Mitogen-induced Expression of the Fibroblast Growth Factor-binding Protein Is Transcriptionally Repressed through a Non-canonical E-box Element

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The fibroblast growth factor-binding protein (FGF-BP) stimulates FGF-2-mediated angiogenesis and is thought to play an important role in the progression of squamous cell, colon, and breast carcinomas. 12-O-Tetradecanoylphorbol-13-acetate (TPA) induction of the FGF-BP gene occurs through transcriptional mechanisms involving Sp1, AP-1, and CCAATT/enhancer-binding protein sites in the proximal FGF-BP gene promoter. The level of TPA induction, however, is limited due to the presence of a repressor element that shows similarity to a non-canonical E-box (AACGTG). Mutation or deletion of the repressor element led to enhanced induction by TPA or epidermal growth factor in cervical squamous cell and breast carcinoma cell lines. Repression was dependent on the adjacent AP-1 site, without discernable alteration in the binding affinity or composition of AP-1. We investigated the following two possible mechanisms for E-box-mediated repression: 1) CpG methylation of the core of the E-box element, and 2) binding of a distinct protein complex to this site. Point mutation of the CpG methylation site in the E-box showed loss of repressor activity. Conversely, in vitro methylation of this site significantly reduced TPA induction. In vitro gel shift analysis revealed distinct and TPA-dependent binding of USF1 and USF2 to the repressor element that required nucleotides within the E-box. Furthermore, chromatin immunoprecipitation assay showed that USF1, c-Myc, and Max proteins were associated with the FGF-BP promoter in vivo. Overall, these findings suggested that the balance between trans-activation by AP-1 and repression through the E-box is an important control mechanism for fine-tuning the angiogenic response to growth factor-activated pathways.

Angiogenesis, or the growth of new blood vessels, is an early and necessary event in the growth of a tumor. Understanding the mechanisms that underlie the switch to an angiogenic phenotype of a tumor is an integral part of the development of anti-angiogenic therapy. One way in which the angiogenic pathway can be stimulated is through the activation of fibroblast growth factors (FGF-1 and FGF-2) that are present at high levels in most tissues where they are bound to heparan sulfate proteoglycans and sequestered in the extracellular matrix (1, 2). Tumor cells can release FGF-2 activity through the induced expression of an FGF-binding protein (FGF-BP), which is secreted from tumor cells and binds and mobilizes stored FGF-2, leading to the activation of FGF-2-dependent processes such as angiogenesis (3, 4).

FGF-BP is found in only a limited number of epithelial tissues where its expression is tightly regulated. During mouse embryonic development, FGF-BP expression is up-regulated in the epithelial layers of the skin, intestine, and lung where it coincides with development of these tissues (5). After peak FGF-BP expression at embryonic day 18, levels drop significantly after birth and remain low in most tissues of the adult mouse (5). In human tissues, FGF-BP expression is low but was found to be significantly up-regulated in certain tumors including squamous cell carcinomas (SCC) derived from skin, cervix, lung, and head and neck region (4). FGF-BP is also highly expressed in some colon cancers (6) and breast adenocarcinomas. A functional role for FGF-BP in these tumors has been shown through the use of ribozyme targeting, where as little as 20% reduction in FGF-BP steady-state mRNA levels led to a decrease in tumor growth and angiogenesis of xenografted cervical SCC and colon tumors (6). Thus it appears that for at least some tumors, FGF-BP expression is rate-limiting for tumor growth and angiogenesis.

A relationship between FGF-BP expression and tumor formation has also been established by the observation that levels of FGF-BP increase during 7,12-dimethylbenz[a]anthracene- and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse skin carcinogenesis (5). We subsequently found that FGF-BP gene transcription is directly induced by TPA or epidermal growth factor (EGF) treatment of SCC cell lines (7, 8). Analysis of the FGF-BP promoter showed that TPA and EGF induction is mediated within the first 118 base pairs of the proximal promoter and requires several positive regulatory cis-elements in the FGF-BP promoter including Sp1, AP-1, and C/EBP (7, 8). In addition, we identified a region of the promoter between the AP-1 and C/EBP sites that mediated a repressive effect on FGF-BP transcription. Deletion or mutation of the

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1 The abbreviations used are: FGF, fibroblast growth factor; FGF-BP, fibroblast growth factor-binding protein; SCC, squamous cell carcinoma; TPA, 12-O-tetradecanoylphorbol-13-acetate; C/EBP, CCAATT/enhancer-binding protein; EGF, epidermal growth factor; USF, upstream stimulatory factor; Arnt, aryl hydrocarbon receptor nuclear translocator; bHLHZip, basic helix-loop-helix leucine zipper; ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction; IMEM, improved minimum essential medium; mAP-1, mutated AP-1.

2 A. T. Riegel and A. Wellstein, unpublished data.
representer region had no effect on basal activity of the promoter but significantly enhanced the level of TPA induction (7), indicating that this region of the promoter functions to limit the overall response to TPA induction of FGF-BP gene expression. In this study, we investigate more closely the mechanisms by which the FGF-BP representer region can limit transcriptional induction of this gene in response to either TPA or EGF.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The human cell lines ME-180 and HeLa (cervical squamous cell carcinoma), BT-549 (dental breast carcinoma), and MCF-7 (breast adenocarcinoma) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in improved minimum essential medium (IMEM) (Biofluids Inc., Rockville, MD) without phenol red and supplemented with 10% fetal bovine serum (Life Technologies, Inc.).

**Plasmids**—Human FGF-BP promoter fragments were cloned into the pSP1 promoterless luciferase reporter vector and have been described elsewhere (7). The FGF-BP promoter constructs from −118 to +62 carrying a mutated AP-1 site (mAP-1 and mAP-1/m-58) or mutated E-box (m-55/−56) were generated by PCR-based site-directed mutagenesis as described previously (7). Briefly, point mutations were introduced into complementary overlapping PCR primers that convert the AP-1 and E-box sites in the pGTA GTAA CGGT (-66 to −55) to TGGAGCAAC GTG or TGGACGATGTG to generate the mAP-1 Luciferase construct. For each transfection, cells were plated in 6-well plates at a density of either 1.0 × 10^5 of USF-1 and USF-2 were also purchased from Santa Cruz Biotechnology. Anti-ARNT polyclonal antibody was kindly provided by Dr. N. Copeland (NCI Center for Biotechnology). Supershift antibodies were purchased from Santa Cruz Biotechnology. Anti-ARNT polyclonal antibody was kindly provided by Dr. N. Copeland (NCI Center for Biotechnology).

**RESULTS**

**Identification of a Repressor Element in the FGF-BP Promoter**—We previously discovered that deletion of the region between −57 and −47, situated between the AP-1 and C/EBP sites, resulted in enhanced TPA induction of transcription (7), indicating the involvement of a negative regulatory element in the TPA regulation of FGF-BP. This region has no homology to any known transcription factor binding sites except for the presence of an imperfect E-box between −60 and −55. To characterize further the FGF-BP representer element, we tested whether mutations in this region would disrupt representer activity and lead to enhanced TPA or EGF induction of the promoter. The region of the FGF-BP promoter between −118 and +62 harbors all of the necessary elements for full induction by both TPA (7) and EGF (8). Internal deletion from −57 to −47 generated in the context of the −118 to +62 promoter showed significantly increased induction by both TPA and EGF when transfected into ME-180 cervical squamous carcinoma cells (Fig. 1A). In addition, introduction of a C to T point mutation at position −58 within the E-box showed a dramatic 16-fold increased response to TPA, being found on a 7- to 18-fold induction (Fig. 1A). EGF induction of the −58 mutant was also highly increased, going from a 5.5- to 10-fold induction (Fig. 1A). Loss of repression was not reflected at the level of basal promoter activity since the representer mutant constructs −57/−47 and m-58 had the same uninduced promoter activity as the wild-type −118/−62 (7, 8). Therefore, the region between −58 and −47 of the FGF-BP promoter appears to function in limiting the overall transcriptional response to both TPA and EGF.

In order to determine whether representer activity on the FGF-BP promoter existed in other cell types, we tested the TPA

...phenol/chloroform and ethanol precipitation prior to transcription. Complete CpG methylation was confirmed by digestion with the methyltransferase-sensitive restriction enzyme HpaII (New England Biolabs).

**Formaldehyde Cross-linking and Chromatin Immunoprecipitation (ChIP Assay)**—Approximately 10^6 ME-180 cells were serum-starved for 16 h with 10^-7 M TPA and cross-linked to DNA by formaldehyde directly to culture medium to a final concentration of 1% for 15 min at room temperature. Cells were subsequently washed and scraped into 1 ml of 1× phosphate-buffered saline containing 1× protease inhibitor mixture (Roche Molecular Biochemicals). Cell pellets were lysed in 200 μl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1× protease inhibitor mixture) for 4 h on ice. Cross-links were reversed by incubating eluates at 65 °C for 4 h. 2 ml of NaCl, followed by digestion with 40 ng/ml proteinase K in 10 mM EDTA, 40 mM Tris-HCl, pH 6.5, for 2 h at 45 °C. DNA was recovered by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation.

PCRs contained 4% of input DNA or 20% of immunoprecipitated DNA along with 10 pmol of primers, 1.5 mM MgCl2, 0.2 mM dNTPs, 1× PCR buffer (Life Technologies, Inc.), and 5 units of Taq DNA polymerase (Life Technologies, Inc.). Primers used were from −369 to −350 and from −47 to −73 of the FGF-BP promoter (7). Plasmid containing −1829 to +62 of the FGF-BP promoter was used as a control template. After 22 cycles of PCR, samples were run on a 1% agarose gel, transferred to nylon membrane, and probed with an FGF-BP-specific primer from −118 to −99 end-labeled with T4 kinase. Band intensities were quantitated by PhosphorImager, and the amount immunoprecipitated was expressed as percent of total input.

The results of the ChIP assay confirmed the in vivo representer activity of the −118 to +62 region of the FGF-BP promoter.
and EGF response of the wild-type −118/+62 or the E-box point mutant (m-58) promoter constructs in other cell lines. The −58 mutant showed significantly enhanced TPA induction in HeLa (cervical squamous carcinoma) and MCF-7 (breast cancer) cell lines (Fig. 1B). In cell lines where the FGF-BP promoter was EGF-responsive, such as HeLa and BT-549 (breast), the fold EGF induction of the −58 mutant was also consistently higher (Fig. 1C). These data indicate that repression of the FGF-BP promoter is a general mechanism of FGF-BP transcriptional regulation in response to TPA or EGF stimulation.

AP-1 Dependence of Repressor Activity—The proximity of the AP-1 site to the repressor mutations raised the possibility that the observed increase in the TPA response was due to its impact on the juxtaposed AP-1 site. To test the possible influence that the −58 mutation may have on the AP-1 site, we generated double mutant constructs carrying the −58 mutation in conjunction with mutations in either the AP-1 site or the C/EBP site. We have shown previously that EGF or TPA induction of FGF-BP partly depends on the AP-1 and C/EBP sites in the promoter, since mutation of the AP-1 site (mAP-1), or deletion of C/EBP (delta C/EBP) results in a significant decrease in the amount of induction by EGF and TPA with no effect on basal promoter activity (Fig. 2 and Refs. 7 and 8). Mutations in the AP-1 site have also been shown to disrupt AP-1 binding (7) (Fig. 5B). As shown in Fig. 2, the −58 mutation alone showed an enhanced TPA response, whereas mutation of the −58 and AP-1 sites together showed no loss of repression. Instead, the m-58/mAP-1 double mutant displayed a similar level of TPA or EGF induction as that of the mAP-1 single mutant construct. Conversely, the −58 mutation in combination with a deleted C/EBP site resulted in loss of repression (Fig. 2). Therefore, the effect of the −58 mutation is not dependent on the C/EBP site but is dependent on an intact AP-1 site since the m-58/mAP-1 construct does not result in a loss of repression.

The AP-1 dependence of repressor activity prompted us to confirm that the −58 mutant phenotype was not simply a consequence of altered AP-1 binding affinity to the FGF-BP AP-1 site. We carried out gel shift competition analysis of the AP-1 site in the presence or absence of the −58 mutation. By using a promoter fragment that spans the AP-1/repressor element (−70 to −51) as a probe, we could detect AP-1 binding (upper complex) and binding of additional proteins that are

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**Fig. 1.** Effect of repressor site mutations on TPA and EGF induction of the FGF-BP promoter. A, TPA and EGF induction of the wild-type FGF-BP promoter construct −118/+62 compared with the repressor deletion mutant Δ−57/−47 and the repressor point mutant m-58 in ME-180 cells. ME-180 cells were transiently transfected with the indicated FGF-BP promoter luciferase (LUC) construct and were either untreated or treated for 18 h with 100 nM TPA (black bars) or 5 ng/ml EGF (white bars). B, TPA induction of the −118/+62 construct compared with the repressor mutant construct m-58 in HeLa and MCF-7 cells. C, EGF induction of −118/+62 and m-58 constructs in HeLa and BT549 cells. All cells were transiently transfected and treated as in A. Luciferase activity is expressed as fold induction of treated over untreated for each construct. Values represent the mean and S.E. from at least three experiments, each done in triplicate wells. In all cell lines, difference in induction of either Δ−57/−47 or m-58 constructs is statistically significant from the −118/+62 construct (p < 0.01).
specific to the repressor element (Fig. 3A, upper panel and Refs. 7 and 8). When the −58 point mutation was introduced into the repressor site, the lower complexes disappeared, and only the AP-1 complex was bound (Fig. 3A, lower panel). Competition with an unlabeled consensus AP-1 element (Fig. 3A, lanes 2–4) or with the FGF-BP AP-1 element (lanes 5–7) could effectively compete for AP-1 binding. Both competitors reduced AP-1 binding to the probe at concentrations between 10- and 20-fold molar excess, as determined by quantitation of the AP-1 band intensity. Similarity, competition for AP-1 bound in the presence of the −58 mutation also occurred at concentrations between 10- and 20-fold molar excess of unlabeled AP-1 elements (Fig. 3A, lanes 8–14). Although quantitation of AP-1 binding to the m-58 probe suggests a decreased AP-1 binding affinity, this may be due to the difficulty in obtaining accurate quantitation of band intensity in the presence of multiple bands (Fig. 3A, upper panel). Nevertheless, these results indicate that the enhanced TPA induction caused by the −58 mutation cannot be explained by increased AP-1 binding affinity.

Alternatively, the −58 mutation could potentially alter the composition of AP-1 by flanking the AP-1 site with nucleotides that favor binding of different AP-1 family members, leading to increased transcriptional activation. We have published elsewhere that EGF activation of ME-180 cells led to increased binding of c-Fos and Jun proteins to the FGF-BP AP-1 site (8). We asked which AP-1 family members were bound to the FGF-BP promoter after TPA treatment and whether the composition of AP-1 changed in the presence of the −58 mutation. Gel supershift analysis was carried out using the −70/+51 FGF-BP promoter fragment as a probe in the presence of TPA-treated ME-180 extracts and antibodies specific for individual members of the AP-1 family (Fig. 3B, lanes 1–10). Supershift of the AP-1 complex occurred in the presence of cross-reactive Fos and Jun antibodies (lanes 2 and 7, respectively) as well as with specific antibodies for c-Fos (lane 3), Fra2 (lane 6), and JunD (lane 10). The binding of c-Fos, Fra1, and JunD was also prevalent in the presence of the −58 mutation (Fig. 3B, lanes 13, 16, and 20). Overall, these experiments demonstrate that although repressor activity mediated through the −58 site is dependent on the adjacent AP-1 site, the −58 mutation had no obvious impact on either AP-1 binding affinity or the composition of the AP-1 complex.

Methylation of the FGF-BP Promoter Represses TPA Induction—We hypothesized that one possible effect of the −58 mutation could be that the C to T point mutation leads to loss of transcriptional repression through disruption of a CpG methylation site. The −57/−47 deletion is consistent with this hypothesis since this mutation also destroys the −58 CpG methylation site. In vivo cytosine methylation occurs preferably at CpG dinucleotides and is closely associated with transcriptional repression of genes with CpG sites in their promoter region (10). In order to determine whether methylation of the −58 CpG site could mediate repression of FGF-BP promoter induction, we tested the effect of in vitro methylation of the FGF-BP promoter constructs on their transcriptional response to TPA. The wild-type −118/+62 and the m-58 promoter constructs were methylated in vitro with SssI methylase so that each plasmid would differ in its CpG methylation pattern only at the −58 site (Fig. 4). Complete methylation of each plasmid construct was confirmed by digestion with HpaII (data not shown). Transfection of the methylated −118/+62 plasmid into ME-180 cells resulted in a significant 50% decrease in the level of TPA induction compared with the unmethylated −118/+62 plasmid (Fig. 4). On the other hand, methylation of the m-58 promoter construct, which is unmethylated at position −58, demonstrated a similar level of TPA induction compared with the unmethylated m-58 construct (Fig. 4). Both methylated constructs displayed an equivalent decrease in basal promoter activity which was 20% lower than the unmethylated plasmids (data not shown) and was independent of the −58 mutation. These results demonstrate that methylation of the −58 CpG site significantly represses TPA induction and indicate that methylation of this site is a potential mechanism for limiting the transcriptional response to growth factor or TPA regulation in vivo.

Transcription Factor Binding to the FGF-BP Repressor Element—Although methylation causes repression through the −58 site, there was still the question of how methylation affects protein binding to the promoter. Furthermore, we also wondered how the C to T point mutation at −58 might affect protein binding to the unmethylated FGF-BP promoter. We examined protein binding to the FGF-BP repressor element by electrophoretic gel mobility shift assays using a fragment of the promoter from −70 to −51 spanning the AP-1/repressor element. The various promoter fragments used are depicted in Fig. 5A, with their sequences shown in Fig. 6A. In the presence of ME-180 nuclear extracts, gel shift with the −70/−51 probe resulted in four distinct protein complexes (Fig. 5B, lane 1, and
see Ref. 7). Protein binding of all four complexes increased in the presence of nuclear extracts from TPA-treated ME-180 cells (Fig. 5B, lane 2). The largest increase was in the uppermost band (complex 1) which represents AP-1 as shown by supershift analysis (Fig. 5B (8)) and by lack of competition with unlabeled −70/−51 fragment harboring point mutations in the AP-1 site (Fig. 5B, lane 3). To determine whether the −58 repressor mutation could affect protein binding to the promoter, competition was carried out with the FGF-BP promoter fragment harboring the C to T point mutation at −58. The m-58 fragment could effectively compete for binding to the AP-1 band (complex 1) since this fragment retains an intact AP-1 element (Fig. 5B, lane 4). In contrast, mutation of position −58 resulted in loss of binding to complexes 2–4 (lane 4) suggesting that these bands represent factors that interact with the FGF-BP repressor element.

Due to the observation that methylation of the −58 site caused repression of TPA induction, we wondered whether methylation itself alters protein binding to the FGF-BP promoter. As seen in Fig. 5C, an unlabeled promoter fragment that was methylated at the −58 CpG site on both strands (methyl-58) was able to compete for AP-1 binding but was a less efficient competitor for the other complexes (lane 2). Similarly, when the methylated fragment was used as a probe, only AP-1 binding was detected (lane 3). This result demonstrates that methylation of the −58 site does not interfere with AP-1 binding to the promoter in vivo, and thus decreased AP-1 binding does not account for methylation-mediated repression. In contrast, methylation does interfere with binding of complexes 2–4, suggesting that the bindings of these proteins are methylation-sensitive. Furthermore, under in vitro gel shift conditions, methylation does not induce binding of additional protein complexes that also might account for transcriptional repression.

We next examined the binding of distinct factors to the unmethylated repressor element in order to investigate whether any of these factors might play a role in repression through the −58 site. In Fig. 5D, the mAP-1 promoter fragment was used as a probe in order to eliminate binding of the AP-1 band (complex 1) and to better resolve the lower bands (complexes 2–4). Surprisingly, only one distinct complex was found to bind to the mAP-1 fragment that co-migrated with complex 2 in the gel shift with the −70/−51 promoter fragment (Fig. 5D). The amount of complex 2 binding to the repressor element was significantly increased in the presence of extracts from TPA-treated cells (lane 3). Competition analysis demonstrated that complex 2 was competed by the wild-type −70/−51 (lane 4) or mAP-1 (lane 5) promoter fragments such that the resulting band intensities were 22% and 26% of uncomplexed binding, respectively. In contrast, complex 2 binding was not efficiently competed by the m-58 fragment (lane 6), which was 63% of uncomplexed binding. These results show that binding of complex 2 is independent of AP-1 and is dependent on the

![Fig. 3. Effect of −58 mutation on AP-1 binding to the FGF-BP promoter.](image)

![Fig. 4. Cpg methylation at position −58 inhibits TPA induction of the FGF-BP promoter.](image)
cytosine at position −58, demonstrating an interaction between a distinct complex with the repressor element of the FGF-BP promoter.

**Mapping of Repressor Binding to the E-box**—In order to determine precisely which nucleotides were necessary for complex 2 binding to the FGF-BP repressor element, we carried out a complete mutational analysis of the −70/−51 promoter fragment. Single point mutations were introduced into the repressor element from −60 to −51 (Fig. 6A) and tested for their ability to compete for binding to complex 2. By using the mAP-1 promoter fragment as a probe, gel shift competition showed that point mutations introduced between −60 and −55, includ-
point mutations at the 3' end of the fragment from the unlabeled promoter fragments as indicated. Complex 2 is indicated by reactions were incubated in the presence of 50-fold molar excess of nuclear extracts from TPA-treated ME-180 cells is shown. Binding analysis of the FGF-BP E-box factor binding to complex 2 (Fig. 6B, lanes 3–8), whereas point mutations at the 3' end of the fragment from −54 to −51 could effectively compete for complex 2 binding (lanes 9–12). It is noteworthy that only mutations within the non-canonical E-box AACGTG had an effect on complex 2 binding.

We next tested whether disruption of complex 2 binding to the FGF-BP E-box correlated with the loss of repressor activity on the FGF-BP promoter. Point mutations were introduced into the promoter at positions −55 and −56 in the 3' end of the FGF-BP E-box which convert the E-box from AACGTG to AACCAC (Fig. 6A). Like the −58 mutation, the −55/−56 E-box mutation was unable to compete for complex 2 binding in a gel shift assay (Fig. 7A). The functional impact of the −55/−56 E-box mutation was then tested in the context of the −118 to +62 promoter construct by transient transfection. Like the m-58 E-box mutant, the −55/−56 promoter mutant displayed significant loss of repression compared with the wild-type promoter in response to TPA (Fig. 7B) and to EGF (Fig. 7C). These results are consistent with the correlation between protein binding to the E-box and transcriptional repression. It should be noted that the m-55/−56 mutation does not disrupt the CpG core of the E-box, indicating that loss of repression through E-box mutation can also occur independently of methylation. Thus, two separate mutations in the FGF-BP E-box disrupt complex 2 binding and display enhanced TPA induction of the FGF-BP promoter.

Identification of USF and c-Myc Binding to FGF-BP Promoter—E-box promoter elements are recognized by a number of basic helix-loop-helix leucine zipper (bHLHZip) transcription factors (11–13). To determine whether any known members of this transcription factor family were present in complex 2, supershift analysis was carried out using antibodies specific for certain bHLHZip factors. The presence of antibodies for c-Myc or Max had no effect on complex 2 binding (Fig. 8, lanes 3 and 4). Similarly, antibodies recognizing the Mad proteins Mad-1 (lane 5) or Mad-2 (Mxi1), Mad-3, and Mad-4 (data not shown) had no effect on complex 2 binding. Although the bHLHZip transcription factor aryl hydrocarbon receptor nuclear translocator (Arnt) has been shown to bind as homodimers in vitro to an AACGTG variant E-box (14), antibodies specific for Arnt had no effect on complex 2 binding to the FGF-BP promoter (Fig. 8, lane 6). On the other hand, antibodies specific for the bHLHZip factors USF-1 and USF-2 completely blocked complex 2 binding to the FGF-BP promoter (Fig. 8, lanes 7 and 9). The ability of the USF-1 or USF-2 antibodies to block complex 2 binding was reversed in the presence of competing USF-1 or USF-2 peptides, respectively (lanes 8 and 10), demonstrating antibody specificity. The TPA-induced binding of USF-1 and USF-2 to a non-canonical E-box AACGTG in the FGF-BP promoter indicated that USF may play a distinct role in the transcriptional regulation of this gene.

Whereas USF is ubiquitously expressed and is easily detected by gel shift assay, the binding of Myc/Max/Mad proteins from cellular extracts is practically undetectable under these conditions (15). Therefore, the in vitro binding assays are biased toward detecting USF. To get a more realistic picture of E-box factor binding to the FGF-BP promoter, we decided to analyze the in vivo binding of USF or Myc/Max/Mad proteins by formaldehyde cross-linking followed by chromatin immunoprecipitation and PCR amplification of the FGF-BP promoter. This type of analysis (also known as the ChIP assay) has proved successful in determining the binding characteristics of c-Myc and USF to the endogenous cad promoter (16–18). We therefore used a similar approach to determine whether the binding of c-Myc family members could be detected on the endogenous FGF-BP promoter in ME-180 cells. After immunoprecipitation, a fragment of the promoter from −369 to −47 was amplified by PCR and detected by Southern analysis. As seen in Fig. 9, antibodies against c-Myc, Max, or USF-1 could effectively immunoprecipitate the FGF-BP promoter. After correction for the amount of input DNA, we determined the amount of FGF-BP promoter immunoprecipitated was 3.0–3.8-fold higher than the no antibody control. This is in contrast to immunoprecipitation with the Mad-2 (Fig. 9) or Mad-1 (data not shown) antibodies that were similar to the control. Immunoprecipitation of the FGF-BP promoter with c-Myc and USF-1 was reproducible over multiple experiments and was also detected after amplification of a shorter FGF-BP promoter fragment (−118 to +62) to the same degree over the control (data not shown). These results suggest that both USF as well as c-Myc-Max complexes can associate with the endogenous FGF-BP promoter in ME-180 cells and could potentially play a functional role in modulating TPA or EGF induction of FGF-BP gene expression.
DISCUSSION

In this study, we show that repression of FGF-BP transcription occurs through a promoter element situated between the AP-1 and C/EBP sites and containing homology to an E-box element. Mutation or deletion of this site substantially increased the level of both TPA and EGF induction of FGF-BP promoter activity in multiple cell lines, implying that this region normally limits the extent of the response to growth factor stimulation. In addition, FGF-BP promoter activity was repressed even further upon CpG methylation of the E-box.

Overall our data suggest that the transcriptional response to TPA can be limited by an E-box site that operates via mechanisms involving methylation and/or interaction with USF and c-Myc transcription factors.

Promoter mutations introduced between the AP-1 and C/EBP sites that exhibited enhanced TPA or EGF induction were consistent with the disruption of the E-box motif from −260 to −255. E-box elements are generally recognized by the family of bHLHZip transcription factors that bind to the consensus E-box element CACGTG. However, certain bHLHZip factors also recognize non-canonical E-box elements similar to the AACGTG E-box found in the FGF-BP promoter. For example, studies using in vitro binding site selection (11) or in vivo chromatin immunoprecipitation (12) found that c-Myc/Max heterodimers are able to recognize the sequence CAACGTG. USF was also found to bind the CAACGTG heptamer (11) as well as the AACGTG hexamer (13), albeit with lowered affinity compared with the consensus CACGTG E-box under in vitro binding conditions (13). Additionally, the bHLH transcription factor Arnt has been shown to bind as homodimers in vitro to an AACGTG variant E-box (14). However, it is unclear whether Arnt homodimers occur in vivo, and we were unable to detect Arnt binding to the FGF-BP E-box in an in vitro gel shift assay.

In our experiments we found that the non-canonical FGF-BP transcription is an E-box element that is involved in the repression of FGF-BP transcription. This E-box element is recognized by USF and c-Myc transcription factors, and its methylation further represses FGF-BP transcription. The E-box element is situated between the AP-1 and C/EBP sites, which are known to be involved in the regulation of gene expression. The repression of FGF-BP transcription by the E-box element is not only due to its recognition by USF and c-Myc, but also due to its interaction with other transcription factors.
E-box is recognized by USF-1 and USF-2 in vitro, suggesting that these factors may play a role in the regulation of this gene. The lack of binding by c-Myc family members in vitro is not surprising since c-Myc binding activity is undetectable in most cells (15). However, using in vivo chromatin immunoprecipitation we were able to detect significant binding of USF, c-Myc, and Max to the region of the promoter (between −118 and +62) containing the AACGTG E-box. Although this region contains no additional E-box or Inr elements, we cannot rule out the possibility that c-Myc-Max complexes bind to an as yet undetermined sequence within this region or that they interact with the promoter through other proteins, such as C/EBP (19). In an attempt to determine whether FGF-BP is indeed a target gene for USF and/or c-Myc we carried out preliminary experiments testing the effect of transient overexpression of USF or c-Myc on FGF-BP promoter activity. Due to indirect effects on cell growth, however, we were unable to detect selective c-Myc- or USF-dependent regulation of FGF-BP, which was consistent with other studies examining c-Myc target genes (15).

The exact mechanism of E-box-mediated repression is complex. Although Mad-Max complexes have been shown to actively repress transcriptional activity through E-box binding and recruitment of mSin3 (20), this mechanism is unlikely to be responsible for FGF-BP repression since we were unable to detect Mad-1 or Mad-2 binding to the promoter in vivo. Repression of FGF-BP was dependent on the juxtaposition of AP-1 site since the phenotype of the m-58 repressor mutation (i.e. increased TPA induction) was lost in the presence of an AP-1 site mutation. This finding implies a mechanism by which action through the E-box element (such as transcription factor binding or methylation) impinges on the trans-activation ability of AP-1. This interaction occurs without affecting the binding affinity of AP-1 or the composition of the AP-1 complex. USF and AP-1 family members have been shown to functionally interact through their leucine zipper domains, resulting in either AP-1 stimulation, as in the case of USF-2-c-Fos complexes (21), or AP-1 inhibition, as in the case of USF-1-Fra1 complexes (22). Whether or not these complexes require DNA binding, however, was unclear. The close proximity between AP-1 and E-box elements on the FGF-BP promoter suggests that E-box factor binding could functionally inhibit AP-1 trans-activation through a specific protein-protein interaction with AP-1. Similarly, the αA-crystallin promoter is repressed in a tissue-specific manner through a composite USF/AP-1 site involving JunD and Fra2 family members (23). Thus, transcriptional repression via interactions between USF and AP-1 may be a common mechanism by which AP-1 and/or USF activity is fine-tuned in response to growth factor stimulation or tissue-specific expression.

The presence of both USF and c-Myc binding to the FGF-BP promoter suggest that the relative binding levels of each of these factors may play a role in FGF-BP regulation. Such a model has been proposed for the cad promoter, where both c-Myc and USF are bound to the promoter E-box in vivo (16, 17). Induction of cad gene expression, however, is dependent on the presence of c-Myc and not USF (16, 17). Therefore, discrimination at the FGF-BP E-box may depend on the relative protein levels through a mechanism involving interplay between c-Myc, USF, and possibly other bHLHZip family members. Alternatively, binding discrimination could occur post-translationally. In this regard, we found that USF binding to the FGF-BP E-box increases significantly after TPA treatment, suggesting that its DNA binding (and perhaps repressor activity) is regulated through a phosphorylation event.

Another aspect of this study is the possibility that methylation of the E-box effectively represses TPA induction of FGF-BP. Aberrant methylation is often associated with tumor progression. Hypermethylation of CpG islands is correlated with the silencing of tumor suppressor genes such as p16 (24). On the other hand, global hypomethylation has been associated with formation of colon cancer (25), and the hypomethylation of individual CpG sites can lead to expression of oncogenes such as ras (26). Methylation is generally associated with transcriptional repression that can occur via indirect mechanisms through the binding of factors, such as MeCP1 and MeCP2, which recognize methylated CpG dinucleotides and recruit histone deacetylases, thus leading to a more compact chromatin structure and transcriptional repression (10). Methylation can also lead to site-specific repression by preventing binding of transcription factors containing CpG dinucleotides in their recognition sites. For example, an inverse relationship between methylation and bHLHZip factor binding to E-box elements has been demonstrated for c-Myc (27) as well as USF (28). Our experiments also show that binding of USF to the FGF-BP E-box in an in vitro assay is sensitive to methylation, whereas methylation had no discernible effect on AP-1 binding. This suggests that the mechanism by which methylation inhibits FGF-BP transcription is distinct from alterations in protein (i.e. USF) binding. This is further supported by the finding that the m-55/−56 mutant disrupts repression without affecting the methylation site, suggesting multiple mechanisms by which the E-box mediates transcription. Our finding suggests a possible correlation between hypomethylation and an increase in FGF-BP gene expression, an event that could significantly contribute to tumor growth and angiogenesis in certain cancer types.

Overall, this study further defines the transcriptional mechanisms by which the angiogenic modulator FGF-BP is regulated in response to stimulation by EGF or TPA. An important aspect of FGF-BP regulation is the presence of an E-box that mediates AP-1-dependent transcriptional repression. Differences in USF and/or c-Myc binding in conjunction with changes in the methylation status of the promoter may be important mechanisms by which the extent of repression exerted on the FGF-BP promoter is de-regulated, thus leading to increased
FGF-BP gene expression and activation of an angiogenic phenotype.

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Page 28544: Figure 5D is incorrect. The corrected Figure 5 is shown below.

**Fig. 5.** Characterization of transcription factor binding to the FGF-BP promoter repressor element. A, diagram illustrating protein binding to FGF-BP promoter fragments used as probes in gel shift analysis. The indicated binding of AP-1 (complex 1) and binding to the repressor element (complex 2) represents results from gel shifts (B–D). Sequences of all promoter fragments used are shown in Fig. 6A. B, gel shift analysis of protein binding to the FGF-BP promoter fragment between –70 and –51. Labeled probe was incubated in the presence of nuclear extracts from untreated (lane 1) or TPA-treated (lanes 2–4) ME-180 cells. Binding reactions were incubated in the presence of 50-fold molar excess of unlabeled mutated promoter fragments as indicated. Complexes 1–4 are indicated to the side of the panel. C, gel shift analysis of protein binding to the FGF-BP promoter fragment which is cytosine-methylated at position –58 (top strand) and –57 (bottom strand). Labeled unmethylated (lanes 1 and 2) and methylated (lanes 3 and 4) probes were incubated in the presence of nuclear extracts from TPA-treated ME-180 cells. Binding reactions were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 50-fold molar excess of unlabeled methylated promoter fragment. D, gel shift analysis of protein binding to the FGF-BP repressor element. Labeled –70/–51 (lane 1) or mAP-1 (lanes 2–6) probes were incubated in the presence of nuclear extracts from untreated (lanes 1 and 2) or TPA-treated (lanes 3–6) ME-180 cells. Competition for complex 2 binding was carried out in the presence of 50-fold unlabeled promoter fragment as indicated. Comp, competitor DNA.

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