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Mitogenic Signaling By Colony-Stimulating Factor 1 and *ras* Is Suppressed by the *ets-2* DNA-Binding Domain and Restored by *myc* Overexpression

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The activity of p21^{ras} is required for the proliferative response to colony-stimulating factor 1 (CSF-1), and signals transduced by both the CSF-1 receptor (CSF-1R) and p21^{ras} stimulate transcription from promoter elements containing overlapping binding sites for Fos/Jun- and Ets-related proteins. A sequence encoding the DNA-binding domain and nuclear localization signal of human c-*ets-2*, which lacked portions of the c-*ets-2* gene product necessary for *trans* activation, was fused to the bacterial *lacZ* gene and expressed from an actin promoter in NIH 3T3 cells expressing either the v-*ras* oncogene or human CSF-1R. Nuclear expression of the Ets-LacZ protein, confirmed by histochemical staining of β-galactosidase, inhibited the activity of *ras*-responsive enhancer elements and suppressed morphologic transformation by v-*ras* as well as CSF-1R-dependent colony formation in semisolid medium. When CSF-1R-bearing cells expressing the Ets-LacZ protein were stimulated by CSF-1, induction of c-*ets-2*, c-*jun*, and c-*fos* ensued, but the c-*myc* response was impaired. Enforced expression of the c-*myc* gene overrode the suppressive effect of *ets-lacZ* and restored the ability of these cells to form colonies in response to CSF-1. NIH 3T3 cells engineered to express a CSF-1R (Phe-809) mutant similarly cannot form CSF-1-dependent colonies in semisolid medium and exhibit an impaired c-*myc* response, but expression of an exogenous *myc* gene resensitizes these cells to CSF-1 [M. F. Roussel, J. L. Cleveland, S. A. Shurtleff, and C. J. Sherr, Nature (London) 353:361–363, 1991]. The ability of these cells to respond to CSF-1 was also rescued by enforced expression of an endogenous c-*ets-2* gene. The *ets* family of transcription factors therefore plays a central role in integrating both CSF-1R and *ras*-induced mitogenic signals and in modulating the *myc* response to CSF-1 stimulation.

The *ras* genes encode small GTP-binding proteins whose functions are important for both cell growth and differentiation (1). These proteins are critical regulators of developmental decisions in nematodes (2, 14) and fruit flies (32), where they act downstream of growth factor receptors in signal transduction. In mammalian cells, activation of growth factor receptor tyrosine kinases augments the amount of GTP bound to p21^{ras} (9, 11, 24, 30, 31), possibly by increasing the rate of exchange of GTP for p21^{ras}-bound GDP or by inhibiting GTP hydrolysis. Antibodies to *ras* and dominant suppressive *ras* mutants can block serum-stimulated mitogenesis in NIH 3T3 cells (6, 20) as well as neurite outgrowth in PC12 cells induced by nerve growth factor (13, 18, 34). Moreover, introduction of the p21^{ras} GTPase activating protein (GAP) into cells can suppress transformation induced by constitutive receptor or cytoplasmic tyrosine kinases (3, 7, 21). Although the proximal effectors of *ras* in mammalian cells have not been well defined, *ras* action can modulate the activities of *raf-1*, MAP, and ribosomal S6 (RSK) serine/threonine kinases (35, 40), which in turn affect the expression of genes required for cell proliferation (5, 16). Microinjected antibodies to p21^{ras} do not suppress cell transformation by an oncogenic v-*raf* allele, consistent with the concept that the *raf-1* kinase acts downstream of p21^{ras} (33).

The study of *trans*-activating factors and *cis*-regulatory promoter elements responsible for governing gene expression in response to *ras* activation has helped to pinpoint other distal elements in these signaling pathways (12, 22, 23, 37). A *ras*-responsive element (RRE) related to oncogene-responsive enhancers defined in polyomavirus (PEA3) (37) is shared by the mouse NVL-3 retrotransposon, the human transforming growth factor β-1 gene, and several other acute-phase and mitogen-responsive genes (22, 23, 37). The RRE is composed of overlapping binding sites for nuclear transcription factors of the Fos/Jun and Ets families (12, 25, 36) and binds p68$^{c\text{-}ets\text{-}1}$ (36) as well as a 120-kDa *ras*-responsive factor functionally related to known *ets*-coded proteins (25). The presence of this DNA-binding motif may help to define a set of genes that are transcriptionally regulated by both *ras* and tyrosine kinases (12, 22, 23, 25, 36, 37). The *ets-1* and *ets-2* gene products bind to DNA sequences closely related to the RRE consensus CAGGAAGT (12, 15, 25, 37) and can collaborate with FosJun heterodimers to activate the polyomavirus oncogene-responsive element (36). Both the Ets-1 and Ets-2 proteins contain distinct domains for *trans* activation and DNA binding, located in their amino- and carboxyl-terminal portions, respectively, thereby allowing these functions to be separated (12, 36). Therefore, by introducing an Ets DNA-binding domain without its *trans*-activating sequences into susceptible target cells, it should be possible to dominantly suppress the transcription of *ets*-responsive genes.

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The growth of fibroblasts transformed by a constitutively activated colony-stimulating factor 1 (CSF-1) receptor (CSF-1R) kinase encoded by the v-*fms* oncogene can be suppressed either by microinjection of antibodies to p21^{ras} (33) or by overexpression of the catalytic domain of GAP (3). Similarly, the enforced expression of the GAP catalytic domain in NIH 3T3 cells expressing human CSF-1R inhibited the activation of genes controlled by RREs and suppressed CSF-1-induced mitogenesis (3). To explore whether such responses might indeed be mediated, at least in part, by proteins of the Ets family, we attempted to use the Ets DNA-binding segment without its *trans*-activating domain to dominantly suppress signal transduction and mitogenesis induced by both *ras* and CSF-1R. We fused the DNA binding domain of c-*ets-2* to the prokaryotic *lacZ* gene and introduced it into NIH 3T3 cells expressing either human CSF-1R or v-*ras*. The *ets-lacZ* gene suppressed the activity of both the NVL-3 *ras*-responsive enhancer and an RRE fused to a reporter gene; selectively inhibited CSF-1 induced c-*myc* expression without affecting the immediate-early responses of c-*jun*, c-*fos*, and c-*ets-2* itself; induced morphological reversion of *ras*-transformed cells; and blocked CSF-1-stimulated colony formation in semisolid medium. The inhibitory effects of *ets-lacZ* on CSF-1-induced colony formation were overridden by constitutive c-*myc* expression, implying that proteins of the *ets* gene family act to directly or indirectly modulate *myc* function during the proliferative response to growth factor stimulation.

MATERIALS AND METHODS

Cells and culture conditions. NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 9.5 mM glutamine, and 100 μg of mezlocillin per ml. Purified recombinant human CSF-1 was provided by Steven C. Clark (Genetics Institute, Cambridge, Mass.). For stimulation of NIH 3T3 cells expressing either exogenous human CSF-1R or a CSF-1R mutation containing a Phe for Tyr substitution at residue 809 (29), subconfluent cultures were incubated in complete medium containing 0.1% fetal bovine serum for 48 h in order to induce quiescence prior to CSF-1 treatment. Unless otherwise indicated, cells were stimulated with 2,000 U (1 U = 0.44 fmol) of CSF-1 per ml, which is sufficient for maximal mitogenicity. Colony formation in agar was performed as previously described (27) by using concentrations of CSF-1 from 20 to 2,000 U/ml, the latter representing a maximal mitogenic dose.

Expression vectors. The 3′ region of human c-*ets-2* DNA encoding C-terminal amino acids 334 to 466 (39) was cloned by the polymerase chain reaction with cDNA prepared from the EJ human bladder carcinoma cell line (3). The primers 5′-CCGGATCCAACATGGATTACATCCAAGAGAGGA-3′ and 5′-GGAGATCTGGCTGGACGCCC-3′ were used to place a methionine initiation codon (underlined above) in frame with aspartic acid residue 334 in c-*ets-2* (39). The identity of the polymerase chain reaction product was confirmed by determining its nucleotide sequence, and the DNA was digested with *Bam*HI and *Bgl*II and cloned into the *Bam*HI site of the collagenase linker–β-galactosidase fusion protein expression vector, pHβApr-1 neo-CB-17 (10), and designated pAPr-etsZ-neo. This plasmid contains the actin promoter (Apr) and the neomycin resistance gene (*neo*), which encodes resistance to the drug G418 (Geneticin; Sigma Chemical Co., St. Louis, Mo.). As a control, *ets-2* sequences were also cloned in the antisense (AS) orientation into the same vector, yielding pAPr-etsZ/AS-neo. To enable transfections in which neomycin resistance could instead be specified by an accompanying plasmid, the c-*ets-2* DNA fragment was cloned downstream of the actin promoter in a derivative plasmid lacking simian virus 40 promoter-driven *neo* (pAPrZ, obtained from Elwood Linney) and designated pAPr-etsZ. A Moloney murine leukemia virus-based vector containing the complete human c-*ets-2* coding sequence was generously provided by Jacques Ghysdael, Institut Curie, Orsay, France. pZIP6-RAS (8) is a Harvey v-*ras* retrovirus expression vector that also produces a spliced *neo* transcript. The *ras*-responsive luciferase reporter construct, pRD053, was described previously (22).

Expression of the Ets-LacZ fusion protein. Plasmid DNAs were introduced into NIH 3T3 cells after precipitation by calcium phosphate (22). For *ras* transformation assays, 9 μg of either the pAPr-etsZ plasmid or the parental pAPr-Z vector lacking the *ets* moiety was cotransfected with 1 μg of pZIP6-RAS. The cells were allowed to recover for 24 h, after which each 60-mm-diameter culture was split into three 100-mm-diameter dishes. Neomycin-resistant colonies recovered 2 weeks after selection in 400 μg of G418 per ml were scored microscopically as either morphologically flat or transformed. Cells expressing both human CSF-1R and Ets-LacZ were generated by cotransfection of NIH 3T3 cells with pAPr-etsZ-neo and a CSF-1R retrovirus vector lacking a dominant selectable gene (27). Drug-resistant colonies selected in 400 μg of G418 per ml were pooled and sorted for CSF-1R cell surface expression by using a monoclonal antibody specific for the extracellular domain of the CSF-1R (28). In both cases, cells were fixed and histochemically stained for β-galactosidase (10), and cell lysates were immunoblotted by using a mouse monoclonal antibody to β-galactosidase (Promega Biotechnology, Madison, Wis.) followed by binding with a peroxidase-conjugated rabbit antiserum to mouse immunoglobulin G and enhanced fluorographic detection (ECL kit; Amersham, Arlington Heights, Ill.). The expression of p21^{ras} was confirmed by Western blotting (immunoblotting) with rat monoclonal antibody Y13-259 to p21 (Oncogene Sciences Inc., Uniondale, N.Y.) followed by binding with a peroxidase-conjugated sheep anti-rat immunoglobulin G (Amersham) and enhanced chemiluminescence (ECL) detection. Transfections utilizing other retrovirus vector plasmids were performed as previously described (27).

Transient expression assays. A total of 3 μg of pRD053 plasmid DNA and either 13 μg of pAPr-etsZ-neo or pAPr-etsZ/AS-neo was cotransfected in the absence or presence of 1 μg of an activated Ha-c-*ras* expression vector (22) onto cells in 100-mm-diameter culture dishes (3). As an internal control, 3 μg of a vector encoding secreted alkaline phosphatase (SEAP) was included (22). Luciferase activity is represented as relative luminescence per microgram of protein extract and SEAP activity in milliunits per milliliter of culture medium (22).

RNA analysis. RNA was isolated and analyzed by S1 mapping with a 483-bp NVL-3 probe and a 419-bp probe to the neomycin resistance gene as previously described (3). Northern (RNA) blotting of mRNAs isolated after stimulation of quiescent cells with CSF-1 was performed by using uniformly labeled c-*fos*, c-*jun*, c-*ets-2*, c-*myc*, and K-*rev-1* cDNA probes (3).

TABLE 1. Cotransfection of *ets-lacZ* and Ha-v-*ras* into NIH 3T3 cells[a]

Vectors	Colony phenotype and frequency (no. [% β-Gal+[b]])		% Flat[c]
	Transformed	Flat	
Expt 1			
v-*ras* + pAPr	207 (0)	0 (0)	0
v-*ras* + pAPr-etsZ	88 (0)	67 (100)	43
Expt 2			
v-*ras* + pAPr	302 (0)	1 (0)	<1
v-*ras* + pAPr-etsZ	110 (0)	71 (100)	39

[a] A vector encoding both v-*ras* and a linked neomycin resistance gene was cotransfected into NIH 3T3 cells together with a second plasmid containing (pAPr-etsZ) or lacking (pAPr) a chimeric *ets-lacZ* gene. After selection in G418, surviving colonies were scored as morphologically flat or transformed and stained for β-galactosidase (β-Gal) activity.

[b] Percentage of colonies that stained for β-galactosidase activity.

[c] Percentage of total colonies that were not transformed.

RESULTS

The c-*ets-2* DNA-binding domain fused to *lacZ* suppresses v-*ras* transformation. A DNA segment encoding the C-terminal 130 amino acids of the human c-*ets-2* protein, including only its DNA-binding domain and sequences necessary for nuclear localization (4, 12, 15), was cloned in frame with the 5′ end of the *lacZ* gene under the control of the human β-actin promoter. This plasmid, termed pAPr-etsZ, was cotransfected into NIH 3T3 cells with a vector encoding both Harvey v-*ras* cDNA and a linked neomycin resistance gene. After 2 weeks of selection in G418, drug-resistant colonies were fixed and morphologically analyzed. In two independent experiments, approximately 40% of the colonies exhibited a flat, nontransformed phenotype characteristic of the parental NIH 3T3 cells, whereas the remainder were morphologically transformed (Table 1). In contrast, when the v-*ras*-containing plasmid was cotransfected with a β-actin-*lacZ* vector lacking the C-terminal c-*ets-2* domain (pAPr-Z), all drug-resistant colonies were morphologically transformed. These data suggested that expression of the *ets* DNA-binding domain could suppress transformation by v-*ras*.

Cells in representative morphologically flat or transformed colonies are shown in panels a and b, respectively, of Fig. 1. These cells were histochemically stained for β-galactosidase activity. Cells in flat colonies were stained exclusively in their nuclei (Fig. 1a), whereas independently arising transformed colonies derived from the same culture dish lacked detectable enzyme activity (Fig. 1b). When representative flat and transformed colonies were expanded and assayed for v-*ras* and Ets-2-LacZ protein by immunoblotting, both cell types showed elevated levels of $p21^{ras}$ protein compared with those in untransfected cells, but only flat colonies expressed a 143-kDa polypeptide reactive with antibodies to β-galactosidase (Fig. 1c). The size of this *lacZ* immunoreactive polypeptide is in good agreement with the predicted mass (145 kDa) of the Ets-LacZ fusion protein. The colonies assayed for $p21^{ras}$ and Ets-LacZ expression were tested for their abilities to grow in semisolid media. The cells that expressed only $p21^{ras}$ grew in soft agar, while those that expressed both $p21^{ras}$ and the Ets-LacZ polypeptide did not (data not shown). Thus, all G418-resistant colonies, regardless of their phenotype, expressed v-*ras*, but only those that coexpressed Ets-LacZ in the nucleus were nontransformed.

Ets-LacZ inhibits CSF-1-mediated colony formation. Engagement of *ras* signaling pathways is also important in the mitogenic response to CSF-1 (3, 11), so that introduction of the *ets-lacZ* gene into cells expressing CSF-1R should block certain downstream steps in the receptor signaling pathway. NIH 3T3 cells were therefore cotransfected with a vector encoding human CSF-1R together with pAPr-etsZ-neo, an *ets-lacZ* plasmid that directly confers G418 resistance. In order to select cells expressing genes encoded by both vectors, drug-resistant colonies were pooled and subjected to fluorescence-activated cell sorting by using a monoclonal antibody to the extracellular domain of CSF-1R (28). Introduction of the *ets-lacZ* plasmid had no significant effect on the growth of pooled, G418-resistant NIH 3T3 cells in serum-containing medium and therefore did not prevent subsequent selection of receptor-positive cells.

NIH 3T3 cells expressing CSF-1R can form colonies in agar in the presence of CSF-1, and their number is dependent upon the CSF-1 concentration at limiting doses (27). However, in cells expressing both CSF-1R and Ets-LacZ, colony formation in response to CSF-1 was severely inhibited (Table 2). As a control, the c-*ets-2* DNA segment was inserted into the same vector in the antisense orientation (pAPr-etsZ/AS-neo) and coexpressed with the CSF-1R plasmid by using the same strategy. The antisense *ets* plasmid had no effect upon the CSF-1-dependent growth of G418-resistant, receptor-bearing cells.

Extracts prepared from cells coexpressing CSF-1R and the *ets-lacZ* gene were analyzed by immunoblotting with a monoclonal antibody directed to β-galactosidase. These cells expressed the immunoreactive 143-kDa polypeptide seen in the v-*ras* revertant cell lines (Fig. 1d). This protein was not detected in cells transfected with the parental expression vector, pAPr. When cells expressing *ets-lacZ* were fixed and stained for β-galactosidase activity, staining was found to be exclusively nuclear, and none was observed in control cells transfected with the antisense *ets* construct (data not shown). The frequency and intensity of staining did not change after prolonged passage of the cells in culture, indicating that expression of the transfected *ets-lacZ* gene was stable.

The *ets-2* DNA-binding domain suppresses the activity of a *ras*-responsive enhancer. If the *ets-2* DNA-binding domain exerts its biologic effects by inhibiting the induction of *ras*- and CSF-1R-responsive genes, the activity of a reporter gene driven by a transcriptional control element containing the RRE should be inhibited in cells expressing the *ets-lacZ* gene. NIH 3T3 cells were therefore cotransfected with a luciferase reporter gene regulated by a functional RRE (pRDO53) together with a control plasmid containing a SEAP reporter gene regulated by the *ras*-nonresponsive Rous sarcoma virus promoter. The relative activities of luciferase (Fig. 2a, right panel) and SEAP (Fig. 2a, left panel) were determined in cells expressing the *ets-lacZ* gene introduced in either the sense (hatched bars) or antisense (solid bars) orientation. To confirm the response of the RRE to *ras*, the Ha-c-*ras* oncogene was introduced into some of the cultures (as indicated by the plus signs below the bar graph in Fig. 2a). High activity of the functional RRE, observed only in the presence of Ha-c-*ras*, was reduced sevenfold in cells transfected with a 2:1 molar excess of the sense *ets-lacZ* vector compared with that in its antisense counterpart (Fig. 2a, right panel). As expected, RRE-driven luciferase activity was considerably lower in cells lacking the H-c-*ras* oncogene, but a 2.7-fold decrease in relative luciferase activity was still observed in the presence of *ets-*

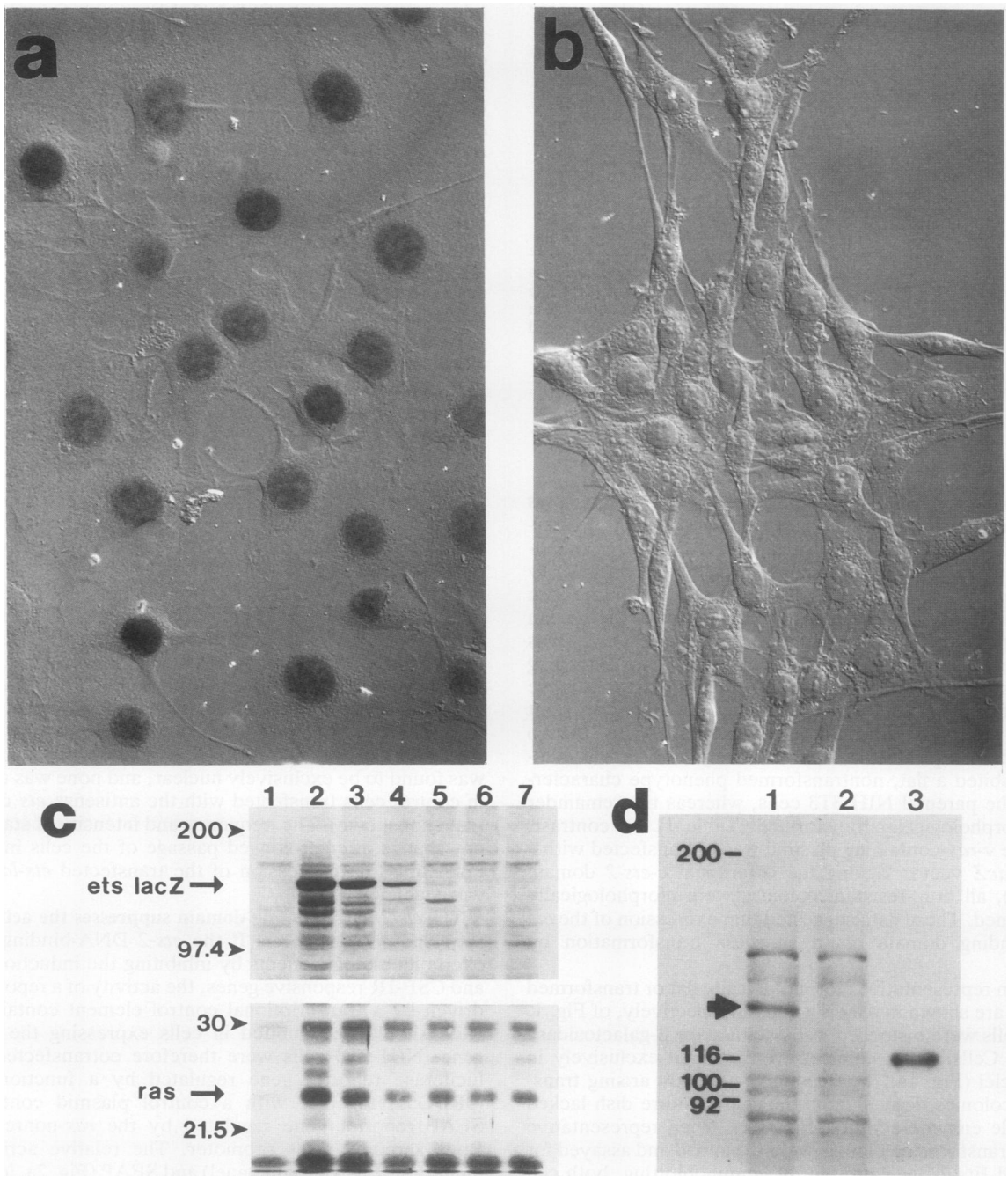

FIG. 1. Inhibition of *ras* transformation by *ets-lacZ* and expression of Ets-LacZ fusion protein. NIH 3T3 cells transfected with pZIP6-RAS and pAPr-etsZ expression vectors were selected in G418, and cells expanded from both flat and transformed colonies were stained for β-galactosidase activity. Shown are representative flat (a) and transformed (b) colonies; the dark nuclei in cells shown in panel a result from positive histochemical staining. (c) Western blots performed on lysates from normal 3T3 cells (lane 1) or flat (lanes 2 to 4) or transformed (lanes 5 to 7) pZIP6-RAS-containing cells. The top blot was incubated with an anti-β-galactosidase antibody, while the bottom blot was incubated with an anti-$p21^{ras}$ monoclonal antibody. Arrows show the positions of immunoreactive bands, and arrowheads show the positions (in kilodaltons) of rainbow protein markers (Amersham). The lysates used in lanes 2 and 7 were derived from the cells depicted in panels a and b, respectively. (d) Western blot performed on lysates from cells transfected with the CSF-1R and *ets-lacZ* genes (lane 1) or with the CSF-1R gene and the pAPr vector lacking *ets* sequences (lane 2). Lane 3 contains authentic β-galactosidase. The arrow designates the location of the fusion protein, and the positions (in kilodaltons) of ^{14}C-labeled protein standards (Amersham) are also indicated. For both panel c and panel d, denatured proteins were electrophoretically separated on polyacrylamide gels containing sodium dodecyl sulfate, transferred to nitrocellulose, and probed with antibodies to either β-galactosidase (c [top] and d) or $p21^{ras}$ (c [bottom]).

TABLE 2. Effects of *ets-lacZ* on CSF-1-induced colony formation[a]

Vector(s)	Efficiency of colony formation (% of cells plated) at following CSF-1 concn (U/ml):				
	0	2,000	100	50	20
CSF-1R alone	0	16.1	9.8	2.4	1.1
CSF-1R + *ets-lacZ*	0	0.9	0	0	0
CSF-1R + *ets*/AS-*lacZ*	0	16.8	7.0	5.6	2.0
CSF-1R + *myc*	0	14.6	6.0	4.5	0.5
CSF-1R + *ets-lacZ* + *myc* (mass population)	0	3.1	0.2	0	0
CSF-1R + *ets-lacZ* + *myc* (subclones)					
Clone 1	0	7.0	ND	ND	ND
Clone 2	0	9.2	ND	ND	ND
Clone 3	0	10.4	ND	ND	ND
Clone 4	0	8.9	ND	ND	ND
CSF-1R (Phe-809)	0	0	0	0	0
CSF-1R (Phe-809) + *myc* (mass population)	0	9.4	3.8	ND	ND
CSF-1R (Phe-809) + *ets-2* (mass population)	0	7.1	1.4	0	0
CSF-1R (Phe-809) + *ets-2* (subclones)					
Clone 1	0	16.1	ND	ND	ND
Clone 2	0	5.8	ND	ND	ND
Clone 3	0	8.9	ND	ND	ND

[a] Vectors containing *ets-lacZ* in the sense or antisense (AS) orientation also contained the *neo* gene. The *ets-lacZ* vectors were cotransfected with a second plasmid encoding human CSF-1R, and G418-resistant cells were subjected to fluorescence-activated cell sorting with a monoclonal antibody to the receptor extracellular domain (28). About 60% of the original G418-resistant population was receptor positive by these criteria. Cells expressing human CSF-1R alone or the Phe-809 mutant were obtained by cotransfection of retroviral c-*fms* plasmids with a second plasmid containing the *neo* gene. Where indicated, cells expressing either wild-type or mutant CSF-1R were infected for 7 days prior to plating in semisolid medium with a *myc*-containing retrovirus in order to generate uncloned (mass) populations expressing exogenous c-*myc* (26). Alternatively, G418-resistant cells expressing wild-type or mutant CSF-1R were cotransfected with a retroviral vector containing the complete *ets-2* coding sequence together with a second plasmid encoding hygromycin resistance. In the latter case, uncloned (mass) populations of colonies were obtained after 3 weeks of selection with 100 μg of hygromycin per ml. Cells expressing the indicated genes were plated in soft agar (5,000 cells per dish) in medium containing the indicated concentrations of CSF-1 (1 U = 0.44 fmol), and colonies were enumerated after 2 weeks (27). Representative colonies derived from single cells were picked from semisolid medium and expanded, and the clones were replated. Northern blotting analysis confirmed high levels of expression of either exogenous *myc* or *ets-2* mRNAs in the CSF-1-responsive subclones. ND, not determined.

lacZ. In contrast, the activity of the control, *ras*-nonresponsive reporter plasmid was not significantly affected by *ets-lacZ*, either in the presence or absence of *ras* (Fig. 2a, left panel). Inhibition of the functional RRE by the sense *ets-lacZ* gene was also observed when stably expressed v-*fms* was substituted for transiently expressed *ras* in this assay (data not shown).

Induction of retrotransposon NVL-3 mRNA in CSF-1R-bearing NIH 3T3 cells occurs as part of the delayed early response to CSF-1 stimulation (3). S1 nuclease mapping was therefore used to determine whether CSF-1 could activate the NVL-3 gene in cells coexpressing CSF-1R and the Ets-LacZ protein (Fig. 2b). For the interval shown, CSF-1 stimulation of NVL-3 RNA synthesis in cells containing *ets-lacZ* was decreased 10-fold compared with the RNA levels in cells expressing CSF-1R alone. However, expression of the simian virus 40 early promoter-driven *neo* gene present in both cell types was not affected. In cells expressing *ets-lacZ*, no further increase in NVL-3 RNA was seen between 8 and 24 h after CSF-1 stimulation, whereas a further four- to fivefold increase was observed in the control cells (data not shown). Thus, repression of genes containing RREs represents one mechanism by which the *ets-2* C-terminal domain might block signaling by both *ras* and *fms*/CSF-1R.

Constitutive c-*myc* expression enables cells suppressed by *ets-lacZ* to respond to CSF-1. To further determine the effects of the Ets-LacZ protein on gene expression, we analyzed the ability of ligand-activated CSF-1R to stimulate the expression of canonical immediate-early genes. NIH 3T3 cells expressing either CSF-1R alone or CSF-1R plus the Ets-LacZ protein were stimulated with CSF-1, and RNA extracted at various times after growth factor treatment was analyzed by Northern blotting (Fig. 3). In each case, blots hybridized with radiolabeled probes that detected the genes of interest were rehybridized with a probe specific for the constitutively expressed K-*rev-1* gene (3) to demonstrate that similar quantities of RNA were analyzed at each point after CSF-1 stimulation. Expression of c-*jun*, c-*fos*, and c-*ets-2* mRNAs was augmented by CSF-1 in the two cell lines, but the induction of c-*myc* mRNA was markedly impaired in cells expressing *ets-lacZ* (Fig. 3).

Because c-*myc* expression is essential for the proliferative response to CSF-1 (26), we reasoned that the enforced expression of c-*myc* in cells bearing both CSF-1R and *ets-lacZ* might reconstitute their biologic responsiveness to the growth factor. These cells were therefore infected with a c-*myc*-containing mouse retrovirus, and 1 week after infection, they were seeded in semisolid medium in the presence or absence of CSF-1. Cells from the c-*myc*-infected cultures conditionally formed colonies only in medium containing the growth factor (Table 2), whereas cells infected with the retroviral vector alone did not respond (data not shown). When four representative CSF-1-responsive colonies were expanded and subjected to Northern blotting analysis, each subclone expressed large amounts of c-*myc* mRNA and continued to stably express the Ets-LacZ protein (data not shown). The subclones yielded agar colonies only in the presence of CSF-1 at two- to threefold-higher frequencies than that observed with the uncloned, mass-infected parental population (Table 2). Thus, *myc* expression can override the inhibitory effects of *ets-lacZ* on CSF-1-induced colony formation.

***ets-2*-mediated rescue of the CSF-1 response in cells bearing a partially defective CSF-1R signal transduction mutant.** A CSF-1R mutant containing a phenylalanine for tyrosine

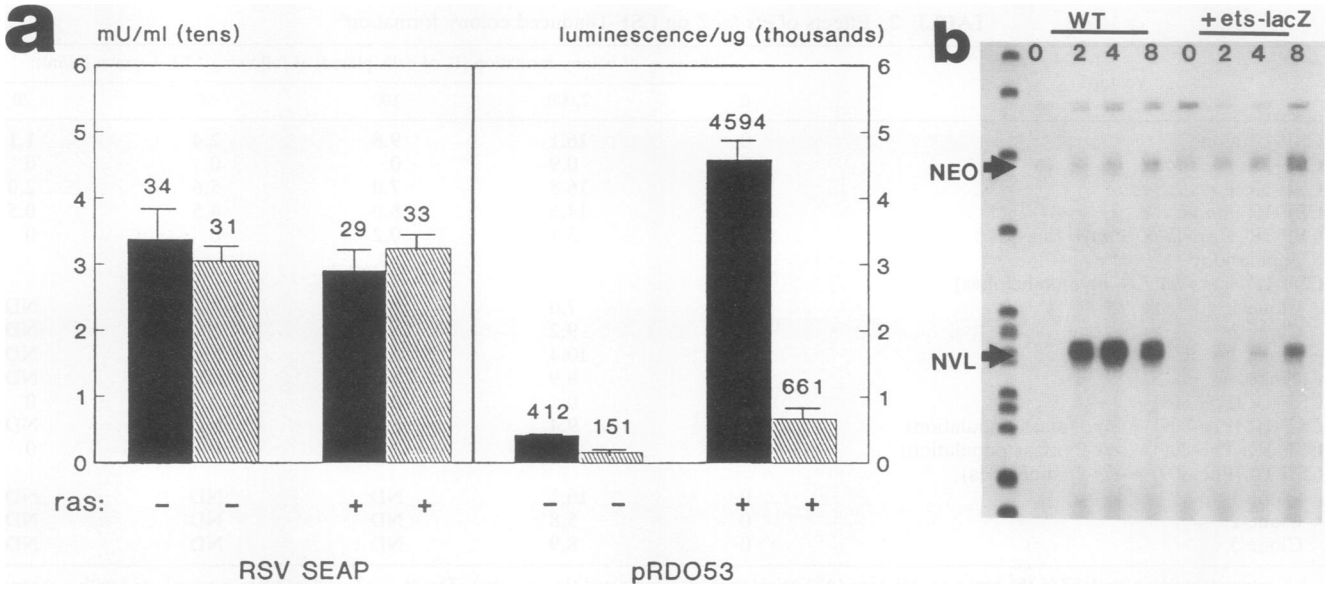

FIG. 2. Inhibition of NVL-3 activation by *ets-lacZ*. (a) Transient transfections were performed by using reporter plasmids which contained (pRDO53) or lacked (RSV SEAP) a functional RRE. Each 100-mm-diameter culture dish was transfected with 3 μg (each) of luciferase and SEAP reporter plasmids, 13 μg of either the *ets-lacZ* expression vector (hatched bars) or a control vector containing *ets* sequences in the inverse orientation (solid bars), and 1 μg of an activated Ha-c-*ras* expression vector (+). The values on the left ordinate indicate secreted alkaline phosphatase activity (milliunits/milliliter of culture medium), and on the right ordinate are the luciferase activities (presented as relative luminescence per microgram of protein). The average values measured for the experiments are given at the tops of the individual bar graphs and represent the average of three independent experiments, each involving duplicate measurements. Error bars indicate standard error of the experiments. (b) NIH 3T3 cells expressing human CSF-1R and containing pAPr (WT) or coexpressing CSF-1R and *ets-lacZ* (+ets-lacZ) were stimulated with CSF-1 for the times (in hours) indicated above each lane. Total cellular RNA was isolated at the indicated times, and 10 μg was subjected to S1 nuclease mapping with NVL-3 or SV-neo probes. Arrows designate the protected fragments.

substitution at residue 809 within the intracellular tyrosine kinase domain is defective in transducing a proliferative response to CSF-1, despite the fact that its stimulation induces a series of immediate-early response genes, including c-*fos* and *junB* (29). As in cells that expressed wild-type CSF-1R (with or without *ets-lacZ*) (Fig. 3), *ets-2* mRNA was constitutively expressed in cells bearing CSF-1R (Phe-809) and was weakly induced within 1 h of CSF-1 treatment (data not shown). In contrast, the CSF-1-induced c-*myc* response in these cells is severely impaired, but enforced expression of an exogenous *myc* gene resensitizes the cells to CSF-1 stimulation (26) (cf. Table 2). Because constitutive *myc* expression enabled cells suppressed by *ets-lacZ* to respond to CSF-1, we reasoned that cells expressing CSF-1R (Phe-809) might also be rendered CSF-1 responsive if they were engineered to express an exogenous *ets* gene at high levels. Indeed, insertion of *ets-2* into these cells rescued their CSF-1 response (Table 2). When individual agar subclones were expanded and analyzed by Northern blotting, relatively high levels of vector-driven *ets-2* mRNA were constitutively expressed (data not shown). In general, the frequencies of CSF-1-dependent colonies formed with subclones expressing CSF-1R (Phe-809) plus exogenous *ets-2* (range 6 to 16%) (Table 2) were less than those of subclones expressing both CSF-1R (Phe-809) and exogenous *myc* (range, 16 to 25%) (26). This suggests that *ets* family members other than *ets-2* might normally function as the key regulators of the CSF-1 response.

DISCUSSION

ras-responsive enhancer elements are composed of binding sites for two classes of nuclear factors, including Ets family members and Fos/Jun heterodimers, with both sites being required for oncogene responsiveness (12, 25, 36, 37). Activation of *ras*-responsive genes appears necessary for the morphological transformation of NIH 3T3 cells by v-*ras*, because a chimeric gene containing the *ets* DNA-binding domain fused to *lacZ* can dominantly suppress both effects. Moreover, the *raf-1* kinase, which functions downstream of $p21^{ras}$ (16, 20, 33, 35, 40), *trans* activates the polyomavirus oncogene-responsive enhancer through an AP1 (*fos/jun*) binding site (5, 38), and dominant negative mutations of either *raf-1* (16) or c-*jun* (19) can also act as antioncogenes in *ras*-transformed NIH 3T3 cells. Thus, *ras* action appears to be central to the cooperative *trans* activation of oncogene-responsive enhancers by both Ets and Fos/Jun proteins. Suppression of *ras* action by microinjection of antibodies to $p21^{ras}$ (33), by overexpression of the catalytic domain of GAP (3), or by the enforced expression of *ets-lacZ* also inhibits the proliferative response to CSF-1, implying that $p21^{ras}$ functions downstream of CSF-1R in relaying mitogenic signals.

Although our results indicate that the mechanism of transformation suppression by the Ets-LacZ protein involves a selective inhibition of gene expression, they do not address which *ets*-related factors are the actual targets of the CSF-1R and *ras*-mediated signal transduction pathways. The 120-kDa *ras*-responsive factor 1 (25) may be one such component, but the involvement of other *ets* family members (15) cannot be precluded. Indeed, the Ets-LacZ fusion protein may act less specifically in competing with many such proteins for DNA binding, thereby accounting for its rather surprising effectiveness in suppressing *ras* transformation and CSF-1-induced colony formation. However, it is un-

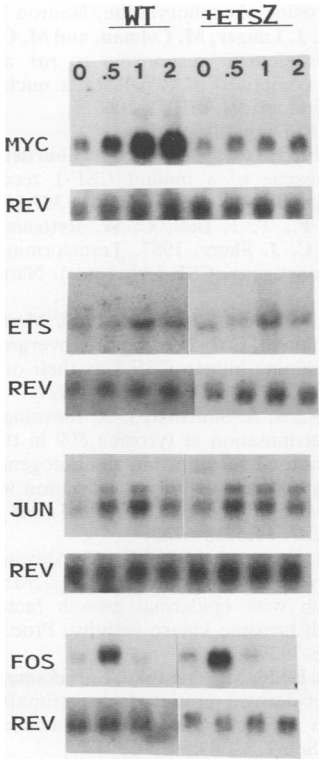

FIG. 3. Effects of *ets-lacZ* on the CSF-1-stimulated immediate-early response. NIH 3T3 cells expressing human CSF-1R alone (WT) or coexpressing *ets-lacZ* (+ETSZ) were rendered quiescent and then stimulated with human recombinant CSF-1 for the times (in hours) indicated above each lane. Total cellular RNA was extracted, separated on parallel gels (10 μg per lane), and transferred to filters. Individual filters hybridized with c-*myc*, c-*ets-2*, c-*jun*, and c-*fos* probes were rehybridized with a human K-*rev-1* probe (panels below each blot [REV]) to control for variations in RNA loading.

likely that the *lacZ* moiety was relevant to the effects observed, because a vector containing the same *ets-2* domains but lacking the *lacZ* gene functioned indistinguishably from the *ets-lacZ* construct in the various assays used (data not shown).

Introduction of the *ets-lacZ* gene can inhibit expression of both immediate-early (e.g., c-*myc*) and delayed-early (NVL-3) response genes, but its inability to affect the expression of other immediate-early genes, including c-*jun*, c-*fos*, and c-*ets-2* itself, underscores the selectivity of its effects during the proliferative response to CSF-1. Clearly, the modest increases in endogenous c-*ets-2* mRNA levels in stimulated cells were insufficient to override the suppressive effects of exogenous *ets-lacZ*, which was constitutively expressed at much higher levels. Conversely, although *ets-lacZ* expression markedly inhibited the accumulation of c-*myc* mRNA following CSF-1 stimulation, the enforced expression of an exogenous *myc* gene in these cells restored their biologic responsiveness and enabled the cells to form colonies in semisolid medium containing CSF-1. Preliminary data indicate that a reporter gene regulated by a c-*myc* promoter can be transactivated in intact cells by either *ets-1* or *ets-2* and that recombinant Ets proteins can bind in vitro to radiolabeled DNA fragments from the *myc* promoter that contain the putative *ets*-binding elements (25a). Although the inhibition of c-*myc* expression by *ets-lacZ* might yet be mediated indirectly, we think it more likely that the effect reflects a requirement for an *ets* family member in *myc* induction.

The requirement for c-*myc* in the proliferative response to CSF-1 was previously revealed by studies with a mitogenically defective CSF-1R mutant in which a site of receptor autophosphorylation, tyrosine 809, was replaced by a phenylalanine residue (26, 29). This mutant receptor is only partially defective in signal transduction, and although it is capable of inducing c-*fos* and *junB* mRNAs, it fails to activate c-*myc*. As in cells expressing Ets-LacZ, the introduction of a constitutively transcribed c-*myc* gene into NIH 3T3 cells expressing the CSF-1R (Phe-809) mutant restores their ability to proliferate in response to CSF-1 (26). Our results indicate that the growth response of cells bearing CSF-1R (Phe-809) can also be rescued, albeit somewhat less efficiently, by introducing an intact exogenous c-*ets-2* gene into them. The ability of *myc* to override the suppressive effects of the *ets-lacZ* suppressor coupled with observations that both *myc* and *ets-2* can rescue CSF-1-induced colony formation in cells expressing a partially defective CSF-1R mutant are consistent with the concept that an *ets* family member and *myc* function cooperatively in the same signal transduction pathway. Conversely, the inability of *ets-lacZ* to suppress c-*fos*, c-*jun*, and *junB* expression and the unperturbed induction of the latter set of genes in response to CSF-1 in cells bearing CSF-1R (Phe-809) indicate that they represent independent targets of CSF-1 action.

Our working model is that ligand stimulation of CSF-1R results in a flow of information from a cytoplasmic effector that interacts with the receptor in the region surrounding P-Tyr-809, through a GTP exchange factor to $p21^{ras}$, through *ets* transcription factors and, ultimately, through c-*myc*. Given that *fos/jun* function is required to complement *ets* (12, 26, 37), that a c-*jun* suppressor can also inhibit *ras* transformation (19), and that microinjection of antibodies to *fos* and *jun* can block S-phase entry in serum-stimulated cells (17), activation of the proposed *ras/ets/myc* pathway is most likely not sufficient for the proliferative response to CSF-1. However, it appears to be necessary.

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