Different Domains of the Transcription Factor ELF3 Are Required in a Promoter-specific Manner and Multiple Domains Control Its Binding to DNA*

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Elf3 is an epithelially restricted member of the ETS transcription factor family, which is involved in a wide range of normal cellular processes. Elf3 is also aberrantly expressed in several cancers, including breast cancer. To better understand the molecular mechanisms by which Elf3 regulates these processes, we created a large series of Elf3 mutant proteins with specific domains deleted or targeted by point mutations. The modified forms of Elf3 were used to analyze the contribution of each domain to DNA binding and the activation of gene expression. Our work demonstrates that three regions of Elf3, in addition to its DNA binding domain (ETS domain), influence Elf3 binding to DNA, including the transactivation domain that behaves as an autoinhibitory domain. Interestingly, disruption of the transactivation domain relieves the autoinhibition of Elf3 and enhances Elf3 binding to DNA. On the basis of these studies, we suggest a model for autoinhibition of Elf3 involving intramolecular interactions. Importantly, this model is consistent with our finding that the N-terminal region of Elf3, which contains the transactivation domain, interacts with its C terminus, which contains the ETS domain. In parallel studies, we demonstrate that residues flanking the N- and C-terminal sides of the ETS domain of Elf3 are crucial for its binding to DNA. Our studies also show that an AT-hook domain, as well as the serine- and aspartic acid-rich domain but not the pointed domain, is necessary for Elf3 activation of promoter activity. Unexpectedly, we determined that one of the AT-hook domains is required in a promoter-specific manner.

The ETS transcription factor family is comprised of nearly 30 members. They are characterized by a highly conserved DNA binding domain (DBD), known as the ETS domain. During the past several years, interest in one of its family members, Elf3, has grown considerably. Elf3 (also known as Ese-1, ESX, ERT, and jen) belongs to the Elf subfamily of ETS proteins whose expression is restricted to epithelial tissues (1–4). Gene knock-out studies have shown that Elf3 plays essential roles during development, in particular the development of the gut. Thirty percent of Elf3 null fetuses die in utero, and the remaining 70% die shortly after birth. These mice exhibit severe alterations in the cellular architecture of the small intestine (5). Defects in the development and function of other tissues, especially those needed later in life, are likely to be masked by the early demise of these mice.

Other studies have directly implicated Elf3 in the normal physiology of the breast, as well as in breast cancer (3, 4, 6–10). In the normal breast, Elf3 is believed to be expressed in a subset of pluripotent ductal epithelial cells, which are retained after involution of the breast (8). Given that pluripotent ductal epithelial cells are likely targets of carcinogenesis, it is not surprising that 40% of ductal carcinoma in situ samples display high levels of Elf3 expression (4, 8). Furthermore, increased expression of Elf3 correlates closely with elevated expression of HER2/neu in ductal carcinoma in situ (4). Interestingly, the disruption of Elf3 activity leads to significant decreases in HER2 expression, which strongly suggests that Elf3 influences HER2 expression (6). Although it is unclear whether the effect of Elf3 on HER2 is direct or indirect, Elf3 has been shown to directly regulate other promoters. This has been shown most clearly in the case of the type II TGF-β-receptor (TβR-II) promoter. Elf3 synergistically activates the TβR-II promoter by binding to two overlapping ets sites located just downstream of the major transcription start site of the gene (11–13). Elf3 not only binds to the promoter of TβR-II in vivo, both over- and underexpression studies have directly implicated Elf3 in the regulation of the endogenous TβR-II gene (11, 14, 15).

Elf3 has also been implicated in the expression of at least 10 other genes, including the collagenase-1 gene (MMP-1, interstitial collagenase, and collagenase type 1 (7), a gene important in extracellular matrix remodeling and tumorigenesis (16). The collagenase-1 promoter contains closely spaced AP-1 and ets sites, which allow cooperation between transcription factors (17, 18). The cooperative action of Elf3 with other transcription factors further enhances the transcription of these genes.

GFP, green fluorescent protein; P/R, promoter/reporter; TBP, TATA box-binding protein; ChIP, chromatin immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDB, Protein Data Bank.
Factors has also been observed in the case of the nitric-oxide synthetase (NOS2) gene, where Elf3 cooperates with NF-κB, by binding to adjacent ets and κB sites (19). These and other studies demonstrate that Elf3 works cooperatively with other transcription factors to achieve specificity and to regulate genes involved in inflammation, differentiation, tumorigenesis, and metastasis (6, 7, 14, 19–25).

Although there is growing evidence that Elf3 regulates a number of important genes that are involved in a wide range of cellular activities, Elf3 structure and function have not been studied in-depth. The work described in this study focuses on the domains of Elf3 required for DNA binding and the activation of gene transcription. Based in part on sequence analysis, Elf3 contains five defined domains (Fig. 1). The N terminus contains a pointed domain (PNT), which is located in residues 63–127 (1). This domain has been implicated in protein–protein interaction for other ETS proteins (26, 27). Thus far, the PNT domain in Elf3 has not been assigned a functional role. The transactivation domain (TAD), which has been studied more extensively, is located in residues 129–159 (6, 28). Unlike TADs of many other transcription factors, the TAD of Elf3 appears to contain an α-helix (6), and point mutations in this α-helix disrupt its ability to activate promoter activity as well as its interaction with the coactivator Med23 (CRSP3, Drip130, and Sur2) and the TATA box-binding protein (TBP) (6, 28). Elf3 also contains a serine- and aspartic acid-rich (SAR) domain located in residues 189–229 (4, 29), which is unique. This domain appears to influence cellular transformation when Elf3 is localized to the cytoplasm, but a nuclear function for this domain has not been identified (29). Unlike other ETS proteins, Elf3 appears to contain two AT-hook domains located within residues 236–267, which have not been examined in detail. AT-hook domains were initially identified in high mobility group (HMG) family members, where they are believed to be involved in nonsequence-specific binding to AT-rich regions of DNA, as well as protein–protein interactions (30, 31). This region of Elf3 also contains a bipartite nuclear localization signal (NLS) (29, 32). Finally, the ETS domain, which is required for DNA binding, is located in residues 272–354 (1). The ETS domain of Elf3 has not been characterized, but it is expected to be structurally similar to those of other ETS family members (1).

Earlier studies argue that Elf3, like eight other ETS proteins, possesses an autoinhibitory domain (AID), which limits its binding to DNA (11) and how autoinhibition is regulated mechanistically. The primary AID affecting DNA binding is within the TAD of Elk1, and phosphorylation of the TAD affects the domain-domain interaction within Elk1 for release of autoinhibition (50). Although previous studies argue that Elf3 contains an AID, it is unclear what region(s) of the protein is involved in limiting its binding to DNA (11) and how autoinhibition is regulated mechanistically.

To identify the regions of Elf3 that affect autoinhibition, as well as the regions that affect its activation of transcription, we created and tested an extensive series of Elf3 mutant proteins with specific domains deleted or targeted by point mutations. We determined that the PNT domain does not appear to be needed for Elf3 to activate the promoter of either the TβR-II or collagenase-1 gene, whereas the SAR domain is needed for both promoters. Interestingly, we show that one of the AT-hook domains is required to activate transcription in a promoter-specific manner. We also determined that several regions outside the ETS domain influence Elf3 binding to DNA both in vitro and in vivo. On the basis of our findings, we present a model of autoinhibition that implicates intramolecular interactions in the creation of the autoinhibited state. Moreover, we propose that the release of autoinhibition is facilitated by interactions with a coactivator, which disrupts intramolecular interactions within Elf3 and increases the affinity of the ETS domain for DNA.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium was obtained from Invitrogen. Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT). Unless indicated otherwise, all chemicals were obtained from Sigma.

**Cell Culture**—As described previously, Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum was used in the culture of each cell line, including F9 embryonal carcinoma (EC) (11, 52) and 293T (11). F9 EC cells were cultured on gelatin-coated tissue culture plastic and induced to differentiate by a 72-h treatment with 5 μM retinoic acid, as described previously (11, 52).

**Promoter/Reporter (P/R) Gene Constructs**—The P/R constructs mTβR-II −108/+56(B/A), mTβR-II −108/+56(B/x), and CMV-β-gal have been described previously (11, 52, 53). P/R construct −517/+63 hCollagenase-1 was a generous gift from Peter Angel (54).

**Expression Plasmids**—Plasmid expression vectors for FLAG-Elf3, FLAG-Elf3ΔN233, FLAG-Elf3ΔN270, FLAG-Elf3L142P, Ets1, and the green fluorescent fusion (GFP) protein, GFP-Elf3, have been described previously (12). The plasmid expression vector for the Med23 (352–625) fragment was obtained from
M. Uesugi (see Ref. 6). The expression vector for full-length Med23 was received from R. Roeder.

We utilized site-directed mutagenesis to create several point mutations and one deletion mutation of five amino acids (FLAG-Elf3Δ246–250) within FLAG-Elf3 or GFP-Elf3 by the “QuickChange” method, as described previously (11). The primers, template, and restriction enzyme sites utilized to obtain several new expression plasmids are listed in Table 1.

FLAG-Elf3Δ173 was created using the primer pair 5’ TTGAGGATCCGCCGACGAGCTTATACTAC 3’ and 5’ CGAGGACCCTAGATTTAGTGAC 3’. The primers amplified a PCR fragment containing residues 174–371. Restriction sites for BamHI (underlined) and XbaI were used to amplify the entire plasmid except residues 173–241. We utilized primers that, upon digesting with the appropriate restriction enzyme and religation, resulted in the incorporation of the amino acid sequence TGDD between residues 172 and 242. FLAG-Elf3Δ235–270 was created by a similar method. PCR was used to amplify the entire plasmid except residues 235–270, and upon cutting with the appropriate restriction enzyme and religation to form a circular plasmid, the amino acid sequence TGTG was incorporated between residues 234 and 271. We used a similar method to create FLAG-Elf3Δ237–241, FLAG-Elf3Δ237–241Δ246–250, FLAG-Elf3Δ232–250, FLAG-Elf3Δ129–159, FLAG-Elf3Δ60–127, and FLAG-Elf3Δ355–362 using the restriction enzyme Xhol and the primer pairs and plasmid templates that are listed in Table 2. FLAG-Elf3Δ235 was created in making FLAG-Elf3Δ235–270 by a frameshift mutation that inserted five amino acids, TGDDR, and a stop codon.

**Table 1**

| Construct       | Template   | Restriction enzyme | Primer used* |
|-----------------|------------|--------------------|--------------|
| FLAG-Elf3Δ237–241 | FLAG-Elf3 | BamHI              | 5’ CGCTCTGCTCAAGGGTTTAGGGACCTCTACTAC 3’ |
| FLAG-Elf3Δ237–241 | FLAG-Elf3 | XhoI               | 5’ TTGAGGATCCGCCGACGAGCTTATACTAC 3’ |
| Δ246–250, FLAG-Elf3Δ235–260 | FLAG-Elf3 | Nael               | 5’ GCCACACACGAGCTTATACTAC 3’ |
| FLAG-Elf3Δ129–159 | FLAG-Elf3 | EcoRI              | 5’ GTTACCTCATGATTTAGTGAC 3’ |
| FLAG-Elf3Δ60–127 | FLAG-Elf3 | BglII             | 5’ CCAACAGCGGAGGAAGCTTATACTAC 3’ |
| FLAG-Elf3Δ355–362 | FLAG-Elf3 | Removed PvuII      | 5’ GCTGAGATATCGCTGAGTTCATCAGAAG 3’ |

* Complementary primers are not shown.

**Table 2**

| Construct       | Template   | Primer pair used |
|-----------------|------------|------------------|
| FLAG-Elf3Δ237–241 | FLAG-Elf3 | 5’ GAAGTACCTGGAGCCGAGGAGAGAAGGGG 3’ |
| Δ246–250, FLAG-Elf3Δ235–260 | FLAG-Elf3 | 5’ GTTACCTCATGATTTAGTGAC 3’ |
| FLAG-Elf3Δ129–159 | FLAG-Elf3 | 5’ GCTGAGATATCGCTGAGTTCATCAGAAG 3’ |
| FLAG-Elf3Δ60–127 | FLAG-Elf3 | 5’ CCAACAGCGGAGGAAGCTTATACTAC 3’ |
| FLAG-Elf3Δ355–362 | FLAG-Elf3 | 5’ GCTGAGATATCGCTGAGTTCATCAGAAG 3’ |
and analyzed for the fluorescent intensity per cell expressing GFP.

Molecular Modeling—A structural model of the Elf3 ETS domain (aa 272–354) was generated using BIO POLYMER and COMPOSER modules of the SYBYL software package (Tripos, Inc., St. Louis, MO). The quality of the model was evaluated with the ProTable module of SYBYL. The ETS domains of ETS proteins GABPα, Sap-1, Ets1, Pdef, and Elf5 from the Protein Data Bank (PDB) (56) were used as structural templates (PDB codes are as follows: 1AWC (57), 1BC8 (58), 1K79 (59), 1YO5 (60), and 1FLI (61), respectively).

Extract Preparation and Western Blotting—293T cells were transiently transfected by calcium phosphate precipitation with 20 μg of the relevant expression plasmid. Forty eight hours after transfection, the cells were harvested, and nuclear extracts were prepared using the Pierce NE-PER™ nuclear and cytoplasmic extraction kit, as described in the manufacturer’s protocol (Pierce). Extracts were supplemented with protease and phosphatase inhibitors and stored at −80 °C. Western blot analysis was performed using the anti-FLAG M2 antibody (Pierce). Extracts were supplemented with protease and phosphatase inhibitors and stored at −80 °C. Western blot analysis was performed using the anti-FLAG M2 antibody against blots in which 10 μl of each nuclear extract was separated by SDS-PAGE. Proteins were detected using the enhanced chemifluorescence (ECF) kit (GE Healthcare) and scanned on a Storm phosphorImager (GE Healthcare). Quantitation was performed using the ImageQuant 5.0 analysis software (GE Healthcare).

Electrophoretic Mobility Shift Assays—To use equal amounts of each FLAG tagged Elf3 protein, the volume of each nuclear extract added was adjusted based on Western blot analysis. Annealed double-stranded oligodeoxynucleotide (ODN) probes (see below) were labeled by a fill-in reaction using the Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA). Nuclear extracts were incubated for 20 min at room temperature in binding buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 0.8% polyvinyl alcohol, 10% bovine serum albumin, ~40,000 cpm of probe, and 0.02 μg/μl of mutETS-annealed double-stranded ODN. The mutETS ODN was utilized in place of traditional nonspecific competitors to reduce nonspecific binding and to allow better visualization of protein-DNA complexes. In supershift assays, the monoclonal antibody to the FLAG epitope (M2) was preincubated with the nuclear extract for 40 min on ice before addition of the labeled probe. DNA-protein complexes were resolved on 4% nondenaturing polyacrylamide gel in 0.5× Tris/glycine/EDTA buffer as described previously (52). The gels were dried and visualized by PhosphorImager (GE Healthcare). ODN probes corresponding to +3 to +34 of the TβR-II gene were as follows: wild type, 5′-TGGCGAGGAGTTTTCACTCAGCCC-3′ and its complement; mutETS, 5′-TGGCGAGGAGTTTTCACTCAGCCC-3′ and its complement (underline indicates the two overlapping ets sites, and italicized letters indicate the mutated sequence). In the case of the wild-type probe, the sequence TAGC was added to the 5′ end of each ODN for use in radiolabeling.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays with 293T cells were carried out as described previously (11). Briefly, 24 h after seeding, cells were cotransfected by the calcium phosphate precipitation method with the mTβR-II –108/+56(B/A) P/R construct and FLAG tagged expression vectors for Elf3 proteins. Approximately 42 h after transfection, proteins were cross-linked with formaldehyde, and then nuclei were isolated and lysed, and chromatin was sheared by sonication. The supernatant was cleared by protein G-agarose (pre-blocked with salmon sperm DNA and bovine serum albumin; Upstate, Charlotteville, VA) for 1 h at 4 °C. The sample was then split into aliquots for incubation with the M2 antibody (FLAG-specific; Sigma) or the Gal4 DBD antibody (nonspecific; Santa Cruz Biotechnologies, Santa Cruz, CA) overnight at 4 °C. Protein complexes were collected with protein G-agarose (Upstate) and washed extensively. Complexes were eluted, de-cross-linked, and treated with RNase A and proteinase K, and the DNA was purified using the Gene Clean Turbo kit (Qbiogene, Irvine, CA). DNA enrichment was determined by quantitative real time PCR analysis using a Cepheid Smart Cycler Version 2.0c Detection System, and SYBR Green Master Mix (SuperArray, Frederick, MD). Relative occupancy values were calculated by normalizing the amount of specific DNA (mTβR-II –108/+56(B/A) P/R) immunoprecipitated to the amount of nonspecific DNA (GAPDH promoter) immunoprecipitated and evaluating enrichment of the specific antibody, M2, as compared with the nonspecific antibody, Gal4. mTβR-II –108/+56(B/A) P/R-specific primers were as published previously (11), and GAPDH primer pairs were supplied in the EZ ChIP kit from Upstate Biotechnology, Inc. The sequence is as follows: forward 5′ TACTAGCGGTGGTTACGCGGCG 3′ and reverse 5′ TCGAACAGAGGAGCAAGGCGA 3′.

FLAG Pulldown Assay—293T cells were cotransfected with 10 μg of an expression vector for a FLAG tagged protein, 5 μg for a GFP fusion protein, and 10 μg of the coactivator expression plasmid as indicated. Vector DNA was added, if needed, to equal 25 μg of total DNA. Twenty four hours after transfection cells were frozen on liquid nitrogen and solubilized in a cell lysis solution containing 50 mM Hepes, pH 7.8, 1% Triton X-100, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM vanadate, 30 mM NaPp, 25 mM benzamidine, and 20 μg/ml aprotinin. Insoluble matter was removed by centrifugation for 10 min at 14,000 rpm. Input fractions were removed for analysis of protein expression. Then FLAG proteins were immunoadsorbed by EZ-Red M2 affinity gel overnight at 4 °C. The affinity gel was then washed three times with RIPA buffer and one time with a solution containing 10 mM Hepes, pH 7.8, 1.5 mM MgCl₂, 0.1 mM NaCl, 0.05% Triton X-100. M2-specific complexes were eluted using 150 ng/μl of 3X FLAG peptide in the previous solution at 4 °C. Western blot analysis was utilized to detect the amount of GFP-tagged protein coimmunoprecipitated by FLAG-Elf3ΔC235. The M2 and GFP (sc-8334; Santa Cruz Biotechnology) antibody were used at a 1:2000 dilution in 3% bovine serum albumin in TBST. Proteins were detected using the enhanced chemifluorescence (ECF) kit (GE Healthcare) and scanned on a Storm phosphorImager (GE Healthcare). Quantitation was performed using the ImageQuant 5.0 analysis software (GE Healthcare).
The ETS Domain of Elf3 Is Similar to the ETS Domain of Ets1—Functional Domains of ELF3

To test our molecular model for the ETS domain of Elf3, we created a large number of Elf3 mutants. Initially, we hypothesized that these mutations should disrupt the secondary structure of the ETS domain and disrupt its activity. A proline was inserted in the putative helix 1 (Leu275-Leu283) in place of isoleucine 279 by site-directed mutagenesis of the overall function of the protein, we created a large number of Elf3 mutant proteins. Initially, we addressed the function of the ETS domain. This domain is highly conserved within the ETS transcription factor family and has been well characterized structurally for other family members but not for Elf3. To examine the structure of the ETS domain of Elf3, we generated a homology model of its ETS domain using PDB structural files corresponding to the ETS domains of GABPα, Sap-1, Ets1, Pdef, and Elf5 as templates. Pdef and Elf5 ETS domains share 42 and 66% sequence similarity with the ETS domain of Elf3, respectively. The resulting molecular model of Elf3 contained three α-helices, four β-sheets, and a turn connecting helix 2 and 3. This structure corresponds to the winged-helix-loop-helix structure of the typical ETS domain, with the third helix as the DNA recognition helix. The molecular model of the ETS domain of Elf3 when overlaid with the crystal structure of the ETS domain of Ets1 (Fig. 1B, white) demonstrated high similarity (the root mean square deviation between the backbone atoms was equal to 0.46 Å). This suggests that although the primary sequences of the ETS domains of Elf3 and Ets1 share only a 35% similarity, the tertiary structure of the ETS domain is highly conserved. Hence, it appears the tertiary structure of the ETS domain is more highly conserved evolutionarily than its amino acid sequence would suggest.

To test our molecular model for the ETS domain of Elf3, proline substitutions were introduced to disrupt the predicted α-helices. We hypothesized that these mutations should disrupt the secondary structure of the ETS domain and disrupt its activity. A proline was inserted in the putative helix 1 (Leu275-Leu283) in place of isoleucine 279 by site-directed mutagenesis of the overall function of the protein, we created a large number of Elf3 mutant proteins. Initially, we addressed the function of the ETS domain. This domain is highly conserved within the ETS transcription factor family and has been well characterized structurally for other family members but not for Elf3. To examine the structure of the ETS domain of Elf3, we generated a homology model of its ETS domain using PDB structural files corresponding to the ETS domains of GABPα, Sap-1, Ets1, Pdef, and Elf5 as templates. Pdef and Elf5 ETS domains share 42 and 66% sequence similarity with the ETS domain of Elf3, respectively. The resulting molecular model of Elf3 contained three α-helices, four β-sheets, and a turn connecting helix 2 and 3. This structure corresponds to the winged-helix-loop-helix structure of the typical ETS domain, with the third helix as the DNA recognition helix. The molecular model of the ETS domain of Elf3 when overlaid with the crystal structure of the ETS domain of Ets1 (Fig. 1B, white) demonstrated high similarity (the root mean square deviation between the backbone atoms was equal to 0.46 Å). This suggests that although the primary sequences of the ETS domains of Elf3 and Ets1 share only a 35% similarity, the tertiary structure of the ETS domain is highly conserved. Hence, it appears the tertiary structure of the ETS domain is more highly conserved evolutionarily than its amino acid sequence would suggest.
Functional Domains of ELF3

Because of binding to both domain and the 17-amino acid C-terminal tail of Elf3, displays protein Elf3 binding of the full-length protein, we utilized the truncated T has been characterized extensively in the F9-differentiated cells/H11002 the second and third helix (S308A and S330A, respectively) did mutations within the turn between helix 2 and 3 (K320E) and within P/R gene construct (Fig. 1C). As predicted, all three helix mutations (GFP-Elf3I279P, GFP-Elf3A312P, and GFP-Elf3R331P) resulted in a complete loss of Elf3 stimulation of mTβR-II −108/+56(B/A). In contrast, other non-helix breaking mutations within the turn between helix 2 and 3 (K320E) and within the second and third helix (S308A and S330A, respectively) did not greatly affect the ability of Elf3 to stimulate mTβR-II −108/+56(B/A) (Fig. 1C and data not shown). This suggested that the loss of activity is because of a disruption of the ability of the ETS domain to bind to DNA as a result of structural abnormalities.

To more directly test the ability of these mutations to affect DNA binding, the same point mutations were made within a mammalian expression vector of an N-terminally truncated form of Elf3 (Elf3ΔN270) (Fig. 1D, top). Earlier studies demonstrated that Elf3 contains an AID, which limits its binding to DNA in vitro, because Elf3ΔN270, which contains only the ETS domain and the 17-amino acid C-terminal tail of Elf3, displays increased affinity for DNA in vitro (11). In light of the limited binding of the full-length protein, we utilized the truncated protein Elf3ΔN270, which forms two specific complexes because of binding to both mTβR-II ets sites, to examine how the mutations affected DNA binding independent of the possible influence of an AID. EMSA using equal amounts of Elf3ΔN270 with or without the ETS domain mutations detected a loss of binding with mutants Elf3ΔN270I279P, Elf3ΔN270A312P, and Elf3ΔN270R331P to the TβR-II probe but not with the Elf3ΔN270K320E mutant (Fig. 1D). We validated the results for the I279P mutant by ChIP. Specifically, we demonstrated that the mutant protein, I279P, in contrast to its wild-type counterpart (Elf3ΔN270), exhibited little or no association with the TβR-II promoter in vivo (Fig. 1E). These findings support the premise that the predicted α-helical regions, which would be disrupted by prolines, are necessary for the binding of Elf3 to DNA. Moreover, these findings also suggest that the winged helix-loop-helix structure of the ETS domain is conserved within Elf3 even though the sequence homology between Ets1 and Elf3 is relatively low.

Requirement for Regions Flanking the ETS Domain—Regions flanking the defined ETS domain have also been reported to affect the ability of ETS proteins to bind to DNA. For example, removal of the C-terminal tail of Ets1 or Ets2, which flanks the C-terminal side of the ETS domain, results in enhanced binding to DNA in vitro (33). To test the possibility that removing the C-terminal tail of Elf3 would increase binding to DNA and thus increase its ability to activate transcription, we inserted a stop codon at amino acid 355 and created Elf3ΔC354 (Fig. 2A). However, when increasing amounts of Elf3ΔC354 and wild-type Elf3 were compared, Elf3ΔC354 failed to stimulate mTβR-II −108/+56(B/A) (Fig. 2B). Using a GFP-Elf3ΔC354 fusion protein, we

FIGURE 2. Removal of the last 17 aa of Elf3 disrupts the ETS domain. A, diagram of FLAG-Elf3 deletion constructs. Elf3 represents the full-length protein (aa 1–371). Residues 355–362, 363–371, or 354–371 were deleted from Elf3 to create Elf3Δ355–362, Elf3ΔC363, or Elf3ΔC354, respectively. B, F9-differentiated cells were transiently transfected with 15 μg of the mTβR-II −108/+56(B/A) P/R construct. Where indicated, the cells were also transfected with expression vectors for Elf3 or Elf3ΔC354 at the amounts shown. Activities were assayed and normalized as described under “Experimental Procedures.” The promoter activity of each construct is calculated relative to the CAT activity observed with mTβR-II −108/+56(B/A) alone, which was set to 1. This experiment was performed two times, and similar results were obtained. C, F9-differentiated cells were transiently transfected and assayed as in B, except where indicated, and the cells were transfected with 0.5 or 1 μg of an N-terminal GFP fusion expression vector for Elf3, Elf3Δ355–362, or Elf3ΔC363. This experiment was performed three times, and similar results were obtained.
determined the Elf3ΔC354 protein was present in the nuclear compartment and that it was expressed at levels similar to wild-type Elf3 (data not shown). There are two possible explanations for the lack of activity of Elf3ΔC354 as follows: 1) removal of the C-terminal tail eliminates part of the protein needed for transcription; 2) removal of the C-terminal tail resulted in a protein unable to bind DNA. We tested these possibilities by determining whether Elf3ΔC354 acts as a dominant negative. In this regard, a protein that is able to bind to the ets sites, but is unable to activate transcription, will compete with wild-type Elf3 for binding to the TBR-II promoter and interfere with its ability to activate the promoter. We have shown a similar dominant negative activity for an N-terminally truncated Elf3 protein (Elf3ΔN270) (12). For this purpose, we transfected F9-differentiated cells with increasing amounts of mutant Elf3ΔC354 in the presence of a fixed amount of the wild-type Elf3. Elf3ΔC354 did not reduce the activation of mTβR-II −108/+56(B/A) by wild-type Elf3 (Fig. 2B). This argues that removal of the C-terminal 17 amino acids of Elf3 disrupts its ability to bind to DNA in vivo.

To elucidate which residues of the C-terminal tail were critical for maintaining the ability of Elf3 to bind to DNA, we created two smaller deletions, Elf3Δ355–362 and Elf3ΔC363 (Fig. 2A). Removal of the last eight amino acids of Elf3 (Elf3ΔC363) reduced the ability of Elf3 to activate transcription from the mTβR-II −108/+56(B/A) P/R construct by 30–40% (Fig. 2C). However, when adjusted for GFP-Elf3ΔC363 expression, which was ∼20–30% lower than wild-type GFP-Elf3 (data not shown), the activity of Elf3ΔC363 appears to be only about 20% lower than Elf3. In contrast, Elf3Δ355–362, which lacks the 8 amino acids adjacent to the ETS domain, failed to stimulate mTβR-II −108/+56(B/A) (Fig. 2C). Furthermore, Elf3Δ355–362, like Elf3ΔC354, did not act as a dominant negative, was localized in the nucleus (data not shown), and was expressed at levels similar to wild-type Elf3 (data not shown). We also examined the ability of Elf3Δ355–362 to occupy the TBR-II promoter in vivo by ChIP, and we found that its presence is greatly reduced when compared with wild-type Elf3 (Fig. 1E). Taken together these findings suggest that Elf3Δ355–362 lacks amino acids essential for the activity of the ETS domain. Hence, amino acids directly flanking the C terminus of the ETS domain of Elf3 appear to be critical for binding to DNA.

We also tested whether residues flanking the N-terminal side of the ETS domain were necessary for maintaining binding to DNA. Recent studies indicated that this region contains a bipartite NLS (29, 32). Indeed, we confirmed by fluorescent microscopy using GFP fusion Elf3 proteins that removal of residues 235–260 or 235–270 resulted in a protein that was primarily located within the cytoplasm (data not shown). To overcome the loss of nuclear localization, we created the mutations Δ235–260 and Δ235–270 within a mammalian Elf3 expression vector that contained the SV40 NLS and a FLAG tag at the N terminus of Elf3 (FLAG-Elf3Δ235–260 or FLAG-Elf3Δ235–270) (Fig. 3A). By using Western blot analysis, we confirmed that the resulting proteins were located within nuclear extracts at similar levels (data not shown). FLAG-Elf3Δ235–260 displayed 70–80% of wild-type FLAG-Elf3 activity when tested with mTβR-II −108/+56(B/A) (Fig. 3B). Unexpectedly, FLAG-Elf3Δ235–270 retained only 30% of the activity of wild-type Elf3 (Fig. 3B). This raised the question of whether removal of residues 235–270, like the removal of 355–362, disrupts the ability of Elf3 to bind to DNA. To address this question, we utilized the FLAG-Elf3Δ235–270 expression construct and tested its ability to disrupt wild-type Elf3 stimulation of mTβR-II −108/+56(B/A) and act as a dominant negative. However, when FLAG-Elf3Δ235–270 was added in addition to Elf3, it did not reduce the ability of wild-type Elf3 to stimulate mTβR-II −108/+56(B/A) (Fig. 3C). Thus, FLAG-Elf3Δ235–270 does not appear to act as a dominant negative. These results suggest that removal of residues 235–270, similar to removal of residues 355–362, influences the ETS domain of Elf3 and results in a
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protein that exhibits reduced binding to DNA in vivo. Collectively, these findings argue that residues (aa 261–270 and aa 354–362) that flank the canonical ETS domain (aa 272–354) are necessary for a functional ETS domain.

The SAR Domain but Not the PNT Domain Is Needed for Elf3 Activity—In view of the importance of the regions flanking the ETS domain, we also examined the contribution of the N-terminal domains to the activity of Elf3. The TAD (aa 129–159) of Elf3 has been extensively characterized for its ability to stimulate promoter activity (6, 28). However, the contribution of the PNT and SAR domains to the transcriptional activity of Elf3 has not been examined. Therefore, we created Elf3 expression vectors in which the PNT (Elf3Δ60–127) or SAR (Elf3Δ173–241) domains were removed (Fig. 4A). We then examined the contribution of each domain to the ability of Elf3 to stimulate the TβR-II promoter. Removal of the PNT domain did not affect the ability of Elf3 to stimulate the mTβR-II −108/+56(B/A) P/R construct (Fig. 4B). This is not entirely unexpected given that the residues in Ets1 required for recognition and phosphorylation by ERK are not conserved in Elf3 (1). Interestingly, the effect of this mutation may be slightly underestimated, because the expression of this protein was ~20–30% lower than wild-type Elf3 (Fig. 4C). In contrast to the PNT domain, removal of the SAR domain did not affect the expression level of Elf3 (Fig. 4C), but it significantly decreased the ability of Elf3 to stimulate transcription, ~30–40% (p < 0.05) upon comparing multiple experiments (Fig. 4B). Taken together, these findings argue that Elf3 does not require the PNT domain to stimulate the promoter of the TβR-II gene, whereas the SAR domain exerts a subtle, but significant, effect on Elf3 transactivation.

The Collagenase-1 Promoter Requires Different Elf3 Domains—To determine whether the findings described above with mTβR-II −108/+56(B/A) apply to another Elf3-responsive promoter, we examined the effects of the Elf3 mutants on the human collagenase-1 gene promoter. The collagenase-1 gene, like the TβR-II gene, is up-regulated upon differentiation of the F9 EC cells with retinoic acid (64). The collagenase-1 promoter contains a single ets site located at −85 to −88 relative to the transcription start site, which responds to ETS family members (17, 65). In HeLa cells, Elf3 has been shown to increase transcription of a CAT reporter gene that was driven by the −516/+63 region of the collagenase-1 gene promoter (7). In F9-differentiated cells, Elf3 stimulated the collagenase-1 P/R construct as much 15–20-fold (Fig. 5). As with the mTβR-II −108/+56(B/A) P/R construct, removal of the PNT domain (Elf3Δ60–127) did not affect the stimulation of the collagenase-1 P/R construct by Elf3 (Fig. 5). Similarly, removal of the residues flanking the ETS domain of Elf3 (aa 355–362, 235–270, or 354–371) resulted in a loss of stimulation of the collagenase-1 P/R construct (data not shown). In addition, Elf3Δ173–241 exhibited a decrease in the ability to stimulate the collagenase-1 promoter when compared with wild type (Fig. 5). Interestingly, Elf3Δ173–241 is 60–70% less active with the collagenase-1 promoter but only 30–40% less active with the mTβR-II −108/+56(B/A) P/R construct (Fig. 4B). These findings suggest that the SAR domain may play a more important role in the activation of the collagenase-1 promoter.

Intriguingly, removal of residues 235–260 from Elf3 resulted in a more dramatic decrease with the collagenase-1 promoter than with the mTβR-II promoter. FLAG-Elf3Δ235–260, which contains an exogenous NLS, retains 70% of the wild-type Elf3 activity with the mTβR-II −108/+56(B/A) P/R construct (Fig. 6A). However, nearly all activity is lost when FLAG-Elf3Δ235–260 was tested with the collagenase-1 P/R construct (Fig. 6A). Our findings argue that residues 235–260 are essential for activation of the collagenase-1 promoter but not for the mTβR-II promoter. When comparing the two promoters, it was noted that the collagenase-1 promoter contains a single ets site in close proximity to an AP-1 site; however the mTβR-II promoter

FIGURE 4. The SAR domain, but not the PNT domain, influences the activity of the TβR-II promoter. A, diagram of FLAG-Elf3 deletion constructs. Elf3 represents the full-length protein (aa 1–371). Residues 60–127 or 173–241 were deleted from Elf3 to create Elf3Δ60–127 or Elf3Δ173–241, respectively. B, F9-differentiated cells were transiently transfected with 15 μg of the mTβR-II −108/+56(B/A) P/R construct. Where indicated, the cells were also transfected with 0.5 or 1 μg of an N-terminal GFP fusion Elf3Δ60–127, or Elf3Δ173–241 expression vector. Activities were assayed and normalized as described under “Experimental Procedures.” The promoter activity of each construct is calculated relative to the CAT activity observed with mTβR-II −108/+56(B/A) alone, which was set to 1. This experiment was performed four times, and similar results were obtained. Analysis of the data by the Student’s t test indicated that the activity of Elf3Δ173–241 was significantly less than the activity of Elf3 with a p value of less than 0.05. C, F9-differentiated cells were transiently transfected with 1.5 μg of the GFP fusion expression vector indicated below the bar using Lipofectamine 2000, and GFP fluorescence was measured by flow cytometry, as described under “Experimental Procedures.” Level of expression is based on the intensity of GFP per cell. Data shown are means ± S.D. for quadruplicate samples from one representative experiment. This experiment was performed three times, and similar results were obtained.
possesses two overlapping ets sites. Given this difference, we hypothesized that residues 235–260 are needed in cases where a single ets site is present, although this region is not necessary for regulatory regions that contain two adjacent ets sites. To test this hypothesis, we tested the mTβR-II P/R construct in which one of the ets sites was removed. Specifically, we utilized the mTβR-II –108/+56(B/x) P/R construct in which the nonconsensus A site is mutated (12). mTβR-II –108/+56(B/x), which retains the consensus B site, was ~40% less active than the wild-type mTβR-II –108/+56(B/A) P/R construct (12). In this study, we directly compared the ability of FLAG-Elf3 and FLAG-Elf3Δ235–260, which both contain an exogenous NLS, to stimulate mTβR-II –108/+56(B/A) and mTβR-II –108/+56(B/x). As shown earlier (Fig. 3B), FLAG-Elf3Δ235–260 displayed ~60–70% of wild-type FLAG-Elf3 activity on the wild-type mTβR-II –108/+56(B/A) P/R construct (Fig. 6B). However, FLAG-Elf3Δ235–260 displayed only about 25% of wild-type FLAG-Elf3 activity when tested with the mutant mTβR-II –108/+56(B/x) P/R construct (Fig. 6B). Taken together, these experiments argue that residues 235–260 are important for stimulation of promoters that contain only a single ets site.

Promoter-specific Effects of an AT-hook Domain of Elf3—Elf3 contains two AT-hook motifs from residues 237–241 and 246–250, which are identical to the HMG-1 and HMG-I/Y protein AT-hooks, respectively (1). This protein motif is unique to Elf3 within the ETS transcription factor family (1), but it is found within other transcription factors, mainly HMG proteins. To further elucidate the reason why Elf3Δ235–260 exhibits reduced activity with the collagenase-1 gene P/R construct, we deleted each of the AT-hook domains with five amino acid deletions either alone or in combination (FLAG-Elf3Δ237–241, FLAG-Elf3Δ246–250, or FLAG-Elf3Δ237–241Δ246–250) (Fig. 7A). These domains are not necessary for Elf3 stimulation of mTβR-II –108/+56(B/A) (data not shown and Fig. 7B). Moreover, FLAG-Elf3Δ237–241, which removed the first AT-hook, stimulated the collagenase-1 P/R construct to levels similar to the wild-type FLAG-Elf3 (Fig. 7C). In contrast, FLAG-Elf3Δ246–250 exhibited only about 20–30% of the activity of wild-type FLAG-Elf3 (Fig. 7C). These findings argue that the second AT-hook plays a significant role in allowing Elf3 to stimulate the collagenase-1 promoter.

Dual Roles of TAD—Multiple regions of Elf3 affect binding to DNA. Earlier in this study, we presented evidence that the ETS domain, as well as the regions directly flanking the ETS domain, are necessary for Elf3 to bind to DNA. Furthermore, previous studies suggested that Elf3 also contains an AID, which negatively affects the ability of Elf3 to bind to DNA in vitro (11, 12). AIDs are not unusual in the ETS transcription factor family, and the best described are found in Elk1 and Ets1. To further characterize the AID of Elf3, which alters its in vitro binding to
DNA, we utilized a FLAG tagged Elf3 mammalian expression construct to express full-length Elf3 in 293T cells. We prepared nuclear extracts from these cells and utilized these extracts to test the binding of Elf3 to a double-stranded ODN probe containing the \( \text{ets}^-\text{H}11001 \) to \( \text{ets}^-\text{H}11001 \) region of the \( \text{T}^-\text{H}9252 \text{R-II} \) promoter. This region contains two overlapping \( \text{ets}^-\text{binding sites} \) to which Elf3 had been shown to bind in vivo (11). However, full-length Elf3 binds poorly to a DNA probe containing these sites in vitro (11). Remarkably, upon addition of the anti-FLAG antibody, which recognizes the FLAG tag at the N terminus of Elf3, a significant increase in binding was observed (Fig. 8B). This was specific for Elf3 and the M2 antibody, because an increase in FLAG-Elf3 binding did not occur in the presence of a nonspecific IgG antibody or with mock-transfected extracts (data not shown). This phenomenon has also been described for PEA3 and its AID (38) and argues that Elf3 does not bind well to DNA in vitro and that the M2 antibody can stabilize its binding in vitro, possibly by artificially dimerizing or reinforcing intrinsic dimerization of Elf3 and/or creating an
artificial release of autoinhibition, which may lead to increased stability of DNA binding.

To elucidate the location of the AID of Elf3, we used 293T cells to express several Elf3 mutant proteins with specific domains deleted. Western blot analysis was used to determine the relative amounts of Elf3 in the nuclear extracts. This enabled us to use equal amounts of Elf3 in our EMSA studies. As shown previously, removal of residues 1–233 or 1–270 from Elf3 enhanced its binding to DNA (Fig. 8B). Interestingly, Elf3ΔN173, like Elf3ΔN270 but not Elf3ΔN233, forms two DNA-protein complexes (Fig. 8B), suggesting that Elf3ΔN173 and Elf3ΔN270 adopt a conformation that allows them to bind to both DNA (Elf3ΔN173; Fig. 8B). Furthermore, a smaller deletion of residues 1–173 also displayed enhanced binding compared with the ability of full-length Elf3 to bind to DNA (11). Therefore, Elf3ΔN270 contains the TAD and PNT domains. The faster migrating complex in this EMSA is believed to contain only one Elf3 molecule (binary complex), whereas the slower migrating complex is believed to be a ternary complex of two molecules of Elf3 bound to the DNA probe. These findings also suggested that autoinhibition could be relieved by removal of the N terminus of Elf3, which contains the TAD and PNT domains.

Next, we examined which Elf3 domain within residues 1–173 led to increased DNA binding upon its removal. For this purpose, we examined the effect of removing the PNT domain (Elf3Δ60–127) or the TAD (Elf3Δ129–159). Deleting the PNT domain did not increase Elf3 binding to DNA in vitro (data not shown). However, removal of the TAD caused a prominent increase in binding to DNA when compared with wild-type Elf3 (Fig. 8B). This suggests that removal of the TAD disrupts autoinhibition and led us to hypothesize that the action of the AID is linked with promoter activation via the TAD. Previous studies have reported that the TAD of Elf3 has potent activity (11). To test this, we created point mutations within the TAD previously shown to affect function. As predicted, point mutations within the TAD, which are known to disrupt the TAD, were used to determine that the GFP-Elf3ΔN270 protein was immunoprecipitated with the M2 antibody (Fig. 9A). We determined that the GFP-Elf3ΔN270 protein was pulled down when FLAG-Elf3ΔC235 was immunoprecipitated with the M2 antibody (Fig. 9B). This argues that the N terminus of Elf3 is able to interact with its C terminus.

These data, combined with our knowledge that the TAD is involved in autoinhibition, transactivation, and interaction with the coactivator Med23, led us to test the hypothesis that the interaction of the coactivator Med23 with the TAD of Elf3 in its N-terminal region could influence the ability of its N terminus to interact with its C terminus. For this purpose, 293T cells were cotransfected with an expression construct for full-length Med23 or a fragment of Med23, which interacts with the

**FIGURE 9.** Intramolecular interactions between the N- and C-terminal halves of Elf3 are competed by addition of the coactivator Med23. A. Diagram of the Elf3 expression constructs created for communoprecipitation studies. B. 293T cells were transfected with 5 μg of the indicated GFP-based expression vectors and 10 μg of the FLAG-based expression plasmids. After 24 h, extracts were made and exposed to M2-anti-FLAG affinity agarose overnight. The agarose was washed extensively, and FLAG proteins were then eluted with 150 ng/μl 3× FLAG peptide and analyzed for GFP and FLAG protein content. Input samples were taken before addition of the M2-anti-FLAG affinity agarose for analysis of protein expression and normalization of GFP-Elf3ΔN270 content present before immunoprecipitation (IP).
TAD of Elf3 (6), along with expression vectors for FLAG-Elf3ΔC235 and GFP-Elf3ΔN270. Importantly, the presence of Med23 or Med23-(352–625) reduced the ability of GFP-Elf3ΔN270 to be coimmunoprecipitated with FLAG-Elf3ΔC235 when the M2 antibody was used (Fig. 9B). After normalization to the amount of FLAG-Elf3ΔC235 immunoprecipitated and the expression of GFP-Elf3ΔN270 (input), we determined that there was a 40–60% decrease in interaction between GFP-Elf3ΔN270 and FLAG-Elf3ΔC235 in the presence of the coactivator as compared with the amount immunoprecipitated in the absence of the coactivator. This suggests that the coactivator interaction with the TAD can alter the interaction between the N terminus and C terminus. Below, we suggest that this may serve as a mechanism to relieve autoinhibition in vivo.

DISCUSSION

Previous studies demonstrated that Elf3 influences a wide range of biological functions (6, 7, 14, 19–25). In the work described here, we examined how the five domains of Elf3 regulate its ability to bind to DNA and activate transcription. The SAR domain contributes to Elf3 transcriptional activity; however, the mechanism by which this happens is unclear. The PNT domain (Elf3Δ60–127) and the last eight amino acids of the C-terminal tail (Elf3ΔC363), however, do not contribute to Elf3 activation of the promoter activity. Intriguingly, removal of the second AT-hook domain differentially affects the ability of Elf3 to stimulate the collagenase-1 and TBR-II promoters. We also demonstrate that regions flanking the ETS domain are essential for maintaining transcriptional activity of Elf3. Removal of these regions disturbs the ability of Elf3 to bind to DNA, therefore affecting transcription. It is also evident that a domain distant from the ETS domain affects DNA binding. Specifically, our data argue that the TAD region is responsible, at least in part, for the AID of Elf3. The TAD is also needed for transcription activity arguing that the two functions may be related, as discussed below.

Promoter-specific Effects of the AT-hook Domain—Elf3 appears to be a unique protein in the ETS family, because it contains two AT-hook domains that are similar to the AT-hook domains of HMG-1 and HMG-I(Y), respectively (1). AT-hook domains are typically the DBDs of HMG proteins (30). The HMG proteins usually contain more than one AT-hook domain, which they utilize to bind in the minor groove of AT-rich DNA and/or to interact with other proteins (31). However, few studies have directly addressed the role(s) of the AT-hook domains of Elf3 (19, 21). The two AT-hook domains of Elf3 are located N-terminal to the ETS domain from residues 237–241 and 246–250. Our studies demonstrate the second AT-hook domain of Elf3, which is conserved between mouse and human, plays an important role in activating transcription but in a promoter-dependent manner. Importantly, Elf3Δ246–250, which removes the second AT-hook domain, is significantly less active with the collagenase-1 promoter than with the TBR-II promoter.

We examined two likely explanations for the disparity in the function of this AT-hook domain between the collagenase-1 promoter and the TBR-II promoter. The first possibility is that DNA-protein interactions between an AT-rich site directly downstream of the ets site and the AT-hook are necessary for Elf3 stimulation of the collagenase-1 promoter. However, this is unlikely because the AT-hook domain is also needed for maximal Elf3 activation of the TBR-II (B/a) promoter, which contains only one ets site (data not shown), and an AT-rich region was not created when the second ets site was destroyed in the TBR-II (B/A) P/R construct. Moreover, when we tested this possibility further by mutating the AT-rich base pairs downstream of the ets site of the collagenase-1 promoter, the ability of the modified promoter to respond to Elf-3 was not affected, nor did it alter the relative difference in the response to Elf-3 and Elf3Δ246–250 (data not shown). This argues that the AT-rich sequence is unlikely to be responsible for the promoter-specific effects of the AT-hook domain. Therefore, we examined a second possibility whereby AP-1 and Elf3 interact via the AT-hook domain to stimulate the collagenase-1 promoter. In this regard, the AP-1 site and the ets site of the collagenase-1 promoter are known to cooperate (17, 18). Moreover, the HMG-I(Y) protein interacts with NF-κB, SRF, and AP-1 through its AT-hook domain (31, 67–69). We tested the connection between the AT-hook domain of Elf3 and AP-1 site in the collagenase-1 promoter by mutating the AP-1 site and examining whether mutation of this site removed the requirement for the AT-hook domain. Although removal of the AP-1 site reduced the activity of the collagenase promoter, it did not eliminate the requirement for the AT-hook domain of Elf3 (data not shown). These results suggest that another, as yet unrecognized, mechanism is responsible for the requirement for the AT-hook domain for stimulation of the collagenase-1 promoter but not the TBR-II promoter.

Requirement for Areas Flanking the ETS Domain—Our studies show that residues outside of the typical 80–85 amino acids of the ETS domain are necessary for Elf3 to bind to DNA. Specifically, we show that removal of residues 260–270 or 355–362 disrupts binding to DNA. Removal of these residues may influence the overall structure of the ETS domain, thereby disrupting protein-DNA interactions within the ETS domain. Alternatively, the regions flanking the ETS domain may themselves make direct contacts with DNA. We favor the former possibility, because other ETS proteins, namely of the Ets1 and PEA3 subfamilies, contain regions flanking their ETS domains that affect its structure (33, 40, 41). In these cases, the regions flanking the ETS domain are believed to stabilize a conformation that has a lower affinity for DNA (40, 41). However, in the case of Elf3, the regions that directly flank the ETS domain may be necessary to maintain the overall structure of the ETS domain and allow Elf3 to bind to DNA once autoinhibition is relieved or disrupted (see below). Future structural studies will be necessary to determine how these regions contribute to the structure of the ETS domain of Elf3 and its binding to DNA.

Model of Elf3 Autoinhibition—Previous studies suggested that Elf3 contains an important AID that affects its binding to DNA in vitro (11). Autoinhibition of transcription factors is often demonstrated by the inability of the protein to bind to DNA in vitro (39, 41). In vivo, autoinhibition is believed to prevent promiscuous DNA binding and adds another layer of control over gene regulation (39). Many proteins are reported to
contain an AID. Germane to this discussion, many transcription factors contain AIDs that affect their ability to bind DNA (39), including nine ETS transcription factor family members (11, 33–38). Thus far, two models have been proposed to explain autoinhibition of ETS proteins. The first, the Ets1-based model, involves regions directly flanking the ETS domain inhibiting DNA binding and direct interaction with other activators within these regions or the ETS domain itself to release autoinhibition (39). The second, the Elk1-based model, involves distant intramolecular interactions to create autoinhibition and release occurs by post-translational modification (50).

Based on three key findings described in this study, we propose a novel model for autoinhibition that differs from the models proposed for Ets1 and Elk1. First, we show that mutation within the α-helix of the TAD increases Elf3 binding to DNA, which argues that its TAD is responsible, at least in part, for Elf3 autoinhibition. Second, we show that the N terminus of Elf3 interacts with its C terminus. Third, we demonstrate that the coactivator Med23, which interacts with the TAD of Elf3 (6), can reduce the interaction between the N and C terminus of Elf3. On the basis of these findings, we propose a model for Elf3 autoinhibition (Fig. 10). Specifically, we propose that Elf3 is held in a conformation that is unable to interact with DNA, because of an interaction between the N terminus and the C terminus, possibly directly through the ETS domain and the TAD (Fig. 10A, left). This would be a reversible state in which the autoinhibited state would predominate, but a limited amount could bind to DNA (Fig 10B, left). In the presence of a coactivator, such as Med23, which interacts with the TAD α-helix, the interaction between the N and C terminus of Elf3 would be weakened or altered shifting the conformation of Elf3 to the DNA binding form. Therefore, in the presence of the coactivator the DNA binding form would predominate (Fig 10A, right). As a result, the interaction of the coactivator with Elf3 would release autoinhibition and simultaneously promote DNA binding and transcription.

There are two possible spatial locations in which coactivator interaction and release of Elf3 autoinhibition may occur. 1) The coactivator may interact with Elf3 in the absence of DNA, which stabilizes the DNA binding form, and this coactivator-Elf3 complex then binds to gene regulatory regions to affect gene transcription (Fig 10B, middle). Alternatively, if the coactivator is present at the promoter prior to Elf3, it could stabilize the transient binding of Elf3 to gene regulatory regions (Fig 10B, right).

This model is similar, in part, to the autoinhibition model described for the ETS transcription factor Elk1. Elk1 is reported to contain three AIDs as follows: the TAD, the R-domain, and the B-box (50, 51). The main AID of Elk1, which affects DNA binding, is located within the TAD (50). The TAD of Elk1 is reported to bind directly to the ETS domain, limiting interaction with DNA until the TAD is phosphorylated (50). In this case, phosphorylation triggers the release of autoinhibition in Elk1, whereas we propose the triggering mechanism(s) for release of autoinhibition in Elf3 is an interaction with a coactivator. Importantly, several reports suggest that protein-protein interactions with cofactors or coactivators can result in a release of autoinhibition in vivo (40, 43–45, 59, 70). For example, TBP is reported to increase DNA binding of p53 independent of the interaction of TBP with DNA (70). Furthermore, several transcription factors display increased affinity for DNA in vitro upon the removal of their TADs, including Sox11, IRF-3, and Ets2 (12, 71, 72). In combination, these studies suggest that other transcription factors may contain an AID that coordinates transcriptional activation and binding to DNA. Furthermore, these studies suggest the interaction of coactivators/cofactors, and the TADs of transcription factors may be important for increasing transcription factor binding in vivo. Future studies will be needed to determine whether this is a common mechanism utilized to enhance the binding of many transcription factors in vivo.

In summary, our work provides a comprehensive examination of the domains utilized by Elf3 to regulate DNA binding and gene expression. Furthermore, we propose a novel model by which transcription and DNA binding may be intimately connected. Future investigation into the autoinhibition may find that multiple mechanisms are used to relieve autoinhibition of individual ETS factors. Moreover, our model for the release of autoinhibition may be applicable to a wide range of transcription factors. However, further work will be needed to test this possibility. Finally, it will be important to identify the cellular cues that regulate the transcriptional activation and/or autoinhibition of Elf3. In the future, this information could prove useful for manipulating Elf3 in disease states, such as cancer.
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