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ras-Induced Neuronal Differentiation of PC12 Cells: Possible Involvement of *fos* and *jun*

PAOLO SASSONE-CORSI,[1†] CHANNING J. DER,[2] AND INDER M. VERMA[1*]

Molecular Biology and Virology Laboratory, The Salk Institute, P.O. Box 85800, San Diego, California 92138,[1] and La Jolla Cancer Research Foundation, La Jolla, California 92037[2]

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Rat pheochromocytoma PC12 cells differentiate to sympathetic neuron-like cells upon treatment with nerve growth factor (NGF). The *ras* and *src* transforming proteins also induce PC12 neuronal differentiation and are likely to involve the protein kinase C signal transduction pathway. Using a number of *ras* mutants, we have established that the domains of oncogenic *ras* protein responsible for PC12 differentiation overlap those required for cellular transformation. All of the *ras* mutants that induced neuronal differentiation also activated c-*fos* transcription through the dyad symmetry element (DSE). Transforming *ras* protein activated an intracellular signal pathway, which led to the induction of 12-*O*-tetradecanoyl phorbol-13-acetate-responsive elements; activation was enhanced by coexpression of the proto-oncogene *jun* (encoding AP-1) and was further augmented by *fos*. Nuclear extracts from *ras*-infected PC12 cells showed an increased AP-1 DNA-binding activity. Transcriptional activation by *ras* was independent of the cyclic AMP-dependent pathway of signal transduction. We propose a possible involvement of *fos* and *jun* in *ras*-induced differentiation.

Understanding of the molecular mechanisms that govern the differentiation program of a cell is a central problem in biology. Extensive studies have been carried out in a number of in vitro cultured cell lines that have the ability to differentiate in response to growth factors, retinols, cyclic AMP (cAMP), phorbol esters, and other agents. Often differentiation is accompanied by cessation of proliferation. Rat pheochromocytoma PC12 cells are adult chromaffinlike cells that, in the presence of nerve growth factor (NGF), acquire the properties of sympathetic neuron-like cells, including neurite outgrowth, increased electrical excitability, and changes in neurotransmitter synthesis (23). Neuronal differentiation of PC12 cells can be induced by *ras* or *src* transforming proteins (2, 5, 37). Since the inductive effects of *ras* and *src* on differentiation resemble the action of NGF, it appears that a common signal transduction pathway is used (32, 35, 36). The differentiation-inducing properties of c-Ha-*ras* were tested either by infections with sarcoma viruses carrying the *ras* oncogene or by microinjection of purified normal or activated Ha-*ras* proteins (5, 34). There is no effect of normal c-*ras* on PC12 differentiation, but activated Ha-*ras* products induce differentiation in both cases (5, 26). Antibody to $p21^{ras}$ microinjected into PC12 cells inhibits NGF-induced differentiation (26). In contrast to the effect of NGF, when PC12 cells are grown in the presence of dexamethasone, they acquire the characteristics of chromaffinlike cells (24). Neither *ras* or *src* is able to mimic the effect of steroids on growth or differentiation of PC12 cells (2, 5, 26, 37).

Because both *ras* and *src* transforming proteins have been postulated to mimic the action of phorbol esters that activate the protein kinase C (PKC) transduction pathway (35, 36), it is likely that NGF-directed neurite outgrowth may be PKC dependent. This expectation has recently been confirmed by the use of sphingosine, a specific pharmacological inhibitor of PKC that blocks NGF-induced differentiation of PC12 cells (27). Thus, the possibility exists that the differentiation program initiated by NGF, *ras*, or *src* involves the transcriptional activation of genes playing a crucial role in determination of the cellular phenotype. Good candidate genes induced by the PKC pathway are proto-oncogenes that have been implicated in the regulation of cell growth, differentiation, and development (7, 9, 54). In particular, the transcription of proto-oncogene *fos* is rapidly and transiently induced when either *ras* or NGF is added to PC12 cells (14, 23, 29, 46, 48). Interestingly, c-*fos* transcription is not induced by dexamethasone treatment of PC12 cells, suggesting a role only in the neuronal differentiation pathway (29).

We undertook studies to determine the molecular mechanisms involved in the neuronal differentiation of PC12 cells induced by the *ras* oncogene. Using a variety of mutants of the *ras* gene product p21, we have established that the protein domains responsible for induction of the differentiated phenotype in PC12 cells overlap those required for the transformation of NIH 3T3 fibroblasts. Transcription of the c-*fos* gene was induced at least 20-fold by expression of the oncogenic *ras* protein. All of the *ras* mutants that induced neuronal differentiation of PC12 cells also activated c-*fos* transcription. This strong correlation suggests a possible role of *fos* in the differentiation process. The same c-*fos* promoter elements responsive to *ras* induction were inducible by serum and by 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). Interestingly, transforming *ras* proteins also activated transcription from a TPA-responsive element (TRE); this activity was enhanced by co-expression of the proto-oncogene *jun*, which encodes a protein with characteristics of the transcription factor AP-1 (3, 10). Binding studies using nuclear extracts from PC12 cells infected by *ras* viruses were performed by using a TRE oligodeoxynucleotide. We observed an increase in DNA-binding activity only in the extract from cells infected with an oncogenic *ras* retrovirus, which is in agreement with the report that *ras* protein influences transcription from regulatory elements containing AP-1-binding sites (58). Finally, the activated *ras* protein did

* Corresponding author.
† Present address: Laboratoire de Genetique Moleculaire des Eucaryotes, Centre National du Recherche Scientifique, 11, rue Humann, 67085 Strasbourg, France.

not induce transcription directed from a cAMP-responsive element (CRE).

MATERIALS AND METHODS

Cell culture. NIH 3T3 and ψ2 cells were maintained in Temin modified minimal essential medium supplemented with 10% calf serum. PC12 and A126-1B2 cells were maintained in Temin modified minimal essential medium supplemented with 10% fetal calf serum and 2.5% horse serum.

Oligonucleotide-directed mutagenesis. Oligonucleotide-directed mutagenesis and generation of molecular constructs were performed as described previously (16, 17). Briefly, 17-base synthetic oligonucleotides were synthesized by the phosphoramidite method on an Applied Biosystems 380A DNA synthesizer and were used to introduce base substitutions into the Ha-*ras* cDNA sequences. The Ser-186 mutation was introduced to change Cys (TGT) to Ser (AGT), and the Ala-35 mutation was introduced to change Thr (ACT) to Ala (GCT). Generation of the remaining mutants has been described elsewhere (18).

Transfection of NIH 3T3 cells. Purified plasmid DNA of each pZIP-*ras* mutant was used for DNA transfection into NIH 3T3 cells by the calcium phosphate precipitation technique as described previously (18). Briefly, 10 ng of plasmid DNA was mixed with 20 μg of calf thymus DNA, and the calcium phosphate precipitate was added to 10^6 cells per 60-mm-diameter dish. The appearance of transformed foci was quantitated after 12 to 14 days.

Virus infection of PC12 cells. The procedures used for preparing retrovirus stocks of the different pZIP-*ras* constructs were essentially as described by Cepko et al. (12). After transfection, stable transfectants were selected in growth medium containing 400 μg of G418 (Geneticin; GIBCO Laboratories) per ml. Multiple G418-resistant colonies were pooled for isolation of helper-free stocks of recombinant virus. Retrovirus supernatants were titered for G418-resistant CFU per milliliter on NIH 3T3 cells. For infection of PC12 cells, 1×10^5 to 5×10^5 CFU was added to cells (5×10^5/60-mm-diameter dish) in Temin modified minimal essential medium containing 8 μg of Polybrene per ml. After 16 h, the virus was removed, and fresh growth medium containing 400 μg of G418 per ml was added to the cultures. The appearance of neurite outgrowths was monitored for 2 weeks. G418-selected populations were monitored for up to 4 weeks for the presence of proliferating, nondifferentiated cells.

Transient-expression assay. PC12 and A126-1B2 cells were plated at 10^6/10-cm-diameter tissue culture dish 24 h before DNA transfection. Cells were transfected by the calcium phosphate coprecipitation technique (18) and exposed to the precipitate for 12 h. Fresh medium was added after a wash with phosphate-buffered saline, and cells were harvested after 24 h. When less than 20 μg of specific DNA was used per 10-cm-diameter culture dish, GEM-3 plasmid DNA was added to give 20 μg of total DNA. Equimolar ratios of cotransfected plasmids were maintained in all experiments. Chloramphenicol acetyltransferase (CAT) activity was determined as described previously (22). In several experiments, a plasmid containing the Rous sarcoma virus long terminal repeat (LTR) linked to the bacterial luciferase gene was cotransfected to monitor the efficiency of transfection.

Recombinant plasmids. Mutated and normal *ras* gene constructs used for transfection and infection were generated in retrovirus vector pZIP-NeoSV(x)1 (12). The resulting pZIP-*ras* constructs contain human cDNA Ha-*ras* or N-*ras* under the control of the Moloney murine leukemia virus LTR. pSV-c-*jun* contains a mouse cDNA (30) under the control of the early simian virus 40 promoter region. BK28 contains the full-length human c-*fos* cDNA clone under the control of the murine sarcoma virus FBJ LTR (42, 44). FC4 is a c-*fos* promoter-CAT fusion gene containing the human c-*fos* genomic segment from −404 to +42. TK-CAT contains the herpes simplex virus thymidine kinase (TK) promoter from positions −109 to +57 linked to the CAT structural gene (42, 44; see Fig. 3). pFtk contains the c-*fos* promoter region from −323 to −276 linked upstream of TK-CAT. TRE/TK-CAT contains one 18-base-pair (bp) synthetic oligodeoxynucleotide complementary to the human metallothionein IIA TRE sequence cloned upstream of the TK promoter. Similarly, CRE/TK-CAT contains a 20-bp synthetic oligodeoxynucleotide complementary to the rat somatostatin CRE sequence (33).

Northern (RNA) analysis. RNA from *ras* virus-infected cells was prepared 2 h after infection. Total RNA was purified as described above and analyzed on formamide-denaturing agarose gels (50). c-*fos* RNA was detected by using a nick-translated probe encompassing the full cDNA sequence.

Nuclear extracts and gel retardation assay. Nuclear extracts from NIH 3T3 and PC12 cells were prepared essentially as described by Dignam et al. (19). Final concentrations were typically 1 to 2 μg/ml. DNA binding was conducted in 20-μl final volumes. Nuclear extract (1 to 3 μl) was incubated with 1 μg of poly(dI-dC) (Boehringer Mannheim Biochemicals) in TM buffer (50 mM Tris hydrochloride [pH 7.9], 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol) for 20 min at room temperature. Nuclear extracts from infected PC12 cells were prepared after 2 h of infection. A synthetic 18-bp oligodeoxynucleotide containing the human metallothionein IIA TRE was end labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase. Approximately 0.1 ng of ^{32}P-labeled DNA (<10,000 cpm) was added to the preincubated nuclear extract or protein fractions. Unlabeled competitor DNA was added to the binding reaction 2 min before the labeled oligomer. DNA-protein complexes were resolved on a 4% polyacrylamide gel (39:1, acrylamide to bisacrylamide) in 0.25× TBE (1× TBE is 50 mM Tris borate [pH 8.3] plus 1 mM EDTA). The gels were dried and autoradiographed with intensifying screens at −70°C.

Antibodies to *fos* and *jun*. Affinity-purified antibodies to the M2 peptide of mouse c-*fos* have been described (15). Anti-*jun* PEP-1 peptide antiserum was a kind gift of T. J. Bos and P. Vogt (10). Inhibition of DNA-protein complex binding with antibodies was performed as described elsewhere (20, 39, 44). In particular, experiments using c-*fos* M2 peptide antibodies (amino acids 127 to 154; 15) were conducted by preincubating the antibodies (0.5 μg) for 2 h at 4°C with nuclear extracts from PC12 cells. No disruption of the nucleoprotein complex binding was observed when a five-fold excess of M2 peptide was added together with the antibody to the extract. Inhibition of DNA binding by using anti-*jun* PEP-1 and PEP-2 (10) peptide antisera was obtained under similar conditions, but the preincubation time with the nuclear extract was longer (8 h). As previously observed (44), no disruption of the nucleoprotein complex binding was observed when preimmune PEP-1 and PEP-2 sera were used.

TABLE 1. Biochemical and biological activities of Ha-*ras* mutants

ras mutant and substitution(s)[a]	NIH 3T3 foci (10^3)[b]	PC12 differentiation[c]	*fos* activation[d]
Ha-*ras*			
Normal	0.00	−	1.4
12R	4.75	+	23.2
61L	5.96	+	25.4
59T	5.35	+	21.6
12R, 59T	5.04	+	16.4
61P	0.53	±	4.8
117E	4.59	+	ND
61L, 35A	0.00	−	1.7
61L, 117E	6.26	+	14.5
61L, 116H, 119H	0.00	−	1.5
61L, 186S	0.00	−	1.2
N-*ras*			
Normal	0.00	−	1.5
12D	6.85	+	12.7

[a] Amino acid substitution(s) in normal *ras* protein.
[b] Number of transformed foci per microgram of DNA transfected; values are averages of multiple transfection assays.
[c] Induction of neurite formation and terminal differentiation of PC12 cells. +, Neurite extensions; −, no neurite extensions; ±, partial neurite extensions.
[d] Fold induction of the *fos* promoter-CAT gene in transfected PC12 cells. Values are averages of three independent experiments. ND, Not determined.

RESULTS

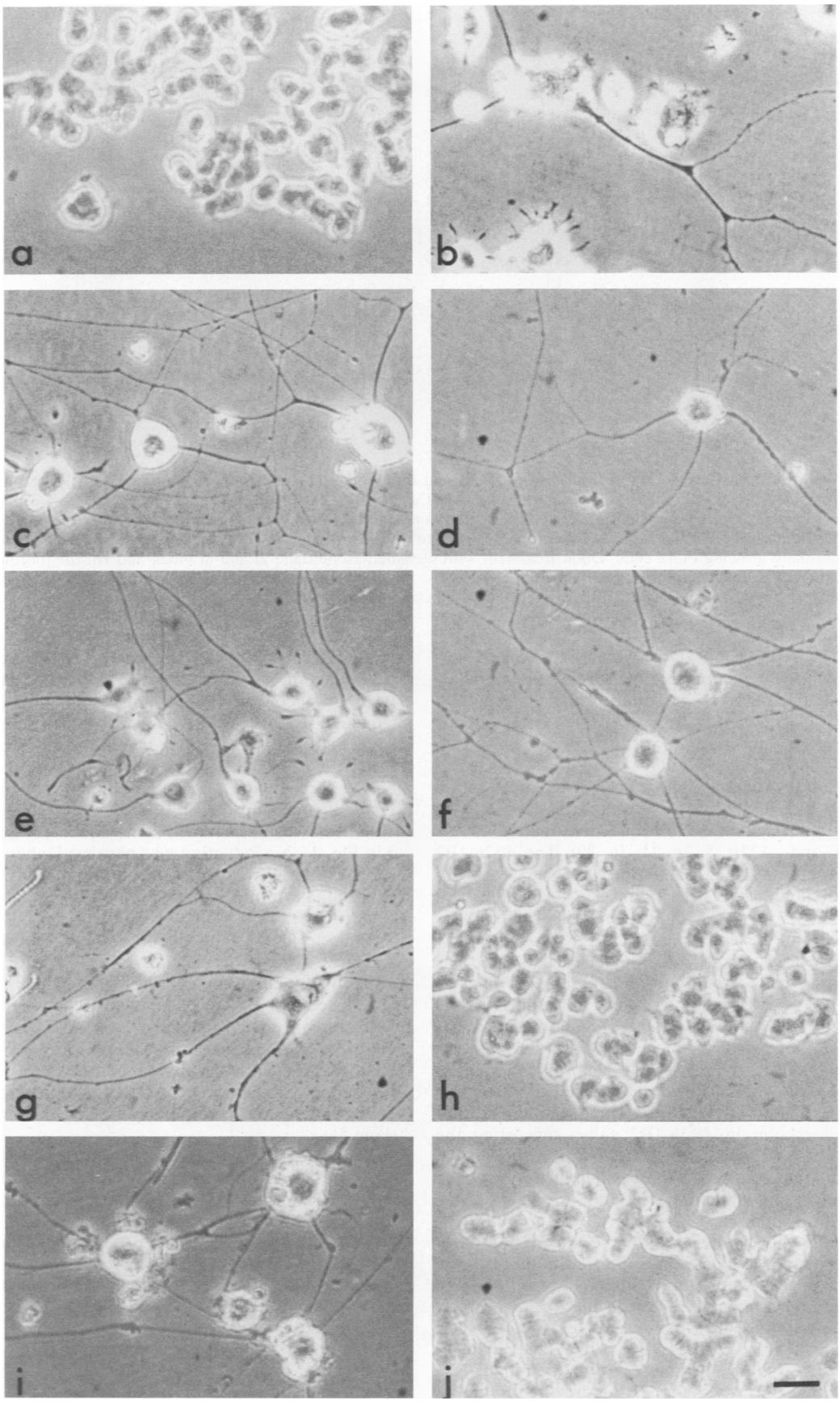
Transforming and differentiating activities of *ras* have the same functional domains. A series of structural and biochemical variants of the human Ha-*ras* protein were compared for their ability to transform NIH 3T3 fibroblasts and to induce differentiation of PC12 rat pheochromocytoma cells (Table 1 and Fig. 1). Whereas normal Ha-*ras* was unable to induce differentiation (Fig. 1a), the strongly transforming variants of Ha-*ras* that were activated by different lesions (12R, 59T, 61L, and 117E) were all equally effective in inducing PC12 neurite formation (Fig. 1b to e). Typically, neurite extensions were visible within 24 h of retrovirus infection, with continued development for up to 10 days, when an extensive network of neurites was visible (18). Consistent with its weak focus-forming activity on NIH 3T3 cells, the Ha-*ras* variant containing a proline substitution at position 61 (61P; 17) also displayed a slower rate of induction of neurite extensions (Fig. 1f). PC12 cells expressing Ha-*ras*(61P) displayed neurite extensions that were 3 to 5 days behind the neurite extensions seen for the strongly differentiating Ha-*ras* mutants. Thus, a direct relationship between Ha-*ras* transforming and differentiating activities was observed. A similar relationship was seen for normal and oncogenic forms of the human N-*ras* protein (Fig. 1g and Table 1).

The inability of a nonpalmitylated, soluble form of Ha-*ras* (61L, 186S; Fig. 1h) to induce differentiation of PC12 cells demonstrated the requirement for membrane localization for both *ras* transforming (59, 60) and differentiating activities. A GTP-binding variant of oncogenic Ha-*ras* (61L, 117E; Fig. 1i) (18), which displayed a significantly impaired ability to bind guanine nucleotides (10,000-fold reduced affinity), still effectively transformed NIH 3T3 cells and induced differentiation of PC12 cells. Therefore, a high affinity of binding for guanine nucleotides is not required for either transforming or differentiating functions of Ha-*ras*. The lack of neurite development in cells expressing the nontransforming Ha-*ras* (61L, 35A) demonstrated that the pathway for transformation and differentiation may share a common downstream effector target (Fig. 1j). Since the 35A substitution prevents stimulation by the GTPase-activating protein (1, 11), this proposed effector target for transformation may also be the downstream target for the *ras*-induced differentiation pathway in PC12 cells.

***ras* induces the *fos* gene.** It has previously been shown that (i) c-*fos* gene transcription is activated by NGF-induced differentiation of PC12 cells (for review, see reference 55) and (ii) microinjection of *ras* protein into NIH 3T3 cells also induces c-*fos* protein (48). Therefore, we wanted to study the effects of various *ras* mutants on c-*fos* gene transcription. We cotransfected in PC12 cells plasmids in which the c-*fos* promoter was linked to the CAT gene, together with each of the *ras* mutants (Table 1 and Fig. 2A). Transcription of the Ha-*ras* gene was under the control of Moloney murine leukemia virus LTR. In general, we observed that the Ha-*ras* mutants that encoded an activated p21 also induced c-*fos* transcription, whereas the nontransforming and nondifferentiating Ha-*ras* proteins did not affect c-*fos* promoter activity. Similarly, only the oncogenic form of N-*ras* activated c-*fos* transcription (Table 1). This strong correlation suggests a role of the *fos* protein in the PC12 neuronal differentiation pathway. We also tested whether transcription of the endogenous *fos* gene would be enhanced by an activated p21 protein. The results of Northern blot analyses of RNA purified from PC12 cells infected with retrovirus vectors carrying either the normal Ha-*ras* (Fig. 2B, lane 4) or an activated Ha-*ras* (61L; Fig. 2B, lane 5) form of p21 supported the data obtained from the cotransfection assay. Similar induction was observed after a 30-min treatment of cells with NGF (lane 6), whereas no effect of dexamethasone was detectable (lane 2). We also found that *ras* could activate *fos* transcription in other cell types, such as mouse NIH 3T3 fibroblasts, F9 embryonal carcinoma cells, and human choriocarcinoma JEG-3 cells, albeit to different extents (data not shown).

Using several heterologous recombinants in which subfragments of the c-*fos* promoter were linked to a herpesvirus TK-CAT fusion gene (44), we also defined the sequence element involved in the *ras*-induced *fos* response (Fig. 3A). The sequence of the *ras*-responsive region between positions −323 and −277 (pFtk; Fig. 3A) overlapped the dyad symmetry element (DSE), which is part of the serum-responsive element and has previously been described to bind nuclear factor SRF (21, 38, 51, 52, 56). Because plasmid pFtk contains both the DSE and AP-1 sites, it is not clear which sequences mediate the induction by *ras*. A plasmid containing only the DSE (region from −323 to −294) was sufficient to induce CAT activity (Fig. 3B, lanes 1 and 2) by mutated *ras*; this activity was completely abolished if an altered DSE was used (lanes 3 and 4). No activation was observed with

FIG. 1. Induction of neurite development in PC12 cells. Photomicrographs of PC12 cells infected with retrovirus supernatants from psi-2 cells transfected with different pZIP-*ras* mutant DNAs. Cells were infected with pZIP-Ha-*ras*(normal) (a), pZIP-Ha-*ras*(61L) (b), pZIP-Ha-*ras*(12R) (c), pZIP-Ha-*ras*(59T) (d), pZIP-Ha-*ras*(117E) (e), pZIP-Ha-*ras*(61P) (f), pZIP-N-*ras*(12D) (g), pZIP-Ha-*ras*(61L, 186S) (h), pZIP-Ha-*ras*(61L, 117E) (i), and pZIP-Ha-*ras*(61L, 35A) (j). Photomicrographs were taken 5 (a to e and g to j) or 10 (f) days after infection. Bar = 20 μm.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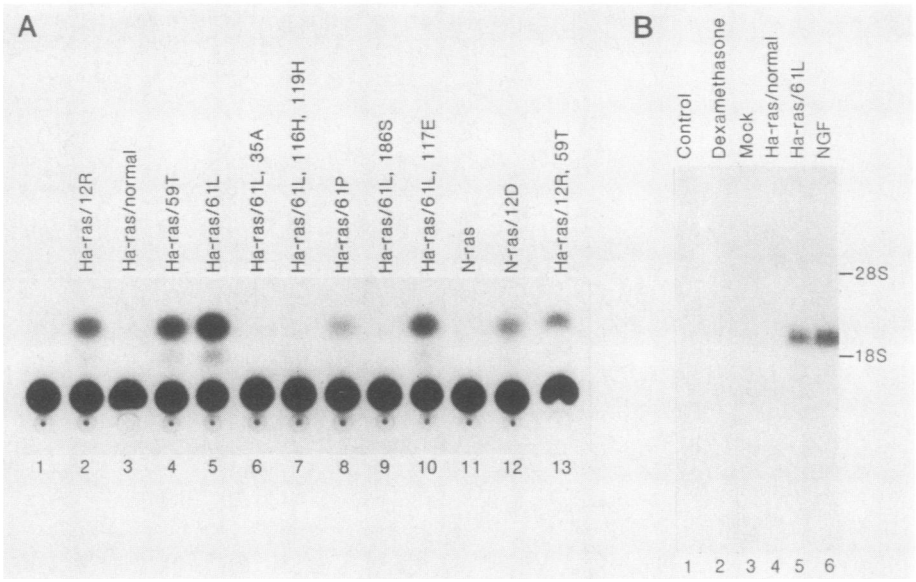

FIG. 2. Activation of c-*fos* transcription by transformation- and differentiation-competent *ras* mutants. (A) Effect of coexpression of normal or mutated *ras* proteins with the *fos*-CAT reporter plasmid FC4 (44) in PC12 cells. Basal expression of *fos*-CAT is represented in lane 1. Several Ha-*ras*-carrying expression vectors were cotransfected in equimolar ratios with the reporter plasmid. Results of several of these experiments are summarized in Table 1. (B) Northern blot analysis of c-*fos* expression in PC12 cells. Lane 1 (control) shows the basal c-*fos* RNA level in normally growing PC12 cells. Upon treatment with dexamethasone (lane 2) and in mock and Ha-*ras* (normal)-infected cells (lanes 3 and 4), there was no detectable increase in *fos* mRNA levels. Infection with Ha-*ras*(61L)-carrying virus and treatment with NGF for 30 min provoked an evident increase in c-*fos* mRNA synthesis (lanes 5 and 6).

the control TK-CAT plasmid (lanes 5 and 6). The DSE has also been shown to be responsible for serum, TPA, epidermal growth factor, and NGF induction of the *fos* gene (38, 47, 51, 52, 57). Consequently, it is likely that activated *ras* protein utilizes the same signal transduction pathway as do these inducers.

***ras*-induced differentiation and *fos* promoter activation are independent of the cAMP pathway.** Differentiation of PC12 cells to neuronlike cells can be induced by activated *ras* protein, NGF, and cAMP (5, 24, 25, 26, 29). The A126-1B2 cell line is a mutant PC12 cell line that lacks cAMP-dependent protein kinase II activity but has normal levels of the type I activity (53). NGF-induced neuronal differentiation is unaffected in these cells (53), and the c-*fos* promoter transcriptionally responds to NGF (57). Because the *ras* proteins in yeast cells are involved in adenylate cyclase regulation (49), we tested whether c-*fos* transcription would still be activated by *ras* in these cells. Induction of the c-*fos* promoter by an activated *ras* protein in A126-1B2 mutant cells was equivalent to that observed in normal PC12 cells (Fig. 3C). Previous studies have shown that somatostatin and c-*fos* CREs linked to a heterologous promoter are not inducible by forskolin in A126-1B2 cells, suggesting the requirement for cAMP-dependent protein kinase II (33, 45). Thus, it appears that activated *ras* protein induces c-*fos* gene expression via a pathway that does not involve cAMP-related signal transduction.

Activation of a TRE and cooperativity with the *jun* oncogene. Several lines of evidence indicate that both TPA and oncogenic *ras* proteins activate the PKC pathway (35). We found that the *ras*-responsive region in the c-*fos* promoter was also the serum- and TPA-responsive element located at −300 containing the DSE (Fig. 3B). Other TREs bind to transcription factor AP-1, which was first identified as a TPA-inducible nuclear protein (4, 31). AP-1 is the product of proto-oncogene *jun*, which itself is a serum- and TPA-inducible gene (30, 40, 41). Because of the apparent similarity of action between *ras* and TPA, we decided to test whether oncogenic *ras* protein could induce transcription from a TRE (binding site of AP-1). For this purpose we used TRE/TK-CAT, a plasmid in which the mouse metallothionein IIA TRE is linked to a TK-CAT fusion gene (Fig. 3A), cotransfected with a *ras* expression vector into PC12 cells. Only oncogenic *ras* (61L) activated transcription from a TRE, whereas normal *ras* had no effect (compare lanes 2 and 3 in Fig. 4). No induction was observed when a mutant TRE (Δ-72/TK-CAT; 42) was used (data not shown). Therefore, *ras* and TPA appear to act in an analogous manner in activating specific promoter elements.

Since a TRE is the binding site of transcription factor AP-1, we tested whether *jun* and *ras* products could cooperate in activating transcription from a TRE. Therefore, we cotransfected pSV-c-*jun*, a mouse *jun* expression vector (42) with either normal or oncogenic *ras*. As expected, pSV-c-*jun* activated transcription from TRE/TK-CAT (Fig. 4A, lane 4). We observed a further increase in transcription only when pSV-c-*jun* was cotransfected with an activated *ras* (61L) expression vector (lane 6) but not with the normal *ras* vector (lane 5).

It has previously been shown that the products of *fos* and *jun* proto-oncogenes associate and cooperate in activating transcription from a TRE (13, 20, 39, 42, 44). Therefore, we tested the effect of *fos* in conjunction with *ras* and *jun*. The presence of *fos* protein further augmented induction by an activated *ras* product (Fig. 4A, lanes 10 and 12).

Because of the sequence similarity between a TRE and a CRE (TRE, GTGACTCAG; CRE, GTGACGTCAG), we also tested whether oncogenic *ras* protein would induce transcription from such a promoter element. A rat somatostatin CRE linked to the TK-CAT promoter was not transcriptionally activated by either *ras* protein or TPA (Fig. 4B, lanes 3 to 5), whereas forskolin treatment produced 20-fold

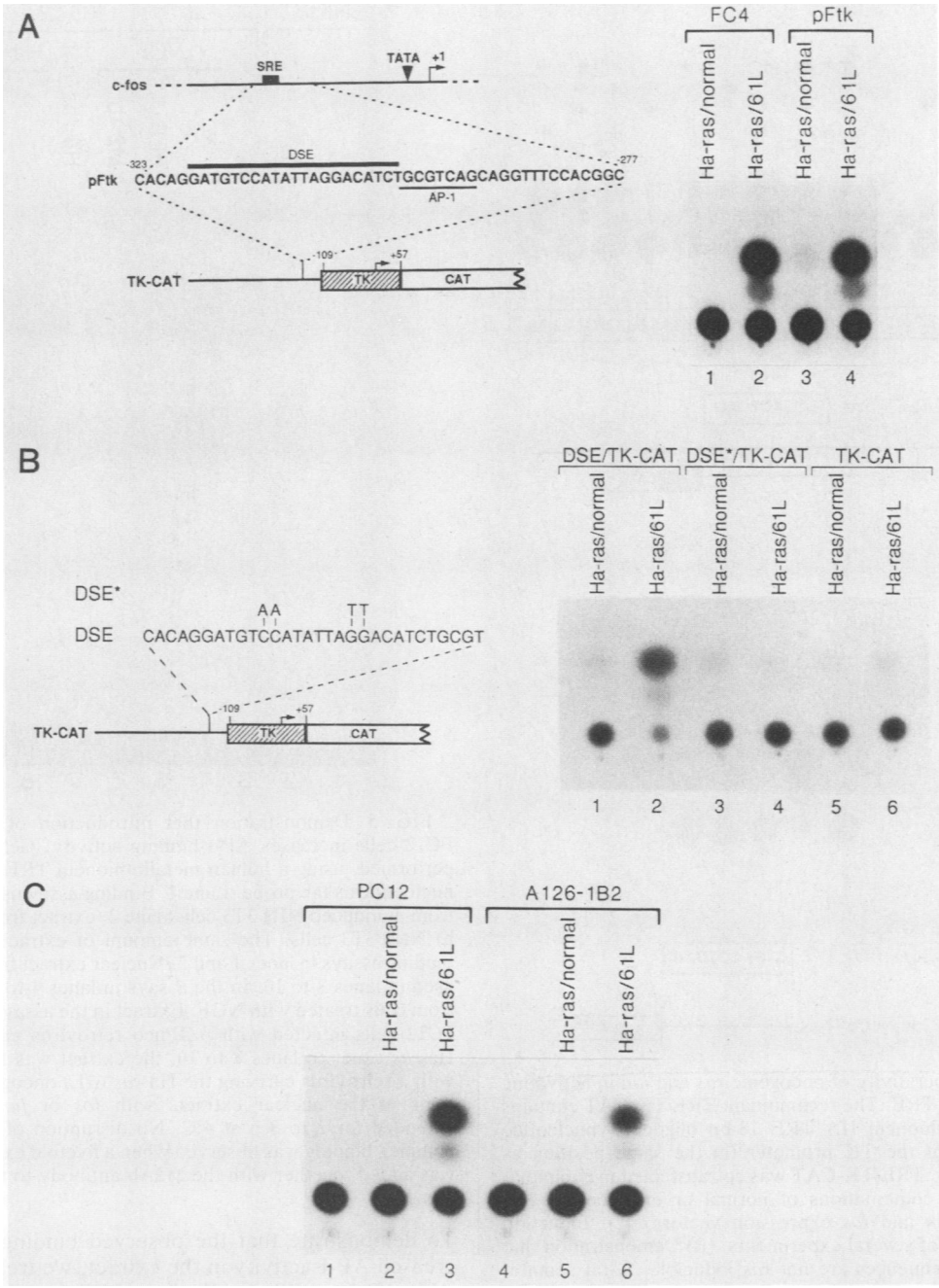

FIG. 3. (A) Ha-*ras*-responsive promoter element in the c-*fos* gene. Several subfragments of the c-*fos* promoter were cloned in the TK-CAT reporter vector (44). pFtk contains the fragment with sequences between −323 and −277 (fragment F in reference 44) and was found to respond to *ras* as efficiently as did the full c-*fos* promoter in recombinant FC4 (which contains the c-*fos* promoter region from −404 to +42) (compare lanes 1 to 4). (B) Demonstration that the DSE is sufficient for induction. A fragment of the DSE (−323 to −294) described previously (57) linked to the TK-CAT reporter vector was cotransfected as described above (lanes 1 and 2). The mutated DSE and TK-CAT plasmids were used as controls (lanes 3 to 6). (C) Demonstration that Ha-*ras* function does not require cAMP-dependent protein kinase. Shown is stimulation of *fos* promoter activity (FC4) in PC12 cells (lanes 1 to 3) and in the mutant cell line A126-1B2 (lanes 4 to 6), which lacks the cAMP-dependent protein kinase II.

induction (lane 2). Similar results were obtained with c-*fos* and α-chorionic gonadotropin CREs (not shown).

Oncogenic *ras* induces nuclear protein binding to a TRE. TPA treatment of HepG2 cells increases the binding of a specific nuclear factor, presumably AP-1, to a TRE (4). Since both TPA and oncogenic *ras* activate transcription from a TRE, we tested whether the same increase in DNA binding would be observed upon expression of oncogenic *ras*. After infection of PC12 cells with retroviruses carrying either a normal or an oncogenic-form (61L) c-Ha-*ras* gene, nuclear extracts were prepared and tested for DNA binding by using an oligodeoxynucleotide bearing the TRE homology of the human metallothionein IIA gene. Extracts from PC12 cells infected with an oncogenic *ras* virus showed a

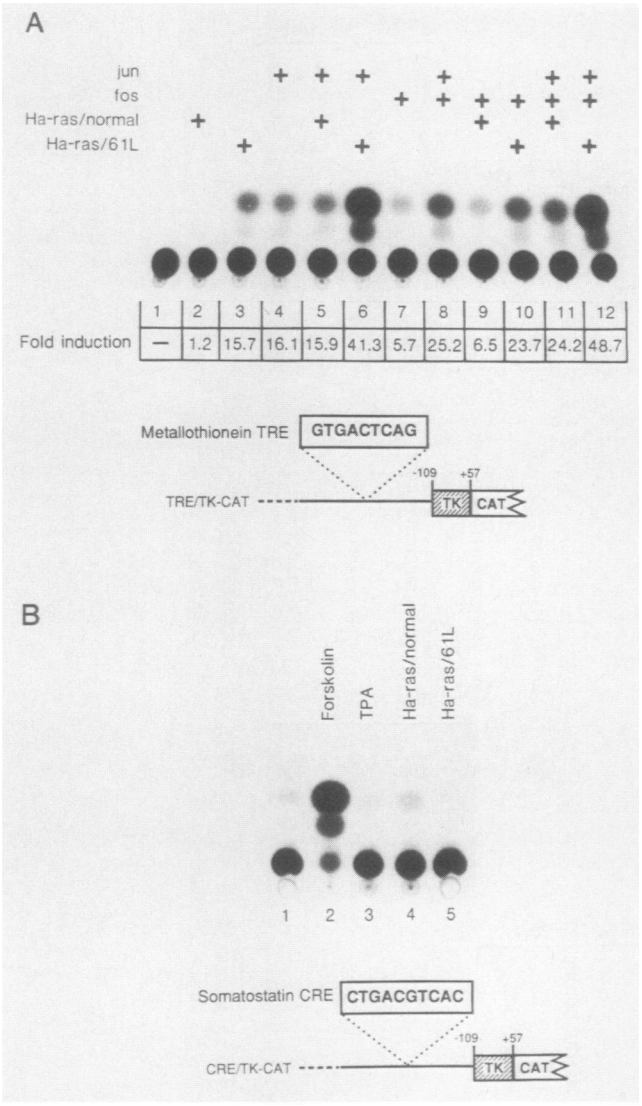

FIG. 4. (A) Cooperativity of oncogenic *ras* and *jun* in activating transcription from a TRE. The recombinant TRE/TK-CAT contains the human metallothionein IIA TRE 18-bp oligodeoxynucleotide inserted upstream of the TK promoter (in the same position as fragment F in Fig. 2). TRE/TK-CAT was cotransfected in equimolar ratios with several combinations of normal or oncogenic *ras* in conjunction with *jun* and *fos* expression vectors (42). Induction values are averages of several experiments. (B) Demonstration that cAMP-responsive sequences are not *ras* inducible. A rat somatostatin CRE oligodeoxynucleotide was cloned in the same position as the TRE in TRE/TK-CAT, producing a CRE/TK-CAT reporter plasmid. Although inducible by cAMP agonists such as forskolin (lane 2). CRE/TK-CAT did not respond to the coexpression of a *ras* oncogenic protein.

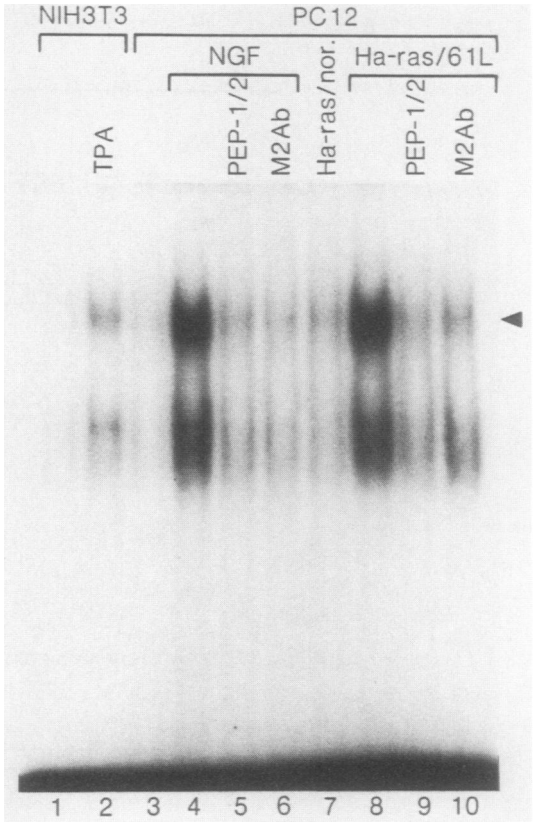

FIG. 5. Demonstration that introduction of oncogenic *ras* in PC12 cells increases AP-1-binding activity. Gel shift assays were performed, using a human metallothionein TRE 18-bp oligodeoxynucleotide as the probe. Lane 1, Binding assay using nuclear extract from uninduced NIH 3T3 cells; lane 2, extract from TPA-induced (1 h) NIH 3T3 cells. The same amount of extract was used for the binding assays in lanes 1 and 2. Nuclear extract from PC12 cells was used in lanes 3 to 10. In the assays in lanes 4 to 6, the extract was from cells treated with NGF. Extract in the assay in lane 7 was from PC12 cells infected with pZIPneo retrovirus carrying the normal Ha-*ras* gene. In lanes 8 to 10, the extract was from cells infected with a retrovirus carrying the Ha-*ras*(61L) oncogene. Immunoreactions of the nuclear extracts with *fos* or *jun* antibodies were extended for 2 to 8 h at 4°C. No disruption of the nucleoprotein complex binding was observed when a fivefold excess of M2 peptide was added together with the M2Ab antibody to the extract.

three- to fourfold increase in TRE binding (Fig. 5, lane 8) in comparison with extracts of uninfected cells (lane 3) or cells infected with the normal *ras* virus (lane 7). Because of the functional similarity between oncogenic *ras* and NGF with regard to inducing differentiation of PC12 cells, we also tested nuclear extracts from PC12 cells treated for 30 min with NGF (lanes 4 to 6). Even in this case, enhanced binding was observed (compare lanes 3 and 4). Interestingly, similar enhancement in DNA binding was obtained with an extract from PC12 cells treated for 8 h with NGF (data not shown).

To demonstrate that the observed binding was due to increased AP-1 activity in the extract, we treated the extracts with AP-1-specific antibodies (PEP-1 and PEP-2; 10). As expected, inhibition of AP-1 binding was observed (lanes 5 and 9). Because the binding activity of AP-1 is modulated by the *fos* product (p55^{fos}) (43), treatment of the extracts with anti-*fos* antibody (M2Ab; 15) also produced an inhibition of TRE binding (lanes 6 and 10).

DISCUSSION

Concordance between differentiation and *fos* induction. Cells proliferate or differentiate in response to internal or external signals. Generally, cells undergoing terminal differentiation stop dividing. What are the molecular mechanisms that trigger the commitment of a cell to these two seemingly disparate pathways? Oncogenes are apparently good candidates for study of these mechanisms because their aberrant expression can lead to both cell transformation and cell

differentiation (9). The ability of oncogenic *ras* protein to cause cellular transformation of one cell type (NIH 3T3) and differentiation of another (PC12) offered a good model with which to study common elements in these two processes.

A consequence of cell proliferation, transformation, and differentiation is modulation of gene expression (54). In particular, nuclear proto-oncogenes are induced in response to a variety of mitogenic and differentiation-inducing agents (23, 42a, 55). We chose to study transcription of the *fos* gene because (i) microinjection of *ras* protein has been shown to induce the expression of *fos* protein (48), (ii) activated *ras* protein can induce differentiation of PC12 cells similar to that observed after addition of NGF (5, 26), and (iii) NGF differentiation of PC12 cells is accompanied by expression of the *fos* gene (14, 23, 29). Results shown here indicate that differentiation of PC12 cells by activated *ras* protein is also accompanied by expression of the *fos* gene. Furthermore, there is a complete correlation between the ability of *ras* protein to transform fibroblasts and differentiate PC12 cells to neuronlike cells and its ability to induce expression of the *fos* gene (Table 1). In contrast, the normal c-Ha-*ras* gene product is unable to perform either function or induce c-*fos* gene expression (Fig. 1 and Table 1). It is therefore tempting to speculate that transformation and differentiation induced by *ras* may be mediated by *fos* protein.

Delineation of a *ras*-responsive element in c-*fos*. What are the target sequences in the c-*fos* promoter that respond to induction by activated *ras* protein and NGF? The DSE in the c-*fos* promoter is sufficient for induction with both agents (Fig. 3). The DSE is also responsible for c-*fos* induction with TPA and growth factors such as NGF and epidermal growth factor and is the binding site for nuclear factor SRF (21, 38, 47, 51, 52, 56). A common characteristic of the inducible agents is the ability to activate the PKC pathway (35). For instance, it has been shown that in both *ras*-transformed cells and NGF-stimulated PC12 cells, there is increased phosphatidylinositol turnover, which leads to the formation of 1,2-diacylglycerol, an activator of PKC and inositol 1,4,5-triphosphate, which mobilizes intracellular Ca^{2+} (35). Since *ras* proteins share homology to the regulatory G proteins such as Gs, which modulates the adenylate cyclase pathway, it was interesting that induction of the *fos* gene by activated *ras* was unaffected in a cell line (A126-1B2) defective in cAMP-dependent protein kinase II (Fig. 3C). Furthermore, a CRE linked to a heterologous promoter and CAT gene was not induced by either normal or oncogenic *ras* protein (Fig. 4B). It is therefore likely that, in contrast to the yeast *ras* protein, mammalian *ras* proteins may not require the adenylate cyclase pathway to manifest their biochemical functions (6, 8).

***ras* modulation of AP-1 activity.** Both oncogenic *ras* and the phorbol ester TPA activate the PKC pathway (35). Furthermore, like TPA, oncogenic *ras* also induces transcription from a TRE (Fig. 4), presumably by activating the nuclear factor AP-1, the product of nuclear oncogene *jun* (28, 46). The binding of AP-1 to its cognate TRE is enhanced in the presence of oncogenic *ras* protein but not by the normal cellular homolog (Fig. 5). Accordingly, activated $p21^{ras}$ augments transcription from the TRE when cotransfected with a c-*jun* expression vector (Fig. 4A). Since *fos* and *jun* oncoproteins cooperatively bind and increase transcription from a TRE (42, 43), we tested whether an oncogenic *ras* protein would increase transcription even further. Cotransfections of oncogenic *ras* (p21), *fos*, and *jun* (AP-1) proteins led to higher activation of a TRE (Fig. 4A). Again, the normal *ras* protein failed to exhibit any enhancement of TRE-dependent transcription.

The precise mechanism of transcriptional TRE activation by mutated $p21^{ras}$ is still unclear. However, one scenario is that oncogenic *ras* induces the transcription of *fos* and possibly *jun*, which then functionally cooperate to increase transcription of TRE-containing genes. Thus, the molecular mechanism governing transformation by *ras* would entail increased gene expression by virtue of activation or induction of *fos* and *jun* oncoproteins. While induction involves de novo transcription of *fos* and *jun*, activation may also occur by modification of existing proteins (for instance, by phosphorylation).

Signal transduction and differentiation. Is activation of the PKC pathway sufficient to induce neuronal differentiation of PC12 cells? Apparently not, because, unlike NGF and oncogenic *ras*, TPA by itself cannot induce differentiation of PC12 to neuronlike cells despite the fact that it can (i) activate the PKC pathway (35, 36); (ii) increase binding of *jun*-encoded AP-1 to a TRE (4, 31); (iii) induce the c-*fos* gene through the DSE, the same element that is responsive to NGF (47, 56); and (iv) induce c-*jun*-encoded AP-1 expression (30, 40, 41). Similarly, NGF, which can induce differentiation, is unable to induce transcription from a TRE (unpublished data) even though it increases AP-1 binding to a TRE (Fig. 5; 39). It is therefore likely that agents which induce proliferation or differentiation operate through both convergent and divergent pathways, leading to gene expression. Presumably, mutated *ras* proteins with either decreased GTPase activity or reduced GTP binding are able to influence the signal transduction pathway leading to altered gene expression. Since a soluble form of oncogenic *ras* (61L, 186S) is both transformation (59, 60) and differentiation deficient and is unable to induce *fos* expression, membrane localization is apparently crucial to initiate all of these *ras* functions. Furthermore, the inability of an oncogenic *ras* with an effector domain mutation to induce either differentiation or *fos* is consistent with the GTPase-binding protein being the downstream target for both transformation and differentiation. Together, these results suggest that *ras* may modulate a common signal transduction pathway that is responsible for both its transforming and differentiating activities. We believe that modulations of the activity of factors such as SRF and AP-1 are likely to be crucial in understanding the links between signal transduction and transcriptional regulation.

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