The PEA3 Ets Transcription Factor Comprises Multiple Domains That Regulate Transactivation and DNA Binding*

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PEA3, a member of the Ets family of transcription factors, is a nuclear phosphoprotein capable of activating transcription. Mouse PEA3 comprises 480 amino acids and bears an ~85-amino acid ETS domain near its carboxyl terminus. Whereas analyses of bacterially expressed PEA3 revealed that the ETS domain is required for sequence-specific DNA binding, little is known of the functional domains in the protein required for its activity in mammalian cells. To this end, we defined the location of the PEA3 functional domains in COS cells. PEA3 bears a strong activation domain near its amino terminus, which is flanked by two regions that independently negatively regulate its activity. PEA3 expressed in COS cells was incapable of binding to DNA in vitro. However, DNA binding activity could be unmasked by incubation with a PEA3-specific antibody. Analyses of the DNA binding activity of PEA3 deletion mutants revealed that two regions flanking the ETS domain independently inhibited DNA binding; deletion of both regions was required to detect DNA binding in the absence of a PEA3-specific antibody. Under these conditions, the ETS domain was sufficient for sequence-specific DNA binding. These findings suggest that the activity of PEA3 is exquisitely controlled at multiple functional levels.

The Ets family of transcription factors are defined by an evolutionarily conserved ~85-amino ETS domain (1). These proteins are found exclusively in multicellular organisms and are thought to play cardinal roles in development and oncogenesis (2). Multiple ets genes have been identified in individual organisms; over 20 mammalian genes have been discovered thus far (3). Ets proteins are sequence-specific DNA-binding proteins that regulate transcription (reviewed in Ref. 2). Generally, these proteins activate transcription, but several members of the family are known to repress this process. DNA binding is achieved by interaction between the ETS domain and an ~10-base pair sequence element termed the ETS binding site comprising a highly conserved central core sequence, 5’-GGA(ATT)-3’. Individual Ets proteins demonstrate specificity for sequences flanking this core, but it is not uncommon for different Ets proteins to bind to the same ETS binding site. Structural analyses of the ETS domain reveal a winged-helix-turn-helix structure akin to that of the Escherichia coli catabolite activator protein and the HNF3/forkhead and heat shock transcription factors (4–7).

Mouse pea3 (8) (the human gene is named ETV4 and has also been termed EIA-F (9, 10)) is the founding member of the pea3 subfamily of ets genes. This subfamily also includes er81 (ETV1) (11–13) and erm (ETV5) (14, 15). Each of these genes is located on a different chromosome (16), but all three genes share a common architecture comprising 14 equivalently sized exons that encode similar sequences of the respective proteins (16–18). The overall amino acid sequence similarity of the PEA3 subfamily is ~50% (17). The three longest stretches of greatest sequence similarity include the ~85-amino acid ETS domain (95% sequence identity); an acidic region near the amino terminus composed of 32 amino acids (85% sequence identity); and a region at the carboxyl terminus comprising ~60 amino acids (50% sequence identity) (17).

PEA3 is overexpressed in breast tumors both in humans and mice, suggesting a role for PEA3 in this malignancy (19, 20). 76% of all human breast tumors contain elevated levels of PEA3 RNA; 93% of the c-ERB-B2/Her2-positive subclass of these tumors overexpress PEA3 (20). PEA3 is also overexpressed in mouse mammary tumors arising in transgenic mice engineered to express murine c-erb-B2/Her2 in their mammary glands (19).

Chromosomal translocations involving the PEA3 subfamily genes have been implicated in a minority of Ewing’s sarcomas in humans (12, 21, 22). The same region of EWS is translocated and juxtaposed to sequences encoding the ETS domain of the FLI-1 ETS gene, which is the most common translocation in this disease (23). EWS-FLI-1 chimeric genes encode EWS-FLI-1 fusion proteins that bear the ETS domain. The transcriptional activity of these chimeric proteins significantly supersedes that of FLI-1, and, unlike FLI-1, they induce transformation of a mouse 3T3 fibroblast cell line (24–27). These findings imply that the transcriptional activity of these chimeras is associated with their transforming and oncogenic potential.

Like other Ets proteins that have been studied, PEA3 binds to DNA with specificity and activates transcription of reporter plasmids bearing PEA3-responsive promoters (8). Analyses of glutathione S-transferase-PEA3 chimeras expressed in and purified from E. coli demonstrate that the ETS domain is required and sufficient for sequence-specific DNA binding as assessed in vitro by EMSA (8). However, the location of sequences required for transactivation and DNA binding in mammalian cells has not been investigated. To this end we constructed and measured the specific activity of a series of unidirectional amino and...
C-terminal deletion mutants of PEA3 and of GAL4-PEA3 chimeras in mammalian COS cells. Our analyses revealed that PEA3 bears a strong activation domain near its amino terminus that is flanked by two negative regulatory regions, which markedly repress its activity. PEA3 expressed in COS cells was incapable of binding to DNA. However, DNA binding activity could be recovered by incubation with PEA3-specific antibodies or by deleting two regulatory regions that flank the ETS domain. Hence, both the transactivation and DNA binding functions of PEA3 are negatively controlled, implying that a mechanism(s) exists to exquisitely regulate its activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (penicillin, streptomycin, and fungizone). The cells were grown at 37 °C in a humidified 5% CO2 atmosphere.

**Construction of Recombinant Plasmids and Isolation of Deletion Mutants**—The PEA3 expression vector (pRSV-PEA3) was constructed by cloning PEA3 cDNA sequences (8) between the HindIII and XbaI sites of pRc/RSV (Invitrogen). The amino-terminal deletion mutants were derived from pRSV-PEA3 by polymerase chain reaction amplification using suitable primers followed by molecular cloning of the product into HindIII–XbaI-cleaved pRc/RSV. The primers used to derive the amino-terminal deletion mutants all comprised a HindIII site and a translation initiation consensus sequence 5′-ACATGG-3′ (28, 29); the invariant sequence of all of the primers is 5′-GGAGGCTCACCAGT-3′ (the HindIII recognition site and the translation initiation codon are underlined). All of the polymerase chain reaction primers used to generate the C-terminal deletion mutants contained an XbaI restriction site and a stop codon (5′-TAG-3′); the complementary sequence of this primer is 5′-TAGAGATCTCC-3′ (the stop codon and XbaI cleavage site are underlined). A naturally occurring PEA3 codon precedes the stop codon in the mRNA of each C-terminal deletion mutant. The variable sequence of each primer used corresponded to that which flanks the deletions described above.

GA4-PEA3 chimeras were constructed by cloning polymerase chain reaction-amplified PEA3 sequences from RSV-PEA3 in frame between the BamHI and XbaI sites of pSG424 (30). Primers containing a stop codon were used to derive GAL4-PEA3 chimeras bearing C-terminal deletion mutants as described above.

The PEA3 response unit of the reporter plasmid (PEA3-luciferase) contained five copies of an optimal, high affinity, BM2 binding site, 5′-TGGCCGGAACCG-3′ (the core sequence of the Ets binding site is underlined). These sequences were cloned into the XbaI site of pGL3-Ad MLP. This reporter was derived by cloning a synthetic double-stranded oligonucleotide (5′-AGATCTCGAGCTCGAGGCGCATATAAAGGGGGATCTGAATTCGAGAAGCTT-3′) bearing the adenovirus type 2 major late promoter TATA box and flanking sequences between the BglII and HindIII sites of the pGL3 basic luciferase reporter plasmid (Promega).

The GA4 reporter plasmid (GAL4-luciferase) contained five copies of a GAL4 DNA binding sites cloned into the Smal I site of the pGL3-Ad MLP reporter (31). The junctions and inserts of all recombinant plasmids were sequenced on both strands to ensure that mutations were not introduced during polymerase chain reaction and molecular cloning.

**Assessment of the Transcriptional Activity of PEA3 and of GAL4-PEA3**—The transcriptional activity of PEA3, GAL4-PEA3, or their derivatives was assessed indirectly by measuring luciferase activity in equivalent amounts of lysate protein isolated from COS cells transfected with the appropriate luciferase reporter plasmid and either PEA3 or GAL4-PEA3 effector plasmid. This value was normalized to the amount of PEA3 or GAL4-PEA3 expression plasmid encoded by pRc/RSV-PEA3 encoding WT PEA3. The total amount of effector DNA was maintained at 0.8 μg by adding empty effector DNA (pRc/RSV). The total DNA concentration of each transfection mixture was adjusted to 2 μg with sheared salmon sperm DNA. The activity of the PEA3 deletion mutants was determined after cotransfection of 0.5 μg of the reporter plasmid with a single dose (0.8 μg) of the effector plasmid (pRc/RSV-PEA3) encoding WT PEA3. The specific activity of the various PEA3 deletion mutants was expressed relative to that of WT PEA3, which was set at 100%.

The activity of GAL4-PEA3 chimeras was assessed similarly, except that 0.25 μg of the GAL4 reporter plasmid was cotransfected with up to 0.5 μg of effector DNA encoding the various GAL4-PEA3 chimeric proteins. The total concentration of pSG424 DNA was adjusted to 0.5 μg with empty effector DNA. The specific activity of the various GAL4-PEA3 chimeras was assessed by measuring luciferase activity obtained from cells transfected with the reporter to that obtained from cells transfected with the reporter and effector plasmid encoding a particular GAL4-PEA3 chimera following normalization of the abundance of the chimera in equal amounts of lysate protein as described below.

Within an experiment, each DNA preparation was transfected in triplicate; two wells of a six-well dish were independently processed to measure luciferase activity, and two wells were independently used to determine the abundance of PEA3 or its derivatives by immunoblot analysis. Cell extracts for luciferase assays were prepared from each well using 200 μl of reporter lysis buffer (Promega catalog no. E3971) according to the manufacturer’s instructions. Luciferase activity was measured using luciferase assay reagent (Promega catalog no. E1950) in a luminometer (model TD-20/20, Turner Designs). The luminometer was set at a fixed emission channel of 800 nM with total light units per μg of total protein after subtracting the activity of the empty effector plasmid control.

To determine the abundance of PEA3 or its derivatives, the cell monolayers were washed in the wells with ice-cold phosphate-buffered saline and then scraped from the plates in 1 ml of phosphate-buffered saline. The cells were concentrated by centrifugation, resuspended in 50 μl of lysis buffer (1% Nonidet P-40, 50 mm Tris-HCl, pH 7.4, 5 mM EDTA, 400 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 2 μg/ml phenylmethylsulfonyl fluoride), and then incubated on ice for 25 min. The lysate was centrifuged for 5 min at 13,000 rpm at 4 °C in a microcentrifuge, and the supernatant was used for immunoblot analysis.

The total protein concentration of each sample was determined by the Bradford dye method using a kit provided by Bio-Rad with bovine serum albumin (Sigma) as the standard. Equal amounts of protein (20 μg of total protein from cell extracts or 8 μg of protein from nuclear extracts) from the duplicate samples were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane by electroblotting. The membrane was washed in distilled water and then blocked for 20 min at room temperature with phosphate-buffered saline containing 5% nonfat dry milk.

PEA3 and its deleted derivatives were detected on the membrane by use of one of several antibodies including one monoclonal antibody and two different polyclonal antibodies. The PEA3-specific monoclonal antibody is named MP16 and recognizes determinants between residues 173 and 226 of PEA3. The rabbit polyclonal antibodies PC2 and PC3 recognize epitopes at the extreme amino and carboxyl terminus of PEA3, respectively. PN1 was derived by injecting rabbits with a key-hole limpet hemocyanin-conjugated peptide corresponding to the first 22 amino acids of PEA3—

226 and 245.3 The rabbit polyclonal antibodies are termed PN1 and PC2. The PEA3-specific monoclonal antibody was assessed by measuring the ratio of luciferase activity obtained from cells transfected with the reporter to that obtained from cells transfected with the reporter and effector plasmid encoding a particular GAL4-PEA3 chimera following normalization of the abundance of the chimera in equal amounts of lysate protein as described below.

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2. S. Bowman and J. A. Hassell, manuscript in preparation.

3. L. Hastings and J. A. Hassell, unpublished results.

4. S. Arber and T. Jessell, personal communication.
15 amino acids of mouse PEA3, whereas PC2 was raised by injecting rabbits with a keyhole limpet hemocyanin-conjugated peptide bearing the C-terminal 15 amino acids of mouse PEA3.

A commercial rabbit polyclonal antibody reactive with the DNA binding domain of GAL4 was used to quantify the abundance of GAL4-PEA3 on immunoblots (Upstate Biotechnology, Lake Placid, NY, catalog no. 06-262). 

125I-Labeled goat anti-mouse or goat anti-rabbit IgG were used as secondary antibodies. Quantification of expression levels of PEA3 and GAL4-PEA3 chimeras was performed by use of a PhosphorImager (Molecular Dynamics) equipped with ImageQuant 3.3 software. In one series of experiments described here, the abundance of several GAL4-PEA3 chimeras (GAL4-PEA3dlC100, GAL4-PEA3dlN42dlC100, GAL4-PEA3dlC85 and GAL4-PEA3dlN42dlC85) was also measured using a rabbit polyclonal primary antibody reactive with the DNA binding domain of GAL4 (BAbCo; PBR-255C) and a secondary goat anti-rabbit antibody coupled to peroxidase (KPL; 474-1506) using chemiluminescence (PerkinElmer Life Sciences). Quantification of the amount of the chimeric proteins using these reagents was accomplished with a Kodak ImageStation and Kodak ID image analysis software version 3.0. Unless indicated otherwise in the figure legends, analyses of two amino-terminal truncation mutants revealed that deletion of 29 or 45 amino acids from this terminus reproducibly increased the specific transcriptional activity of the resulting proteins 2–3-fold, implying that these sequences negatively regulate PEA3 activity (Fig. 1B). Mutants with amino-terminal end points at residues 55, 65, 74, and 85 were progressively compromised in their capacity to stimulate luciferase expression by comparison with WT. Indeed, the specific activity of dln85 was only 10% that of the WT protein. These observations are consistent with the presence of positively acting sequences, perhaps an activation domain, in this amino-terminal region. Surprisingly, a mutant lacking the first 106 amino acids of PEA3 was 5-fold more active than dln85 and only 2-fold less active than WT PEA3. Hence, residues between amino acids 85 and 106 may comprise another negative regulatory region. The specific activity of several other progressively larger amino-terminal deletion mutants (dln120, dln130, and dln145) was similar to that of dln106, implying that residues between 106 and 145 play little role in the activity of PEA3. However, amino-terminal deletions extending to residues 226, 245, and 326 were increasingly reduced in their activity, consistent with the occurrence of another activation domain in this region. The ETS domain in PEA3 is located between residues 334 and 417; deletions impinging on the ETS domain abolished transcriptional activity probably by compromising DNA binding and consequently were not characterized further (Ref. 41; data not shown).

Four C-terminal deletion mutants were isolated and characterized (dIC450, dIC440, dIC430, and dIC420). Each of these was reproducibly more active (~2-fold) than the WT protein, implying that sequences between residues 480 (the C-terminal residue of PEA3) and 450 negatively regulate PEA3 activity. Taken together, the results of the analyses of both the amino- and C-terminal deletion mutants suggested the occurrence of both positively and negatively acting regions located outside of the borders of the PEA3 ETS DNA binding domain.

Analyses of GAL4-PEA3 Chimeras—The various deletion mutants described previously may have lesions affecting either the transactivation or the DNA binding function of PEA3. Deletions that increased or decreased PEA3 activity may have compromised negatively or positively acting domains affecting transactivation or negatively acting regions regulating DNA binding. To distinguish the effect of the mutations on each of these activities, we independently defined the borders of the PEA3 activation domain and those of its DNA binding domain. To map the location of the activation domain, we constructed GAL4-PEA3 chimeras and measured their capacity to effect the
expression of luciferase from a reporter plasmid containing repeats of a GAL4 DNA binding site located upstream of a minimal promoter. We could not detect the expression of the GAL4 DNA binding domain encoded by the expression plasmid pSG424 by immunoblotting. In consequence, we always included a transcriptionally active chimera (GAL4-PEA3dlC85) as a positive control in each experiment.

The GAL4-PEA3 chimera comprising full-length PEA3 was transcriptionally inert (Fig. 2). As demonstrated subsequently, this was true independent of its abundance in cells. The inability of GAL4-PEA3 to stimulate luciferase expression was not surprising, because it has been commonly observed that GAL4 chimeras harboring one of several different DNA binding domains are poor transcriptional activators. However, two different C-terminal deletion mutants (dlC317 and dlC267) lacking the ETS DNA binding domain were also impaired in their capacity to stimulate luciferase expression from the reporter. Unlike GAL4-PEA3, which was inactive, these C-terminal deletion mutants possessed measurable but low activity. Hence, the mere presence of the ETS domain in the GAL4-PEA3 chimera was not solely responsible for its lack of transcriptional activity.

Deletion of C-terminal residues distal to amino acid 267 increased the specific activity of the resulting chimeras and commensurately decreased their expression levels (Fig. 2, A and B). Indeed, a dramatic increase in transcriptional activity of nearly 50-fold occurred when C-terminal sequences between residues 120 and 85 were deleted. One interpretation of these findings is that there is a strong activation domain between residues 1 and 85 in PEA3, whose activity can be substantially inhibited by a regulatory region located between amino acids 85 and 120.

It is noteworthy that analyses of amino-terminal deletion mutants of PEA3 also suggested the occurrence of a negative regulatory region between residues 85 and 106 capable of suppressing the activity of an activation domain located C-terminal of residue 106 (Fig. 1, A and B).

We noted that transcriptionally inactive GAL4-PEA3 was expressed at 20-fold higher levels than transcriptionally active
FIG. 2. Transcriptional activity of GAL4-PEA3 chimeras. A, schematic illustrating the structure of the GAL4-PEA3 chimeras. Each chimera bears the GAL4 DNA binding domain and either all of PEA3 or various amino-terminal portions of PEA3. The abundance (relative expression) of the various GAL4-PEA3 chimeras relative to that of GAL4-PEA3diC85 (ΔC85), which was set as 1.0, is shown. Similarly, the specific activity of the GAL4-PEA3 chimeras by comparison with that of GAL4-PEA3diC85 is also shown. The data from four independent experiments were averaged. B, representative immunoblot of the abundance of the GAL4-PEA3 chimeras in COS cell lysates resulting from a single experiment. Equal amounts of total cellular protein (30 μg) was loaded onto the gel prior to immunoblotting. Protein samples were prepared from COS cells transfected with 0.5 μg of the pSG424-PEA3 expression vectors encoding ΔC317 (lane 1), ΔC267 (lane 2), ΔC177 (lane 3), ΔC128 (lane 4), ΔC120 (lane 5), ΔC85 (lane 6), ΔC55 (lane 7), and GAL4-PEA3 (lane 8). The GAL4-PEA3 chimeras were detected with an antibody that recognizes determinants in the GAL4 DNA binding domain.

GAL4-PEA3diC85 (Fig. 2B). This suggested the potential that the apparent inactivity of GAL4-PEA3 may have been due to transcriptional squelching when expressed in cells at high levels. To test this possibility, we varied the amount of the expression vector encoding these two GAL4-PEA3 fusion proteins in transfection assays and compared their capacity to stimulate luciferase expression when present in cells at equivalent levels (Fig. 3). This comparison showed that under conditions where both proteins were expressed at similar levels, GAL4-PEA3diC85 was active, whereas GAL4-PEA3 was not. Indeed, GAL4-PEA3 was inactive when expressed over a 10-fold range. Hence, it would appear that GAL4-PEA3 is inert and that its inability to transactivate transcription does not result from squelching.

Two Regions Flanking the Amino-terminal Activation Domain Independently Negatively Regulate Its Activity—To further refine the location of the PEA3 activation domain and putative negative regulatory regions affecting transactivation, we isolated amino-terminal deletion mutants of the GAL4-PEA3 chimera bearing the first 85 amino acids of PEA3 (Fig. 4, A and B). This chimera, GAL4-PEA3diC85, possesses the putative amino-terminal negative regulatory region but lacks the candidate inhibitory sequences flanking the C-terminal border of the activation domain. Deletion of the first 23 or 42 residues from GAL4-PEA3diC85 progressively increased the activity of the resulting GAL4-PEA3 chimeras to levels 6- and 20-fold greater than that of GAL4-PEA3diC85. It is noteworthy that deletion of this region from PEA3 also increased the activity of the resulting amino-terminal deletion mutants (Fig. 1, A and B; compare PEA3ΔN29 and PEA3ΔN45 with PEA3). Another amino-terminal deletion mutant of GAL4-PEA3diC85 lacking the first 55 residues of PEA3 (GAL4PEA3ΔN55diC85) was substantially reduced in its capacity to activate transcription, suggesting that this deletion impinged on the activation domain (Fig. 4A). We interpret these results to indicate that sequences between residues 1 and 42 independently inhibit the activity of an activation domain whose amino-terminal border lies between residues 42 and 55 and whose C-terminal border is located near amino acid 85.

To learn whether the candidate negative regulatory region flanking the C-terminal border of the activation domain could also function to independently inhibit the activity of the activation domain and to refine the location of these sequences, we isolated additional C-terminal GAL4-PEA3 chimeras and measured their specific activity (Fig. 4, C and D). By comparison with GAL4-PEA3diC85, GAL4-PEA3diC100 was severely impaired in its capacity to activate luciferase expression. Indeed, GAL4-PEA3diC100 possessed very low activity akin to that of GAL4-PEA3diC120 (Fig. 2A). This suggests that the C-terminal border of this negative regulatory region is located between residues 100 and 85.

FIG. 3. GAL4-PEA3 is transcriptionally inactive. A, table illustrating the activity and abundance of GAL4-PEA3 chimeras. COS cells were transfected in duplicate with 500 ng of pSG424, which codes for the GAL4 DNA binding domain (Gal4 DBD), with 20, 50, or 100 ng of the vector encoding GAL4-PEA3 (PEA3) or with 100, 250, or 500 ng of that encoding GAL4PEA3diC85 (PEA3ΔC85). Cell lysates from the transfected cells was used to measure luciferase activity, which was recorded as relative light units. The abundance of the GAL4 chimeras in 30 μg of cell protein from cells transfected in the same experiment was expressed in arbitrary units relative to that of GAL4-PEA3diC85 (set to 1) following PhosphorImager analysis. B, representative immunoblot of a single experiment carried out in duplicate. COS cells were transfected with various amounts of the expression vector encoding either GAL4-PEA3 or GAL4-PEA3diC85 as described above. The chimeras were detected with an antibody that recognizes determinants in the GAL4 DNA binding domain.
Deletion of the amino-terminal 42 residues of PEA3, corresponding to the amino-terminal negative regulatory region, from GAL4-PEA3Δ1C100 stimulated the activation potential of the resulting chimera 15-fold (Fig. 4C). However, this chimera (GAL4-PEA3ΔN42ΔC100) was only 1% as active as GAL4-PEA3ΔN42ΔC85, which lacks both negatively acting regulatory regions (Fig. 4, compare A and C). Hence, the 15 residues between amino acids 85 and 100 potently and independently inhibit the activity of the activation domain. Taken together, these findings suggest that two negatively acting regions residing approximately between amino acids 1 and 42 and between amino acids 85 and 100 act independently to suppress the activity of a strong activation domain located between residues 42 and 85.

We noted that several of the aforementioned GAL4-PEA3 chimeras were expressed at comparatively different levels in transfected COS cells; this might have affected assessment of their specific transcriptional activity. Hence, to verify the results reported above, we determined the activity of these chimeras when expressed at approximately the same concentration in COS cells. This condition was achieved by varying the amount of transfected DNA encoding each GAL4-PEA3 chimera (Fig. 5). Transfection of differing amounts of the expression vectors encoding each GAL4-PEA3 chimera (Fig. 5A) led to a linear dose-dependent increase in the abundance of each chimera (Fig. 5B) and to a dose-dependent increase in luciferase activity expressed from the GAL4 reporter plasmid. These findings substantiate our contention that the amino-terminal 100 residues of PEA3 comprise two negative regulatory elements that flank a central activation domain; each negative regulatory region was independently capable of inhibiting the activity of the activation domain.

**Only the Amino-terminal Activation Domain Functions in the Context of the GAL4 DNA Binding Domain to Activate Transcription**—The analysis of the transcription activity of PEA3 deletion mutants suggested the potential that PEA3 might possess at least two activation domains, one near its amino terminus (residues 42–85) and another in the central portion of the protein. The occurrence of the centrally located activation domain was inferred from the observation that PEA3 deletion mutants lacking the amino-terminal activation domain (and both flanking inhibitory regions) were only 2-fold reduced in activity compared with WT PEA3 (Fig. 2). For example, PEA3ΔN106, ΔN120, ΔN130, and ΔN145 were ~50% as active as WT PEA3. Furthermore, progressive deletion of amino-terminal residues from amino acids 145–226 reduced the activity of the resulting deletion mutant 10-fold to ~5% that of WT. Other amino-terminal deletion mutants with end points at residues 245, 278, and 326 were similarly debilitated. These findings are consistent with the occurrence of another activation domain in PEA3 with an amino-terminal border between residues 145 and 226.

To learn whether this central portion of the protein harbored an activation domain, we isolated a GAL4-PEA3 chimera devoid of the amino-terminal activation domain and flanking...
was recorded as relative light units expressed per 100, 250, or 500 ng of the indicated expression vector. The abundance of these chimeras following transfection of cells with differing amounts of the various expression vectors. COS cells were transfected with 100, 250, or 500 ng of the indicated expression vector. The abundance of the GAL4-PEA3 chimeras in 50 ng of cell protein was assessed following transfection by use of a chemiluminescence protocol as described under “Experimental Procedures.” The abundance of the chimeras was expressed in arbitrary units. C, table illustrating the activity and abundance of the various GAL4-PEA3 proteins following transfection of COS cells. An approximately equal amount (5 μg) of cell protein from transfected cells was used to measure luciferase activity, which was recorded as relative light units expressed per μg of cell protein. The abundance of the GAL4 chimeras in 50 μg of cell protein from cells transfected in the same experiment was expressed in arbitrary units.

negative regulatory regions but including residues bearing this putative activation domain. This chimera (GAL4-PEA3Δ147) contained the region between residues 147 and 480 in PEA3. We also isolated five carboxyl deletion mutants of this chimera with end points at residues 420, 375, 326, 276, and 226. All of these chimeras were expressed, but none of them stimulated luciferase expression from the GAL4-responsive reporter plasmid as assessed by this assay. It is conceivable that this central portion of PEA3 apparently does not possess an independent activation domain as assessed by this assay. It is conceivable that this central region and others in the ETS domain or in the C-terminal region of PEA3 are required together to constitute an activation domain. However, because all the GAL4 chimeras bearing the ETS domain that we have constructed are inactive, we have been unable to verify this possibility.

The Latent DNA Binding Activity of PEA3 Is Unmasked by PEA3-specific Antibodies—We showed previously that the PEA3 ETS domain, expressed as a GST fusion protein in E. coli, is necessary and sufficient for sequence-specific DNA binding in vitro (8). To learn whether this was also true of the native PEA3 protein isolated from mammalian cells, we measured the capacity of the WT and various amino and C-terminal deletion mutants expressed in COS cells to bind to an optimized PEA3 binding site by EMSA. Initially, we compared the DNA binding activity present in untransfected COS cells with that present in COS cells transfected with the PEA3 expression vector. An activity capable of binding to an optimized PEA3 binding was observed in nuclear lysates of untransfected COS cells (Fig. 6; band labeled B). However, COS cells do not express PEA3 protein at levels detectable by immunoblot analysis (Fig. 1, C and D), and hence this species is unlikely to be due to endogenous simian PEA3. We suspect that it represents the binding of some other Els protein endogenous to COS cells. Unexpectedly, no new protein-DNA complexes were detected when lysates from COS cells transfected with the PEA3 effector plasmid were used in these assays (Fig. 6, lane 5). Indeed, the same endogenous protein-DNA species was detected at the same relative abundance in nuclear lysates prepared from COS cells transfected with the PEA3 expression vector as assessed in the EMSA. Our inability to detect a novel PEA3-DNA complex using lysates from transfected COS cells in this assay was not due to the failure of PEA3 to be expressed in these cells (Fig. 1C; data not shown). Hence, despite the presence of PEA3 in the lysates, this protein was apparently incapable of binding to DNA.

A number of different explanations could account for this observation. For example, the PEA3-DNA complex and that formed between the endogenous protein and DNA may comi-
gate in the gel system used here. Alternatively, PEA3 may not have been expressed at high enough levels to detect its binding to DNA using this assay. Or PEA3 ectopically expressed in COS cells may not bind to DNA. In an initial attempt to distinguish among these possibilities, we included a PEA3-specific antibody in the DNA binding assays. Preincubation of nuclear lysates from untransfected COS cells with a PEA3-specific monoclonal antibody (MP16), a PEA3-specific polyclonal antibody (PC2) or a control Myc-specific monoclonal antibody did not alter the electrophoretic mobility of the protein-DNA complex due to the endogenous activity (Fig. 6, compare lane 1 with lanes 2–4). This finding too suggests the absence of PEA3 in COS cell lysates. By contrast, both PEA3-specific antibodies effected or stabilized the binding of a nuclear protein to the optimized PEA3 binding site after incubation with nuclear extracts prepared from transfected COS cells (compare lane 5 with lanes 6 and 7). This species was not detected when a Myc-specific antibody was preincubated with lysates containing PEA3 (lane 8). Therefore, the novel protein-DNA species detected in lysates from COS cells transfected with the PEA3 effector plasmid probably represents a PEA3-antibody complex bound to DNA. We show subsequently that the electrophoretic mobility of this complex varies with the size of PEA3 encoded by various deletion mutants, providing additional evidence that it comprises PEA3, the PEA3-specific antibody, and DNA.

The requirement for PEA3-specific antibodies to detect DNA binding by PEA3 in COS cell nuclear lysates may have been due to the abundance of PEA3 in these extracts. The antibody may stabilize the binding of low quantities of PEA3 in the lysates to DNA. To investigate this potential, we compared the binding of equivalent amounts of PEA3 expressed in COS cells with that of GST-dlN145PEA3 isolated from E. coli (Fig. 7). We used an amino-terminal truncated version of GST-PEA3 lacking the first 144 residues of PEA3 in these analyses primarily because it is more stable during isolation from E. coli than is a GST fusion protein comprising full-length PEA3, thereby facilitating calculation of its specific DNA binding activity.

GST-dlN145PEA3 bound to the optimized PEA3 binding site in a dose-dependent fashion without a requirement for preincubation with a PEA3-specific antibody (Fig. 7A). Measurement of the specific DNA binding activity of this GST-PEA3 fusion protein in the absence and presence of this antibody revealed that preincubation of the fusion protein with the PEA3-specific antibody increased DNA binding between 2- and 3-fold (Fig. 7, A–C). By comparison, the same amount of PEA3 in nuclear lysates from COS cells was incapable of binding to DNA unless it was preincubated with a PEA3-specific antibody. These observations suggest that the DNA binding activity of PEA3 in COS cell nuclear lysates is inhibited and that preincubation of the lysate with PEA3-specific antibodies unmasks its DNA binding activity.

Two Modules Flanking the PEA3 ETS Domain Independently Negatively Regulate DNA Binding—Quantification of the data presented in Fig. 7 revealed that expression of PEA3 in COS cells over a 10-fold range resulted in a linear increase in DNA binding activity that was proportional to the amount of PEA3 protein in the nuclear lysates (Fig. 7C). None of the PEA3 amino- and carboxyl-truncated deletion mutants analyzed previously (Fig. 1) were expressed at less than 10% the level of the WT protein, suggesting that we could quantitatively compare the DNA binding activity of PEA3 with that of its mutant forms. In consequence, we transfected expression vectors encoding PEA3 and its deleted derivatives into COS cells and tested their DNA binding capacity by EMSA in the presence of a PEA3-specific antibody. A polyclonal antibody recognizing the C-terminal residues of PEA3 was used to analyze the amino-terminal deletion mutants, whereas a monoclonal antibody, which recognizes a centrally located epitope, was used to characterize the C-terminal deletion mutants. The results of three separate experiments are illustrated in Fig. 8, A–C. The top portion of each panel illustrates an EMSA, whereas the bottom portion of each panel represents a corresponding immunoblot of PEA3 levels in the lysates from the same experiment. Duplicate samples bearing equal amounts of total nuclear protein from independently transfected cultures were assayed. The structure of the PEA3 deletion mutants is illustrated in D, and a summary of the results of several independent repetitions of each experiment is shown in E.

All but one of seven amino-terminal deletion mutants was capable of binding to DNA as well as the WT protein. Only
PEA3dIN85, which was expressed at ~10% the level of WT PEA3, was reduced in its DNA binding activity to levels 50% that of PEA3. Deletion of amino-terminal residues up to amino acid 226 did not affect DNA binding, suggesting that these sequences play little role in this process. Interestingly, three mutants, PEA3dIN245, PEA3dIN278, and PEA3dIN326, reproducibly bound DNA 2–4-fold better than did the WT protein, suggesting the occurrence of sequences that negatively regulate DNA binding between residue 245 and the amino-terminal border of the ETS domain. It is also noteworthy that whereas these four amino-terminal deletion mutants (PEA3dIN226, PEA3dIN245, PEA3dIN278, and PEA3dIN326) bound to DNA as well as or better than WT PEA3, each of these was nonetheless severely compromised in its capacity to activate transcription (compare Fig. 1, A and B, with Fig. 7, D and E). This suggests that residues between 226 and 480, comprising the C-terminal half of PEA3, do not harbor an independent activation domain.

We also analyzed the DNA binding activity of four C-terminal deletion mutants (Fig. 9). Deletion of the C-terminal 30 amino acids of PEA3 increased DNA binding ~2-fold. Three other C-terminal deletion mutants displayed similarly increased DNA binding capacity that varied between 2- and 3-fold that of WT PEA3. It is noteworthy that each of these mutants also possessed commensurately increased transcriptional activity (2–3-fold) by comparison with PEA3 (Fig. 1). Hence, residues C-terminal of the ETS domain of PEA3 also appeared to negatively regulate DNA binding and apparently play no role in transcription activation.

We inferred the occurrence of two regions in the PEA3 protein flanking the ETS domain that inhibit DNA binding. The amino-terminal border of one of these is located between residues 245 and 278, whereas the carboxyl terminus of the other is located between residues 480 and 450. In independent experiments, we found that the DNA binding activity of deletion mutants bearing either of these negative regulatory regions was manifest only in the presence of PEA3 specific antibody (data not shown). Hence, deletion of only one of these two regions did not relieve the requirement for a PEA3-specific antibody to uncover PEA3 DNA binding activity. To learn whether a PEA3 mutant lacking both inhibitory sequences could bind to DNA in the absence of a PEA3-specific antibody, we isolated such a mutant, PEA3dIN315dlC417, and measured the capacity of the encoded protein to bind to DNA by EMSA following its expression in COS cells.

As shown previously, WT PEA3 in COS cell nuclear lysates was incapable of binding to DNA unless preincubated with a PEA3-specific antibody (Fig. 10, compare lanes 1 and 2 with...
lanes 3 and 4). By contrast, PEA3ΔN315ΔC417, comprising essentially only the PEA3 ETS domain, was capable of binding to DNA without prior incubation with a PEA3-specific antibody (lanes 5 and 6). Hence, our analyses of the DNA binding activity of PEA3 deletion mutants suggest the occurrence of two DNA binding inhibitory regions flanking the ETS domain that function independently to block DNA binding. Deletion of both regions is required to unmask PEA3 DNA binding activity.

**DISCUSSION**

**PEA3 Comprises an Amino-terminal Acidic Activation Domain, Which Is Flanked by Modules That Independently Negatively Regulate Its Activity**—A summary of our analyses of both the specific transcriptional and DNA binding activity of PEA3 deletion mutants is shown in Fig. 11. Deletion of the first 45 amino acids of PEA3 increased its transcriptional activity but did not affect its DNA binding activity. This finding is consistent with the occurrence of sequences that negatively regulate transcription activation in this region (residues 1–45). By contrast, amino-terminal truncations with end points at residues 55, 65, 74, and 85 were progressively and markedly reduced in their capacity to activate transcription but were essentially unaffected in their capacity to bind to DNA. Hence, the region between residues 45 and 85 probably comprises an activation domain. Like several other activation domains, this region is rich in acidic amino acids. Surprisingly, extension of the amino-terminal deletions to residues 106, 120, 130, and 145 progressively increased the transcription activity of PEA3 by comparison with PEA3ΔN85, which was essentially devoid of activity. Indeed, the transcriptional activity of PEA3ΔN106, PEA3ΔN120, PEA3ΔN130, and PEA3ΔN145 varied between 40 and 60% that of the WT protein. None of these mutants were compromised in their capacity to bind to DNA. These observations are consistent with yet another negative regulatory region C-terminal of residue 85 affecting transcription activation but not DNA binding. This finding also suggested the occurrence of another activation domain C-terminal of residue 145. This second activation domain must include sequences between residues 145 and 226, because PEA3ΔN226, unlike PEA3ΔN145, was significantly reduced in its capacity to activate transcription but possessed DNA binding activity equivalent to that of WT PEA3. Collectively, our observations are consistent with the occurrence of two negative regulatory regions and two activation domains within the first 226 amino acids of PEA3. These sequences appear to play no role in DNA binding, because their deletion did not affect this process.

This interpretation is largely supported and extended by our analyses of GAL4-PEA3 chimeras. Analyses of such chimeras confirmed the occurrence of two negative regulatory regions affecting transcription activation flanking a strong activation domain.
domain in PEA3. The negative regulatory regions mapped between residues 1 and 42 and between residues 85 and 100, respectively, whereas the activation domain was located between residues 42 and 85. Chimeras harboring this acidic activation domain and either one or the other negative regulatory module (GAL4-PEA3dlC85 or GAL4-PEA3dlN42dlC100) were much less active than the chimeras bearing only the acidic activation domain (GAL4-PEA3dlN42dlC85) (Figs. 4 and 5). Therefore, the function of the activation domain was potently and independently inhibited by each of the negative regulatory regions. It will be interesting to learn whether these regulatory regions function through an intramolecular or intermolecular mechanism and whether they demonstrate specificity for particular activation domains.

Analyses of amino-terminal deletion mutants of PEA3 suggested the occurrence of a second activation domain located broadly between residues 106 and 226. However, we were unable to verify the occurrence of an activation domain in this region by use of GAL4-PEA3 chimeras. Whereas these negative findings may have numerous explanations, one possibility is that the central portion of PEA3 serves a function unrelated to either transactivation or DNA binding per se. We are currently investigating this potential. A schematic illustrating the location of these various functional motifs is shown in Fig. 12.

The activation domains of the two PEA3 subfamily members, human ERM and mouse ER81, have also been broadly localized. Analyses of GAL4-ERM chimeras in human HeLa cells and a rabbit kidney cell line (RK13) revealed the occurrence of an activation domain in the first 198 residues of the 510-amino acid ERM protein and another in the C-terminal 61 residues (33). The amino-terminal 72 amino acids, but not the C-terminal 61 residues, function to stimulate transcription in the budding yeast, *S. cerevisiae*, when fused to the GAL4 DNA binding domain (34). Similar analyses of GAL4-ER81 chimeras in rabbit kidney cells also revealed the occurrence of an activation domain within the amino-terminal 182 residues of mouse ER81 (35). Hence, it would appear that all three PEA3 subfamily members possess an amino-terminal activation domain.

The amino-terminal activation domain and the acidic region are coincident in PEA3. The acidic region is highly similar in sequence (85% sequence identity) among PEA3 subfamily members and may constitute the activation domain of all three proteins (17). The acidic region of each PEA3 subfamily member is predicted to comprise an $\alpha$-helix (36, 37). Indeed, a structural analysis (circular dichroism) of a peptide bearing this region of human ER81 demonstrates that it can assume an $\alpha$-helix, albeit in a nonphysiological hydrophobic solvent (34). However, NMR analyses of a 128-residue amino-terminal fragment of PEA3 comprising the acidic activation domain and

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**Fig. 11.** Summary of the transcriptional and DNA binding activities of the amino and C-terminal deletion mutants of PEA3. A, schematic representation of the structure of the PEA3 deletion mutants. B, bar diagram illustrating the specific transcriptional and DNA binding activity of the PEA3 deletion mutants. The data represented in this figure was taken from Figs. 1, 8, and 9.

**Fig. 12.** Schematic illustrating the functional domains, negative regulatory regions, and sequence motifs in mouse PEA3. AD, acidic domain; ETS, ETS domain.
both negative regulatory modules suggest that this region is unstructured in physiological solutions. Hence, if this region assumes an α-helical structure in vivo, then this may be induced by its interaction with a partner protein.

There is little data to suggest the occurrence of negative regulatory domains affecting the function of the amino-terminal activation domains of ERM and ER81. On the contrary, there is evidence that ERM does not possess a negative regulatory module at its amino terminus (33). Deletion of the amino-terminal 42 residues of ERM from a GAL4-ERM chimera bearing residues 1–72 reduced the activity of the resulting chimera up to 10-fold.

Our analyses of deletion mutants of PEA3 and of GAL4-PEA3 chimeras provided little support for the occurrence of a C-terminal activation domain in PEA3 similar to that in ERM (33). Deletion of this region (residues C-terminal of the ETS domain) from PEA3 stimulated the capacity of the resulting mutants to activate transcription directly commensurate with their increased DNA binding activity (Fig. 11). Moreover, deletion mutants of PEA3 harboring these sequences but lacking the amino-terminal activation domain (i.e. PEA3ΔN245 and PEA3ΔN326) did not activate transcription, yet these mutants bound DNA as well as the WT protein (Fig. 11). Hence, analyses of PEA3 deletion mutants provided no evidence for an activation domain in the C-terminal region of PEA3. Indeed, direct test of the potential of a GAL4-PEA3 chimera bearing only the C-terminal residues of PEA3 (residues 419–480) to activate transcription in COS cells revealed that it did so at levels only 0.2% that of the chimera bearing the amino-terminal acidic activation domain (residues 42–85) (data not shown).

Hence, it seems unlikely that this weak C-terminal activation domain plays a significant role in transactivation by PEA3.

Two Regions Flanking the PEA3 ETS Domain Negatively Regulate DNA Binding—The DNA binding activity of PEA3 expressed in COS cells could not be detected unless nuclear lysates bearing the protein were preincubated with antibodies directed against PEA3. Two different PEA3-specific antibodies reactive with epitopes located in either the center or the carboxyl terminus of the protein were independently capable of unmasking its DNA binding activity. The latent DNA binding activity of COS cell-expressed PEA3 could also be uncovered by deleting two inhibitory regions flanking the ETS DNA binding domain. The amino-terminal border of one such negative regulatory module was located between residues 245 and 278, and the C-terminal border of the other was located between residues 425 and 450 (Fig. 12). Deletion of either negative regulatory region separately did not overcome the requirement for a PEA3-specific antibody to uncover DNA binding activity, implying that each region acts independently to repress binding of the ETS domain to DNA. Interestingly, a GST-PEA3 fusion protein isolated from E. coli and bearing both negative regulatory modules and the ETS domain bound to DNA in the absence of a PEA3-specific antibody. Incubation of these proteins with a PEA3-specific antibody modestly (2–3-fold) increased DNA binding. Hence, fusion of GST to PEA3 simultaneously abrogates the function of both negative regulatory modules.

Taken together, these observations suggest that native PEA3 in mammalian cells exists in a dynamic equilibrium between two states, one capable and another incapable of binding to DNA. We suspect that the transition between these two states is normally regulated and is accompanied by structural changes in the protein. This equilibrium may be perturbed artificially by interaction with an antibody, linkage to GST, or mutation. In vivo, the transition between the inactive and active states for DNA binding is likely to be governed by post-translational modification (i.e. phosphorylation, acetylation, proteolysis, etc.) and/or interaction with one or more partner proteins.

We reported previously that mouse PEA3 synthesized in vitro is incapable of binding to DNA (41). This finding has been confirmed and extended using zebrafish PEA3 synthesized in vitro in reticulocyte lysates (10). Deletion of either one of two regions flanking its ETS domain improves DNA binding, but this effect is greatest when both regions are deleted. These negatively acting regions bordering the zebrafish ETS domain comprise about 90 amino acids (amino-terminal region) and 60 amino acids (C-terminal region) and approximate the locations of those reported herein for mouse PEA3. Expectedly, these negative regulatory regions in mouse and zebrafish PEA3 share significant amino acid identity, 49% identity between the two amino-terminal negative regulatory regions and 59% between the two C-terminal negative regulatory regions. By comparison, the overall sequence identity between mouse and zebrafish PEA3 is 54%, and that between their ETS domains is 96%.

The sequences affecting DNA binding by the PEA3-related Ets protein ERM have also been located (33). Full-length ERM synthesized in vitro in reticulocyte lysates binds to DNA, but to a lesser extent than do deleted versions of the protein lacking sequences flanking the ETS domain. One of the negative regulatory regions at the carboxyl terminus of human ERM occurs at the same position and shares sequence similarity to the corresponding negative regulatory region of mouse and zebrafish PEA3. However, the negative regulatory region amino-terminal of the ERM ETS domain does not correspond in either location or sequence to that of the mouse and zebrafish proteins. The reason for this difference is not clear.

Several other Ets proteins including Ets-1 (5, 38), Elk-1 (39), and PU.1 (40) also possess regulatory modules flanking their ETS domain that negatively affect DNA binding. The location and structure of these regions have been extensively characterized for Ets-1, which has served as a model for studies of auto inhibition of DNA binding by sequence-specific transcription factors (41). The regulatory modules in Ets-1 comprise α-helical regions that are thought to allosterically inhibit the function of another α-helical motif, termed helix 1, in the ETS domain that is required for DNA binding. There are no obvious sequence similarities among the inhibitory regions bordering the ETS domains of these various Ets proteins, but the potential that they comprise similar structural modules seems a likely possibility.

PEA3 Transcription Activity Is Regulated at Multiple Levels—Our deletion analyses reveal that the activity of PEA3 is negatively regulated at the level of both transactivation and DNA binding. This suggests that its activity is tightly regulated by mechanisms that are currently poorly understood. We imagine that the interaction of PEA3 with other proteins and/or its post-translational modification may affect both of these functions of the protein. In this regard, it is noteworthy that PEA3 is phosphorylated at serine residues by mitogen-activated protein kinases and that some of these sites occur within the negative regulatory regions identified here. Moreover, we have recently identified several proteins that physically interact with PEA3, although the functional consequences of these interactions have yet to be uncovered. Clearly, it will be of interest to unravel the mechanisms accounting for the regula-
tion of PEA3 activity and to learn more of its developmental and physiological roles.

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The PEA3 Ets Transcription Factor Comprises Multiple Domains That Regulate Transactivation and DNA Binding
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