A Composite Ets/Pit-1 Binding Site in the Prolactin Gene Can Mediate Transcriptional Responses to Multiple Signal Transduction Pathways*

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Binding sites for the tissue-specific transcription factor, Pit-1, are required for basal and hormonally induced prolactin gene transcription. Although Pit-1 is phosphorylated in response to several signaling pathways, the mechanism by which Pit-1 contributes to hormonal induction of gene transcription has not been defined. Recent reports suggest that phosphorylation of Pit-1 may not be required for hormonal regulation of the prolactin promoter. Analysis of the contribution of individual Pit-1 binding sites has been complicated due to the fact that some of the elements appear to be redundant. To better understand the role of Pit-1 sites in mediating hormonal regulation of the prolactin gene, we have performed enhancer tests using the three most proximal Pit-1 binding sites of the rat prolactin gene which are designated the 1P, 2P, and 3P sites. The results demonstrate that multimers of the 3P Pit-1 binding site are much more responsive to several hormonal and intracellular signaling pathways than multimers of the 1P or 2P sites. The 3P DNA element was found to contain a consensus binding site for the Ets family of proteins. Mutation of the Ets binding site greatly decreased the ability of epidermal growth factor, phorbol esters, Ras, or the Raf kinase to induce reporter gene activity. Mutation of the Ets site had little effect on basal enhancer activity. In contrast, mutation of the consensus Pit-1 binding site in the 3P element essentially abolished all basal enhancer activity. Overexpression of Ets-1 in GH3 pituitary cells enhanced both basal and Ras induced activity from the 3P enhancer. These data describe a composite element in the prolactin gene containing binding sites for two different factors and the studies suggest a mechanism by which Ets proteins and Pit-1 functionally cooperate to permit transcriptional regulation by different signaling pathways.

The transcription of the prolactin gene is modulated by a number of hormones which bind to plasma membrane receptors. Hormones which regulate the transcription of the prolactin gene include dopamine (1), epidermal growth factor (2, 3), and thyrotropin releasing hormone (4, 5). The transcriptional effects of these hormones likely involve the activation of protein kinases leading to the phosphorylation of specific transcription factors. It has been demonstrated that activation of the cAMP-dependent protein kinase (6), the Ca2+/calmodulin-dependent protein kinase type II (7), or the MAPK cascade (8) is sufficient to stimulate transcription of the prolactin gene. Despite rather extensive studies of the prolactin promoter, the mechanisms which permit transcriptional responses to different hormones and signal transduction pathways have not been clearly defined. It is clear that the prolactin promoter contains multiple binding sites for the tissue-specific transcription factor Pit-1 (9, 10), and there is evidence that Pit-1 binding sites may contribute to both basal and hormonally regulated transcription (11–16). The observation that treatment of GH3 cells with cAMP or phorbol esters stimulates phosphorylation of Pit-1 (17) is consistent with a role for Pit-1 in mediating hormonal regulation of transcription. However, recent studies have shown that phosphorylation of Pit-1 may not be necessary for hormonal induction of prolactin gene transcription (18–20). This led to the suggestion that other factors which interact with Pit-1 binding sites may mediate transcriptional regulation of the prolactin gene or that co-activators may interact with Pit-1 in a regulated fashion (21). There is evidence that cooperative interactions between Pit-1 and other transcription factors may be crucial for some transcriptional responses (12, 15, 22, 23). It has also been shown that factors other than Pit-1 can interact with Pit-1 binding sites. For instance, Oct-1 (24), Zn-15 (25), and TEF (26) can all interact with Pit-1 binding sites and activate transcription through these sites. However, it is not clear if any of these other factors play a role in mediating hormonally regulated transcriptional activation.

In the present study we have examined the ability of individual Pit-1 binding sites to permit responses to hormones and activators of specific signaling pathways. Previous studies have suggested that the 5'-flanking region of the rat prolactin gene contains multiple, redundant DNA elements which mediate responses to TRH (11). This functional redundancy has complicated analysis of the role of specific DNA elements in mediating hormonal responsiveness. Because of this problem, we have used an enhancer test to compare the ability of the three most proximal Pit-1 binding sites of the prolactin gene to respond to hormones, intracellular second messengers and activated components of signal transduction pathways. We find that one site, the 3P site, is particularly responsive to several signal transduction pathways including activation of the MAPK cascade. We have further examined the DNA sequences of the 3P DNA element which are important for transcriptional regulation. These studies suggest that the 3P element contains a binding site for a member of the Ets family

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1The abbreviations used are: MAPK, mitogen activated protein kinase; TRH, thyrotropin releasing hormone; PMA, phorbol myristate acetate; EGF, epidermal growth factor.
of transcription factors in addition to a Pit-1 binding site. The Ets site in the 3P DNA element is crucial for transcriptional response to hormones or second messengers.

**MATERIALS AND METHODS**

Transfection of GH3 and Rat-1 Cells—GH3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 15% equine serum and 2.5% fetal bovine serum. Rat-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. Reporter genes containing 0.6 kilobase pairs of 5’-flanking region of the rat prolactin gene fused to the firefly luciferase coding sequence (11) and five copies of a GAL4 binding site upstream of the E1b TATA box linked to luciferase (27) have been described previously. To prepare a luciferase reporter gene (28) containing multiple copies of the prolactin gene proximal Pit-1 binding sites, the rat prolactin 5’-flanking region was truncated at position -33 and a non-palindromic Avai site was placed at the upstream termini. Multiple, tandem copies of Pit-1 binding sites with Avai cohesive termini were ligated upstream of the minimal prolactin promoter and luciferase gene. GH3 and Rat-1 cells were transfected by electroporation with 10–15 μg of luciferase reporter genes using conditions described previously (29, 30). In some experiments the cells were transfected with expression vectors encoding glo-

**RESULTS**

Enhancer Activity of Multimers of Pit-1 Binding Sites—In an effort to provide a relatively simple test system for analysis of possible mechanisms mediating hormonal responsiveness of the prolactin promoter, we tested the ability of individual Pit-1 binding sites to function as hormone-responsive enhancers. This approach is based on the observation that there appears to be functional redundancy in the DNA elements which permit hormonal regulation of the prolactin promoter (11, 35, 36) and the finding that multimers of individual Pit-1 binding sites can support transcriptional responses to some hormones or second messengers (14, 18). The proximal region of the prolactin gene is sufficient to mediate transcriptional responses to several different hormones (2, 35, 37, 38), and this region contains four Pit-1 binding sites which are designated P1 through P4 (9, 10). We have focused our attention on the three most proximal Pit-1 binding sites (P1, P2, and P3) as we previously found that mutation of the P4 Pit-1 binding site had little effect on hormonal responsiveness (11). We prepared multimerized, direct repeats of the P1, P2, and P3 Pit-1 binding sites which were placed upstream of the minimal TATA box of the prolactin gene and the luciferase coding sequence. The reporter genes containing the multimers were then tested for hormonal regulation by transfection of the GH3, rat pituitary cell line (Fig. 1). The response of the multimers was compared to a reporter gene containing 600 base pairs of the proximal region and promoter of the rat prolactin gene (0.6PRL). A reporter gene containing the thymidine kinase (TK) promoter was included as a control for nonspecific effects of agonist treatments. The minimal prolactin promoter (TATA box alone) was essentially inactive. All three Pit-1 binding site multimers enhanced basal expression of the reporter gene. The 1P site is the highest affinity Pit-1 binding site in the proximal prolactin promoter (9), and the 1P multimers were the strongest basal enhancer. Multimers of the 1P site also supported a modest response to cAMP as reported previously (18). Although it has been reported that multimers of the 1P site are able to permit a response to TRH (14), we could not confirm this observation. This may reflect differences in culture conditions as the previous studies used serum-free medium while we used serum-containing medium. Multimers of the 2P site were found to be a rather weak basal enhancer and did not appear to permit responses to hormones or second messenger treatments. Although Pit-1 binding to the 2P site has been demonstrated (10), there is also evidence that this DNA element can interact with a ubiquitous factor (39) and that it plays a role in repressing the activity of the prolactin promoter in non-pituitary cell types (40). Multimers of the 3P site demonstrated substantial basal enhancer activity and also permitted responses to cAMP, TRH, and PMA. Indeed, a reporter gene containing seven copies of the 3P site was substantially more responsive to phorbol esters than the native prolactin promoter. These findings demonstrate that multimers of the 3P element are sufficient to confer responses to at least two different signal transduction pathways and suggest that this element may contribute to the ability of the prolactin promoter to respond to several different hormones. We were particularly interested in the finding that the 3P multimer was more responsive to TRH than the 1P multimer. TRH has been reported to activate the MAPK pathway in GH3 cells (41), and the prolactin promoter has been shown to be responsive to activation of MAPK cascade (8). Therefore, we tested the ability of the multimerized DNA elements to mediate responses to activation of the MAPK pathway (Fig. 1B). For these studies we used expression vectors encoding activated forms of ras (33) or the Raf kinase (31) which would be expected to stimulate the activity of the MAPK cascade. As previously reported (8), the pro-

![Fig. 1. Enhancer activity in rat GH3 pituitary tumor cells of Pit-1 binding site multimers.](image-url)
lactin promoter is quite responsive to expression vectors for activated Ras or Raf. While neither the 1P nor 2P multimers permitted a substantial response to the Ras or Raf expression vectors, the 3P multimers conferred a vigorous response. The results shown in Fig. 1 were obtained using multimers containing seven copies of the 1P, 2P, and 3P elements. Similar results have been obtained using multimers containing four copies of the DNA elements (data not shown). These enhancer tests suggest that the 3P element is particularly capable of responding to multiple signal transduction pathways including the MAPK cascade.

Presumably, the ability of the 3P multimer to facilitate transcriptional responses is dependent on binding of Pit-1 to these elements. On the other hand, although Pit-1 is known to bind to both the 1P and 3P elements, the 3P element multimer was much more responsive to activation of the MAPK pathway. Pit-1 expression is restricted to somatotrophs, lactotrophs, and a subset of thyrotroph cells of the anterior pituitary (42–44). To directly assess the role in Pit-1 in mediating transcriptional responses, we transfected the same reporter genes in the presence or absence of a Pit-1 expression vector into Rat-1 cells which do not contain endogenous Pit-1 (Fig. 2). In the absence of the Pit-1 expression vector, the proximal prolactin promoter, the 1P multimer construct and the 2P multimer construct were all essentially inactive in Rat-1 cells. The 3P multimer did support a very low level of basal expression. Transfection of the Pit-1 expression vector substantially activated the proximal prolactin reporter gene (23-fold) as well as the constructs containing the 1P (49-fold) or the 3P multimers (43-fold). The 2P multimer did not respond to the Pit-1 expression vector in Rat-1 cells. Transfection of the Pit-1 expression vector enabled the 1P multimer to support a modest response to cAMP as has been reported previously (18). Although both the proximal prolactin promoter and the 3P multimer supported responses to phorbol esters in GH3 cells, these reporter genes did not respond to phorbol esters in Rat-1 cells even in the presence of the Pit-1 expression vector. This difference may indicate that tissue-specific factors other than Pit-1 are required for these regulatory responses. Alternatively, there may be differences in the signal transduction machinery of GH3 and Rat-1 cells.

Analysis of Pit-1 Binding to the 1P and 3P Elements—Multimers of either the 1P or 3P Pit-1 binding site can provide basal enhancer activity. However, only the 3P multimer can support substantial transcriptional responses to TRH, phorbol esters, or activation of the MAPK cascade. It is certainly likely that this difference in transcriptional regulation mediated by the 1P and 3P multimers would involve differences in protein binding to these specific elements. This could reflect quantitative or qualitative differences in the binding of Pit-1 to these elements. Alternatively, the differential regulation could involve the binding of factors other than Pit-1 to the DNA elements. The possibility of qualitative differences in Pit-1 binding to these two sites is suggested by the organization of the two DNA elements (Fig. 3A). Comparison of the 1P and 3P DNA elements to the consensus Pit-1 binding site, TATTCCAT (9, 45, 46) demonstrates that the 1P site contains two Pit-1 binding sites.
monomer sites arranged in an imperfect palindrome. The 3P site can be considered to contain a direct repeat of the monomer binding site. Previous studies have shown that Pit-1 interacts with the 1P element as both a monomer and a dimer with the dimer forming cooperatively as the concentration of Pit-1 is increased (47). A detailed analysis of Pit-1 binding to the 3P element has not been performed. Therefore, we compared the ability of purified Pit-1 to bind to the 1P and 3P elements using a mobility shift assay (Fig. 3, B and C). With the 1P element, addition of increasing concentrations of Pit-1 resulted in the formation of two complexes (C1 and C2). Previous studies have shown that the C1 complex appears to represent the binding of Pit-1 monomers while the C2 complex represents binding of Pit-1 dimers (47). As observed by Ingraham et al. (47), the formation of the C2 complex on the 1P element is greatly facilitated over a narrow range of Pit-1 concentrations consistent with cooperative formation of Pit-1 dimers. Binding of Pit-1 to the 3P element also demonstrated the formation of two complexes which were similar in mobility to those observed with the 1P probe. However, the C1 complex persisted at much higher concentrations of Pit-1, and there was reduced formation of the C2 complex at all concentrations of Pit-1. These findings suggest reduced cooperativity in the binding of Pit-1 to the 3P element. Quantitative analysis of the mobility shifts shown in Fig. 2, A and B, by PhosphorImager confirmed these conclusions. The overall affinity of Pit-1 for the 3P element was about 60% of that for the 1P element. Binding to the 1P element occurred with a Hill coefficient of 1.5 consistent with substantial cooperativity while a Hill coefficient of 1.1 was obtained for the 3P DNA element. Similar results have been obtained in several independent binding experiments. The reduced cooperativity of binding and decreased apparent formation of Pit-1 dimers at the 3P site raises the possibility that Pit-1 may interact with this site as a monomer.

We also explored the binding of endogenous factors from GH3 cells to the 1P and 3P sites. Radiolabeled probes representing the 1P and 3P sites were tested for their ability to interact with GH3 cell nuclear proteins in a gel mobility shift assay (Fig. 4). Multiple protein-DNA complexes were detected with both probes. Two major complexes observed with the 1P probe were designated complex-1 (C1) and complex-2 (C2). These complexes are similar in mobility to the C1 and C2 complexes formed with purified Pit-1 (data not shown) and likely represent binding of Pit-1 monomers and dimers, respectively. However, the multiple bands observed with total nuclear proteins prevent definitive assignments. The C1 and C2 complexes were also observed with the 3P probe, but the C2 signal was much weaker. In an effort to enhance detection of factors other than Pit-1 which bind to the 1P and 3P DNA elements, antisera to Pit-1 was included in the binding reactions. Formation of protein-DNA complexes with either the 1P or the 3P probe was completely disrupted by antisera to Pit-1. Preimmune serum had little or no effect on binding. The Pit-1 antisera had no effect on the binding of GH3 nuclear proteins to a cyclic AMP response element (data not shown), further confirming the specific effect of the antisera. Unfortunately, this approach did not permit detection of any protein-DNA complexes which were resistant to the antisera. This finding does provide strong evidence that Pit-1 is a major component of the endogenous GH3 factors which bind to both the 1P and 3P DNA elements. Of course, we cannot rule out the possibility that factors other than Pit-1 may be included in the endogenous proteins which bind to the 1P and 3P elements but that these other factors require the presence of Pit-1 for stable interaction with the 1P and 3P elements.

Mutational Analysis of the 3P DNA Element—The preceding experiments provided evidence that multimers of the 3P DNA element are much more capable of mediating transcriptional responses to TRH, phorbol esters, or activation of MAPK than are multimers of the 1P element. Furthermore, Pit-1 binds cooperatively to the 1P element but with little or no detectable cooperativity at the 3P element. To further explore the relationship between Pit-1 binding and hormonal responsiveness of the 3P site, a series of clustered point mutations were introduced into this DNA element (Fig. 5A). The binding of Pit-1 to the wild type and mutant DNA elements was then tested by performing mobility shift analysis with increasing concentrations of Pit-1 for each DNA and calculation of a relative affinity from the quantitative titration data (Fig. 5B). Multimers of each mutant DNA were also prepared and tested for basal and regulated enhancer activity (Fig. 5, C and D). Interestingly, two of the mutations (mut1 and mut2) had either very little or no effect on Pit-1 binding but dramatically reduced the ability of multimers to mediate transcriptional regulation in response to PMA, EGF, or Raf. One of these mutations (mut1) is completely outside of the two putative Pit-1 monomer binding sites. We noticed that both mut1 and mut2 disrupted a DNA sequence, TTCC, which represents a core site for the Ets family of transcription factors. Another set of mutations (mut4, mut5, and mut6) substantially decreased Pit-1 binding and reduced basal and regulated enhancer activity of DNA multimers. All of the mutations which reduced both Pit-1 binding and basal enhancer activity were found to disrupt DNA sequences within the downstream Pit-1 monomer binding site (Pit-1 Site-a, indicated in Fig. 5A). One of the mutations, mut3, disrupts sequences within the center of the upstream putative Pit-1 binding site (Pit-1 Site-b). As mut3 has very little effect on either basal or inducible enhancer activity, it seems likely that Pit-1 does not bind to this site in vivo. The clustered point mutation analysis of the 3P element provides evidence that binding of Pit-1 is necessary but not sufficient to permit inducible transcription. All of the mutations which substantially reduce Pit-1 binding, also reduce basal and inducible reporter gene expression. Although mut1 did not disrupt Pit-1 binding, it greatly reduced the ability of 3P multimers to mediate responses to phorbol esters, EGF, and the Raf kinase. These findings suggest a possible model in which the 3P DNA element contains binding sites for Pit-1 and another factor and both of these binding sites are required for transcriptional responses to activation of MAPK.
An Ets Factor Can Interact with the 3P Enhancer Element—

The presence of a putative Ets binding site within the 3P DNA element raised the possibility that this site may serve as an Ets factor binding site. To test the ability of Ets factors to interact with the 3P enhancer, the DNA binding domain of an Ets factor, human ER81 (48), was produced in E. coli and tested for binding to wild type and mutant 3P DNA elements (Fig. 6). The results demonstrate that the DNA binding domain of ER81 is able to bind to the wild type 3P element. This binding appears to be specific as a 3-base pair mutation which disrupts the core Ets 1 binding site (mut1) greatly reduces binding of ER81. In contrast, a mutation which disrupts the consensus Pit-1 binding site (mut4) had no effect on the binding of ER81. In mixing experiments, we found that the presence of Pit-1 did not enhance the binding of ER81 to the 3P element (data not shown).

To provide a functional test for the interaction of Ets factors with the 3P element, an expression vector for human c-Ets-1 was cotransfected with reporter genes containing multimers of the 3P site (Fig. 7). Overexpression of Ets-1 stimulated expression of the reporter gene containing four copies of the wild type 3P enhancer. As shown earlier, the wild type 3P element can support a response to Ras. The ability of a reporter gene to respond to Ets-1 or Ras was essentially eliminated by mutation of the putative Ets binding site (mut1). These studies demonstrate that the putative Ets factor binding site of the 3P element is essential for mediating responses to both Ets-1 and Ras.

Several Different Signal Transduction Pathways Can Activate the Elk1 Transcription Factor in GH3 Cells—

The preceding studies have shown that an Ets factor binding site in the 3P element is essential to permit transcriptional regulation by several different signal transduction pathways. As the Ets family member Elk1 can mediate transcriptional activation in response to growth factors and other activators of the MAPK pathway (49), we explored the possibility that TRH, EGF, phorbol esters, and cAMP might be able to activate Elk1 in GH3 cells. An expression vector encoding the DNA binding domain of GAL4 fused to the carboxyl-terminal transcriptional activation domain of Elk1 was cotransfected with a reporter gene containing five copies of the GAL4 binding site upstream of a minimal TATA box and the luciferase coding sequence. The GAL4-Elk1 fusion protein supported transcriptional responses to TRH, EGF, phorbol esters, and cAMP (Fig. 8). These studies demonstrate that diverse signal transduction pathways which can mediate transcriptional activation through the 3P DNA element can also activate a specific member of the Ets family of transcription factors. Of course, we have not identified the endogenous Ets factor which binds to the 3P DNA element, and this study has simply used Elk1 as a model to determine if these signal pathways can activate an Ets factor. As Elk1 is known to be responsive to activation of the MAPK pathway (49), these studies raise the possibility that the ability of the 3P DNA element to permit multihormonal regulation may involve convergent regulation of the MAPK pathway.

**DISCUSSION**

These studies have shown that multiple copies of the 3P DNA element of the rat prolactin gene can function as an inducible enhancer mediating transcriptional activation in re-
A number of previous studies led to the view that Pit-1 is important for mediating hormonal regulation of the prolactin gene. In several studies, Pit-1 binding sites were shown to be important for mediating hormonal regulation of the prolactin promoter (13, 14, 16). The observation that Pit-1 is phosphorylated in vivo after treatment with cAMP, PMA, or TRH (17, 20) is also consistent with a role for Pit-1 in mediating transcriptional responses to these agents. This might suggest a simple model in which phosphorylation of Pit-1 modulates its transcriptional activity. However, several recent studies have reached the surprising conclusion that although Pit-1 is required for hormonal regulation of the prolactin gene, phosphorylation of Pit-1 is apparently not required (18–20). What then is the role of Pit-1 in mediating hormonal regulation of transcription? It is clear that Pit-1 is essential for tissue-specific expression of the prolactin gene (10, 42, 50–52). As has been previously suggested, the participation of Pit-1 in hormonal regulation of the prolactin gene may provide a mechanism which permits the linking of ubiquitous signal transduction pathways to cell-specific transcriptional responses (21, 36). The present studies suggest that cooperation between Pit-1 and an Ets factor likely provides a mechanism permitting a tissue-specific transcriptional response to activation of the MAPK pathway.

Ets factors appear to frequently act in concert with other transcription factors and a subset of Ets factors mediate transcriptional responses to the MAPK pathway. There are now numerous examples in which an Ets factor appears to cooperate with another transcription factor to mediate transcriptional responses. For instance, the Ets factor Elk1 interacts with the serum response factor as a crucial step in mediating the ability of the fos promoter to respond to growth factors (49, 53, 54). Similarly, other members of the Ets family have also been found to functionally interact with other transcription factors, often to permit synergistic activation of specific genes (31, 55–62). In view of the ability of the prolactin 3P element to permit responses to activators of the MAPK cascade, it is particularly interesting that Elk1 contains several MAPK phosphorylation sites. The use of fusions in which the carboxy-terminal domain of Elk1 was linked to a heterologous DNA binding domain have demonstrated that the Elk1 contains a transcriptional activation domain that is regulated by MAPK phosphorylation (49). SAP1 and Net which are related to Elk1 have also been shown to be regulated by Ras and the MAPK pathway (63, 64). A combination of genetic and biochemical studies have clearly demonstrated that the Drosophila Ets factors, Yan and Pointed, are regulated by the Ras/MAPK pathway (65, 66). Thus, it is well established that Ets factors can participate in mediating MAPK-induced transcriptional activation.

Multimers of the 3P site can support transcriptional responses to a number of diverse signaling pathways including TRH, EGF, phorbol esters, cAMP, Ras and Raf. It is possible that activation of the MAPK pathway leading to phosphorylation of an Ets factor could be involved in all of these responses. The finding that the Ets site is required for all of the responses is consistent with this model. However, is there evidence for activation of MAPK by all of these pathways? Of course, EGF, Ras, and Raf would be expected to activate MAPK. Recent studies have shown that TRH treatment activates MAPK in GH3 cells through a mechanism which is at least partially dependent on protein kinase C (41). This is consistent with the observation that protein kinase C can phosphorylate and activate Raf (67, 68) and that TRH treatment leads to activation of protein kinase C (69, 70). While it might be surprising to suggest that cAMP could also activate MAPK, recent studies provide evidence for tissue-specific differences in the effects of cAMP on MAPK activity. Although cAMP decreases MAPK activity in many cells, there is evidence that cAMP can increase MAPK activity in at least some cells (71, 72). The present studies have shown that cAMP as well as EGF, TRH, and phorbol ester treatment of GH3 cells can increase the transcriptional response to the 3P site. The finding that the Ets site is required for all of the responses to Ras and the MAPK pathway (63, 64). A combination of genetic and biochemical studies have clearly demonstrated that the Drosophila Ets factors, Yan and Pointed, are regulated by the Ras/MAPK pathway (65, 66). Thus, it is well established that Ets factors can participate in mediating MAPK-induced transcriptional activation.

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tion stimulating activity of a GAL4-Elk1 fusion protein. As GAL4-Elk1 has been shown to mediate transcriptional responses to MAPK, these results raise the possibility that cAMP may stimulate MAPK activity in GH3 cells. Thus it is conceivable that the multihormonal regulation of the 3P element involves convergent activation of MAPK. Further studies of cAMP effects on MAPK activity in GH3 cells will be required to further address this issue.

In a number of respects the present findings are similar to a recent report from Bradford et al. (73) which examined the interaction of Ets-1 and Pit-1 in mediating the ability of Ras to activate the prolactin promoter. Bradford et al. focused their attention on a different Pit-1 binding site, the 4P site. They demonstrated that there is an Ets factor binding adjacent to the 4P Pit-1 binding site. They found that overexpression of both Pit-1 and Ets-1 enhanced responsiveness of the prolactin promoter to Ras. Our studies demonstrate that the 3P site also contains an Ets factor binding site which is adjacent to a Pit-1 binding site. Taken together, the two studies provide strong evidence that Pit-1 can functionally synergize with Ets factors and that this interaction appears to be important for mediating transcriptional responses to the MAPK pathway. As noted above, the identity of the endogenous Ets factor which binds to the 3P element has not yet been determined. Further understanding of transcriptional regulation of the prolactin gene will require identification of the endogenous proteins which interact with these sequences and characterization of their phosphorylation in response to hormonal stimulation.

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