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Selective Repression of Rat Prolactin Gene by Stable Expression of Dominant-Negative Ets in GH4 Pituitary Cells

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Members of the Ets family of transcription factors are key regulators controlling prolactin (PRL) gene expression. Utilizing a transient transfection approach and the GH4 rat pituitary cell line, we have shown that Ets-1 acts synergistically with the pituitary-specific POU homeodomain transcription factor, Pit-1, to mediate basal and Ras-induced regulation of the proximal (–425) rat PRL (rPRL) promoter. Although the transient transfection approach has provided important information regarding rPRL proximal promoter regulation, the role of Ets factors in the regulation of the intact, endogenous PRL promoter has not been explored. To address this area of question, we created several clonal GH4 cell lines that stably express either dominant-negative Ets (dn-EtsZ) or dominant-active Ets (VP16 Ets) constructs and used these cell lines as a model system to analyze the role of Ets factors on endogenous PRL gene expression. Northern blot analysis of these cells showed that PRL mRNA levels were dramatically reduced, by an average of 80%, in the cell lines expressing dn-Ets compared to vector-only controls. Conversely, stable expression of the dominant-active VP16 Ets led to an average threefold increase in PRL mRNA. GH4 cells expressing dn-EtsZ displayed significantly lower levels of intracellular PRL protein content and greatly diminished secretion of PRL into the cell culture medium, compared to vector-only controls. Consistent with our previous observations, the mRNA levels for growth hormone were unaffected by either dn-EtsZ or VP16 Ets expression. Expression of dn-EtsZ reduced Pit-1 mRNA levels by about 30%; however, the intracellular levels of Pit-1 protein were unchanged. Taken together, these results verify and strengthen the view that Ets factors play a critical role in the regulation of endogenous PRL gene expression and PRL protein production.

Key Words: Prolactin; Ets factors; Ets-binding site; DNA-binding domain; GH4 cells.

Introduction

The Ets family of transcription factors play important roles in the control of cell proliferation and differentiation, as well as oncogenic transformation *(1,2)*. The family is defined by the highly conserved ETS domain, which comprises 84–90 amino acids folded into a winged helix-turn-helix DNA-binding structure *(3)*. Most Ets proteins bind as monomers to DNA motifs containing a core 5'GGA(A/T)3' sequence *(4)*. Further binding specificity and affinity is dictated by nucleotides flanking the core sequence *(4,5)*. As an additional level of transcriptional control, the DNA-binding activity of several Ets factors is also subject to intramolecular autoinhibition by protein domains flanking the Ets domain *(5)*. Ets factors typically act in conjunction with other transcription factor partner proteins, resulting in cooperative interactions at composite DNA elements and synergistic transcriptional responses *(1,4)*.

Utilizing GH4 rat pituitary cells, HeLa nonpituitary cells, and a transient cotransfection approach, our laboratory and others have previously identified Ets proteins as key regulatory factors involved in basal and growth factor/Ras-regulated activity of the rat prolactin (rPRL) promoter *(6-9)*. Regarding the role of Ets factors regulating basal rPRL promoter activity, we have shown that the activity of the proximal (–425) rPRL promoter–luciferase reporter in GH4 cells was diminished in a dose-responsive fashion with increasing amounts of transiently transfected dominant-negative EtsZ (dn-EtsZ), resulting in a maximal inhibition of 70% at the highest dose of dn-EtsZ *(6)*. Similarly, the Ets-2 repressor factor, ERF, also resulted in a dose-responsive, selective, and potent inhibition of a rPRL promoter–luciferase reporter in GH3 cells, with maximal inhibition reaching about 90% *(10)*. Reconstitution studies demonstrated that both Pit-1 and Ets-1 were required for the rPRL promoter activity in HeLa nonpituitary cells to achieve optimal levels that were equivalent to that seen in GH4 pituitary cells *(6)*. Moreover, dn-EtsZ and ERF blocked the reconstitution effects of Ets-1 plus Pit-1 in HeLa cells *(6)*. Finally, the basal transcription element (BTE), a *cis*-acting element that had previously been shown to be critical for regulation of basal rPRL promoter activity *(12,13)*, has recently been shown to be a functional Ets-binding site (EBS) targeted by several growth factor signaling pathways *(8,14,15)*.

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While the transient transfection approach has provided valuable insights into regulation of the proximal –425 rPRL promoter, the role of Ets factors in the regulation of the full-length, endogenous PRL promoter has not been explored. Furthermore, studies to determine the effects of Ets factors on intracellular content and secretion of PRL protein are not amenable to transient transfection experiments. To address these issues, we generated GH4 cell lines that stably express either a dominant-negative or a dominant-active Ets construct. The dn-Ets construct comprises the Ets-2 DNA-binding domain (DBD) fused to LacZ (dn-EtsZ), or a dominant-active Ets construct, consisting of the chicken Ets-1 DBD fused to the viral VP16 transactivating domain (VP16 Ets). Individual clones were isolated, expanded, and analyzed for PRL, growth hormone (GH) and Pit-1 gene expression, and PRL protein production.

In the present study, we show that expression of the dn-Ets causes a dramatic decrease in steady-state levels of PRL mRNA in GH4 cells, reducing expression by 80% compared to vector-only controls. Conversely, stable expression of VP16 Ets led to an average threefold increase in PRL mRNA. Consistent with our previous observations, the mRNA levels for GH were unaffected by either dn-EtsZ or VP16 Ets expression. A smaller decrease in Pit-1 mRNA levels was also observed, though the functional significance of this decrease is questionable since we observed no alterations in the intracellular levels of Pit-1 protein. Furthermore, the cells expressing the dn-EtsZ construct exhibit significantly lower intracellular PRL protein levels, and greatly diminished secretion of PRL into the cell culture medium, compared to controls. Thus, we have confirmed and expanded our previous observations that implicate Ets factors as critical elements in the basal regulation of the endogenous PRL promoter.

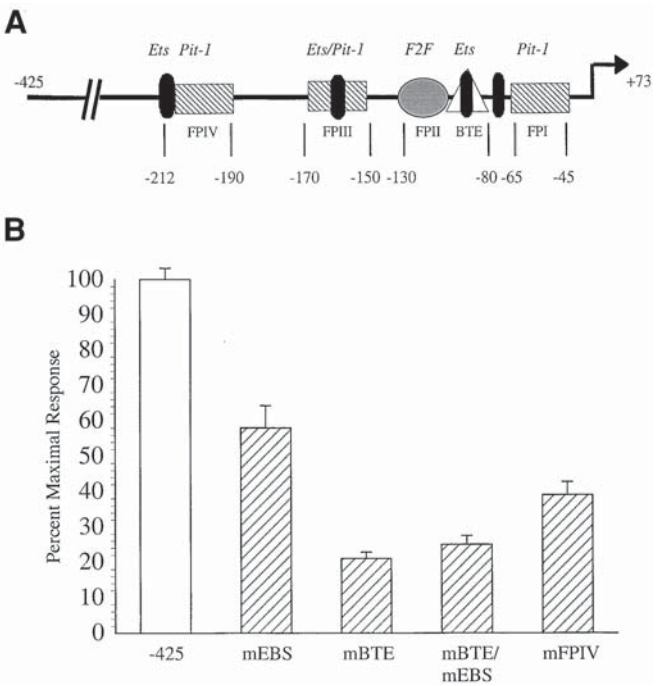

Fig. 1. Site-specific mutation of EBSs within proximal –425 PRL promoter. (**A**) Structural organization of the proximal –425 rPRL promoter. The proximal –425 rPRL promoter is depicted. Solid ovals indicate EBSs; rectangles indicate Pit-1 footprints (FP I, III, and IV), as determined by DNaseI protection; and the triangle and circle indicate the BTE and FPII repressor sites, respectively. Site-specific mutations of FPIV and the EBSs within the RRE (mEBS), BTE (mBTE), and both the BTE and EBS (mBTE/EBS) have been previously described *(7,8)*. (**B**) Effects of site-specific mutations of EBSs within BTE and RRE of rPRL promoter. GH4 cells were transfected with 3 μg of each mutant rPRL promoter construct (mEBS, mBTE, and mBTE/mEBS), where indicated, along with 0.3 μg of pCMV β-gal. Cells were harvested and assayed as described in Materials and Methods. The basal activity of the intact –425 rPRL promoter was set to 100%, and the results are the mean ± SD of three transfections.

Results

Ets Factors Are Critical for Basal PRL Promoter Activity

Our laboratory and others have previously identified consensus EBSs within the proximal –425 rPRL promoter, including the EBS-Ras response element (EBS-RRE), centered at –212 *(16)*, and the BTE, centered at –96 (Fig. 1A) *(7,8,12, 13,17)*. The EBS-RRE is part of a composite EBS and Pit-1 element termed footprint 4 (FP IV) that constitutes the RRE, and the BTE is similarly juxtaposed to the FP II repressor site at –125 and an adjacent EBS at –76 (Fig. 1A).

To determine the functional contribution of the EBS-RRE and BTE EBSs within the rPRL promoter on basal promoter activity, rPRL promoter constructs containing individual and combined site-specific mutations within these sites were tested for basal activity in transient transfection experiments in GH4 rat pituitary cells (Fig. 1B). For these experiments, the basal activity of the intact –425 promoter was set to 100%. Figure 1 demonstrates that mutation of the EBS within the RRE (mEBS) creates a rPRL promoter construct that retains only 60% of basal promoter activity, compared to the intact promoter (Fig. 1B). By contrast, mutation of the EBS within the BTE (mBTE) resulted in a more dramatic reduction, to 20% of wild-type levels (Fig. 1B). The mBTE/mEBS double-mutant rPRL promoter construct showed the same level of reduction as the single mBTE mutant alone (Fig. 1B). For comparison, a rPRL promoter construct containing a site-specific mutation in FPIV (mFPIV) was also tested, and this construct demonstrated 40% basal rPRL promoter activity. Although these mutant EBS promoter constructs exhibit lower basal activities than the intact –425 rPRL promoter, these mutant promoters do retain detectable basal activity that is significantly higher compared to the empty vector, pA3luc (data not shown). Taken together, these data demonstrate an important role for EBSs within the RRE and BTE, and that the EBS within the BTE is the most critical site regulating basal activity of the rPRL promoter.

Construction and Selection of Stable Cell Lines

To examine the functional role of Ets transcription factors in basal regulation of the endogenous rPRL gene, we generated GH4 cells that were stably transfected with plasmid constructs encoding either dominant-negative or dominant-active forms of Ets proteins as well as empty vector controls. The dn-Ets construct consists of the DBD of human Ets-2 fused to the β-galactosidase (β-gal) reporter gene. This construct also truncates portions of the autoinhibitory domains of Ets-2 that attenuate DNA binding, giving it a higher affinity for DNA than the full-length protein and thus interfering with the action of endogenous Ets factors in this subfamily *(18)*. The dn-Ets construct consists of the DBD of chicken Ets-1 fused to the viral VP16 transactivation domain, creating a very potent transactivating protein that is targeted to Ets DNA-binding sites. Since the Ets-1 and Ets-2 DBDs are 96% identical at the amino acid level and select essentially the same DNA-binding sites, the dn-EtsZ and the VP16 Ets very likely modulate the same target genes *(5)*.

The transfected plasmid constructs also contain a neomycin resistance cassette, allowing for positive drug selection of transfected cells. After 3 wk of selection with 500 μg/mL of the neomycin analog G418, individual clones were isolated from each group. In the case of the dominant-negative Ets construct, selection of cells expressing the transfected plasmid to a high degree was facilitated by direct staining of the cells with the β-gal substrate, S-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal), which was scored by the presence of blue coloration of the cells (data not shown). Additionally, liquid assay measurement of β-gal in the cell extracts was performed to assess expression of the dn-EtsZ construct in the individual clonal isolates. The results of the β-gal assay are depicted in Fig. 2. As expected, the three vector-only clones, which do not contain the Lac-Z gene, showed only background levels of β-gal activity whereas the dn-EtsZ clones selected had varying levels of β-gal expression, from 3 to 10 units (Fig. 2). These variations are likely owing to differences in copy number and/or chromosomal integration of the dn-EtsZ DNA among the cell clones selected. The levels of the Lac-Z reporter gene were subsequently used to select clones that were most likely to express the dn-Ets construct.

Expression of dn-Ets Selectively Represses PRL mRNA Levels

To determine the effects of dn-EtsZ expression on PRL gene expression in GH4 rat pituitary cells, we performed Northern blot analysis on total RNA extracted from individual clonal isolates that were stably transfected with vector alone or the dn-EtsZ construct (Fig. 3). The expression levels of the dn-EtsZ construct in the individual clones were detected by first probing the Northern blot with a ^{32}P-labeled cDNA fragment encoding the DBD of Ets-2. As shown in the first panel in Fig. 3, the exogenously expressed Ets-2

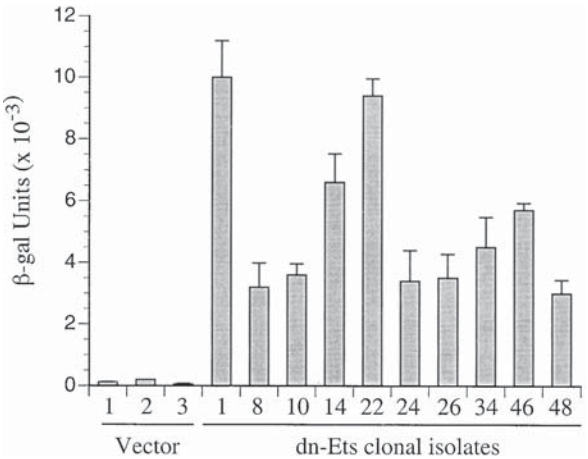

Fig. 2. β-Gal activity in vector and dn-EtsZ stable cell lines. Two vector-only clones and 10 dn-Ets clones were grown to 80% confluency in 60-mm culture dishes. Cells were harvested, lysed, and assayed for β-gal activity as described in Materials and Methods. Data are expressed as mean ± SD of three separate experiments.

DBD was not detected in the two vector-only clones tested (Fig. 3, lanes 1 and 2). By contrast, there were varying levels of Ets-2 DBD expression among the 10 dn-EtsZ clones tested (Fig. 3, lanes 3–12), which generally correlated with the β-gal results shown in Fig. 2. The same blot was stripped and reprobed with full-length rPRL cDNA, shown in the second panel in Fig. 3. A strong signal for PRL is evident in the two vector-only clones, while there was a marked diminution of the steady-state levels of PRL mRNA in all of the dn-EtsZ clones tested to an average 80% reduction, compared to the controls. There was a correlation between the expression levels of the dn-EtsZ construct and the decrease in PRL mRNA, in that cells expressing high levels of Ets-2 DBD had the lowest levels of steady-state PRL mRNA (compare Ets-LacZ with PRL in dn-Ets clones 1, 22, and 46 in Fig. 3). However, many of the other clones showed only modest levels of dn-EtsZ mRNA, yet showed repression of PRL mRNA equal to those that expressed higher levels of dn-EtsZ. This may be owing to the fact that all of these clones expressed at least threefold higher levels of dn-Ets/lacZ protein than vector-only controls (Fig. 2), which may represent a threshold level for PRL gene suppression.

The third panel in Fig. 3 shows the same blot probed with a cDNA encoding rGH. GH and PRL are closely related genes that are believed to be derived from a common ancestral gene via a gene duplication event *(19)*. Furthermore, the GH promoter contains putative binding sites for both Pit-1 and Ets factors; however, these are not juxtaposed into a functional composite element as they are in the rPRL promoter. Consistent with the results our laboratory has previously reported using a transient transfection approach *(7)*, blocking Ets factor function via a dn-Ets construct had no significant effect on rGH gene expression levels in any of

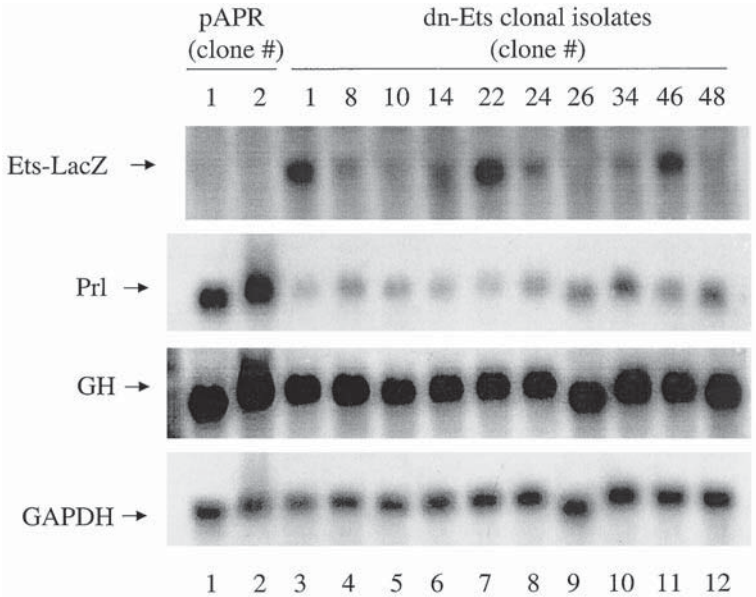

Fig. 3. Northern blot analysis of vector and dn-EtsZ stable cell lines. Total RNA (10 μg) from two vector-only GH4 clones (Vector) and 10 dn-Ets clones was extracted and electrophoresed on a 1.4% agarose-formaldehyde gel and transferred to a nylon-reinforced nitrocellulose membrane. The membrane was sequentially probed with radiolabeled cDNAs encoding the DBD of Ets-2 (Ets-LacZ, first panel), rat PRL (Prl, second panel), rat GH (GH, third panel) or glyceraldehyde phosphate dehydrogenase (GAPDH) (fourth panel), to control for equal loading of RNA. Representative autoradiographs are presented.

the clones tested (Fig. 3). Finally, the fourth panel in Fig. 3 shows the same blot probed for GAPDH and indicates that dn-Ets expression has no effect on this gene and that relatively equal amounts of total RNA were loaded in each lane.

The bar graph in Fig. 4 depicts a scanning densitometric quantification of the PRL and dn-EtsZ mRNA bands from the Northern blot shown in Fig. 3, normalized to GAPDH levels. This graph highlights the diminished PRL mRNA levels and the corresponding expression levels of dn-EtsZ and indicates that the clones with the lowest levels of PRL mRNA typically show the highest levels of dn-Ets. In subsequent experiments, we focused on a few selected clones that showed the highest levels of dn-Ets expression, namely clones 1, 22, and 46.

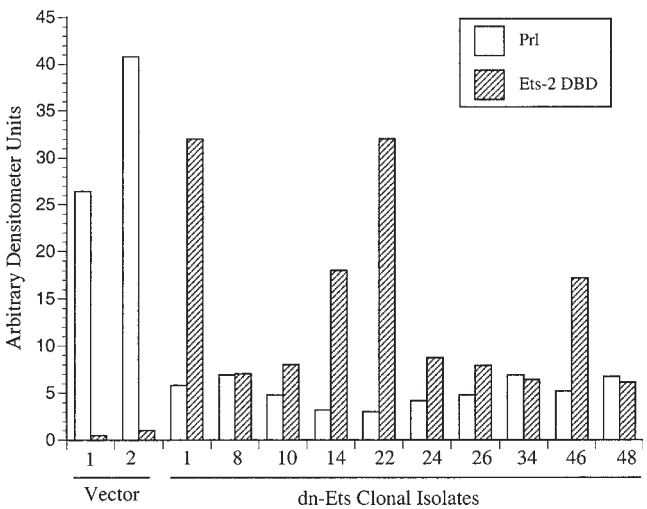

Fig. 4. Quantitation of Northern analysis of stable cell lines. The autoradiographs for PRL and Ets-LacZ depicted in the Northern blot in Fig. 2 were quantified by scanning laser densitometry (Molecular Dynamics). Data represent arbitrary densitometer units for each band, divided by the density of GAPDH in each lane, to correct for differences in loading of RNA.

Stable Expression of Dominant-Active Ets (VP16 Ets) Stimulates PRL Gene Transcription

Northern blot analysis was also performed to determine the effects of the dominant-active Ets construct (VP16 Ets) on endogenous PRL gene expression. As shown in Fig. 5, the VP16 Ets construct led to an increase in PRL mRNA levels that are approximately threefold greater than the vector-only controls (cf. lanes 1 and 5, Fig. 5). Again, three selected dn-Ets clones (lanes 2–4, Fig. 5) showed decreased basal PRL mRNA levels compared to vector-only controls. A probe for GAPDH shows relatively equal loading of total RNA in all the lanes (Fig. 5, bottom panel). This blot was also probed for GH, and just as the levels of this message were unaffected by dn-Ets expression, we also observed no effects on this gene in the dominant-active stable cell lines (data not shown). Thus, we have demonstrated by two separate means (i.e., blocking Ets function with a dominant-negative construct and utilizing a potent activating form of an Ets protein) that Ets factors are critical regulators of endogenous basal PRL expression in GH4 pituitary cells.

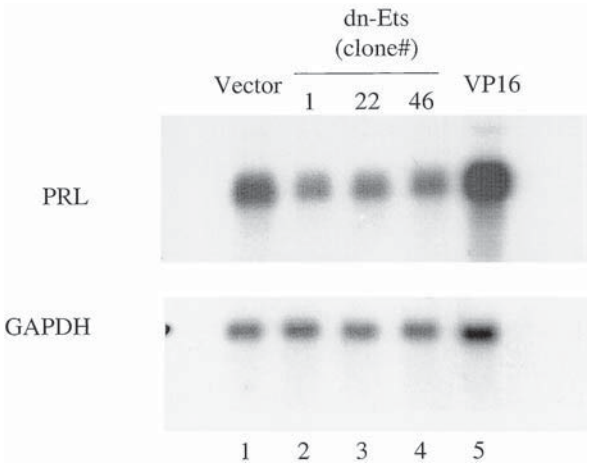

Fig. 5. Northern blot analysis of dn-EtsZ and VP16 Ets stable cell lines. Total RNA (10 μg) from vector-only (Vector), three dn-EtsZ, and one dominant-active (VP16) clone were extracted and electrophoresed on a 1.4% agarose-formaldehyde gel and transferred to a nylon-reinforced nitrocellulose membrane. The membrane was sequentially probed with radiolabeled cDNAs encoding rPRL prolactin (PRL, top panel), or GAPDH (bottom panel), to control for equal loading of RNA. Representative autoradiographs are presented.

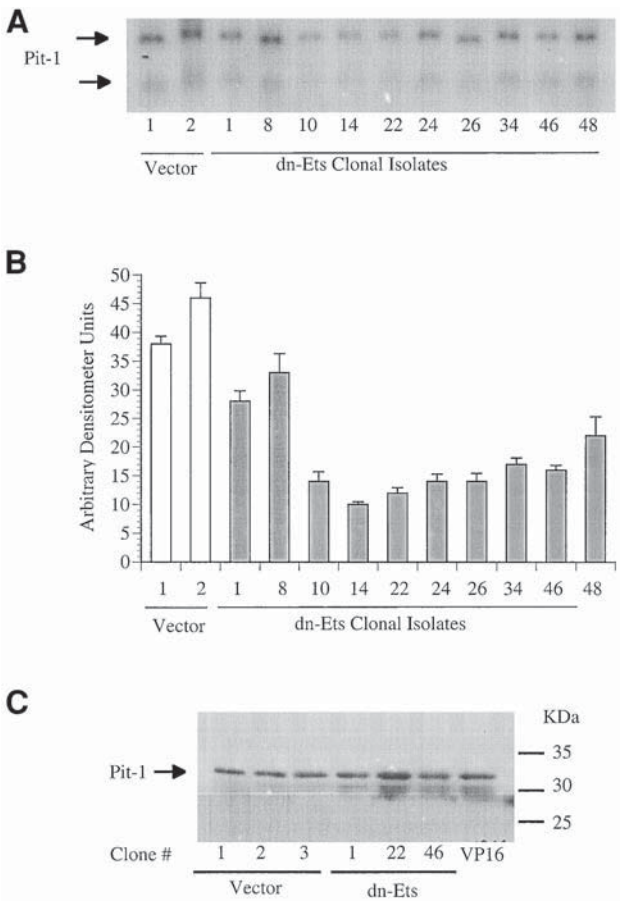

Fig. 6. Effect of dn-EtsZ expression on endogenous Pit-1 RNA and protein (**A**) Northern blot of stable cell lines. Ten micrograms of total RNA from vector-only and dn-Ets clones were extracted and electrophoresed on a 1.4% agarose-formaldehyde gel and transferred to a nylon-reinforced nitrocellulose membrane. The membrane was probed with radiolabeled cDNAs encoding Pit-1. (**B**) Densitometric quantification of Pit-1 Northern blots. The Pit-1 signal was quantified by scanning laser densitometry (Molecular Dynamics). Data represent arbitrary densitometer units from two separate experiments for each band, divided by the density of GAPDH in each lane (data not shown), to correct for differences in loading of RNA. (**C**) Western blot of intracellular Pit-1 protein. Vector-only (Vector), dn-EtsZ, or dominant-active Ets (VP16) stable clones were cultured in DMEM with 10% fetal calf serum (FCS) and 100 μg/mL of G418 and grown to 80% confluency in 60-mm plates. Cells were lysed and equal amounts of whole cell extract (approx 25 μg/lane) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting to polyvinyl difluoride (PVDF) membrane. The blot was probed with an antibody specific for Pit-1.

dn-Ets Expression Reduces Pit-1 mRNA Levels but Not Pit-1 Protein

Sequence analysis of the proximal 200 bp of the rat Pit-1 promoter reveals putative EBSs; however, to our knowledge, these sites have not been characterized or shown to be functional Ets sites. To determine whether the effects of dn-EtsZ on PRL gene expression are owing to direct actions at the PRL promoter, or whether they are indirect effects through the inhibition of Pit-1, the Northern blot shown in Fig. 3 was stripped and reprobed with a labeled cDNA for Pit-1. As shown in Fig. 6A, and in the densitometric quantification of two separate experiments, shown in Fig. 6B, we observed a modest and variable decrease in Pit-1 mRNA in most of the dn-Ets clones tested, compared to vector-only controls. However, Western blot analysis of intracellular Pit-1 protein levels in selected dn-Ets clones expressing low to near-control Pit-1 mRNA levels indicates that this modest decrease in mRNA does not result in diminished Pit-1 protein content (Fig. 6C). These results indicate that the effects of dn-EtsZ expression on PRL transcription are likely to be direct effects at the level of the PRL promoter and not mediated by Pit-1.

dn-EtsZ Expression Selectively Inhibits Transfected PRL Promoter Constructs

As a further test of the specificity of dn-EtsZ action on pituitary promoters, we transiently transfected vector-only and selected dn-EtsZ stable clones with either the –425 proximal PRL promoter; the larger, –2.5-kb rat PRL promoter, containing the distal enhancer, or the GH promoter, fused to the luciferase reporter gene. As shown in Fig. 7, basal expression from the two PRL promoters was inhibited approx 40–50% in the dn-EtsZ clones compared to vector-only controls. However, no significant decrease in effects was observed on the GH promoter. These results confirm our previous observations and those seen in the Northern blot shown in Fig. 3, indicating that the inhibitory actions

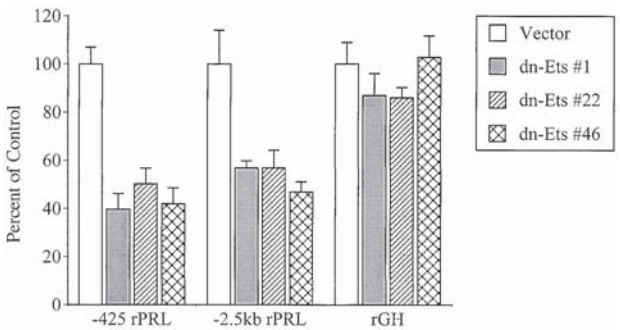

Fig. 7. Effect of stable expression of dn-EtsZ on transiently transfected pituitary hormone promoters. Vector-only and selected dn-Ets stable cell lines were cotransfected with either 3 μg of pA3rPRLluc (–425 rPRL), or 3 μg of pA3(-2.5)rPRLluc (-2.5-kb rPRL), 3 μg of pA3rGHluc (rGH), as indicated, and 0.3 μg of pCMV β-gal by electroporation. Promoter activity was calculated as described previously *(24)* and expressed as percentage of activity of vector-only control. Results are the mean of ± SD of three transfections.

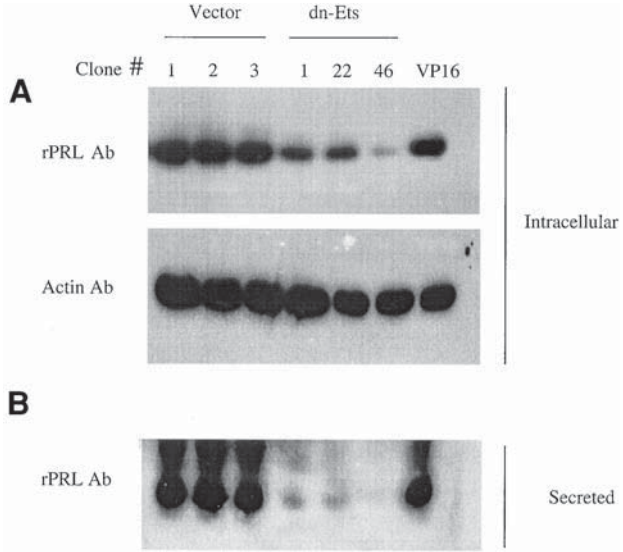

Fig. 8. Western blot analysis of intracellular and secreted PRL protein in stable cell lines. (**A**) Intracellular PRL protein. Vector-only (Vector), dn-EtsZ, or dominant-active Ets (VP16) stable clones were cultured in DMEM with 10% FCS and 100 μg/mL of G418 and grown to 80% confluency in 60-mm plates. Cells were lysed, and equal amounts of whole cell extract (approx 25 μg/lane) were resolved by SDS-PAGE followed by Western blotting to PVDF membrane. The blot was probed with an antibody specific for rPRL (top panel). The blot was subsequently stripped and reprobed with an antibody to actin (bottom panel) to correct for loading differences. (**B**) Secreted PRL protein. Vector-only (Vector), dn-Ets, or dominant-active Ets (VP16) stable clones were cultured in serum-free DMEM with 100 μg/mL of G418 for 16 h. One-milliliter aliquots of medium were harvested and equal amounts (approx 100 μg/lane) were fractionated on a 10% SDS-PAGE gel, subjected to Western blotting, and probed with an antibody to rPRL as described in CA).

of dn-Ets are specific to the PRL promoter and have no effects on the related GH promoter.

PRL Protein Levels Are Reduced in GH4 Cells Expressing dn-Ets

To determine whether the reduced basal PRL mRNA levels in the dn-EtsZ stable cell lines correlated with reduced intracellular and secreted PRL protein, we performed Western blot analysis on total cell extracts and medium from vector-only, selected dn-EtsZ, and VP16 Ets clones. Fig. 8A, top panel, shows the results of a representative Western blot for intracellular PRL protein. Approximately 10 μg of total cell extract was loaded in each lane of a 12% SDS-PAGE gel and probed with an rPRL antibody. After correcting for loading with an actin control antibody (Fig. 8A, bottom panel), we observed a variable 40–80% reduction in intracellular PRL protein levels between the three dn-EtsZ clones tested, compared to vector-only controls. Of note, we did not observe any significant increase in PRL protein content in the cells stably expressing the dominant-active Ets (VP16 Ets; Fig. 8A), despite the increased levels of PRL mRNA seen in these cells (Fig. 4).

Figure 8B shows a Western blot analysis of PRL protein secretion into the cell culture medium. For this study, GH4 cells were cultured in serum-free conditions for 24 h in order to reduce potential crossreactivity of the PRL antibody with proteins present in FCS. After 24 h, medium was collected from plates containing vector-only, dn-EtsZ, and VP16 Ets clones and assayed for PRL protein by Western blot. As shown in Fig. 8B, expression of the dn-EtsZ construct led to a dramatic decrease in the amount of PRL secreted into the cell culture medium in all three dn-EtsZ stable clones tested compared to vector-only controls. Similar to the observations seen in Fig. 8A, there was no significant increase in the amount of secreted PRL in the VP16 Ets clone.

Discussion

While a number of previous reports have demonstrated the importance of Ets transcription factors in the regulation of basal and growth factor–induced activity of transfected rPRL promoter constructs, the precise role of Ets factors in controling endogenous rPRL gene activity remained unknown. The work presented here has shown that two separate EBSs in the proximal rPRL promoter, and Ets factors of the Ets-1/2 subfamily, are critical for basal promoter activity. More important, we have shown that the stable expression of a dn-Ets construct in GH4 rat pituitary cells significantly diminished the expression of endogenous PRL mRNA and intracellular protein and reduced the secretion of PRL peptide hormone.

The role of Ets factors in regulating exogenous basal rPRL promoter activity has been previously shown using several experimental approaches, including transient transfection of dominant-negative and repressor Ets constructs in GH4 and GH3 pituitary cells, and reconstitution assays of rPRL promoter activity in nonpituitary HeLa and COS-1 cells *(6,*

10). The data presented in Fig. 1 are an extension of these types of transient transfection studies and show that the EBSs within the RRE and the BTE, at positions –212 and –96, respectively, each contribute to the basal activity of the rPRL promoter. The more proximal EBS contained within the BTE is the more important element regarding to basal rPRL promoter activity, contributing 80% of total rPRL promoter activity (Fig. 1). Moreover, the mBTE site appears to be the dominant site, since mutation of both the BTE and the RRE-EBS shows a loss of promoter activity that is equivalent to the single mBTE mutant promoter (Fig. 1). The data presented here, using a site-specific mutation to address the role of the BTE in regulating basal rPRL promoter activity, are consistent with previously published data whereby the BTE region was either internally deleted or mutated by linker-scanner methods *(12,13,20)*. While these and other previously published studies strongly implicate Ets factors as key regulators of basal rPRL promoter activity, the biologic significance of these types of transient transfection approaches using rPRL promoter–luciferase reporter constructs is subject to question, particularly if the type of more physiologically relevant studies such as those presented here are not included.

The data from our study, showing that the stable expression of dn-EtsZ and constitutively active VP16 Ets constructs result in repression and activation of the endogenous rPRL gene, respectively, confirm these previous studies in more physiologically relevant conditions. Specifically, in our experiments, we did not simply study 425 bp of the proximal rPRL promoter in plasmid DNAs that may not be appropriately chromatinized, but, rather, we analyzed the effects of dn-EtsZ and VP16 Ets on the entire endogenous rPRL gene in the context of its native chromatin organization. In this regard, it is noteworthy that all 10 of the dn-EtsZ clones that were isolated displayed significant reduction in PRL mRNA expression (Fig. 3). These data suggest that dn-EtsZ is capable of accessing the chromatinized PRL gene and reveal that the inhibitory effects of dn-EtsZ are very consistent and reproducible. Moreover, the data show that the proximal EBSs on the rPRL promoter are key contributors to basal rPRL promoter activity, since the strong effects of the distal enhancer *(21)* and estrogen response element *(11, 22)* located in the −1713 to −1532 region cannot override the repressor effects of dn-EtsZ.

While the dn-EtsZ clones were initially selected on the basis of β-gal production, the degree of PRL gene repression was not strictly correlated with β-gal activity (Fig. 4). The level of β-gal activity should directly correlate with the amount of dn-Ets expressed, since this enzymatic function is fused to the Ets-2 DBD *(18)*. These results suggest that even the low levels of β-gal activity produced (and therefore dn-EtsZ expressed) represent near-maximal levels of dn-EtsZ protein with respect to its ability to repress the rPRL gene. By contrast, there was, in general, a positive correlation between the amount of dn-EtsZ mRNA expressed and the amount of β-gal activity generated (Figs. 2 and 3). For this reason, we chose clones 1, 22, and 46 for further study, since they displayed maximal levels of dn-EtsZ mRNA and β-gal activity, associated with the lowest levels of PRL mRNA.

Although these levels of dn-EtsZ achieved maximal inhibition of rPRL gene expression, they nevertheless displayed significant gene selectivity. Thus, none of the stable dn-EtsZ-expressing clones revealed any alteration of GH or GAPDH gene expression (Fig. 3). The rGH promoter does contain several putative EBSs; however, they have not been functional when previously assayed in oncogenic Ras response assays and are only minimally responsive in HeLa reconstitution assays *(6,7)*. Additionally, transient transfection of each of three dn-EtsZ stable cell lines with two rPRL promoter constructs (−425 and –2.5 kb) and an rGH promoter (−560) verified that expression of dn-EtsZ also selectively represses an exogenous rPRL promoter (Fig. 7).

The strong inhibitory effect of dn-EtsZ on endogenous and exogenous rPRL promoters, while having no effect on endogenous or exogenous rGH promoters, validates the selectivity of the Ets response on these two Pit-1-dependent and ancestrally related pituitary-specific genes. In this regard, we were somewhat surprised to find that the expression of the Pit-1 gene, which is also Pit-1 dependent and pituitary specific, displayed variable levels of transcription inhibition in the various dn-EtsZ clones (Fig. 6A). This variability in Pit-1 mRNA is not simply owing to loading differences, since normalization of Pit-1 mRNA to GAPDH mRNA levels also revealed moderate alterations in Pit-1 mRNA in the dn-EtsZ clones (Fig. 6B). The proximal rat Pit-1 promoter contains a putative Ets-1-binding site (CCAGG AGGGT) *(23)*. However, the functional role of this site in transient transfection assays has not been reported. Despite the variable inhibitory effects of dn-EtsZ on Pit-1 mRNA expression, dn-EtsZ did not inhibit Pit-1 protein production (Fig. 6C). Similarly, VP16 Ets did not increase Pit-1 protein production (data not shown). Thus, the effects of these two Ets constructs on PRL gene expression cannot be explained by alterations in Pit-1 protein levels.

To validate further the biologic significance of the inhibitory effects of dn-EtsZ on rPRL gene expression, we also showed that the dn-EtsZ stable cell lines produced lower levels of PRL peptide hormone and secreted negligible amounts of PRL hormone (Fig. 8). These data substantiate the notion that dn-EtsZ repression of rPRL gene transcription ultimately results in lowered levels of steady-state PRL mRNA and intracellular peptide hormone. By contrast, the VP16 Ets stable cell line increased levels (approximately threefold) of PRL mRNA, yet produced wild-type levels of intracellular and secreted PRL peptide hormone. Although this result would suggest a dissociation between PRL mRNA and protein production in this cell line, another possibility is that VP16 Ets cells have reached the maximal level of intracellular and secreted PRL allowed.

We have shown that an Ets factor of the Ets-1/2 subfamily is required for optimal basal rPRL promoter activity. However, since the dominant-negative approach we used interferes with several Ets factors, particularly those in the Ets-1/2 subfamily, the precise identity of the specific Ets factor (or factors) that mediates this effect remains unknown. GH4 cells express multiple Ets proteins (including Ets-1, Ets-2, ER81, GABP, and Ehf), and Ets-1 appears to preferentially bind to the EBS-RRE, whereas GABP appears to preferentially bind to the BTE (unpublished data). In transient transfection HeLa reconstitution studies, several Ets factors are able to activate the rPRL promoter, with Ets-1, Ets-2, and ER81 resulting in the strongest effect (unpublished data). However, transfection of Ets factors alone is insufficient to yield optimal rPRL promoter activity, and cotransfection of Pit-1 is required to achieve levels that are equivalent to that seen in GH4 pituitary cells *(6)*. Indeed, Ets-1 and Pit-1 function synergistically via a composite EBS juxtaposed to a Pit-1-binding site at position –212 *(6)*. Recent data indicate that GABP may be functioning in a similar manner via the BTE, centered at position –96 (unpublished data). Of note, the Ets-2 DBD in the dn-EtsZ is capable of interfering with Ets-1 and GABP action, since the binding sites for these two Ets factors are very similar *(5)*. Thus, despite the complex expression of various Ets factors in GH4 cells, our study shows that interference with the Ets-1/2 subfamily is sufficient to significantly abrogate endogenous basal rPRL promoter activity, and that other Ets factors are unable to compensate for this function.

Materials and Methods

Plasmid Constructs

The reporter constructs pA3-425 rPRL luc, pA3-2.5 rPRL luc, rGH luc, and pCMV βgal have been described previously *(24,25)*. The dn-Ets expression plasmid, pAPrEts-Z/neo, encoding the DBD of Ets-2 fused to lac Z, which also contains a nuclear localization signal *(18)*, was obtained from Michael Ostrowski (Ohio State University, Columbus, OH). The VP16 Ets construct was generously supplied by Dr. Bohdan Wasylyk (INSERM, Illkirch, France). Plasmid DNAs were purified and quantified as described previously *(25)*.

Cell Culture and Transient Transfections

GH4T2 rat pituitary tumor cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% FCS (Hyclone, Logan, UT). Cells were maintained at 37ºC in 5% CO_2. Medium was changed 4–12 h prior to transfection, and cells were harvested at 50–70% confluency. For transfection, cells were harvested in 0.05% trypsin, 0.5 m*M* EDTA and resuspended in DMEM supplemented with 10% FCS. Aliquots of approx 4×10^6 cells in 200 µL of medium were added to plasmid DNA and transfected by electroporation at 220 V and 500 µF using a Bio-Rad gene pulser with 0.4 mm cuvets. All transfections included pCMVβ-gal as an internal control for transfection efficiency. Following transfection, cells were plated in DMEM with 10% FCS and incubated for 24 h. Electroporation was performed in triplicate for each condition within a single experiment and experiments were repeated twice.

Stable Cell Line Construction

GH4T2 rat pituitary somatolactotroph cells were transfected by electroporation with empty vector or dn-Ets or VP16 Ets plasmids. All plasmids contained the neomycin resistance cassette. The cells were first plated in 10-cm tissue culture plates containing DMEM (Gibco) for 16 h to allow for production of the neomycin resistance gene product. Selection for stable transfectants was carried out by the addition of 500 µg/mL of the neomycin analog, G418 (Geneticin; Gibco), for a period of 3 wk. Individual clones were picked from each group and transferred to 24-well tissue culture plates. Further selection of dn-Ets clonal isolates was performed by histochemical detection of β-gal activity *in situ* using the substrate X-gal. Briefly, medium was removed from cells, followed by two rinses with 1X phosphate-buffered saline (PBS). The cells were fixed in a 2% paraformaldehyde/0.2% glutaraldehyde solution in PBS at 4ºC for 5 min. The fixative was aspirated, and fixed cells were rinsed gently two times at room temperature with 1X PBS. The fixed cells were then overlaid with 1 mL of X-gal staining solution (100 m*M* sodium phosphate, pH 7.3; 1.3 m*M* $MgCl_2$; 3 m*M* K_3 FE $[CN]_6$; and 1 mg/mL of X-gal). X-gal is hydrolyzed by β-gal to generate galactose and soluble indoxyl molecules, which, in turn, are converted to insoluble indigo. Clonal isolates were chosen based on intensity of indigo color and expanded from duplicate, untreated plates.

Luciferase and β-Gal Assays

Transfected cells were harvested in PBS containing 3 m*M* EDTA, and extracts were prepared by three sequential freeze-thaw cycles in 100 m*M* potassium phosphate pH 7.8, 1 m*M* dithiothreitol. Cell lysis was increased by vortexing between cycles. Cell debris was pelleted by centrifuging at 10,000*g* for 10 min at 4ºC, and aliquots of supernatant were used in subsequent assays. Luciferase was assayed as previously described *(24)*. Samples were measured in duplicate using a Monolight 2010 luminometer (Analytical Luminescence, San Diego, CA). β-Gal activity was determined spectrophotometrically using the chromogenic substrate *o*-nitro phenyl-β-D-galactopyranoside as described previously *(24)*. Total light units were normalized to total β-gal activity.

Northern Blot Analysis

Total cellular RNA was prepared from the indicated clones with the RNA Stat 60 Kit (Tel Test). Samples (10 µg of total RNA) were electrophoresed on a 1.4% agarose-formaldehyde gel and transferred to a nylon-reinforced nitrocellulose membrane (Duralon-UV; Stratagene). Blots were

prehybridized for 2 h at 42ºC in Ultrahyb buffer (Ambion) and then incubated overnight with an [α-^{32}P] dCTP-labeled hybridization probe prepared from PRL, GH, Pit-1, or GAPDH cDNA templates by random primer synthesis (Prime-it II; Stratagene). After hybridization, the membrane was washed three times for 30 min each in high-stringency wash buffer (0.1X saline sodium citrate, 0.1% SDS) at 65ºC and exposed to autoradiographic film.

Western Blot Analysis

Cell extracts for Western blotting were prepared from 80% confluent 60-mm dishes. Cells were harvested with Laemmli-SDS sample buffer and cell scraping. Cell extracts were boiled for 5 min, and DNA was sheared by passage through a 22-gage needle. Samples were resolved on 12% SDS polyacrylamide gels and transferred to Immobilon P as described previously *(8)*. Western blots were blocked in 5% nonfat milk, 0.2% Tween-20; probed with antibodies to PRL (generously provided by Dr. A. F. Parlow, National Hormone and Pituitary Program) and actin (Boehringer Mannheim); and developed using ECL (Amersham) according to the manufacturer's protocols. The Pit-1 Western blot was performed in a similar fashion using the Pit-1 (214–230) polyclonal antibody (BAbCO).

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