MOLECULAR AND CELLULAR BIOLOGY, Sept. 1996, p. 4621–4631 Vol. 16, No. 9
0270-7306/96/$04.00+0

Activation of Codependent Transcription Factors Is Required for Transcriptional Induction of the *vgf* Gene by Nerve Growth Factor and Ras

GABRIELLA D'ARCANGELO,[1]† RAYMOND HABAS,[1] SU WANG,[2] SIMON HALEGOUA,[1]* AND STEPHEN R. J. SALTON[2]

Department of Neurobiology and Behavior and Institute for Cell and Developmental Biology, State University of New York at Stony Brook, Stony Brook, New York 11794-5230,[1] and Fishberg Research Center for Neurobiology, Mount Sinai School of Medicine, New York, New York 10029[2]

Received 14 December 1995/Returned for modification 1 February 1996/Accepted 5 June 1996

Nerve growth factor (NGF) treatment of PC12 cells leads to the elaboration of a neuronal phenotype, including the induction of neuronally expressed genes such as *vgf*. To study *vgf* transcription, we have created chimeric *vgf*/β-globin genes in which *vgf* promoter sequences drive the expression of the β-globin reporter gene or of a chimeric β-globin gene fused to 3′ untranslated *vgf* gene sequences. We have found that the level of inducibility of the latter construct by NGF resembles that of the endogenous *vgf* gene. Using transient transfection of the chimeric reporter genes into PC12 cells, into PC12 subclones expressing activated or dominantly interfering mutant Ras proteins, and into PC12 variants expressing specific NGF receptor/Trk mutants, we show that transcriptional regulation of the *vgf* promoter by NGF is mediated through a Ras-dependent signaling pathway. By mutational analysis of the *vgf* promoter, we have identified three promoter elements involved in mediating transcriptional induction by NGF and Ras. In addition to the cyclic AMP-responsive element (CRE), which binds to ATF-1, ATF-2, and CRE-binding protein in PC12 nuclear extracts, a novel CCAAT element and its binding proteins were identified, which, like the CRE, is necessary but not sufficient for the Ras-dependent induction of the *vgf* gene by NGF. We also identify a G(S)G element unusually located between the TATA box and transcriptional start site, which binds the NGF- and Ras-induced transcription factor, NGFI-A, and amplifies the transcriptional response. Integrating data from studies of *vgf* promoter regulation and NGF signal transduction, we present a model for *vgf* gene induction in which transcriptional activation is achieved through the persistent, direct activation of multiple interacting transcription factors binding to CRE and CCAAT elements, coordinated with the delayed transcription factor action at a G(S)G element resulting from the induced expression of NGFI-A.

Neurotrophins regulate the survival and differentiation of a variety of neuronal populations in the vertebrate central and peripheral nervous systems. Nerve growth factor (NGF) is the best-characterized member of this family of neuronal growth factors. The differentiating activity of NGF has been investigated at the molecular level with the PC12 clonal cell line, derived from a rat pheochromocytoma (20). PC12 cells undergo dramatic phenotypic changes in response to NGF, acquiring many characteristics of sympathetic neurons. The addition of NGF to these cells triggers the activation of a tyrosine kinase-containing receptor, including the proto-oncoprotein Trk (27, 29, 31), and the activation of a variety of signal transduction pathways, leading to the expression of a multiplicity of genes (reviewed in reference 25). Several cytoplasmic proto-oncoproteins, including c-Src (1, 32, 48, 63), c-Shc (50), c-Ras (2, 23, 43, 60), and Raf kinases (44, 64, 69), have been implicated in NGF action. A host of studies in various cell types indicate that Ras is a pivotal mediator of receptor and nonreceptor tyrosine kinase signaling. Several tyrosine kinase pathways lead to Ras activation (reviewed in references 30, 37, and 56), and Ras in turn mediates the activation of various serine/threonine kinases, including Raf and the mitogen-activated protein kinases (42, 62, 65, 67, 70). The persistent activation of Ras and its targets has been proposed to mediate NGF-induced changes in gene expression (11, 25, 36).

We have previously suggested that a linear cascade of Src, Ras, and Raf proto-oncoprotein activities mediates neurite growth and the activation of several NGF-inducible genes (11; see also references 32, 63, and 69). The neuronal gene *vgf* (also known as NGF33.1 and a2) provides one example of a gene which is regulated by NGF, at least in part through the activation of this Ras-dependent pathway. The *vgf* gene was first identified as a cDNA which was induced robustly by NGF in PC12 cells (34). The expression of this gene is restricted almost exclusively to central and peripheral neuronal populations and is developmentally regulated (55). In PC12 cells, *vgf* gene expression is maximally induced by NGF and, to a much lesser extent, by cyclic AMP (cAMP) and other non-differentiating agents (53, 55). The partial protein synthesis dependence (3, 46) and the delayed response to growth factors are features of *vgf* gene regulation which are intermediate between those of the immediate-early and the late-response gene classes. The increase in *vgf* expression by NGF is due to an increase in the rate of transcription (3, 46), which appears to overcompensate for a decrease in mRNA stability (3). It has recently been shown that the first 250 bp of the cloned promoter contains sequences which are responsive to NGF (26, 46). At least one regulatory element, the cAMP-responsive element (CRE) located at bp −71 from the transcription initiation site, has been shown to be required for NGF inducibility in PC12 cells and

* Corresponding author. Mailing address: Department of Neurobiology and Behavior, SUNY at Stony Brook, Stony Brook, NY 11794-5230. Phone: (516) 632-8640. Fax: (516) 632-9714.

† Present address: Department of Developmental Neurobiology, St. Jude's Childrens Hospital, Memphis, TN 38101.

brain-derived neurotrophic factor inducibility in both primary cortical and hippocampal neurons (5, 26).

To understand how the induction of gene expression by NGF is coupled to the activation of specific signal transduction pathways, we have examined the regulatory elements present in the *vgf* gene and determined their role in the responsiveness to NGF and to the Ras-dependent signaling pathway. In addition, we have identified transcription factors that may bind to these elements in nuclear extracts from NGF-treated cells. We propose that *vgf* transcriptional activation by NGF and Ras is achieved through the activation of proteins which constitutively bind to CRE and CCAAT and is potentiated by the induction of a transcription factor binding to the G(S)G element.

MATERIALS AND METHODS

Cell culture. PC12 (19), PC12-derived GSras1 and GSrasDN6 transfectant lines (11), the PC12-derived mutant line nnr-5 (18), and stable transfectant sublines of nnr-5 (35, 59) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) in tissue culture dishes as previously described (11). The oncogenic RasL61 mutant (13) in GSras1 and GSras2 cells and the dominant negative RasN17 mutant (15) in GSrasDN1 and GSrasDN6 cells were induced from the mouse mammary tumor virus promoter by addition of 0.5 μM dexamethasone to the culture medium. Ratios of wild-type and Y490P/Y785P double-mutant Trk levels expressed in the nnr-5 transfectants (kindly provided by L. A. Greene), tested by Western blot (immunoblot) analysis with anti-Trk antibody, were found to be about 1:2 (data not shown). Dexamethasone (Sigma) and forskolin (Sigma) were dissolved in dimethyl sulfoxide and diluted so that the final content of dimethyl sulfoxide in the culture medium did not exceed 0.1%. NGF, the 2.5S form (β-NGF), was purified from mouse submaxillary glands (41) and added to the culture medium at final concentrations of 50 ng/ml. N^6,2′-*O*-dibutyryl-AMP (dbcAMP) was dissolved in DMEM at 40 mM and added to the culture medium at a final concentration of 1 mM. Forskolin was added to the culture medium at a final concentration of 1 μM.

Generation of plasmid constructs. The *vgf*–β-globin chimeric plasmids were constructed by fusing portions of the rat *vgf* promoter (55), generated from an initial fragment which spanned from bp −2500 to +42 relative to the *vgf* transcription start site, to a rabbit β-globin reporter gene (68) (pOVEC plasmids, the generous gift of W. Schaffner, were modified by J. R. Lundblad). In some cases, a 1,070-bp fragment of the *vgf* 3′ untranslated region (UTR), extending from the translation stop codon to a position 620 bp 3′ of the polyadenylation signal, was fused to rabbit β-globin exon III sequences, resulting in the replacement of the β-globin polyadenylation signal with that found in the *vgf* 3′ region. A plasmid containing the 250-bp *Nhe*I-*Pvu*II fragment of the rat *vgf* promoter, from −205 to +42 relative to the transcription start site, was used as a template for site-directed mutagenesis by overlap extension by PCR (58). Promoter fragments containing either mutated CCAAT (plasmid mCCAAT in which CCAAT was changed to AAAAT), CRE (plasmid mCRE in which TGACGTCA was changed to TAAAGTCA), G(S)G [plasmids G(S)Gmut1 and G(S)Gmut2 in which GCG CGGGCG was changed to TATCGGGCG and GCGCTTTCG, respectively] or E-box (plasmids Ebox mut1A in which CACGTG was changed to ACTAGT) elements were synthesized and subcloned into the appropriate pOVEC reporter plasmid, and the promoter sequences and mutations were verified by DNA sequence analysis. Plasmids were isolated with a commercial kit (Qiagen) as specified by the manufacturer, with the final addition of a phenol-chloroform extraction step.

Transient transfection. Approximately 10^7 cells were suspended in 0.4 ml of DMEM, containing 20 μg of *vgf*–β-globin constructs. When indicated, 1 μg of pRSV-β-*gl, expressing a deleted β-globin gene from the Rous sarcoma virus promoter (68), was added to the cell suspension. Cells were placed on ice for 5 min and then electroporated in a 0.4-ml cuvette with a Gene Pulser (Bio-Rad) with settings of 250 V and 500 μF. Transfected cells were immediately set on ice for approximately 5 min and plated on tissue culture dishes containing 10 ml of DMEM supplemented with serum, except for the G(S)G mutant experiments, in which the cells were plated in DMEM supplemented with only 5% donor horse serum. Treatment agents were added to the incubation medium 24 h after transfection. The cells were lysed after an additional 24 h.

RNase protection. Transfected cells were resuspended in the incubation medium, centrifuged, washed with 1 ml of phosphate-buffered saline, and lysed in 10 mM Tris (pH 7.5)–10 mM NaCl–3 mM $MgCl_2$–0.5% Nonidet P-40. Total RNA was isolated as previously described (54). [^{32}P]UTP-labeled antisense β-globin and cyclophilin probes were generated from linearized plasmids pNBSKII, encoding a fragment of the *vgf*–β-globin fusion gene subcloned into Bluescript SKII, or pIB15, encoding cyclophilin (10). Riboprobes were synthesized with Promega kits as specified by the manufacturer and were gel purified before hybridization. Each sample RNA (10 μg) was hybridized to 5×10^6 cpm of probe (*vgf*–β-globin) and 10^5 cpm of probe (cyclophilin) where indicated and digested for 1 h with RNase A (40 μg/ml) and RNase T_1 (2 μg/ml) as previously described (54). The protected fragments were resolved on denaturing 6% polyacrylamide gels (Sequagel sequencing system; National Diagnostics). Quantification was done either with a Phosphorimager (Molecular Dynamics) or after densitometric analysis of autoradiographic exposures. The induction of *vgf*–β-globin expression was normalized to that of RSV-β-*gl (to control for transfection efficiency) or to the content of cyclophilin mRNA (to control for RNA loading). The values reported in the text refer to the experiment shown in the indicated figure and were representative of those obtained in three or four additional experiments.

Electrophoretic mobility shift analysis. Nuclear extracts were prepared by the method of Dignam et al. (14) from untreated PC12 cells and those treated with 50 ng of 2.5S NGF per ml for 1 h and from similarly treated stable transfectant sublines of nnr-5 cells that expressed wild-type (T14) or double-mutant Trk (Y490P/Y785P) (59). Nuclear extracts were dialyzed against buffer D (14), which in addition contained the phosphatase inhibitors NaF and Na_2MoO_4 (each at 10 mM), and aliquots were flash frozen in liquid N_2 and stored at −70°C. Complementary oligonucleotides were annealed and labeled with [^{32}P]ATP by using polynucleotide kinase (New England BioLabs). Each DNA-binding assay mixture contained 1 to 2 ng of ^{32}P-labeled oligonucleotide and as nonspecific competitors both a 100-fold molar excess of an unrelated double-stranded oligonucleotide of similar size and 2 μg of poly(dI-dC) (Boehringer Mannheim). Competitive inhibitors were added to selected assay mixtures in either 10-, 50-, 100-, or 500-fold molar excesses, after which 15 μg of nuclear extract was added and the assay mixtures were incubated at room temperature for 20 min. In supershift experiments, the following antisera (unless noted otherwise, obtained from Santa Cruz Biotechnology) were added concurrent with the addition of nuclear extract, and the reaction mixtures were incubated for 20 min at room temperature: anti-ATF1, anti-ATF2, anti-ATF3, anti-ATF4, anti-CREB2, anti-CREB (generous gift of M. E. Greenberg), anti-NGFIA (monoclonal antibodies 6H10 and 1H4; generous gift of J. Milbrandt), anti-CRP1, anti-C/EBPα, anti-C/EBPβ, and anti-C/EBPδ. Plasmids encoding recombinant ATF-1 and CRE-binding protein (CREB) were provided by J. R. Lundblad and R. Goodman. The oligonucleotides used were CRE_{WT}, 5′ CCCATGAATGAACATTGACGTCA ATGGGGCGGG 3′; CRE_M, 5′ CCCATGAATGAACATTAAAGTCAATGG GGCGGG 3′; $CCAAT_{WT}$ 5′ GAGCGACGCTTATCCTCCAATCATTGGAC TTCC 3′; $CCAAT_{MT}$, 5′ GAGCGACGCTTATCCTAAAATCATTGGACT TCC 3′; $CCAAT_{con}$, 5′ TCGACTCCCTGATTGCGCAATAGGCTCC 3′ (generously provided by P. F. Johnson); NGF-IA_{WT}, 5′ AAAGCAGCGGTGG CGCGGGCGCTGTC 3′; $(NGF\text{-}IA)_3$, 5′ GCGCGGGCGGCGCGGGCGGCG CGGGCG 3′; NGF-IA_{M1}, 5′ AAAGCAGCGGTGTATCGGGCGCTGTC 3′, NGF-IA_{M2}, 5′ AAAGCAGCGGTGGCGCTTTCGCTGTC 3′; $Ebox_{WT}$, 5′ GGGGCGGGGCGAGACCACGTGACCCCGCGCGCTCCC 3′; and $Ebox_M$, 5′ GGGGCGGGGCGAGACACTAGTACCCCGCGCGCTCCC. Formation of specific complexes was inhibited by a 100-fold molar excess of wild-type but not mutant or unrelated oligonucleotide. Binding-assay mixtures were analyzed on 4% low-ionic-strength polyacrylamide gels, essentially as described previously (57), followed by autoradiography.

RESULTS

We have previously shown that the increase in the steady-state level of *vgf* mRNA by NGF is largely dependent upon Ras activity (11). Since NGF induction of the *vgf* gene occurs at the level of transcription (3, 7, 46), we sought to determine whether activation of the Ras-dependent pathway could mediate the increase in transcriptional activity.

Initially, *vgf* promoter sequences containing 206 bp upstream and 44 bp downstream of the transcription start site, were cloned 5′ to the β-globin reporter gene (Fig. 1). The *vgf*–β-globin chimeric constructs were transfected into PC12-derived subclones expressing either the activated RasL61 (GSras1 cells) or the dominant negative RasN17, which dominantly interferes with endogenous Ras function (GSrasDN6 cells). Both PC12 subclones express the stably transfected mutant *ras* genes driven by the dexamethasone-inducible mouse mammary tumor virus promoter (11). The transcriptional activity of the *vgf* promoter region was determined by measuring the production of correctly initiated *vgf*–β-globin transcripts in the RNase protection assay. To normalize for a potentially general increase in transcription by inducing agents, expression of the *vgf*–β-globin fusion genes was compared with that of a shortened cotransfected β-globin reference gene driven by the RSV promoter (RSV-β-*gl) (68) and/or with the amount of endogenous cyclophilin mRNA (10).

To determine the role of Ras in the *vgf* gene induction by NGF, we first compared the effects of NGF and the activated

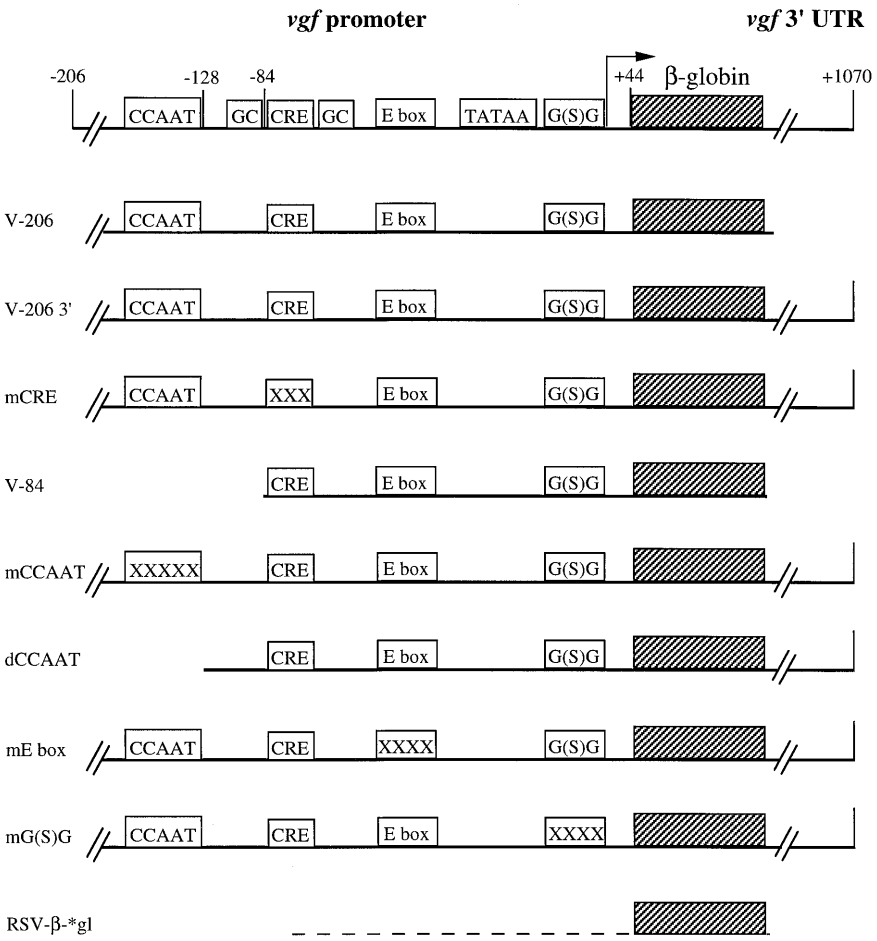

FIG. 1. Schematic representation of *vgf*–β-globin chimeric constructs. The *vgf* promoter contains putative consensus sequences (boxed) for binding to basal and inducible transcription factors. In the *vgf*–β-globin chimeric construct (V-206), the indicated *vgf* promoter regions were cloned upstream of the β-globin reporter gene, whereas in the construct V-206 3′, approximately 1 kb of *vgf* 3′ UTR was additionally cloned downstream of β-globin. Mutated CCAAT, CRE, E-box and G(S)G elements, in the mCCAAT, mCRE, mE-box, and mG(S)G constructs, respectively, are indicated as XXX. In the mCRE construct, the CRE was mutated from TGACGTCA to TAAAGTCA. In the mCCAAT construct, the element was mutated from CCAAT to AAAAT. In the dCCAAT construct, the CCAAT element was deleted by removing *vgf* sequences upstream of bp −128. In the mE-box construct, the element was mutated from CACGTG to ACTAGT. In the mG(S)G constructs, the element was mutated from GCGCGGGCG to TATCGGGCG [G(S)Gmut1] and GCGCTTTCG [G(S)Gmut2]. The promoter and 3′ UTR nucleotide sequences have been reported previously (55).

mutant RasL61 on the activity of the transfected *vgf* promoter in the GSras1 cells. When the construct containing the proximal 250 bp of the *vgf* promoter was transfected in these cells, NGF treatment resulted in a fivefold induction of β-globin transcripts (Fig. 2A). This result is consistent with those of previous studies in which a similar *vgf* promoter region was linked to the CAT reporter gene and transfected into PC12 cells (26, 46). When GSras1 cells were treated with dexamethasone to induce the expression of RasL61, a 3.5-fold induction of the reporter gene was observed (Fig. 2A). As was shown for the endogenous *vgf* gene (11), a similar dexamethasone treatment had no effect on expression from the *vgf* promoter when the construct was transfected into parental PC12 cells (results not shown). These results indicate that NGF, or the activated Ras protein, can induce transcription from the *vgf* promoter.

To determine if the transcriptional activation from the *vgf* promoter by NGF was mediated through Ras, we examined the ability of the dominant negative RasN17 to inhibit NGF induction of *vgf* promoter activity in GSrasDN6 cells. NGF treatment of transfected GSrasDN6 cells resulted in a sixfold induction of the reporter gene expression (Fig. 2A). However, after dexamethasone treatment, the NGF stimulation was reduced by 40% (Fig. 2A). Because a similar dexamethasone treatment of parental PC12 cells did not impair the activation of the reporter gene (results not shown) or of the endogenous *vgf* gene (11) by NGF, the inhibitory effect seen in dexamethasone-treated GSrasDN6 cells can be attributed to the expression of the dominant negative mutant, RasN17.

In the present study, as in all previous reports (26, 46), the level of induction of the transfected *vgf* promoter by NGF (between 2.5- and 6-fold) was significantly lower than that seen for the endogenous gene. This was true whether the increase in steady-state mRNA levels (20- to 50-fold) (34, 55) or the rate of transcription (10- to 12-fold) (3, 46) was measured. We and others have found that sequences further upstream of bp −200 do not further contribute to the level of inducibility (references 26 and 46 and data not shown). However, additional regulatory elements could be present in other regions of the gene. To examine this possibility, we created the construct V-206 3′, which contains, in addition to the proximal 250 bp of the *vgf* promoter, approximately 1,000 bp of the *vgf* 3′ UTR (Fig. 1). When GSras1 cells were transfected with the new *vgf* construct,

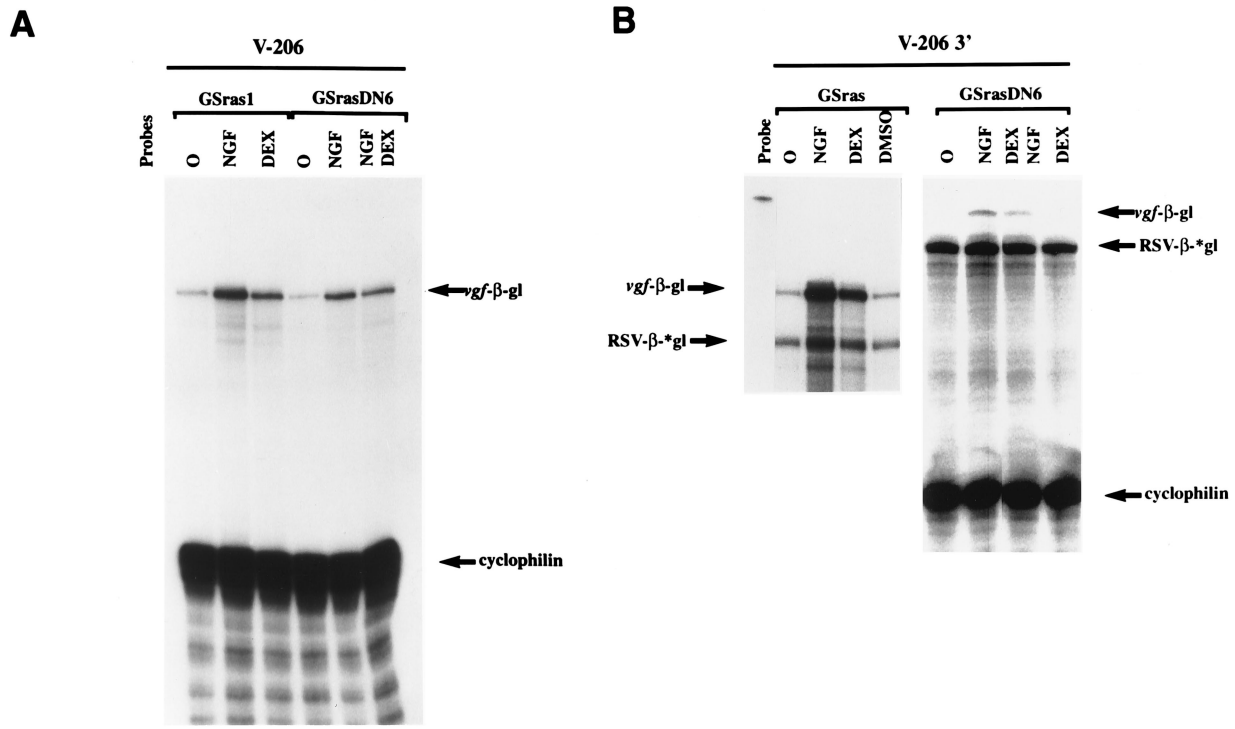

FIG. 2. Ras-mediated transcriptional induction of the *vgf* promoter by NGF. GSras1 or GSrasDN6 cells were transfected with the V-206 promoter construct (A) or with both the V-206 3′ construct and RSV-β-*gl construct (B), indicated in Fig. 1. Transfected cells were treated with NGF, dexamethasone (DEX), or dimethyl sulfoxide (DMSO) for 24 h or preincubated with dexamethasone for 24 h and then also treated with NGF for an additional 24 h (DEX NGF). Total RNA was isolated and then subjected to RNase protection analysis as described in Materials and Methods. (A) RNA samples were cohybridized with antisense probes directed against the β-globin reporter gene (upper band) and the internal control cyclophilin (lower band). Similar induction levels were obtained when the RSV-β-*gl construct was cotransfected. (B) Samples were hybridized with the β-globin probe only (left panel), or with both β-globin and cyclophilin probes (right panel). The sizes of protected bands are 250 bp (*vgf*–β-gl), 220 bp (RSV-β-*gl), and 70 bp (cyclophilin).

basal levels of reporter RNA were reduced by 30% and a more robust induction was measured in response to either NGF (between 6- and 13-fold) or dexamethasone-induced RasL61 (between 5- and 10-fold), whereas the vehicle dimethyl sulfoxide had no effect (NGF induction, 11-fold; dexamethasone induction, 9-fold [Fig. 2B]). A similar NGF induction was seen in parental PC12 cells, whereas dexamethasone treatment had no effect (data not shown), suggesting that the dexamethasone-stimulated induction in GSras1 cells was mediated through RasL61. When the V-206 3′ construct was transfected in GSrasDN1 cells, NGF treatment of cells resulted in an eight-fold induction of reporter gene expression (Fig. 2B). After dexamethasone treatment to express RasN17, the NGF stimulation was reduced by 60% in both GSrasDN1 cells (Fig. 2B) and GSrasDN6 cells (results not shown). Since the presence of the 3′ UTR increases the apparent transcriptional response of the chimeric gene to approximate the level of endogenous *vgf* gene induction, we fused it to all the constructs used in the promoter mutational analysis described below, to facilitate the identification of regulatory elements.

The partial inhibition of NGF induction of both the endogenous *vgf* gene (11) and the transfected *vgf* reporter gene by RasN17 could be due to an incomplete inhibition of Ras activity or to the involvement of additional Ras-independent pathways. To distinguish between these possibilities, we compared the abilities of the Trk receptor and the Trk double mutant, Y490P/Y785P, which is defective in mediating Ras activation (59), to mediate NGF induction of the *vgf* reporter gene V-206 3′ in stably transfected nnr-5 cells (a PC12 variant which lacks Trk) (18). As shown in Fig. 3, NGF was able to

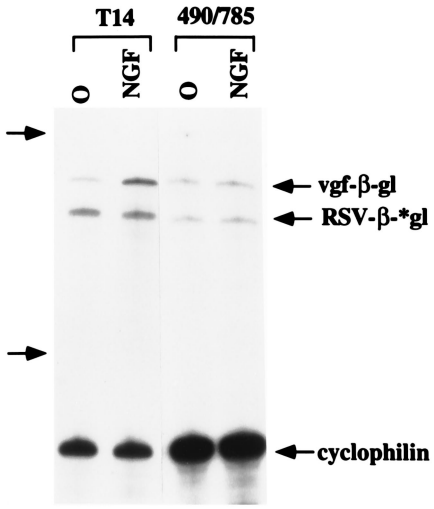

FIG. 3. A Trk signaling mutant is ineffective at mediating *vgf* induction. nnr-5 cells stably transfected with wild-type Trk (T14) or the Trk double-mutant Y490P/Y785P (490/785) were transfected with the V-206 3′ construct, together with the control RSV-β-*gl, and treated with NGF as indicated. Total RNA was isolated and subjected to RNase protection analysis as described in Materials and Methods. RNA samples were cohybridized with antisense probes directed against the β-globin reporter gene and the internal control cyclophilin, as described in the legend to Fig. 2.

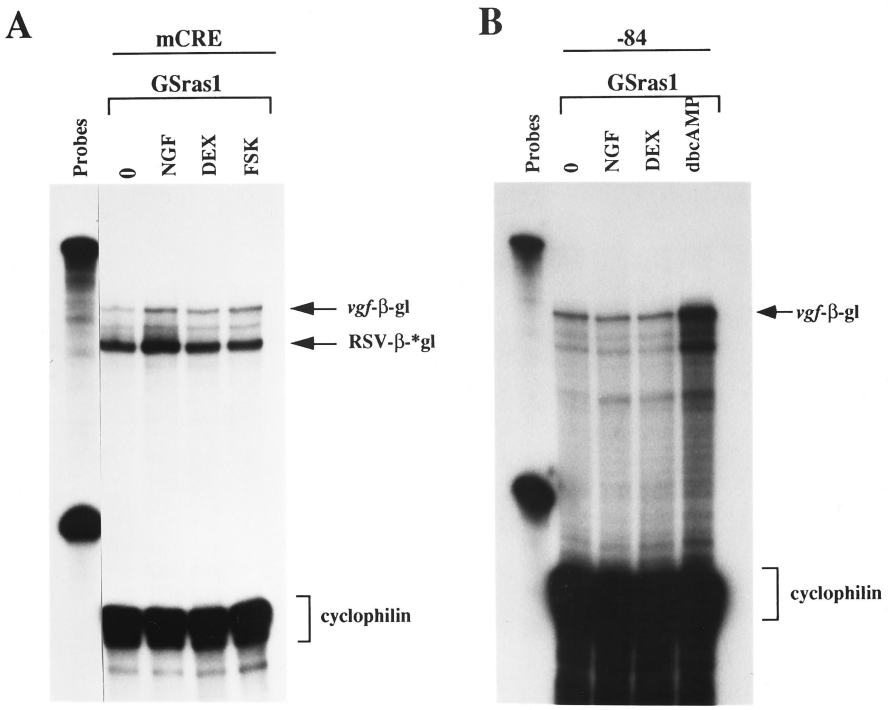

FIG. 4. CRE is required but not sufficient for *vgf* promoter induction by NGF and activated Ras. GSras1 cells were transfected with the mCRE construct and the control RSV-β-*gl (A) or with the −84 construct (B). Transfected cells were incubated in the presence of NGF, dexamethasone (DEX), forskolin (FSK), or dbcAMP for 24 h. Total RNA was isolated and subjected to RNase protection analysis as described in Materials and Methods. RNA samples were cohybridized with antisense probes directed against the β-globin reporter gene and the internal control cyclophilin, as in Fig. 2.

induce reporter expression by sixfold in wild-type Trk-transfected cells. However in stable transfectants expressing the Trk double mutant, NGF treatment was unable to mediate any increase in reporter expression (Fig. 3) and to induce endogenous *vgf* expression (52). Using Western blot analysis with anti-Trk antibody, we verified that the level of Trk mutant receptor expression was at least as high as that of wild-type Trk in the different nnr-5 transfectants used (data not shown).

The role of the CRE in mediating NGF or activated-Ras induction of the *vgf* promoter was examined. The *vgf* promoter contains a perfect consensus CRE, located at bp −71 upstream of the transcriptional start site. A construct containing the sequences between bp −205 and +44 of the *vgf* promoter in which the CRE element was mutated from TGACGTCA to TAAAGTCA was created (mCRE; Fig. 1). We initially assessed whether this particular mutation of the CRE would eliminate the binding of factors in PC12 cell nuclear extracts. Incubation of nuclear extracts isolated from NGF-treated PC12 cells with ^{32}P-labeled CRE_{WT} oligonucleotide resulted in the formation of two major shifted complexes (see Fig. 8A). Formation of these two ^{32}P-labeled complexes was efficiently inhibited by the addition of excess unlabeled CRE_{WT} oligonucleotide but not by excess CRE_M, and neither complex was visualized when nuclear extract was incubated with the ^{32}P-labeled CRE_M oligonucleotide, indicating that this mutation destroys the basic requirement for binding to the CRE (data not shown).

When the mCRE construct was cotransfected into GSras1 cells, together with a control RSV-β-*gl construct, basal levels of reporter transcripts were detectable. However, the levels of β-globin transcripts were reduced (by approximately 15-fold) compared with the wild-type promoter construct (compare Fig. 4A with Fig. 2B). Forskolin treatment of GSras1 cells resulted in only minimal activation of the mutated construct (13% of wild-type induction [Fig. 4A]), and NGF treatment resulted in no specific induction of the reporter gene (Fig. 4A), as expected from previous studies with the CRE (26, 46). When the GSras1 cells transfected with the mCRE were treated with dexamethasone to induce the production of activated RasL61, steady-state levels of reporter transcripts did not significantly increase (1.3-fold over that of RSV-β-*gl [Fig. 4A]). This result suggests that as with NGF, the induction of *vgf* promoter activity by activated Ras also requires an intact CRE.

To further examine the role of the CRE in mediating NGF and Ras inducibility, a minimal *vgf* promoter containing the sequences to bp −84 was transfected into GSras1 cells. A previous study had shown that the first 75 bp of the *vgf* promoter (containing the intact CRE element as the most distal sequence) did not confer NGF responsiveness (46). However, in that study, the 14-bp palindromic sequence which included the CRE was disrupted. Given the potential importance of palindromic sequences in mediating transcriptional activation, we created the −84 construct, in which the palindrome around the CRE was maintained. Neither NGF treatment nor treatment with dexamethasone was effective in stimulating the production of reporter transcripts (Fig. 4B), although dbcAMP treatment resulted in a considerable (fivefold) induction of reporter expression (Fig. 4B). Thus, the CRE in the *vgf* promoter is not sufficient to confer transcriptional induction in response to activated RasL61 expression, indicating that in addition to the CRE, other regulatory elements must be present within the first 250 bp of the *vgf* promoter to confer NGF and RasL61 inducibility.

We noticed the presence at bp −130 of an inverted repeat containing CCAAT, a sequence found in palindromic elements binding to the C/EBP family of transcription factors (28). To

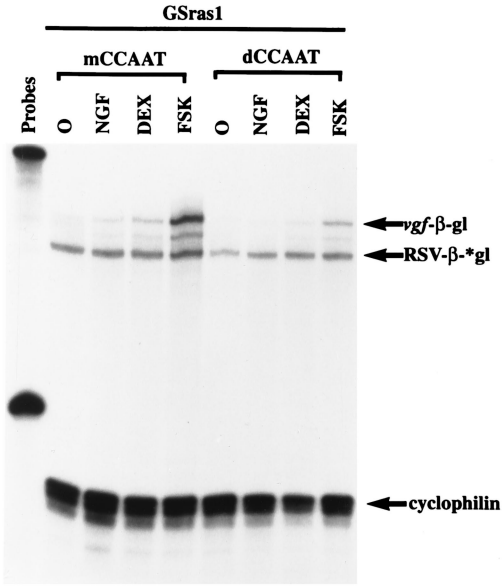

FIG. 5. The CCAAT element is required for *vgf* promoter induction by NGF and activated Ras. GSras1 cells were cotransfected with the mCCAAT construct, containing a mutated CCAAT element, or with the dCCAAT construct, in which the CCAAT element was deleted, together with control RSV-β-*gl. Cells were incubated in the presence of NGF, dexamethasone (DEX), or forskolin (FSK) for 24 h. Total RNA was isolated and subjected to RNase protection analysis as described in Materials and Methods. RNA samples were cohybridized with antisense probes directed against the β-globin reporter gene and the internal control cyclophilin, as in Fig. 2.

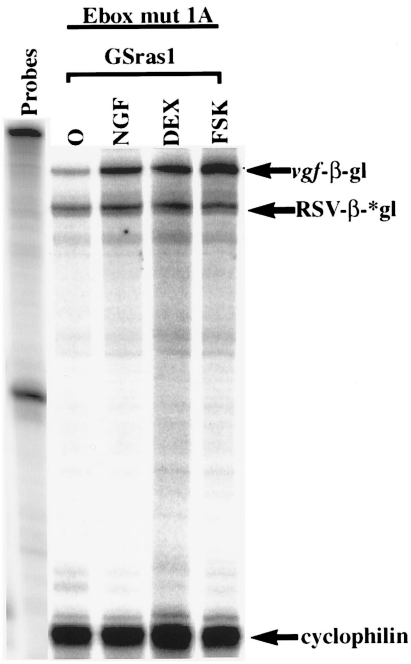

FIG. 6. The E-box element is not required for *vgf* promoter induction by NGF or activated Ras. GSras1 cells were cotransfected with the mE-box construct together with the RSV-β-*gl construct. The cells were incubated in the presence of NGF, dexamethasone (DEX), or forskolin (FSK) for 24 h. Total RNA was isolated and subjected to RNase protection analysis as described in Materials and Methods. RNA samples were cohybridized with antisense probes directed against the β-globin reporter gene (upper bands) and the internal control cyclophilin (lower band), as in Fig. 2.

assess the role of this putative element, we created two constructs in which the putative CCAAT element was either mutated from CCAAT to AAAAT (mCCAAT) in the context of the 250-bp *vgf* promoter or entirely deleted (dCCAAT) (Fig. 1). To verify the effectiveness of these mutations, electrophoretic mobility shift analysis was carried out with oligonucleotides containing the intact sequence ($CCAAT_{WT}$) or the mutated one ($CCAAT_M$). Formation of one major shifted complex was visualized after incubation of nuclear extract with the ^{32}P-labeled $CCAAT_{WT}$ oligonucleotide (see Fig. 8B). This complex was inhibited much more efficiently by the addition of as little as 10-fold excess unlabeled $CCAAT_{WT}$ than by similar amounts of excess unlabeled $CCAAT_M$. In addition, incubation of nuclear extract with ^{32}P-labeled $CCAAT_M$ did not result in the formation of significant amounts of this shifted complex, indicating that this particular mutation substantially reduces factor binding to the CCAAT site (data not shown). When the mCCAAT and the dCCAAT constructs were transfected into GSras1 cells (Fig. 5), induction of reporter transcripts was nearly abolished (NGF induction, 1.5- and 0.7-fold, and dexamethasone induction, 2.4- and 1.1-fold, for mCCAAT and dCCAAT, respectively [Fig. 5]). The mutations affect NGF- and Ras-mediated inductions specifically, because elevation of cAMP levels by forskolin treatment still caused induction of reporter transcripts (>20-fold for mCCAAT and 5-fold for dCCAAT [Fig. 5]). As noted above for the CRE mutation, the CCAAT mutation or deletion also dramatically reduced the basal expression of the reporter gene (by 15-fold compared with wild-type V-250 3′).

NGF induction of the *vgf* gene is partially dependent upon protein synthesis (3, 46), suggesting the possible involvement of immediate-early gene products. We noticed the presence at bp −48 of a consensus E box, the core sequence of a binding element for the product of the c-*myc* proto-oncogene (47). A double-stranded oligonucleotide (5′ GGGGCGAGACCACG TGACCCCGCG 3′) containing this sequence neither competed with binding of the myc/myn (max) heterodimer to its optimal binding element nor directly bound to in vitro-synthesized myc and myn (max) (51; unpublished data). Interestingly, the formation of a major shifted complex was detected after incubation of PC12 nuclear extracts with a similar oligonucleotide, $Ebox_{WT}$, although binding was unchanged when using nuclear extracts from NGF treated cells (data not shown). The formation of this complex was inhibited by a 10-fold excess of unlabeled $Ebox_{WT}$ but not at all by a 100-fold excess of a mutant oligonucleotide, $Ebox_M$, indicating that the mutation eliminates binding to the E-box element. To determine if the *vgf* E box was required for mediating promoter activation by NGF or Ras, we created a construct in which the consensus E box was mutated from CACGTG to ACTAGT (mE box) in the context of the 250-bp *vgf* promoter. When the mutated E-box construct was transfected into GSras1 cells (Fig. 6), induction of reporter transcripts was unaffected (NGF induction, sevenfold; dexamethasone induction, sevenfold), indicating that the E box was not essential for induction in these cells.

We also identified a consensus G(S)G element, located at −6, between the TATA box and transcriptional start, which may bind to NGFI-A, an immediate-early gene product that is robustly induced by NGF and Ras (11, 39). Gel shift analysis was carried out by incubating PC12 cell nuclear extracts with a ^{32}P-labeled oligonucleotide, $NGFI\text{-}A_{WT}$, containing the G(S)G element or with $(NGFI\text{-}A)_3$, containing three repeats of this G(S)G element, and competitor oligonucleotides that contained either wild-type or mutated sequences (see Fig. 8

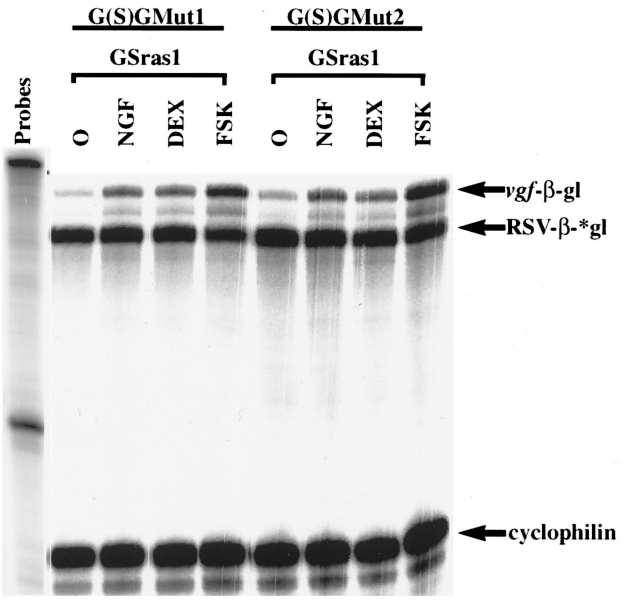

FIG. 7. The G(S)G element amplifies but is not required for *vgf* promoter induction by NGF and activated Ras. GSras1 cells were cotransfected with the G(S)Gmut1 construct or with the G(S)Gmut2 construct, together with the control RSV-β-*gl. Cells were incubated in the presence of NGF, dexamethasone (DEX), or forskolin (FSK) for 24 h. Total RNA was isolated and subjected to RNase protection analysis as described in Materials and Methods. RNA samples were cohybridized with antisense probes directed against the β-globin reporter gene (upper bands) and the internal control cyclophilin (lower band), as in Fig. 2.

and Materials and Methods). One major shifted complex was visualized after incubation of nuclear extract prepared from NGF-treated PC12 cells with ^{32}P-labeled NGFI-A_{WT} or $(NGFI\text{-}A)_3$ oligonucleotide (see Fig. 8C). Complex formation was inhibited by excess NGFI-A_{WT}, which contained the wild-type *vgf* sequence, but not by excess NGFI-A_{M1} or NGFI-A_{M2}, which contained mutations that were previously shown to disrupt Krox 24 protein binding (reference 33 and data not shown). To examine the role of the G(S)G element in transcriptional activation of the *vgf* promoter, we created a construct in which the G(S)G element was mutated from GCGC GGGCG to TATCGGGCG [G(S)Gmut1] and GCGCTTTCG [G(S)Gmut2] in the context of the *vgf* promoter (Fig. 1). When the G(S)Gmut1 and G(S)Gmut2 constructs were transfected into GSras1 cells (Fig. 7), the basal expression of the reporter gene was unaffected compared with the wild-type V-206 3′ promoter. NGF or dexamethasone treatment (to express activated RasL61) induced the expression of reporter transcripts from the wild-type construct V-206 3′ (NGF fold induction, 7.4 ± 0.8, averaged over four experiments; dexamethasone induction, 6.8 ± 1.9, averaged over four experiments [Fig. 7]). However, induction of the mutant constructs was reduced [NGF induction, 3.0 ± 0.2 for G(S)Gmut1 and 2.6 ± 0.9 for G(S)Gmut2, averaged over four experiments; dexamethasone induction, 2.7 ± 0.3 for G(S)Gmut1 and 2.5 ± 1.4 for G(S)Gmut2, averaged over three experiments (Fig. 7)]. Forskolin treatment resulted in a greatly increased expression of reporter transcripts [9.3 ± 1.6 for G(S)Gmut1 and 9.7 ± 2.8 for G(S)Gmut2, averaged over four experiments (Fig. 7)], indicating that the G(S)G element is not required for cAMP-induced transcription from the *vgf* promoter.

To identify the transcription factors which might mediate *vgf* induction, we conducted electrophoretic mobility supershift analysis with antibodies to putative binding proteins and compared the mobilities of the complexes obtained with PC12 or T14 cell nuclear extracts with those obtained with the recombinant proteins (Fig. 8). Two major complexes were formed with the CRE_{WT} oligonucleotide (Fig. 8A). Formation of the lower-mobility complex and higher-mobility complex was preferentially inhibited by anti-ATF2 and anti-ATF1, respectively (Fig. 8A, panel I). The higher-mobility complex also comigrated with a complex formed by recombinant ATF1, and anti-ATF1 was found to selectively inhibit the formation of both of these complexes but to have no effect on a complex of similar mobility formed by recombinant CREB (Fig. 8A, panels II and III). In addition, anti-CREB was found to preferentially inhibit formation of the faster complex and to specifically supershift a complex of similar mobility formed by recombinant CREB (Fig. 8A, panel III). Note that the supershifted CREB complex migrates with approximately the same mobility as the ATF2-CRE_{WT} complex, making detection of the supershifted CREB complex more difficult when PC12 cell nuclear extracts were used. The faster complex could not be visualized following addition of both anti-ATF1 and anti-CREB antisera (Fig. 8A, panel III). Addition of antisera to ATF3, ATF4, and CREB-2 had no visible effect on complexes formed between PC12 nuclear proteins and CRE_{WT} (data not shown).

Because the CCAAT element at −130 contains partial sequence similarity to the C/EBP-binding palindrome, we examined the possible binding in PC12 nuclear extracts of C/EBP proteins to the CCAAT element in the *vgf* promoter. As shown in Fig. 8B, anti-C/EBPβ immunoglobulin G did not supershift the specific complex formed with the *vgf* $CCAAT_{WT}$ oligonucleotide but did supershift a specific complex formed by incubating PC12 cell nuclear extract with a consensus C/EBP binding-element oligonucleotide (C/EBP_{con} was generously provided by P. F. Johnson, Frederick Cancer Research Center [28]). Furthermore, addition of as much as 100-fold excess unlabeled C/EBP_{con} oligonucleotide did not inhibit the formation of the shifted complex with $CCAAT_{WT}$ (data not shown). Affinity-purified antisera against C/EBPα, C/EBPδ, and CRP1 did not supershift either the $CCAAT_{WT}$ or C/EBP_{con} complex (data not shown), suggesting that C/EBP family members are not contained in the $CCAAT_{WT}$-binding complex.

Because NGF induces NGFI-A (39), a member of a family of transcription factors which bind to the G(S)G element (33) in a Ras-dependent manner (11), we examined whether this factor is present in the complexes formed from PC12 nuclear extract and the $(NGFI\text{-}A)_3$ and NGFI-A_{WT} oligonucleotides (Fig. 8C). Unlike the binding of proteins to the CRE and CCAAT element, the formation of a specific complex with either $(NGFI\text{-}A)_3$ or NGFI-A_{WT} was greatly induced by NGF treatment of T14 cells (Fig. 8C). As expected, the shifted complex formed with $(NGFI\text{-}A)_3$ was more abundant than was the one with NGFI-A_{WT}. Since NGF-treatment of stable nnr-5 transfectants expressing the Trk double mutant did not mediate an increase in *vgf*-reporter expression (Fig. 3) or induce endogenous *vgf* expression (52), we examined whether the $(NGF\text{-}IA)_3$ oligonucleotide complexed with proteins in extracts isolated from NGF-treated cells stably transfected with wild-type (T14) or double-mutant (V490P/V785P) Trk. Formation of the shifted complex with NGF-IA_{WT} or with $(NGF\text{-}IA)_3$ was greatly increased when extracts from NGF-treated T14 cells were used compared with those from V490P/V785P cells (Fig. 8C). Hybridoma-conditioned media that contained anti-NGFI-A monoclonal antibodies 6H10 and 1H4 inhibited the formation of supershifted complexes formed with the $(NGFI\text{-}A)_3$ and NGFI-A_{WT} oligonucleotides, suggesting that

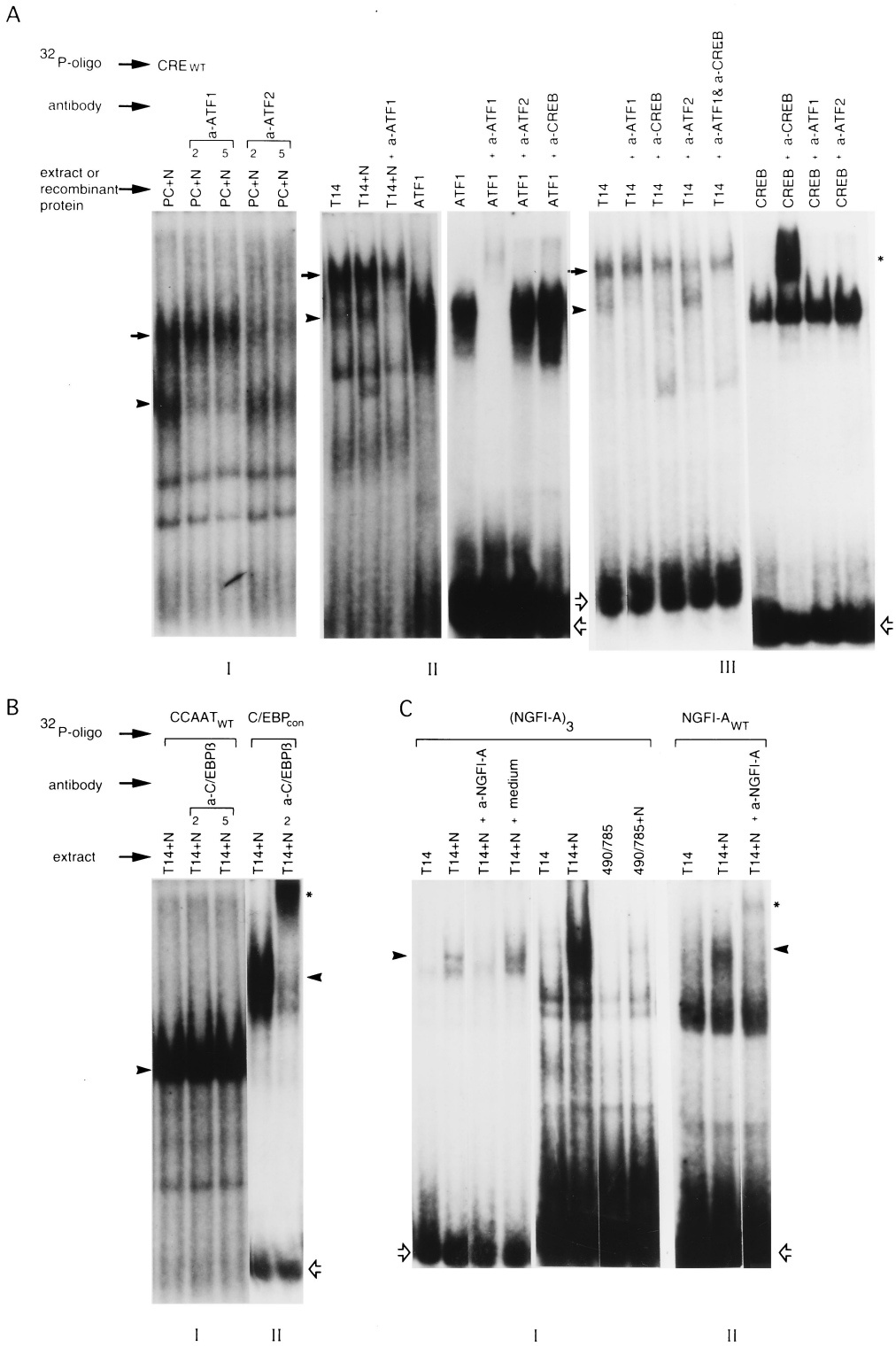

FIG. 8. Effect of specific antibodies on electrophoretic mobility shift of PC12 proteins complexed with CRE, CCAAT, and G(S)G elements. (A) PC12 or T14 nuclear extracts or recombinant ATF1 or CREB proteins were incubated with ^{32}P-labeled CRE_{WT} oligonucleotide, and anti-ATF1, anti-ATF2, or anti-CREB antisera were added as indicated (+, antibody added; 2, 2 μg of immunoglobulin G added; 5, 5 μg of immunoglobulin G added). The less mobile complex is indicated by the solid arrow, and the more mobile complex is indicated by the arrowhead. (B) Nuclear extracts from NGF-treated T14 cells were incubated with ^{32}P-labeled $CCAAT_{WT}$ or C/EBP_{con} as indicated, and 2 or 5 μg of anti-C/EBPβ immunoglobulin G was added as shown. (C) ^{32}P-labeled $(NGFI\text{-}A)_3$ (panel I) or $NGFI\text{-}A_{WT}$ (panel II) oligonucleotides were incubated with nuclear extracts prepared from NGF-treated or untreated T14 or Trk mutant 490/785 cells. Where indicated, anti-NGFI-A hybridoma supernatants 6H10 and 1H4 (2 μl of each) or, as a control, unconditioned medium were added to the assays. The formation of specific complexes, indicated by the solid arrows and arrowheads, was inhibited by a 100-fold excess of the same unlabeled oligonucleotide but not by a 100-fold excess of an unrelated or mutant oligonucleotide (results not shown); free oligonucleotide is indicated by the open arrow, and supershifted complexes are indicated by the asterisk. Abbreviations: PC, PC12; T14, nnr-5 expressing wild-type Trk; 490/785, nnr-5 expressing Trk Y490P/Y785P; +N, treated with NGF for 1 h. CRE_{WT}, $CCAAT_{WT}$, and $NGFI\text{-}A_{WT}$ include the respective elements of the wild-type (WT) rat *vgf* promoter; C/EBP_{con} contains a consensus C/EBP-binding site; $(NGFI\text{-}A)_3$ includes three tandem copies of the rat *vgf* NGFI-A-binding site (see Materials and Methods for oligonucleotide sequences).

NGFI-A is the major protein component of this NGF-inducible complex (Fig. 8C).

DISCUSSION

We have shown that activation of a Ras-dependent signaling pathway by NGF induces transcription from the *vgf* promoter and that at least three distinct promoter elements play a role in the transcriptional response. A CRE and the CCAAT element are necessary but not sufficient for induction, whereas a G(S)G element is required for maximal induction. The identification of these regulatory elements was made possible by the establishment of chimeric reporter genes containing both 5′ and 3′ *vgf* gene sequences and by the sensitivity of the RNase protection assay used to measure the levels of reporter transcripts. This chimeric gene, unlike those previously used to map promoter elements (26, 46), mediates a level of gene induction to NGF which is comparable to the level of transcriptional induction of the endogenous *vgf* gene. The enhanced induction may in part be a consequence of the lower level of basal expression, as a result of the presence of RNA-destabilizing sequences in the *vgf* 3′ UTR (53). Because NGF does not increase but, rather, decreases the stability of the *vgf* mRNA (3), the 3′ UTR should not confer NGF-induced stabilization of the reporter gene product. We have addressed the potential involvement of enhancer sequences in the 3′ UTR, such as the sequence TTTTGCTA, located immediately upstream of the polyadenylation signal (53). This sequence closely resembles the 7-mer enhancer element (TTTTGTA) found in the 3′ UTR of the Je gene, as well as many other immediate-early genes, which enhances growth factor inducibility (16). However, replacement of *vgf* 3′ UTR sequences in our *vgf*–β-globin reporter constructs with 30-bp oligonucleotides containing either the *vgf* TTTTGCTA element and polyadenylation signal or a mutated element and polyadenylation signal did not affect their NGF inducibility (12). Further studies are required to identify possible enhancer elements in the *vgf* 3′ UTR. It is also possible that the role of the 3′ region of *vgf* is cell type dependent, because brain-derived neurotrophic factor induction in rat primary cortical neurons appears to require only promoter sequences (5).

The ability of an activated form of Ras (RasL61) to mimic and of a dominantly interfering form of Ras (RasN17) to inhibit NGF induction of *vgf* indicated that NGF acts primarily through a Ras-mediated pathway. However, the inhibition by RasN17 of NGF induction of the chimeric gene or the endogenous gene (11) was incomplete. The inability of a Trk double mutant, defective in binding to signaling proteins involved in Ras-activation (59), to mediate NGF induction of the chimeric gene suggests that NGF induction may be mediated primarily through Ras but that this activity may be only partially blocked by RasN17 expression in the GSrasDN6 cell line. Alternatively, additional Ras-independent pathways, mediated through proteins that may bind to the same phosphorylated residues that were mutated in the Y490P/Y785P mutant Trk, such as Shc and phospholipase C-γ (59), could participate in the NGF response. Although we have previously demonstrated that induction of the *vgf* gene by NGF is unaffected by single mutations of Y-490 or Y-785 in Trk (52), the possibility of cooperation among the different pathways is still left open.

We have demonstrated that the NGF- and Ras-mediated activation of *vgf* gene transcription requires both the CRE and CCAAT elements. This is consistent with previous studies in which the CRE was shown to be essential for *vgf* induction by NGF in PC12 cells (26) and by brain-derived neurotrophic factor in cultured rat cortical neurons (5). Our data further indicate that Ras activity impinges at least in part on the CRE, probably by activating a member of the CREB/ATF family. Oncogenic Ras has been previously linked to promoter elements other than the CRE, such as AP-1 (reviewed in reference 21), AP-1/Ets (6), or distinct Ras-responsive elements (45). Recently, an NGF-stimulated, Ras-dependent kinase that phosphorylates CREB has been described, which might stimulate c-*fos* transcription (17). It has been suggested that CREB may act in conjunction with the serum response element complex to stimulate c-*fos* transcription (5). We propose here that the role of the CRE in the Ras-dependent induction of *vgf* is to bring an activated CREB/ATF factor into a functional complex with other factors, some of which bind to the upstream CCAAT element. CREB/ATF activation could be achieved through phosphorylation (71), binding to CBP (8), and/or heterodimer formation with Ras-responsive transcription factors such as c-Jun (4, 24). It is also conceivable that *vgf* induction by depolarization and NGF treatment may be transduced by distinct CREB/ATF family members and/or distinct CBPs. We have found that the activation of CRE-binding factors is sufficient to mediate induction of *vgf* by cAMP but not by NGF or Ras, suggesting that Ras acts in a cAMP- and A kinase-independent manner. This is consistent with our finding that NGF-stimulated expression of the endogenous *vgf* gene is not impaired in the A126-1B2 cell line (52), which is deficient in A kinase activity (66).

We have identified the CCAAT box located at bp −130 in the *vgf* promoter as a component of the NGF and RasL61 responsive unit. Palindromic sequences containing a GCAAT sequence are known to bind to transcription factors of the C/EBP family (38). Interestingly, we have found that an oligonucleotide derived from the *vgf* promoter and containing the CCAAT box was able to bind proteins in nuclear extracts of PC12 but that the complex formed did not contain a C/EBP family member. This is consistent with the fact that the CCAAT inverted repeat contained in the *vgf* promoter is not a consensus sequence for C/EBP binding (28). Together, these findings suggest that a novel CCAAT-binding protein is required for transcriptional induction by NGF and Ras.

In addition to the CRE and CCAAT elements discussed above, we identified a G(S)G element in the *vgf* promoter located between the TATA box and the transcriptional start site. A similar unusual location is occupied by a G(S)G element, which represses transcription from the herpes simplex virus latency-associated transcript (LAT) promoter (49, 61). In this context, however, the G(S)G element mediates repression of latency-associated transcript transcription. Two lines of evidence support the idea that the G(S)G element acts as a nonessential amplifier of *vgf* induction through the Ras signaling pathway. First, NGF and Ras stimulate NGFI-A expression (11) and its subsequent binding to this site. Second, unlike mutations in the CRE or CCAAT element, mutations of the G(S)G element do not affect basal expression and reduce but do not abolish the NGF and Ras inducibility of the *vgf* chimera. We have shown that NGF can induce NGFI-A binding to the G(S)G element, but full stimulation of *vgf* transcription requires the activation of other factors binding at the CRE and CCAAT element. Consistent with this, expression of NGFI-A from a cotransfected plasmid is insufficient to induce expression of the transfected *vgf*-reporter (22). The G(S)G element may also bind other family members such as NGFI-C (9); however, NGFI-A appears to be stimulated to levels that are at least 10-fold greater than those of NGFI-C in PC12 cells (40). The role of the G(S)G element in NGFI-A-stimulated amplification may explain the reported partial protein synthesis dependence of the *vgf* gene induction by NGF (3). The E box

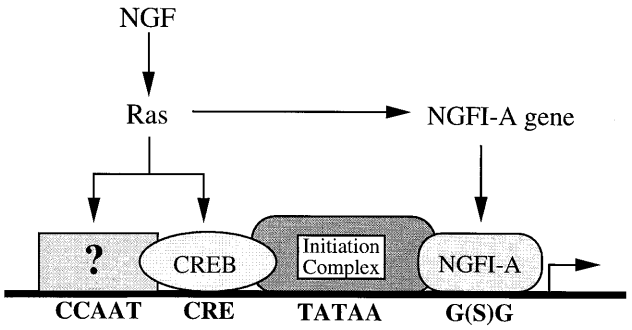

FIG. 9. Model for *vgf* transcriptional regulation. NGF treatment results in the activation of a Ras-dependent signaling pathway and in the transcriptional activation of the *vgf* gene. At least two promoter elements, the CCAAT element located at bp −130 and the CRE located at bp −75 from the transcription initiation site, are required for induced gene expression through this pathway. The G(S)G element located between the TATA box and transcriptional start site amplifies the induction. The Ras-dependent NGF induction of the *vgf* gene might result from complex interactions between activated transcription factors which are bound to the CRE and CCAAT element, induced NGF1A binding to the G(S)G element, and basal factors at the initiation complex binding at the TATAA box. The CREBs may be distinct members of the CREB/ATF family, while the CCAAT-binding protein(s) is novel.

identified at bp −48 in the *vgf* promoter is another potential site for the binding of immediate-early proteins induced by NGF. Because mutation of this site did not influence the ability of NGF or Ras to induce *vgf*-reporter transcripts, the binding of proteins to this site is not essential for induction in PC12 cells. This site could be involved in NGF inducibility in different neuronal subtypes (5), in which NGF may induce the proper array of myc/max factors which could bind to the E box and activate transcription.

The present results are summarized in a model (Fig. 9) which also takes into account previous studies (3, 5, 26, 46, 52). The transcriptional activity of the *vgf* promoter is induced by NGF through stimulation of a Ras-dependent signaling pathway and independently of A kinase activity. Unlike the cAMP-dependent pathway, which requires the presence of only CRE-binding proteins, the NGF signaling pathway impinges on at least two classes of transcription factors, a novel factor which binds to the CCAAT box and a member of the CREB/ATF family which binds to the CRE. These transcription factors could act in concert, through either direct or indirect interaction, to mediate transcriptional activation by the Ras-dependent signaling pathway. Because NGF does not increase the binding of proteins to either the CRE or CCAAT element, it is likely that the transcription factors functionally interact via synergism of their activation domains rather than by cooperative DNA binding. NGFI-A and possibly other members of this family could bind to the G(S)G site. Induced binding at this site, a result of prior activation of the NGFI-A gene, may increase the level of *vgf* transcription even further. Because the delayed binding of NGFI-A to the G(S)G element required both the CRE and CCAAT element to mediate increased transcriptional activation, we suggest that persistent activation of factors binding to these elements is required for the long-term induction of *vgf*. This idea is supported by the reported prolonged activation of Ras (36) and phosphorylation of CREB (5) stimulated by NGF and could explain the relatively poor *vgf* induction by EGF, which only transiently activates Ras signaling. This model does not preclude the potential involvement of additional transcription factors binding to as yet unidentified elements in the *vgf* promoter. Further studies are required to identify the transcription factors binding to the CRE and CCAAT element and to understand the modality of their activation by NGF and Ras.

ACKNOWLEDGMENTS

We thank Gail Mandel for critical review of the manuscript and Peter Johnson for advice and help with the analyses of C/EBP.

This work was supported by grants from the National Institute of Health (S.H., S.R.J.S.), the Dysautonomia Foundation (S.R.J.S.), and Trophix Pharmaceuticals, Inc. (S.H.); a predoctoral fellowship from Hoffmann-La Roche (G.D.); a predoctoral fellowship from the Starr Foundation (R.H.); and a Pew Scholar Award in the Biomedical Sciences (S.R.J.S.).

G. D'Arcangelo and R. Habas contributed equally to this work.

REFERENCES

1. **Alemà, S., P. Casalbore, E. Agostini, and F. Tatò.** 1985. Differentiation of PC12 phaeochromocytoma cells induced by v-*src* oncogene. Nature (London) **316:**557–559.
2. **Bar-Sagi, D., and J. R. Feramisco.** 1985. Microinjection of the *ras* oncogene protein into PC12 cells induces morphological differentiation. Cell **42:**841–848.
3. **Baybis, M., and S. R. J. Salton.** 1992. Nerve growth factor rapidly regulates VGF gene transcription through cycloheximide sensitive and insensitive pathways. FEBS Lett. **308:**202–206.
4. **Binetruy, B., T. Smeal, and M. Karin.** 1991. Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. Nature (London) **351:**122–127.
5. **Bonni, A., D. D. Ginty, H. Dudek, and M. E. Greenberg.** 1995. Serine 133-phosphorylated CREB induces transcription via a cooperative mechanism that may confer specificity to neurotrophin signals. Mol. Cell. Neurosci. **6:**168–183.
6. **Bruder, J. T., G. Heidecker, and U. R. Rapp.** 1992. Serum-, TPA-, and Ras-induced expression from Ap-1/Ets-driven promoters requires Raf-1 kinase. Genes Dev. **6:**545–556.
7. **Cho, K.-O., W. C. Skarnes, B. Minsk, S. Palmieri, L. Jackson-Grusby, and J. A. Wagner.** 1989. Nerve growth factor regulates gene expression by several distinct mechanisms. Mol. Cell. Biol. **9:**135–143.
8. **Chrivia, J. C., R. P. Kwok, N. Lamb, M. Hagiwara, M. R. Montminy, and R. H. Goodman.** 1993. Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature (London) **365:**855–859.
9. **Crosby, S. D., J. J. Puetz, K. S. Simburger, T. J. Fahrner, and J. Milbrandt.** 1991. The early response gene NGFI-C encodes a zinc finger transcriptional activator and is a member of the GCGGGGGCG (GSG) element-binding protein family. Mol. Cell. Biol. **11:**3835–3841.
10. **Danielson, P. E., S. Forss-Petter, M. A. Brau, L. Calavetta, J. Douglass, R. J. Milner, and J. G. Sutcliffe.** 1988. pIB15: a cDNA clone of the rat RNA encoding cyclophilin. DNA **7:**261–267.
11. **D'Arcangelo, G., and S. Halegoua.** 1993. A branched signaling pathway for nerve growth factor is revealed by Src-, Ras-, and Raf-mediated gene inductions. Mol. Cell. Biol. **13:**3146–3155.
12. **D'Arcangelo, G., and S. Salton.** Unpublished data.
13. **Der, C. J., T. Finkel, and G. M. Cooper.** 1986. Biological and biochemical properties of human ras^{H} genes mutated at codon 61. Cell **44:**167–176.
14. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
15. **Feig, L. A., and G. M. Cooper.** 1988. Inhibition of NIH/3T3 cell proliferation by a mutant *ras* protein with preferential affinity for GDP. Mol. Cell. Biol. **8:**3235–3243.
16. **Freter, R. R., J.-C. Irminger, J. A. Porter, S. D. Jones, and C. D. Stiles.** 1992. A novel 7-nucleotide motif located in 3′ untranslated sequences of the immediate-early gene set mediates platelet-derived growth factor induction of the Je gene. Mol. Cell. Biol. **12:**5288–5300.
17. **Ginty, D. D., A. Bonni, and M. E. Greenberg.** 1994. Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. Cell **77:**713–725.
18. **Green, S. H., R. E. Rydel, J. L. Connolly, and L. A. Greene.** 1986. PC12 cell mutants that possess low- but not high-affinity nerve growth factor receptors neither respond to nor internalize nerve growth factor. J. Cell Biol. **102:**830–843.
19. **Greene, L. A., and A. S. Tischler.** 1976. Establishment of a noradrenergic clonal cell line of rat pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA **73:**2424–2428.
20. **Greene, L. A., and A. S. Tischler.** 1982. PC12 pheochromocytoma cultures in neurobiological research. Adv. Cell. Neurobiol. **3:**373–414.
21. **Gutman, A., and B. Wasylyk.** 1991. Nuclear targets for transcription regulation by oncogenes. Trends Genet. **7:**49–54.
22. **Habas, R., and S. Salton.** Unpublished data.

23. **Hagag, N., S. Halegoua, and M. Viola.** 1986. Inhibition of growth factor-induced differentiation of PC12 cells by microinjection of antibodies to *ras* p21. Nature (London) **319:**680–682.
24. **Hai, T., and T. Curran.** 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. Proc. Natl. Acad. Sci. USA **88:**3720–3724.
25. **Halegoua, S., R. C. Armstrong, and N. E. Kremer.** 1991. Dissecting the mode of action of a neuronal growth factor. Curr. Top. Microbiol. Immunol. **165:**119–170.
26. **Hawley, R. J., R. J. Scheibe, and J. A. Wagner.** 1992. NGF induces the expression of the VGF gene through a cAMP response element. J. Neurosci. **12:**2573–2581.
27. **Hempstead, B. L., D. Martin-Zanca, D. R. Kaplan, L. F. Parada, and M. V. Chao.** 1991. High-affinity NGF binding requires coexpression of the *trk* proto-oncogene and the low-affinity NGF receptor. Nature (London) **350:** 678–683.
28. **Johnson, P. F.** 1993. Identification of C/EBP basic region residues involved in DNA sequence recognition and half-site spacing preference. Mol. Cell. Biol. **13:**6919–6930.
29. **Kaplan, D. R., B. L. Hempstead, D. Martin-Zanca, M. V. Chao, and L. F. Parada.** 1991. The *trk* proto-oncogene product: a signal transducing receptor for nerve growth factor. Science **252:**554–558.
30. **Keegan, K., and S. Halegoua.** 1993. Signal transduction pathways in neuronal differentiation. Curr. Opin. Neurobiol. **3:**14–19.
31. **Klein, R., S. Jing, V. Nanduri, E. O'Rourke, and M. Barbacid.** 1991. The trk proto-oncogene encodes a receptor for nerve growth factor. Cell **65:**189–197.
32. **Kremer, N. E., G. D'Arcangelo, S. M. Thomas, M. DeMarco, J. S. Brugge, and S. Halegoua.** 1991. Signal transduction by nerve growth factor and fibroblast growth factor in PC12 cells requires a sequence of src and ras actions. J. Cell Biol. **115:**809–819.
33. **Lemaire, P., C. Vesque, J. Schmitt, H. Stunnenberg, R. Frank, and P. Charnay.** 1990. The serum-inducible mouse gene *Krox-24* encodes a sequence-specific transcriptional activator. Mol. Cell. Biol. **10:**3456–3467.
34. **Levi, A., J. D. Eldridge, and B. M. Paterson.** 1985. Molecular cloning of a gene sequence regulated by nerve growth factor. Science **229:**3469–3473.
35. **Loeb, D. M., R. M. Stephens, T. Copeland, D. R. Kaplan, and L. A. Greene.** 1994. A Trk nerve growth factor (NGF) receptor point mutation affecting interaction with phospholipase C-gamma 1 abolishes NGF-promoted peripherin induction but not neurite outgrowth. J. Biol. Chem. **269:**8901–8910.
36. **Marshall, C. J.** 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell **80:**179–185.
37. **McCormick, F.** 1993. How receptors turn Ras on. Nature (London) **363:**15–16.
38. **McKnight, S. L.** 1992. CCAAT/enhancer binding protein, p. 771–795. *In* S. L. McKnight and K. R. Yamamoto (ed.), Transcriptional regulation. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Milbrandt, J.** 1987. A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science **238:**797–799.
40. **Milbrandt, J.** Personal communication.
41. **Mobley, W. C., A. Schenker, and E. M. Shooter.** 1976. Characterization and isolation of proteolytically modified nerve growth factor. Biochemistry **15:** 5543–5552.
42. **Moodie, S. A., B. M. Willumsen, M. J. Weber, and A. Wolfman.** 1993. Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. Science **260:**1658–1661.
43. **Noda, M., M. Ko, A. Ogura, D. G. Liu, T. Amano, T. Takano, and Y. Ikawa.** 1985. Sarcoma viruses carrying *ras* oncogenes induce differentiation-associated properties in a neuronal cell line. Nature (London) **318:**73–75.
44. **Oshima, M., G. Sithanandam, U. R. Rapp, and G. Guroff.** 1991. The phosphorylation and activation of B-raf in PC12 cells stimulated by nerve growth factor. J. Biol. Chem. **266:**23753–23760.
45. **Owen, R. D., and M. C. Ostrowski.** 1990. Transcriptional activation of a conserved sequence element by ras requires a nuclear factor distinct from c-fos or c-jun. Proc. Natl. Acad. Sci. USA **87:**3866–3870.
46. **Possenti, R., G. Di Rocco, S. Nasi, and A. Levi.** 1992. Regulatory elements in the promoter region of vgf, a nerve growth factor-inducible gene. Proc. Natl. Acad. Sci. USA **89:**3815–3819.
47. **Prendergast, G. C., and E. B. Ziff.** 1992. A new bind for Myc. Trends Genet. **8:**91–96.
48. **Rausch, D. M., G. Dickens, S. Doll, K. Fujita, S. Koizumi, B. B. Rudkin, M. Tocco, L. E. Eiden, and G. Guroff.** 1989. Differentiation of PC12 cells with v-src: comparison with nerve growth factor. J. Neurosci. Res. **24:**49–58.
49. **Rauscher, F. J.** Personal communication.
50. **Rozakis-Adcock, A., J. McGlade, G. Mbamalu, G. Pelicci, R. Daly, W. Li, A. Batzer, S. Thomas, J. Brugge, P. Pelicci, J. Schlessinger, and T. Pawson.** 1992. Association of the SHC and GRB2/sem-5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. Nature (London) **360:**689–692.
51. **Salton, S. R., G. Prendergast, and E. B. Ziff.** Unpublished data.
52. **Salton, S. R., C. Volonte, and G. D'Arcangelo.** 1995. Stimulation of vgf gene expression by NGF is mediated through multiple signal transduction pathways involving protein phosphorylation. FEBS Lett. **360:**106–110.
53. **Salton, S. R. J.** 1991. Nucleotide sequence and regulatory studies of VGF, a nervous system-specific mRNA that is rapidly and relatively selectively induced by nerve growth factor. J. Neurochem. **57:**991–996.
54. **Salton, S. R. J., M. Blum, J. A. Jonassen, R. N. Clayton, and J. L. Roberts.** 1988. Stimulation of pituitary luteinizing hormone secretion by gonadotropin-releasing hormone is not coupled to beta-luteinizing hormone gene transcription. Mol. Endocrinol. **2:**1033–1042.
55. **Salton, S. R. J., D. J. Fischberg, and K. W. Dong.** 1991. Structure of the gene encoding VGF, a nervous system-specific mRNA that is rapidly and selectively induced by nerve growth factor in PC12 cells. Mol. Cell. Biol. **11:**2335–2349.
56. **Schlessinger, J.** 1993. How receptor tyrosine kinases activate Ras. Trends Biochem. Sci. **18:**273–275.
57. **Singh, H., R. Sen, D. Baltimore, and P. A. Sharp.** 1986. A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. Nature (London) **319:**154–158.
58. **Steffan, N. H., H. D. Hunt, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
59. **Stephens, R. M., D. M. Loeb, T. D. Copeland, T. Pawson, L. A. Greene, and D. R. Kaplan.** 1994. Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. Neuron **12:**691–705.
60. **Szeberenyi, J., H. Cai, and G. M. Cooper.** 1990. Effect of a dominant inhibitory Ha-*ras* mutation on neuronal differentiation of PC12 cells. Mol. Cell. Biol. **10:**5324–5332.
61. **Tatarowicz, W. A., et al.** Submitted for publication.
62. **Thomas, S. M., M. DeMarco, G. D'Arcangelo, S. Halegoua, and J. Brugge.** 1992. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. Cell **68:**1031–1040.
63. **Thomas, S. M., M. Hayes, G. D'Arcangelo, R. C. Armstrong, B. E. Meyer, A. Zilberstein, J. Brugge, and S. Halegoua.** 1991. Induction of neurite outgrowth by v-*src* mimics critical aspects of nerve growth factor-induced differentiation. Mol. Cell. Biol. **11:**4739–4750.
64. **Troppmair, J., J. T. Bruder, H. App, H. Cai, L. Liptak, J. Szeberenyi, G. M. Cooper, and U. R. Rapp.** 1992. Ras controls coupling of growth factors receptors and protein kinase C in the membrane to Raf-1 and B-Raf protein serine kinases in the cytosol. Oncogene **7:**1867–1873.
65. **Van Aesle, L., M. Barr, S. Marcus, A. Polverino, and M. Wigler.** 1993. Complex formation between RAS and RAF and other protein kinases. Proc. Natl. Acad. Sci. USA **90:**6213–6217.
66. **Van Buskirk, R., T. Corcoran, and J. A. Wagner.** 1985. Clonal variants of PC12 pheochromocytoma cells with defects in cAMP-dependent protein kinases induce ornithine decarboxylase in response to nerve growth factor but not adenosine agonists. Mol. Cell. Biol. **5:**1984–1992.
67. **Vojtek, A. B., S. M. Hollenberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:**205–214.
68. **Westin, G., T. Gerster, M. M. Muller, G. Schaffner, and W. Schaffner.** 1987. OVEC, a versatile system to study transcription in mammalian cells and cell-free extracts. Nucleic Acids Res. **15:**6787–6798.
69. **Wood, K. W., H. Qi, G. D'Arcangelo, R. C. Armstrong, T. M. Roberts, and S. Halegoua.** 1993. The cytoplasmic *raf* oncogene induces the neuronal differentiation of PC12 cells: a potential role for cellular raf kinases in neuronal growth factor signal transduction. Proc. Natl. Acad. Sci. USA **90:**5016–5020.
70. **Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis.** 1992. *ras* mediates nerve growth factor receptor modulation of three signal transduction protein kinases: MAP kinase, Raf-1 and RSK. Cell **68:**1041–1050.
71. **Yamamoto, K. K., G. A. Gonzales, W. H. I. Biggs, and M. R. Montminy.** 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature (London) **334:**494–498.