto-oncogene function by expression of a dominant inhibitory mutation in c-Ha-*ras* which changes Ser-17 to Asn-17 in the gene product (the Ha-*ras* Asn-17 mutation) inhibits the proliferation of NIH 3T3 cells (5). In both cases, the growth-inhibitory effect of interfering with normal *ras* function can be bypassed by transformation with the *raf* and *mos* oncogenes, which encode protein-serine/threonine kinases, but not by transformation with *src* or other protein-tyrosine kinase oncogenes (5, 25).

In *Saccharomyces cerevisiae*, *RAS* proteins are also required for growth, acting to stimulate the activity of adenylate cyclase (30). However, no effect of *ras* proteins on adenylate cyclase has been found in vertebrate cells (1). A number of studies with both mammalian cells and *Xenopus laevis* oocytes have suggested that *ras* proteins function by regulating the metabolism of phospholipid-derived second messengers, particularly inositol phosphates and diacylglycerol (7, 14, 16, 18, 20, 22, 31, 35). However, these effects have not been shown to be mediated directly by the *ras* proteins. In addition, the functional relationship between the activities of *ras* and protein kinase C is unclear. In several studies, inhibition of protein kinase C has been found to block the ability of *ras* proteins to induce cell proliferation, consistent with the possibility that *ras* acts to stimulate production of a second messenger (e.g., diacylglycerol) that activates protein kinase C (11, 15, 18). On the other hand, the mitogenic effect of stimulating protein kinase C directly with phorbol esters has been reported to be inhibited by microinjection of anti-Ras antibody, suggesting that *ras* function is required for protein kinase C-mediated mitogenesis (36). Thus, some indications suggest that p21 may affect phospholipid metabolism, but the role of p21 in these second-messenger systems requires further definition.

In the present study, we used the dominant inhibitory Ha-*ras* Asn-17 mutation (5) to further investigate the role of *ras* proto-oncogene proteins in the response of NIH 3T3 cells to mitogenic growth factors. *RAS* genes with similar mutations have also been found to inhibit *RAS* function in *S. cerevisiae*, apparently by blocking the interaction of *RAS* with its upstream regulator *CDC25* (21). Our results indicate that *ras* function is required for induction of mitogenesis either by growth factors or by direct stimulation of protein kinase C with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Inhibition of *ras* function also inhibited *fos* induction by growth factors but did not inhibit *fos* induction by TPA. Conversely, downregulation of protein kinase C did not inhibit *fos* induction by an activated *ras* oncogene. These

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findings suggest the involvement of *ras* proto-oncogenes in at least two parallel pathways of mitogenic signal transduction, one of which may involve activation of protein kinase C and one of which appears to be protein kinase C independent. Although either pathway alone appears to be sufficient to induce *fos* expression, the activity of both pathways appears to be necessary to induce the full mitogenic response.

MATERIALS AND METHODS

Plasmid DNAs. The Ha-*ras* Asn-17 gene was expressed via two different vectors. pZIP M17 contains Ha-*ras* Asn-17 inserted into the *Bam*HI site of pZIPneoSV(X) so that the mutant *ras* gene is coexpressed with Neo^r from the Moloney murine leukemia virus long terminal repeat (LTR) (5). pMMTVrasH(Asn-17) is an expression plasmid in which the Ha-*ras* Asn-17 gene is under control of a mouse mammary tumor virus (MMTV) LTR and is inducible by dexamethasone, while a Neo^r gene in the same plasmid is constitutively expressed from the simian virus 40 (SV40) early promoter. It was constructed from pLTL3 (37) by replacing the thymidine kinase (*tk*) gene with Ha-*ras* and replacing the pBR322 sequences with pSV2neo (26).

Expression of bacterial chloramphenicol acetyltransferase (CAT) was assayed in transient expression experiments. The *fos-cat* plasmid pFC700 is a pUC19 construct that contains the *cat* gene under control of the *fos* −700 to +42 promoter region, including the dyad symmetry element between −317 and −298, followed by an SV40 polyadenylation site (23). pSV2CAT is a construct in which the *cat* gene is expressed via the SV40 early promoter (12).

The v-Ha-*ras* gene of Harvey sarcoma virus was expressed in pZIPneoSV(X) (5). Other oncogenes were expressed from retroviral LTRs in previously described plasmids: the v-*src* gene of Rous sarcoma virus in pSrc11 (24), the v-*mos* gene of Moloney sarcoma virus in pHT25 (2), and the human c-*raf* gene activated by amino-terminal deletion in pLTRraf24G (29).

Transfection assays. NIH 3T3 cells were transfected with plasmid DNAs together with 20 μg of carrier NIH 3T3 DNA as described before (4). For isolation of stable transformants, cells were subcultured 3 days after transfection into medium containing G418 (400 μg/ml), and drug-resistant colonies were isolated.

Northern (RNA) blot analysis. Total cytoplasmic RNA was isolated as described before (5). RNA (15 μg) was electrophoresed in 1% agarose–formaldehyde gels, transferred to Gene Screen Plus membranes (NEN Research Products), and hybridized with nick-translated DNA fragments. Hybridization and washing conditions were those recommended by the manufacturer. The DNA fragments used as probes for *ras*, *fos*, *myc*, and *jun* were prepared as described before (34).

Western immunoblot analysis. Protein samples (200 μg) were first immunoprecipitated with anti-Ras monoclonal antibody YA6-259 (10). The immunoprecipitated proteins were then electrophoresed in 7.5 to 15% sodium dodecyl sulfate (SDS)–polyacrylamide gels, transferred to nitrocellulose filters, and analyzed with anti-Ras monoclonal antibody ras-10 (3). Blots were developed by using goat anti-mouse immunoglobulin G horseradish peroxidase conjugate (Bio-Rad Laboratories).

Mitogen-stimulated DNA synthesis. Cells (2×10^5) were plated in 60-mm dishes in medium containing 10% calf serum. The next day, the medium was changed to 0.5% calf serum, and cells were incubated for 24 h to induce quiescence. Where indicated, 5×10^{-7} M dexamethasone was then added, and the cells were stimulated by addition of serum or growth factors 8 h later. DNA synthesis was assayed 16 h after growth factor addition by labeling with [^{3}H]thymidine (5 μCi per plate, 6.7 Ci/mmol; New England Nuclear) for 3 h. The cells were then washed three times with phosphate-buffered saline, and 2 ml of 0.2 N NaOH plus calf thymus carrier DNA (40 μg/ml) was added to each plate. Samples were collected by filtration through Whatman GF/C glass fiber filters and washed with 10% trichloroacetic acid. [^{3}H]thymidine incorporation was determined by scintillation counting.

CAT transient expression assays. NIH 3T3 cells (1×10^6 cells per 60-mm plate) were transfected with plasmid DNAs plus 20 μg of carrier NIH 3T3 DNA in medium containing 10% calf serum. Immediately after transfection, the medium was changed to 0.5% calf serum, and cells were incubated for 48 h. Serum, growth factors, or TPA was then added as indicated in individual experiments, and the cells were incubated for an additional 4 h. The cells were then trypsinized and collected by centrifugation. The cell pellet was suspended in 50 μl of 100 mM Tris hydrochloride (pH 7.6), and the cells were lysed by four cycles of freezing and thawing. Debris was removed by centrifugation, and 25 μl of the extract was assayed for CAT activity as described before (12). The samples were then extracted with 1 ml of ethyl acetate, dried under nitrogen, and analyzed by thin-layer chromatography with 95% chloroform–5% methanol (12).

RESULTS

Isolation of a cell line with inducible Ha-*ras* Asn-17 expression. In previous studies, cotransfection assays were used to demonstrate that expression of Ha-*ras* Asn-17 was inhibitory to proliferation of NIH 3T3 cells, apparently as a consequence of interfering with normal *ras* proto-oncogene function (5). In order to undertake a more detailed analysis of the effect of this dominant inhibitory *ras* mutant, we sought to isolate stably transformed cells in which Ha-*ras* Asn-17 expression was controlled by an inducible promoter. For this purpose, NIH 3T3 cells were transfected with a construct [pMMTVrasH(Asn-17)] in which Ha-*ras* Asn-17 is expressed via the glucocorticoid-regulated MMTV LTR and a Neo^r gene is constitutively expressed from the SV40 early promoter. Results of a representative experiment in which transfected cells were assayed for G418 resistance in the presence or absence of dexamethasone are illustrated in Fig. 1.

Cells transfected with the pMMTVrasH(Asn-17) construct yielded approximately fivefold more G418-resistant transformants in the absence of dexamethasone (compare Fig. 1C and D), consistent with the growth-inhibitory activity of p21(Asn-17)$^{Ha\text{-}ras}$. In contrast, cells transfected with a normal Ha-*ras* gene in the pMMTVrasH vector yielded similar numbers of G418-resistant transformants in either the presence (Fig. 1B) or absence (Fig. 1A) of dexamethasone. Since dexamethasone induced high-level expression of the normal Ha-*ras* gene, most colonies transfected with this construct displayed the transformed phenotype in the presence of dexamethasone and were consequently stained more darkly.

In order to isolate NIH 3T3 transformants with inducible Ha-*ras* Asn-17 expression, G418-resistant colonies were picked from cultures that had been transfected with pMMTVrasH(Asn-17) and selected for G418 resistance in the absence of dexamethasone. Over 100 such transformants

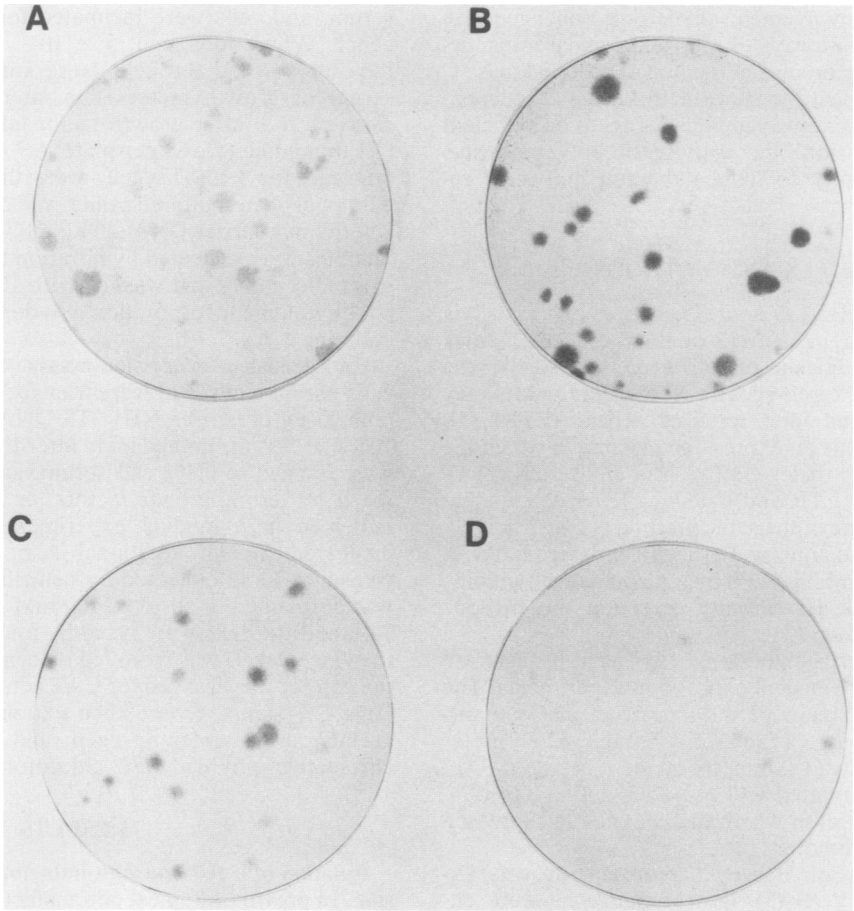

FIG. 1. Transfection of NIH 3T3 cells with pMMTVrasH(Asn-17). NIH 3T3 cells were transfected with 0.1 μg of pMMTVrasH (A and B) or pMMTVrasH(Asn-17) (C and D). Three days after transfection, cells were subcultured into medium containing G418 either with (B and D) or without (A and C) 5×10^{-7} M dexamethasone. Colonies were stained and photographed 2 weeks after transfection.

were screened for growth with and without dexamethasone. The majority were unaffected by dexamethasone, but five showed reduced proliferation in the presence of dexamethasone. One subclone designated NIH(M17), which displayed the strongest growth inhibition by dexamethasone, was selected for further studies.

The effect of dexamethasone on proliferation of NIH(M17) cells is shown in Fig. 2. Dexamethasone did not affect proliferation of NIH 3T3 cells. In the absence of dexamethasone, the growth rate of NIH(M17) cells was similar to that of NIH 3T3 cells. However, induction of Ha-*ras* Asn-17 by addition of dexamethasone significantly inhibited NIH(M17) proliferation.

To determine whether the inhibitory effect of Ha-*ras* Asn-17 expression was reversible, cultures of 5×10^5 NIH(M17) cells were maintained in the presence or absence of dexamethasone for 48 h. Cell viability was then determined by plating 100 and 1,000 cells in medium without dexamethasone. Similar efficiencies of colony formation (~20 to 25%) were obtained for both dexamethasone-treated and untreated cultures (data not shown), indicating that inhibition of cell proliferation by p21(Asn-17) is not cytocidal.

Expression of Ha-*ras* Asn-17 in NIH(M17) cells was analyzed by both Northern and Western blotting (Fig. 3). These experiments indicated that *ras* expression at both the RNA and protein levels was inducible by dexamethasone in NIH(M17) cells as well as in NIH 3T3 cells that had been transfected by a control pMMTVrasH plasmid containing normal Ha-*ras* [NIH(c-rasH) cells] (Fig. 3). Interestingly, the level of expression of p21 was at least 10-fold lower in NIH(M17) cells than in NIH(c-rasH) cells. This result, together with the rarity of pMMTVrasH(Asn-17) transformants that displayed dexamethasone-inhibited proliferation (see above), suggests that the level of Ha-*ras* Asn-17 expression was critical to obtaining a cell line with the dexamethasone-inhibited phenotype displayed by NIH(M17) cells. Since addition of dexamethasone results in only about a 10- to 20-fold increase in gene expression, it is likely that cell lines expressing much higher levels of Ha-*ras* Asn-17 would be growth-inhibited even in the absence of dexamethasone, whereas cell lines expressing significantly lower levels might not produce sufficient amounts of the mutant protein to effectively interfere with normal *ras* function.

Mitogenic response of NIH(M17) cells to growth factors and TPA. Previous studies indicated that microinjection of anti-p21 antibodies blocked the mitogenic response of quiescent NIH 3T3 cells, suggesting that *ras* is involved in regulation of cell proliferation at the G0/G1 transition (19). We therefore examined the effect of Ha-*ras* Asn-17 expression on the response of quiescent NIH(M17) cells to mitogens. NIH(M17) cells and normal NIH 3T3 cells were cultured in medium containing 10% calf serum without dexamethasone. The cells were arrested in G0 by incubation in medium

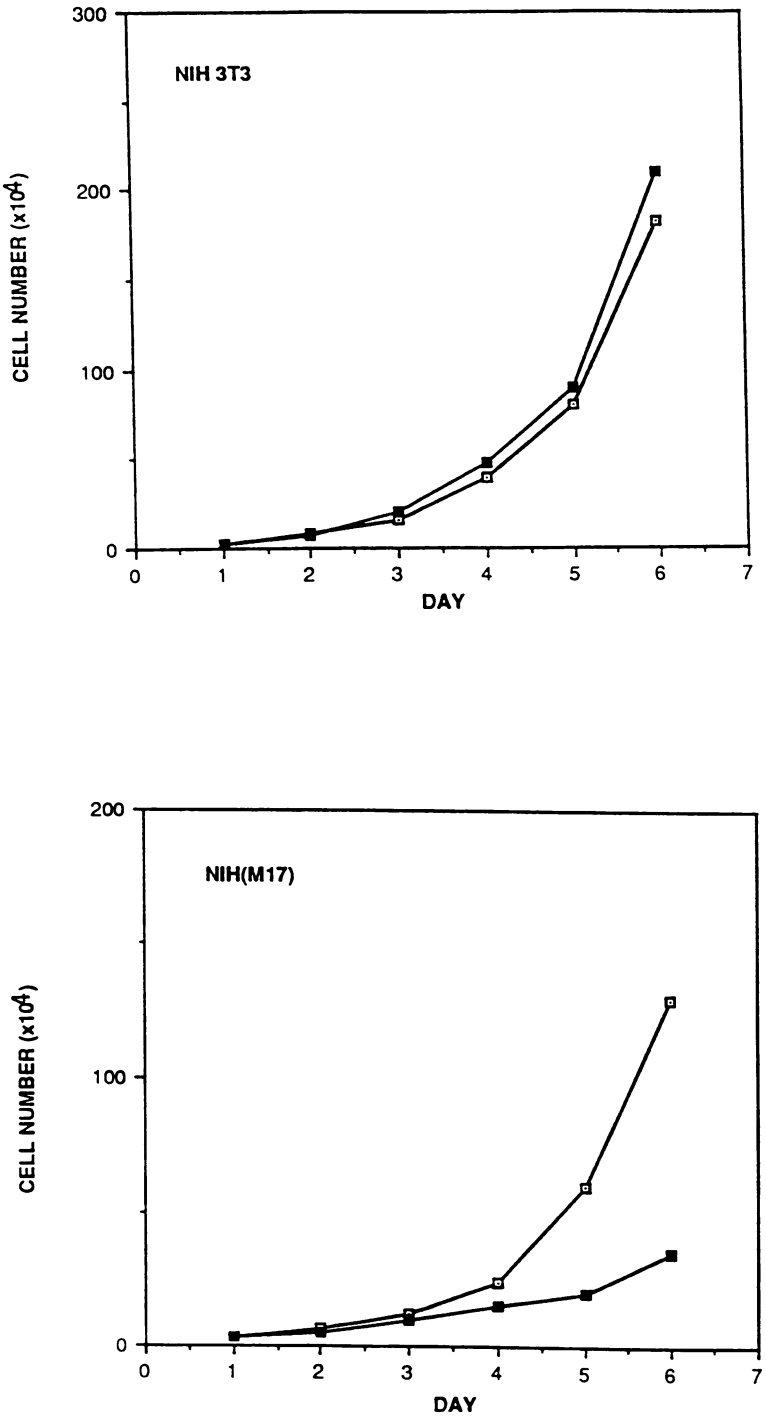

FIG. 2. Growth of NIH(M17) cells. NIH 3T3 or NIH(M17) cells (10^4) were plated in 60-mm dishes in medium containing 10% calf serum (day 0). Cells were cultured either with (■) or without (□) 5×10^{-7} M dexamethasone added on day 1. Cell numbers were determined by counting cells in duplicate plates.

containing 0.5% calf serum for 24 h. The cells were then incubated for an additional 8 h in either the presence or absence of dexamethasone and stimulated by the addition of either 10% calf serum or individual growth factors. Mitogenic response was determined by assaying the entry of cells into S phase, monitored by [^{3}H]thymidine incorporation 16 h after growth factor addition. Control experiments showed that [^{3}H]thymidine incorporation increased by 12 h after addition of serum or growth factors, with maximum incorporation at 16 h after mitogenic stimulation (data not shown).

[^{3}H]thymidine incorporation was stimulated approximately 10- to 20-fold by addition of serum, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), or epidermal growth factor (EGF) to quiescent NIH 3T3 cells in either the presence or absence of dexamethasone (Table 1). A similar mitogenic response was observed for NIH(M17)

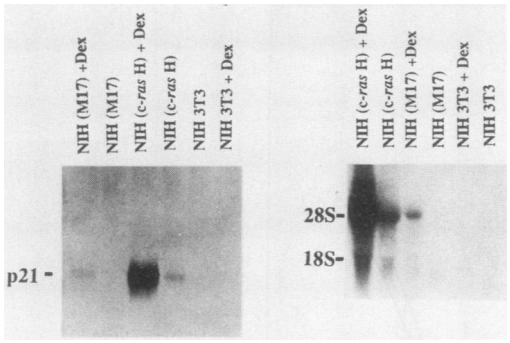

FIG. 3. Expression of Ha-*ras* Asn-17 in NIH(M17) cells. NIH 3T3 cells, NIH(M17) cells, and NIH 3T3 cells transfected with the pMMTVrasH vector containing a normal c-Ha-*ras* gene [NIH(c-rasH) cells] were analyzed for *ras* expression by Northern and Western blotting. Dexamethasone (5×10^{-7} M) was added 24 h prior to sample preparation as indicated (+Dex). For Northern blot analysis, 15 μg of total cytoplasmic RNA was electrophoresed in each lane, and filters were hybridized with nick-translated Ha-*ras* probe. Ethidium bromide staining revealed comparable amounts of 28S and 18S rRNAs in each lane. For Western blot analysis, 200 μg of protein was immunoprecipitated with anti-p21 monoclonal antibody YA6-259. Immunoprecipitated proteins were then electrophoresed in a 7.5 to 15% SDS–polyacrylamide gel, transferred to a nitrocellulose filter, and analyzed with anti-p21 monoclonal antibody ras-10.

cells in the absence of dexamethasone (Table 1). Induction of Ha-*ras* Asn-17 expression by addition of dexamethasone, however, inhibited the mitogenic response of NIH(M17) cells to both serum and individual growth factors, although to different extents. In particular, Ha-*ras* Asn-17 expression resulted in approximately a twofold inhibition of the response to serum and FGF, a three- to fourfold inhibition of response to PDGF, and a virtually complete inhibition of response to EGF (Table 1).

Since Ha-*ras* Asn-17 expression inhibited the mitogenic response of NIH(M17) cells to growth factors, we were interested in determining its effect on mitogenesis induced by direct stimulation of protein kinase C with the phorbol ester TPA. In normal NIH 3T3 cells, TPA stimulated [^{3}H]thymidine incorporation by about eightfold in either the presence or absence of dexamethasone (Fig. 4). Similar results were obtained for NIH(M17) cells in the absence of dexamethasone (Fig 4). However, induction of Ha-*ras* Asn-17 by dexamethasone treatment of NIH(M17) cells completely abolished the mitogenic response to TPA (Fig. 4). These results are consistent with those of antibody microinjection experiments (36) and indicate that *ras* function is required for both growth factor- and TPA-induced mitogenesis.

Induction of nuclear proto-oncogenes in NIH(M17) cells. The nuclear proto-oncogenes *fos*, *myc*, and *jun* are early-response genes which are rapidly induced following mitogenic stimulation of quiescent cells. Since the stimulation of DNA synthesis by EGF and TPA was strongly inhibited by expression of Ha-*ras* Asn-17 in NIH(M17) cells, we investigated the effect of expression of this mutant *ras* gene on nuclear proto-oncogene induction. RNAs were extracted from quiescent NIH 3T3 and NIH(M17) cells that had been treated with EGF or TPA in either the presence or absence of dexamethasone. Exposure of quiescent NIH 3T3 cells to either EGF or TPA resulted in a rapid transient increase in *fos* mRNA, followed by a later induction of *myc* and *jun* (Fig. 5). The induction of *fos* and *myc* by both EGF and TPA in NIH(M17) cells was similar whether or not expression of Ha-*ras* Asn-17 was induced by addition of dexamethasone (Fig. 5). Induction of *jun* in response to EGF was partially inhibited by dexamethasone addition in NIH(M17) cells, but its induction by TPA was not affected by Ha-*ras* Asn-17 expression (Fig. 5). It thus appeared that expression of the Ha-*ras* Asn-17 mutant protein blocked the proliferation of NIH(M17) cells induced by EGF and TPA without significantly inhibiting nuclear proto-oncogene induction.

Effect of Ha-*ras* Asn-17 on *fos-cat* induction by growth factors and TPA in transient expression assays. As discussed above, the NIH(M17) cell line expressed a relatively low level of p21(Asn-17), most likely due to selection for normal growth in the absence of dexamethasone. To investigate the possible effects of higher levels of the mutant protein on nuclear proto-oncogene induction and to extend our experiments beyond a single transformant line, we investigated the activity of Ha-*ras* Asn-17 in transient expression assays in NIH 3T3 cells. In these experiments the *fos* promoter, including the dyad symmetry element which confers inducibility by serum, growth factors, and TPA, was linked to *cat* as a reporter gene (23). NIH 3T3 cells were transfected with the *fos-cat* plasmid pFC700, cultured in medium containing 0.5% serum for 48 h, and then exposed to mitogens. CAT activity was strongly induced by serum, EGF, and TPA (Fig. 6). The effect of Ha-*ras* Asn-17 expression was then tested by cotransfection of NIH 3T3 cells with pFC700 plus either pZIP M17 or pZIPneoSV(X) as a control. Expression of the Ha-*ras* Asn-17 gene in these cotransfection assays blocked the induction of CAT activity by EGF, partially inhibited its

TABLE 1. Stimulation of DNA synthesis by growth factors[a]

Mitogen	Relative [^{3}H]thymidine incorporation					
	NIH(M17) cells			NIH 3T3 cells		
	−Dex	+Dex	Ratio[b]	−Dex	+Dex	Ratio
Calf serum	23 ± 3	10 ± 1	0.5	18 ± 5	18 ± 1	1.0
FGF	14 ± 2	8 ± 1	0.6	15 ± 6	15 ± 6	1.0
PDGF	16 ± 8	4.5 ± 2	0.3	14 ± 4	14 ± 6	1.0
EGF	22 ± 6	1.4 ± 0.1	0.06	8 ± 1	10 ± 2	1.3

[a] NIH 3T3 or NIH(M17) cells (2×10^5) were plated in 60-mm dishes in medium containing 10% calf serum. Medium was changed to 0.5% serum the next day, and cells were incubated for 24 h to induce quiescence. Dexamethasone (Dex; 5×10^{-7} M) was then added to some cultures, and the cells were stimulated 8 h later by addition of calf serum (10%), FGF (20 ng/ml), PDGF (5 U/ml), or EGF (10 ng/ml). [^{3}H]thymidine incorporation was assayed 16 h after growth factor addition. Duplicate plates were used for each assay, and the ratio of [^{3}H]thymidine incorporation in stimulated cultures to that in unstimulated controls is presented. Data are the average of three independent experiments ± standard deviation.

[b] Ratio of stimulation with dexamethasone/without dexamethasone.

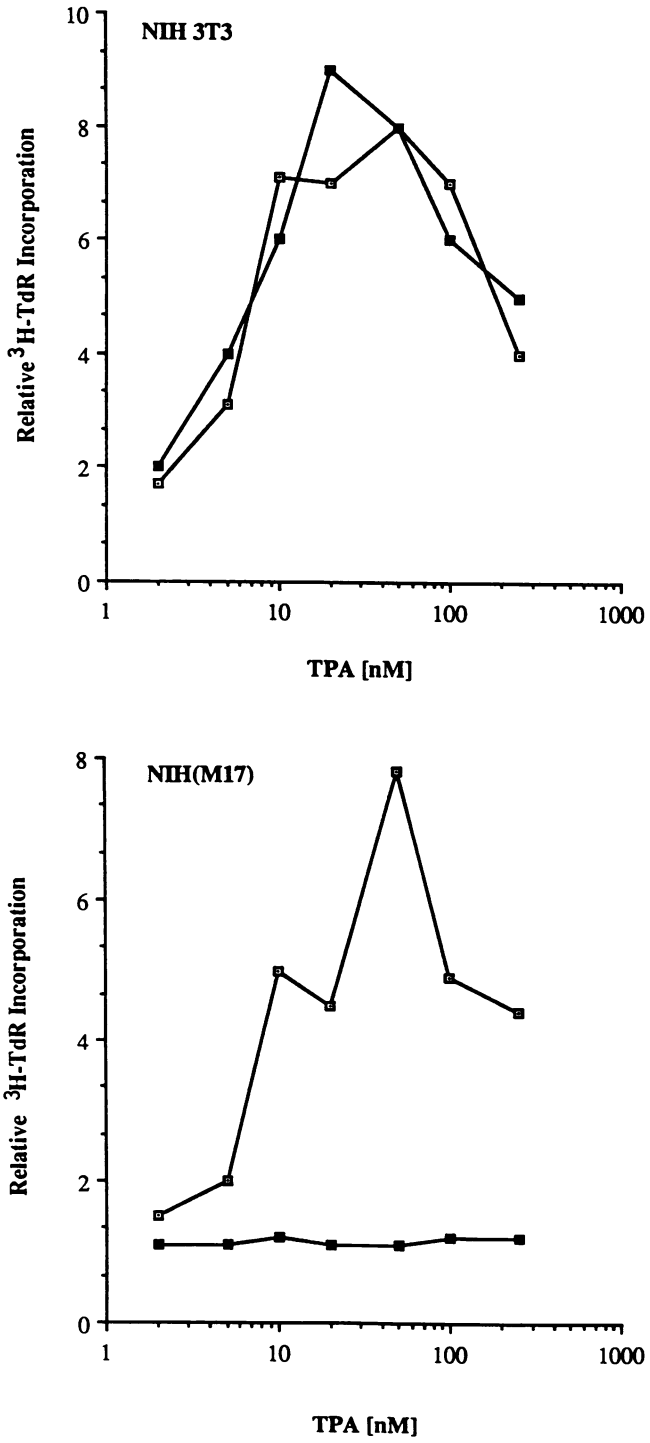

FIG. 4. Stimulation of DNA synthesis by TPA. Quiescent NIH 3T3 and NIH(M17) cells were stimulated by addition of TPA in the presence (■) or absence (□) of dexamethasone as described in Table 1, footnote *a*. Data are presented as the ratio of [^{3}H]thymidine (^{3}H-TdR) incorporation in treated cultures to that in untreated controls and represent the average of two independent experiments.

induction by serum, but did not affect induction by TPA (Fig. 6). This inhibitory effect of Ha-*ras* Asn-17 expression appeared to be specific for the inducible *fos* promoter, since constitutive expression from the SV40 early promoter was

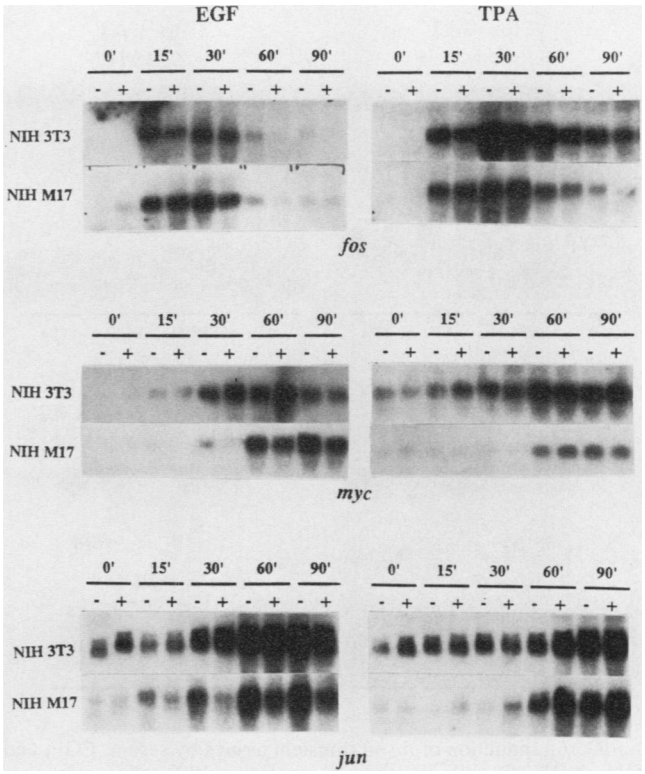

FIG. 5. Induction of early-response genes by EGF and TPA in NIH(M17) cells. NIH 3T3 and NIH(M17) cells were grown to ~90% confluence in medium containing 10% calf serum. Medium was then changed to 0.5% calf serum, and cells were incubated for 24 h to induce quiescence. Where indicated (lanes marked +), 5×10^{-7} M dexamethasone was added at the time of medium change. Cells were then stimulated by addition of EGF (10 ng/ml) or TPA (50 nM) and harvested for RNA extraction at the indicated times (in minutes) after treatment. Total cytoplasmic RNAs (15 μg) were analyzed by Northern blot hybridization with *fos*, *myc*, and *jun* probes. Each lane contained similar amounts of 28S and 18S rRNAs.

not inhibited by cotransfection with pZIP M17 (Fig. 6). Cotransfection of pZIP M17 also inhibited *fos-cat* expression induced by PDGF and FGF, although to a lesser extent than induction by EGF (Fig. 7). Thus, in transient assays in NIH 3T3 cells, expression of Ha-*ras* Asn-17 inhibited *fos-cat* induction by growth factors. In contrast to its effect on mitogenesis, however, Ha-*ras* Asn-17 did not significantly affect *fos-cat* induction by TPA.

The lack of effect of Ha-*ras* Asn-17 on *fos* induction in the stably transformed NIH(M17) cell line contrasts with the results of these transient expression assays. This difference could reflect higher levels of Ha-*ras* Asn-17 expression in transiently transfected cells compared with NIH(M17) cells, or it could be due to a difference in regulation of genomic *fos* compared with the *fos-cat* construct. To test the latter possibility, NIH(M17) cells were transfected with the *fos-cat* plasmid, and induction of CAT activity by EGF was assayed in the presence or absence of dexamethasone. Dexamethasone induction of Ha-*ras* Asn-17 expression had no detectable effect on CAT expression (data not shown), indicating that regulation of the *fos-cat* plasmid was similar to that of genomic *fos* in NIH(M17) cells. It thus appears likely that a higher level of Ha-*ras* Asn-17 expression, obtained in the transient expression assays, is required to block *fos* induction.

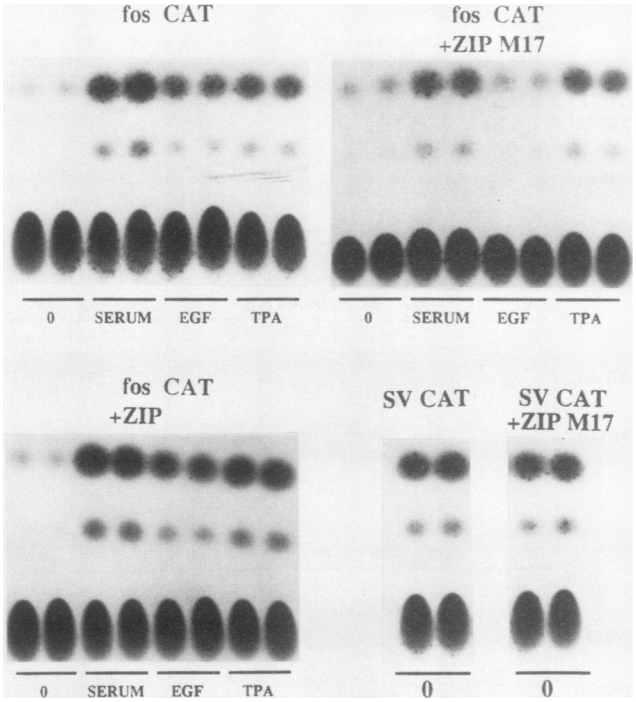

FIG. 6. Induction of *fos* in transient assays by serum, EGF, and TPA. NIH 3T3 cells in medium containing 10% calf serum were transfected with 1 μg of pFC700 (*fos-cat*) or pSV2CAT (SVCAT) plasmid DNA either alone or together with 3 μg of pZIP M17 or pZIPneoSV(X) plasmid (ZIP) DNA. Medium was changed to 0.5% calf serum immediately after transfection, and cells were incubated for 48 h. Cells were either maintained in the absence of mitogenic stimulation (lanes 0) or stimulated by addition of calf serum (20%), EGF (10 ng/ml), or TPA (50 nM). Cells were incubated an additional 4 h, harvested, and assayed for CAT activity. Duplicate cultures were assayed in adjacent lanes.

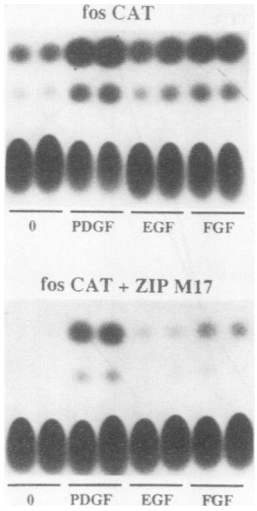

FIG. 7. Transient *fos* induction by serum, EGF, FGF, and PDGF. The experiment was performed as described in the legend to Fig. 6, except that *fos* induction by FGF (10 ng/ml) and PDGF (5 U/ml) was also assayed.

Induction of *fos-cat* by *ras* and other oncogenes. Previous studies showed that the growth-inhibitory effect of Ha-*ras* Asn-17 could be reversed by coexpression of an activated *ras* gene (5). We therefore tested whether the inhibition of *fos* induction by Ha-*ras* Asn-17 could be overcome by expression of v-Ha-*ras* (Fig. 8). Cotransfection of quiescent NIH 3T3 cells with *fos-cat* plus a v-Ha-*ras* expression plasmid induced CAT expression in the absence of mitogen stimulation (Fig. 8). Cotransfection of the *fos-cat* plus v-Ha-*ras* plus pZIP M17 plasmids revealed a competitive relationship between v-Ha-*ras* and Ha-*ras* Asn-17. Cotransfection of 3 μg of Ha-*ras* Asn-17 plus 0.5 μg of v-Ha-*ras* blocked *fos* induction; cotransfection of 3 μg of Ha-*ras* Asn-17 plus 3 μg of v-Ha-*ras* yielded partial *fos* induction; and cotransfection of 3 μg of Ha-*ras* Asn-17 plus 10 μg of v-Ha-*ras* resulted in full *fos* induction (Fig. 8). These results indicate that coexpression of an active *ras* gene can overcome the inhibitory effect of Ha-*ras* Asn-17. The apparent competition between v-Ha-*ras* and Ha-*ras* Asn-17 suggests that Ha-*ras* Asn-17 may block the interaction of v-Ha-*ras* with an effector molecule(s) in addition to blocking upstream activation of normal Ha-*ras* (21).

Inhibition of NIH 3T3 cell proliferation by either microinjection of anti-Ras antibody (25) or expression of Ha-*ras* Asn-17 (5) can also be overcome by transformation with *mos* and *raf*, but not with *src* or other tyrosine kinase oncogenes. A similar relationship between these oncogenes was observed in assays of *fos-cat* induction. Consistent with previous studies (8, 13, 32, 33), cotransfection of quiescent NIH 3T3 cells with either *src*, *mos*, or *raf* efficiently induced *fos-cat* expression in the absence of growth factor stimulation (Fig. 9). Cotransfection with Ha-*ras* Asn-17 blocked *fos-cat* induction by *src* but did not affect induction by *mos* or *raf* (Fig. 9). These results are consistent with the notion that the action of tyrosine kinase oncogenes requires *ras* function, whereas the serine/threonine kinase oncogenes *mos* and *raf* may act either downstream or independently of *ras* in signal transduction pathways leading to both induction of *fos* and cell proliferation.

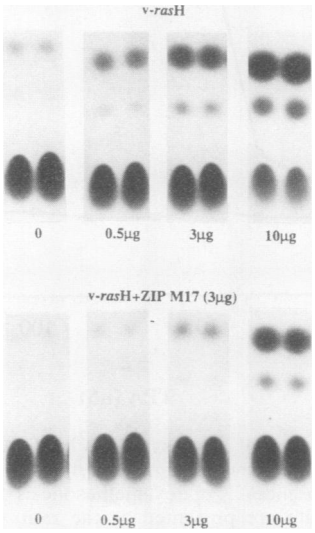

FIG. 8. Competition between v-Ha-*ras* (v-*ras*H) and Ha-*ras* Asn-17. NIH 3T3 cells were cotransfected with *fos-cat* (1 μg) plus pZIPv-rasH (0, 0.5, 3, or 10 μg as indicated at the bottom of each panel). Where indicated (bottom half of the figure), cells were also cotransfected with pZIP M17 (3 μg). Transfected cells were incubated for 48 h in medium containing 0.5% calf serum and assayed for CAT activity without addition of exogenous mitogens. Duplicate cultures were analyzed in adjacent lanes.

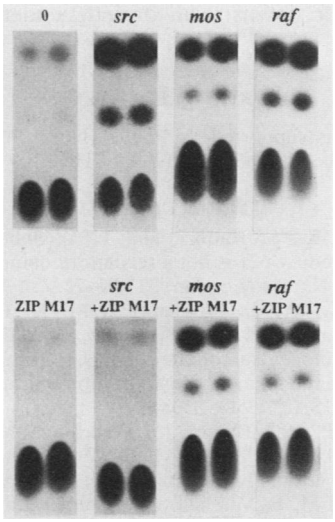

FIG. 9. Transient *fos* induction by *src*, *raf*, and *mos* oncogenes. NIH 3T3 cells were cotransfected with *fos-cat* (1 μg) plus *src*, *mos*, or *raf* expression plasmid DNA (5 μg). Where indicated (bottom), cells were also cotransfected with pZIP M17 (3 μg). Cells were incubated for 48 h in medium containing 0.5% calf serum, and duplicate cultures were assayed for CAT activity.

Since *fos-cat* induction by TPA did not appear to require *ras* function, it was of interest to determine whether protein kinase C was required for *fos-cat* induction by *ras*. The possible requirement for protein kinase C activity for *fos-cat* induction was therefore tested by downregulation of protein kinase C in recipient NIH 3T3 cells (Fig. 10). In parallel control cell cultures, *fos-cat* expression was readily induced by TPA (data not shown). Downregulation of protein kinase C by pretreatment of recipient cells with 400 nM TPA for 48 h blocked *fos-cat* induction by TPA, indicating that protein kinase C activity had been effectively depleted (Fig. 10). However, *fos-cat* induction by serum, v-Ha-*ras*, *src*, *mos*, or *raf* was not affected by TPA pretreatment (Fig. 10). These results indicate that protein kinase C activity was not required for *fos-cat* induction by *ras* or the other oncogenes tested.

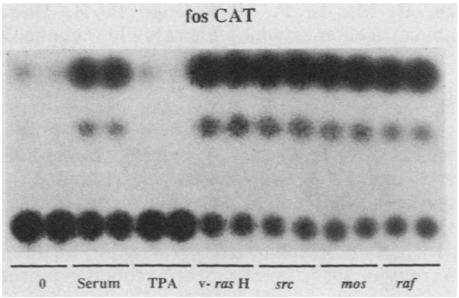

FIG. 10. Effect of downregulation of protein kinase C on *fos* induction by oncogenes. NIH 3T3 cells were incubated for 48 h in the presence of 400 nM TPA. Cells were then transfected with *fos-cat* (1 μg) plus v-Ha-*ras* (v-*ras* H), *src*, *mos*, or *raf* expression plasmid DNA (5 μg) where indicated. Cells were incubated for 48 h in medium containing 0.5% calf serum. Calf serum (20%) or TPA (50 nM) was then added to the indicated cultures that had been transfected with *fos-cat* alone. After 4 h of further incubation, duplicate cultures were assayed for CAT activity.

DISCUSSION

In the present study, we used the dominant inhibitory Ha-*ras* Asn-17 mutation to investigate the role of *ras* in mitogenic signal transduction. This mutant has provided a means of blocking normal *ras* function in cultured cells so that the effects of interference with *ras*-mediated signal transduction can be analyzed.

Our initial approach was to isolate a stably transfected cell line [NIH(M17) cells] in which the Ha-*ras* Asn-17 gene was expressed via the dexamethasone-inducible MMTV LTR. These cells proliferated normally in the absence of dexamethasone, but growth was significantly inhibited when Ha-*ras* Asn-17 expression was induced by dexamethasone addition. Results obtained with NIH(M17) cells were extended beyond this single transformant cell line by transient expression assays in NIH 3T3 cells.

In quiescent NIH(M17) cells, progression from G0 to S phase in response to mitogenic stimulation by serum and growth factors, particularly EGF, was inhibited by Ha-*ras* Asn-17 expression. In addition, expression of Ha-*ras* Asn-17 completely blocked DNA synthesis induced by the phorbol ester TPA. These results are consistent with experiments in which *ras* function was inhibited by antibody microinjection (19, 36) and indicate that *ras* proto-oncogene function is required for induction of mitogenesis not only by growth factors, but also by direct activation of protein kinase C.

An early event in the response of cells to mitogenic stimulation is the induction of *fos*, followed by induction of *jun* and *myc*. Surprisingly, expression of Ha-*ras* Asn-17 had little effect on induction of these nuclear proto-oncogenes by EGF or TPA in NIH(M17) cells. Induction of *fos* and *myc* by either EGF or TPA was not detectably affected by Ha-*ras* Asn-17 expression, although the induction of *jun* by EGF but not TPA was partially inhibited. Induction of these early-response genes could thus be uncoupled from progression to S phase, indicating that expression of these nuclear proto-oncogenes is not sufficient to induce cell proliferation.

Inhibition of *fos* induction by Ha-*ras* Asn-17 was, however, observed in transient expression assays in which NIH 3T3 cells were cotransfected with a *fos-cat* reporter plasmid plus a Ha-*ras* Asn-17 expression vector. In these experiments, Ha-*ras* Asn-17 inhibited CAT expression from the *fos* promoter in response to stimulation of quiescent cells by EGF, PDGF, and FGF. The inhibition of *fos* induction in this assay is consistent with previous findings that induction of *fos* by growth factors was inhibited by microinjection of anti-Ras antibody (28). The difference between the effect of Ha-*ras* Asn-17 on *fos* induction in the stably transformed NIH(M17) cells and in the transient assays was not due to differences in regulation of the endogenous *fos* gene compared with the *fos-cat* construct, since expression of Ha-*ras* Asn-17 in NIH(M17) cells did not inhibit *fos-cat* induction. Rather, it appears that the low level of Ha-*ras* Asn-17 expression in the stable NIH(M17) transformant line, although sufficient to inhibit mitogenesis, was not sufficient to inhibit induction of *fos*. However, *fos* induction was blocked by the presumably higher levels of Ha-*ras* Asn-17 expression resulting from transient transfection with the mutant *ras* gene in the pZIP M17 vector. Similar results have been obtained in recent studies of the effect of Ha-*ras* Asn-17 expression on morphological differentiation of PC12 cells (29a). In this case, a low level of Ha-*ras* Asn-17 expression was sufficient to block morphological differentiation without affecting induction of *fos* or other early-response genes,

although early-response gene induction was blocked by higher levels of the mutant protein.

Transfection of quiescent NIH 3T3 cells with activated Ha-*ras*, *src*, *mos*, or *raf* oncogenes also induced *fos-cat* expression in the absence of growth factor stimulation. The induction of *fos-cat* by a v-Ha-*ras* expression plasmid was inhibited in a competitive manner by cotransfection with Ha-*ras* Asn-17. In addition, induction of *fos-cat* expression by *src* but not by *mos* or *raf* was inhibited by Ha-*ras* Asn-17 expression. These results are also consistent with previous studies of the activity of these oncogenes in inducing cell proliferation and suggest that *ras* function is required for *src*-initiated signal transduction, whereas the *mos* and *raf* protein-serine/threonine kinase oncogenes apparently act either downstream or independently of *ras* in signal transduction pathways (5, 25).

In contrast to the results obtained with EGF and other growth factors, the induction of *fos-cat* by TPA in transient assays was not inhibited by Ha-*ras* Asn-17 expression. Expression of Ha-*ras* Asn-17 in PC12 cells similarly inhibits *fos* induction by nerve growth factor but not by TPA (29a). Therefore, it appears that direct activation of protein kinase C is sufficient to induce *fos* expression in the absence of functional *ras*. This is not the case for induction of DNA synthesis by TPA, which is blocked by either anti-Ras antibody microinjection (36) or Ha-*ras* Asn-17 expression (present study).

A number of studies have indicated that *ras* proteins stimulate phospholipid turnover, leading to production of diacylglycerol and activation of protein kinase C (7, 14, 16, 18, 20, 22, 31, 35). Furthermore, several investigators have reported that downregulation of protein kinase C inhibits *ras*-induced mitogenesis (11, 15, 18). In our experiments, however, downregulation of protein kinase C did not inhibit *fos-cat* induction by *ras* or other activated oncogenes. Therefore, *ras* can apparently induce *fos* expression via a protein kinase C-independent pathway. Similar studies in PC12 cells have also recently shown that functional protein kinase C is not required for induction of *fos* by *ras* protein (9). In addition, whereas protein kinase C was required for induction of DNA synthesis by *ras* protein introduced into Swiss 3T3 cells by scrapeloading (18), protein kinase C was not required for induction of *myc* in the same system (17). On the other hand, protein kinase C has been reported to be required for both induction of *fos* and DNA synthesis by *ras* protein in rat embryo fibroblasts (11). Although the basis for this discrepancy is unclear, it appears that, in at least some cell types, *ras* can induce nuclear proto-oncogenes via a protein kinase C-independent pathway.

Taken together, these findings indicate that either *ras* or protein kinase C can independently induce *fos*, but the activities of both *ras* and protein kinase C are required to induce DNA synthesis. A model in which *ras* and protein kinase C are viewed as elements in a single linear signal transduction pathway cannot readily accommodate these results. Instead, it appears more likely that *ras* proteins are involved in at least two parallel pathways of signal transduction. One of these pathways may involve activation of protein kinase C, whereas a second, currently unidentified, pathway is protein kinase C independent. The activity of either pathway alone is sufficient to induce *fos*, but both pathways are needed to induce DNA synthesis. Growth factors and tyrosine kinase oncogenes (e.g., *src*) apparently act upstream of *ras* in both pathways, since inhibition of *ras* function inhibits both induction of *fos* and mitogenesis by these agents. The *mos* and *raf* proteins, in contrast, apparently act either downstream or independently of the *ras* proteins.

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