Ras Signaling and Transcriptional Synergy at a Flexible Ets-1/Pit-1 Composite DNA Element Is Defined by the Assembly of Selective Activation Domains*

Received for publication, March 10, 2003, and in revised form, July 25, 2003
Published, JBC Papers in Press, August 5, 2003, DOI 10.1074/jbc.M302433200

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Pit-1 and Ets-1 binding to a composite element synergistically activates and targets Ras-mitogen-activated protein kinase signaling to the rat prolactin promoter. These transcriptional responses appear to depend on three molecular features: organization of the Ets-1/Pit-1 composite element, physical interaction of these two factors via the Pit-1 homeodomain (amino acids 199–291) and the Ets-1 regulatory III domain (amino acids 190–257), and assembly of their transcriptional activation domains (TADs). Here we show that the organization of the Ets-1/Pit-1 composite element tolerates significant flexibility with regard to Ras stimulation and synergy. Specifically, the putative monomeric Pit-1 binding site can be substituted with bona fide binding sites for either a Pit-1 monomer or dimer, and these sites tolerated a separation of 28 bp. Additionally, we show that the physical interaction of Ets-1 and Pit-1 is not required for Ras responsiveness or synergy because block mutations of the Pit-1 interaction surface in Ets-1, which reduced Ets-1/Pit-1 binding in vitro, did not significantly affect Ets-1 stimulation of Ras responsiveness or synergy. We also show differential use of distinct TAD subtypes and Pit-1 TAD subregions to mediate either synergy or Ras responsiveness. Specifically, TADs from Gal4, VP16, or Ets-2 regulatory III domain linked to Ets-1 DNA binding domain constructs restored synergy to these TAD/Ets-1 DNA binding domain fusions. Conversely, deletion of the defined Pit-1 TAD (amino acids 2–80) retained synergy, but not Ras responsiveness. Consequently, we further defined the Pit-1 amino-terminal TAD into region 1 (R1, amino acids 2–45) and region 2 (R2, amino acids 46–80). R1 appears to regulate basal and synergistic responses, whereas the Ras response was mapped to R2. In summary, Ras responsiveness and Pit-1/Ets-1 synergy are mediated through the assembly of distinct TADs at a flexible composite element, indicating that different mechanisms underlie these two transcriptional responses and that the Pit-1 R2 subregion represents a novel, tissue-specific Ras-responsive TAD.

Pit-1/GHF-1 is a POU homeodomain transcription factor specifically expressed in the anterior pituitary which is responsible for determining the somatotroph, lactotroph, and thyrotroph lineages, as well as the regulated expression of their respective hormones: growth hormone, prolactin, and thyroid-stimulating hormone. Combinatorial interactions between Pit-1 and other trans-acting factors at composite or adjacent response elements have been shown to allow Pit-1 to serve as a cell-specific integrator of signaling pathways. As such, Pit-1 has been shown to physically interact with itself, ER, thyroid hormone receptor, c-Jun, Oct-1, GATA-2, P-Lim, Ptx-1, and Ets-1 (1–9).

In precisely this manner, an Ets/Pit-1 combination, acting via the Ets binding site (EBS)/footprint IV (FPIV) composite element spanning positions –217 to –190, is critical for both basal lactotroph-specific expression of the rPRL gene and for the targeting of the Ras-MAP kinase pathway to the prolactin promoter (3, 10). Several lines of evidence indicate that both Ets-1 and Pit-1 factors are required for the synergistic activation and Ras responsiveness. For example, transient transfection of pituitary-derived GH4T2 somatolactotrope cells with a dominant-negative Ets factor encoding only the Ets DNA binding domain (DBD), or with Pit-1β (an alternatively spliced isoform that functions as a dominant-negative effector in pituitary cells), results in diminished activity of the rPRL promoter in an effector-dependent manner (3). Similarly, transient transfection of rPRL promoter constructs containing site-specific mutations of either the EBS or FPIV sites within the composite element results in a loss of Ras responsiveness (10, 11). In non-pituitary HeLa cells, it has been shown that although individual Ets-1 or Pit-1 effectors strongly activate the rPRL promoter, the combination results in a response that is 6-fold greater than the sum of the individual responses (i.e. 6-fold synergy) (3). In addition, Ets-1 and Pit-1 have been shown to bind physically to each other in dilute solution and in the absence of DNA by utilizing the Ets-1 regulatory III (RII) transcriptional activation domain (TAD) and Pit-1 homeodomain (3, 12, 13). Finally, Thr-82 of chicken p68 c-Ets-1 was identified as a MAP kinase phosphorylation site, and site-specific mutation of Thr-82 to alanine resulted in loss of the Ets-1-mediated enhancement of the Ras response (14). Taken

§ This work was supported by National Institutes of Health Grants DK46868 (to A. G.-H.) and DK02946 (to D. L. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: ER, estrogen receptor; BPV, bovine papilloma virus; CBP, cAMP-responsive element-binding protein-bind- ing protein; CMV, cytomegalovirus; DBD, DNA binding domain; EBS, Ets binding site; FPIV, footprint IV; GST, glutathione S-transferase; In1, In2, inhibitory domains 1 and 2, respectively; Lue, luciferase; m, mutant; MAP, mitogen-activated protein; P1, P2, pointed domains 1 and 2, respectively; PBS, phosphate-buffered saline; PRL, prolactin; r, rat; R1 and R2, amino acids 1–45 functioning as a modular domain; rPRL promoter, the combination results in a response that is 6-fold greater than the sum of the individual responses (i.e. 6-fold synergy) (3). In addition, Ets-1 and Pit-1 have been shown to bind physically to each other in dilute solution and in the absence of DNA by utilizing the Ets-1 regulatory III (RII) transcriptional activation domain (TAD) and Pit-1 homeodomain (3, 12, 13). Finally, Thr-82 of chicken p68 c-Ets-1 was identified as a MAP kinase phosphorylation site, and site-specific mutation of Thr-82 to alanine resulted in loss of the Ets-1-mediated enhancement of the Ras response (14).Taken
MATERIALS AND METHODS
Plasmid Construction

pA3–425PRL-Luc containing the proximal 425 bp of 5′-flanking sequence from the rPRL promoter in the pA3Luc reporter has been described previously (28). Mutations mEBS and mFPIV have been previously described (11). Mutations of pA3–425PRL-Luc including monomer, dimer, 12 bp, 16 bp, and 24 bp were generated by overlap extension PCR amplification from −425PRLpGEM7 using mutant internal primers and SP6 and T7 primers with dual rounds of amplification. The 12-bp and 16-bp inserts incorporate an SoII site into the space between the EBS and FPIV Pit-1 binding site. The 24-bp insertion incorporates SoI, SpeI, and NheI sites. The mutant primers are as follows: monomer S, GCA TTA AAA GAT CAT TTA CTG CTA AAT GTG CCA GCA ATG TGA TTC CTG CTA AAG AAG ACC CTT ATG CTA GTG CTA GCT GCT GTA ATT AAT CAA AAT CCT CTG AGT TCT CTG; 24-bp S, TCG ACA CTA GTG CTA GCT GTA ATT AAT CAA AAT CCT CTG AGT TCT CTG; 12-bp S, TCC TCT TGT GCT GTA ATT AAT CAA AAT CCT CTG AGT TCT CTG; 16-bp S, TGT GGA GAC TCT GTA ATT AAT CAA AAT CCT CTG AGT TCT CTG; 28-bp S, CAT GCA CTA GTG TCG ACA AAA GGA AAT GAG AGA. Mutant promoter 28 bp was generated by digesting the 24-bp insertion construct with SpeI, filling in the overhangs by Klenow treatment, and religating the vector. Mutant promoter 32 bp was generated by digesting 28 bp with SoII, filling in the overhangs by Klenow treatment, and religating the vector. Sequences were verified by sequencing.

Plasmid pSG5s-Ets-1 and Ets-2 encode the p68 chicken Ets-1 and chicken Ets-2, respectively, under control of the SV40 early promoter. These constructs as well as the amino-terminal deletions of Ets-1, pSG5s-Ets-1-L5–4 and Δ5–6, n70 Ets-1DBD (EtsDBD), VP16TAD-n70Ets-1DBD (VP16EtsDBD), VP16Ets280–440, and VP16Ets330–440 (29) were generously provided by Dr. Bohdan Wasylyk (Institut de Genetique et de Biologie Moleculaire et Cellulaire, CBRE/INSERM/ULP, Illkirch Cedex, France). The pSG5s Ets-1 BPV-1, BPV-2, and BPV-3 are mutant constructs in which overlapping regions of p68 chicken Ets-1 amino acids 190–257 are replaced by 26 amino acids from the bovine papilloma virus type 1 (amino acids 415–440 including the AU1 epitope). These mutations were made utilizing overlap extension PCR to amplify mutated fragments of Ets-1 from nucleotides 401–1054. The fragments were cloned into PCR 2.1 and subcloned into pSG5s Ets-1 by digesting at internal BglII and ApI III sites. PCR primers utilized are: BPV-1 S, CTA AAT GTG CGA GCA ATG TGA TCG CTA AAG AAG ACC CTT ATG CCG CCT CTG AGT TCT GTG; BPV-1 AS, TTG CTG GCA CAT TTA GTT GCA GGA GAC GAC TCT ATA TAG CGA TAG TCT GCC ACC TTG CCT GGG; BPV-2 S, CTA AAT GTG CGA ATG TGA TCG CTA AAG AAG ACC CTT ATG CTT GC; BPV-2 AS, TTG CTG GCA CAT TTA GTT GCA GGA GAC GAC TCT ATA TAG CGA TAG TCT GCC ACC TTG CCT GGG; BPV-3 S, CTA AAT GTG CGA ATG TGA TCG CTA AAG AAG ACC CTT ATG CCG CCT CTG AGT TCT GTG; BPV-3 AS, TTG CTG GCA CAT TTA GTT GCA GGA GAC GAC TCT ATA TAG CGA TAG TCT GCC ACC TTG CCT GGG; pSG5s Ets-1 401 S, TGA AGG GAG TGG ATT TCC; pSG5s Ets-1 1054 AS, CAT ACT CCT TGA AGG TCC; Gal4AD Δ4–5 and Δ5–6 were constructed by subcloning the Gal4AD from pACT2 into
pBluescript utilizing HindIII and XhoI restriction sites. The Gal4AD was then isolated from pBluescript by a partial digest with EcoRI and complete digest with XhoI and ligated into either pSG5 Δ5-4 or Δ5-6 that had also been partially digested with EcoRI and completely digested with XhoI.

The mutant Ets-1/2 chimera was generated using overlap extension PCR to replace amino acids 185–257 of Ets-1 with amino acids 178–251 of Ets-2. The resulting PCR product was digested at internal BglII and AflIII restriction sites and ligated into a pSG5 Ets-1 vector in which the BglII site in the multiple cloning site was destroyed. This vector was partially digested with AflIII and completely digested with BglII. The primers used for PCR were as follows: (C) CCGGATCCTACGCAAGTTACCGTACACTAAATTAGGT (N) to eliminate binding of any contaminating DNA (31). The bacterial pellets were resuspended in 5 ml of Bugbuster Reagent (Amersham Biosciences) for 1 hour at room temperature. The Sepharose was washed extensively with 1 ml of medium was added to plasmid DNA as described in the figure legends and transfected by electroporation at 220 V and 500 microfarads using a Bio-Rad Gene Pulser with 4-mm gap cuvettes. Following transfection, cells were plated on 60-mm tissue culture plates in culture medium and incubated for 24 h. All electroporations included 0.3 μg of CMV-β-galactosidase (Clontech) as an internal control for transfection efficiency. Total DNA was kept constant, and nonspecific effects of viral promoters were controlled for by transfecting appropriate control vectors. Approximately 24 h following the electroporation, the cells were harvested, and luciferase and β-galactosidase activities were measured. The transfected cells were harvested in PBS containing 3 ml EDTA, pelleted by centrifugation, and lysed by three freeze-thaw cycles in 100 μl of 100 mM potassium phosphate, pH 7.5 with 1 mM dithiothreitol. Cell debris was pelleted by centrifugation at 10,000 g for 10 min at 4 °C, and aliquots of the supernatant were used in subsequent assays. Samples were assayed in duplicate for luciferase activity as described previously (28) using a Monolight 3010 luminometer (Analytical Luminescence Laboratories). β-Galactosidase activity was determined spectrophotometrically using the chromogenic substrate o-nitrophenyl-β-D-galactopyranoside as described (28).

**Lipid-mediated Transfection—**HeLa cells were transiently transfected by lipid-mediated DNA transfer using Effectene (Qiagen) according to the manufacturer's instructions. Briefly, on day 1, HeLa or GH4T2 cells were harvested in 0.05% trypsin with 0.5 mM EDTA and plated on 96-well plates at a concentration of 20,000 or 45,000 cells/well in 100 μl of medium, respectively. On day 2, plasmid DNA for transfection was prepared by mixing 0.8 μl/well of Enhancer and 2.5 μl/well Effectene reagent diluted in 30 μl of medium were added to plasmid DNA as described in the figure legends and transfected by electroporation at 220 V and 500 microfarads using a Bio-Rad Gene Pulser with 4-mm gap cuvettes. Following transfection, cells were harvested with 1× PBS supplemented with 1× EDTA and resuspended in 1 ml of medium supplemented with 20% fetal calf serum (Invitrogen). Cells were grown at 37 °C in 5% CO2. The medium was changed 4–16 h prior to transfection.

**Cell Culture**

HeLa and GH4T2 cells were maintained in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 15% horse serum and 2.5% fetal calf serum (Invitrogen). Cells were grown at 37 °C in 5% CO2. The medium was changed 4–16 h prior to transfection.

**Transient Transfection**

Electroporation—Cells were harvested with 1× PBS and grown at 37 °C to an absorbance of 0.5 at 600 nm. The bacterial pellets were resuspended in 5 ml of Bugbuster Reagent (Amersham Biosciences), transformed with pGex plasmids, and incubated for 30 min at 4 °C on a rotator. 35S-Labeled proteins (5 μg) were eluted from the beads by boiling in SDS-sample buffer and analyzed by SDS-PAGE and autoradiography. Bands were quantified using a Molecular Dynamics PhosphorImager with ImageQuant software.
sient transfections of GH4 cells. Block mutations of either EBS or FPIV were tested as positive controls for loss of function (10, 11). Neither of the mutations significantly reduced the ability of the −425PRL promoter to respond to Ras stimulation as determined by Student’s t test, p < 0.05 (Fig. 1B). However, in agreement with previously published work, block mutations in either the EBS or FPIV site significantly reduced the Ras response (11).

To characterize the synergistic response of each monomer/dimer substitution construct in the HeLa reconstitution assay, we first tested the response of these constructs in the absence of either Pit-1 or Ets-1 (Fig. 1C, Vector), to Pit-1 alone (+Pit-1), Ets-1 alone (+Ets-1), and finally to Pit-1 plus Ets-1 (Both). Because the activity of the rPRL promoter is highly restricted to Pit-1-positive pituitary cells, transfection of each rPRL promoter-Luc reporter construct alone resulted in minimal activity, with relative light units (RLU; firefly luciferase divided by Renilla luciferase light units) in the range of 0.01 (Fig. 1C, Vector), as reported previously (3). However, coexpression of Pit-1 resulted in a 170-fold stimulation of the WT rPRL promoter, increasing the RLU from 0.01 in the absence of Pit-1 to 1.7 RLU in the presence of Pit-1 (Fig. 1C, +Pit-1). The Pit-1-enhanced activity of the monomer, dimer, and mEBS reporter constructs revealed a slight reduction in promoter activity compared with WT which was not statistically significant. However, the effects of Pit-1 on the mFPIV construct resulted in ~0.7 RLU, or about 70-fold stimulation, which was significantly reduced compared with the WT rPRL promoter, indicating that this Pit-1 binding site, one of three found in the −425PRL promoter, is required for optimal Pit-1 stimulation. Cotransfection with an Ets-1 expression vector resulted in ~30-fold stimulation of the WT and dimer rPRL promoters, increasing the RLU from −0.01 in the absence of Ets-1 to −0.3 RLU in the presence of Ets-1 (Fig. 1C, +Ets-1). The monomer construct was about twice as responsive to Ets-1, whereas the mEBS and mFPIV promoters displayed somewhat reduced activities compared with WT (Fig. 1C, +Ets-1). Finally, the combination of Pit-1 plus Ets-1 resulted in the expected strong synergistic, ~570-fold response of the WT promoter construct, increasing the RLU from −0.01 in the absence of Pit-1/Ets-1 to ~5.7 RLU in the presence of Pit-1/Ets-1 (Fig. 1C, +Both). The monomer and mFPIV promoter constructs displayed modest increases in the synergistic response (Fig. 1C, +Both). These HeLa reconstitution data reveal that the Ets-1/Pit-1 composite
were assayed for luciferase and empty pSG5 control. After 24 h, the cells were cotransfected with 3 μg of plasmid preparations were used. Asterisks represent values significantly different from WT promoter (p < 0.05 by Student’s t test). C, HeLa cells were transiently cotransfected in 96-well plates with 75 ng of each promoter construct and 1 ng of pRLC-β-gal. Either 50 ng of pSG5-ets-1, 50 ng of pBSV-Pit-1, or both vectors was added where indicated. Total DNA was held constant with the addition of appropriate control vector DNA. After 24 h, the cells were assayed for luciferase and β-galactosidase activity. Values represent the mean ± S.E. of triplicate samples in three or more transfections. At least two different plasmid preparations were used. Asterisks represent values significantly different from WT promoter (p < 0.05 by Student’s t test). C, HeLa cells were transiently cotransfected in 96-well plates with 75 ng of each promoter construct and 1 ng of pRLC-β-gal. Either 50 ng of pSG5-ets-1, 50 ng of pBSV-Pit-1, or both vectors was added where indicated. Total DNA was held constant with the addition of appropriate control vector DNA. After 24 h, the cells were assayed for luciferase and β-galactosidase activity. Values represent the mean ± S.E. of triplicate samples in three or more transfections. Asterisks represent values significantly different from WT promoter (p < 0.05 by Student’s t test).

Fig. 2. Effect of Ets/Pit-1 site spacing on Ras responsiveness or synergistic activation of rPRL promoter constructs. A, outlined are the sequences of the WT and Ets/Pit-1 site spacer mutants and mutant EBS and FPIV of the EBS/FPIV composite element. Inserted spacers are shown below the element in lowercase text. All mutations were generated in the context of 425 bp of 5'-flanking sequence of the rPRL promoter. B, GH1T2 cells were transiently cotransfected with 3 μg of promoter constructs, 0.3 μg of pCMV-β-galactosidase, and either 3 μg of pSV-Ras or empty pSG5 control. After 24 h, the cells were assayed for luciferase and β-galactosidase activity. Values represent the mean ± S.E. of triplicate samples in three transfections. At least two different plasmid preparations were used. Asterisks represent values significantly different from WT promoter (p < 0.05 by Student’s t test). C, HeLa cells were transiently cotransfected in 96-well plates with 75 ng of each promoter construct and 1 ng of pRLC-β-gal. Either 50 ng of pSG5-Ets-1, 50 ng of pBSV-Pit-1, or both vectors was added where indicated. Total DNA was held constant with the addition of appropriate control vector DNA. After 24 h, the cells were assayed for luciferase and β-galactosidase activity. Values represent the mean ± S.E. of triplicate samples in three or more transfections. Asterisks represent values significantly different from WT promoter (p < 0.05 by Student’s t test).

Separation of the Ets-1 and Pit-1 Sites by 32 Base Pairs Is Required to Reduce Ras and Synergistic Responses—We next asked how spacing of the EBS/FPIV binding sites affected Ras responsiveness in GH4 pituitary cells and the synergistic response in the HeLa reconstitution assay, by increasing the spacing between the sites from 8 bp to 12, 16, 24, 28, and 32 bp (Fig. 2A). We found that increasing the spacing to 12 bp resulted in a significant increase in Ras responsiveness, from 7- to 12-fold (Fig. 2B). Although responsiveness was still elevated at 16 bp, the response was no longer significant. Subsequent increases in spacing resulted in a gradual loss in Ras responsiveness up to 32 bp with 16 bp being the most significant. ETS-1 and PTS-1 binding sites resulted in a gradual decrease in the activation of the PRL promoter by Pit-1 alone, with the 12-, 16-, 24-, 28-, and 32-bp insertions resulting in −95, −80, −70, −50, and −30-fold stimulation, respectively (Fig. 2C, +Pit-1). In contrast, ETS-1 stimulated all of the spacing mutants to the same extent as the WT promoter (−15-fold), as assessed by Student’s t test (Fig. 2C, +ETS-1). Finally, the synergistic stimulation by the combination of Pit-1 and p68 e-ETS-1 resulted in an apparent gradual decrease from −600-fold for the WT promoter to −200-fold for the 32-bp insertion (Fig. 2C, +Both). These data reveal that the ETS-1/Pit-1 composite element allows modest spacing between the two sites for both the Ras and HeLa synergistic responses, yet there is a maximum spacing of 28 bp which is tolerated in these two functional assays.

Scanning Mutations of ETS-1 Amino Acids 190–257 Reduce Pit-1 Binding but Not Ras or Synergy Responses—Previously, studies in our laboratory using GST pull-down analysis have shown that Pit-1 physically binds to ETS-1 in the absence of DNA. In Pit-1, this physical interaction has been mapped to amino acids 199–291, which define the Pit-1 homeodomain. GST interaction assays and NMR studies have shown that both amino- and carboxyl-terminal contacts in the Pit-1 homeodomain are required for interaction with ETS-1 (12, 13). Carboxyl-
and amino-terminal deletion analysis of Ets-1 has shown that the Pit-1 interaction domain maps to amino acids 190–257 within the RIII TAD (3, 12). To map further the domain in Ets-1 responsible for binding to the Pit-1 homeodomain, we replaced overlapping 26-amino acid segments of p68 c-Ets-1 in the region from 190 to 257 with amino acids 415–440 of the BPV L-1 capsid (including the AU1 epitope) to generate a series of three block mutations that “scan” across this 68-amino acid region (BPV-1–3, Fig. 3A) (32). GST pull-down analysis was used to assess binding of these constructs to the Pit-1 homeodomain. Fig. 3B shows that WT Ets-1 bound 17% of input, whereas mutant BPV-1 and BPV-2 proteins bound 8–9% of input, a decrease of ~50%. Mutation of amino acids 232–257 (BPV-3) resulted in only 5% binding, or a 71% reduction compared with WT Ets-1 (Fig. 3B). These results suggest that there are multiple contact points for Pit-1 within amino acids 190–257, with a major site located in the region mutated by BPV-3. Despite these reductions in the in vitro interaction assay, these mutations had minimal effects, if any, in the in vivo functional assays (Fig. 4). For example, in the absence of Ras stimulation, these BPV mutant Ets constructs resulted in an apparent slight increase in basal rPRL promoter activity compared with WT Ets-1; however, these increases were not statistically significant (Fig. 4A, black bars). With regard to the Ras response, WT Ets-1 enhanced the Ras response from ~7-fold in its absence to ~14-fold in its presence (Fig. 4A, white bars). Each BPV Ets-1 mutant enhanced the Ras response in a similar fashion, ranging from 12- to 14-fold and did not differ from the WT Ets-1 construct (Fig. 4A). The intrinsic transcriptional potency of each Ets construct, as measured in the HeLa reconstitution assay, revealed that they all maintained essentially the same level of activity (~22-fold), although BPV-3 was reduced slightly (~15-fold) (Fig. 4B, black bars). With regard to the synergistic response, Pit-1 alone yields a ~60-fold response, Ets-1 alone yields a ~30-fold response, and together Pit-1 + Ets-1 yield a ~325-fold response, which is 5.7 times the sum of the Pit-1 and Ets-1 responses alone (calculated as 90-fold). Similarly, each of these BPV mutant Ets constructs also synergized with Pit-1, yielding 6.2-, 6.7-, and 4–9-fold synergy for the BPV-1, BPV-2, and BPV-3 constructs, respectively (Fig. 4B, white bars). Although the synergistic response of BPV-3 is reduced, it is not statistically significant (p = 0.083). Finally, Fig. 4C shows that the expression levels of transiently transfected WT Ets-1 and the BPV mutant Ets-1 proteins were all equivalent. COS-1 cells were utilized to assess Ets protein expression in a background with lower endogenous protein levels. Thus, significant reduction of the physical interaction between Ets-1 and Pit-1 does not appear to interfere with the functional cooperativity of Ets-1 and Pit-1 in vivo, suggesting that DNA contacts and higher order transcription complexes also contribute to these functional responses in the intact cell.

Ets-1/Ets-2 RIII Substitution Abrogates Pit-1 Binding but Not Synergy—To determine better the role of the Ets-1/Pit-1 physical interaction for the synergistic response in HeLa cells, we took advantage of the similarities and dissimilarities of the Ets-1 and Ets-2 proteins which would allow alterations of the amino acids 190–257 region, yet maintain an intact TAD. Specifically, Ets-1 and Ets-2 are highly related and retain similar protein sequences and functional structures, except in amino acids 180–300 of the RIII TAD, which are only 6% conserved. Thus, we replaced the Pit-1 interaction face of Ets-1 (amino acids 190–257) with the corresponding 67-amino acid region of Ets-2, generating a chimeric Ets-1/2 protein that retains an intact RIII TAD but does not physically interact with Pit-1 (3). Indeed, assessment of physical binding of radiolabeled Ets-1, Ets-2, or the Ets-1/2 chimera to the GST-Pit-1 homeodomain (amino acids 199–291) showed 52, 3, and 6% binding of input, respectively, corroborating our previously published results that binding of Ets-2 to Pit-1 is reduced by 90–95% (Fig. 5A).

Transfection of these various Ets constructs alone revealed that they all activated the rPRL promoter equivalently in the HeLa synergy assay (each ~25-fold), verifying that they retained transcriptional activity (Fig. 5B, upper panel, left, black bars). As shown above, Pit-1 alone resulted in a ~85-fold response, WT Ets-1 alone yielded a ~30-fold response, and together Pit-1 + Ets-1 yielded a ~730-fold response. Thus, the
Selective Ets-1/Pit-1 TADs Mediate Ras/Synergistic Responses

Fig. 4. Effect of BPV-1 mutations in Ets-1 on Ras responsiveness and synergistic activation of the rPRL promoter. A, GH4T2 cells were transiently cotransfected in 96-well plates with 100 ng of promoter and 1 ng of pRLC-Renilla. 20 ng of pSV-Ras and 50-ng pSG5-Ets-1 constructs were added where indicated. After 24 h, the cells were assayed for luciferase activity. Bars represent the mean ± S.E. of triplicate samples in three transfections. B, HeLa cells were transiently cotransfected in 96-well plates with 75 ng of promoter and 1 ng of pRLC-Renilla. 50 ng of pSG5-Ets-1 constructs and 50 ng of pRSV-Pit-1 were added where indicated. After 24 h, the cells were assayed for luciferase activity. Bars represent the mean ± S.E. of triplicate samples in three transfections. Numbers indicate fold synergy. C, COS-1 cells were transiently transfected with 10 µg of the various Ets-1 constructs. After 24 h the cells were harvested, and 100 µg of total cellular protein from each sample was analyzed by Western blotting utilizing a primary antibody directed against the carboxyl terminus of human Ets-1 (PA-94). The blot was analyzed using pico-West chemiluminescent substrate (Pierce) and autoradiography.

functional cooperativity of Pit-1 with WT Ets-1 yielded ~6.2-fold synergistic response, whereas the WT Ets-2 and the Ets-1/2 chimera yielded similar responses of ~650-fold, yielding 6- and 5.6-fold synergy, respectively (Fig. 5B, upper panel, right). Finally, using an Ets-1/2-specific antibody in Western blot analysis of extracts derived from cells transiently transfected with these various Ets constructs, we found that Ets-1, Ets-1/2, and Ets-2 are each expressed in equivalent amounts (Fig. 5B, lower panel). Taken together, these data indicate that synergistic activation of the rPRL promoter by Ets-1 and Pit-1 is defined primarily by the assembly of their respective TADs at the composite element and that physical interaction of Ets-1 and Pit-1 is not required for this response.

Various TADs Linked to EtsDBD Can Mediate Synergy with Pit-1—Given that physical interaction is not required for the Pit-1/Ets-1 synergy, we next tested the possibility that synergy is determined by the number and/or identity of TADs assembled on the EBS/FPIV composite element. We have reported previously that the Ets-1 RIII TAD is required because an amino-terminal deletion construct that retains this domain (Ets-1Δ35–4) synergizes with Pit-1, but further deletion to remove this domain (Ets-1Δ5–6) results in the loss of both basal activity and synergy with Pit-1 (3). Replacement of Ets-1 RIII with Ets-2 RIII suggests that other Ets factor TADs can replace the Ets-1 RIII TAD. However, this also raises the question of whether any TAD can replace the RIII TAD of Ets-1. To determine whether synergy is governed by an assembly of selective TADs, we linked either the Gal4 or VP16 TADs to various Ets-1 amino-terminal truncations that: 1) retained the minimal TAD/synergy domain (Ets-1Δ35–4), 2) were devoid of this domain (Ets-1Δ5–6), or 3) retained only the EtsDBD (VP16EtsDBD) (Fig. 6A). Again, as previous studies have shown, Ets-1 strongly synergizes with Pit-1 (6.1-fold synergistic response), the Δ5–4 construct retained essentially WT synergy levels (5.6-fold synergy), and the Δ5–6 construct neither activated transcription of PRL alone, nor synergized with Pit-1 (3). Addition of the Gal4 TAD to the Δ5–4 construct had no effect on either the basal or synergistic responses of the rPRL promoter, despite the additional TAD. In contrast, addition of the Gal4 TAD to the Δ5–6 construct restored its ability both to activate the PRL promoter (~15-fold, Fig. 6A, black bars) and synergize with Pit-1 (4.3-fold synergy). Although there is a different magnitude in overall activation by the Pit-1/Gal4ADΔ5–6 combination compared with Pit-1/Ets-1, the fold synergies are not significantly different. Transfection of the Ets-1 DBD, which has increased affinity for the Ets binding site because of the deletion of the upstream inhibitory domain, reduced the Pit-1 activation of rPRL promoter activity to 20% of the activity seen in the presence of Pit-1 alone. Thus, it acted as a dominant-negative regulator of the PRL promoter. However, addition of the VP16 TAD to the EtsDBD construct reversed its dominantly-negative activity, restoring transcriptional activity to the EtsDBD and mediating a 5.9-fold synergistic response in combination with Pit-1, similar to the 6.1-fold synergy of the WT Ets-1 construct (Fig. 6A). It is important to note that Ets-1, Δ5–4, Δ5–6, and EtsDBD progressively delete the Pit-1 interaction surface, with Δ5–6 and the EtsDBD lacking this interaction face completely. Thus, these data separately verify that the Pit-1 interaction face is not required for the synergistic response. To verify that these TAD fusion constructs failed to bind Pit-1, GST pull-down studies were performed. Although [35S]methionine labeling of VP16EtsDBD resulted in a poorly labeled protein because of its smaller size and lower methionine content, these data show that binding of VP16EtsDBD and of GalADΔ5–6 to Pit-1 was significantly reduced at 2 and 3% of input, respectively, compared with Ets-1 (27% of input) (Fig. 6B). Taken together, these data reveal that synergistic activation of the PRL promoter by Ets-1 and Pit-1 can be conferred by the assembly of multiple transcriptional activation domains at the composite element.

A Hierarchy of Cooperating TADs Is Defined by the Pit-1 TAD—Studies of the functional domains of Pit-1 have shown that amino acids 1–80 located at the amino-terminal region of Pit-1 represent its dominant TAD (33). However, previous studies have shown that even with deletion of this putative
Pit-1 TAD, Pit-1 is able to stimulate synergistic activation of the rPRL promoter with Ets-1 (13). This is in direct contrast to the current studies, which suggest that synergy is conferred by the assembly of multiple TADs at the composite element. Thus, we asked whether the Pit-1Δ2–80 construct, devoid of the Pit-1 TAD, could synergize with the Gal4 TAD and VP16 TAD constructs in the same manner as the full-length Pit-1 construct. We transiently transfected HeLa cells with the −425PRL promoter and mammalian expression vectors for Pit-1 or PitΔ2–80 alone and in combination with Ets-1Δ5–6, Gal4ADΔ5–6, EtsDBD, VP16EtsDBD, VP16Ets280–440, and VP16Ets330–440 (Fig. 7A). To address the possibility that the insertion position of the VP16/Gal4 TAD relative to the EtsDBD and the presence of the inhibitory domains flanking the DBD might affect its function (Fig. 3), we chose several available VP16-Ets fusion constructs that placed the VP16 TAD both amino- and carboxyl-terminal to the EtsDBD and contained only the amino-terminal or both inhibitory domains (34). In the absence of Ets-1, Pit-1 stimulated the −425PRL promoter by 87-fold, and Pit-1Δ2–80 stimulated 41-fold. As described previously, both Pit-1 and PitΔ2–80 cooperated functionally with Ets-1, resulting in 3.6 ± 1- and 4.4 ± 1-fold synergy levels, respectively (Fig. 7A). The synergistic activation of Pit-1 and PitΔ2–80 with Gal4ADΔ5–6 was approximately equivalent (1.9 ± 0.5- and 3 ± 1-fold synergy, respectively), although the cooperativity of these Pit-1 proteins with Gal4ADΔ5–6 was slightly lower than with full-length Ets-1 (3.6–4.4-fold synergy). Conversely, the cooperative effects of Pit-1 and PitΔ2–80 with VP16EtsDBD were notably different, resulting in 7.1 ± 0.9-fold and 2.9 ± 0.8-fold synergistic responses, respectively (Fig. 7A). Fusion of the VP16 TAD to the carboxyl-terminal end of an EtsDBD that retains a portion of the In1 domain (Fig. 3A), resulted in a similar synergistic response (8.6 ± 2.9-fold) compared with VP16EtsDBD (7.1 ± 0.9-fold). Like VP16EtsDBD, the synergy level of VP16Ets280–440 was greater than that seen with the Gal4ADΔ5–6, indicating that the inclusion of the In1 region is not responsible for the reduced activity of the Gal4AD fusion. Nevertheless, the differential cooperative effects of Pit-1 and PitΔ2–80 with VP16Ets280–440 were maintained (8.6 ± 2.9-versus 2.7 ± 0.9-fold, respectively). Finally, using a human version of the EtsDBD lacking the In1 inhibitory region with the VP16TAD linked to the carboxyl terminus (VP16Ets330–440), we recapitulated the strong synergistic response (19 ± 8-fold) and differential Pit-1/PitΔ2–80 cooperativity (19 ± 8.1-versus 3.4 ± 0.8-fold) noted with the chicken Ets VP16EtsDBD (Fig. 7A). As shown in Fig. 7B, the various Ets fusion constructs, particularly those that fail to synergize, are expressed at levels that are equivalent to or greater than Ets-1, and the Pit-1 constructs are also expressed at equivalent levels. These data indicate that the enhanced synergy noted with the VP16 TAD requires the amino-terminal TAD in Pit-1. Although these data support our hypothesis that assembly of multiple TADs at the composite element is required for synergistic activation of the rPRL promoter, they also demonstrate that the identity of the specific TAD combinations modulates the level of the synergistic response.

Distinct Subregions of the Pit-1 TAD (2–80) Mediate Synergy and Ras Responsiveness—To gain further insights into the
structure/function mechanisms of the Pit-1 TAD responsible for the Ras- and the Pit-1/Ets-1 synergistic response of the rPRL promoter, we tested WT and two amino-terminal deletions encompassing the Pit-1 TAD (Fig. 8, A and B, WT, Δ2–80, Δ2–45) for their ability to enhance the Ras response (Fig. 8C) and to mediate Pit-1/Ets-1 synergy (Fig. 8D). The data reveal that WT Pit-1 enhances the 10-fold Ras response to 35-fold, whereas the Δ2–80 construct fails to enhance the Ras response and yet the Δ2–45 construct retains WT levels of enhancement of the Ras response to 35-fold (Fig. 8C). These results effectively map the Ras-responsive subdomain of the Pit-1 TAD to amino acids 46–80. Of note, the lack of enhancement of the Δ2–80 construct is not the result of lack of protein expression because these various Pit-1 constructs were expressed in a similar manner (Figs. 7B and 8B). In comparison, the Pit-1 Δ2–80 construct was equally as efficient at promoting synergy with Ets-1 alone was subtracted from total binding, and specific binding is expressed as percent input.

**DISCUSSION**

Interplay of Cis- and Trans-elements in the Regulation of Synergy and Ras Signaling—Pit-1 has been identified as a pituitary-specific signal integrator that directs both positive and negative regulatory stimuli to genes in pituitary somatotropes, lactotropes, and thyrotropes. This signal integration appears to be regulated by the binding of Pit-1 to a variety of composite cis-elements as well as functional interactions with other nuclear regulatory factors. In the current study, we examined the spacing and organization of the individual sites within the EBS/FPIV composite element, the requirement of physical interaction between Pit-1 and Ets-1, and the role of distinct TADs and their physical arrangement which allow for targeting of the rPRL promoter by the Ras signaling pathway and which promote the synergistic response to Ets-1 and Pit-1. Here, we show that although the Pit-1/Ets-1 composite element tolerates a high degree of flexibility and that direct physical interaction between Pit-1 and Ets-1, and the role of distinct TADs and their physical arrangement which allow for targeting of the rPRL promoter by the Ras signaling pathway and which promote the synergistic response to Ets-1 and Pit-1. Here, we show that although the Pit-1/Ets-1 composite element tolerates a high degree of flexibility and that direct physical interaction between Pit-1 and Ets-1 is not required for transcription synergy, an assembly of specific TADs on the composite element is required for optimal synergistic activation of the rPRL promoter. Taken together, these data not only provide important information regarding transcription synergy in general, but also reveal novel mechanistic insights regarding the precise cis- and trans-requirements for transcription synergy for the rPRL promoter. Specifically, the data show a differen-
Selective Ets-1/Pit-1 TADs Mediate Ras/Synergistic Responses

Atial use of distinct TAD subtypes (e.g. Ets-1, Pit-1, Gal4, VP16) and TAD subregions (e.g. Pit-1 TAD R1 versus R2) to mediate either synergy or Ras responsiveness. Finally, because the Pit-1 R2 TAD subregion lacks a proline-directed MAP kinase site, it appears to be a novel Ras-responsive TAD.

Pit-1 Monomer versus Dimer DNA Binding Sites Are Not a Primary Determinant for Ets-1 Synergy or Ras Responsiveness—POU transcription factors are allosterically regulated by the sequence and organization of their DNA binding sites; therefore, the specific DNA-induced conformation of Pit-1 appears to select functional interactions with specific coregulatory proteins generating transcription complexes that mediate distinct responses (25, 27, 35). In the studies shown here, both the monomeric 1D site and the palindromic FPI dimer site serve nearly as well as the endogenous FP1V Pit-1 binding site in mediating the Ras response in GH4 pituitary cells, whereas the 1D monomer site is preferred for transcriptional synergy in HeLa cells (Fig. 1). These data are consistent with a previously published report showing that intact Pit-1 displayed optimal synergy with the ER, regardless of the identity of the Pit-1 site at a composite Pit-1/ER element from the rPRL promoter (26). Additionally, the palindromic FPI dimer site used here has been reported to present Pit-1 in a conformation that preferably binds the CBP coactivator (27, 35). Yet our data reveal that for the Ras response, the precise DNA structure of the Pit-1 binding site as a monomer or dimer, and therefore the precise conformation of Pit-1 appears to be quite resilient. In contrast, the ~30% enhancement of the Pit-1 plus Ets-1 synergistic response of the one-dimensional monomer reporter construct is likely the result of the improved Ets-1 stimulation (Fig. 1C). The WT behavior of the EBS and FP1V site-specific mutants in the synergistic response suggests that additional Ets-1 and Pit-1 sites in the ~425 rPRL promoter function in a redundant manner in this assay, particularly those found in FP1II and the EBS at position ~76 adjacent to FPI at ~65 (see Fig. 1A, top panel). Finally, it is important to note that these data do not preclude a preferred Pit-1 conformation for the responses tested, but rather the data indicate that of the var-

Fig. 7. Role of the Pit-1 TAD on synergistic activation of rPRL promoter. A, HeLa cells were transiently cotransfected with 75 ng of promoter and 1 ng of pRL-C-Renilla. Ets-1 fusions were adjusted as follows to give similar levels of protein expression: 35 ng of pSG5-Ets-1, 11.6 ng of pSG5-Ets-1 ΔΔ5–6, 16.45 ng of pSG5-Gal4AD ΔΔ5–6, 14.7 ng of pSG5-VP16EtsDBD, 12.25 ng of pSG5-VP16Ets280–440, 105 ng of pSG5-VP16Ets330–440, and 105 ng of pSG5-VP16Ets330–440. DNA levels were held constant with the addition of empty pSG5 vector. pCGN2-Pit-1 (2.5 ng) or 0.625 ng of pCGN2-Pit-1 Δ2–80 was added where indicated. After 24 h, the cells were assayed for luciferase activity. Bars represent the mean ± S.E. of triplicate samples in three transfections. B, left and middle panels, COS-1 cells were transiently transfected with the various Ets-1 constructs at the following concentrations: 30 μg of pSG5-VP16Ets330–440, 30 μg of pSG5-VP16Ets280–440, 4.2 μg of pSG5-EtsDBD, 3.5 μg of pSG5-VP16 EtsDBD, 10 μg of pSG5-Ets-1, 3.3 μg of pSG5 Ets-1 ΔΔ5–6, 4.7 μg of pSG5-Gal4AD ΔΔ5–6, 10 μg of pSG5-Ets-1 ΔΔ5–6, and 10 μg of pSG5Gal4AD ΔΔ5–4. After 24 h the cells were harvested, and 100 μg of total cellular protein from each sample was analyzed by Western blot utilizing a primary antibody directed against the carboxyl terminal of human Ets-1 (PA-94). The blots were analyzed using pico-West chemiluminescent substrate (Pierce) and autoradiography or ECL Plus and the Storm system, respectively. Because this antibody also detects endogenous Ets factors the specific expressed protein in each lane is indicated with an asterisk. Right panel, HeLa cells were transiently transfected with 50 ng of pCGN2-Pit-1 and 12.5 ng of pCGN2-Pit-1 Δ2–80. After 24 h the cells were harvested, and 100 μg of total cellular protein from each sample was analyzed by Western blot utilizing a primary antibody directed against the hemagglutinin tag (Santa Cruz Biotechnology). The blot was analyzed using pico-West chemiluminescent substrate and autoradiography.
Selective Ets-1/Pit-1 TADs Mediate Ras/Synergistic Responses

The Spatial Requirements for the Ets-1/Pit-1 Composite Element Are Flexible—The spatial flexibility of an EBS relative to the binding site of its vicinal partner has been examined in detail only in a few composite cis-acting elements (19, 36, 37). The best studied is the serum response element, in which randomized DNA sequences were inserted upstream of a canonical SRF binding sequence (CArG box), and after several rounds of electrophoretic mobility shift assay selection for ternary complex formation, it was found that EBS sequences were enriched in the upstream region. In this in vitro gel shift assay, both the spacing and orientation of the EBS sequences relative to the SRF site in the serum response element could be altered substantially without affecting the efficiency of ternary complex formation (19). By contrast, using functional reporter gene assays, certain artifically constructed Ets composite elements (e.g. Ets/AP-1 or Ets/Ets) displayed a very limited flexibility with respect to spacing and/or orientation, when tested for basal activity and Ras responsiveness (36, 37). The natural separation between the EBS and FPIV is about 8 bp (Fig. 2A), and here we found that both the Ras and synergistic responses of the rPRL promoter were reduced significantly only when the spacing was increased from 28 to 32 bp, suggesting a relatively flexible configuration of the EBS and FPIV sites. Additionally, functional responses did not appear to correlate with the helical phasing of the binding sites relative to each other (Fig. 2, B and C), and reversing the orientation of the Pit-1 binding site also failed to decrease functional responses (data not shown). These spacing data, together with those in the literature (19, 36, 37), raise the question as to which parameters actually define a composite element. For example, the spacing limit of 28 and 32 bp for EBS and FPIV found here is in agreement with the 23–26-bp limit found for the EBS and serum response element in the in vitro ternary complex formation studies (19). However, these results are in contrast to other functional data, showing that spacing and orientation of an EBS relative to either an AP-1 or other EBS sites are quite limited (36, 37). Further challenging the definition of an EBS/Pit-1 composite element is the observation that the ancestrally related rat growth hormone gene promoter also contains Pit-1 binding sites as well as putative Ets binding sites within 32 bp, yet fails to display either Ras or synergistic responses (10, 11). Taken together, these data indicate that promoter context plays a significant role in defining a composite element. Thus, the overall DNA sequence composition of the promoter (AT- versus GC-rich), the position of the composite element relative to other cis-sites, the sequence identity of each component of the composite element, and the specific proximal promoter structure (e.g. CAAT, TATA, and initiator sequences), may all contribute to the maximal synergistic activity of any putative composite element (27, 38–40).

Physical Binding of Ets-1 to Pit-1 Is Not a Primary Determinant for Ras Responsiveness or Synergy—The assembly of transcription factors on composite elements is typically driven by vicinal DNA sites that bind these factors via protein-DNA interactions and stabilized via protein-protein interactions (37, 41, 42). We have shown previously by GST interaction assays and NMR analysis that the homeodomain of Pit-1 (amino acids 199–291) interacts directly with the RIII TAD of chicken c-Ets-1 (amino acids 190–257), albeit weakly (Kd ~300 μM) (12, 13). Here we show by three separate approaches for altering

ious Pit-1 binding sites analyzed thus far, all are functional in the Ras response and synergy assays.

The Spatial Requirements for the Ets-1/Pit-1 Composite Element Are Flexible—The spatial flexibility of an EBS relative to the binding site of its vicinal partner has been examined in detail only in a few composite cis-acting elements (19, 36, 37). The best studied is the serum response element, in which randomized DNA sequences were inserted upstream of a canonical SRF binding sequence (CArG box), and after several rounds of electrophoretic mobility shift assay selection for ternary complex formation, it was found that EBS sequences were enriched in the upstream region. In this in vitro gel shift assay, both the spacing and orientation of the EBS sequences relative to the SRF site in the serum response element could be altered substantially without affecting the efficiency of ternary complex formation (19). By contrast, using functional reporter gene assays, certain artifically constructed Ets composite elements (e.g. Ets/AP-1 or Ets/Ets) displayed a very limited flexibility with respect to spacing and/or orientation, when tested for basal activity and Ras responsiveness (36, 37). The natural separation between the EBS and FPIV is about 8 bp (Fig. 2A), and here we found that both the Ras and synergistic responses of the rPRL promoter were reduced significantly only when the spacing was increased from 28 to 32 bp, suggesting a relatively flexible configuration of the EBS and FPIV sites. Additionally, functional responses did not appear to correlate with the helical phasing of the binding sites relative to each other (Fig. 2, B and C), and reversing the orientation of the Pit-1 binding site also failed to decrease functional responses (data not shown). These spacing data, together with those in the literature (19, 36, 37), raise the question as to which parameters actually define a composite element. For example, the spacing limit of 28 and 32 bp for EBS and FPIV found here is in agreement with the 23–26-bp limit found for the EBS and serum response element in the in vitro ternary complex formation studies (19). However, these results are in contrast to other functional data, showing that spacing and orientation of an EBS relative to either an AP-1 or other EBS sites are quite limited (36, 37). Further challenging the definition of an EBS/Pit-1 composite element is the observation that the ancestrally related rat growth hormone gene promoter also contains Pit-1 binding sites as well as putative Ets binding sites within 32 bp, yet fails to display either Ras or synergistic responses (10, 11). Taken together, these data indicate that promoter context plays a significant role in defining a composite element. Thus, the overall DNA sequence composition of the promoter (AT- versus GC-rich), the position of the composite element relative to other cis-sites, the sequence identity of each component of the composite element, and the specific proximal promoter structure (e.g. CAAT, TATA, and initiator sequences), may all contribute to the maximal synergistic activity of any putative composite element (27, 38–40).

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the Pit-1 interaction motif in Ets-1, a BPV scanning mutagenesis scheme, an Ets-2 substitution method, and a deletion approach, that despite significant loss of physical interaction (~90%) in vitro, these mutants retained essentially WT levels of Ras responsiveness and transcriptional synergy in vivo (Figs. 3–6). One possible interpretation of these data is that the in vivo reporter assays are measuring a complex array of interactions that include chromatin reorganization, complexity of the promoter, transcription factor-DNA and transcription factor-coactivator interactions, making it difficult to identify the functional impact of a single type of interaction, whereas the GST pull-down assay is limited to transcription factor-transcription factor interactions. Alternatively, another interpretation is that direct physical interaction of Ets-1 and Pit-1 is not critical for these responses in vivo.

Optimal Synergy Is Conferred by the Assembly of Select TADs—In general, the key mechanism of action of composite elements appears to be the localized assembly of multiple TADs, generating several juxtaposed activation surfaces that recognize transcriptional coactivators better (43–45). However, the precise contribution and specificity of each TAD for the synergistic response previously have not been examined systematically. Here we show that in the transcriptional synergy assay, retention of a single TAD linked to Ets-1 is sufficient to reconstitute Ets-1/Pit-1 synergy, i.e. the Pit-1 amino-terminal TAD is not required for synergistic responses (Figs. 7 and 8). This suggests that a secondary TAD within the Pit-1 homeodomain may be responsible for the synergistic response.

Moreover, we also demonstrate that the three different classes of TADs represented by Ets-1, Gal4, and VP16 generate distinct hierarchies of cooperativity with Pit-1, based on whether intact Pit-1 or Pit-1Δ2–80, devoid of its TAD, is used as the Ets-1 partner. Specifically, for intact Pit-1, the TAD rank order for synergistic activation is: VP16 = Ets > Gal4. In contrast, for Pit-1Δ2–80, the TAD rank order is: Ets > Gal4 > VP16 (Figs. 6 and 7). These data show that the Pit-1 TAD (amino acids 2–80) is required for the VP16 TAD to display maximal synergy, yet this same domain of Pit-1 is dispensable for optimal synergy with Ets-1 and Gal4. This differential requirement of the Pit-1 TAD for maximal cooperativity with the VP16 TAD implies that a distinct mechanism of transcription activation is used by the VP16-Ets/Pit-1 combination. One possible explanation for the optimal synergy of the VP16-Ets fusion with full-length Pit-1 (7:1-fold synergy) compared with the less than optimal synergy with Pit-1Δ2–80 (2.9-fold synergy) may be that the VP16 TAD linked to Ets-1 is only able to cooperate with the amino-terminal TAD and is unable to cooperate with the homeodomain TAD, whereas the Ets-1 and Gal4 TADs appear to be able to cooperate with the homeodomain TAD alone.

The Pit-1 TAD (Amino Acids 2–80) Can Be Subdivided into Modulatory and Ras-responsive Subdomains—The precise role of the Pit-1 amino-terminal TAD (amino acids 1–80) appears to be quite complex. Based on the summary of our data presented in Fig. 9, we propose that amino acids 1–45 function as a modulatory region 1 (R1) and amino acids 46–80 function as an effector region 2 (R2) (Fig. 9). Specifically, deletion of both R1 and R2 (Pit-1Δ2–80) results in modest basal activity, no Ras response, and essentially a WT synergistic response (Figs. 7–9). Addition of the R2 region alone, however, doubles the basal response, confers the Ras response, but diminishes the synergistic response. Finally, further addition of the R1 region, generating the WT Pit-1, results in a reduction in basal activity, a persistence of the Ras response, and a gain in the strength of the synergistic response. This summary reveals that the R1 region has both negative and positive effects on the basal and synergistic responses, respectively, whereas the R2 region is required for the Ras response.

Taken together, the data show remarkable flexibility in the

| Pit-1Δ2-80 | 81 POU HOMEHO | + | - | ++ |
|-----------|---------------|---|---|----|
| Pit-1Δ2-45 | 46 POU HOMEHO | +++ | + | + |
| Pit-1      | 291 R1 R2     | ++ | + | ++ |

Fig. 9. Summary of basal activity and Ets-1 synergy in HeLa cells and enhancement of Ras responsiveness of GH4T2 cells. Deletion of both R1 and R2 (Pit-1Δ2–80) results in modest basal activity, no Ras response, and essentially a WT synergistic response. Addition of the R2 region alone doubles the basal response, confers the Ras response, but diminishes the synergistic response. Finally, further addition of the R1 region, generating the WT Pit-1, results in a reduction in basal activity, a persistence of the Ras response, and a gain in the strength of the synergistic response. Thus, the R1 region has both negative and positive effects on the basal and synergistic responses, respectively, whereas the R2 region is required for the Ras response.

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Taken together, the data show remarkable flexibility in the
organization of the Ets-1/Pit-1 composite element with regard to both Ras responsiveness and Ets-1/Pit-1 transcription synergies. However, the data also reveal that the Ras and synergistic responses are mediated by the specific assembly of select activation domains on this composite element, indicating that distinct mechanisms underlie these two transcriptional effects.

Acknowledgments—We acknowledge gratefully Anne Hartley, Mostafa Mohammed, and Kelley Brodsky for technical assistance in these studies and James Hagman, James Goodrich, and David Gordon for critical reading and discussions of this manuscript.

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