GHF-1/Pit-1 Functions as a Cell-specific Integrator of Ras Signaling by Targeting the Ras Pathway to a Composite Ets-1/GHF-1 Response Element*

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Activation of the rat prolactin (rPRL) promoter by Ras is a prototypical example of tissue-specific transcriptional regulation in a highly differentiated cell type. Using a series of site-specific mutations and deletions of the proximal rPRL promoter we have mapped the major Ras/Raf response element (RRE) to a composite Ets-1/GHF-1 binding site located between positions −217 and −190. Mutation of either the Ets-1 or GHF-1 binding sites inhibits Ras and Raf activation of the rPRL promoter, and insertion of this RRE into the rat growth hormone promoter confers Ras responsiveness. We show that Ets-1 is expressed in GH4 cells and, consistent with their functional synergistic interaction, both Ets-1 and GHF-1 are able to bind specifically to this bipartite RRE. We confirm that Ets-1 or a related Ets factor is the nuclear target of the Ras pathway leading to activation of the rPRL promoter and demonstrate that Elk-1 and Net do not mediate the Ras response. Thus, the pituitary-specific POU homeodomain transcription factor, GHF-1, serves as a cell-specific signal integrator by functionally interacting with an Ets-1-like factor, at uniquely juxtaposed binding sites, thereby targeting an otherwise ubiquitous Ras signaling pathway to a select subset of cell-specific GHF-1-dependent genes.

The p21 Ras proto-oncogene is a critical component of a network of signaling pathways that mediate the control of cell growth, metabolism, and differentiation (1) Signals initiated at transmembrane receptors are transduced via Ras and propagated, by a phosphorylation cascade, to the nucleus, resulting in changes in the activity of specific transcription factors (2, 3). Distinct signaling components of the Ras pathway may be present in different cell types, allowing the signal to be interpreted in a cell-specific manner (4, 5) Indeed, cell-specific, phenotypic sequelae of Ras activation are exemplified by the differential effects of oncogenic Ras in PC12 pheochromocytoma, TT medullary carcinoma, and FRTL5 thyroid cells (6–8), whereby V-12 Ras induces terminal differentiation of the first two cell lines but causes transformation of the last. Thus, the characterization of cell-specific endogenous nuclear factors that may act as effectors of the Ras signaling pathway and the identification of specific Ras-responsive cis-acting DNA elements are important unanswered questions. Several Ras/Raf response elements (RREs) or oncogene response units have been identified to date, implicating members of the Ets, AP-1, and ATF/CREB families of transcription factors as nuclear components of the Ras signaling pathway (2, 3).

Tandem c-Ets-2 binding sites have been shown to confer Ras responsiveness in NIH-3T3 cells (9), and dominant-negative Ets constructs inhibit both Ras-induced mitogenesis (10) and transformation (11). The serum response is governed by MAP kinase phosphorylation of Elk-1, a member of the Ets family of transcription factors (12, 13), thus facilitating its interaction with serum response factor (SRF). Similarly, the Drosophila Ets factors, Pointed and Yan (14, 15), the negative regulator Net (16), and the repressor Erf (17) are also regulated by the Ras/Raf/MAP kinase pathway.

A number of RREs contain AP-1-like elements (18–20). Furthermore, the transcription potency of both c-Fos and c-Jun is enhanced by phosphorylation catalyzed by Ras-activated protein kinases (21, 22), and a transdominant Jun factor is able to suppress transformation of cells by Ras (23). Other RREs contain a cAMP response element (CRE) (24, 25), and Ras-dependent phosphorylation of CREB/ATF, leading to increased transcription of the c-fos proto-oncogene, has been demonstrated in PC12 cells (26). Finally, the oncogene response units of certain cellular genes consists of binding sites for both AP-1 and Ets factors (27, 28), and these transcription factors have been shown to cooperate functionally in mediating a Ras response (29, 30).

Regulation of the rat prolactin (rPRL) gene in the rat GH4 pituitary cell line is an excellent model system in which to study cell-specific aspects of the Ras signaling pathway (31–34). GH4 cells are a highly differentiated neuroendocrine line, which retain cell-specific functions and hormonal responses (35–37) and express the phenotypic marker PRL under control of the pituitary-specific, POU homeodomain transcription fact
tor, GFP-1/Pit-1 (38, 39). We have previously shown that oncogenic V-12 Ras selectively activates the rPRL promoter in GH3 cells and that the Ras signal is not transduced via protein kinase C or protein kinase A. Indeed, in our pituitary model system, the Ras and protein kinase A/protein kinase C signaling pathways are mutually antagonistic (33, 34). Furthermore, overexpression of c-Jun inhibits Ras activation of the proximal rPRL promoter (34), which does not contain any DNA sequences homologous to canonical CRE or AP-1 sites (32). Recent evidence from this laboratory indicates a critical role for the transcription factor Ets-1 in Ras activation of the rPRL promoter (32) and demonstrates that a functional interaction of Ets-1 and GFP-1 is required for an optimal Ras response (31). Additionally we have proposed that an Ets binding site (EBS) adjacent to a GFP-1 binding site (footprint IV (FPIV)), spanning positions −217 to −190, functions as a composite rPRL promoter RRE. However, a recent report has implicated a more proximal region of the rPRL promoter, (−165 to −150), also containing an Ets site (EBS) in a luciferase reporter construct (40). Thus, in the studies presented here, we determine the precise cis-acting DNA sequences and transacting factors, within the context of the proximal rPRL promoter, that are necessary and sufficient for Ras activation. We utilized a series of site-specific mutations and deletions in the proximal rPRL promoter to precisely map the principal and physiologically relevant RRE to the composite Ets-1/GFP-1 binding site spanning positions −217 to −190. Furthermore, we show, using immunological and electrophoretic mobility shift techniques, that GH3 rat pituitary cells express both GFP-1 and c-Ets-1 and that both factors exhibit specific binding to this composite RRE. In contrast, the more proximal element (−165 to −150) (40) was not functionally relevant for Ras or Raf activation in the context of an intact rPRL promoter.

Thus, the interaction of a widely expressed transcription factor, Ets-1, with the pituitary-specific POU homeodomain factor GFP-1 at a unique cis-element provides a molecular mechanism by which the ubiquitous Ras signaling pathway can selectively regulate expression of a cell-specific gene.

Experimental Procedures

Cell Culture—GH3/T2 rat pituitary tumor cells were repassaged through rats as described (33) and grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and penicillin-streptomycin. Cells were maintained at 37°C in 5% CO2. Medium was changed 4–12 h prior to use. Cells were rinsed through rats as described (33) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics for 24 h. Electroporations were performed in triplicate for each condition within a single experiment, and experiments were repeated using different plasmid preparations of each construct.

Luciferase and β-Galactosidase Assays—Transfected cells were harvested in phosphate-buffered saline (Life Technologies, Inc.) 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 described previously (31, 33). Samples were measured in duplicate using a Monolight 2010 luminometer (Analytical Luminescence Laboratories, San Diego, CA). β-Galactosidase activity was deter-

ined spectrophotometrically using the chromogenic substrate o-nitrophenyl-β-D-galactopyranoside essentially as described (31, 33). 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.

Electrophoretic Mobility Shift Assays—Whole cell extracts were prepared by harvesting confluent GH3 cells in phosphate-buffered saline containing 3 mM EDTA and resuspending pellets in 50 mM Tris, 150 mM NaCl, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 0.1% Triton X-100, pH 7.4. Cells were lysed by sonication, and extracts were clarified by centrifugation at 15,000 × g for 10 min at 4°C. Extracts of DH5α E. coli expressing GST fusion proteins were prepared essentially as described previously (42). Double-stranded oligonucleotides (see figure legends) were labeled using T4 polynucleotide kinase or Klenow DNA polymerase. Probes (∼30,000 cpm, 0.2–0.5 ng) were incubated for 30 min at 4°C, with the indicated E. coli or GH3 cell extracts in 10 mM Hepes, 70 mM KCl, 4% glycerol, 1 mM EDTA, and 2 mM dithiothreitol, pH 7.9, containing 500 ng/ml sheared herring sperm DNA or 1 mg/ml poly(dI,dC) in a final volume of 20 μl. Loading buffer

Composite Ets-1/GHF-1 Ras Response Element

The reporter construct pA3PRL luciferase contains a 593-base pair fragment encompassing positions −528 to +65 of the rGH gene has been described elsewhere (33). The plasmid pA rGH/RRE/luc was generated by PCR site-directed mutagenesis of the −214 to −209 EBS in pG7rPRL. The core AAGGAA is changed to CTCCGAG, generating a unique XhoI site, and the resulting construct was cloned into the HindIII restriction site of pA luc. Similarly the pA mpFIVIluc promoter was constructed by mutation of the core GFP-1 binding site (−195 to −200) within FP IV1 (41) from ATTAAT to Sall site GTCGAC. Both promoters were then sequenced to confirm the presence of the mutant EBS or mutant GFP-1 binding site and verify that the promoters were cloned into pA3PRL. The pA rGH luc reporter containing a 593-base pair fragment was sequenced to confirm the presence of the mutant EBS or mutant GFP-1 binding site.
and transfection and assayed for luciferase lesion. Cells were harvested 24 h after missingsymbols tations, and solid black bars by the FPII repressor site and BTE are denoted (Strasbourg).

were generously provided by Dr. B. Wasylyk (IGBMC, CNRS, according to the manufacturer's protocols. Antibodies to Elk-1 and Net (SantaCruzBiotech, CA), and developed using ECL (AmershamCorp.) probed with antibodies to GHF-1 (BAbCO, Berkeley, CA) or c-Ets-1 (2 h. Gels were dried, and DNA-protein complexes were detected by autoradiography.

Western Blots—Cell extracts were prepared from confluent 100-mm dishes. Cells were washed in cold phosphate-buffered saline and harvested with Laemmli SDS sample buffer. Extracts were boiled for 5 min, and viscosity was reduced by shearing through a 22-gauge needle. Samples were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose in 192 mM glycine, 25 mM Tris, 10% methanol at 25°C for 2 h. Gels were dried, and DNA-protein complexes were detected by autoradiography.

Results—Cell extracts were prepared from confluent 100-mm dishes. Cells were washed in cold phosphate-buffered saline and harvested with Laemmli SDS sample buffer. Extracts were boiled for 5 min, and viscosity was reduced by shearing through a 22-gauge needle. Samples were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose in 192 mM glycine, 25 mM Tris, 10% methanol at 100 mA for 16 h. Filters were blocked in 5% nonfat milk, 0.2% Tween 20, probed with antibodies to GHF-1 (BAbCO, Berkeley, CA) or c-Ets-1 (Santa Cruz Biotech, CA), and developed using ECL (AmershamCorp.) according to the manufacturer's protocols. Antibodies to Elk-1 and Net were generously provided by Dr. B. Wasylyk (IGBMC, CNRS, Strasbourg).

Results—Mapping of the Ras-responsive Element of the Rat PRL Promoter—Both the rPRL and rGH genes require the pituitary-specific POU homeodomain transcription factor GHF-1 for their expression (35, 39), and the promoters of both genes contain multiple GHF-1 binding sites (41). Previous studies have shown that the proximal GHF-1 binding sites (Fig. 1) are required for activation of the rPRL promoter in response to several hormones and growth factors (35, 43). FPI and FPIII are necessary for activation of the rPRL promoter by thyrotropin-releasing hormone (44), and FPI is also critical to mediate cAMP induction of PRL transcription (36, 45–47). Interestingly, these DNA cis-elements have also been implicated in the response of the rPRL promoter to both EGF (44, 48) and insulin (37, 49), both of which are known to transduce their signals via the Ras pathway in other cell types (50, 51). Subsequent studies indicate that insulin and EGF activation of the rPRL promoter require the basal transcription element (BTE) and the adjacent repressor binding site, FPII, respectively (see Fig. 1) (44, 49, 52, 53). To investigate the role of specific GHF-1 binding sites in the Ras activation of the rPRL promoter, a series of rPRL promoter constructs (47) that contain site-specific mutations in the proximal GHF-1 binding sites (FPI and/or FPIII), were tested for their ability to respond to the Ras signal. Similarly, to examine the contribution of FPI/FPII and BTE/BTE factors and binding sites to the Ras response, constructs containing a site-specific mutation of FPII, alone or combined with a deletion of the BTE (47), were also tested.

Co-transfection experiments were completed with the site-specific and 5’-deletion mutant rPRL promoter constructs depicted in Fig. 1. The data show that a site-specific mutation in the most proximal GHF-1 binding site, FPI (81), reduces the Ras activation of the rPRL promoter by approximately 40%. The significance of any loss of the Ras activation by mutation of FPI alone is unclear, since the δ1,3 construct, which contains the same FPI mutation in combination with a site-specific mutation in the next upstream GHF-1 site, FPIII, retains a full Ras response (Fig. 1). Additionally, an rPRL promoter containing an internal deletion overlapping FPI, from position −55 to −155, was also fully responsive to Ras (data not shown). Of note, FPI has been shown to bind GHF-1 with the highest affinity, followed by FPIII, with the lowest affinity binding at FPIV (41). Mutation of FPII (δ2) had no effect on Ras activation of the promoter, and similarly, the Ras response is not affected by a BTE deletion (δ2D) in the region extending from −80 to −112 in the δ2 background (Fig. 1). Thus, of the site-specific mutant rPRL promoter constructs tested, only δ1 exhibited any decrease in the Ras response compared with the −425 control. The inability of FPII, FPIII, and BTE mutants to modulate the Ras response demonstrates that these DNA sequences and the factors that bind to them are not required for Ras activation of the rPRL promoter. Thus, the two most proximal GHF-1 binding sites and other, previously defined, proximal transcription factor binding sites are not the primary cis-elements of the rPRL promoter targeted by the Ras-initiated signal. Furthermore, these proximal elements alone are not sufficient to mediate the Ras response. Consistent with these results, the rGH promoter, which is also dependent upon GHF-1 and contains multiple GHF-1 binding sites, is not activated by V-12 Ras (31, 33). However, the possibility that factors that bind to FPI, including GHF-1, may modestly contribute to the Ras response was not ruled out.

The above experiments did not address the role of the most distant and lowest affinity GHF-1 binding site, FPIV. Furthermore, we have previously noted a significant decrease in basal rPRL promoter activity upon removal of the region upstream of FPIV (−425 to −212), despite the fact that no factor binding footprints were detected in that span of rPRL DNA using crude GH3 and GH4 extracts (41). Therefore, it was postulated that this upstream DNA segment may mediate a hormone (or growth factor)-regulated response (36). Sequence examination of the rPRL promoter sequence revealed several potential binding sites for members of the Ets family of transcription factors (54, 55) in both this upstream region (−425 to −212) and in the more proximal promoter (Fig. 2). Although two noncanonical AP-1 sites were also identified in this region, at −240 and −235, it seemed highly unlikely that either of these functioned as a Ras-responsive element, since neither is a consensus binding site and c-Jun had been previously shown to be antagonistic to the rPRL promoter Ras response (33, 34). In contrast, we have previously shown that overexpression of a dominant-negative Ets factor (10) significantly inhibits Ras activation of the rPRL promoter (32) and that co-transfection of intact c-Ets-1
synergistically enhances the Ras response (31), thus implicating an Ets-related factor as a nuclear acceptor of the Ras signal.

In order to more precisely map the cis-elements of the rPRL promoter required for activation by Ras and to elucidate which of the potential Ets binding sites were important, an exonuclease III deletion procedure was used to generate a series of 5′ rPRL promoter deletions. Transfection experiments using this series of deletions, the 5′ end points of which are shown in Fig. 2A, were carried out to determine the region(s) of the rPRL promoter necessary for Ras responsiveness. We have previously identified the protein kinase Raf-1 (27) as a functional, downstream component of the Ras signaling pathway leading to rPRL promoter activation (32). Thus, the indicated rPRL promoter constructs were cotransfected in the presence or absence of constitutively active forms of Ras (V-12 Ras) or the downstream effector Raf-1 (Raf-BXB), since the Ras and Raf responses co-localize. The results from these studies map the Ras and Raf responses to a single region of the rPRL promoter spanning the −217 to −190 region and further suggest that Ras and Raf activation of the rPRL promoter is mediated via a unique composite Ets/GHF-1 binding site.

Site-directed Mutagenesis of the RRE—To verify the functional role of the proposed composite RRE in the Ras activation of the rPRL promoter, both GHF-1 and Ets binding sites were independently mutated, as shown in Fig. 3A, using PCR site-directed mutagenesis. The resulting pA3(mEBS)rPRLuc and pA3(mFPIV)rPRLuc reporter constructs containing mutations in the Ets and GHF-1 binding sites, respectively, were transiently transfected in the presence of activated Ras or Raf to determine the effect of the mutations on rPRL promoter activation. Fig. 3B shows that mutation of the EBS almost completely abrogates both Ras and Raf responses. While there is a greater than 8-fold Ras activation of the −425 rPRL promoter, minimal 1.5-fold activation is observed with the mutant EBS promoter. Similarly, Raf activates the −425 rPRL promoter approximately 6-fold, whereas the mutant EBS promoter exhibits no significant Raf response. Minimal Ras responses of the mEBS promoter in some experiments suggest that the mutation does not simply alter the promoter structure so greatly that it is no longer active, and they are consistent with the residual Ras response noted for the −212 rPRL deletion (31). Additionally, the mEBS promoter retains a protein kinase A response comparable with the intact rPRL promoter (not shown).

In an analogous experiment, the rPRL promoter bearing a mutant FPIV GHF-1 binding site exhibits a significantly attenuated Ras/Raf response, less than 50% of the intact promoter (Fig. 3C). However, in contrast to the pA3(mEBS) rPRLuc construct, the pA3(mFPIV)rPRLuc promoter retains significant residual activation by both Ras and Raf. Thus, the 17-fold Ras activation of the −425 promoter is reduced to 7.8-fold in the FPIV mutant, and the 18-fold activation of the wild type promoter by Raf is correspondingly diminished to 8-fold. The greater -fold activation by Ras and Raf in this experiment reflects the increased amounts of Ras and Raf transacted in order to clearly demonstrate the decreased Ras/Raf activation of the pA3(mFPIV)rPRLuc promoter.

The partial Ras/Raf response of the pA3(mFPIV)rPRLuc promoter could possibly reflect residual, reduced affinity bind-
ing of GHF-1. Thus, to assess the ability of the mutant FPIV site to bind GHF-1, an electrophoretic mobility shift assay using increasing amounts of a bacterial extract expressing a GST-GHF-1 fusion protein was performed (Fig. 4). No specific complexes were observed in the presence of bacterial extract expressing GST alone (lanes 2 and 8). Incubation of GST-GHF-1 with a radiolabeled probe corresponding to the −190 to −220 sequence of the rPRL promoter (RRE) reveals dose-dependent formation of a single major complex, B1 (lanes 3–6). The faster migrating, minor DNA-protein complexes B2 and B3 may be due to degradation products of GST-GHF-1 present in the extract. In contrast, no DNA-protein complexes are formed in the presence of a probe containing the mutant (mFPIV)RRE/FPIV site (lanes 9–12). In a similar experiment, an excess of unlabeled mutant oligonucleotide (mFPIV/RRE) failed to compete with GST-GHF-1 binding to the intact RRE (not shown). These data indicate that the mutant pA$_{r}$GhGHF-1/RRE binding motif has completely lost the ability to bind GHF-1 at this site.

### FIG. 3. Site-directed mutagenesis of the Ets binding site and the GHF-1 binding site of the rPRL promoter RRE significantly reduces Ras and Raf activation. A, sequence of the rPRL promoter Ras-responsive element. Site-directed mutations of the Ets (EBS) and GHF-1 (FPIV) binding sites to generate the pA$_{r}$EBS/PRLuc and pA$_{r}$/mFPIV/PRLuc constructs, respectively, are shown under the wild type sequence. B, GH$_4$ cells were cotransfected with 3 μg of wild type pA$_{r}$/rPRLuc (−425) or 3 μg of mutant pA$_{r}$/mEBS/PRLuc and 0.3 μg of pcMV β in the presence or absence of 2 μg of pSVras or 5 μg of pRSVxB-Raf as indicated. C, GH$_4$ cells were cotransfected with 3 μg of wild type pA$_{r}$/rPRLuc (−425) or 3 μg of mutant pA$_{r}$/mFPIV/PRLuc and 0.3 μg of pcMV β in the presence or absence of 3 μg of pSVras or 10 μg of pRSVxB-Raf as indicated. Promoter activity was calculated as in Fig. 2. Luciferase activity was normalized to β-galactosidase, and the basal activity of each promoter in the absence of Ras/Raf was set to 1. Results are expressed as -fold activation over control and are the mean ± S.E. of nine transfections.

Thus, the results of site-specific mutation of both elements of the composite RRE suggest that, while the Ets binding site is required to mediate the Ras response, the vicinal GHF-1 site is necessary but not sufficient for full activation of the promoter by Ras or Raf. Similarly, the low basal activity of the mutant EBS construct (not shown) suggests that binding of an Ets factor at this site may also be required to maintain basal rPRL promoter activity in pituitary cells.

Insertion of the RRE Renders the rGH Promoter Responsive to Ras—We have previously shown that the GHF-1-dependent rGH promoter, which is homologous to the rPRL promoter and contains both GHF-1 binding sites and several potential Ets sites, does not exhibit a Ras response (31, 33). We proposed that this lack of Ras activation reflected the lack of a composite GHF-1/Ets-1 RRE. To directly test this hypothesis and to verify the physiological role of the rPRL RRE composite element, this RRE was inserted into the rGH promoter. PCR site-directed mutagenesis was used to replace the distal GHF-1 binding site (−135 to −113) in the rGH promoter with the composite Ets/GHF-1 binding sites derived from the rPRL promoter RRE (−217 to −190). The resulting reporter construct, pA$_r$/GH (RRE)luc, was transiently transfected into GH$_4$ cells to determine the effect of oncogenic Ras. As shown in Fig. 5, the PRL promoter exhibits a typical 8-fold Ras response, whereas the rGH promoter is not activated by Ras. However, insertion of the composite RRE into the rGH promoter confers a 4-fold Ras response. These results, in conjunction with the mutagenesis of the RRE (Fig. 3), provide further evidence for the critical physiological role of the composite Ets/GHF-1 rPRL promoter element (−217 to −190) in conferring cell type- and promoter-specific Ras activation of PRL gene expression.

The rPRL Promoter Ras Response Is Not Transduced via Elk-1 or Net—Members of the Ets family of transcription factors appear to act via cooperative interactions with other transcription factors, resulting in synergistic activation of transcription (54, 55). The interaction of the Ets factor Elk-1 with SRF bound to a serum response element (SRE), provides a classic example. This Elk-1/ SRF functional interaction is regulated by the Ras pathway via MAP kinase phosphorylation of
Elk-1 (12, 13, 56). Net is another member of the Elk subfamily able to interact with SRF. In contrast to Elk-1, Net inhibits transcription but is converted to an activator by Ras (16). Since we have previously demonstrated a role for MAP kinase in the rPRL promoter Ras response (32) and the FPIV region bears some similarity to the AT-rich core of the palindromic SRE recognition element (CTCTAATTAGG) (57), the possible role of Elk-1 and Net in the Ras response of the rPRL promoter was investigated. As shown in Fig. 6, and consistent with our previous results (31), co-transfection of c-Ets-1 slightly increases basal rPRL promoter activity (1.5-fold) and enhances the Ras response increasing it from 10.7-to over 15-fold. In contrast, although Elk-1 and Net in the Ras response of the rPRL promoter was investigated. As shown in Fig. 6, and consistent with our previous results (31), co-transfection of c-Ets-1 slightly increases basal rPRL promoter activity (1.5-fold) and enhances the Ras response increasing it from 10.7- to over 15-fold. In contrast, transient transfection of either Elk-1 or Net resulted in significant inhibition of basal rPRL promoter activity (solid bars) and actually reduced the Ras activation from 10.7-fold to 7.4- and 6.6-fold, respectively (shaded bars). Of note, transfection of c-Ets-1, Elk-1, and Net into COS-1 cells resulted in similar levels of protein expression of each factor, as detected by specific antibodies (not shown). Thus, unlike Ets-1, neither Elk-1 nor Net is likely to be a nuclear component of the Ras pathway leading to activation of the rPRL promoter. Although Elk-1 and Net may compete with SRF-1 for binding to the RRE, to inhibit both basal and Ras-stimulated rPRL promoter activity, the precise mechanism of negative modulation of the Ras signal by these Ets factors remains to be investigated.

An Endogenous Ets-1-related Factor Is Present in GH4 Pituitary Cells—The studies presented here clearly show that an Ets-1-like factor, rather than an Elk or Net factor, is a critical component of the Ras signaling pathway leading to selective activation of the rPRL promoter. We have previously documented, by gene transfer experiments, that c-Ets-1, but not the related factor c-Ets-2, activates the basal promoter and enhances the Ras response (Fig. 6 and Ref. 31). Furthermore, c-Ets-1, but not c-Ets-2, functionally interacts with GHF-1 to mediate Ras activation of the rPRL promoter (31). Although c-Ets-1 was originally thought to be present predominantly in hematopoietic and macrophage cells (55), more recent studies have shown that this factor is more widely expressed (58–60). In order to confirm that an endogenous Ets-1-like factor was present in GH4 pituitary cells, whole cell extracts were prepared, separated by SDS-polyacrylamide gel electrophoresis, and analyzed by Western blotting (Fig. 7). Blots were probed with an antibody (ets1 N-276) that recognizes only the Ets-1 isoform (Santa Cruz Biotechnology, CA). Fig. 7, lane 2, shows that GH4 cells appear to contain an Ets-1-like protein, indicated by the presence of a band of approximately 54 kDa, which is consistent with the major Ets-1 isoform (p54). As a control, extracts were also analyzed for the pituitary-specific factor GSHF-1/Pit-1 using a specific antibody (antipeptide antibody 214–230; BabCO) that recognizes a protein of about 33 kDa (lane 1) corresponding to GSHF-1. Thus, in accordance with a composite Ets-1/GHF-1 element being necessary for the Ras activation of the rPRL promoter, GH4 rat pituitary cells express both GSHF-1 and an endogenous protein homologous to the transcription factor c-Ets-1.

To further characterize the endogenous GH4 pituitary Ets factors, electrophoretic mobility shift assays were performed using an oligonucleotide probe containing a known consensus
Ets binding site, derived from the murine sarcoma virus long terminal repeat (MSV-EBS) (61). As shown in Fig. 8, lane 2, a single major complex (B1) is formed upon incubation of GH4 cell extract with the consensus EBS. This binding is eliminated by the addition of excess unlabeled MSV-EBS oligonucleotide (lanes 3 and 4). Complex formation is also abolished by an excess of an oligonucleotide corresponding to the rPRL promoter composite RRE (lanes 5 and 6). An oligonucleotide spanning the FPI high affinity GHF-1 site (FPI), which does not contain an EBS, also fails to compete with binding to the MSV-EBS probe. Together, these results show that GH4 cells contain a factor homologous to c-Ets-1 (Fig. 7), show that a consensus EBS results in a single specific shifted complex (Fig. 8), and suggest that this Ets factor is also able to specifically bind to the EBS site within the rPRL promoter RRE (Fig. 8).

An Ets Family Member Can Bind to the rPRL RRE—To directly assess binding of an Ets family member to the putative (−217 to −209) EBS within the rPRL promoter RRE, we first performed a gel mobility shift assay using an oligonucleotide encompassing the −220 to −190 region of the rPRL promoter and recombinant GST-Ets protein. Labeled probe (RRE) was incubated with the highly conserved Ets DNA binding domain, expressed as a GST fusion protein, as described under "Experimental Procedures." The results of this assay are shown in Fig. 9A. While a nonspecific complex (B3) is observed in the presence of GST alone (lane 2), incubation with the GST-Ets protein results in the formation of a major (B2) and a minor (B1) complex with the RRE (lane 3). This GST-Ets-RRE complex is abolished by the addition of an excess of unlabeled RRE (lanes 6 and 7) but not by the same excess of an oligonucleotide that has a mutation (mEBS RRE) in the consensus EBS (lanes 4 and 5). Additionally, binding of GST-Ets to the RRE is not significantly affected by the addition of excess FPI oligonucleotide, which contains a strong GHF-1 binding site (lanes 8 and 9). This indicates that GST-Ets is not binding to the FPIV GHF-1 site within the RRE. Thus, a bacterial GST-Ets fusion protein encoding the highly conserved DNA binding (ETS) domain is able to specifically bind to the EBS site within the rPRL promoter RRE.

In order to determine whether the EBS within the RRE binds an endogenous Ets factor, contained in GH4 cell extracts, we used a double-stranded DNA oligonucleotide probe spanning positions −220 to −203 of the rPRL promoter (EBS) and incubated it with GH4 nuclear extract (Fig. 9B). Although major (B3) and minor (B2) nonspecific bands are noted, a faint band (B1) that reveals specificity is also evident (lane 2). For-
mation of the B1 complex is selectively inhibited by an excess of unlabeled EBS probe (lanes 3 and 4), whereas a similar EBS probe mutated in the core GGAA Ets binding motif (mEBS) fails to interfere with B1 complex formation (lanes 5 and 6). Additionally, the irrelevant FPI probe does not compete the B1 complex (data not shown).

Together, the results of these gel mobility shift assays (Figs. 8 and 9) confirm that GH4 cells contain an endogenous Ets-1-like factor that is able to bind specifically to the EBS within the PRL promoter RRE. Although we have as yet been unable to demonstrate formation of a stable ternary complex, this RRE is also able to bind GHF-1 (Ref. 41 and Fig. 4), indicating that it serves as a composite signaling element. The juxtaposition of factor binding sites facilitates the functional interaction of an Ets-1-like factor, with a tissue-specific transcription factor, GHF-1, thereby allowing the ubiquitous Ras signal transduction pathway to be harnessed in a pituitary-specific manner (31).

**DISCUSSION**

Identification of the Functionally Relevant Ras Response Element of the rPRL Gene—Although significant progress has been made in the identification of RREs in proto-oncogenes and oligonucleotide sequences fused to minimal promoters, a similar characterization of naturally occurring RREs in cellular genes is generally lacking. In this report, we provide detailed characterization of a functionally relevant RRE in a highly specialized, tissue-specific, cellular gene, rPRL. We used a combination of 5' deletion and site-specific mutagenesis in the context of an intact, proximal rPRL promoter, to localize the RRE to a composite element composed of an EBS and a vicinal GHF-1/Pit-1 binding site spanning positions −217 to −190. We have previously shown that, at the trans-acting factor level, a functional interaction of c-Ets-1 and GHF-1 is required to obtain optimal Ras and Raf responses (31). Here we verify that, at the cis-level, each unit of the composite element contributes to the Ras and Raf responses of the rPRL promoter. Although site-specific mutation of the EBS essentially eliminates both Ras and Raf effects, a similar mutation in the adjacent GHF-1 binding site (FPIV) results in a marked reduction, but not complete elimination, of the Ras/Raf responses (Fig. 3). These data suggest that a c-Ets-1-like factor is the critical nuclear component governing Ras/Raf responses of the rPRL gene, whereas GHF-1 DNA-binding is necessary but not sufficient to mediate Ras-inducible gene transcription. A recent report describes a composite Ets/GHF-1 element encompassing most of FPIII (−165 to −150), which is sufficient to confer multihormonal responses, including Ras, when fused to a minimal heterologous promoter (40). However, this report did not include 5' deletion or site-specific mutations of putative RREs in the context of the intact rPRL promoter. By contrast, in our studies, in the context of an intact proximal rPRL promoter, this FPIII composite element was functionally irrelevant for the Ras and Raf responses. Specifically, site-specific mutation of the GHF-1 binding sites in FPIII does not interfere with the Ras response (Fig. 1), and an intact FPIII region in the −212 and −189 rPRL promoter constructs failed to confer a response to either Ras or Raf (Fig. 2). Also, a single copy of the −220 to −190 RRE mapped here was sufficient to confer Ras activation to an otherwise unresponsive rGH promoter (Fig. 5). Nevertheless, the report of Howard and Maurer (40) stressed the importance of an Ets-related factor for the Ras response, and in this point we are in complete agreement. Together, these data are consistent with our hypothesis that a c-Ets-1-like factor is the nuclear target of the Ras/Raf/MAP kinase pathway and that it is likely to be phosphorylated via this pathway at a consensus MAP kinase site, threonine 82, since site-specific mutation of this amino acid results in a loss of the Ras effect (62).

Although we have not yet identified the precise endogenous Ets factor in GH4 pituitary cells that binds to the RRE, biochemical and functional evidence presented indicate that it is related to Ets-1. For example, Western blot analysis with an Ets-1-specific antibody reveals that GH4 cells contain c-Ets-1 (Fig. 7) and that GH4 nuclear extracts contain an Ets protein that binds the MSV-EBS and the rPRL EBS in the RRE (Figs. 8 and 9). While we have not yet unambiguously shown that c-Ets-1 is the specific Ets factor in the DNA complexes shown in Figs. 8 and 9, it is clear from the functional data presented here (Fig. 6) and that reported previously by us (31) and others (40), that c-Ets-1 is able to enhance the Ras response, whereas the Ets members c-Ets-2, Elk-1, and Net are unable to do so and in some cases even interfere with the Ras response.

The precise role of Ets factors in mediating other hormonal responses of the rPRL promoter remains unresolved and is under current investigation. For example, we have shown that a dominant-negative Ets construct does not interfere with the EGF-mediated stimulation of the intact proximal rPRL promoter (52), whereas a site-specific mutation in the FPIII EBS in the context of a heterologous promoter does result in a loss of EGF response (40). Additionally, a DNA region encompassing the BTE, which contains an EBS (Fig. 2A), has been shown to contribute to the cAMP activation of the human PRL gene (63), and this same BTE-EBS has been shown to be critical for insulin activation of the rPRL promoter (49, 53, 64). Moreover, a dominant-negative Ets construct inhibits the insulin response of the rPRL promoter, and the Ets-related factors Elk-1 and Sap-1 were shown to bind to an oligonucleotide spanning positions −106 to −97, encompassing the BTE (53). Although the reports of Jacob et al. and others have not identified the precise Ets member that is functionally relevant in mediating the specific hormonal response being studied, a role for Elk-1 has been suggested (40, 53). Additionally, a recent report suggests that GABP mediates the insulin response of the rPRL promoter via the BTE site (65). Here we show that site-specific deletion of the BTE does not interfere with the Ras activation of the proximal rPRL promoter (Fig. 1) and that expression of Elk-1 or Net may actually inhibit the Ras response (Fig. 6). Taken together, it is clear that distinct Ets family members may play different roles in mediating diverse hormonal responses of the rPRL promoter.

The RRE Is a Composite Ets/GHF-1 Binding Site—Previously defined Ras response elements have typically included an AP-1 site, either alone or in combination with an EBS (2, 18–20, 27, 29, 30). Additionally, CRE and SRE control elements have been shown to mediate the Ras response in certain promoters (24–26, 28). Although composite elements have been previously defined as RREs, the factors that bind to the specific sites have been ubiquitously expressed proteins. In the case of the rPRL promoter, it is the precise juxtaposition of binding sites for both a c-Ets-1-like factor, and the pituitary-specific factor, GHF-1, that is required for an optimal Ras response. Indeed, the rGH promoter, which is also GHF-1-dependent and contains core Ets binding sites (GGAA), fails to respond to Ras or Raf, most likely because the GHF-1 and Ets binding sites are not in the appropriate vicinal arrangement found in the rPRL promoter (31). However, upon substitution of the distal rGH GHF-1 site with the composite EBS/GHF-1 RRE, the rGH promoter gains Ras responsiveness (Fig. 5). Thus, the requirement for a tripartite regulatory unit, composed of a c-Ets-1-like factor, GHF-1, and a composite cis-acting DNA element, provides an elegant mechanism by which tissue-specific transcrip-

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6 B. Wasylyk and A. Gutierrez-Hartmann, unpublished results.
Figure 10. Model for pituitary-specific signal integration by the homeodomain protein GHF-1/Pit-1. Extracellular signals are targeted to nuclear co-activators such as CREB, the Jun/Fos family (AP-1), members of the Ets family of transcription factors (ETS), thyroid receptor (TR), estrogen receptor (ER), or retinoid receptor (RXR). Such inductive signals alter the transactivation potential of these co-activators. Functional interaction of these signal-dependent co-activators (SDCs), e.g., Ets-1, with GHF-1, at a composite GHF-1/Co-activator DNA binding site, forms a tripartite response unit, which permits highly specific pituitary transcriptional responses to general signaling pathways. Thus, GHF-1 functions as a cell-specific nuclear integrator of diverse extracellular hormone/growth factor signals.

Gene expression factors, such as GHF-1, serve as signal integrators for generalized signaling pathways and by which only a subset of GHF-1-dependent genes are selected to respond to the Ras pathway. In certain respects, this is reminiscent of the SRE in the c-Fos promoter, which requires the interaction of the Ets member, Elk-1, with SRF in order to achieve a growth factor response (12, 13). However, in the case of the rPRL gene, GHF-1 appears to function as a “cell-specific SRF.”

GHF-1 Functions as a Cell-specific Signal Integrator—We (31, 43) and others (66) have proposed a hypothesis whereby homeodomain proteins, such as GHF-1, target signal transduction pathways to selected tissue-specific genes by functionally interacting with a variety of signal-dependent transcription factors, such as Ets-1, AP-1, CREB, thyroid receptor (TR), estrogen receptor (ER), or retinoid receptor (RXR), at composite DNA-regulatory elements (Fig. 10). Synergistic interactions between GHF-1 and other factors may also determine cell phenotype and regulate proliferation during pituitary organogenesis (67). Several examples of such interactions, in addition to the Ets-1/GHF-1 interaction at the composite RRE discussed in this manuscript, can be found among GHF-1-dependent genes, including the GHF-1, GH, PRL, and thyrotropin-β genes. For example, the murine GHF-1 enhancer contains an atypical, cell-specific retinoic acid response element, composed of adjacent GHF-1 and retinoid receptor binding sites, and both GHF-1 and retinoic acid receptor are required to confer retinoid induction of GHF-1 gene transcription (68). Additionally, the coordinate actions of GHF-1 and CREB/ATF-1-related factors, at a cAMP response unit comprising a GHF-1 site flanked by CREs, are necessary to mediate the effects of cAMP on the human GH gene (69). Similarly, the rGH gene is synergistically activated by GHF-1 and thyroid receptor via relatively closely spaced DNA binding sites (70), and direct protein-protein interaction between GHF-1 and thyroid receptor has been demonstrated. Cooperation of GHF-1 and estrogen receptor is required for rPRL distal enhancer activity, and the binding of both factors, at adjacent elements, is required for the estradiol response (71). Finally, an AP-1-like factor functionally cooperates with GHF-1 to mediate forskolin and phorbol-ester activation of the human thyrotrophin-β gene (72). In this case, binding sites for GHF-1 and AP-1 are located somewhat further apart. However, the authors note that both AP-1 and GHF-1 can induce DNA bending, which may facilitate synergistic interactions (72). It is noteworthy that in most of these cases, mutation of the GHF-1 binding site, adjacent to the hormone response element, results in loss of the specific hormonal effect. Thus, in many GHF-1-dependent promoters, the inductive effects of extracellular signals require binding of both GHF-1 and the signal-dependent co-activator at composite DNA binding sites. Specifically, in the context of our model (Fig. 10), we hypothesize that GHF-1 either recruits an Ets-like factor or stabilizes its binding to the adjacent BBS and that the actual Ras/Raf response is transduced via a MAP kinase phosphorylation of the highly conserved threonine 82 in the Ets-1 protein. In summary, we propose that GHF-1 functions as a cell-specific integrator of hormonal and growth factor signaling, resulting in distinct patterns of GHF-1-dependent gene expression in pituitary development, differentiation, and proliferation. Finally, these results imply that other homeodomain proteins may function in a similar manner, providing a novel paradigm that should be considered in future studies of hormonal regulation of tissue-specific gene expression.

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