Selective Inhibition of Prolactin Gene Transcription by the ETS-2 Repressor Factor*

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Regulation of prolactin gene transcription requires cooperative interactions between the pituitary-specific POU domain protein Pit-1 and members of the ETS transcription factor family. We demonstrate here that the ETS-2 repressor factor (ERF) is expressed in pituitary tumor cells and that overexpression of recombinant ERF inhibits prolactin promoter activity, but not the closely related growth hormone promoter. In non-pituitary cell lines, coexpression of ERF disrupts the cooperative interactions between Pit-1 and ETS-1 and blocks the induction of Pit-1-dependent prolactin promoter activity by cAMP. The potential role of ERF in the inhibitory response of the prolactin promoter to dopamine was examined using pituitary tumor cells stably expressing dopamine D2 receptors. The inhibitory responses of the prolactin promoter to ERF and dopamine are additive, suggesting that ERF has a complementary role in this hormonal response. A single Pit-1 DNA-binding element from the prolactin promoter is sufficient to reconstitute the inhibitory response to ERF. DNA binding analysis using either a composite Pit-1/ETS protein-binding site or a Pit-1 element with no known affinity for ETS proteins revealed that ERF interferes with Pit-1 binding. Together, these results demonstrate that ERF is a specific inhibitor of basal and hormone-regulated transcription of the prolactin gene and suggest a new level of complexity for the interaction of ETS factors with Pit-1 target genes.

The transcription of the prolactin (PRL) gene in the lactotroph cells of the anterior pituitary is under predominantly inhibitory control mediated by dopamine released by hypothalamic neurons. Extensive analysis of tissue-specific and hormone-responsive DNA elements in the rat PRL gene promoter, however, has not lead to the identification of specific inhibitory sites. Instead, this analysis has revealed a series of apparently redundant DNA-binding sites for the pituitary-specific transcription factor Pit-1 (GHF-1) that are both necessary and sufficient for multihormonal regulation of transcriptional activity, including the inhibitory response to dopamine (1–6).

Although Pit-1 has been implicated as the mediator of hormonal responses conferred by the PRL gene promoter, the transcriptional regulatory mechanisms involving Pit-1 remain obscure. Previous studies demonstrated that Pit-1 is phosphorylated in response to several different signaling pathways (7–9); however, mutagenesis of the three phosphoacceptor sites within the Pit-1 POU domain showed that this modification is not a key step in hormonal regulation of the PRL gene (10, 11). These data predict that Pit-1 is not the sole mediator of hormonal responses conferred by the PRL gene promoter and that other factors recruited to the Pit-1 DNA elements may function in the capacity of positive or negative regulators of PRL gene transcription.

In this regard, some of the Pit-1 elements in the PRL promoter also serve as binding sites for other transcription factors. For example, members of the ETS transcription factor family interact with two Pit-1-binding sites within the PRL promoter (12–15). The ETS family of proteins mediate transcriptional responses to growth factors and activators of the Ras/mitogen-activated protein kinase (MAPK) pathway, and these composite PRL gene Pit-1/ETS protein DNA elements confer responses to several different hormones acting through diverse signal transduction pathways (12–15). One possible molecular mechanism for multihormonal control of PRL promoter activity is the convergence of different signal transduction pathways on the MAPK pathway, which in turn modulates the cooperative interactions between ETS factors and Pit-1 (13, 14). The cooperative interactions between Pit-1 and ETS family members appear particularly important in establishing pituitary lactotroph-specific PRL gene expression (15) and could account for the differential basal and hormonally induced transcriptional responses of the Pit-1-dependent PRL and growth hormone (GH) genes.

The above model predicts that factors interfering with the interactions between Pit-1 and ETS proteins could selectively inhibit PRL gene transcription. Recently, a novel member of the ETS family was identified that acts as a transcriptional repressor. The ETS-2 repressor factor (ERF) is a 548-amino acid phosphoprotein with an N-terminal DNA-binding domain homologous to that of other ETS family members and a unique C-terminal repressor domain (16, 17). The DNA-binding domain of ERF recognizes ETS-binding sites, and the repressor activity of ERF can abrogate transactivation by ETS proteins.

Given the importance of the interaction of Pit-1 and ETS proteins to PRL gene transcription, we hypothesized that ERF could act to inhibit PRL promoter activity by antagonizing this
interaction. Here we show that the ERF gene transcript is expressed in GH pituitary tumor cells. Using an expression vector encoding the ERF protein, we demonstrate profound and specific suppression of PRL reporter gene expression in both lactotroph cell lines and non-pituitary cells coexpressing the Pit-1 and ETS-1 proteins. Moreover, expression of ERF effectively blocked the induction of PRL gene transcription by the protein kinase A pathway and acted in an additive manner with dopamine to suppress PRL promoter activity. We demonstrate that tandem copies of a single PRL promoter Pit-1 DNA element are sufficient to confer the inhibitory response to ERF and that protein extracts from cells expressing ERF inhibit Pit-1 binding to Pit-1 DNA elements. Together, these results support the view that ERF can act as a potent inhibitor of PRL gene activity.

EXPERIMENTAL PROCEDURES

Cell Culture, Expression Vectors, and Transfection—All cell lines used in these studies were maintained as monolayers in Ham’s F-12/Dulbecco’s modified Eagle’s medium supplemented with 10% new born calf serum. The cell culture medium was changed prior to transfection as indicated for the individual experiments. Cells were harvested by treatment with 0.05% trypsin and 0.53 mM EDTA and washed by centrifugation. The LZR1 cells were transfected using calcium phosphate precipitation as described (4). All other cell lines were transfected by electroporation with the BTX ECM 600 electroporation system using conditions optimized for each cell type. The cells were resuspended Ca2+/Mg2+-free Dulbecco’s phosphate-buffered saline, and 0.4-ml aliquots of the cell suspension (containing ~1 × 10^6 cells) were transferred into 0.2-cm gap electroporation cuvettes containing the indicated luciferase reporter gene and varying concentrations of the indicated expression vector DNAs. The total amount of DNA was kept constant using empty vector DNA. Luciferase reporter plasmids containing rat PRL promoter sequences spanning coordinate +2 to +34 or +16 to +34 relative to the transcription start site (rPRL-Luc) and the rPRL GH promoter (+235 to +8) have been described previously (14, 18, 19). The luciferase reporter gene containing four tandem copies of the rPRL 3P Pit-1 element linked to the rPRL minimal promoter (coordinates −36 to +34) was described previously (13). The expression vector encoding the ERF protein used in these studies was prepared by in-frame insertion of the ERF cDNA sequence from the pSG5-ERF plasmid (16) into the pcDNA3.1-HisA expression vector (Invitrogen, San Diego, CA). The expression vectors encoding Pit-1 (18) and c-ETS-1 (20) have been described.

Analysis of ERF mRNA—Total cellular RNA was isolated from GH4ZR7 cells by the guanidinium thiocyanate/phenol/chloroform method (21), and poly(A)^+ RNA was isolated. The yield and purity of RNA samples were assessed by the ratio of absorbance at 260 and 280 nm. RNA was fractionated by denaturing agarose gel electrophoresis and transferred to nylon membrane. The membranes were sequentially hybridized with 1 × 10^6 cpm/ml 32P-labeled complementary DNA probes to human ERF and glyceraldehyde-3-phosphate dehydrogenase at 45 °C for 16 h. The blots were rinsed in 2× SSC and washed in succession with 2× SSC and 0.1% SDS, 0.5× SSC and 0.1% SDS, and 0.1× SSC and 0.1% SDS at 60 °C and exposed to Kodak XAR-5 film at −70 °C.

Western Blotting and Electrophoretic Mobility Shift Assay (EMSA)—Transiently transfected HeLa cells were lysed at 4 °C in detergent buffer as described previously (22). Samples were fractionated by SDS-polyacrylamide gel electrophoresis on 10% gels. The proteins were transferred to nitrocellulose for 1 h by electroblotting at 100 V and then detected by Ponceau S staining. The membranes were blocked with 5% nonfat dried milk in TBS-T buffer (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20) and incubated with the Anti-Xpress antibody directed against the enterokinase recognition sequence (1:10,000 final dilution; Invitrogen) for 1 h at room temperature. Following washes in TBS-T buffer, the membranes were incubated with a 1:50,000 final dilution of horseradish peroxidase-conjugated anti-rabbit Ig (Pierce). The membranes were washed in TBS-T buffer and incubated in ECL reagents (NEN Life Science Products) for 1 min. The membranes were then exposed to Kodak XAR-5 film for 5–15 min.

EMSA bands were performed on whole cell extracts prepared from transiently transfected HeLa cells as described previously (23). Duplex oligonucleotides corresponding to the PRL 3P andPAL 1P Pit-1 binding sites and the GATA gene EBS were as follows: PRL 3P, 5′-GGCTTC-GCATTCAGATATTCAT-3′; PAL 1P, 5′-GGCTTC-CCTGATTACATGAATATTCAT-3′; GATA EBS, 5′-GGCTTC-GGAAGGTG; and GATA EBS, 5′-GGCTTC-GGAAGGTG, and GATA EBS, 5′-GGCTTC-GGAAGGTG.

RESULTS

Expression of ERF mRNA in PRL-secreting Cells—RNA blot analysis was performed to determine if ERF is expressed in pituitary lactotroph cells. Using RNA from the dopamine-responsive GH4ZR7 cell line probed with the human ERF cDNA, we detected a single transcript ~2.6 kilobases in length (Fig. 1). This result is in agreement with that reported by Sgouras et al. (16) for other tissues and cell lines. The recent cloning of the ERF gene and 5′-regulatory sequences revealed a number of potential promoter elements that could function in hormonal regulation of ERF expression (24). The GH4ZR7 cells were treated with several different hormones and agents known to alter gene expression in GH pituitary cell lines to determine if ERF is transcriptionally regulated. Treatment of GH4ZR7 cells with dopamine for 5 or 24 h or exposure to forskolin, dexamethasone, or retinoic acid had no affect on steady-state levels of ERF mRNA (Fig. 1). These results indicate that expression of the ERF gene in pituitary cells does not appear to be controlled at the level of transcription.

Selective Inhibition of PRL Promoter Activity by ERF—Because cooperative interactions between Pit-1 and ETS-1 are key to the regulation of PRL gene transcription (12–15), we determined the effect of ERF expression on PRL promoter activity. The Pit-1-dependent GH promoter was used for comparison. Cotransfection of GH pituitary cells with an expression vector encoding ERF and the luciferase reporter gene linked to the rat PRL promoter (rPRL-Luc) demonstrated that ERF inhibits the basal activity of the PRL promoter in a dose-

FIG. 1. Constitutive expression of ERF transcripts in PRL-secreting GH4ZR7 cells. Dopamine D2 receptor-expressing GH4ZR7 cells were maintained in cell culture and received no treatment or were treated with hormones and agents known to regulate transcription in this pituitary cell line. The treatment time was for 5 h (except in lane 3, 24 h). Following treatment, poly(A)^+ RNA was isolated, and equivalent amounts (~4 µg) were fractionated by denaturing agarose gel electrophoresis. Following transfer, the RNA blots were hybridized sequentially with full-length 32P-labeled human ERF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. Lane 1, no treatment; lanes 2 and 3, dopamine (1 µM); lane 4, forskolin (5 µM); lane 5, dexamethasone (0.1 µM); lane 6, retinoic acid, (0.3 µM). kb, kilobases.
activity. Values are average luminescence normalized to protein con-
tector encoding the ERF protein. Approximately 18 h after transfection,
electroporation with the rPRL-Luc or rGH-Luc reporter gene in the
GH3 cells were transfected by
moter activity in pituitary cells.
cAMP had no effect on basal rPRL promoter activity in these
cells. Addition of CPT-cAMP increased cAMP hydrolysis (28, 29), and similar to other stimulatory hormonal
responses of the PRL promoter, Pit-1 markedly enhances the

FIG. 2. Expression of ERF inhibits rPRL (but not rGH) pro-
tomer activity in pituitary cells. GH3 cells were transfected by
electroporation with the rPRL-Luc or rGH-Luc reporter gene in the
absence or presence of increasing concentrations of the expression vec-
tor encoding the ERF protein. Approximately 18 h after transfection,
the cells were collected for analysis of total protein and luciferase
activity. Values are average luminescence normalized to protein content ± S.E. of three independent determinations.

dependent manner (Fig. 2A). In contrast, expression of ERF had no significant impact on the rat GH promoter-luciferase reporter gene (rGH-Luc) (Fig. 2B), suggesting selectivity in the inhibitory actions of ERF at these Pit-1-dependent promoters. In this regard, it should be noted that in cells expressing ERF, we also achieved expression of other proteins from several different genetic vectors using the cytomegalovirus, early SV40, or Rous sarcoma virus promoters. This indicates that ERF is not a potent inhibitor of transcription from these promoters.

Cotransfection studies to reconstitute the activation of the
PRL promoter in non-pituitary cells were used to determine the
effect of ERF expression on the functional interactions between
Pit-1 and ETS-1. COS-1 kidney cells were cotransfected with the
rPRL-Luc reporter gene and expression vectors encoding
Pit-1, ETS-1, and ERF. Expression of Pit-1 induced PRL prom-
tomer activity 5-fold, and coexpression of Pit-1 with ETS-1 resulted in ~30-fold induction (Fig. 3). Cotransfection of the
Pit-1 and ETS-1 plasmids with increasing amounts of the ERF
expression vector resulted in the dose-dependent inhibition of the
Pit-1- and ETS-1-mediated induction of PRL promoter ac-
tivity. Together, these results show that expression of the ERF
protein inhibits rPRL (but not rGH) basal promoter activity in
pituitary cells and suggest that the inhibitory activity results from
interference with the ability of Pit-1 and ETS-1 to co-
operatively induce the PRL promoter.

ERF Suppression of cAMP-dependent Stimulation of the PRL Promoter—Previous studies demonstrated that functional in-
teractions between Pit-1 and ETS-1 are required for some
hormonal responses conferred by the PRL promoter (12–14). The
protein kinase A signaling pathway activates the MAPK signaling cascade through B-Raf, resulting in activation of ETS
family transcription factors (25). In pituitary cells, this signaling
cascade could induce the cooperative interaction of an ETS
protein with Pit-1, resulting in protein kinase A-dependent
activation of PRL transcription (13). We examined the effect of
ERF expression on protein kinase A induction of PRL promoter
activity and evaluated the role of p42 MAPK in this response
using the specific inhibitor of MAPK kinase-1 (MEK), the drug
PD098059 (26). Rat-1 fibroblast cells maintained in the ab-
sence of serum were cotransfected with the rPRL-Luc reporter
gene and the indicated protein expression vectors. The trans-
fected cells received no further addition or were treated with the
permeable analog of cAMP, CPT-cAMP. Addition of CPT-
cAMP had no effect on basal rPRL promoter activity in these
non-pituitary cells (Fig. 4). In contrast, coexpression of Pit-1
induced reporter gene activity 5-fold, and treatment with CPT-
cAMP increased this activity an additional 2-fold. This re-
sponse to CPT-cAMP was completely blocked by addition of the
MEK inhibitor PD098059. Under these serum-free conditions,
coexpression of the ETS-1 and Pit-1 proteins did not result in
cooperative induction of rPRL promoter activity (compare with
Fig. 3). However, CPT-cAMP treatment of the cells coex-
pressing Pit-1 and ETS-1 resulted in 8-fold induction of PRL pro-

FIG. 3. Expression of ERF inhibits the cooperative activation
of the PRL promoter by Pit-1 and ETS-1 in COS-1 kidney cells.
The COS-1 cells were cotransfected with the rPRL-Luc reporter
gene and the indicated protein expression vectors. The trans-
fection of serum were cotransfected with the rPRL-Luc reporter
gene and the indicated protein expression vectors. The trans-
fection of reporter gene (rGH-Luc) (Fig. 2). This indicates that the ERG promoter mediates PRL promoter activity. Together, these results support the convergence of the protein kinase A and MAPK signaling pathways in
activation of PRL promoter activity and demonstrate that ERF
can inhibit this response.

ERF Regulation of the PRL Gene Promoter in Dopamine-
responsive Cells—Dopamine binding to lactotroph D2 receptors
results in tonic inhibition of PRL promoter function (4, 27). We
examined the ability of ERF to modulate dopaminergic inhibi-
tion of cAMP-dependent PRL promoter activity in the dopamine D2 receptor-expressing GH4ZR7 cell line. Typical dose-response curves for both dopamine and the ERF expression plasmid are shown in
Fig. 5A. Dopamine treatment of GH4ZR7 cells transfected with
the rPRL-Luc reporter gene caused a 1.7-fold decrease in re-
porter activity. Similarly, in GH4ZR7 cell cultures transfected
with the ERF plasmid, basal promoter activity was reduced
2.6-fold, and the inhibitory response to ERF was enhanced
significantly by addition of dopamine (Fig. 5A).

The inhibitory response of the PRL promoter to D2 receptor
activation is restricted to pituitary cells and several other neu-
roendocrine cell types. In a number of non-pituitary cell lines
stably expressing dopamine D2 receptors, dopamine stimulates
PRL promoter activity (6, 28). This response may result from
increased Ca2+ levels or phosphatidylinositol 4,5-bisphosphate
hydrolysis (28, 29), and similar to other stimulatory hormonal
responses of the PRL promoter, Pit-1 markedly enhances the
ERF Inhibits Prolactin Gene Transcription

**ERF Inhibitory Responses Can Be Conferred by a Single Pit-1 Element**—The results comparing the rGH and rPRL promoters (Fig. 2) indicated that ERF displayed specificity in inhibition of these Pit-1-dependent promoters. The PRL 3P Pit-1 DNA element is sufficient to confer the inhibitory response to dopamine induction in these cell types. A mouse Ltk− cell line stably expressing dopamine D_{2} receptors (LZR1) was used to determine the effect of ERF expression on the stimulatory responses to dopamine conferred by the rPRL promoter. The results shown in Fig. 5B demonstrate that expression of Pit-1 in LZR1 cells induced the rPRL-Luc reporter gene 4-fold over basal levels and that expression of ERF suppressed both basal and Pit-1-activated transcription. Treatment of LZR1 cells with dopamine induced basal rPRL-Luc activity <2-fold, but stimulated the promoter by 3.7-fold in the presence of Pit-1 (Fig. 5B, right panel). Interestingly, expression of ERF failed to suppress the stimulatory response to dopamine, either in the presence or absence of Pit-1 (Fig. 5B). These results indicate that certain transcriptional responses conferred by PRL promoter elements in the context of non-pituitary cells are not inhibited by the ETS repressor, ERF.

**ERF Inhibitory Responses Can Be Conferred by a Single Pit-1 Element**—The results comparing the rGH and rPRL promoters (Fig. 2) indicated that ERF displayed specificity in inhibition of these Pit-1-dependent promoters. The PRL 3P Pit-1 DNA element, a composite Pit-1/ETS site located between positions −165 and −150 base pairs, is sufficient to confer hormonal responsiveness in non-pituitary cells expressing Pit-1 (13). We examined the ability of ERF to inhibit transcriptional responses conferred by tandem copies of the PRL 3P DNA element. HeLa cells were transfected with a luciferase reporter gene coupled to four tandem copies of the PRL 3P site and the minimal PRL promoter (4×3P-Luc). Expression of Pit-1 resulted in a 6-fold induction of the reporter gene, and coexpression of the ERF protein inhibited this response by 60% (Fig. 6). Furthermore, coexpression of Pit-1 and ETS-1 together resulted in a cooperative 25-fold induction of the PRL 3P site reporter gene construct, and this response was reduced 90% by expression of ERF. These results demonstrate that this Pit-1 DNA element is sufficient to confer the inhibitory response to ERF.

**Functional Interaction of Pit-1 and ERF at Pit-1 DNA Elements**—To begin to dissect the mechanisms for ERF-dependent inhibition of PRL promoter activity, extracts were prepared from HeLa cells expressing epitope-tagged Pit-1 and ERF proteins for use in EMSA. Western blot analysis confirmed that similar amounts of the recombinant proteins were made and indicated that the tagged ERF protein migrated as a doublet (Fig. 7). The ERF protein is a substrate for MAPK, and this pattern of migration could indicate that the protein is partially phosphorylated in HeLa cells (16). EMSA was then used to assess the binding of proteins from these transfected cell extracts to the PRL 3P site. An endogenous HeLa cell protein bound to the PRL 3P element, forming a single shifted complex (Fig. 8A, lane 1, solid arrow), and extracts from HeLa cells expressing Pit-1 resulted in the formation of two additional shifted complexes (lane 2, open arrows). This result is consistent with previous reports of Pit-1 binding to DNA elements as both a monomer and dimer (13, 30, 31). Both of these complexes were cleared from the reaction by addition of an antibody specific to Pit-1 (Fig. 8A, lane 3), and all three protein complexes were diminished by competition with increasing amounts of the unlabeled PRL 3P site oligonucleotide. In contrast, only the complex formed by endogenous HeLa cell protein was competed for by the EBS from the GATA gene (32), suggesting that this complex contains an ETS family member (Fig. 8A).

To investigate potential interactions of ERF with the PRL 3P site, EMSA reactions were prepared using increasing amounts of protein extract from HeLa cells expressing ERF. Total pro-
The ERF protein inhibits transcriptional responses conferred by tandem copies of the composite PRL 3P DNA element. HeLa cells were cotransfected with a plasmid containing the luciferase reporter gene linked to the minimal rPRL promoter (coordinates -36 to +34) and four tandem copies of the PRL 3P site (4×3P-Luc) and the indicated protein expression vectors. After overnight incubation, the cultures were collected for analysis of luciferase activity. Values are average -fold change in luminescence over basal levels ± S.E. of three independent determinations.

Expression of epitope-tagged Pit-1 and ERF proteins in HeLa cells. Protein extracts were prepared from HeLa cells transiently transfected with the empty pcDNA3.1 vector (lane 1) or with the Pit-1 (lane 2) or ERF (lane 3) expression vector. The protein extracts (50 μg) were fractionated by denaturing polyacrylamide electrophoresis and transferred to membranes. The Western blots were probed with an antibody directed against the Anti-Xpress epitope tag. The positions of the Pit-1 protein (open arrow) and a doublet formed by the ERF protein (solid arrows) are indicated. A nonspecific protein (NS) was also detected.

The ERF protein interferes with protein binding to the composite PRL 3P DNA element. Whole cell extracts were prepared from HeLa cells transfected with empty vector or with the expression vector encoding either Pit-1 or ERF. EMSA was used to assess binding of proteins to the duplex 32P-labeled PRL 3P Pit-1 site probe. The reactions were fractionated by nondenaturing gel electrophoresis, and the probe was detected by autoradiography. A, a single shifted complex was detected for proteins from the control HeLa cell extract (lane 1, solid arrow). Two additional DNA-protein complexes were formed by extracts from cells expressing Pit-1 (lane 2, open arrowheads), and these complexes were cleared by pretreatment with an antibody directed against Pit-1 (lane 3). Competition with excess unlabeled PRL 3P oligonucleotide (lanes 4–6) or excess unlabeled GATA gene EBS oligonucleotide (lanes 7–9) from 3- to 30-fold (indicated by the wedge) demonstrated the specificity of these DNA-protein complexes. B, to examine potential interactions of ERF with the PRL 3P DNA element, extract from control HeLa cells was mixed with increasing amounts of extract from HeLa cells expressing ERF (left panel). Then, to determine if the ERF protein influenced Pit-1 binding to the PRL 3P site, a constant amount of extract from HeLa cells expressing Pit-1 (3 μg) was mixed with increasing amounts of ERF cell extract (3 or 9 μg); the total amount of HeLa cell protein was kept constant with the control extract (right panel).

DISCUSSION

The predominant physiological control of PRL gene expression in anterior pituitary lactotrophs is inhibitory, and the molecular mechanisms that contribute to the suppression of transcription are not well understood. It has become increasingly clear that specific endocrine regulation of PRL gene transcription requires the cooperative interactions between Pit-1 and other transcription factors, including members of the ETS family of proteins. More important, the closely related GH gene is expressed in distinct pituitary cell types and has a different pattern of endocrine regulation. Whereas the GH promoter contains both Pit-1 DNA elements and potential ETS protein-binding sites, this promotor does not support the cooperative interactions between Pit-1 and ETS factors (14). Thus, it appears that the Pit-1/ETS protein interactions provide a mechanism for differential regulation of the PRL and GH gene.
promoters in anterior pituitary cells. This interaction could also serve as a potential target for inhibitory factors that selectively suppress PRL gene transcription, and the results shown here indicate that the ETS family repressor protein ERF can function in this capacity.

The studies presented here demonstrate that mRNAs homologous to the transcript encoding ERF are present in GH pituitary tumor cells, indicating that these lactotroph cells have the capacity to synthesize the ETS repressor protein. Consistent with the results reported by Sgouros et al. (16), we found no evidence for hormonal regulation of the ERF transcript levels. However, these authors demonstrated post-transcriptional regulation of ERF repressor activity through phosphorylation by MAPK, and our Western blot analysis of the ERF protein expressed in HeLa cells indicated that the protein is modified (see Fig. 7). The differential regulation of gene expression by MAPK pathway modulation of ETS transcription factor activities appears to be of both physiological and developmental importance. For example, in Drosophila, a critical stage in eye development occurs when activation of the MAPK pathway simultaneously inhibits the ETS repressor protein Yan and stimulates the ETS activator Pointed (34). A similar mechanism involving coordinate activation of ETS proteins and reduction of ERF repressor activity by MAPK phosphorylation could mediate transcriptional responses to hormones (16, 17). Given the central role of Pit-1 and ETS proteins in the hormonal regulation of PRL gene transcription, we undertook studies to determine if the ERF protein could influence PRL promoter activity. Our results demonstrate ERF to be a potent inhibitor of the PRL promoter, but not the closely related GH promoter, in pituitary tumor cells. Moreover, expression of the ERF protein in non-pituitary cells blocked the activation of the PRL promoter by the coexpressed Pit-1 protein as well as by the combined expression of Pit-1 and ETS-1. Furthermore, we found that ERF inhibits the cAMP induction of Pit-1/ETS-1-dependent PRL promoter activity in these non-pituitary cells. A convergence of the protein kinase A signaling pathway and the MAPK pathway resulting in the activation of ETS proteins, such as Elk-1, was demonstrated (25), and we show here that the Pit-1-dependent cAMP response is blocked by the MEK inhibitor PD098059. Together, these results support the view that induction of PRL gene transcription by the protein kinase A signaling pathway is mediated by activation of the MAPK pathway and ETS proteins and that the ETS repressor ERF can inhibit this response.

These results implicated ERF as a potential mediator of inhibitory responses conferred by the PRL promoter. Because PRL transcription in pituitary lactotrophs is under the inhibitory control of dopamine D_{2} receptor-coupled signaling, we used the dopamine-responsive GH4C17 pituitary cell line to determine if ERF participated in this inhibitory pathway. Our results indicate that dopamine inhibition and ERF inhibition of PRL promoter activity are additive. Our previous study demonstrated that isolated Pit-1 DNA elements, including the PRL 1P site, which has no known affinity for ETS proteins, are sufficient to confer dopamine inhibition (6). Moreover, site-directed mutagenesis of the EBSs that are part of the composite Pit-1 elements does not prevent dopamine inhibition. These results suggest that ERF and dopamine may function by complementary mechanisms to reduce transcription at the PRL promoter. In D_{2} receptor-expressing mouse L cells, dopamine treatment stimulated PRL promoter activity. We observed here that ERF failed to block dopaminergic stimulation, indicating that not all PRL promoter responses are sensitive to ERF inhibition. It is important to note that not all cis-active sites in the rPRL promoter are part of composite Pit-1 elements. For example, an ETS site located in the promoter region between −101 and −76 confers responsiveness to insulin, insulin-like growth factor 1, and fibroblast growth factor (35–37). This site is also required for responsiveness to the phosphatidylinositol 3-kinase signaling pathway and may specify interactions with ETS family proteins that differ from those directed to the composite Pit-1 DNA elements (36, 37). Together, these results indicate that ERF is unlikely to directly mediate transcriptional effects of dopamine on the PRL promoter. Our present data, however, do not exclude a mechanism whereby dopamine D_{2} receptor signaling could induce post-translational modifications of ERF that enhance repressor activity that may be independent of binding to DNA elements.

We found that tandem copies of the PRL 3P Pit-1 site were sufficient to confer the inhibitory response to ERF (Fig. 6), suggesting that ERF could interact with this composite element. Using EMSA and extracts from HeLa cells expressing Pit-1, we demonstrated binding of an endogenous HeLa cell protein to the PRL 3P site as well as specific shifted complexes formed by Pit-1. Competition studies demonstrated that HeLa cell protein binding to the PRL 3P site also had affinity for the GATA EBS oligonucleotide. More important, although competition with the homologous PRL 3P site reduced both the Pit-1-specific and HeLa protein complexes, competition with the EBS oligonucleotide did not influence Pit-1 binding (Fig. 8). This indicated that in this in vitro system, binding of Pit-1 to the PRL 3P site is independent of the HeLa cell protein binding. Using extracts from HeLa cells expressing ERF, we were unable to detect the formation of any additional shifted complexes. There was, however, diminished binding of both the endogenous HeLa cell protein and Pit-1 to the PRL 3P site in

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the presence of these extracts. This result could be an indication that ERF binding to the EBS of the composite PRL 3P element is capable of displacing both Pit-1 and ETS proteins, suggesting that competition for DNA binding at this composite Pit-1 element is a potential mechanism of ERF inhibitory action.

This interpretation, however, is not supported by our EMSA results using the PAL 1P site, an element with no known affinity for ETS proteins. EMSA studies using extracts from HeLa cells expressing Pit-1 identified both Pit-1-specific complexes and a nonspecific complex formed by HeLa cell protein. Using ERF-containing cell extracts, we again found no evidence for the binding of ERF to this DNA element. However, titration of a constant amount of Pit-1 cell extract with increasing amounts of ERF cell extract resulted in diminished Pit-1 binding. In contrast, the binding of the nonspecific protein complex was not influenced by increasing amounts of ERF cell extract (Fig. 9), demonstrating selective effects of ERF on DNA/protein interactions. It is possible that low affinity binding of ERF to these Pit-1 DNA elements could account for the inhibitory actions of ERF and that the EMSA conditions used here did not allow detection of these interactions. However, this would predict inhibition of all Pit-1-dependent promoters, including the rGH promoter, a result not obtained in the present studies (Fig. 2). An alternative view is that inhibitory activity results from the formation of protein complexes with Pit-1 not requiring the ERF protein to form specific contacts with DNA. Promoter element specificity could arise if the pairing of Pit-1 and coactivator protein partners directs these ERF protein interactions. The potential role of ERF in the pituitary lactotroph has not been defined, nor has its ability to regulate the endogenous PRL gene been determined. One approach to address this issue would be to examine endogenous PRL gene transcription in a stable pituitary cell line in which ERF is expressed under the control of an inducible promoter. Taken together, our results suggest that ERF inhibits Pit-1-dependent transcriptional activity through interference with binding to specific promoter elements. In addition, our results with ERF extend the view that ubiquitous ETS family proteins, through unique partnerships with cell-specific transcription factors, can serve to integrate and coordinate both stimulatory and inhibitory transcriptional responses, leading to the control of tissue-specific gene expression.

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