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Interaction of Ets-1 and the POU-Homeodomain Protein GHF-1/Pit-1 Reconstitutes Pituitary-Specific Gene Expression

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The pituitary-specific, POU-homeodomain factor GHF-1/Pit-1 is necessary, but not sufficient, for cell-specific expression of prolactin (PRL), growth hormone (GH), and thyrotropin. Combinatorial interactions of GHF-1 with other factors are likely to be required; however, such factors and their mechanisms of action remain to be elucidated. Here we identify Ets-1 as a factor that functionally and physically interacts with GHF-1 to fully reconstitute proximal PRL promoter activity. In contrast, Ets-2 has no effect, and the alternatively spliced GHF-2/Pit-1β variant fails to synergize with Ets-1. The Ets-1–GHF-1 synergy requires a composite Ets-1–GHF-1 *cis* element and is dependent on an Ets-1-specific protein domain. Furthermore, the ancestrally related and GHF-1-dependent GH promoter, which lacks this composite element, does not exhibit this response. Finally, Ets-1, but not Ets-2, binds directly to GHF-1 and GHF-2. These data show that a functional interaction of GHF-1 and Ets-1, acting via a composite DNA element, is required to establish lactotroph-specific PRL gene expression, thus providing a molecular mechanism by which GHF-1 can discriminate between the GH and PRL genes. These results underscore the importance of transcription factors that are distinct from, but interact with, homeobox proteins to establish lineage-specific gene expression.

Tissue-specific gene expression is typically governed by combinations of cell type-specific and ubiquitous transcription factors (15, 30). Homeobox genes encode archetypal cell-specific transcription factors that control distinct cell fates (17). However, it has become increasingly clear that homeobox factors alone are not always sufficient to optimally activate tissue-specific target genes (17, 28, 29). GHF-1/Pit-1 is a pituitary-specific POU-homeobox transcription factor that not only specifies somatotroph, lactotroph, and thyrotroph cell lineages but also regulates growth hormone (GH), prolactin (PRL), and thyrotropin (TSHβ) gene expression (25, 35, 51). Differential splicing of the GHF-1 gene results in a functionally distinct isoform, GHF-2, which contains a 26-amino-acid insertion within the transcription activation domain (37, 41, 50). GHF-1 and GHF-2 appear to differentially regulate the activity of the GH, PRL, TSHβ, and GHF-1 promoters, suggesting that these two GHF isoforms interact with different cofactors (26, 37, 41, 50, 51).

PRL gene expression is highly restricted to somatomammotroph and lactotroph cells of the anterior pituitary and is subject to regulation by a variety of hormones and second messengers (13, 19). The rat PRL (rPRL) promoter is comprised of a distal enhancer (−1710 to −1550), containing an estrogen response element, and a proximal (−425) promoter region (13, 19). This proximal region is sufficient to confer tissue-specific expression and to impart both positive and negative hormonal regulation to the rPRL gene (6, 14, 23). Several hormone response elements have been localized to GHF-1/Pit-1 binding sites on the PRL, GH, and TSHβ promoters (22). However, recent data have shown that GHF-1 is not a direct nuclear target of Ras and cyclic AMP-dependent protein kinase A signaling pathways but rather serves as a cell-specific signal integrator by functionally interacting with other transcription factors (3, 4, 22). Nevertheless, the possible physiological role of such interactions in controlling basal cell-specific promoter activity remains to be elucidated. For example, GHF-1 is unlikely to be the sole factor regulating the rPRL gene, since pituitary somatotrophs, lactotrophs, and thyrotrophs all express GHF-1, but only lactotrophs produce PRL (10).

The Ets superfamily is a novel class of *trans*-acting phosphoproteins which have important roles in the control of growth and development (34, 52). The family is defined by a highly conserved ETS domain (52, 56), which encodes a winged helix-turn-helix DNA-binding motif (11, 12). Current evidence indicates that gene expression is modulated by a functional cooperation of Ets family members with other transcription factors, some of which are cell type specific, allowing Ets members to regulate a number of tissue-specific promoters (34, 52). We have previously shown that a functional interaction between GHF-1 and Ets-1, at a composite Ets-1–GHF-1 binding site, is required to mediate the tissue-specific activation of the rPRL promoter by oncogenic Ras (3, 4). As noted above, GHF-1 functions as a cell-specific integrator of the Ras pathway, and activated mitogen-activated protein (MAP) kinase appears to impinge on the Ets-1 component of this complex (3, 4, 54, 58). However, the potential role of Ets factors in governing the tissue-specific restriction of basal rPRL promoter activity, and whether the Ets-1–GHF-1 synergy occurs in the absence of Ras activation, has not been determined.

In this study, we directly addressed this question, and we show that both GHF-1 and Ets-1 are critical for basal activity of the rPRL promoter in GH_4 cells. Moreover, Ets-1 and GHF-1 function in a synergistic manner, independent of Ras, and are both necessary and sufficient to fully and selectively reconstitute rPRL promoter activity in HeLa nonpituitary cells, since the related rat GH (rGH) promoter does not exhibit this response. In contrast, GHF-2 and Ets-2 fail to synergize with Ets-1 and GHF-1, respectively, in the rPRL promoter reconstitution assay. We have identified an Ets-1-specific domain, encompassing amino acids 218 to 390 of Ets-1, as being required to mediate both basal rPRL promoter acti-

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vation and the functional synergism with GHF-1. Finally, we used glutathione *S*-transferase (GST) fusion proteins to show that GHF-1 and GHF-2 physically interact with Ets-1 but not with Ets-2, and we mapped the region of Ets-1 required for this physical interaction to the Ets-1-specific domain. Taken together, the occurrence of functionally distinct factor combinations provides a mechanism to permit diverse yet highly selective responses in the control of cell-specific gene expression, differentiation, and development. Here we present a model by which such combinatorial interactions serve to establish and regulate highly specialized, cell-type-specific patterns of gene expression.

MATERIALS AND METHODS

Cell culture. HeLa, GH_4, and COS-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, Utah) and penicillin-streptomycin. Cells were grown at 37°C in 5% CO_2. Medium was changed 4 to 12 h prior to each transfection, and cells were harvested at 50 to 70% confluency.

Plasmid constructs. The reporter constructs pA_3rPRL*luc*, and pA_3rGH*luc*, and pCMV β have been described previously (3, 7). pSG5Ets-1 and pSG5Ets-2 encode the p68 chicken Ets-1 and chicken Ets-2, respectively (53), under control of the simian virus 40 early promoter. Deletion mutants of Ets-1 (Δ5′ and Δ3′) in pSG5 were constructed as described previously (47). Plasmid pAPrEts-Z encoding the DNA-binding domain of Ets-2 (dominant-negative Ets) was obtained from M. Ostrowski (Duke University, Durham, N.C.). Plasmids pRSVGHF-1 and pRSVGHF-2 (49) encoding the rat GHF-1 and GHF-2 transcription factors were kindly provided by M. Karin (University of California, San Diego). Plasmid DNAs were purified and quantified as described previously (3, 7).

Electroporation. Cells were harvested in 0.05% trypsin and 0.5 mM EDTA and resuspended in DMEM supplemented with 10% FCS. 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 (36) at 220 V and 500 μF, using a Bio-Rad Gene Pulser with 0.4-mm cuvettes. All transfections included 0.3 μg of pCMV β as an internal control for transfection efficiency. Total DNA was kept constant, and nonspecific effects of viral promoters were controlled for by using the appropriate empty vector. Following transfection, cells were plated in DMEM with 10% FCS and incubated for 24 h. Electroporations were performed in triplicate for each condition within a single experiment, and experiments were repeated several times with different plasmid preparations of each construct.

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 (DTT) (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 (7). Samples were measured in duplicate, using a Monolight 2010 Luminometer (Analytical Luminescence Laboratories, San Diego, Calif.). β-Galactosidase activity was determined spectrophotometrically, using the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside essentially as described previously (7). 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.

GST fusion proteins. Bacterial extracts containing the recombinant fusion proteins GST–GHF-1 or GST–GHF-2 were prepared essentially as described previously (48). Overnight cultures of *Escherichia coli* DH5α, transformed with plasmid pGEX-2TrGHF-1 or pGEX2T-rGHF-2 (27, 40), were diluted 1:10 in fresh Luria broth supplemented with ampicillin (50 μg/ml). Upon attaining an optical density at 600 nm of 0.6 to 1.0, cultures were induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM. Growth was continued for a further 2 to 3 h at 37°C. Bacterial cells were harvested by centrifugation at 3,000 × *g* for 5 min at 4°C and resuspended in approximately 1/50 volume of buffer A (16 mM Na_2HPO_4, 4 mm NaH_2PO_4, 150 mM NaCl) containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed by sonication (2- to 3-ml aliquots) on ice twice for 10 s each time, using a Cell Disruptor microprobe (Heat Systems-Ultrasonics, Plainville, N.Y.) on maximum setting. Cellular debris was removed by centrifugation at 10,000 × *g* for 10 min at 4°C. Supernatants were bound to glutathione-agarose (Pharmacia LKB) for 30 min at 4°C and washed extensively in buffer A. Bound protein was determined by the Bio-Rad assay and by analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Coomassie blue staining.

In vitro binding assays. Ets-1, Ets-2, and various truncation mutants of Ets-1 were synthesized and labeled with [^{35}S]methionine (NEN), using a Promega TNT coupled transcription-translation reticulocyte lysate system, with T7 polymerase, according to the manufacturer's protocol. Immobilized GST fusion proteins were prepared as described above and suspended in binding buffer (40 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM PMSF, 0.05% Nonidet P-40, 1 mM DTT [pH 7.5]) supplemented with the protease inhibitors antipain, leupeptin, aprotinin, and bestatin at 2 μg/ml. Labeled, in vitro-translated Ets-1 or Ets-2 was incubated with immobilized GST, GST–GHF-1 or GST–GHF-2 beads (see Fig. 11) in a final volume of 0.5 ml of binding buffer and mixed by rocking for 1 h at room temperature. Beads were collected by centrifugation at 1,000 × *g* for 30 s and washed five times for 5 min each in 0.5 ml of binding buffer containing 0.1% Triton X-100. Bound Ets-1 or Ets-2 was eluted by boiling in SDS sample buffer or by treatment with 50 mM Tris–5 mM glutathione (reduced)–1 mM DTT (pH 7.5) and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Analysis of binding of the ΔEts mutants to GHF-1 and GHF-2 used a column procedure (see Fig. 12). Binding assays were carried out at 4°C, using 40 μl of beads containing approximately 25 μg of GST fusion protein in a micropipette tip column. Aliquots (25 μl) of labeled Ets factors were diluted in 100 μl of buffer (20 mM HEPES, 150 mM KCl, 8.7% glycerol, 0.1 mM EDTA, 0.2 mM PMSF, 400 μg of ethidium bromide per ml, 5 mM DTT [pH 7.4], 5 μg of leupeptin, pepstatin, aprotinin, antipain, and chymostatin per ml) and applied to the beads. The columns were washed three times with 180 μl of buffer, and bound Ets factors were eluted by boiling in SDS-sample buffer and separated by SDS-polyacrylamide gel electrophoresis.

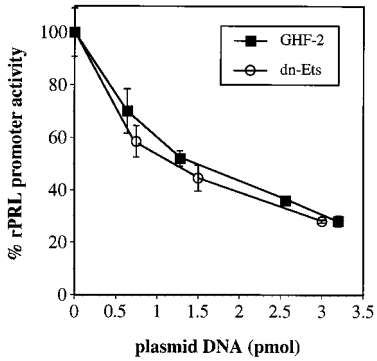


FIG. 1. GHF-2 and dominant-negative Ets inhibit basal rPRL promoter activity in GH_4 cells. GH_4T2 rat pituitary cells were cotransfected with 3 μg of pA_3rPRL*luc* and the indicated amounts of pRSVGHF-2 or pAPrEts-Z (dn-Ets). Cells were harvested after 24 h and assayed for luciferase, and the results were normalized with respect to β-galactosidase activity as described in Materials and Methods. Results are expressed as percent rPRL promoter activity relative to pA_3rPRL*luc* alone. Each data point is the mean of three transfections, and error bars indicate standard deviations.

RESULTS

Inhibition of rPRL promoter activity in GH_4 cells by dominant-negative isoforms of Ets and GHF-1. We have previously shown that both GHF-1 and Ets-1 transcription factors are nuclear components of the oncogenic Ras signaling pathway leading to activation of the rPRL promoter in rat pituitary GH_4 cells (3, 4, 8). We have now used a transient cotransfection approach, employing dominant-negative constructs which interfere with the action of endogenous factors, to examine the role of Ets and GHF-1 in the regulation of basal rPRL promoter activity. As shown in Fig. 1, cotransfection of GHF-2, a splice variant of GHF-1 which behaves as a dominant-negative factor with respect to the rPRL promoter (26, 37, 41, 50), results in a dose-dependent inhibition of basal rPRL promoter activity in GH_4 cells, reducing basal activity by approximately 70%. To examine the role of Ets factors in the regulation of rPRL transcription, GH_4 cells were cotransfected with a dominant-negative Ets construct (pAprEts-Z) lacking a transactivation domain and encoding only the highly conserved DNA-binding ETS domain (38). Expression of this dominant-negative Ets in GH_4 cells resulted in a dose-dependent inhibition of rPRL promoter activity (Fig. 1), similar to that observed upon overexpression of GHF-2. Thus, inhibitory isoforms of both Ets and GHF-1 significantly reduce basal rPRL promoter activity in the rat pituitary GH_4 cell line. Additionally, site-specific mutation of the Ets binding site, at positions

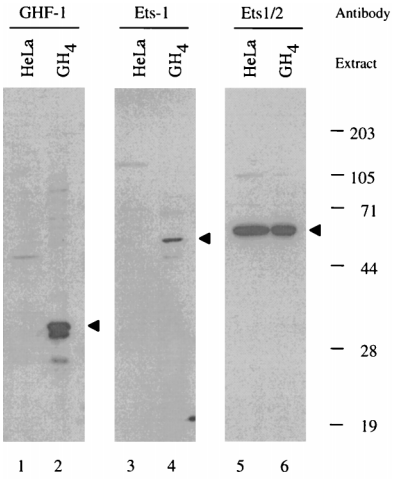


FIG. 2. Analysis of Ets-1 and GHF-1 expression in GH_4 and HeLa cell extracts. Whole-cell extracts were prepared by sonication in lysis buffer containing 100 mM potassium phosphate, 1% Triton X-100, 2 μg each of aprotinin, pepstatin, and leupeptin per ml, 1 mM DTT (pH 7.8). Extracts were clarified by centrifugation at 12,000 × *g* for 10 min at 4°C. Proteins were resolved on an SDS–10% polyacrylamide gel, transferred to nitrocellulose, and probed with either anti-GHF-1 (amino acids 214 to 230) (BAbCo, Richmond, Calif.) or anti-Ets-1 or anti-Ets-1/2 (Santa Cruz Biotechnology) as indicated. Antigen-antibody complexes were detected by enhanced chemiluminescence (Amersham International) using peroxidase-linked secondary antibodies according to the manufacturer's directions. Lanes 1, 3, and 5, 100 μg of HeLa extract; lanes 2, 4, and 6, 100 μg of GH_4 extract. The mobilities of prestained molecular weight markers (Gibco BRL) are indicated in kilodaltons.

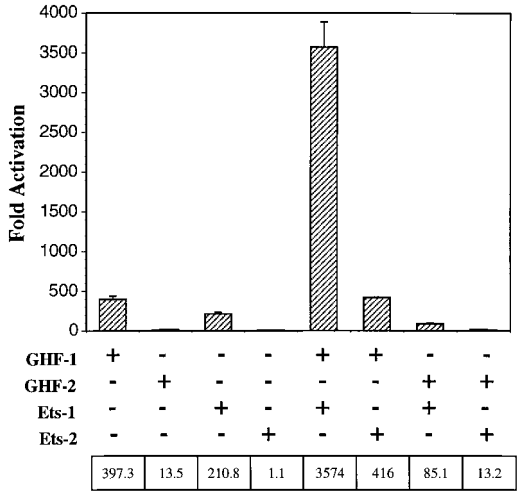


FIG. 3. Reconstitution of rPRL promoter activity in a nonpituitary cell type. HeLa cells were transfected with 3 μg of pA_3rPRL*luc* with or without 5 μg of pRSVGHF-1, 5 μg of pRSVGHF-2, 5 μg of pSG5Ets-1, or 5 μg of pSG5Ets-2 as indicated. Cells were harvested after 24 h and assayed for luciferase and β-galactosidase as described in Materials and Methods. Results are expressed as fold activation relative to basal promoter activity and are the means ± standard errors of the means of four experiments, each consisting of triplicate transfections. Fold activation is also shown at the bottom.

−217 to −209 within the rPRL promoter Ras-responsive element (RRE), reduces basal activity to approximately 30% of the wild-type level (not shown), further corroborating a role for Ets-1 in basal rPRL promoter regulation. Together, these results suggest that both GHF-1 and a member of the Ets family, such as Ets-1, are required for basal rPRL promoter activity and maintenance of rPRL expression in pituitary lactotroph cells.

Analysis of Ets-1 and GHF-1 expression in HeLa and GH_4 cell lines. Consistent with a physiological role for Ets-1 and GHF-1 in rPRL promoter regulation, GH_4 cells contain both transcription factors (Fig. 2). However, overexpression of either protein in these cells has little, if any, effect on basal rPRL promoter activity (3, 26). Thus, to further characterize the role of Ets-1 and GHF-1 in regulation of rPRL gene expression, we used a transient-transfection protocol to reconstitute the rPRL promoter in a nonpituitary HeLa cell line. HeLa cells are derived from a human cervical carcinoma and do not express endogenous PRL or the pituitary-specific factors GHF-1 and GHF-2. Furthermore, Ets-1 mRNA is not detected by Northern analysis in HeLa cells (42). To verify that the HeLa cell line used in these studies did not express either GHF-1 or Ets-1 protein, GH_4 and HeLa whole-cell extracts were analyzed by Western blot using specific antibodies (Fig. 2). As shown in lanes 1 and 2, GHF-1 is not detected in HeLa cells but is clearly present in extracts of GH_4 cells as a closely spaced doublet at approximately 33 kDa. Similarly, antibody specific for Ets-1 (Santa Cruz Biotechnology, Santa Cruz, Calif.) detects a major band at approximately 55 kDa in the pituitary cell line (lane 4), whereas no specific bands are present in HeLa cell extracts (lane 3). In contrast, a pan-Ets antibody which recognizes both Ets-1 and Ets-2 as well as other related isoforms (Santa Cruz Biotechnology) detects a major band at 55 to 60 kDa and several minor bands in both HeLa and GH_4 cell extracts. This result indicates that both GH_4 and HeLa cells express Ets-related proteins distinct from Ets-1 and also verifies that the HeLa cell extracts are not degraded. Thus, HeLa cells do not express detectable levels of either GHF-1, GHF-2, or Ets-1 protein and therefore represent an ideal model system in which to investigate their effects on the rPRL promoter by using the transient-transfection reconstitution system described below.

Selective reconstitution of basal rPRL promoter activity by Ets-1 and GHF-1. The effect of cotransfection of Ets-1 and GHF-1, either alone or in combination, on the reconstitution of rPRL promoter activity in HeLa nonpituitary cells is shown in Fig. 3. As we have reported previously (33, 36), in the absence of exogenous factors, rPRL promoter activity in HeLa cells is very low, exhibiting less than 1% of the basal activity observed in GH_4 cells. Expression of GHF-1 results in an approximately 400-fold activation of the rPRL promoter. Cotransfection of Ets-1 also results in a significant 210-fold activation (Fig. 3). However, cotransfection of both Ets-1 and GHF-1 results in a marked synergistic activation of the promoter of over 3,500-fold (Fig. 3). The actual normalized total light units obtained for the rPRL promoter construct in HeLa cells, in the presence of both GHF-1 and Ets-1 (352,902 ± 39,032; $n = 18$), are similar to those observed upon transfection of GH_4 pituitary cells with the same amount of the identical rPRL promoter-luciferase reporter (348,273 ± 33,477; $n = 18$). These results indicate that full rPRL promoter activity is reconstituted by Ets-1 and GHF-1 in this system but that neither alone is sufficient. In contrast, cotransfection of GHF-2 in HeLa cells results in only minimal (13-fold) activation of the rPRL promoter, and GHF-2 clearly does not synergistically activate the promoter in the presence of Ets-1 (Fig. 3). In fact, GHF-2 apparently inhibits the activation of the rPRL promoter by Ets-1, reducing it from 210- to 85-fold. These results suggest that the 26-amino-acid insert within the transcription activation domain of GHF-2 disrupts a region critical for activation of the rPRL promoter and functional interaction with Ets-1. Similarly, expression of Ets-2, an Ets isoform homologous to Ets-1 but functionally distinct (34, 39, 52), has no

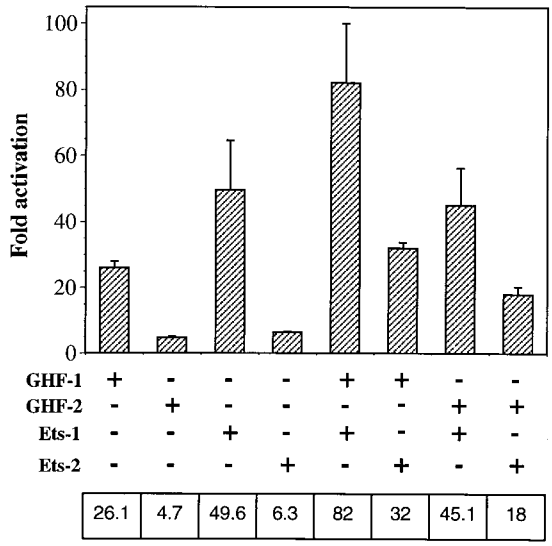


FIG. 4. rGH promoter activity in HeLa cells. HeLa cells were transfected with 3 μg of pA_3rGH*luc* with or without 5 μg of pRSVGHF-1, 5 μg of pRSVGHF-2, 5 μg of pSG5Ets-1, or 5 μg of pSG5Ets-2 as indicated. Cells were harvested after 24 h and assayed for luciferase and β-galactosidase as for Fig. 3. Results are expressed as fold activation over the basal level and are the means ± standard deviations of six to nine transfections. Fold activation is also shown at the bottom.

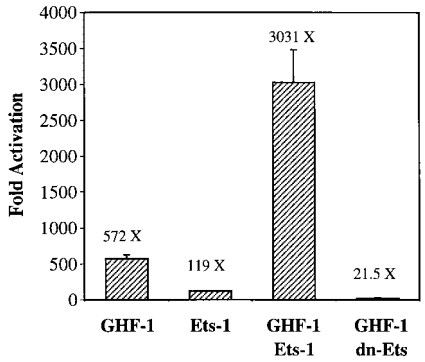


FIG. 5. Dominant-negative Ets inhibits activation of the rPRL promoter by GHF-1. HeLa cells were cotransfected with 3 μg of pA_3rPRL*luc* with or without 5 μg of pRSV GHF-1, 5 μg of pSG5Ets-1, or 10 μg pAprEtz-Z (dn-Ets) as indicated. Promoter activity was determined as for Fig. 3, and the results are expressed as fold activation. Numbers above the bars indicate fold activation. Data are the means ± standard deviations of three transfections.

apparent effect on the rPRL promoter in HeLa cells and does not further enhance the activation by GHF-1 or GHF-2 (Fig. 3), suggesting that structural domains specific for Ets-1 are critical for regulation of rPRL gene expression. Of note, this Ets-2 construct is clearly functional in HeLa cells, since it is able to activate the rGH promoter in this study (Fig. 4) and strongly activates the stromelysin promoter and polyomavirus enhancer (each about 100-fold), as reported previously (55). Thus, both Ets-1 and GHF-1 isoforms specifically and significantly increase rPRL promoter activity in a nonpituitary cell line. Furthermore, expression of both GHF-1 and Ets-1 together results in a synergistic activation of the rPRL promoter, restoring absolute activity comparable to that observed in GH_4 pituitary cells.

In an analogous experiment, we determined the effects of single and combinatorial cotransfection of GHF-1, GHF-2, Ets-1, and Ets-2 on the activity of the homologous, ancestrally related rGH promoter (1) in the HeLa reconstitution system. As shown in Fig. 4, cotransfection of GHF-1 or GHF-2 activates the rGH promoter approximately 26- or 5-fold, respectively. Similarly, rGH promoter activity is also increased upon cotransfection of either Ets-1 (50-fold) or Ets-2 (6-fold). Of note, activation of the rGH promoter by Ets-2 verifies that this construct is expressed in HeLa cells. However, cotransfection of combinations of Ets-1 or Ets-2 and GHF-1 or GHF-2 do not elicit either the dramatic or synergistic activation of the rGH promoter (Fig. 4) as is observed with the rPRL promoter (Fig. 3). In general, the rGH promoter is activated to a significantly diminished extent compared to the rPRL promoter, and cotransfection of Ets-1 or Ets-2, together with GHF-1 or GHF-2, results in additive effects upon rGH promoter activity.

To further investigate the synergistic effects of GHF-1 and Ets-1 on the rPRL promoter, we examined the effect of the dominant-negative Ets construct (pAPrEts-Z) encoding only the DNA-binding ETS domain. As shown in Fig. 5, cotransfection of GHF-1 in the presence of pAPrEts-Z not only fails to induce the synergistic response elicited by intact Ets-1 but also interferes with GHF-1 activation, reducing it from almost 600-fold to approximately 20-fold. The dominant-negative Ets construct alone had no detectable effect on the minimal rPRL promoter activity in HeLa cells (not shown). Taken together

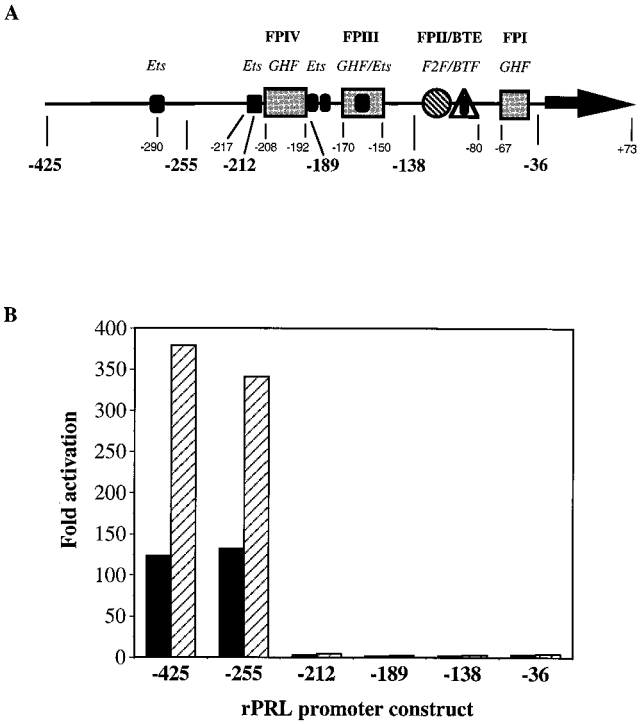


FIG. 6. Mapping of the rPRL promoter Ets-responsive element. (A) Structural organization of the proximal rPRL promoter. The nucleotide sequence of the rPRL gene from −425 to +73 is depicted. The endpoints of exonuclease deletions constructed in pA3luc and verified by dideoxy sequencing are indicated in boldface. GHF-1 sites (footprints I, III, and IV [FPI, FPIII, and FPIV]), as determined by DNase protection (23), are indicated by the shaded rectangles. Putative consensus EBSs are shown by the solid rectangles. The footprint II repressor site (FPII) and the basal transcription element (BTE) (33) are denoted by the circle and triangle, respectively. (B) HeLa cells were cotransfected with 5 μg of the indicated rPRL promoter reporter constructs in pA_3*luc* with or without 5 μg of (solid bars) or 10 μg of pSG5Ets-1 (hatched bars). Assays were carried out after 24 h as described for Fig. 3. Results are expressed as fold activation by Ets-1 relative to the basal activity of each promoter construct and are the means of three transfections.

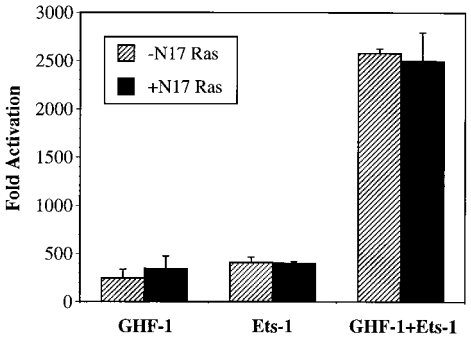


FIG. 7. Dominant-negative N-17 Ras does not inhibit GHF-1–Ets-1 activation of the rPRL promoter. HeLa cells were cotransfected with 3 μg of pA_3rPRL*luc* with or without 5 μg of pRSV GHF-1 and/or 5 μg of pSG5Ets-1 as indicated, in the absence (shaded bars) or presence (solid bars) of 10 μg of pZCRN17Ras. Promoter activity was determined as for Fig. 3, and the results are expressed as fold activation. Data are the means ± standard deviations of three transfections.

with the observed inhibition of Ets-1 activation by GHF-2 (Fig. 3), these results suggest a functional interaction between GHF-1 and Ets-1 to reconstitute basal rPRL promoter activity in HeLa cells and also that this cooperative interaction requires Ets-1-specific sequences located amino terminal to the DNA-binding domain.

The basal Ets *cis* element of the rPRL promoter. The structure of the proximal −425 rPRL promoter is shown in Fig. 6A. Several potential Ets-binding sites (EBSs) containing the core GGAA motif, the GHF-1-binding sites, footprints I, II (a repressor-binding site), III, and IV, and a basal transcription element (32, 33) are indicated. A series of 5′ deletion constructs (Fig. 6A) was used to determine which of the putative Ets sites are required for activation of the basal rPRL promoter in HeLa by Ets-1. Whereas the −425 and −255 promoter constructs exhibit significant dose-dependent activation upon cotransfection with either 5 or 10 μg of Ets-1, deletion at −212 almost completely abolishes the Ets response. Shorter constructs are also not activated by Ets-1 (Fig. 6B). Thus, the Ets response localizes to the region between −255 and −212, which contains a single EBS positioned at −214 to −209, located immediately upstream of the distal and lowest-affinity (24) binding site for GHF-1 (footprint IV). Together with the above-noted inhibition of GHF-1 activation of the rPRL promoter by dominant-negative Ets (Fig. 5) and the inhibition of the Ets-1 response by GHF-2 (Fig. 3), the data provide strong support for a functional interaction between GHF-1 and Ets-1 at this composite element within the rPRL promoter. Interestingly, the rGH promoter contains both GHF-1-binding sites and several putative EBSs; however, it lacks any such composite element, which may explain the lack of synergistic activation by Ets-1 and GHF-1.

We have previously shown that the −214 to −190 composite Ets-GHF binding site in the rPRL promoter functions as an RRE in GH_4 pituitary cells and that a functional interaction of GHF-1 and Ets-1 is required to mediate rPRL promoter activation by oncogenic *ras* (3, 4). Since the studies presented above were performed with cycling cells in the presence of 10% FBS, the possibility remained that the Ets-1–GHF-1 functional interaction, noted in Fig. 3, was Ras dependent and not truly a reflection of basal activity. To examine the role of Ras in the reconstitution of rPRL promoter activity in HeLa cells, we used a dominant-negative N-17 Ras construct (16). Cotransfection of N-17 Ras did not effect the activation of the rPRL promoter by GHF-1 or Ets-1 and did not reduce the synergistic

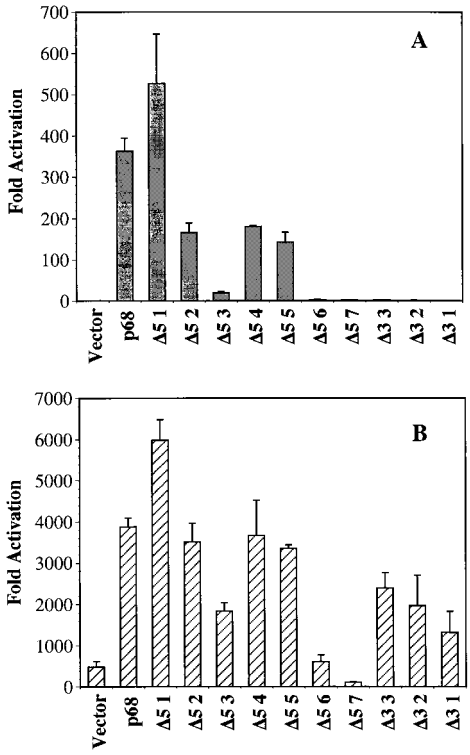
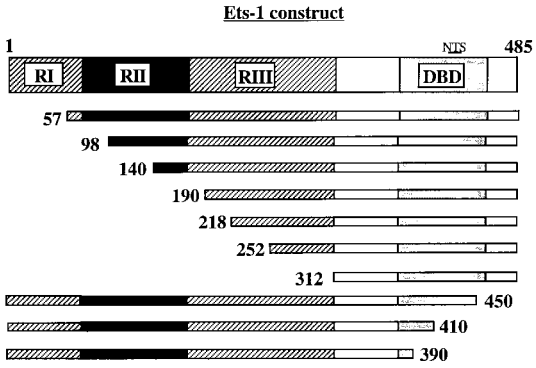


FIG. 8. Mapping of the functional domains of Ets-1. HeLa cells were cotransfected with 3 μg of pA_3rPRL*luc* reporter with or without 5 μg of vector (pSG5) or the indicated Δ5 and Δ3 Ets-1 constructs, in the absence (A) or presence (B) of 5 μg of pRSVGHF-1. rPRL promoter activity was determined as for Fig. 3, and the data are expressed as fold activation. Results are the mean ± standard deviations of six transfections; results of a typical experiment are depicted. The structures of the Δ5 and Δ3 Ets constructs, in pSG5, is shown in Fig. 9.

activation induced by both factors (Fig. 7). This N-17 Ras construct inhibits v-Src activation of the PRL and c-*fos* promoters (8, 45). Moreover, similar levels of Ets-1–GHF-1 synergy were observed by cotransfection of HeLa cells in serum-free medium (data not shown). Thus, the effects of Ets-1 and GHF-1 to reconstitute basal rPRL promoter activity in HeLa cells are independent of the Ras signaling pathway.

Functional domains of Ets-1. The HeLa reconstitution system provides a highly sensitive assay to map the functional domains of Ets-1 involved in both Ets-1-mediated basal rPRL promoter activity and activities required for synergistic interaction with GHF-1. A series of 5′ and 3′ truncations of p68 Ets-1 were used to delineate the functional regions of Ets-1, in comparison to the previously identified transactivation RI and RIII domains and the modulatory RII domain (Fig. 8 and 9) (47). Figure 8 shows the results of a typical cotransfection of the indicated Ets-1 constructs on the reconstitution of rPRL promoter activity, in the absence or presence of GHF-1, and data from four such reconstitution experiments using the Ets deletion constructs are summarized in Fig. 9. We will first discuss the effects of Ets-1 alone; these data are shown as Ets-1 fold activation (Fig. 8A) and as a percentage of the activity of the full-length p68 Ets-1 (Fig. 9). Deletion of the first 57 amino acids (Δ5-1) results in a 43% enhancement of the Ets effect, and this is most likely due to an alteration of the RII negative modulation of RI. Complete deletion of RI (Δ5-2) results in significant loss of Ets-1 activity (Fig. 8A and 9). The ability of the Δ5-3 Ets construct to transactivate the rPRL promoter is substantially reduced (Fig. 8A), perhaps reflecting an inhibi-



	Ets-1 activity (%)	GHF-1/Ets-1 Synergy
p68	100	6.2 ± 1.0
Δ5-1	143 ± 3	6.2 ± 1.4
Δ5-2	48 ± 5	6.1 ± 1.0
Δ5-3	6 ± 1	5.3 ± 1.9
Δ5-4	56 ± 14	5.9 ± 0.6
Δ5-5	40 ± 3	6.7 ± 2.4
Δ5-6	1 ± 0.2	1.6 ± 0.6
Δ5-7	< 0.5	0.4 ± 0.2
Δ3-1	< 0.5	3.5 ± 1.1
Δ3-2	< 0.5	5.3 ± 2.0
Δ3-3	< 0.5	2.9 ± 1.0

FIG. 9. Basal and synergistic activities of Ets-1 constructs. Functional domains of chicken p68 Ets-1 (39) are shown on the left. Numbers indicate amino acid endpoints of each construct. RI and RII, transcription activation domains; RII, regulatory domain; DBD, DNA-binding ETS domain; NTS, putative nuclear targeting signal (47). Ets-1 activity is expressed as a percentage of the ability of intact p68 Ets-1 to transactivate the rPRL promoter determined as for Fig. 8A. Mean p68 Ets-1 activation was 350-fold (100%). GHF-1–Ets-1 synergy is defined as the fold activation induced by each Ets-1 construct plus GHF-1 divided by the sum of the fold activation by each Ets-1 construct and GHF-1 alone, i.e., fold (Ets + GHF-1)/[fold (Ets) + fold (GHF-1)]. Data are the means ± standard errors of the means of four experiments.

tory region of the regulatory domain RII (Fig. 9). However, further deletions (Δ5-4 and Δ5-5) removing the remainder of the RII domain restore 40 to 50% of intact Ets-1 activity (Fig. 8A and 9). The Δ5-6 construct exhibits only residual (1%) Ets activity, and the Δ5-7 construct, which lacks both transactivation domains, is essentially inactive. The carboxy-terminal Δ3 series of deletions are unable to bind DNA (47) and, as predicted, do not transactivate the rPRL promoter (Fig. 8A and 9).

We next examined the ability of each Ets construct to functionally interact with GHF-1 and synergistically activate the rPRL promoter. Figure 8B illustrates the ability of each Ets construct to synergistically increase rPRL promoter activity in the presence of GHF-1. The fold GHF-1–Ets-1 synergy, defined as the fold activation in the presence of Ets plus GHF-1 divided by the sum of the fold activation induced by Ets and GHF-1 alone, is shown in Fig. 9. In the absence of Ets-1, GHF-1 activated the rPRL promoter approximately 450-fold. Cotransfection of intact p68 Ets-1 results in a synergistic, almost 4,000-fold activation (Fig. 8B), approximately sixfold higher than predicted based on the sum of their individual effects (Fig. 9). Consistent with its enhanced Ets transactivation ability (Fig. 8A), the Δ5-1 Ets construct increases GHF-1 activation to almost 6,000-fold over the basal level (Fig. 8B) but exhibits similar sixfold GHF-1–Ets-1 synergy (Fig. 9). Subsequent Ets constructs, Δ5-2 to Δ5-5, despite their significantly reduced Ets activity (Fig. 8A and 9), retain the ability to enhance GHF-1 activation of the rPRL promoter (Fig. 8B) comparably to intact Ets-1, exhibiting approximately sixfold synergy in each case (Fig. 9). In contrast, the minimally active Δ5-6 construct fails to enhance GHF-1 activation of the rPRL promoter, and the Δ5-7 construct, which is devoid of both transactivation domains and similar in structure to the dominant-negative Ets construct, actually inhibits activation of the rPRL promoter induced by cotransfection of GHF-1 (Fig. 8B and 9). The Δ3 Ets C-terminal truncations disrupt the DNA-binding domain and render the constructs essentially inactive on the basal rPRL promoter (Fig. 8A). However, interestingly, these Δ3 constructs retain partial ability to enhance GHF-1 activation approximately threefold (Fig. 8 and 9), suggesting that Ets-1 may be able to directly interact with GHF-1 independent of its binding to DNA. Nevertheless, binding of both factors, at the composite Ets-GHF element, is necessary for optimal functional synergistic activation of the rPRL promoter.

Despite the evident sensitivity of the functional assay in transfected HeLa cells, Ets-1 proteins expressed from transfected DNA vectors have not been detectable by Western blot analysis. To ensure that equivalent amounts of Ets protein was expressed in assays using similar amounts of DNA, each Ets construct was transfected into COS-1 cells and extracts were analyzed by Western blotting with specific antibodies (Fig. 10). Figure 10 illustrates that each Ets deletion mutant is expressed at levels equal to or exceeding that of wild-type full-length Ets-1. Thus, the lack of transactivation by the Ets deletion mutants, shown in Fig. 8 and 9, cannot be attributed to protein instability or absence of Ets factor expression but rather reflects the consequences of the deletion or truncation of key structural and/or functional domains within Ets-1.

In summary, the results shown in Fig. 8 and 9 indicate that full activation of the basal rPRL promoter by Ets-1 requires a

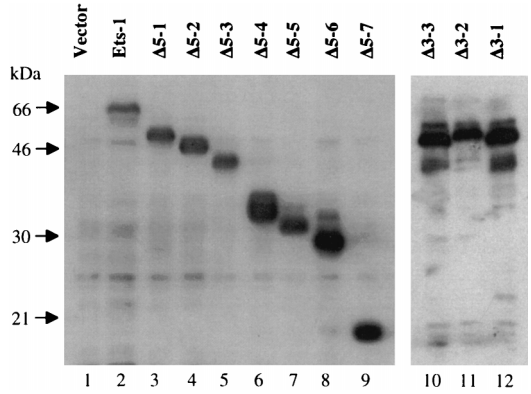


FIG. 10. Expression of Ets-1 truncation mutants. COS-1 cells (~5 × 10^6) were transfected, by electroporation, with 10 μg of the indicated Ets-1 construct or empty vector (pSG5). Cells were incubated for 24 h, washed with cold phosphate-buffered saline, and harvested as described in Materials and Methods. Cell pellets, derived from equal numbers of transfected cells, were resuspended in Laemmli SDS sample buffer containing 5% β-mercaptoethanol, vortexed vigorously, and dissolved by heating to 100°C for 2 min. Extracts were analyzed by SDS-polyacrylamide electrophoresis and Western blotting. Δ5 Ets constructs (lanes 1 to 9) were detected by using an antibody, PA94, directed against the carboxy termini of Ets-1 and Δ3-Ets constructs (lanes 10 to 12), using an amino-terminally directed Ets-1-specific antibody (Santa-Cruz Biotechnology). Immunoblots were developed by enhanced chemiluminescence (Amersham) as described for Fig. 2.

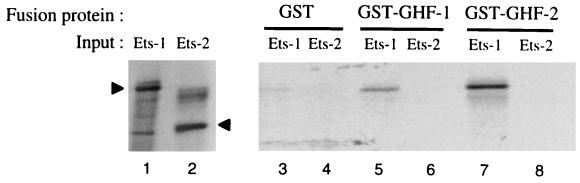


FIG. 11. In vitro binding of Ets-1 to GHF-1 and GHF-2. Binding assays were performed essentially as described in Materials and Methods. The autoradiograph shows fusion protein-Ets complexes resolved by SDS-polyacrylamide gel electrophoresis. Aliquots (10-μl packed volume) of glutathione-Sepharose beads, bound to either ~5 μg of GST (lanes 3 and 4), ~1 μg of GST–GHF-1 (lanes 5 and 6), or ~1 μg of GST–GHF-2 (lanes 7 and 8), were incubated with equal amounts of in vitro-transcribed and -translated ^{35}S-labeled Ets-1 or Ets-2 as indicated. Lanes 1 and 2 show 10% of the amount of methionine-labeled Ets-1 and Ets-2 added to each reaction. Arrows indicate p68 Ets-1 (lane 1) and Ets-2 (lane 2) bands.

region between residues 57 and 98, encompassing the carboxy-terminal part of the transactivation domain RI. However, neither RI nor the regulatory domain RII is required for the functional, synergistic interaction with GHF-1. In contrast, the region of Ets-1 between amino acids 218 and 312, within the second transactivation domain (RIII) (Fig. 9), contains elements critical both for the ability of Ets-1 alone to activate the rPRL promoter and for the cooperation of Ets-1 with GHF-1.

Physical interaction of Ets-1 with GHF-1 and GHF-2. Our transfection studies indicate that Ets-1, but not Ets-2, is able to activate the rPRL promoter and synergize with GHF-1. Similarly, the alternatively spliced isoform GHF-2 not only fails to synergize with Ets-1 but actually inhibits the ability of Ets-1 to transactivate the rPRL promoter (Fig. 3). These results indicated a functional cooperation between GHF-1 and Ets-1 and suggested a possible direct protein-protein interaction between these factors as the molecular mechanism underlying their synergistic activation of the rPRL promoter. Similarly, the inability of GHF-2 and Ets-2 to reconstitute rPRL promoter activity (Fig. 3) may reflect lack of binding to Ets-1 and GHF-1, respectively. To address these questions, bacterial GST fusion proteins of GHF-1 and GHF-2 were immobilized on glutathione-Sepharose beads and used in binding assays with in vitro-transcribed and -translated Ets-1 and Ets-2, labeled with [^{35}S]methionine (Fig. 11). Equal amounts of labeled Ets-1 or Ets-2, based on specific activities of their respective protein bands resolved by polyacrylamide gel electrophoresis (Fig. 11, lanes 1 and 2), were incubated in dilute solution with immobilized GST, GST–GHF-1, or GST–GHF-2. Equal amounts of GHF-1 and GHF-2 immobilized fusion protein, based on protein determination and Coomassie blue staining after SDS-polyacrylamide gel analysis, were used. As shown in Fig. 11, lanes 3 and 4, no specific binding of either Ets-1 or Ets-2 to GST was observed. However, Ets-1 was able to bind to both GST–GHF-1 and GST–GHF-2 (lanes 5 and 7, respectively). Indeed, Ets-1 appeared to bind more efficiently to GHF-2. In contrast, consistent with the lack of effect of Ets-2 in transient transfections, Ets-2 showed no detectable binding to either GHF-1 or GHF-2 (lane 6 or 8, respectively). Complexes of Ets-1 bound to GHF-1 or GHF-2 could also be specifically eluted by 5 mM glutathione (not shown), verifying that Ets-1 bound specifically to the immobilized fusion protein. Additionally, no binding was observed to glutathione-Sepharose beads in the absence of fusion protein or to GST–GHF-1 incubated with mock-transcribed and -translated reticulocyte extracts programmed with empty vector (not shown). Thus, the data indicate that Ets-1 is able to bind specifically and directly to both GHF-1 and GHF-2 fusion proteins, independent of DNA, and suggest that the inhibitory effects of GHF-2 in both GH_4 and HeLa cells (Fig. 1 and 3, respectively) may be due to the sequestration of Ets-1 or a related factor in an inactive or inhibitory complex. In contrast, the lack of effect of Ets-2 on the rPRL promoter may be a reflection of its inability to bind to GHF-1.

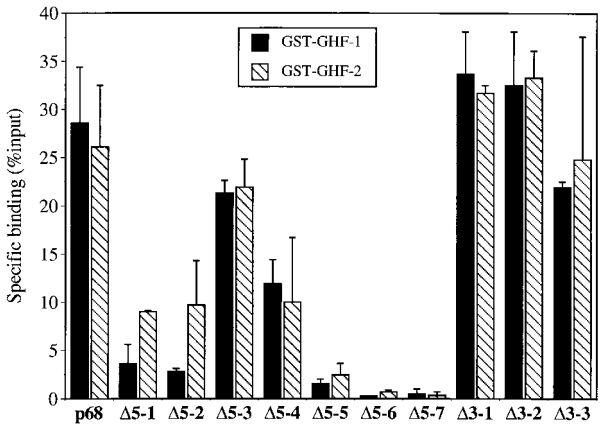


FIG. 12. In vitro binding of Ets-1 and ΔEts mutants to GHF-1 and GHF-2. Full-length (p68) and truncated (Δ5 and Δ3) Ets-1 proteins were synthesized and labeled by in vitro transcription and translation. GST, GST–GHF-1, and GST–GHF-2 were prepared and immobilized on glutathione-Sepharose (see Materials and Methods). Binding assays were carried out as described in Materials and Methods. Ets-GHF complexes were analyzed by SDS-polyacrylamide gel electrophoresis and quantified with a Fuji Bas 2000 phosphorimager. Nonspecific background binding to immobilized GST alone was subtracted from binding to GST–GHF-1 or GST–GHF-2, and specific binding is expressed as the percentage of input Ets protein retained on the GST–GHF-1 or GST–GHF-2 beads. Results are the means ± standard deviations of three separate experiments.

In an effort to determine whether the region of Ets-1 required for transcriptional synergy is also required for physical interaction with GHF-1 and GHF-2, we investigated the ability of amino- and carboxy-terminal truncations of Ets-1 (Fig. 8 and 9) to bind to GST–GHF-1 and GST–GHF-2 (Fig. 12). As shown in Fig. 12, p68 Ets-1 and the carboxy-terminal truncations Δ3-1, Δ3-2, and Δ3-3 show significant and similar levels of binding to both GHF-1 and GHF-2. These results are consistent with the functional data shown in Fig. 8 and 9 and further suggest that the synergistic effects of the Δ3 Ets-1 truncations, which cannot bind DNA, are most likely mediated by recruitment to the composite element via a physical interaction with GHF-1. The Δ5-3 and Δ5-4 amino-terminal truncations retain ~80 and 50%, respectively, of the binding capacity of p68 Ets-1. By contrast, Δ5-1 and Δ-5-2 exhibit reduced binding to GHF-1 yet retain a fair amount of binding to GHF-2, whereas Δ5-5 shows decreased binding to both GHF isoforms. Finally, Δ5-6 and Δ5-7 do not exhibit specific binding to either GHF-1 or GHF-2. While these data generally show a good correlation between the regions of Ets-1 that are required for GHF-1 binding and synergy, certain discrepancies are noted. Thus, Δ5-1, Δ5-2, and Δ5-5 constructs, which exhibit wild-type synergy, show reduced binding to GHF-1 in this assay. This may be due to altered protein structure generated by production of recombinant proteins in vitro or, alternatively, may reflect the greater sensitivity of the functional assay relative to the in vitro binding assay. However, the inability of the Δ5-6 and Δ5-7 proteins to bind to GHF-1 and GHF-2 directly correlates with their loss of synergy.

DISCUSSION

Combinatorial interactions in the control of pituitary-specific gene expression. The control of distinct but related genes

that are selectively expressed in progressively differentiated cell types in a particular developmental lineage requires a combination of cell-specific and general transcription factors (17, 28, 29). Pituitary stem cells develop into five distinct cell types, of which the thyrotroph and somatotroph-lactotroph lineages are GHF-1 dependent. In the somatotroph lineage, pituitary stem cells initially differentiate into somatotrophs (GH^+ PRL^-), and these then give rise to an intermediary somatomammotroph cell type (GH^+ PRL^+), which then terminally differentiates into lactotrophs (GH^- PRL^+) (35, 51). The critical importance of GHF-1 for the ontogeny of these cell fates, and for the cell-specific expression of the GH, PRL, and TSHβ genes, has been well documented (35, 51). However, since GHF-1 is expressed in all of these distinct pituitary cell types, yet they each express a highly specialized and different peptide hormone gene, factors other than GHF-1 must be involved in the regulation of these cell-type-specific genes. Here we show that the selective functional and physical interaction of GHF-1 with a more widely expressed transcription factor, Ets-1, acting via a composite Ets-GHF DNA element, is necessary and sufficient to establish optimal lactotroph-specific PRL promoter activity. These results indicate that a molecular code composed of an elegant combination of distinct transcription factors and a composite *cis*-acting element governs cell-type-specific gene expression and may serve to establish appropriate terminally differentiated cell lineages.

Differential roles of GHF-1 and GHF-2. Pituitary somatotrophs from human, rat, mouse, turkey, and salmon contain both GHF-1 and GHF-2 isoforms, which differ only in a highly conserved, 26-amino-acid insert domain in GHF-2 (9, 37, 41, 44, 50, 57). Previous studies have shown that GHF-2 functions as a dominant inhibitory factor, with respect to PRL and TSHβ promoters, by competing with GHF-1 for binding to target DNA sites (26, 37, 50). Since both GHF-1 and GHF-2 physically interact with Ets-1, here we propose that GHF-2 sequesters the critical cofactor, Ets-1, forming an inactive complex. The inhibitory complex may form either on the composite DNA element or independent of DNA binding, since the physical interaction does not require DNA (Fig. 11 and 12). Additionally, these data imply that the 26-amino-acid insert does not interfere with the GHF–Ets-1 interaction, but rather this insert dictates the functional consequences of the interaction. The versatility of POU-homeodomain protein function in developmental pathways is that POU-homeodomain proteins can bind cooperatively to *cis*-regulatory sites as either homo- or heterodimers, and interactions with heterologous factors further increase their range of effects (29). Indeed, most of these interactions appear to be mediated via the POU domain (29), which is common to both GHF-1 and GHF-2. Particularly relevant to this report is the interaction of the Phox1 and Paired homeodomain proteins with serum response factor, which then recruits the Ets family member Elk-1 to the c-Fos serum response element (20, 21). There is an increasing body of evidence showing that the combinatorial interactions of homeodomain proteins with other transcription factors provide a further level of control of tissue-specific gene expression.

Functional domains of Ets-1. We have previously shown that Ras activation of the rPRL promoter is dependent on a functional interaction of Ets-1 and GHF-1 at a composite DNA element (−217 to −190) that we initially termed the RRE (3, 4). A conserved MAP kinase site, $PLLT^{82}PSS$, is critical for the Ets-1-mediated Ras response of the rPRL promoter (3, 54) and to confer growth factor- and Ras-induced activation of other murine and *Drosophila* Ets proteins (5, 43, 58), indicating that this is a highly conserved mechanism (54).

In this report, we present evidence establishing the importance of Ets-1 in regulating the lactotroph-specific basal activity of the rPRL promoter. First, dominant-negative Ets inhibits rPRL promoter activity in the context of GH_4 rat pituitary cells (Fig. 1). Second, Ets-1 alone is able to partially reconstitute rPRL promoter activity in HeLa nonpituitary cells (Fig. 3) via the EBS in the previously defined RRE (Fig. 6), and Ets-1 functionally interacts with GHF-1 to fully establish rPRL promoter activity in this reconstitution assay (Fig. 3). We used amino- and carboxy-terminal truncations of Ets-1 in the reconstitution system to map the functional domains of Ets-1 required to transactivate the rPRL promoter in the absence of GHF-1 (Fig. 8 and 9). Our results are consistent with those obtained with LexA- and Gal4-Ets fusion constructs, using synthetic promoters (47). The first functional domain encompasses amino acids 57 to 98 and includes the MAP kinase site (T-82) critical for Ras activation of Ets-1 (3, 58). However, phosphorylation of this site is unlikely to be required for the Ets-1 reconstitution of basal PRL promoter activity, since site-specific mutation of T82A (data not shown) and dominant-negative N-17 Ras (Fig. 7) have no detectable effect on the Ets-1 response. Finally, deletion of amino acids 218 to 252, within the RIII domain, and deletions of the carboxy-terminal regions, which abrogate binding to DNA, result in essentially complete loss of basal Ets-1 activity.

The use of these same Ets-1 truncations, in combination with GHF-1, in the reconstitution assay enabled us to map the putative region(s) of Ets-1 required for functional synergy with GHF-1. Surprisingly, deletion of the first 218 amino acids of Ets-1 does not significantly interfere with the synergistic Ets-1–GHF-1 effect (Fig. 8B and 9). This result implies that the T-82 MAP kinase site is not required for the Ets-1–GHF-1 functional interaction in the reconstitution response. Indeed, the functional interaction of Ets-1–GHF-1 occurs independent of Ras, since the magnitude of the Ets-1–GHF-1 reconstitution response is similar in the presence of dominant-negative N-17 Ras (Fig. 7), in quiescent serum-depleted cells (data not shown), or in assays using a T82A site-specific Ets-1 mutant (data not shown). By contrast, deletion of amino acids 218 to 312 eliminates the ability of Ets-1 to synergize with GHF-1 (Fig. 8B and 9). Thus, this Ets-1 region contains elements necessary for both the Ets-1-only and the Ets-1–GHF-1 synergistic responses of the rPRL promoter in HeLa cells. Moreover, the Ets-1 and Ets-2 amino acid sequences diverge within this 218–312 region, resulting in distinct Ets-1- and Ets-2-specific structures, which may explain the inability of Ets-2 to activate the rPRL promoter or bind to GHF-1 (Fig. 3 and 11). Of note, the GST binding assay revealed that the 218–312 region is also important for physical interaction between GHF-1 and Ets-1 (Fig. 12). Interestingly, Ets-1 Δ3 truncations, which affect the DNA-binding ETS domain, retain partial ability to synergize with GHF-1, despite their inability to transactivate the rPRL promoter in the absence of GHF-1 and the fact that the Δ3-2 and Δ3-3 Ets-1 proteins lack a putative nuclear targeting sequence (2). These data show that carboxy-terminal Ets-1 sequences, downstream of amino acid 390, are unnecessary for the synergistic response. These results are consistent with the abilities of Ets-1 and the Δ3 truncated proteins to bind directly to GHF-1 and GHF-2 (Fig. 12) in the absence of DNA and suggest that in the HeLa system, the Δ3 Ets truncated proteins may be translocated to the nucleus and recruited to the composite PRL promoter *cis* element by virtue of their interaction with GHF-1. However, it is important to note that an intact DNA-binding ETS domain is required in order to obtain a complete synergistic effect of Ets-1 and GHF-1 on the rPRL promoter (Fig. 8B and 9), indicating that both an interaction domain (amino acids 218 to 312) and sequences within

the DNA-binding domain (amino acids 390 to 450) are important for optimal synergistic Ets-1–GHF-1 effects on the composite rPRL DNA element.

Model for the role of Ets-1–GHF-1 interactions in establishing basal and Ras-regulated rPRL promoter activity. Although previously defined as an RRE, in this report we demonstrate that the composite Ets-GHF element also serves to establish optimal lactotroph-specific activity of the rPRL promoter. The data that we have accumulated thus far are most consistent with a model whereby Ets-1 and GHF-1 interact via discrete functional motifs and act via a composite Ets-GHF DNA-binding site, not only to control optimal lactotroph-specific rPRL promoter activity but also to mediate the Ras response of this pituitary-specific gene (3, 4, 8). In this model, we propose that the functional interaction of these two factors does not require phosphorylation of the MAP kinase T-82 site but rather the synergistic interaction occurs via the 218–312 Ets-1-specific domain, with contributions by the DNA-binding domain. Moreover, we propose that this Ras-independent combinatorial effect of Ets-1 and GHF-1 serves to establish optimal lactotroph-specific expression of the rPRL gene, and thus Ets-1 may have a developmental role in specifying the lactotroph phenotype. In this regard, it is noteworthy that the ancestrally related and similarly GHF-1-dependent rGH promoter fails to exhibit synergistic Ets-1–GHF-1 effects (Fig. 4 and reference 4). A separate layer of regulation is imposed by the presence of GHF-1 and GHF-2 isoforms in pituitary somatotrophs and lactotrophs, whose interaction with Ets-1 results in distinct functional consequences. Implicit from our dominant-negative Ets data for GH_4 cells and the reconstitution studies in the presence of N-17 Ras in HeLa cells is that this Ets-1–GHF-1 synergy occurs constitutively and does not require any special cellular conditions. Also implicit from our data, the model presented, and current understanding of Ets factor action (34, 52) is that Ets-1 and GHF-1 should form a ternary complex on the composite −217/−190 rPRL DNA element. Although this is the expected result, ternary complex formation has been difficult to document in the PRL and SAP-2/Net systems (3, 18, 31, 46), suggesting that the requisite conditions occurring in vivo have not yet been adequately reproduced in vitro. Finally, the Ras/Raf induction of the rPRL promoter occurs via this same Ets-1–GHF-1 complex and composite DNA element. We have shown that both factors are required for the Ras response (3, 4, 8), implying that the interaction of these two factors occurs first, as proposed above, and that the Ras-activated serine/threonine kinase cascade then impinges on T-82 of Ets-1 to augment the transcription potency of the preformed Ets-1–GHF-1 complex (54). In summary, the combinatorial interactions of Ets-1, GHF-1, and GHF-2 discussed above provide a mechanism to permit both diversity and a high degree of specificity in the transcriptional control of basal and hormone-induced pituitary-specific gene expression.

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