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Functional Interaction of c-Ets-1 and GHF-1/Pit-1 Mediates Ras Activation of Pituitary-Specific Gene Expression: Mapping of the Essential c-Ets-1 Domain

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The mechanism by which activation of common signal transduction pathways can elicit cell-specific responses remains an important question in biology. To elucidate the molecular mechanism by which the Ras signaling pathway activates a cell-type-specific gene, we have used the pituitary-specific rat prolactin (rPRL) promoter as a target of oncogenic Ras and Raf in GH_4 rat pituitary cells. Here we show that expression of either c-Ets-1 or the POU homeo-domain transcription factor GHF-1/Pit-1 enhances the Ras/Raf activation of the rPRL promoter and that coexpression of the two transcription factors results in an even greater synergistic Ras response. By contrast, the related GHF-1-dependent rat growth hormone promoter fails to respond to Ras or Raf, indicating that GHF-1 alone is insufficient to mediate the Ras/Raf effect. Using amino-terminal truncations of c-Ets-1, we have mapped the c-Ets-1 region required to mediate the optimal Ras response to a 40-amino-acid segment which contains a putative mitogen-activated protein kinase site. Finally, dominant-negative Ets and GHF constructs block Ras activation of the rPRL promoter, and each blocks the synergistic activation mediated by the other partner protein, further corroborating that a functional interaction between c-Ets-1 and GHF-1 is required for an optimal Ras response. Thus, the functional interaction of a pituitary-specific transcription factor, GHF-1, with a widely expressed nuclear proto-oncogene product, c-Ets-1, provides one important molecular mechanism by which the general Ras signaling cascade can be interpreted in a cell-type-specific manner.

The process of cell division, growth, and differentiation is under exquisite control mediated by hormones and growth factors. Signals received by transmembrane receptors are propagated through the cytoplasm by a series of phosphorylation and dephosphophorylation events culminating in changes in the activity of specific nuclear transcription factors, which result in changes in gene expression (32, 56). The GTP-binding protein $p21^{ras}$ is a critical component of many signaling pathways and appears to function as a molecular switch (2, 66, 72) to convert the receptor tyrosine kinase signal to a serine phosphorylation cascade mediated by Raf, MEK, and mitogen-activated protein (MAP) kinases (16, 57, 79, 86). Recent evidence indicates that the components of the Ras pathway may be distinct in different cell types (20, 48, 67) and that cells may be programmed to interpret signals in a cell-specific manner (1, 14, 15, 59, 63). However, the molecular mechanisms by which common growth factor/Ras-induced signals result in cell-specific responses remain to be determined (7).

GH_4 rat pituitary tumor cells provide an excellent model with which to address this important question, since they are a highly differentiated line which maintain cell-specific functions and hormonal responses (18, 41–43, 76). These neuroendocrine cells express the phenotypic markers prolactin (PRL) and growth hormone (GH) under transcriptional control of the pituitary-specific factor GHF-1/Pit-1, a POU homeo-domain transcription factor (3, 18, 33). GHF-1 also plays a crucial role in the development of somatotroph, lactotroph, and thyrotroph pituitary cell lineages (6, 80). Differential splicing results in the functionally distinct factor GHF-2, which appears to activate the GH promoter but inhibit the basal activity of the PRL promoter (46, 58, 78).

Identification of the V-12 Ha-*ras* oncogene in a locally invasive lactotroph adenoma, secreting significant amounts of PRL hormone, suggests a physiological role for Ras in lactotroph cell function (39). We have previously demonstrated that oncogenic V-12 Ras selectively activates the rat PRL (rPRL) promoter (14) via the Raf/MAP kinase pathway (15) and that the nuclear acceptor of the Ras response includes a member of the Ets family of transcription factors (15). The Ets superfamily is a novel structural class of *trans*-acting phosphoproteins which have important roles in the control of growth and development (26, 37, 44, 47, 52, 65, 82, 83). The family is defined by a highly conserved ETS domain, which encodes a structurally novel DNA-binding motif (82, 85). Two well-characterized members are c-Ets-1 and c-Ets-2, which both bind to the same purine-rich DNA sequence, 5′-(A/C)GGAA-3′ (52, 82). Ets-binding sites have been identified in the oncogene response unit of several oncogene-responsive promoters (4, 26, 49), including the proximal rPRL promoter (12). Furthermore, recent reports have suggested that activation of gene expression may be modulated by functional cooperation of specific transcription factors with members of the Ets family (11, 17,

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19, 22, 31, 37, 53, 60, 61, 68, 70, 83, 87). In this study, we used a transient-transfection approach with GH_4 rat pituitary cells to show that both c-Ets-1 and GHF-1 act synergistically with oncogenic Ras and Raf to stimulate rPRL promoter activity and that both transcription factors are necessary for an optimal Ras response. Furthermore, a dominant-negative Ets construct inhibits both Ras stimulation of the rPRL promoter and the GHF-1 enhancement of the Ras response. Similarly, a dominant-negative GHF isoform, GHF-2, attenuates both the Ras response and the c-Ets-1 enhancement of the Ras effect. Finally, using amino-terminal truncations of c-Ets-1, we have mapped the c-Ets-1 region required for cooperation with GHF-1 and for mediation of the optimal Ras response to a 40-amino-acid segment which contains a putative MAP kinase site. We have previously shown that MAP kinase is a critical component of the Ras signaling pathway leading to rPRL promoter activation in GH_4 cells (15). Thus, a Ras-, Raf kinase-, and MAP kinase-dependent functional interaction of a widely expressed (45) proto-oncogene product, c-Ets-1, with a pituitary-specific transcription factor, GHF-1, provides a molecular mechanism by which activation of the general Ras signaling pathway is harnessed to mediate transcription regulation of a cell-type-specific gene.

MATERIALS AND METHODS

Cell culture. GH_4T2 rat pituitary tumor cells (76) were repassaged through rats as described previously (14) and grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Hyclone, Logan, Utah) and penicillin-streptomycin. Cells were maintained at 37°C in 5% CO_2. The medium was changed 4 to 12 h prior to each transfection, and the cells were harvested at 50 to 70% confluency.

Plasmid constructs. The reporter construct pA_3PRL*luc* (14) contains a 498-bp fragment from positions −425 to +73 of the rPRL gene ligated upstream of the firefly luciferase gene and downstream of three polyadenylation termination sites in pA_3*luc* (54). The pA_3mEBSrPRL*luc* promoter was created by PCR site-directed mutagenesis of the −214 to −209 Ets-binding site (EBS) in pG7rPRL (to be described in detail elsewhere). The core AAGGAA was changed to CTCGAG, generating a unique *Xho*I restriction site, and the resulting construct was cloned into the *Hin*dIII restriction site of pA_3*luc*. The entire promoter was then sequenced to confirm the presence of the mutant EBS and verify that the remaining promoter is identical to pA_3PRL*luc*. Construction of the pA_3rGH*luc* reporter containing a 593-bp fragment encompassing positions −528 to +65 of the rat growth hormone gene has been described previously (14).

Plasmid pSVras (74) contains the T24 bladder carcinoma Ha-*ras* valine 12 mutant oncogene (V-12 *ras*) under control of the simian virus 40 (SV40) early promoter. Plasmid pRSVRaf-BXB, an amino terminally deleted, constitutively active Raf kinase construct, was generously provided by U. Rapp, National Cancer Institute, Frederick, Md. pSG5Ets-1 and pSG5Ets-2 encode the p68 chicken c-Ets-1 and chicken c-Ets-2, respectively (83), under control of the simian virus 40 early promoter. Mutants with deletions of c-Ets-1 (Δ5′ and Δ3′) in pSG5 were constructed as described previously (73). The plasmid pAPrEts-Z encoding the DNA-binding domain of c-Ets-2 (dominant-negative Ets) was obtained from M. Ostrowski, Duke University, Durham, N.C. Plasmids pRSVGHF-1 and pRSVGHF-2 (77) encoding the rat GHF-1 and GHF-2 transcription factors, respectively, were kindly provided by M. Karin, University of California, San Diego. A plasmid containing the *Escherichia coli* β-galactosidase gene under control of the human cytomegalovirus immediate-early promoter (51), pCMV β (Clontech), was used as an internal control for transfection efficiency. Plasmid DNAs were purified by alkaline sodium dodecyl sulfate extraction and cesium chloride density gradient centrifugation. DNA was quantified by spectrophotometry at 260 nm and by comparison with DNA standards on agarose gel electrophoresis (71).

Electroporation. GH_4 cells were harvested in 0.05% trypsin–0.5 mM EDTA and resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Aliquots of approximately 2×10^6 to 4×10^6 cells in 200 μl of medium were added to plasmid DNA and transfected by electroporation (41) at 220 V and 500 μF in a Bio-Rad Gene Pulser with 0.4-mm cuvettes. All transfections included 0.5 μg of pCMVβ as an internal control for transfection efficiency. The total amount of DNA was kept constant, and nonspecific effects of viral promoters were controlled for by using empty vector or pRSVβglobin. Following transfection cells were plated in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and incubated for 24 h. Electroporations were performed in triplicate for each condition within a single experiment, and experiments were repeated 3 to 20 times with different plasmid preparations of each construct.

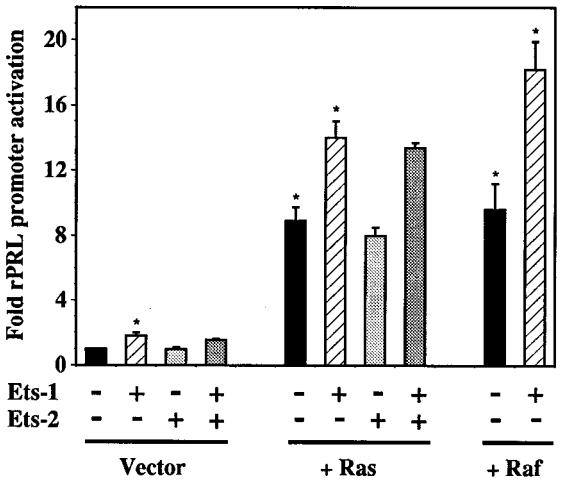

FIG. 1. c-Ets 1 enhances Ras and Raf activation of the rPRL promoter. GH_4 cells were cotransfected with 3 μg of pA_3rPRL*luc*, 10 μg of pSG5c-Ets-1, and/or 10 μg of pSG5c-Ets-2, ± 2 μg of pSVras or 7 μg of pRSVRaf-BXB as indicated. Cells were harvested after 24 h, assayed for luciferase, and standardized with respect to β-galactosidase activity as described in Materials and Methods. Results are expressed as fold activation relative to the basal promoter activity and represent the mean and standard error of the mean of 6 to 20 experiments, each consisting of triplicate transfections. *, $P < 0.005$.

Luciferase and β-galactosidase assays. Transfected cells were harvested in phosphate-buffered saline containing 3 mM EDTA, and extracts were prepared by three sequential freeze-thaw cycles in 100 mM potassium phosphate–1 mM dithiothreitol (pH 7.8). Cell lysis was increased by vortexing between cycles. Cell debris was pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C, and aliquots of the supernatant were used in subsequent assays.

Luciferase was assayed as previously described (14) in 100 mM potassium phosphate (pH 7.8)–1 mM dithiothreitol–15 mM $MgSO_4$–5 mM ATP–0.2 mM luciferin. Samples were measured in duplicate in a Monolight 2010 Luminometer (Analytical Luminescence Laboratories, San Diego, Calif.).

β-Galactosidase activity was determined spectrophotometrically with the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) essentially as described previously (14). Samples were assayed in duplicate in 60 mM sodium phosphate (pH 7.5)–1 mM $MgCl_2$–0.8 mg of ONPG per ml–40 mM 2-mercaptoethanol, and a standard curve was constructed by using a commercial preparation of *E. coli* β galactosidase (Boehringer-Mannheim). Color development occurred at 37°C over 30 to 90 min, and results were linear up to 3 h. Reactions were terminated by addition of 0.5 ml of 1 M sodium carbonate, and the A_{405} was measured (51).

Total luciferase light units were normalized to total β-galactosidase activity. The normalized relative luciferase activity for each control was set to 1, and results were expressed as fold rPRL promoter activation. Data were analyzed by analysis of variance with Sigma Stat (Jandel Scientific, San Rafael, Calif.). Multiple comparisons were carried out by the methods of Bonferroni and Dunn.

RESULTS

c-Ets-1 enhances Ras and Raf activation of the rPRL promoter. Consistent with data for other cell types (16, 57, 79, 86), we have previously demonstrated that the Ras signal in GH_4 cells is propagated through Raf and MAP kinases (14, 15). The ability of a dominant-negative Ets construct encoding only the DNA-binding domain, pAPRetsZ (49), to inhibit both Ras and Raf activation of the rPRL promoter suggested that a member of the Ets family of transcription factors is a nuclear mediator of the Ras response in these neuroendocrine cells (15). On the basis of these results, which predict that an intact Ets should enhance the Ras/Raf response, the ability of full-length chicken c-Ets-1 and c-Ets-2 to activate the rPRL promoter was examined. Cotransfection of GH_4 cells with c-Ets-1 in the absence of Ras results in a modest (1.8 ± 0.2-fold for 60 transfections) but statistically significant ($P < 0.005$) activation of basal rPRL promoter activity (Fig. 1). This activation was linearly dependent on the amount of c-Ets-1 DNA transfected,

up to 20 μg (not shown). Conversely, c-Ets-2, even at the highest doses (30 μg) tested, had no effect on basal rPRL promoter activity. Cotransfection of c-Ets-1 and c-Ets-2 did not activate the rPRL promoter beyond the level obtained with c-Ets-1 alone. Thus, in this transient-transfection system, c-Ets-1 but not c-Ets-2 activates basal rPRL promoter activity.

To determine the effect of c-Ets-1 and c-Ets-2 on the Ras activation of the rPRL promoter, GH_4 pituitary cells were transiently transfected with V-12 Ras and either c-Ets-1, c-Ets-2, or both factors. Expression of c-Ets-1 enhances the 9-fold Ras activation of the rPRL promoter to almost 15-fold (Fig. 1). In multiple experiments, the stimulation by Ras in the presence of c-Ets-1 was consistently greater than the sum of the effects of either factor alone, implying a synergistic response, as previously defined (30). In contrast, cotransfection of c-Ets-2 had no statistically significant effect on either the Ras activation of the rPRL promoter or the enhancement of that response by c-Ets-1 (Fig. 1). Since we have shown that Raf kinase is a component of the Ras signaling pathway leading to activation of the rPRL promoter (15), we next determined whether c-Ets-1 also enhanced the Raf response. Transfection of the constitutively active mutant BXB Raf (4) activates rPRL transcription approximately ninefold. In the presence of c-Ets-1, this effect is increased to 18-fold over the basal level (Fig. 1). Consistent with results obtained with V-12 Ras, cotransfection of c-Ets-2 did not enhance the BXB-Raf activation of the rPRL promoter (not shown).

The c-Ets-1 enhancement of the Ras response colocalizes to the RRE. A diagram of the principal transcription factor-binding sites in the proximal −425 rPRL promoter is shown in Fig. 2A. The proximal rPRL promoter contains three GHF-1-binding sites, designated footprints I, III, and IV (FPI, FPIII, and FPIV; shaded rectangles); a repressor-binding site, termed FPII (hatched circle); and a basal transcription factor-binding site (open triangle) (35). Additionally, there are several putative Ets-binding sites containing the core GGAA sequence (62), which are shown as solid ovals (Fig. 2A). We have previously mapped the *cis* element required for the Ras response (RRE) to an EBS positioned at −217 to −209 (12). Moreover, site-specific mutation of this EBS abrogates the Ras response (see Fig. 5). If c-Ets-1 and V-12 Ras are acting via the same pathway, the c-Ets-1 enhancement of the Ras response should colocalize to this putative RRE. Thus, rPRL 5′ promoter deletion constructs, whose endpoints are shown in boldface type (Fig. 2A), were used to map the *cis* elements responsible for the increased Ras response observed in the presence of c-Ets-1. Of note, only the −425 and −255 promoter constructs displayed any significant enhancement of the Ras response by c-Ets-1, while shorter constructs did not demonstrate this effect (Fig. 2C). Interestingly, the −255 construct consistently exhibited higher basal promoter activity (12) and a greater Ras-Ets synergy (Fig. 2C). This may reflect the presence of an as yet undefined upstream inhibitory element. The −212 promoter construct exhibits a reduced Ras activation, and the c-Ets-1 enhancement is completely lost (Fig. 2C). Although the −212 construct retains the core EBS (GGA), the deletion endpoint disrupts the putative Ets-responsive element by deleting the immediate upstream flanking sequences required for Ets binding (62). Thus, the Ras-Ets cooperative activation of rPRL transcription colocalizes to the Ets-binding site within the RRE. Although a minimal residual Ras response is noted with the −189 construct, other studies (12) clearly show that the majority of the Ras and Raf response is mediated by DNA sequences surrounding position −212.

GHF-1 but not GHF-2 synergistically enhances Ras and Raf activation of the rPRL promoter. Previous experiments have shown that rPRL promoter constructs with site-specific mutations in factor-binding sites, such as FPI, FPII, and FPIII and the basal transcription element (Fig. 2A), alone and in combination, are still responsive to Ras (12). Furthermore, the ancestrally related rat growth hormone (rGH) promoter, which is also GHF-1 dependent, does not respond to Ras or Raf in GH_4 cells (14, 15). These results suggested that GHF-1 alone does not mediate the Ras/Raf activation of the rPRL promoter. However, examination of the rPRL promoter DNA sequence reveals that the EBS at −217 to −209 is immediately adjacent to FPIV, the most distal and lowest-affinity GHF-1-binding site (28) (Fig. 2B), suggesting that deletion at −212 not only destroys the EBS but, by doing so, also interferes with a composite c-Ets-1/GHF-1 Ras/Raf-responsive element. These observations raised the question of the role of GHF-1 in the Ras response, particularly as Ets has been shown to recruit

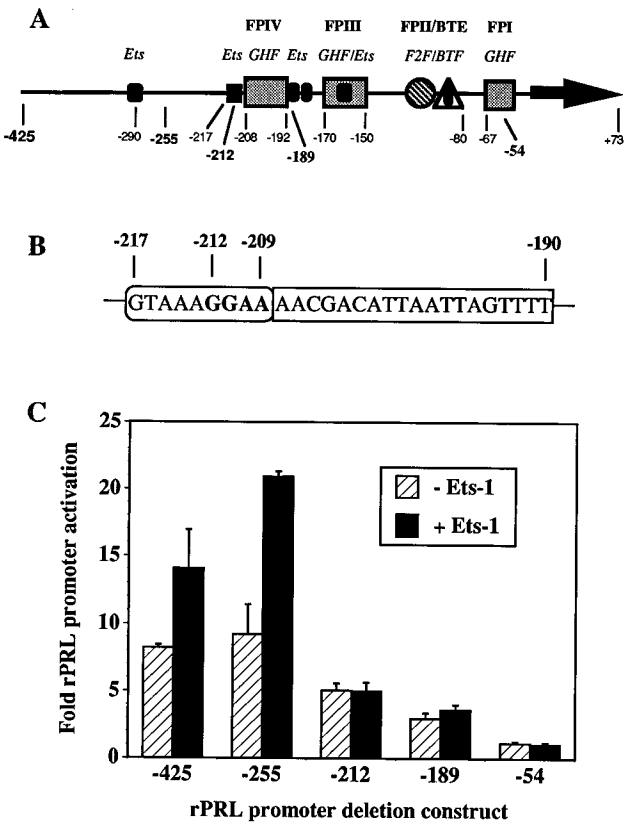

FIG. 2. Mapping of the *cis* elements required for c-Ets-1/Ras activation of the rPRL promoter. (A) Structural organization of the proximal rPRL promoter. The nucleotide sequence of the rPRL gene from −425 to +73 is depicted. The endpoints of exonuclease deletions constructed in pA_3*luc* and verified by dideoxy sequencing (13) are indicated by the numbers in boldface type. GHF-1 sites (FPI, FPIII, and FPIV), as determined by DNase protection (25), are indicated by the stippled rectangles. Putative Ets consensus-binding sites are shown by the solid rectangles. Putative Ets consensus-binding sites are shown by the solid rectangles. The FPII repressor site (F2F) and the basal transcription element (BTE) (35) are denoted by the circle and triangle, respectively. (B) Composite RRE of the rPRL promoter. The diagram shows the nucleotide sequence of the rPRL promoter from −217 to −190. The Ets-binding site is indicated by the oval, and the core GGAA motif is shown in boldface type. The rectangular box denotes the GHF-1 footprint. (C) The indicated series of rPRL promoter deletions (5 μg) in pA_3*luc* were cotransfected with or without 2 μg of Ras and with or without 10 μg of pSG5 c-Ets-1. Cells were harvested after 24 h and assayed for luciferase and β-galactosidase as described in Materials and Methods. Fold activation is determined relative to the basal activity of each individual promoter construct. A representative experiment is depicted; results are the mean and standard deviation of three transfections.

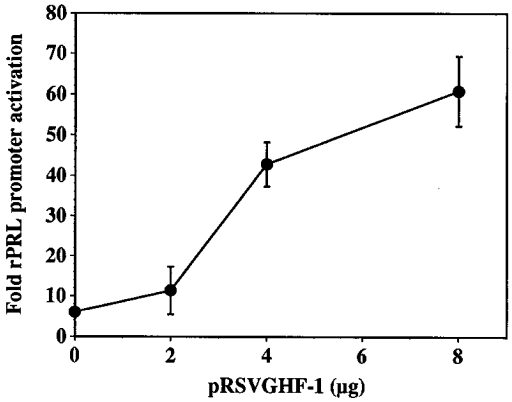

FIG. 3. Ras and GHF-1 synergistically activate the rPRL promoter. GH_4 cells were transiently cotransfected with 3 μg of pA_3rPRL*luc* reporter construct and the indicated increasing amounts of pRSVGHF-1 with or without 2 μg of pSVras. Cells were harvested and assayed for rPRL promoter activity as in Fig. 1. Results are expressed as fold activation induced by V-12 Ras relative to control. Each point is the mean and standard deviation of three transfections.

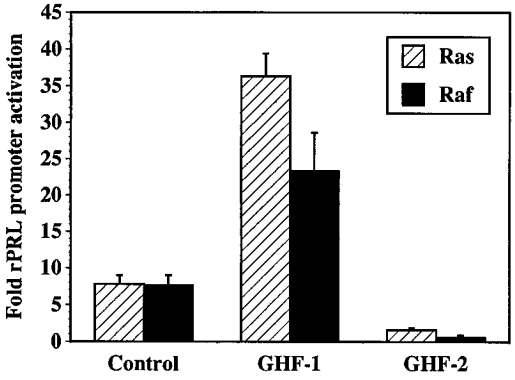

FIG. 4. Effect of GHF-1 and GHF-2 on activation of the rPRL promoter by Ras and Raf. GH_4 cells were transiently cotransfected with 3 μg of pA_3rPRL*luc* reporter construct and either 2 μg of pSVras or 7 μg of pRSVRaf-BXB with or without 10 μg of pRSVGHF-1 or pRSVGHF-2 as indicated. Results are expressed as the fold activation of the rPRL promoter induced by V-12 Ras and by BXB-Raf and are the mean and standard error of the mean of 15 transfections.

other transcription factors to discrete promoter-binding sites (for a review, see reference 37). In an experiment to directly determine the role of GHF-1 in the Ras response of the rPRL promoter, we found that increasing amounts of transfected pRSVrGHF-1 resulted in a dose-dependent synergistic enhancement of the Ras response, from 6-fold in the absence of GHF-1 to over 60-fold in the presence of 8 μg of pRSVGHF-1 (Fig. 3). Of note, similar amounts of pRSVGHF-1 alone had little to no effect on basal rPRL promoter activity in GH_4 pituitary cells (see Fig. 5), consistent with the report of Haugen et al. (29). Since the Ras response of the rPRL promoter is mediated via Raf kinase (15), cotransfected GHF-1 should also enhance the BXB-Raf effect. The data in Fig. 4 show that cotransfected GHF-1 synergistically enhanced the V-12 Ras activation of the rPRL promoter, from 6- to over 35-fold, and that GHF-1 also enhanced the BXB-Raf activation of the rPRL promoter, from 6-fold to over 20-fold. To more precisely control for nonspecific effects of expressed GHF-1 and to verify that the effects were due to a functional GHF-1 isoform, the effect of GHF-2 on the Ras and Raf response was examined. GHF-2 is a splice variant of GHF-1 which contains a 26-amino-acid insertion in the transcription activation domain, resulting in an isoform that behaves as a dominant-negative effector with respect to rPRL promoter activity (29, 46, 58, 78). Cotransfected pRSVGHF-2 not only fails to synergize with V-12 Ras or BXB-Raf but also inhibits activation of the rPRL promoter by both oncogenes, reducing activity to near basal levels (Fig. 4). Together, these data corroborate the importance of GHF-1 in mediating the Ras and Raf responses of the rPRL promoter in GH_4 rat pituitary cells, providing a novel mechanism by which oncogene signaling pathways utilize a cell-specific transcription factor to activate a tissue-specific gene.

Functional interaction of c-Ets-1 and GHF-1 mediates the Ras response. The above results show that both c-Ets-1 and GHF-1 are critical nuclear components of the molecular mechanism mediating the Ras response of the pituitary-specific rPRL promoter. Furthermore, the observations that the critical EBS is vicinal to a GHF-1-binding site (FPIV; Fig. 2B) and that the dominant-negative GHF-2 isoform completely blocks the Ras and Raf response (Fig. 4) suggest that the −217 to −190 region of the rPRL promoter functions as a composite RRE (Fig. 2B). Such a composite element may facilitate a functional interaction between these transcription factors as a key mechanistic feature of the Ras/Raf response (19, 31, 37, 53, 60, 68, 87). Thus, we examined the combined effects of c-Ets-1 and GHF-1 on the Ras response of the rPRL promoter in GH_4 cells, and as a control we used the rGH promoter. The 9-fold Ras activation of the rPRL promoter is enhanced to 15-fold by c-Ets-1 alone, to 40-fold by GHF-1 alone, and to over 60-fold by GHF-1 and c-Ets-1 together (Fig. 5). In the absence of V-12 Ras, c-Ets-1 alone or in combination with GHF-1 results in only a twofold activation of rPRL promoter activity (Fig. 5), and GHF-1 alone has no significant effect on basal promoter activity, as previously discussed (29). Thus, optimal Ras response was observed upon overexpression of both c-Ets-1 and GHF-1. The rGH promoter is also regulated by GHF-1 (40); however, the rGH promoter was not activated by Ras, and, more importantly, c-Ets-1 and/or GHF-1 failed to enhance the Ras response (Fig. 5). Of note, the rGH promoter does not

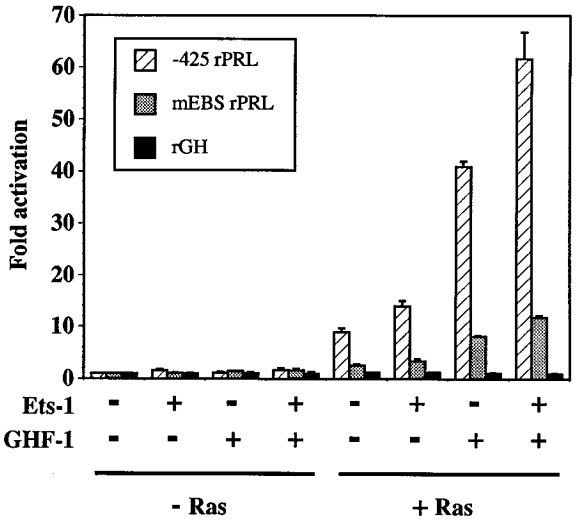

FIG. 5. Optimal Ras activation of the rPRL promoter requires both c-Ets-1 and GHF-1. GH_4 cells were transiently cotransfected with 3 μg of pA_3rPRL*luc*, 3 μg of pA_3mEBSrPRL*luc*, or 5 μg of pA_3rGH*luc* reporter constructs with or without 10 μg of pSG5 c-Ets-1, 10 μg of pRSVGHF-1 and 2 μg of pSVras as indicated. Cells were harvested and assayed for promoter activity as in Fig. 1. Results are expressed as fold activation relative to the basal activity of each promoter and represent the mean and standard deviation of 3 to 15 transfections.

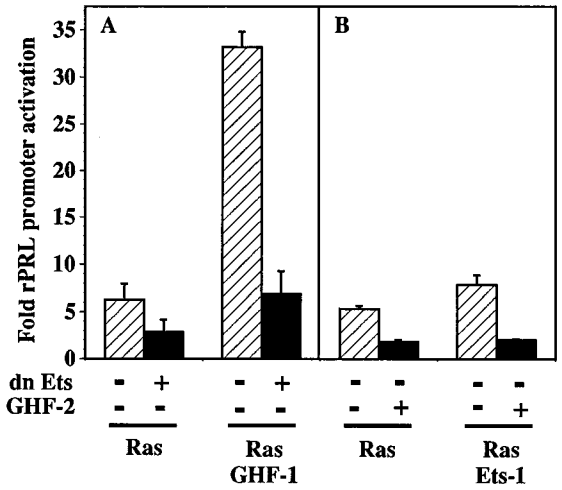

FIG. 6. Dominant-negative (dn) forms of Ets and GHF attenuate the synergistic activation of the Ras response by c-Ets-1 and GHF-1. GH_4 cells were cotransfected with 3 μg of pA_3rPRL*luc* reporter and 2 μg of pSVras, 10 μg of pRSVGHF-1, or 10 μg of pSG5 c-Ets-1 as indicated with or without 10 μg of pRSVGHF-2 (A) or 15 μg of pAPrEts-Z (B). Fold activation of the rPRL promoter was determined as in Fig. 1. Results are the mean and standard deviation of 15 transfections.

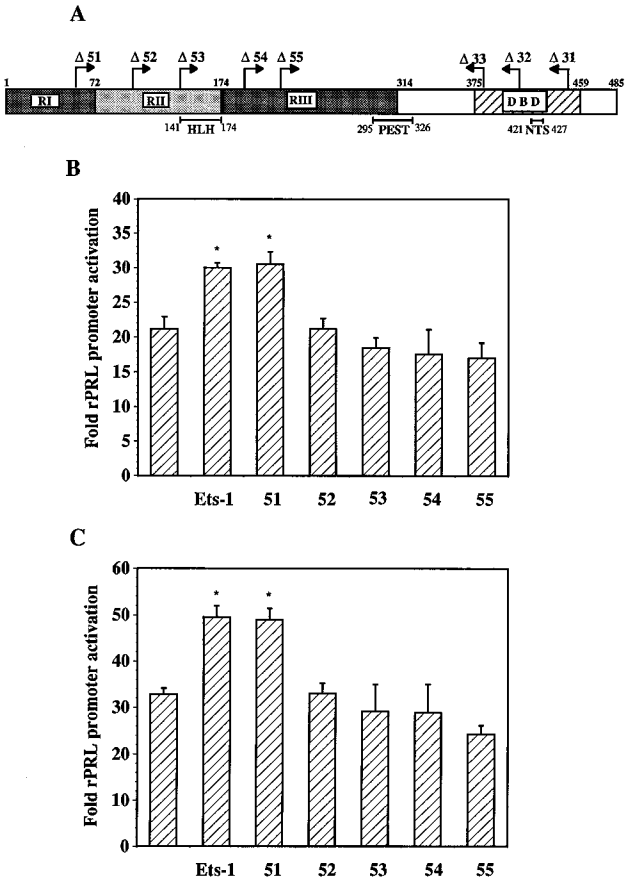

FIG. 7. Mapping of the regions of c-Ets-1 required for functional interaction with Ras and GHF-1. (A) Functional domains of chicken p68 c-Ets-1 (52). Numbers indicate amino acids 1 to 485. RI and RIII, transcription activation domains; RII, regulatory domain; DBD, DNA-binding domain; HLH, helix-loop-helix domain; NTS, nuclear targeting signal; PEST, possible protein cleavage site, rich in Pro, Glu, Ser, and Thr residues. Arrows indicate endpoints of the Δ5 and Δ3 c-Ets-1 deletion constructs in pSG5 (73). (B) GH_4 cells were cotransfected with 3 μg of $pA_3$255rPRL*luc* reporter with or without 2 μg of pSVras and 10 μg of the indicated Δ5 c-Ets-1 construct in pSG5. Activation of the −255 rPRL promoter was determined as in Fig. 1, and the data are expressed as fold activation induced by V-12 Ras. Results are the mean and standard deviation of six transfections. *, $P < 0.005$. (C) GH_4 cells were cotransfected with 3 μg of pA_3rPRL*luc* reporter with or without 5 μg of pRSVGHF-1, 2 μg of pSVras, and 10 μg of the indicated Δ5 c-Ets-1 construct. rPRL promoter activity was determined as in panel B, and the data are expressed as fold activation induced by Ras/GHF-1. Results are the mean and standard deviation of nine transfections. *, $P < 0.005$.

appear to contain a consensus EBS, again suggesting that GHF-1 alone is insufficient and that the Ras enhancement effect of transfected c-Ets-1 requires an EBS in the target promoter. To verify the role of the rPRL promoter −214 to −209 EBS, the core (AAGGAA) motif was altered by PCR site-directed mutagenesis to an *Xho*I restriction site (CTCGAG), thereby generating the mEBS rPRL promoter construct. Mutation of this EBS, which forms part of the composite RRE, almost completely inhibited the Ras activation of the rPRL promoter and the c-Ets-1 enhancement of the Ras response (Fig. 5). Similarly, the mEBS rPRL promoter exhibited a significantly attenuated response to Ras plus GHF-1 (8-fold) compared with the wild-type promoter (>40 fold), and the optimal 60-fold Ras response of the −425 rPRL promoter, obtained in the presence of Ras, c-Ets-1 and GHF-1, was reduced to only 11-fold in the mutant mEBS rPRL promoter (Fig. 5).

Taken together, these data suggest that Ras activation of the rPRL promoter is mediated by a Ras-dependent functional interaction between GHF-1 and c-Ets-1. To investigate this hypothesis, the effects of dominant-negative Ets and GHF-2 on the Ras response and the c-Ets-1/GHF-1 enhancement were examined. Expression of dominant-negative Ets inhibited the Ras response and almost completely abrogated the synergistic activation of rPRL promoter activity in response to Ras plus GHF-1 (Fig. 6A). Similarly, expression of GHF-2 blocked both the Ras stimulation of the rPRL promoter and the synergistic response mediated by c-Ets-1, reducing activity to near basal levels (Fig. 6B). This mutual "cross-inhibition" not only provides further evidence for a functional interaction between c-Ets-1 and GHF-1 in mediating the Ras response but also strongly argues for a composite element mediating the c-Ets-1/GHF-1 effect, since dominant-negative Ets and GHF-2 block by virtue of nonproductive binding to their respective DNA sites (15, 29, 49, 78).

A 40-amino-acid region (amino acids 57 to 98) of chicken c-Ets-1 mediates the cooperativity with GHF-1. A series of amino-terminal (Δ5) and carboxy-terminal (Δ3) deletion constructs of p68 chicken c-Ets-1 (73) were used to identify the regions of c-Ets-1 necessary to mediate the Ras-dependent, functional synergism with GHF-1. The endpoints of the deletions relative to the known functional domains of c-Ets-1 are shown (Fig. 7A). On the basis of the observed enhancement of the Ras response by cotransfected c-Ets-1 (Fig. 1), we determined the effects of each of the Δ5 Ets truncation mutants on the Ras activation of the −255 rPRL promoter. The 21-fold Ras activation of the −255 rPRL promoter is significantly enhanced to similar levels (over 30-fold) by both full-length c-Ets-1 and the Δ51 Ets (amino acid 57) (Fig. 7B). However, the Δ52 (amino acid 98) construct, and the remaining constructs with progressive amino-terminal deletions, Δ53 (amino acid 140) to Δ55 (amino acid 218), fail to increase the Ras response (Fig. 7B). These data show that the region between amino acids 57 and 98 is critical for c-Ets-1-mediated activation of the rPRL promoter in GH_4 pituitary cells. Since GH_4

cells contain endogenous GHF-1, and since we have shown that c-Ets-1 and GHF-1 appear to functionally interact (Fig. 5 and 6), these data imply that the region of c-Ets-1 between amino acids 57 and 98 is also critical for its functional interaction with GHF-1. To directly test this possibility, we determined the effects of each of the Δ5 Ets truncation mutants on the synergistic effect mediated by exogenous GHF-1 on the V-12 Ras activation of the −425 rPRL promoter (Fig. 7C). Full-length p68 c-Ets-1 increases the Ras/GHF-1-induced activation of the rPRL promoter from 33- to almost 50-fold in GH_4 cells. As in Fig. 7B, the amino-terminal 56 residues (Δ51) of c-Ets-1 are dispensable for the c-Ets-1-mediated enhancement, and identical regions (amino acids 57 to 98) of c-Ets-1 are required for both the enhancement of the Ras response (Fig. 7B) and the enhancement of Ras/GHF-1 (Fig. 7C). As expected, carboxy-terminal deletions of c-Ets-1 (Δ31, Δ32, and Δ33), which disrupt the DNA-binding domain (Fig. 7A), are also unable to increase the Ras/GHF-1 activation of the PRL promoter (results not shown). Therefore, these data verify that the region of c-Ets-1 required for the Ras-induced c-Ets-1/GHF-1 synergy maps between amino acids 57 and 98. Although phosphorylation of c-Ets-1 by MAP kinase is yet to be documented, it is noteworthy that this region contains a putative MAP kinase site (PLLT^{82}PSS), which we (15) and others (11) have speculated may be important in V-12 Ras augmentation of c-Ets-1 transactivation potency.

DISCUSSION

Although oncogene and growth factor activation of gene transcription has been the subject of extensive investigation (32, 56), many of the nuclear targets studied to date are transcription factors that are present in most cell types. Thus, the question how the oncogene and growth factor pathways regulate cell-type-specific genes has not been addressed. Here, we show that the Ras/Raf pathway regulates pituitary-specific gene expression at two different levels: (i) at the level of *trans*-acting factors, both a widely expressed factor, c-Ets-1, and a tissue-specific factor, GHF-1, are required for optimal rPRL promoter activation; and (ii) at the *cis*-acting level, the presence of an EBS/GHF-1 composite RRE in the rPRL promoter and its absence in the rGH promoter further restrict the Ras/Raf response to a subset of pituitary-specific, GHF-1-dependent promoters. These studies therefore provide novel and important mechanistic insights into how the general growth factor and oncogene signaling cascade is harnessed in a cell-specific and gene-specific manner to result in exquisite control of gene expression.

In the studies shown here, we have verified, using several different experimental paradigms, that the proto-oncogene c-Ets-1 is required to mediate the Ras/Raf responses of the pituitary-specific rPRL promoter. By contrast, the observations that intact c-Ets-2 has no effect while the dominant-negative Ets construct, which encodes only the ETS-binding domain of human c-Ets-2 (49), inhibits Ras/Raf activation of the rPRL promoter suggest that c-Ets-2 contains domains which may modulate its function. Indeed, conserved sequences flanking the ETS domain of c-Ets-1 and c-Ets-2 have been shown to inhibit DNA binding and serve as regulatory regions (10, 27, 85). While the differential ability of c-Ets-1 and c-Ets-2 to *trans*-activate target promoters could be due to differences in DNA binding, the possibility remains that differences in Ets protein stability could also account for the observations reported here. However, it is important to note that the chicken c-Ets-2 expressed from the pSG5-Ets-2 vector used here can function as a nuclear target of the oncogenic Ras response in F9 teratocarcinoma cells (84). Additionally, both chicken c-Ets-1 and human c-Ets-2 are able to stimulate basal *junB* promoter activity and enhance its activation by oncogenic Ha*ras*, although c-Ets-1 is clearly the better transactivator (11). The basis for the differential activity of c-Ets-2 may be due to cell-type-specific influences on its activity. For example, structural differences between c-Ets-1 and c-Ets-2 may render the latter unable to functionally interact with GHF-1 (see below). Indeed, distinct Ets family members may have selective effects because of their ability to recruit different factors to specific promoters (37, 52, 82). Thus, although c-Ets-1 and c-Ets-2 bind to identical consensus sequences in vitro, specific effects of each factor have been reported (17, 69, 83, 87), which are dependent on promoter context and Ets isoform-specific interactions with other transcription factors (37, 52, 82).

Although in our previous studies we questioned the role of the pituitary-specific, POU homeo-domain transcription factor, GHF-1, in the activation of the rPRL promoter by Ras/Raf (14, 15), here we present several lines of evidence documenting that GHF-1 is a critical component of the Ras/Raf response of the rPRL promoter in GH_4 pituitary cells. First, intact rat GHF-1 cooperates with Ras and Raf to synergistically activate the rPRL promoter in a dose-dependent manner (Fig. 3 and 4). Second, GHF-1 synergizes with intact c-Ets-1 to enhance the Ras activation of the rPRL promoter (Fig. 5). Third, the GHF-2 isoform, which contains a 26-amino-acid insertion within the transcription activation domain (34, 46, 77, 78), functions as a dominant-negative effector with regard to basal rPRL and rGHF-1 promoter activation (46, 78) and blocks the effects of both Ras and Raf on the rPRL promoter (Fig. 4 and 6). However, GHF-2 reconstitutes rGH promoter activity in nonpituitary cells with about equal potency to that of GHF-1 (46, 78), indicating that, like the various Ets isoforms, promoter context and interactions with other proteins may dictate the transcription activation function of the GHF isoforms. Indeed, we have preliminary data indicating that GHF-2 is equally as active as GHF-1 in reconstituting the protein kinase A response of the rPRL promoter in HeLa nonpituitary cells (9). Nevertheless, in contradistinction to the lack of effect of the c-Ets-2 isoform, GHF-2 significantly inhibits both the Ras and Raf responses (Fig. 4 and 6). This finding is consistent with previous reports showing that GHF-2 can bind DNA as well as GHF-1 but has differential transcription potency which is specific for the target promoter (46, 78). Taken together, these data suggest either that the 26-amino-acid insertion present at position 48 in GHF-2 disrupts a domain critical for its interaction with other factors, such as c-Ets-1, or that GHF-2 forms a transcriptionally inactive complex with c-Ets-1 or another transcription factor(s) required for rPRL promoter activation. Finally, these data verify that GHF-1 is a necessary but insufficient nuclear component of the Ras/Raf pathway in pituitary cells, since the evolutionarily related and GHF-1-dependent rGH promoter fails to respond to either Ras, Raf, GHF-1, c-Ets-1, or combinations thereof (Fig. 5) (14, 15). Thus, optimal Ras/Raf activation of the rPRL promoter requires both c-Ets-1 and GHF-1.

The key mode of action of the Ets family members appears to be via cooperative interactions with other transcription factors, which results in synergistic activation of gene transcription (17, 19, 22, 31, 37, 53, 60, 61, 68, 81–83, 87). Although these cumulative data imply that Ets factors recruit other transcription factors to adjacent DNA-binding sites, resulting in a ternary complex (37), to date there are few such examples (31, 68, 87). The serum response is governed by the interaction of the Ets member Elk-1 with serum response element-bound serum response factor, providing the prototypical example of a

functional interaction between Ets members and other transcription factors (31, 36, 53). In the studies reported here, we show that transfection of either c-Ets-1 or GHF-1 expression vectors alone is able to enhance the Ras or Raf responses of the rPRL promoter (Fig. 1, 3, and 4), although not to maximal levels. These data indicate that one or both of these factors becomes a rate-limiting component of the pathway upon overexpression of oncogenic Ras or Raf; however, this limitation is apparently circumvented by providing both factors exogenously. Moreover, the fact that either GHF-1 or c-Ets-1 is able to enhance the Ras response implies that interactions of exogenous with endogenous partners is occurring, since GH_4 pituitary cells contain both GHF-1 and c-Ets-1 (12). Verifying that c-Ets-1 and GHF-1 cooperate is the observation that neither c-Ets-1 nor GHF-1 alone is sufficient to mediate the maximal Ras/Raf response but, instead, that both factors are required (Fig. 5). Further support for this interpretation is provided by the crossover inhibition of either the GHF-1 or c-Ets-1 enhancement of the Ras response by their dominant-negative partner (Fig. 6). Thus, dominant-negative Ets inhibits both Ras activation and the synergistic response of Ras plus GHF-1, and, similarly, GHF-2 inhibits both the Ras response and the c-Ets-1-mediated enhancement (Fig. 6). Since the dominant-negative isoforms function by virtue of their nonproductive binding to DNA, these data suggest that formation of a ternary complex of GHF-1 and c-Ets-1 on a composite DNA element is required to mediate optimal Ras/Raf regulation of the rPRL promoter. Furthermore, mutation of the −217 to −209 EBS significantly blunts the effects of V-12 Ras alone and inhibits the enhancing effects of c-Ets-1 and/or GHF-1 on the Ras response of the rPRL promoter (Fig. 5). This also corroborates the notion that the RRE functions as a composite element requiring both factors. These findings are in agreement with previous reports, which provide similar functional data that show (i) that there is cooperativity between an Ets member and another transcription factor and (ii) that the *cis*-acting element typically contains adjacent binding sites for these factors, in support of a composite element (36). The requirement for two distinct factors and a bipartite DNA-binding region to interact in combination provides a multicomponent mechanism to permit an extremely high degree of transcription control. Indeed, in this system, the lack of a putative EBS in the GHF-1-dependent rGH promoter results in its total inability to respond to either oncogenic Ras or Raf, whereas the combination of V-12 Ras, GHF-1, and c-Ets-1 results in over 60-fold stimulation of the related rPRL promoter (Fig. 5). Interestingly, another GHF-1-dependent gene, rGHF-1 itself, also contains a GHF-1 footprint (8, 55), which is flanked by several putative Ets-binding sites, suggesting that this GHF-1-dependent promoter may also be regulated by the Ras/Raf oncogene-signaling pathway.

While the data indicate that a Ras/Raf-dependent functional interaction between these factors occurs to mediate a maximal Ras/Raf response of the rPRL promoter, it is important to stress that our findings fail to show any evidence for direct protein-protein interactions between GHF-1 and c-Ets-1. To date, there are three clear examples of functionally relevant, direct physical interactions between an Ets protein and another factor: (i) PU.1 interacts with the B-cell-restricted factor NF-EM5, and this interaction requires PU.1 phosphorylation (68); (ii) Elf-1 interacts only with dephosphorylated forms of Rb (81); and (iii) Elk-1 and serum response factor interact directly and in the absence of the composite DNA-binding site (75). By contrast, the vast majority of examples published to date show that an Ets member cooperates with another transcription factor only at the level of promoter activation, and they fail to provide biochemical evidence for any direct physical interactions (11, 17, 19, 22, 60, 61, 70, 83, 87). In this respect, the data presented here are in keeping with these published reports. However, it is important to note that, distinct from these previous studies, the c-Ets-1/GHF-1 functional interaction is conditional upon activation of the Ras/Raf pathway (Fig. 6) and that exogenous c-Ets-1, GHF-1, or both, had minimal effects on rPRL promoter activity (Fig. 1 and 5). From these data, it is likely that diverse molecular mechanisms, such as direct and indirect, and signal dependent and independent, may be responsible for the observed functional interactions of Ets members with other transcription factors. Therefore, in cases when a direct protein-protein interaction has not been detected, the possibility remains that a bridging factor actually stabilizes the indirect interaction of Ets with other factors, much like the proposed stabilization of the DRTF1/E2F heterodimer by the putative bridging factor Orf 6/7 (50). Irrespective of the precise molecular mechanism, we have taken advantage of our assay system to map the domain of c-Ets-1 required to mediate either the Ras- or the Ras–plus–GHF-1-mediated enhancement of rPRL promoter activation and found that a 40-amino-acid segment in the amino-terminal region, including residues 57 to 98, is necessary for both of these effects (Fig. 7). Of note, this segment colocalizes precisely with the amino terminus of the two separate transcription activation domains of c-Ets-1 previously mapped by using a LexA operator reporter construct and LexA fusions of these same c-Ets-1 amino-terminal deletions (73). Thus, we have been able to functionally dissect the structure-function relationships of both c-Ets-1 and GHF-1, which are required for their synergistic effect, by using different isoforms (e.g., c-Ets-2 and GHF-2) and amino-terminal deletion mutants of c-Ets-1 and hence to define critical regions of each molecule to mediate the Ras and Raf effect.

Members of the Ets family of transcription factors are typically found in the cell as phosphorylated proteins, and it has been suggested that such phosphorylations may play a central role in the regulation of their transcription potency (37, 52, 82). For example, increased gene transcription in response to proliferative signals that are mediated by the serum response element involves MAP kinase phosphorylation of Elk-1 (31, 36, 53). Similarly, the heterodimerization of PU.1 with the B-cell-restricted factor NF-EM5 is dependent on PU.1 phosphorylation, although the in vivo signaling pathway which mediates this effect has not yet been identified (68). Finally, one of the more elegant examples of the Ras/Raf/MAP kinase pathway regulating Ets transcription factor activity is the role of the Ets proteins pointed and yan in establishing the fate of R7 photoreceptor cells in *Drosophila* eye development (5, 65). In these studies, Ras- and MAP kinase-dependent signaling inhibited yan activity, whereas a critical MAP kinase phosphorylation site, $PLT^{151}P$, was identified in the positive regulator pointed (5, 65). We have previously shown that expression of a kinase-deficient, inhibitory form of MAP kinase significantly reduces oncogenic Ras and Raf activation of the rPRL promoter (15), implying that MAP kinase is a component of the Ras/Raf/c-Ets-1/GHF-1 signaling pathway leading to rPRL promoter activation. Consistent with these results, a consensus MAP kinase phosphorylation site ($PLLT^{82}P$) is contained in the region of c-Ets-1 required to mediate its enhancement of the Ras effect upon rPRL promoter activity and for the Ras-dependent functional interaction of c-Ets-1 with GHF-1. Of note, this putative c-Ets-1 MAP kinase site is analogous to the critical site identified in the pointed gene (5, 63) and has been proposed to be important for the Ras response of the *junB* promoter (11). However, biochemical evidence documenting

c-Ets-1 phosphorylation, in vivo, by MAP kinase in a growth factor- or Ras-dependent manner has not yet been reported.

The role of phosphorylation of GHF-1 in regulating its *trans*-acting capacity has been much less clear. Although an early study (38) indicated that phosphorylation of serine 115 and threonine 220, catalyzed by either protein kinase A or protein kinase C, altered the DNA-binding activity of GHF-1, subsequent site-specific mutations of these two inducible phosphorylation sites were shown to have no effect on the protein kinase A- or protein kinase C-mediated increase in GHF-1 transcription potency (21, 24, 64). Interestingly, however, amino acid sequence analysis of GHF-1 reveals a putative proline-directed kinase site ($TLT^{75}PC$) located within the *trans*-activation domain. Nevertheless, whether Ras induces GHF-1 phosphorylation at all, the precise functional role of any such phosphorylation and the effects of the GHF-2 insert domain on its suitability as a kinase substrate remain to be defined.

In summary, the Ras/Raf-dependent functional interaction of c-Ets-1 and GHF-1 on a composite DNA element, which contains an EBS juxtaposed to a GHF-1-binding site, provides an important and novel mechanism by which general signaling pathways can activate highly specialized and cell-specific genes. Moreover, the requirement for a combination of all three components, c-Ets-1, GHF-1, and the composite DNA element, to mediate the Ras/Raf activation of the rPRL promoter, provides molecular details which significantly further our understanding of the complexity and exquisite specificity of gene control mechanisms. The insights gained are likely to be applicable to other systems and therefore to be of general significance. Indeed, on the basis of the observation that homeodomain proteins heterodimerize with serum response factor, it has been proposed that such interactions provide a means by which cell-specific genes may be activated in response to a generic signal (23). The Ras-dependent functional interaction of c-Ets-1 and the homeo-box factor GHF-1 reported here provide direct evidence in support of this elegant hypothesis.

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REFERENCES

1. **Avvedimento, V. E., A. M. Musti, M. Ueffing, S. Obici, A. Gallo, M. Sanchez, D. DeBrasi, and M. E. Gottesman.** 1991. Reversible inhibition of a thyroid-specific *trans*-acting factor by Ras. Genes Dev. **5:**22–28.
2. **Barbacid, M.** 1987. Ras genes. Annu. Rev. Biochem. **56:**779–827.
3. **Bodner, M., J. L. Castrillo, L. E. Theill, T. Deerinck, M. Ellisman, and M. Karin.** 1988. The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. Cell **55:**505–518.
4. **Bruder, J. T., G. Heidecker, and U. R. Rapp.** 1992. Serum-, TPA-, and *ras*-induced expression from AP-1/*ets*-drives promoters requires *raf-1* kinase. Genes Dev. **6:**545–556.
5. **Brunner, D., K. Ducker, N. Oellers, E. Hafen, H. Scholz, and C. Klambt.** 1994. The ETS domain protein Pointed-P2 is a target of MAP kinase in the Sevenless signal transduction pathway. Nature (London) **370:**386–389.
6. **Castrillo, J. L., L. E. Theill, and M. Karin.** 1991. Function of the homeodomain protein GHF1 in pituitary cell proliferation. Science **253:**197–199.
7. **Chao, M. V.** 1992. Growth factor signaling: where is the specificity? Cell **68:**995–997.
8. **Chen, R. P., H. A. Ingraham, M. N. Treacy, V. R. Albert, L. Wilson, and M. G. Rosenfeld.** 1990. Autoregulation of Pit-1 gene expression mediated by two *cis*-active promoter elements. Nature (London) **346:**583–586.
9. **Chiono, M., and A. Gutierrez-Hartmann.** Unpublished data.
10. **Chumakov, A. M., D. L. Chen, E. A. Chumakova, and H. P. Koeffler.** 1993. Localization of the c-Ets-2 transactivation domain. J. Virol. **67:**2421–2425.
11. **Coffer, P., M. de Jonge, A. Mettouchi, B. Binetruy, J. Ghysdael, and W. Kruijer.** 1994. *junB* promoter regulation: Ras mediated transactivation by c-Ets- and c-Ets-2. Oncogene **9:**911–921.
12. **Conrad, K. E., A. P. Bradford, and A. Gutierrez-Hartmann.** Unpublished data.
13. **Conrad, K. E., A. P. Bradford, P. Tran, L. Nguyen, M. C. Ostrowski, and A. Gutierrez-Hartmann.** Unpublished data.
14. **Conrad, K. E., and A. Gutierrez-Hartmann.** 1992. The *ras* and protein kinase A pathways are mutually antagonistic in regulating rat prolactin promoter activity. Oncogene **7:**1279–1286.
15. **Conrad, K. E., J. M. Oberwetter, R. Vaillancourt, G. L. Johnson, and A. Gutierrez-Hartmann.** 1994. Identification of the functional components of the Ras signaling pathway regulating pituitary cell-specific gene expression. Mol. Cell. Biol. **14:**1553–1565.
16. **Dent, P., W. Haser, T. A. J. Haystead, L. A. Vincent, T. M. Roberts, and T. W. Sturgill.** 1992. Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cell in vitro. Science **257:**1404–1406.
17. **Dudek, H., R. V. Tantravahi, V. N. Rao, E. S. P. Reddy, and E. P. Reddy.** 1992. Myb and Ets proteins cooperate in transcriptional activation of the Mim-1 promoter. Proc. Natl. Acad. Sci. USA **89:**1291–1295.
18. **Elsholtz, H. P.** 1992. Molecular biology of prolactin: cell-specific and endocrine regulators of the prolactin gene. Semin. Reprod. Endocrinol. **10:**183–195.
19. **Ernst, P., K. Hahm, and S. T. Smale.** 1993. Both LyF-1 and an Ets protein interact with a critical promoter element in the murine terminal transferase gene. Mol. Cell. Biol. **13:**2982–2992.
20. **Feig, L. A.** 1993. The many roads that lead to Ras. Science **260:**767–768.
21. **Fischberg, D. J., X.-H. Chen, and C. Bancroft.** 1994. A Pit-1 phosphorylation mutant can mediate either basal or induced prolactin or growth hormone promoter activity. Mol. Endocrinol. **8:**1566–1573.
22. **Gegonne, A., R. Bosselut, R.-A. Bailly, and J. Ghysdael.** 1993. Synergistic activation of the HTLV1 LTR Ets-responsive region by transcription factors Ets-1 and Sp-1. EMBO J. **12:**1169–1178.
23. **Grueneberg, D. A., S. Natesan, C. Alexandre, and M. Z. Gilman.** 1992. Human and Drosophila homeodomain proteins that enhance the DNA-binding activity of serum response factor. Science **257:**1089–1095.
24. **Gutierrez-Hartmann, A.** 1994. Pit-1/GHF-1: a pituitary-specific transcription factor linking general signaling pathways to cell-specific gene expression. Mol. Endocrinol. **8:**1447–1449.
25. **Gutierrez-Hartmann, A., S. Siddiqui, and S. Loukin.** 1987. Selective transcription and DNase I protection of the rat prolactin gene by GH3 pituitary cell-free extracts. Proc. Natl. Acad. Sci. USA **84:**5211–5215.
26. **Gutman, A., and B. Wasylyk.** 1991. Nuclear targets for transcription regulation by oncogenes. Trends Genet. **7:**49–54.
27. **Hagman, J., and R. Grosschedl.** 1992. An inhibitory carboxyl-terminal domain of Ets-1 and Ets-2 mediates differential binding of ETS family factors to promoter sequences of the mb-1 gene. Proc. Natl. Acad. Sci. USA **89:**8889–8893.
28. **Harvey, C., S. M. Jackson, S. K. Siddiqui, and A. Gutierrez-Hartmann.** 1991. Structure-function analysis of the rat prolactin promoter: phasing requirements of proximal cell-specific elements. Mol. Endocrinol. **5:**836–843.
29. **Haugen, B. R., D. F. Gordon, W. W. Wood, and E. C. Ridgway.** 1994. The combination of Pit-1 and Pit-1T have a synergistic stimulatory effect on the thyrotropin β subunit promoter but not the growth hormone or prolactin promoters. Mol. Endocrinol. **8:**1574–1582.
30. **Herschlag, D., and F. B. Johnson.** 1993. Synergism in transcriptional activation: a kinetic view. Genes Dev. **7:**173–179.
31. **Hill, C. S., R. Marais, S. John, J. Wynne, S. Dalton, and R. Treisman.** 1993. Functional analysis of a growth factor-responsive transcription complex. Cell **73:**395–406.
32. **Hunter, T., and M. Karin.** 1992. The regulation of transcription by phosphorylation. Cell **70:**375–387.
33. **Ingraham, H. A., R. P. Chen, H. J. Mangalam, H. P. Elsholtz, S. E. Flynn, C. R. Lin, D. M. Simmons, L. Swanson, and M. G. Rosenfeld.** 1988. A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. Cell **55:**519–529.
34. **Ingraham, H. A., S. E. Flynn, J. W. Voss, V. R. Albert, M. S. Kapiloff, L. Wilson, and M. G. Rosenfeld.** 1990. The POU-specific domain of Pit-1 is essential for sequence-specific, high affinity DNA binding and DNA-dependent Pit-1-Pit-1 interactions. Cell **61:**1021–1033.
35. **Jackson, S. M., C. A. Keech, D. J. Williamson, and A. Gutierrez-Hartmann.** 1992. Interaction of positive and negative transcription elements controls repression of the proximal rat prolactin promoter in nonpituitary cells. Mol. Cell. Biol. **12:**2708–2719.
36. **Janknecht, R., W. H. Ernst, V. Pingoud, and A. Nordheim.** 1993. Activation of ternary complex factor Elk-1 by MAP kinases. EMBO J. **12:**5097–5104.
37. **Janknecht, R., and A. Nordheim.** 1993. Gene regulation by Ets proteins.

Biochim. Biophys. Acta **1155:**346–356.
38. **Kapiloff, M. S., Y. Farkash, M. Wegner, and M. G. Rosenfeld.** 1991. Variable effects of phosphorylation of Pit-1 dictated by the DNA response elements. Science **253:**786–789.
39. **Karga, H. J., J. M. Alexander, E. T. Hedley-Whyte, A. Klibanski, and J. L. Jameson.** 1992. Ras mutations in human pituitary tumors. J. Clin. Endocrinol. Metab. **74:**914–919.
40. **Karin, M., L. Theill, J. L. Castrillo, A. McCormick, and H. Brady.** 1990. Tissue-specific expression of the growth hormone gene and its control by growth hormone factor-1. Recent Prog. Horm. Res. **46:**43–57.
41. **Keech, C. A., and A. Gutierrez-Hartmann.** 1989. Analysis of rat prolactin promoter sequences that mediate pituitary-specific and 3′,5′-cyclic adenosine monophosphate-regulated gene expression *in vivo*. Mol. Endocrinol. **3:**832–839.
42. **Keech, C. A., and A. Gutierrez-Hartmann.** 1991. Insulin activation of rat prolactin promoter activity. Mol. Cell. Endocrinol. **78:**55–60.
43. **Keech, C. A., S. M. Jackson, S. K. Siddiqui, K. W. Ocran, and A. Gutierrez-Hartmann.** 1992. Cyclic AMP activation of the rat prolactin promoter is restricted to the pituitary-specific cell type. Mol. Endocrinol. **6:**2059–2070.
44. **Klaes, A., T. Menne, A. Stollewerk, H. Scholz, and C. Klambt.** 1994. The Ets transcription factors encoded by the Drosophila gene *pointed* direct glial cell differentiation in the embryonic CNS. Cell **78:**149–160.
45. **Kola, I., S. Brookes, A. R. Green, R. Garber, M. Tymms, T. S. Papas, and A. Seth.** 1993. The Ets-1 transcription factor is widely expressed during murine embryo development and is associated with mesodermal cells involved in morphogenetic processes such as organ formation. Proc. Natl. Acad. Sci. USA **90:**7588–7592.
46. **Konzak, K. E., and D. D. Moore.** 1992. Functional isoforms of Pit-1 generated by alternative mRNA splicing. Mol. Endocrinol. **6:**241–247.
47. **Lai, Z.-C. and G. M. Rubin.** 1992. Negative control of photoreceptor development in *Drosophila* by the product of the *yan* gene, an ETS domain protein. Cell **70:**609–620.
48. **Lange-Carter, C. A., C. M. Pleiman, A. M. Gardner, K. J. Blumer, and G. L. Johnson.** 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science **260:**315–319.
49. **Langer, S. J., D. M. Bortner, M. F. Roussel, C. J. Sherr, and M. C. Ostrowski.** 1992. Mitogenic signaling by colony-stimulating factor 1 and *ras* is suppressed by the *ets-2* DNA-binding domain and restored by *myc* overexpression. Mol. Cell. Biol. **12:**5355–5362.
50. **La Thangue, N. B.** 1994. DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. Trends Biochem. Sci. **14:**108–114.
51. **MacGregor, G. R., and C. T. Caskey.** 1989. Construction of plasmids that express *E. coli* beta-galactosidase in mammalian cells. Nucleic Acids Res. **17:**2365.
52. **Macleod, K., D. Leprince, and D. Stehelin.** 1992. The *ets* gene family. Trends Biochem. Sci. **17:**251–256.
53. **Marais, R., J. Wynne, and R. Treisman.** 1993. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell **73:**381–393.
54. **Maxwell, I. H., G. S. Harrison, W. M. Wood, and F. Maxwell.** 1989. A DNA cassette containing a trimerized SV40 polyadenylation signal which efficiently blocks spurious plasmid-initiated transcription. BioTechniques **7:**276–280.
55. **McCormick, A., H. Brady, L. E. Theill, and M. Karin.** 1990. Regulation of the pituitary-specific homeobox gene GHF1 by cell-autonomous and environmental cues. Nature (London) **345:**829–832.
56. **Meek, D. W., and A. J. Street.** 1992. Nuclear protein phosphorylation and growth control. Biochem. J. **287:**1–15.
57. **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.
58. **Morris, A. E., B. Kloss, R. E. McChesney, C. Bancroft, and L. A. Chasin.** 1992. An alternatively spliced Pit-1 isoform altered in its ability to transactivate. Nucleic Acids Res. **20:**1355–1361.
59. **Nakagawa, T., M. Mabry, A. deBustros, J. N. Ihle, B. D. Nelkin, and S. B. Baylin.** 1987. Introduction of v-Ha-Ras oncogene induces differentiation of cultured human medullary thyroid carcinoma cells. Proc. Natl. Acad. Sci. USA **84:**5923–5927.
60. **Nakajima, K., T. Kusafuka, T. Takeda, Y. Fujiani, K. Nakae, and T. Hirano.** 1993. Identification of a novel interleukin-6 response element containing an Ets binding site and a CRE-like site in the *junB* promoter. Mol. Cell. Biol. **13:**3027–3041.
61. **Nelsen, B., G. Tian, B. Erman, J. Gregoire, R. Maki, B. Graves, and R. Sen.** 1993. Regulation of lymphoid specific immunoglobulin mu heavy chain gene enhancer by ETS-domain proteins. Science **261:**82–86.
62. **Nye, J. A., J. M. Petersen, C. V. Gunther, M. D. Jonsen, and B. J. Graves.** 1992. Interaction of murine Ets-1 with GGA-binding sites establishes the ETS domain as a new DNA-binding motif. Genes Dev. **6:**975–990.
63. **Oberwetter, J. M., K. E. Conrad, and A. Gutierrez-Hartmann.** 1993. The Ras and protein kinase C signaling pathways are functionally antagonistic in GH4 neuroendocrine cells. Mol. Endocrinol. **7:**915–923.
64. **Okimura, Y., P. W. Howard, and R. A. Maurer.** 1994. Pit-1 binding sites mediate transcriptional responses to cAMP through a mechanism which does not require inducible phosphorylation of Pit-1. Mol. Endocrinol. **8:**1559–1565.
65. **O'Neill, E. M., I. Rebay, R. Tjian, and G. M. Rubin.** 1994. The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. Cell **78:**137–147.
66. **Pazin, M. J., and L. T. Williams.** 1992. Triggering signaling cascades by receptor tyrosine kinases. Trends Biochem. Sci. **17:**374–378.
67. **Pelech, S. L., and J. S. Sanghera.** 1992. Mitogen-activated protein kinases: versatile transducers for cell signaling. Trends Biochem. Sci. **17:**233–238.
68. **Pongubala, J. M. R., C. V. Beveren, S. Nagulapalli, M. J. Klemsz, S. R. McKercher, R. A. Maki, and M. L. Atchison.** 1993. Effect of PU.1 phosphorylation of interaction with NF-EM5 and transcriptional activation. Science **259:**1622–1625.
69. **Prosser, H. M., D. Wotton, A. Gegonne, J. Ghysdael, S. Wang, N. A. Speck, and M. J. Owen.** 1992. A phorbol ester response element within the human T-cell receptor β-chain enhancer. Proc. Natl. Acad. Sci. USA **89:**9934–9938.
70. **Reddy, M. A., S. J. Langer, M. S. Colman, and M. C. Ostrowski.** 1992. An enhancer element responsive to *ras* and *fms* signaling pathways is composed of two distinct nuclear factor binding sites. Mol. Endocrinol. **6:**1051–1060.
71. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
72. **Satoh, T., M. Nakafuku, and Y. Kaziro.** 1992. Function of Ras as a molecular switch in signal transduction. J. Biol. Chem. **267:**24149–24152.
73. **Schneikert, J., Y. Lutz, and B. Wasylyk.** 1992. Two independent activation domains in cEts1 and cEts2 located in nonconserved sequences of the ets gene family. EMBO J. **7:**249–256.
74. **Shih, C., and R. A. Weinberg.** 1982. Isolation of a transforming sequence from a human bladder carcinoma cell line. Cell **29:**161–169.
75. **Shore, P., and A. D. Sharrocks.** 1994. The transcription factors Elk-1 and serum response factor interact by direct protein-protein contacts mediated by a short region of Elk-1. Mol. Cell. Biol. **14:**3283–3291.
76. **Tashjian, A. H. J., Y. Yasumura, L. Levine, G. H. Sato, and M. L. Parker.** 1968. Establishment of clonal strains of rat pituitary tumor cells that secrete growth hormone. Endocrinology **82:**342–352.
77. **Theill, L. E., J. L. Castrillo, D. Wu, and M. Karin.** 1989. Dissection of functional domains of the pituitary-specific transcription factor GHF-1. Nature (London) **342:**945–948.
78. **Theill, L. E., K. Hattori, D. Domenico, J.-L. Castrillo, and M. Karin.** 1992. Differential splicing of the GHF1 primary transcript gives rise to two functionally distinct homeodomain proteins. EMBO J. **11:**2261–2269.
79. **Thomas, S. M., M. DeMarco, G. D'Arcangelo, S. Halegoua, and J. S. Brugge.** 1992. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. Cell **68:**1031–1040.
80. **Voss, J. W., and M. G. Rosenfeld.** 1992. Anterior pituitary development: short tales from dwarf mice. Cell **70:**527–530.
81. **Wang, C.-Y., B. Petryniak, C. B. Thompson, W. G. Kaelin, and J. M. Leiden.** 1993. Regulation of the Ets-related transcription factor Elf-1 by binding to the retinoblastoma protein. Science **260:**1330–1335.
82. **Wasylyk, B., S. H. Hahn, and A. Giovane.** 1993. The Ets family of transcription factors. Eur. J. Biochem. **211:**7–18.
83. **Wasylyk, B., C. Wasylyk, P. Flores, A. Begue, D. Leprince, and D. Stehelin.** 1990. The c-*ets* proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. Nature (London) **346:**191–193.
84. **Wasylyk, C.** Unpublished data.
85. **Wasylyk, C., J.-P. Kerckaert, and B. Wasylyk.** 1992. A novel modulator domain of Ets transcription factors. Genes Dev. **6:**965–974.
86. **Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis.** 1992. Ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell **68:**1041–1050.
87. **Wu, H., K. Moulton, A. Horval, S. Parik, and C. K. Glass.** 1994. Combinatorial interactions between AP-1 and Ets domain proteins contribute to the developmental regulation of the macrophage scavenger receptor gene. Mol. Cell. Biol. **14:**2129–2139.