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Fibroblast growth factors are highly potent angiogenic factors. However, their role in cancer is not clear since they are frequently expressed but then stored in a non-secreted form. Recently a binding protein for FGF has been described, FGF-BP, which can activate dormant FGF during tumorigenesis. FGF-BP is aberrantly expressed in tumors and causes the development of tumors in vivo. In this grant the role of FGF-BP in breast cancer is examined. In order to understand the regulation of FGF-BP more clearly the PI has cloned the gene promoter for FGF-BP and analyzed the transcription factors necessary for its activation in cancer cell lines (28).In addition the FGF-BP gene was found to be highly inducible by phorbol esters and by epidermal growth factor(EGF) in breast and other cancer cell lines. (33). This induction occurs selectively through the MEK2/ERK2 and p38 kinase pathways and suggests that selective pharmacological inhibition of these pathways could be useful for cancer therapy. During the analysis of the FGF-BP promoter a novel repressor site was found which controls the level of EGF and TPA regulation of this gene. Future studies will determine the role of this factor in breast tumorigenesis.

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P.I. Violaine K. Harris

Title: A Modulator of FGF's in Breast Cancer

I. Introduction

Growth factors, tumor angiogenesis and metastasis

One of the pivotal roles of locally-acting polypeptide growth factors is the induction of new blood vessels in a healing wound as well as in growing tumors. It has been shown in numerous studies with different approaches that a solid tumor cannot grow beyond a few millimeters in size without sufficient blood supply. In addition to the nourishing function of tumor blood vessels, they provide a pathway for the tumor cells to metastasize to distant organs [1-5]. A direct correlation between blood vessel density in primary tumors and their metastasis has been reported for breast cancer [6-10]. Most interestingly tumor angiogenesis as reflected in microvessel density is an independent prognostic indicator in breast cancer patients when tested against known parameters (e.g. tumor size, estrogen receptor, lymph node status, c-erbB-2 expression). Since angiogenesis is such an important feature of *in vivo* tumor biology, the driving forces behind this process need to be understood.

The role of fibroblast growth factors (FGFs) and a potential function for a novel binding protein (BP) for FGF

The most prominent and best studied angiogenesis factors are members of the fibroblast growth factor (FGF) family of polypeptides [11,12]. Several members of the FGF family, mainly bFGF and aFGF are very effective angiogenesis factors and have thus been a focus of research in tumor vascularization in the past decade. The biological activities of both FGFs can be quenched by tight binding to heparansulfate proteoglycans present in the extracellular matrix [13-16]. It is only partly understood how these FGFs become solubilized and thus activated in embryonic or in tumor tissues that require angiogenesis for their growth. One established mechanism that can solubilize bFGF from this storage site is the digestion of the glycosaminoglycan portion of the cell attachment molecule by heparanases [17-20].

An alternate mode of delivering active FGF from the storage site to its receptor could be binding to a secreted carrier protein. Recently, a secreted protein has been described binds to aFGF and bFGF in a non-covalent, reversible manner [21]. Furthermore, bFGF bound to this protein was prevented from degradation and retained its mitogenic activity [21]. These characteristics make this **FGF-binding protein (BP)** an excellent candidate carrier molecule for FGFs. We hypothesized that this BP could be an important regulator that releases immobilized FGFs from their matrix storage site and thus activates them *in vivo* [22,23].

The secreted binding protein for FGF (BP) as a carrier for immobilized FGFs

Recently, it was demonstrated by our laboratory that expression of this binding protein in cell lines which express bFGF leads to a tumorigenic and angiogenic phenotype of these cells [22]. We also showed that BP-transfected cells release the protein into their media together with bFGF in a non-covalently bound form. This released bFGF becomes activated biologically. In addition, *in vivo* tumor growth of a BP-positive squamous cell carcinoma (SCC) cell line and a colon cancer cell line was inhibited by a reduction of endogenous BP using BP-targeted ribozymes [24]. This supports the notion of an activating step for the locally stored bFGF due to expression and secretion of BP.

BP mRNA is expressed in tumor cell lines and primary tumor tissue of squamous cell carcinomas, colon cancers, and breast cancers [22]. Using skin carcinogenesis as a model for epithelial cancers, we studied the role of BP expression during tumor progression. We found that BP mRNA is upregulated in the skin during development but drops to low levels in the adult mouse skin. Interestingly, in both mouse and human skin, BP mRNA and protein levels increase more than 3-fold upon treatment with the PKC-activating agent TPA (12-O-tetradecanoylphorbol-13-acetate), and is further upregulated (4-7 fold) in DMBA/TPA induced papillomas and carcinomas [25,26]. The correlation between BP expression and tumor promotion by TPA strongly suggests a role for BP in tumorigenesis. The study of BP regulation in the mouse skin, which I co-authored, was published in 1997 [25].

Using RT-PCR to screen for FGF-BP expression, we found that FGF-BP is expressed in a 4 out of 6 clinical samples of primary human breast cancers, and in 9 out of 14 breast cancer cell lines. We also detected BP mRNA expression in the mammary gland of the human and the mouse. This pattern of expression suggests that BP may be involved in breast cancer progression, where its deregulated expression could potentially contribute to tumor growth and angiogenesis. During my thesis research I plan experiments that will test the role of BP in human breast cancer cell progression, and its mechanism of regulation by the tumor promoter TPA.

The aims of my grant application were the following:
Aim 1: To study the tumor growth effects of BP expression in breast cancer cells. and **Aim 2:** To study the mechanisms of regulation of BP by Protein Kinase C (PKC).

II. Summary of Results

Isolation and Characterization of the Human FGF-BP Promoter. In order to study the transcriptional regulation of the human FGF-BP gene, 1.8kb of genomic sequence upstream to the known 5'UTR sequence of human FGF-BP cDNA was isolated from a human genomic library. Sequence analysis of the promoter demonstrated the presence of numerous consensus transcription factor binding sites which were conserved between mouse and human FGF-BP promoters and which may have functional importance in FGF-BP regulation. Consensus binding sites included a TATA box (required for transcriptional initiation), a binding site for the C/EBP (CCAAT/enhancer binding protein) family of leucine zipper transcription factors which plays a central role during inflammation and differentiation [27]. In addition, an AP-1 consensus binding site and two Sp1 binding sites were located in this region of the promoter and also play a role in transcriptional regulation of FGF-BP. **The analysis of the FGF-BP promoter was published in the Journal of Biological Chemistry in July 1998 [28], with the P.I. of this grant (Violaine Harris) as first author.**

TPA regulation of the FGF-BP promoter involves a repressor element juxtaposed to the AP-1 site. Phorbol esters, such as TPA, act as potent tumor promoters through the activation of protein kinase C (PKC). We found that FGF-BP gene expression is dramatically upregulated in response to TPA treatment. To study which promoter elements might be involved in TPA regulation, we generated a series of promoter deletion mutants and analyzed the ability of these mutants to drive expression of a luciferase reporter gene. This functional promoter analysis revealed that TPA induction required an interplay between several regulatory elements, including the juxtaposed Sp1/AP-1 site as well as the C/EBP site. The involvement of Sp1, AP-1, and C/EBP transcription factors in the regulation of FGF-BP was further confirmed using gel shift analysis. These results demonstrated distinct binding of each of these factors to their respective promoter elements. **The TPA regulation of the FGF-BP promoter was published in JBC 1998 [28].**

EGF regulation of FGF-BP transcription. Epidermal growth factors (EGF) are potent regulators of cell proliferation and differentiation of many tissue types. Deregulation of the EGF-induced signaling network is known to play an important role in the tumorigenesis for several human cancers, including neoplasms of the brain, lung, breast, ovary, pancreas, prostate, colon, and squamous cell carcinoma (SCC) of the skin and cervix [29-32]. The EGF family of growth factors plays an especially important role in the development of the mammary gland and in the pathogenesis of breast cancer.

We examined the regulation of FGF-BP by EGF and found that EGF treatment caused a rapid induction of FGF-BP mRNA and transcription. Promoter analysis using the deletion constructs described above demonstrated that EGF induction was mediated through the AP-1 and C/EBP elements in the FGF-BP promoter. The identity of these factors was confirmed using gel shift analysis, which demonstrated the EGF-induced binding of c-Fos and JunD to the AP-1 site, as well as C/EBPβ and C/EBPδ to the C/EBP site. These results convincingly identified transcription factor targets which are important in the regulation of FGF-BP gene expression and the stimulation of angiogenesis by EGF. **The work describing EGF regulation of FGF-BP transcription has been submitted for publication [33].**

MAP kinase signal transduction pathways which mediate EGF induction of FGF-BP. EGF signaling occurs binding to its receptor EGFR (ErbB1) and its dimerization partner ErbB2 (Her2/Neu). ErbB2 plays a significant role in the progression of breast cancer, is frequently amplified in more aggressive breast cancers, and is currently a target for breast cancer therapy [34]. Autophosphorylation of activated EGF receptor stimulates a number of signal transduction pathways, including the classical MAP kinase pathway

Ras/Raf/MEK/ERK, which is known to phopsphorylate and activate AP-1 transcription factors [35]. Protein kinase C (PKC), which is stimulated by TPA, can mediate this pathway through Ras-dependent or Ras-independent mechanisms [36,37]. Other signaling pathways induced by EGF include the stress-activated protein kinases (SAPKs) such as JNK (SAPK1) and p38 (SAPK2) [38], the PI3 kinase pathway [39], and the JAK/STAT pathway [40].

In order to differentiate between the possible signaling pathways involved in EGF induction of FGF-BP, we chose to test pharmacological inhibitors of signal transduction components for their effect on FGF-BP regulation. We found that treatment with the EGFR tyrosine kinase inhibitor Tyrphostin AG1478 reduced EGF induction of FGF-BP mRNA. Therefore, as expected, EGFR tyrosine kinase activity is essential for the EGF effect on the FGF-BP gene. In addition, we have shown previously that TPA induction of FGF-BP transcription was mediated through a PKC-dependent pathway [28]. To establish whether PKC activation was also required for the EGF effects on FGF-BP, we treated ME-180 cells with the specific PKC inhibitor Calphostin C [41] and found that this completely blocked EGF induction of FGF-BP mRNA. **This finding demonstrates that PKC activation is central in mediating FGF-BP transcriptional activation upon either EGF or TPA stimulation.**

To determine the contribution of the MAP kinase kinases (MEK1 and MEK2) to FGF-BP regulation, we tested the effects of pharmacological inhibitors of MEK1 and MEK2 on EGF signaling. Treatment with the PD98059 at a concentration which fully inhibits MEK1 but not MEK2 had no effect on EGF induction of FGF-BP mRNA indicating that MEK1 activation does not play a significant role in the regulation of FGF-BP. In contrast, pretreatment with the drug U1026, which is a potent inhibitor of both MEK1 and MEK2, could effectively block EGF induction of FGF-BP mRNA. Consistent with this was the observation that expression of dominant-negative constructs of MEK2 blocked EGF induction of the FGF-BP promoter, whereas expression of dominant-negative MEK1 had no effect. In addition, expression of dominant-negative ERK2 (a substrate of MEK) inhibited EGF induction of FGF-BP, whereas expression of dominant-negative ERK1 had no effect. **These results indicated that selective activation of MEK2 and ERK2 is necessary for FGF-BP gene regulation.**

EGF is also known to stimulate intracellular signaling via the PI3 kinase pathway [39]. In order to test the contribution of PI3 kinase to FGF-BP regulation, we used the PI3 kinase specific inhibitor Wortmannin. Pretreatment with Wortmannin had no effect on EGF induction of FGF-BP mRNA, ruling out a role for PI3 kinase in the regulation of FGF-BP.

Stimulation of the JNK/p38 MAP kinase pathway has also been shown to regulate AP-1 activity in response to mitogens and stress [38]. We therefore tested whether JNK or p38 activation could induce FGF-BP gene expression by treating with the antibiotic anisomycin. Anisomycin treatment at concentrations below 200 nM is known to be an effective stimulator of both JNK and p38 [42]. Treatment of ME-180 cells with anisomycin alone resulted in a significant and dose-dependent increase of FGF-BP mRNA levels up to 2.3-fold.

Because anisomycin is capable of activating both p38 and JNK MAP kinase pathways, we tested the contribution of p38 to FGF-BP induction using the p38-specific inhibitor SB203580, which has no inhibitory activity for JNK or ERK1/2 [43]. Treatment with SB203580 reduced EGF and anisomycin induction of FGF-BP mRNA by 60% in a dose-dependent manner. Consistent with this was the observation that expression of dominant-negative constructs for p38 blocked EGF induction of the FGF-BP promoter, whereas expression of dominant-negative JNK had no effect. **These results demonstrate that both anisomycin and EGF induction of FGF-BP mRNA require p38 activation.**

Overall these data indicate that two important MAP kinase pathways, MEK2/ERK2 and p38, are necessary for full induction of FGF-BP transcription by EGF. This work has been recently submitted for publication [33].

Identification of a repressor element in the regulation of FGF-BP. Between the AP-1 site and the C/EBP site lies a region of low homology between the human and mouse BP promoter sequences. Because this region was not suspected to have any effect on TPA induction, an internal deletion removing this region (-57 to -47) was tested as a control. Surprisingly, in the Δ57/47 construct, TPA induction of the FGF-BP promoter increased from approximately 7- fold to 14-fold, suggesting the presence of a possible repressor which may interact with this site. Loss of repression of this construct was also observed with EGF, with promoter induction going from 5-fold to 8-fold in the repressor mutant. The -57 to -47 deletion disrupts an AACGTG (-60 to -55) which is juxtaposed to the 3' end of the AP-1 site and which shows some similarity to the CACGTG E-box element recognized by a number of basic helix-loop-helix leucine zipper (bHLHZip) factors [44]. To test this

imperfect E-box for repressor activity, a C to T point mutation at position -58 was introduced into the -118/+62 BP promoter construct. **The m-58 construct showed a dramatic increase in TPA induction up to 18-fold above background, and EGF induction up to 10-fold. This data shows that the point mutation at position -58, as well as the internal deletion from -57 to -47, disrupts repression of the FGF-BP promoter which normally limits the response to TPA or EGF.**

Importantly, the activity of the repressor element on the FGF-BP promoter was not limited to one cell line. The mutant repressor promoter construct (m-58) showed increased TPA and EGF induction in breast cancer cell lines (BT549, MCF7) as well as in cervical squamous cell carcinoma cell lines (ME-180, HeLa).

Binding of USF to the FGF-BP repressor element. In order to understand the mechanisms of repression through the FGF-BP E-box, we looked to see whether we could detect the binding of a protein complex using gel shift analysis. We identified binding of a distinct factor to the FGF-BP E-box element which was independent of the AP-1 complex. Mutational analysis showed convincingly that the nucleotides within the E-box element (AACGTG), including the C at position -58, were all required for repressor binding. Using antibodies against different transcription factors within the bHLHZip family, we identified a complex containing USF1 (upstream stimulatory factor) and USF2 binding to the FGF-BP E-box. Interestingly, USF binding to this site was induced after TPA or EGF treatment. **USF binding to the FGF-BP promoter is hypothesized to repress transcriptional induction by TPA and EGF.**

There are at least two possible mechanisms by which USF binding could repress transcriptional induction of the FGF-BP promoter. First, USF could act as an active repressor through recruitment of co-repressors which interfere with the efficiency of transcription. Second, USF binding itself might interfere with other positive regulatory transcription factors, such as AP-1. To test the second possibility, we generated a number of promoter mutants containing double mutations in the AP-1/repressor(-58) sites or in the C/EBP/repressor(-58) sites and tested for loss of repression. Whereas the promoter containing both C/EBP and repressor mutations was still highly inducible (loss of repression), the promoter containing double AP-1 and repressor mutations was not. This result demonstrated that repression through the USF binding site was dependent on an intact AP-1 site. Furthermore, the repressor mutation had no effect on AP-1-binding or on the composition of AP-1, suggesting instead that USF interferes somehow with AP-1 trans-activating ability. **This result demonstrates that repression through the USF binding site is AP-1-dependent.**

Repression of the FGF-BP promoter through methylation of the USF binding site. Aberrant methylation is known to be closely associated with cancer progression. Cytosine methylation of CpG dinucleotides is often correlated with repression of genes containing isolated CpG dinucleotides in the regulatory regions of their promoters. Due to the presence of a CpG dinucleotide at the core of the FGF-BP E-box at position -58, we tested whether methylation of this site could have a repressive effect on FGF-BP transcription. Loss of methylation of this site, in the -58 (C to T) promoter mutant for example, would be an alternate mechanism explaining the enhanced response of this construct to TPA and EGF. We conducted *in vitro* methylation of the wild-type FGF-BP promoter construct vs. the m-58 promoter construct so that the methylation pattern of each these promoters differed only at the E-box CpG. **When the FGF-BP E-box was methylated, TPA induction was dramatically reduced. This shows that methylation of the repressor site may be a potential mechanism for limiting the transcriptional response to growth factor induction of the FGF-BP gene during cancer formation.**

The work describing repression of the FGF-BP promoter through the USF binding site is in manuscript form and will soon be submitted for publication [45].

III. Publications and Abstracts

Harris, V.K., Liaudet-Coopman, E.D.E., Boyle, B.J., Wellstein, A., Riegel, A.T. Phorbol ester-induced transcription of a FGF-binding protein is modulated by a complex interplay of positive and negative regulatory promoter elements. *J Biol Chem* 273: 19130-19139. 1998 July.

Harris, V.K., Liaudet-Coopman, E., Wellstein, A., Riegel, A.T. A fibroblast growth factor binding protein (FGF-BP) is transcriptionally regulated by phorbol esters and retinoic acid. *Proceedings of the American Association for Cancer Research*, 89th Annual Meeting, New Orleans, LA, March 28-April 1, 1998

Harris, V. K., Coticchia, C. M., Kagan, B. L., Wellstein, A., Riegel, A. T. Induction of the angiogenic modulator FGF-BP by EGF is mediated through the MEK2/ERK2 and p38 signaling pathways. 1999 (submitted)

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Phorbol Ester-induced Transcription of a Fibroblast Growth Factor-binding Protein Is Modulated by a Complex Interplay of Positive and Negative Regulatory Promoter Elements*

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Earlier studies from our laboratory showed that a secreted binding protein for fibroblast growth factors (FGF-BP) is expressed at high levels in squamous cell carcinoma (SCC) cell lines. Overexpression studies or conversely reduced expression of FGF-BP by ribozyme targeting have elucidated a direct role of this protein in angiogenesis during tumor development. We have also observed a significant up-regulation of FGF-BP during TPA (12-*O*-tetradecanoylphorbol-13-acetate) promotion of skin cancer. Here we investigate the mechanism of TPA induction of FGF-BP gene expression in the human ME-180 SCC cell line. We found that TPA increased FGF-BP mRNA levels in a time- and dose-dependent manner mediated via the protein kinase C signal transduction pathway. Results from actinomycin D and cycloheximide experiments as well as nuclear transcription assays revealed that TPA up-regulated the steady-state levels of FGF-BP mRNA by increasing its rate of gene transcription independently of *de novo* protein synthesis. We isolated the human FGF-BP promoter and determined by deletion analysis that TPA regulatory elements were all contained in the first 118 base pairs upstream of the transcription start site. Further mutational analysis revealed that full TPA induction required interplay between several regulatory elements with homology to Ets, AP-1, and CAATT/enhancer binding protein C/EBP sites. In addition, deletion or mutation of a 10-base pair region juxtaposed to the AP-1 site dramatically increased TPA induced FGF-BP gene expression. This region represses the extent of the FGF-BP promoter response to TPA and contained sequences recognized by the family of E box helix-loop-helix transcription factors. Gel shift analysis showed specific and TPA-inducible protein binding to the Ets, AP-1, and C/EBP sites. Furthermore, distinct, specific, and TPA-inducible binding to the imperfect E box repressor element was also apparent. Overall, our data indicate that TPA effects on FGF-BP gene transcription are tightly controlled by a complex interplay of positive elements and a novel negative regulatory element.

FGF-BP[1] is a secreted protein that binds to acidic FGF and basic FGF in a non-covalent reversible manner (1). FGF-BP mRNA has been found to be up-regulated in squamous cell carcinoma (SCC) cell lines of different origin, in SCC tumor samples from the head and neck, and in some colon cancer cell lines (1, 2). More recently, developmental expression of the mouse FGF-BP gene was found to be prominent in the skin and intestine during the perinatal phase and is down-regulated in adult mice (3). We previously described that expression of FGF-BP in a non-tumorigenic human cell line (SW-13) which expresses bFGF leads to a tumorigenic and angiogenic phenotype (2). Expression of FGF-BP in these cells solubilizes their endogenous bFGF from its extracellular storage and allows it to reach its receptor, suggesting that FGF-BP serves as an extracellular carrier molecule for bFGF (2, 4). Expression of FGF-BP under the control of a tetracycline-responsive promoter system in SW-13 cells revealed its role during the early phase of tumor growth (5). To assess the significance of FGF-BP endogenously expressed in tumors, we depleted human SCC (ME-180) and colon carcinoma (LS174T) cell lines of their FGF-BP by targeting with specific ribozymes (6). This study showed that the reduction of FGF-BP reduced the release of biologically active bFGF from cells in culture. In addition, the growth and angiogenesis of xenografted tumors in mice was decreased in parallel with the reduction of FGF-BP, suggesting that some human tumors can utilize FGF-BP as an angiogenic switch molecule.

The fact that FGF-BP has been detected in only a few types of tumors, where it seems to play a crucial role in angiogenesis, led us to investigate the mechanisms responsible for turning its expression on or off. Studying the regulation of FGF-BP in SCC cell lines, we showed that all-*trans*-retinoic acid, used as a chemotherapeutic agent against SCCs, down-regulates FGF-BP gene expression *in vitro* by both transcriptional and post-transcriptional mechanisms (7). *In vivo* all-*trans*-retinoic acid treatment reduces FGF-BP expression in SCC xenografts and inhibits their tumor growth and angiogenesis (8). On the other hand, FGF-BP mRNA expression in the adult mouse skin was found to be dramatically increased during the early stages

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF062639.

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[1] The abbreviations used are: FGF-BP, fibroblast growth factor-binding protein; bFGF, basic FGF; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SCC, squamous cell carcinoma; PKC, protein kinase C; C/EBP, CAATT/enhancer binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IMEM, improved minimum essential medium; kb, kilobase pair(s); DTT, dithiothreitol; PCR, polymerase chain reaction; UTR, untranslated region; CMV, cytomegalovirus.

of 7,12-dimethylbenz[*a*]anthracene/TPA-induced mouse skin papilloma formation (3), as well as in 7,12-dimethylbenz[*a*]anthracene/TPA-treated human skin grafted onto SCID mice.[2] Similarly, FGF-BP expression *in vitro* was up-regulated in epidermal cell lines carrying an activated *ras* gene, implicating the *ras*/PKC pathway in the regulation of FGF-BP (3).

In this context, and given the fact that FGF-BP could play a critical role in the development of human skin cancer, we decided to investigate the effects of the tumor promoter TPA on FGF-BP gene regulation. Our results show that FGF-BP mRNA expression is up-regulated by TPA in the ME-180 SCC cell line and that this induction is mediated by direct transcriptional mechanisms. Analysis of the human FGF-BP promoter reveals that the TPA induction is mediated by cooperation of several inducible regulatory elements. Furthermore, the induction of gene expression by TPA can be modified by a repressor element juxtaposed to the AP-1 site which contains sequences recognized by E box element factors.

MATERIALS AND METHODS

Cell Culture—The ME-180 squamous cell carcinoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in improved minimum essential medium (Biofluids Inc., Rockville, MD) with 10% fetal bovine serum (Life Technologies, Inc.).

Northern Analysis—ME-180 cells were grown to 80% confluence on 150-mm tissue culture dishes, washed three times in serum-free IMEM, and then treated 16 h later with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma) in serum-free IMEM. Total RNA was isolated with the RNA STAT-60 method using commercially available reagents and protocols (RNA STAT-60™, Tel-Test, Friendswood, TX). 30 μg of total RNA were separated by electrophoresis in 1.2% formaldehyde-agarose gel and then blotted onto nylon membranes (MSI, Westboro, MA). The blots were prehybridized in 6× SSC (0.9 M sodium chloride, 0.09 M sodium citrate, pH 7.0), 0.5% (w/v) SDS, 5× Denhardt's solution (0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin, 100 μg/ml sonicated salmon sperm DNA) (Life Technologies, Inc.) for 4 h at 42 °C. Hybridization was carried out overnight at 42 °C in the same buffer. After hybridization, blots were washed three times with 2× SSC and 0.1% SDS for 10 min at 42 °C and finally once with 1× SSC and 0.1% SDS for 20 min at 65 °C. Autoradiography was performed using intensifying screens at −70 °C. Blots were stripped by boiling 2 × for 10 min in 1× SSC and 0.1% SDS. Hybridization probes were prepared by random-primed DNA labeling (Amersham Pharmacia Biotech) of purified insert fragments from human FGF-BP (2) and human GAPDH (CLONTECH). The final concentration of the labeled probes was always greater than 10^6 cpm/ml hybridization solution. Quantitation of mRNA levels was performed using a PhosphorImager (Molecular Dynamics).

In Vitro Transcription on Isolated Nuclei—ME-180 cells were grown to 80% confluence on 150-mm tissue culture dishes. Cells were washed three times in serum-free IMEM and then treated 16 h later with TPA in serum-free IMEM for indicated times. Nuclei from 10^7 cells for each time point were isolated after incubation in lysis buffer containing 0.5% Nonidet P-40 as described (7). Nuclear transcription assays were performed with [α-^{32}P]UTP (Amersham Pharmacia Biotech) as described (7). Equal amounts of radioactivity (0.5–1 × 10^7 cpm) were hybridized to nitrocellulose filters containing 3 μg of each plasmid. After hybridization for 4 days at 42 °C, the filters were washed 4 times with 2X SSPE, 0.1% SDS for 5 min at 25 °C and treated for 30 min at 25 °C in 2X SSPE containing 20 μg/ml RNase A. The filters were then washed 4 times for 30 min in 1X SSPE, 1% SDS at 65 °C. The amount of radioactivity present in each slot was determined using a PhosphorImager after overnight exposure, and autoradiograms were exposed for 1–3 days with intensifying screens.

Primer Extension—Primer 1 was designed from the coding region of the human FGF-BP cDNA (5′-GTGAGGCTACAGATCTTC-3′), primer 2 from the FGF-BP 5′-UTR (5′-GTTCACCTTGTTCTGAGCACACG-GATCCA-3′), and a control primer for the 1.2-kb kanamycin RNA (Promega). 10 pmol of each primer was labeled with T4 polynucleotide kinase (Promega) and 30 μCi of [γ-^{32}P]ATP (Amersham Pharmacia Biotech) for 1 h, and labeled primers were purified over a ChromaSpin-10 gel filtration column (CLONTECH). Total RNA from ME-180 cells was isolated as described for Northern analysis. ME-180 mRNA was purified from total RNA over an oligo(dT)-cellulose column (Life Technologies, Inc). 100 fmol of each FGF-BP-specific primer was incubated with or without 7 μg of ME-180 mRNA or control primer with or without 2 ng of 1.2-kb kanamycin control RNA (Promega) in the presence of avian myeloblastosis virus reverse transcriptase buffer (50 mM Tris-HCl, 8 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, pH 8.5, Boehringer Mannheim) and allowed to anneal for 1 h at 50 °C. Annealed mixtures were then incubated in the presence of 2 mM dNTPs, 50 units of RNase inhibitor (Boehringer Mannheim), and 40 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) for 1 h at 42 °C. Samples were then run on a 6% polyacrylamide sequencing gel along with radiolabeled *Hin*fI markers and exposed overnight for autoradiography.

Cloning of FGF-BP Gene Promoter—1.8 kb of genomic sequence lying upstream of the human FGF-BP gene was isolated from a human genomic library using the PCR-based PromoterFinder DNA Walking Kit (CLONTECH) according to the manufacturer's recommendations using rTth XL DNA polymerase (Perkin-Elmer). Gene-specific primers derived from the 5′-UTR of the human FGF-BP cDNA were 5′-ACACG-GATCCAGTGCAATCC-3′ (+91 to +72) for the primary round of PCR and 5′-GGAGTGAATTGCAGGCTGCAGCTGTGTCAG-3′ (+62 to +33) for secondary PCR. Secondary PCR products from a *Dra*I library (1.1 kb) and *Pvu*II library (1.8 kb) were cloned into a TA Cloning Vector pCR2.1 (Invitrogen), sequenced by automated cycle sequencing (ABI PRISM Dye Terminator Cycle Sequencing, Perkin-Elmer), and confirmed to contain contiguous genomic sequence. This sequence has been submitted to GenBank™ (accession number AF062639).

Plasmid Constructs—Promoter fragments were cloned into the PXP1 promoterless luciferase reporter vector (9). PCR products from *Pvu*II (−1829/+62) and *Dra*I (−1060/+62) libraries were removed from pCR2.1 using *Hin*dIII and *Xho*I sites in the pCR2.1 multiple cloning site and ligated into the *Hin*dIII and *Xho*I site of PXP1 to generate pX-1829/+62Luc and pX-1060/+62Luc. Both constructs contained a *Bam*HI site carried over from the pCR2.1 vector and located 3′ of the FGF-BP sequence and 5′ of the luciferase gene. The 5′ promoter deletion constructs pX-118/+62Luc, pX-93/+62Luc, pX-77/+62Luc, pX-67/+62Luc, pX-56/+62Luc, and pX-31/+62Luc were generated by PCR from pX-1060/+62 Luc template using upstream primers spanning −118 to −100, −93 to −76, −77 to −59, −67 to −49, −56 to −35, and −31 to −11, respectively, all containing a 5′-linked *Bam*HI site. Downstream primer was derived from the luciferase gene 5′-CCATCCTCTAGAG-GATAGAATGGCGCCGGGCC-3′. PCR was carried out using *Taq* DNA Polymerase (Boehringer Mannheim). Products were cut with *Bam*HI, gel-purified, and cloned into the PXP1 *Bam*HI site. Correct sequence and orientation were verified by dideoxynucleotide chain termination sequencing with a Sequenase kit 2.0 (U.S. Biochemical).

Internal promoter deletions were generated by PCR-based site-directed mutagenesis. Complementary overlapping oligonucleotides containing specific promoter deletions were generated as follows. Ets site deletion from −76 to −67 was incorporated into primers spanning −58 to −93 and −85 to −47; AP-1 site deletion from −65 to −58 was incorporated into primers spanning −47 to −83 and −72 to −35; Ets/AP-1 site deletion from −76 to −58 was incorporated into primers spanning −49 to −93 and −85 to −36; C/EBPβ site deletion from −47 to −33 was incorporated into primers spanning −24 to −66 and −57 to −11; and deletion from −57 to −47 was incorporated into primers spanning −35 to −75 and −69 to −27. The −58 point mutant was made by incorporating a C to T point mutation at position −58 into primers representing both strands from −70 to −51. For each construct, two separate PCR reactions were carried out with either the *Bam*HI-linked −118 to −99 upstream primer or the luciferase-specific downstream primer. The products were separated from excess primers and mixed, denatured, and allowed to reanneal. Amplification of the heteroduplex with overlapping 3′ ends was carried out by 3′ extension in the absence of primers followed by amplification using outside primers (*Bam*HI-linked −118 to −99 primer and Luciferase-specific primer) in a secondary round of PCR. Final PCR products were digested with *Bam*HI, gel-purified, and ligated into the PXP1 *Bam*HI site. Correct sequence and orientation were verified by dideoxynucleotide chain termination sequencing with a Sequenase kit 2.0 (U. S. Biochemical Corp.). To control for TPA effects on vector sequences unrelated to the FGF-BP promoter insert, we tested a number of non-TPA responsive promoters inserted into the PXP 1 vector for their response to TPA under the conditions used in our experiments. Control vectors, as seen in Fig. 6, consisted of fragments of the pro-opiomelanocortin promoter (10), the thymidine kinase minimal promoter (9), or the CMV minimal promoter

[2] A. Aigner and A. Wellstein, unpublished data.

which were cloned into PXP1 vector. All these vectors demonstrated an approximately 2-fold induction after TPA treatment (see "Results").

Transient Transfections and Reporter Gene Assays—24 h before transfection, ME-180 cells were plated in 6-well plates in IMEM, 10% FBS at a density of 750,000 cells/well. For each transfection, 1.0 μg FGF-BP-luciferase construct and 10 μl of LipofectAMINE Reagent (Life Technologies, Inc.) were combined in 200 μl of IMEM, and liposome-DNA complexes were allowed to form at room temperature for 30 min. Volume was increased to 1 ml with IMEM, added to rinsed cells, and incubated for 3 h at 37 °C. Cells were washed and incubated in IMEM for 3 h and then treated for 18 h with vehicle alone (Me_2SO, final concentration 0.1%) or 10^{-7} M TPA. Transfection efficiency for each construct was determined by co-transfection with 1.0 ng of a CMV-driven *Renilla* luciferase reporter vector pRL-CMV (Promega) and found to be the same for all BP-PXP1 constructs. However, due to a 2-fold background TPA induction of pRL-CMV (see above), results were normalized for protein content and not for *Renilla* luciferase activity. Cells were lysed by scraping into 150 μl of Passive Lysis buffer (Promega), and cell debris was removed by brief centrifugation. 20 μl of extract was assayed for both firefly and *Renilla* luciferase activity using the Dual-Luciferase™ Reporter assay system (Promega). Light intensity was measured in a Monolight 2010 luminometer. Light units are expressed firefly light units/μg of protein. Protein content of cell extracts was determined by Bradford assay (Bio-Rad).

Gel Shift Assays—ME-180 cells were grown to 80% confluency on 150-mm dishes, serum-starved for 6 h, and treated with or without 10^{-7} M TPA for 90 min. Nuclear extracts were prepared according to Dignam *et al.* (11) with the following modifications. Pelleted cells were resuspended in 1 ml of buffer A (15 mM KCl, 10 mM HEPES, pH 7.6, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, 1 mM sodium orthovanadate) (12) with 1× Complete™ protease inhibitor mixture (Boehringer Mannheim) and incubated on ice for 10 min. Crude nuclei were pelleted at 700 g and resuspended in 50 μl of ice-cold buffer C (0.42 M NaCl, 20 mM HEPES, pH 7.9, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1 mM sodium orthovanadate, 1× Complete™ protease inhibitor mixture) and vortexed at 4 °C for 15 min. After centrifugation for 10 min at 1000 × *g*, supernatant was used directly in binding assays and stored at −70 °C.

4.8 pmol of each synthetic double-stranded oligonucleotide was labeled with T4 polynucleotide kinase (Promega) and 50 μCi of [γ-^{32}P]ATP (Amersham Pharmacia Biotech) for 30 min, and labeled primers were purified over a G-25 Sephadex column (Boehringer Mannheim). Binding reactions consisted of 2.5 μg (−80/−63 probe) or 5 μg of ME-180 nuclear extracts, binding buffer (10 mM Tris, pH 7.5, 10 mM KCl, 5% glycerol, 0.1 mM DTT, 0.1 mM EDTA), and 200 ng of poly(dI-dC) (−80/−63 and −70/−51 probes) or 1.0 μg of poly(dA-dT) (−55/−30 probe) and incubated for 10 min on ice. Unlabeled competitor oligonucleotides were added and incubated for another 10 min before adding 20 fmol of labeled probe. Reactions were carried out 45 min on ice and analyzed by 6% polyacrylamide gel electrophoresis in 1× TBE buffer at 80 V. The AP-1 consensus sequence was 5′-CTAGTGATGAGTCAGCC-GGATC-3′.

RESULTS

TPA Increases FGF-BP mRNA in SCCs—We have previously detected an up-regulation of FGF-BP mRNA following TPA treatment of mouse skin during the development of skin tumors (3) and also in human skin xenografts.[2] These data suggest that the control of this angiogenic switch factor may play an important role in skin carcinogenesis. To examine this further we studied the effect of the tumor promoter TPA on FGF-BP gene expression in ME-180 cells which express high levels of the FGF-BP transcript (7). Cells were treated with 10^{-7} M TPA from 1 to 24 h which resulted in an increase in the steady-state levels of FGF-BP mRNA detectable 1 h after treatment (Fig. 1*A*). PhosphorImager analysis showed that the induction was maximal after 6 h by 452 ± 44% (Fig. 1*A*). GAPDH mRNA remained unaffected by TPA treatment, as judged relative to the total amount of RNA loaded and was used to standardize FGF-BP mRNA. The dose dependence of TPA induction of FGF-BP mRNA in ME-180 is shown in Fig. 1*B*. We estimated the half-maximal effective concentration as 1 nM. The inductive effect of TPA on FGF-BP mRNA was also observed in two other SCC cell lines, FaDu and A431 (data not shown) demonstrating that TPA induction of FGF-BP mRNA is generally preserved in SCC cell lines.

To establish whether TPA induction of FGF-BP mRNA was mediated through a PKC-dependent pathway, ME-180 cells were pretreated or not pretreated for 2 h with 100 nM highly specific PKC inhibitor, calphostin C (13), and then treated with or without 10^{-7} M TPA for 4 h. As can be seen in Fig. 1*C*, pretreatment of the cells with calphostin C prior to TPA treatment totally blocked the TPA effect, demonstrating that the induction of FGF-BP transcript by TPA is mediated via a PKC-dependent mechanism. In addition, it is known that TPA causes an immediate up-regulation of PKC followed by long term down-regulation of PKC activity. In contrast, although long term calphostin C appears to be able to down-regulate protein kinase C, it does not cause early induction of PKC activation but rather blocks the inductive effects of TPA on PKC (14). Thus the fact that Calphostin C causes no induction of FGF-BP mRNA and blocks the TPA effect argues that induction of FGF-BP mRNA is through an up-regulation of PKC activity rather than a consequence of long term down-regulation.

Mechanism of TPA Induction of FGF-BP mRNA—We have previously shown that FGF-BP gene expression can be regulated through both transcriptional and post-transcriptional mechanisms (7). Therefore we next attempted to determine whether the TPA induction of FGF-BP mRNA was at the transcriptional or post-transcriptional level. We first assessed whether TPA treatment affected the stability of the FGF-BP mRNA. Experiments were performed to determine whether addition of inhibitors of transcription (actinomycin D) or translation (cycloheximide) could inhibit the TPA induction of FGF-BP mRNA. Actinomycin D (5 μg/ml) or cycloheximide (10 μg/ml) were added with or without TPA (10^{-7} M), and FGF-BP mRNA levels were determined 6 h after treatment. As shown in Fig. 2*A*, simultaneous addition of TPA and actinomycin D completely blocked the TPA induction, whereas simultaneous addition of TPA and cycloheximide had no effect. These data suggest that TPA directly increased the rate of FGF-BP gene transcription independently of *de novo* protein synthesis and did not affect the stability of the FGF-BP transcript. To verify further that the stability of the FGF-BP transcript was not modified by TPA treatment, ME-180 cells were pretreated for 2 h with TPA and then actinomycin D was added to inhibit transcription. As shown in Fig. 2*B*, pretreatment of cells with TPA did not increase the half-life of the FGF-BP mRNA indicating that the stability of the FGF-BP transcript is not affected by TPA.

To prove directly that TPA increases the rate of transcription of the FGF-BP gene, we then performed nuclear transcription run-on assays. ME-180 cells were treated with or without 10^{-7} M TPA for various periods. ^{32}P-Labeled nascent transcripts were prepared from isolated ME-180 cell nuclei and hybridized to a nylon membrane bearing immobilized target DNA sequences. As shown in Fig. 3, TPA increased FGF-BP transcript levels maximally after 1 h of treatment. Quantitation and normalization to β-actin showed that FGF-BP transcription was up-regulated by 647 ± 1, 448 ± 16, and 197 ± 31% after 1, 4, and 24 h of treatment, respectively (Fig. 3). β-Actin plasmid DNA was used as a control since transcription of this gene remained constant. These findings are consistent with the above results studying steady-state mRNA and the effects of actinomycin D and cycloheximide treatment. Clearly the induction of FGF-BP mRNA by TPA in ME-180 cells is directly due to a rapid up-regulation of transcription.

Isolation and Characterization of the Human FGF-BP Promoter—In order to understand better the transcriptional reg-

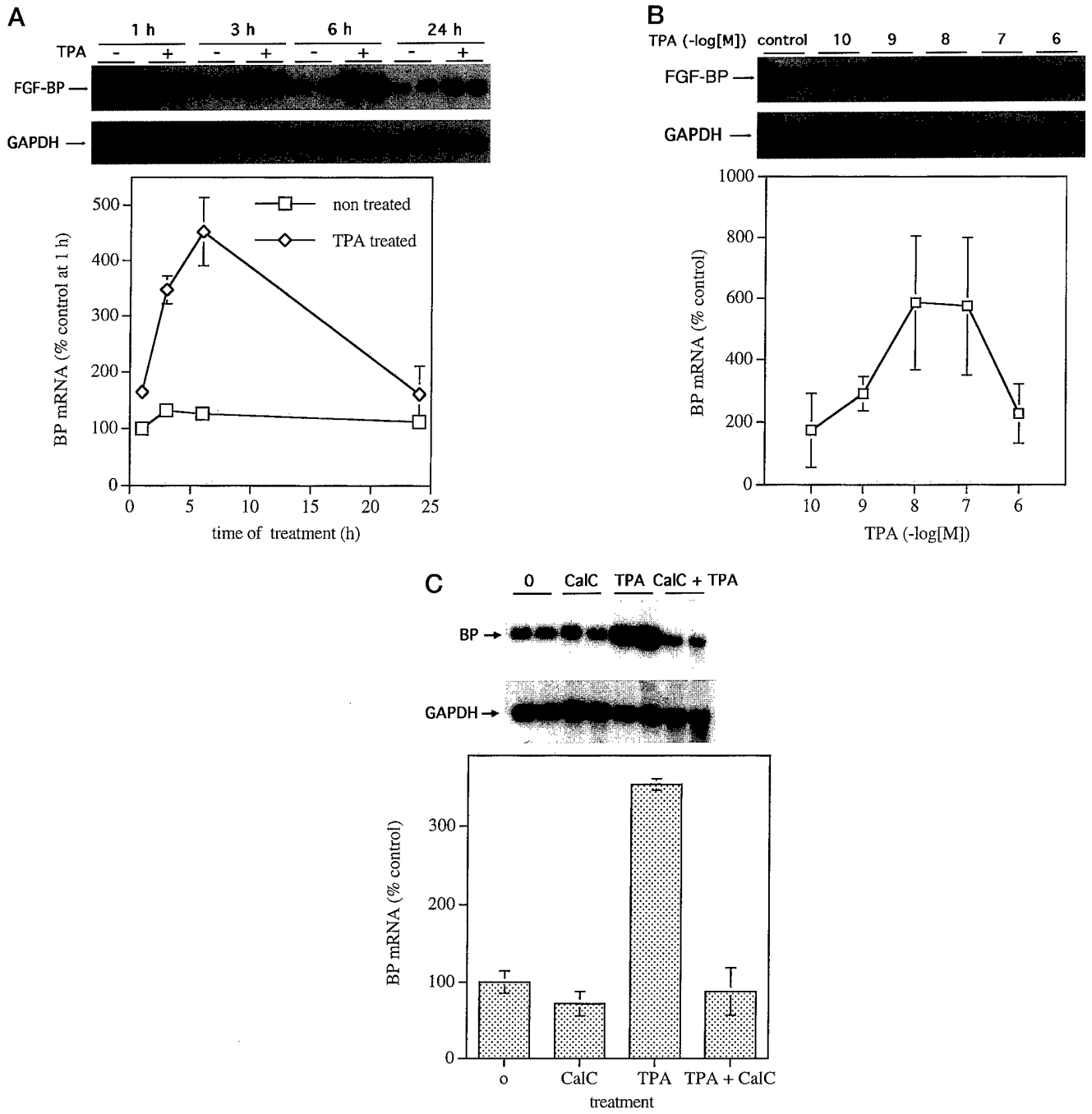

FIG. 1. **TPA effect on FGF-BP mRNA in the ME-180 SCC cell line.** The respective *upper panels* for (*A–C*) are representative Northern blot analyses performed with 30 μg of total RNA/lane as described under "Materials and Methods." Bands corresponding to FGF-BP mRNA (1.2 kb) and the control gene GAPDH mRNA (1.3 kb) are indicated. The respective *lower panels* (*A–C*) show results from quantitation of Northern blots. Signal intensities were quantified by phosphorimaging and normalized to the control gene, GAPDH. The mean ± S.D. of two separate experiments is given. *A*, time dependence of FGF-BP mRNA induction after exposure to 10^{-7} M TPA. *B*, concentration dependence of FGF-BP mRNA after 6 h induction by TPA. *C*, the effect of calphostin C on the TPA induction of FGF-BP mRNA. ME-180 cells were pretreated or not pretreated for 2 h with 10^{-7} M calphostin C (*CalC*) and then treated for 4 h with or without 10^{-7} M TPA in absence or presence of calphostin C.

ulation of the human FGF-BP gene, 1.8 kb of genomic sequence upstream to the known 5′-UTR sequence of human FGF-BP cDNA was isolated from a human genomic library and sequenced. The transcription start site of the human gene was determined using primer extension analysis with nested primers derived from known cDNA sequence (Fig. 4). The precise start site compatible with the primer extension results is indicated in Fig. 5. Alignment between the human and mouse FGF-BP promoter which we cloned previously (3) revealed a region of high homology with 70% nucleotide identity within the first 200 nucleotides upstream from the transcription start (Fig. 5). Nucleotide homology dropped significantly in more upstream sequences, suggesting that the proximal conserved 200 nucleotides of the promoter could be important for transcriptional regulation of FGF-BP in both species.

Sequence analysis of the promoter demonstrated the presence of numerous consensus transcription factor binding sites that were conserved between mouse and human FGF-BP promoters and that may have functional importance in FGF-BP regulation. As shown in Fig. 5, a consensus TATA box is located at about −25 base pairs upstream from the transcription start for both promoters. Between −48 and −40 of the human

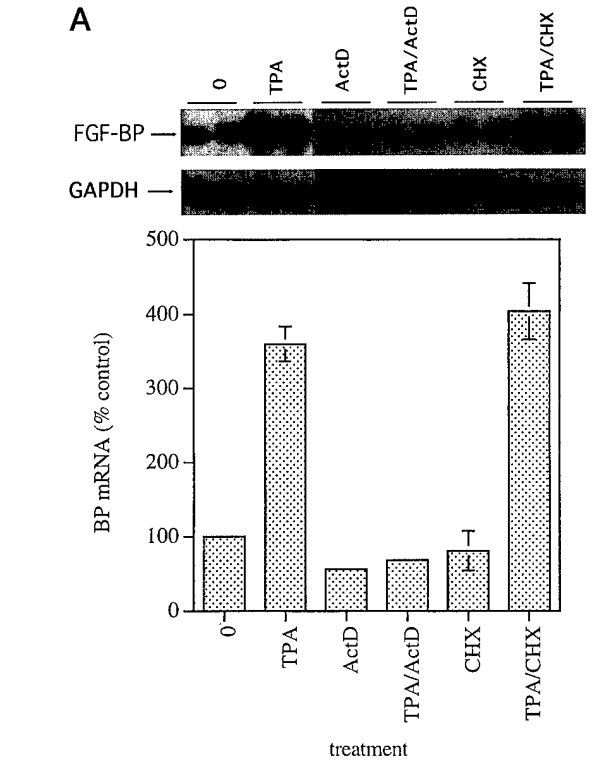

FIG. 2. **Mechanism of TPA induction of FGF-BP mRNA levels.** The respective *upper panels* of *A* and *B* are representative Northern blot analyses performed as described in the legend to Fig. 1. The respective *lower panels* of *A* and *B* represent quantification of data in *upper panels*. Signal intensities were quantified by phosphorimaging and normalized to GAPDH. Results represent mean ± S.D. of two independent experiments. *A*, effect of actinomycin D and cycloheximide on the FGF-BP mRNA induction by TPA. ME-180 cells were treated for 6 h in the absence or presence of 10^{-7} M TPA in combination with 5 μg/ml actinomycin D (*ActD*) or 10 μg/ml cycloheximide (*CHX*). *B*, analysis of turnover of FGF-BP mRNA in TPA-treated cells. ME-180 cells were treated with vehicle alone or 10^{-7} M TPA for 2 h, and 5 μg/ml actinomycin D was then added to control and to TPA-treated cells for 0–16 h. Total RNA was isolated and hybridized sequentially with FGF-BP and GAPDH probes as described in Fig. 1.

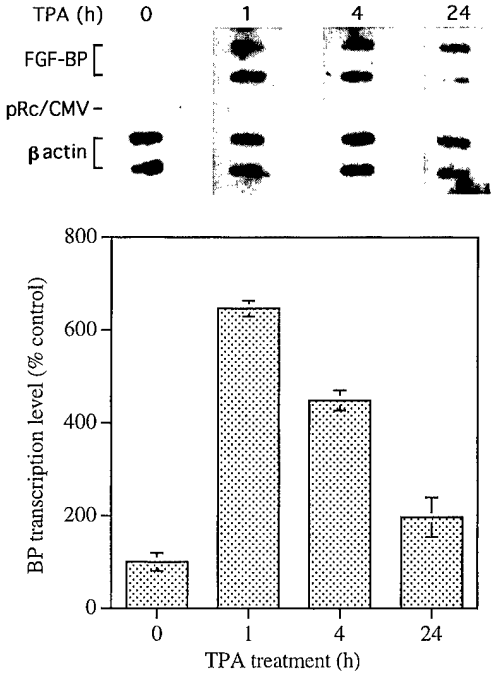

FIG. 3. **Transcription run-on analysis of the effects of TPA on FGF-BP gene expression.** Nuclei were isolated from subconfluent layers of ME-180 cells treated or not with 10^{-7} M TPA for 1, 4, or 24 h, and nascent RNA was extended *in vitro* as described under "Materials and Methods." Labeled RNAs (10^7 cpm) were hybridized to nylon membranes on which 3 μg of the pRC/FGF-BP vector was immobilized. β-Actin was used as an internal control and pRC/CMV as a background control vector. Signal intensities were quantified by phosphorimaging and normalized to the control gene β-actin. Hybridization data are shown in the *upper panel* and quantitative data (mean ± S.D.) derived from two independent experiments in the *lower panel*.

FGF-BP promoter we found a highly conserved consensus binding site for C/EBPβ, a member of the CCAAT/enhancer binding protein (C/EBP) family of leucine zipper transcription factors which play a central role in the acute phase response and in a number of cell differentiation pathways (15–17). An AP-1 consensus binding site (−65 to −59) lies juxtaposed to a sequence with homology to an Ets factor binding motif (−76 to −68), suggesting potential functional similarity to the juxtaposed Ets/AP-1 site found in the polyoma virus enhancer and in the collagenase promoter (18, 19). In addition, a consensus Sp1 factor binding site (−90 to −80), an additional Ets factor binding motif (−107 to −100), and a potential NF-κB-binding site (−185 to −176) are located in the conserved region of the promoter and may play a role in transcriptional regulation of FGF-BP as well.

Functional Analysis of the Human FGF-BP Promoter—To identify the functional promoter elements involved in FGF-BP gene regulation by TPA, progressive 5′ deletion mutants were constructed based on the location of consensus factor binding sites on the promoter. Deletion constructs were transiently transfected into ME-180 cells, and their relative luciferase activity was assayed in the absence or presence of TPA (Fig. 6). The basal activity of each vector is shown in the *left panel* of Figs. 6 and 7. The empty PXP1 vector had no detectable luciferase activity either in the absence or presence of TPA (data not shown). However, we did observe a background, approximately 2-fold, TPA induction of the PXP1 vector when several unrelated minimal promoters (*i.e.* thymidine kinase minimal promoter, the CMV minimal promoter, and the pro-opiomelanocortin minimal promoter) were treated with TPA after transfection into ME-180 cells (Fig. 6, *control vector*). Background induction by TPA of a variety of vectors has been described previously and is presumably mediated through cryptic sites in the PXP1 plasmid (20). The TPA induction due to the inserted FGF-BP promoter was considered to be that observed above the background control vector induction. The FGF-BP promoter from −1060 to +62 was induced about 4-fold above control vector in the presence of TPA and showed the same TPA inducibility as the full-length 1.8-kb promoter construct (data not shown). Deletion from −1060 to −118, which removed 950 base pairs of promoter sequence including the potential NF-κB

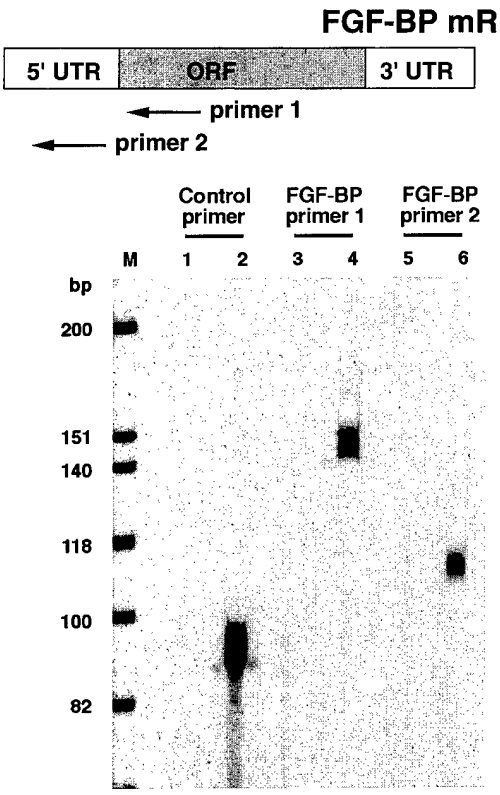

FIG. 4. **Mapping of the FGF-BP transcription start site.** Primer extension of the FGF-BP transcript. *Upper panel* shows location of primers derived from the coding region (FGF-BP primer 1, *lower panel, lanes 3* and *4*) and 5′-UTR (FGF-BP primer 2, *lower panel, lanes 5* and *6*) of the human FGF-BP cDNA. Extension reaction was carried out in the absence (*lanes 3* and *5*) or presence (*lanes 4* and *6*) of mRNA isolated from ME-180 cells. A control primer was used in the absence (*lane 1*) or presence (*lane 2*) of 1.2-kb kanamycin control RNA. The transcription start site derived from these results is shown as an *asterisk* in Fig. 5.

site, had no effect on TPA induction and was also induced 5-fold above background (Fig. 6). Similarly, deletion from −118 to −93, which removed one of the potential Ets-binding sites, retained full TPA induction. Removal of the consensus Sp1 binding site from −93 to −77 had no effect on TPA induction of the FGF-BP promoter. However, the 5′ deletion to −77 caused an 80% decrease in basal activity of the promoter (Fig. 6, *left panel*) suggesting that the Sp1 consensus site is a predominant mediator of basal promoter activity of FGF-BP but is not required for TPA induction.

TPA induction was significantly reduced upon deletion of the potential Ets factor binding site from −77 to −67 and is reduced even further upon deletion of the AP-1 site from −67 to −56 (Fig. 6), indicating that each of these sites contributes to some degree in TPA induction. The basal activity of these constructs was similar at about 10–20% of the −118 construct. Finally, deletion from −56 to −31, which removes sequences containing homology to a C/EBPβ-binding site, abolished any remaining TPA induction to the background control vector level. The basal activity of this vector was 5% that of the −118/+62 vector.

Contribution of Ets, AP-1, and C/EBPβ Sites to TPA Induction—In order to better understand the contribution of each individual consensus binding site to TPA induction, internal promoter deletions were introduced and tested for TPA inducibility within the context of the promoter from −118 to +62. Deletion of the Ets site alone (−76 to −67) or deletion of the AP-1 site alone (−65 to −58) reduced TPA induction slightly to the intact promoter (Fig. 7). Deletion of both Ets and AP-1 (−76 to −58), however, resulted in a significant decrease in both basal activity and in TPA induction, indicating that both sites act in cooperation for full promoter activity. However, loss of the juxtaposed Ets/AP-1 site does not completely abolish TPA induction, suggesting that additional sites are also involved.

The contribution of the C/EBPβ binding motif to TPA induction of the FGF-BP promoter was determined by an internal deletion from −47 to −33 (Fig. 7, *ΔC/EBPβ*). Consistent with the 5′ deletion construct which contained only the C/EBPβ site and retained some TPA inducibility (Fig. 6, *−56/+62*), an internal deletion of this site showed a significant decrease in TPA induction. Activation of C/EBPβ has been shown to occur through *ras*-dependent phosphorylation and is involved in phorbol ester induction of genes such as MDR1 (21–23). Similarly, C/EBPβ seems to play a role in TPA induction of the FGF-BP promoter since deletion of this site reduces the overall induction by TPA.

TPA Regulation of the FGF-BP Promoter Involves a Repressor Element Juxtaposed to the AP-1 Site—Between the AP-1 site and the C/EBPβ site lies a region of low homology between the human and mouse EGF-BP promoter sequences. Because this region was not suspected to have any effect on TPA induction, an internal deletion removing this region (−57 to −47) was tested as a control. Surprisingly, in the Δ57/47 construct, TPA induction of the FGF-BP promoter increased from approximately 5 to 11-fold, suggesting the presence of a possible repressor which may interact with this site. The lack of sequence conservation between the human and mouse in this region may reflect a difference in the regulation of FGF-BP between the two species. The −57 to −47 deletion disrupts an AACGTG (−60 to −55) which is juxtaposed to the 3′ end of the AP-1 site and which shows some similarity to the CACGTG E box element recognized by a number of helix-loop-helix factors (24). To test this imperfect E box for repressor activity, a C to T point mutation at position −58 was introduced into the −118/+62 BP promoter construct (Fig. 7). This mutant-58 (m-58) construct showed a dramatic increase in TPA induction up to 16-fold above background. Moreover, when the −58 point mutation is introduced into the ΔC/EBPβ construct (Fig. 7, *ΔC/EBPβ*/m-58), this promoter mutant also showed increased fold induction by TPA, suggesting that repression mediated by this site is not dependent on the C/EBPβ site. These data show that the point mutation at position −58, as well as the internal deletion from −57 to −47, disrupts repression of the FGF-BP promoter which normally limits the response to TPA.

Transcription Factor Binding to FGF-BP Promoter Elements—In order to ascertain that TPA induction of FGF-BP was due to direct activation by transcription factors, we performed gel retardation analysis to show transcription factor binding to FGF-BP promoter elements. By using labeled promoter sequence from −80 to −63 containing the putative Ets-binding site as a probe (Fig. 8*A*), the binding of three specific protein complexes in the presence of ME-180 nuclear extracts was detected (Fig. 8*B*). Protein binding to all three complexes was increased in the presence of TPA (Fig. 8*B, lane 3*) and was specifically competed away in the presence of excess unlabeled −80/−63 oligonucleotides (*lanes 4* and *5*). Further competition analysis showed that the factors binding to the −80/−63 element were only weakly competed by consensus Ets elements from the collagenase promoter (25) and polyoma virus enhancer (26), requiring over 100-fold excess in order to compete for binding (data not shown). It has previously been described that specific residues flanking the GGA trinucleotide motif of the Ets site are required for high affinity sequence-specific binding of individual Ets family members (27–29). Therefore, our data suggest that the −80/−63 element on the FGF-BP

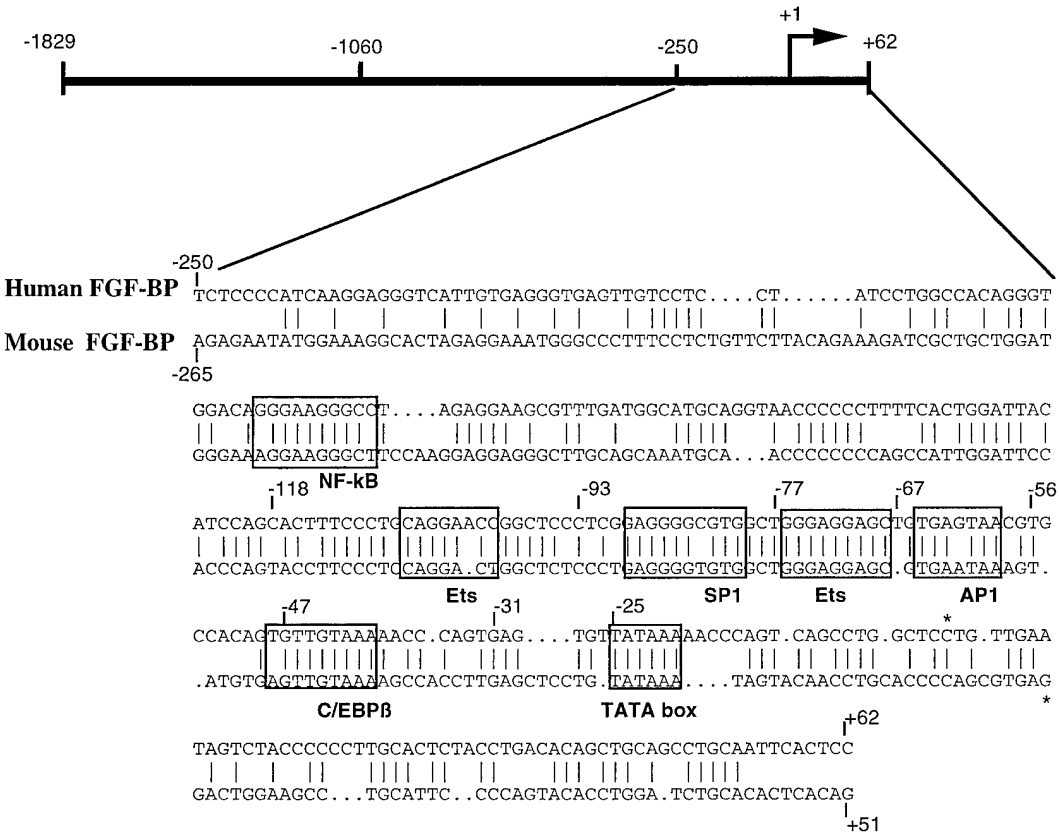

FIG. 5. **Structure and homology of the human FGF-BP promoter.** *Top*, the structure of the human FGF-BP promoter from −1829 to +62 is shown schematically. *Bottom*, nucleotide homology between promoter sequences of human (*top strand*) and mouse (*bottom strand*) FGF-BP. Transcription start sites are indicated by an *asterisk*. *Vertical lines* indicate homologous nucleotides between the mouse and human, and *dotted lines* represent gaps in the homology. Consensus transcription factor binding sites are *boxed*. *Numbers* on the *top strand* correspond to the numbering of the human FGF-BP promoter and show the location of the distal end points used to create the luciferase promoter constructs in Fig. 6.

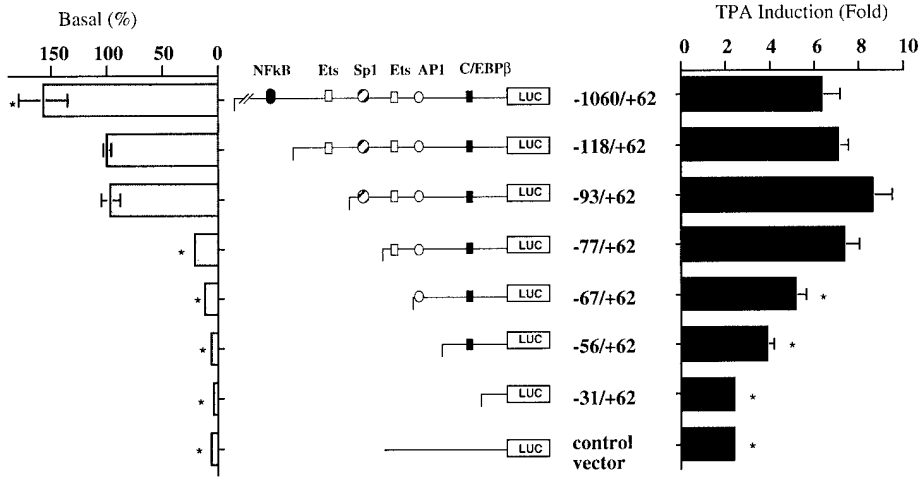

FIG. 6. **Effect of progressive 5′ deletions on basal activity and TPA induction of FGF-BP promoter.** The *left* histogram indicates the impact of the promoter deletion on the basal activity of each construct. The basal activity of the −118/+62 construct was set at 100%. The control vector shown is the thymidine kinase minimal promoter in PXP1. The *right* histogram shows the transcriptional activity in the presence of TPA 10^{-7} M for each FGF-BP promoter deletion construct (*center*). Each promoter construct was transiently transfected into ME-180 cells, and luciferase activity is expressed as fold induction of TPA-treated over untreated for each construct. The mean basal activity of the −118/+62 construct was 15,000 light units per μg of protein and the mean TPA-induced level was 100,000 light units/μg of protein. Values represent the mean ± S.E. from at least three separate experiments, each done in triplicate wells. *Asterisk* indicates significant difference ($p < 0.05$) from the −118/+62 promoter construct.

promoter could bind an Ets family member other than the Ets-1 or Ets-2 proto-oncogenes (26).

To determine transcription factor binding to the C/EBPβ site, gel shift analysis was carried out using labeled promoter sequence from −55 to −30 (Fig. 8*A*) as a probe. In the presence of ME-180 extracts, the −55/−30 element bound one predominant complex (Fig. 8*C*), which demonstrated increased binding in the presence of TPA (*lane 3*). The majority of the complex was competed away in the presence of excess unlabeled −55/−30 oligonucleotide (*lanes 4* and *5*), indicating that binding

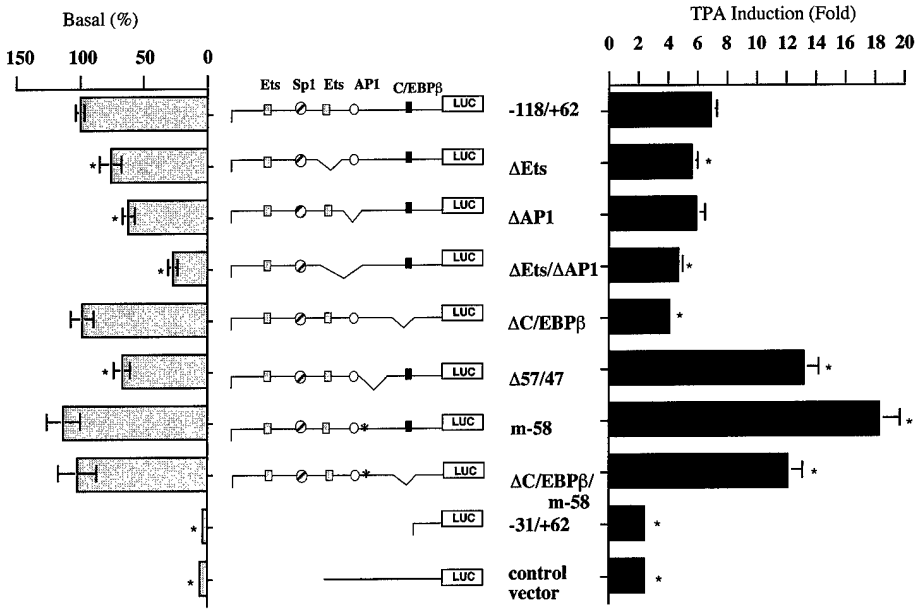

FIG. 7. **Effect of internal deletions on basal activity and TPA-induced activity of the FGF-BP promoter.** The *left* histogram shows the impact of each mutation on basal activity of the construct (*center*). The control vector shown is the thymidine kinase minimal promoter in PXP1. The *right* histogram shows the transcriptional activity in the presence of TPA 10^{-7} M for each internal deletion of FGF-BP promoter. Each promoter construct was transiently transfected into ME-180 cells, and luciferase activity is expressed as fold induction of TPA-treated over untreated for each construct. Values represent the mean ± S.E. from at least three separate experiments, each done in triplicate wells. *Asterisk* indicates significant difference ($p < 0.05$) from the −118/+62 promoter construct.

was specific. Competition by the C/EBPβ site from the $p21^{WAF1/CIP1}$ gene promoter (30) for the specific complex was effective only at high molar excess (data not shown) indicating that the FGF-BP −55/−30 element may bind a different C/EBPβ-related factor.

To investigate further the transcriptional activation of the FGF-BP promoter by AP-1 and the involvement of the variant E box repressor element, gel shift experiments were carried out using the labeled promoter sequence spanning the juxtaposed AP-1/repressor element as a probe (−70/−51, Fig. 8*A*). In the presence of ME-180 nuclear extracts, the −70/−51 element bound an upper complex (Fig. 8*D, arrow*) and a lower doublet (*bracket*). The binding of all three complexes was highly induced by TPA (Fig. 8*D, lane 3*) and was effectively competed by molar excess of the unlabeled −70/−51 oligonucleotide (*lanes 4* and *5*). To understand better the specific composition of these complexes, point mutations were introduced in either the AP-1 site (mut AP-1) or the repressor site (mut −58) and tested for their abilities to compete for binding. Competition with the mutant AP-1 site resulted in a decrease of only the bottom doublet and no competition for the upper band (*lanes 6–8*), suggesting that the upper band corresponds to factors bound specifically to the AP-1 site. Conversely, when competition was carried out with the repressor mutant (mut −58), binding of the doublet on the probe remains intact, whereas binding to the AP-1 site is reduced (*lanes 9–11*), indicating that the lower two bands represent distinct protein binding to the repressor element. Furthermore, when competition was carried out with an AP-1 consensus, which contains an AP-1 site flanked by sequences which are not homologous to the FGF-BP promoter, competition for only the upper AP-1 complex was observed (*lanes 12* and *13*). These results show that the AP-1 site and the repressor site of the FGF-BP promoter bind distinct and specific transcription factor complexes that are induced in the presence of TPA. Taken together, our data show that sequences between −77 and −33 of the FGF-BP promoter form a novel TPA regulatory cassette consisting of interacting positive and negative control elements.

DISCUSSION

In this report we demonstrate that TPA induction of FGF-BP mRNA levels is primarily through stimulation of gene transcription. This is in contrast to the retinoid repression of FGF-BP gene expression which we have previously shown is mediated through post-transcriptional and transcriptional mechanisms (7). In fact, at least at early time points after retinoid administration, the post- transcriptional mechanism which is dependent on new protein synthesis predominates since the half-life of the FGF-BP mRNA is greater than 16 h (7). Our studies show that the TPA induction of FGF-BP mRNA is rapid, requiring no new protein synthesis and involves direct activation by transcription factors whose site of action is clustered in the first 118 base pairs upstream of the transcription start site. Within this region the majority of the TPA stimulation of the FGF-BP promoter can be explained by the additive effects of two sites positioned between −76 to −58 and from −47 to −33.

The −76 to −58 site harbors a perfect consensus to the AP-1 transcription factor binding site NTGAGTCA (31). The AP-1 transcription factor complex comprises the c-*fos* and c-*jun* proto-oncogenes which are known to be activated as a result of TPA stimulation of PKC-dependent pathways (32). However, deletion of the AP-1 site alone in the FGF-BP promoter caused only a slight reduction in TPA effects on the FGF-BP promoter. This result is consistent with the emerging picture that AP-1 acts synergistically with other transcription factors, such as the Ets family of transcription factors, to mediate gene expression in response to TPA and other stimuli (28, 29). In the FGF-BP promoter deletion of sequences 5′ to the AP-1 consensus significantly decreases the TPA stimulation in comparison with deletion of the AP-1 site alone. These 5′ sequences contain the core GGA found in the center of the Ets family DNA consensus recognition site (29). Considering the body of evidence that suggests that Ets/AP-1 cooperate for full transcriptional activation, it seems likely that this may be the function of the −76/−58 element. For instance similar cooperation between Ets and AP-1 occurs through a juxtaposed Ets/AP-1-binding site in the polyoma virus enhancer (18) and has subsequently been implicated in the regulation of genes involved in invasion and metastasis, including collagenase and urokinase plasminogen activator (19, 33–37). Although we found that the collagenase Ets element or the polyoma virus Ets element did not effectively compete for binding to the FGF-BP Ets element, this may reflect the binding of another Ets family member to the FGF-BP promoter whose recognition site could be determined by sequences flanking the GGA core (27).

Deletion of the −47 to −33 FGF-BP promoter region also

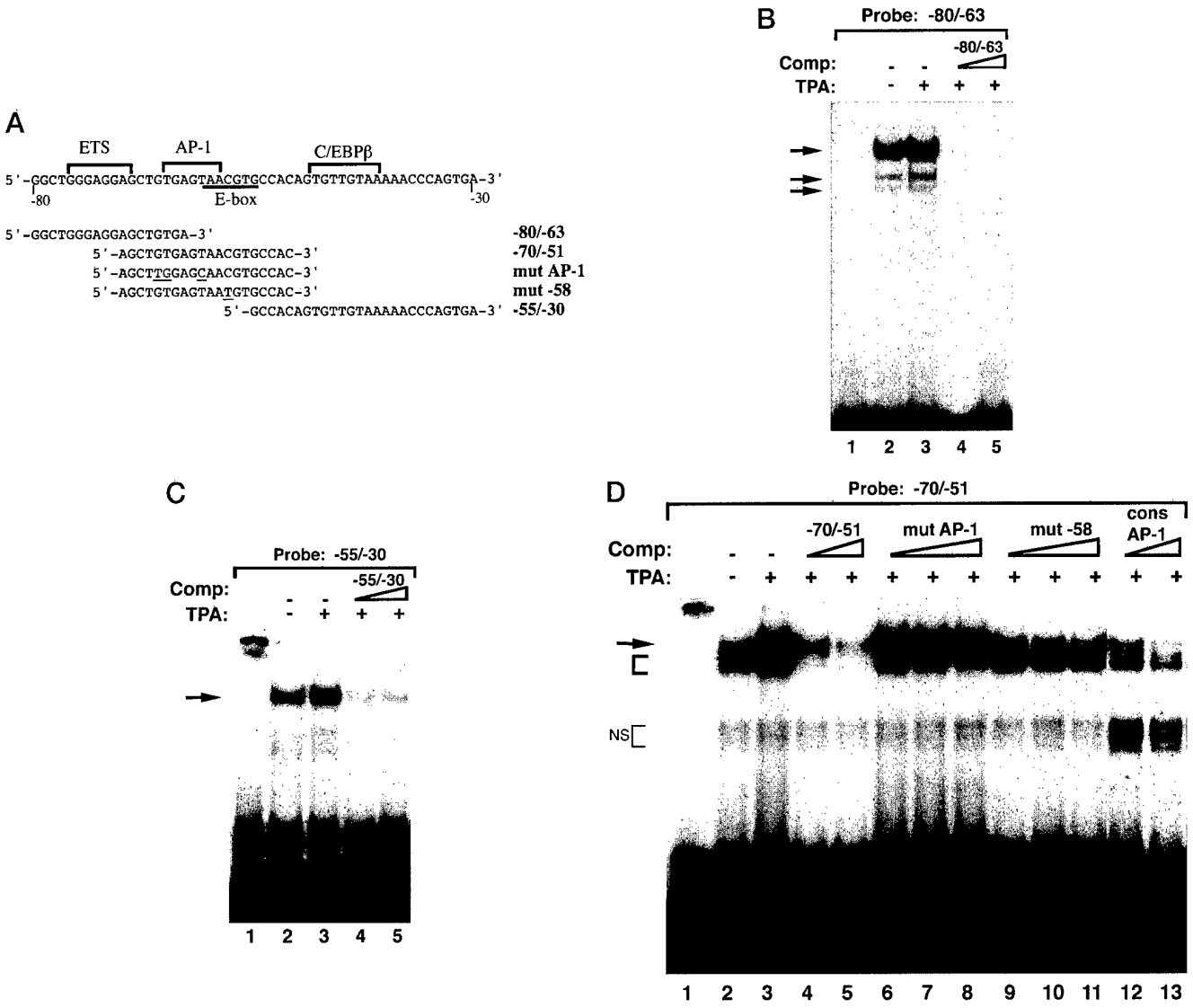

FIG. 8. **Transcription factor binding to FGF-BP promoter elements.** *A*, double-stranded oligonucleotide sequences used for gel shift analysis. Point mutations are *underlined*. *B*, gel shift assay with ^{32}P-labeled FGF-BP promoter sequence from −80 to −63 containing the putative Ets site either alone (*lane 1*) or in the presence of untreated (*lane 2*) or TPA-treated (*lanes 3–5*) ME-180 nuclear extracts. Binding reactions were incubated in the presence of 20-fold (*lane 4*) or 50-fold (*lane 5*) molar excess of the unlabeled −80/−63 oligonucleotide. *C*, gel shift assay with labeled promoter sequence from −55 to −30 containing the putative C/EBPβ-binding site either alone (*lane 1*) or in the presence of untreated (*lane 2*) or TPA-treated (*lanes 3–5*) ME-180 nuclear extracts. Competition for binding was carried out with 20-fold (*lane 4*) and 50-fold (*lane 5*) molar excess of unlabeled −55/−30 oligonucleotide. *D*, gel shift assay with labeled promoter sequence from −70 to −51 containing the AP-1 and repressor sites either alone (*lane 1*) or in the presence of untreated (*lane 2*) or TPA-treated (*lanes 3–13*) ME-180 nuclear extracts. Competition in the presence of 10-fold (*lanes 6* and *9*), 20-fold (*lanes 4, 7, 10,* and *12*), or 50-fold (*lanes 5, 8, 11,* and *13*) molar excess of unlabeled oligonucleotides as indicated. Specific complexes are indicated by an *arrow* on the *left* of each panel and nonspecific complexes are labeled *NS*.

substantially reduces the TPA effects on the FGF-BP promoter. Sequence analysis revealed that a site homologous to the C/EBPβ-binding site is centered in this region of the promoter. The factors binding to FGF-BP C/EBPβ element, however, are not effectively competed by the C/EBPβ site from the p21$^{WAF1/CIP1}$ gene promoter, suggesting that transcription of FGF-BP may be mediated by a different C/EBPβ-related factor. The published consensus for C/EBPβ is T(T/G)NNGNAA(T/G) (38) which is identical in eight positions (underlined) to the site between −48 to −41 differing only in the most 3′-nucleotide of the consensus. In addition, the involvement of C/EBPβ in TPA-mediated responses has been shown previously. For instance, induction by phorbol esters has been shown to cause increased C/EBPβ synthesis, phosphorylation, and DNA binding to promoters of a number of genes including MDR1 and collagenase 1 (21–23, 39). Thus, C/EBPβ or a family member is involved in the activity of the −47 to −33 element. Like other leucine zipper family members, C/EBPβ acts cooperatively with other transcription factors to modulate the level of gene expression in response to extracellular stimuli. For example, C/EBPβ has been shown to associate with Fos/Jun *in vitro* (40) and can cooperate *in vivo* to induce expression of the *TSG-6* gene in response to interleukin-1 and tumor necrosis factor-α which is mediated through distinct AP-1 and C/EBPβ-binding sites in the *TSG-6* promoter (41). Similarly, our data show that the C/EBPβ consensus element is a major mediator of TPA-induced gene expression of FGF-BP. However, because removal of the C/EBPβ site alone does not completely abolish TPA induction, this suggests that like other TPA-induced genes, the C/EBPβ site acts in cooperation with other promoter elements.

A novel aspect of TPA regulation of the FGF-BP promoter is the role of the region −57 to −47 between the AP-1 site and the C/EBPβ site. Deletion of this region substantially increases the TPA response, implying that this region normally represses the

extent of the response to TPA. A point mutation in this region also abrogates repression thus making it unlikely that the effect of the deletion is simply to bring the AP-1 and C/EBPβ sites in closer proximity leading to their increased responsiveness to TPA. In fact, the relief of repression obtained with the −58 point mutant is observed in the presence of the C/EBPβ deletion suggesting that the repression impacts on the AP-1 element rather than the C/EBPβ site. An alternate possibility is that the factor bound to the −57 to −47 site interacts with the general transcription machinery in a manner similar to the NC2 repressor (42). However, this seems less likely because we observe no increase in basal activity of the promoter after deletion or mutation of the repressor site in comparison to the −118 construct (Fig. 7). The −57 to −47 deletion destroys an AACGTG (−60 to −55) which is a variant of the CACGTG E box element recognized by a number of helix-loop-helix factors (24). The −58 mutant changes the AACGTG to AATGTG and would perturb the 5′ part of the dimer recognition sequence (24). However, the wild type sequence alone does not predict which member of the helix-loop-helix family would interact with this site. Interestingly, binding to an AACGTG recognition element has been described *in vitro* to a homodimer of the aryl hydrocarbon receptor nuclear translocator helix-loop-helix factor (43), and aryl hydrocarbon receptor nuclear translocator-deficient embryonic stem cells have a defective angiogenesis process (44). However, it is unclear whether aryl hydrocarbon receptor nuclear translocator homodimers interact with promoters *in vivo*. Alternatively, other helix-loop-helix factors are known to function as transcriptional repressors, such as the Mad family of proteins that bind related E box sequences during TPA-induced macrophage differentiation (45, 46) and recruit the mSin3-histone deacetylase corepressor complex, leading to a more closed chromatin structure and transcriptional repression (47).

Through gel retardation analysis, we show distinct factor binding to the AP-1 site and to the E box repressor site. Interestingly, factor binding to both of these sites is increased upon stimulation with TPA. TPA-induced transcription factor binding to E box elements has been described for a number of different promoters including c-*fos* (48–50). The observation that TPA induces factors which both stimulate and limit induction of FGF-BP suggests a mechanism by which transcription of the FGF-BP gene could be tightly regulated and may reflect a level of tissue-specific expression of this gene.

Overall, our data suggest that the TPA induction of the FGF-BP promoter is induced through both Ets/AP-1 site and a C/EBPβ site and that the extent of induction is moderated by factors that bind to an E box repressor element which lies adjacent to the AP-1 site. It is known that TPA also induces the expression of genes involved in proteolytic degradation of the extracellular matrix such as stromelysin, collagenase, and urokinase plasminogen activator (33, 51, 52). Interestingly, these promoters are regulated by similar transcription factors as those which we show are involved in FGF-BP promoter induction, *e.g.* Ets/AP-1 and C/EBPβ. Thus, our data would support the argument that a specific subset of transcription factors may be induced (or derepressed) to specifically stimulate a panel of genes involved in invasion, angiogenesis, and metastasis during skin tumor development.

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Induction of the angiogenic modulator FGF-BP by epidermal growth factor is mediated through both MEK2/ERK2 and p38 signal transduction pathways.

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Running Title: EGF induction of FGF-binding protein

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SUMMARY

FGF-BP is a secreted protein which binds and activates fibroblast growth factors (aFGF and bFGF) and induces angiogenesis in some human cancers. FGF-BP is expressed at high levels in squamous cell carcinoma (SCC) cell lines and tumor samples and has been shown to be rate limiting for the growth of SCC tumors *in vivo*. In this study, we examine the regulation of FGF-BP by epidermal growth factor (EGF) and the signal transduction mechanisms which mediate this effect. We found that EGF treatment of the ME-180 SCC cell line caused a rapid induction of the FGF-BP gene. This induction was mediated transcriptionally through the AP-1 (c-Fos/Jun D) and C/EBP elements, as well as through an E-box repressor site in the proximal regulatory region of the FGF-BP promoter. Pharmacological inhibition of protein kinase C and MEK2 completely blocked EGF induction of FGF-BP mRNA, whereas inhibitors of MEK1 or PI3 kinase had no effect. In addition, treatment with the JNK/p38 activator anisomycin resulted in a dose-dependent increase in FGF-BP mRNA. Inhibition of p38 with the inhibitor SB203580 blocked both anisomycin and EGF induction, suggesting a role for p38 in the regulation of FGF-BP. Co-transfection of the FGF-BP promoter with dominant negative forms of MEK2, ERK2, and p38 significantly decreased the level of EGF induction, whereas dominant negative forms of MEK1, ERK1, or JNK had no effect. These results demonstrate that EGF induction of FGF-BP occurs selectively through dual activation of the stress-activated p38 pathway and the MEK2/ERK2 MAP kinase pathway, which ultimately leads to AP-1 and C/EBP activation of the FGF-BP promoter.

INTRODUCTION

A pivotal process in a healing wound as well as in a growing tumor is the development of new blood vessels, or angiogenesis. Some of the most potent angiogenic stimulators are the fibroblast growth factors, including the classical angiogenic activators of this family, aFGF[1] and bFGF [1]. High concentrations of biologically active aFGF and bFGF are found in extracts of normal human tissues which are not necessarily undergoing active new blood vessel growth [1]. This is due to the storage of aFGF and bFGF in the extracellular matrix (ECM) where they are found tightly bound to membrane-attached heparansulfate proteoglycans [2] which quenches their biological activity. One mechanism by which aFGF and bFGFs are released from the ECM is through the secretion of the carrier protein FGF-BP which binds to aFGF and bFGF in a non-covalent and reversible manner [3]. FGF-BP is actively secreted from the cell [3] and, once bound, prevents degradation of aFGF and bFGF [3,4]. The importance of FGF-BP secretion in promoting tumor growth was shown in studies using the non-tumorigenic adrenal carcinoma cell line SW-13 which has high expression of bFGF but is negative for FGF-BP. Stable overexpression of FGF-BP in SW-13 cells led to a dramatic increase in bFGF-dependent colony formation and formation of highly vascularized tumors in nude mice [4]. Additional studies were carried out to characterize the biological role of FGF-BP in highly tumorigenic cell lines such as ME-180 (human cervical squamous cell carcinoma) and LS174T (colon adenocarcinoma) which express high levels of endogenous FGF-BP [4,5]. Reduction of FGF-BP mRNA levels in these cell lines using ribozyme targeting significantly inhibited tumor development and angiogenesis [6]. These studies demonstrated that FGF-BP can serve as a rate-limiting angiogenic modulator for some tumor types [6,7].

FGF-BP is highly expressed in squamous cell carcinoma (SCC) cell lines from lung, bladder, skin and cervix, and is positive in primary SCC tumor samples [4]. Furthermore, its expression has been shown to be upregulated during mouse embryonic development of the skin, lung, and intestine and is low in most adult tissues [8]. A potential role for FGF-BP during skin carcinogenesis was described in studies showing dramatic FGF-BP upregulation in human[2] and

mouse skin treated topically with DMBA and TPA [8]. These observations suggested several mechanisms which might be involved in the direct regulation of the FGF-BP gene. First, DMBA treatment has been shown to cause a specific point mutation in the ras oncogene [9], suggesting that the ras signal transduction pathway might regulate FGF-BP expression. This is also indicated by the observation that FGF-BP is induced in ras-transformed keratinocytes [8]. Second, the role of TPA as a direct regulator of FGF-BP gene expression was confirmed upon TPA treatment of several SCC cell lines, including ME180, which caused rapid transcriptional induction the of the FGF-BP gene [10]. We further discovered that Sp1, AP-1, and C/EBP sites within the proximal FGF-BP promoter are all required for TPA regulation of FGF-BP [10].

SCC cell lines and tumors, including the ME-180 cell line, typically express high levels of EGF receptors (EGFR) [11,12] and overexpression of EGFR in SCC has been shown to confer greater tumorigenicity [13], which led us to investigate the possible role and mechanisms of EGF regulation of FGF-BP. EGF signaling occurs predominantly through binding to its receptor EGFR (ErbB1) and its dimerization partner ErbB2 (Her2/Neu). Autophosphorylation of activated EGF receptors stimulates a number of signal transduction pathways, including the classical Ras/Raf/MAPKK (MEK)/MAPK (ERK) pathway which is known to phosphorylate and activate AP-1 [14]. MEK/ERK activation can occur either through phosphorylated EGFR recruitment of the Shc/Grb2/SOS complex and subsequent Ras activation, or through recruitment of PLCγ and subsequent PKC activation. PKC can in turn modulate Raf through both Ras-dependent and -independent mechanisms [15,16]. Other signaling pathways induced by EGF include the stress-activated protein kinases (SAPKs) such as JNK (SAPK1) and the p38 (SAPK2) [17], the PI3 kinase pathway [18], and the JAK/STAT pathway [19].

Here we show a possible link between EGF signaling and angiogenic activation through the regulation of the FGF-BP gene in SCC. We find that EGF treatment induces rapid upregulation of FGF-BP transcription occurring through the AP-1 and C/EBP sites in the FGF-BP promoter. Furthermore, inhibition of both the MEK2/ERK2 pathway or the p38 pathway block

EGF induction, implicating dual activation of these MAP kinases as an important step in FGF-BP regulation.

EXPERIMENTAL PROCEDURES

Cell Culture and reagents. The ME-180 cervical squamous cell carcinoma cell line was obtained from American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in improved minimum essential medium (IMEM) (Biofluids Inc., Rockville, MD) with 10% fetal bovine serum (Life Technologies, Inc.). Actinomycin D, Calphostin C, and Wortmannin were purchased from Sigma. PD98059 was from New England Biolabs and Anisomycin, SB 203580, and Tyrphostin AG 1478 were from Alexis Corp. U1026 was purchased from RBI (Natick, MA). All compounds were dissolved in Me_2SO.

Northern Analysis. ME-180 were grown to 80% confluence in 10 cm dishes, washed twice in serum-free IMEM, and incubated 16 hours in serum-free IMEM prior to treatment. Cells were pretreated for 1 hour with the indicated drug or with vehicle alone (Me_2SO, final concentration 0.1%). EGF or Anisomycin treatment was for 6 hours unless otherwise indicated. Total RNA was isolated with RNA STAT-60™ (Tel-Test Inc.) and Northern analysis carried out as previously described [10]. Hybridization probes were prepared by random-primed DNA labeling (Amersham) of purified insert fragments from human FGF-BP [4] and human GAPDH (Clontech). Quantitation of mRNA levels was performed using a PhosphorImager (Molecular Dynamics).

Plasmids. Human FGF-BP promoter fragments were cloned into the pXP1 promoterless luciferase reporter vector and have been described previously [10]. The mutant AP-1 (mAP-1) FGF-BP promoter construct was generated by PCR as previously described [10] introducing point mutations which convert the AP-1 site from GTGAGTAA (-66 to -59) to TGGAGCAA. pRL-CMV *Renilla* luciferase reporter vector was purchased from Promega. The dominant negative expression vector for MEK1 (MEK1eeCMV), and the empty vector (eeCMV) were provided by Dr. D. Templeton (Case Western) [20]. The MEK2 (K101A) dominant negative was provided by Dr. J. Holt (Vanderbilt University) [21]. The dominant negatives of ERK1 (K71R), ERK2 (K52R), and the empty vector pCEP4 were provided by Dr. M. Cobb (University of Texas Southwestern) [22]. Dominant negatives of JNK1 (APF) and p38α (AGF) were provided by Dr.

R. Davis (University of Massachusetts) [23,24]. All effects of dominant negatives were compared to their empty vector control or to the empty vector pCDNA3 (Invitrogen).

Transient Transfections and Reporter Gene Assays. 24 hours before transfection, ME-180 cells were plated in 6 well plates at a density of 750,000 cells/well. For each transfection, 1.0 μg FGF-BP promoter-luciferase construct, 0.2 ng pRL-CMV (transfection efficiency control), and 8 μl Lipofectamine (Gibco-BRL) were combined and added to cells for 3 hours in serum free conditions as described previously [10]. For co-transfections, 750 ng of –118/+62Luc FGF-BP promoter construct, 1.0 μg expression vector, 0.2 ng pRL-CMV, and 10 μl Lipofectamine were added to cells. Transfection was treated for 18 hours with EGF (5 ng/ml) in serum free IMEM before cell lysis in 150 μl passive lysis buffer (Promega). 20 μl of extract was assayed for both firefly and *Renilla* luciferase activity using Dual-Luciferase™ Reporter assay system (Promega). Due to a small background induction (1.5-2 fold) of the pRL-CMV plasmid by EGF, all luciferase values were normalized for protein content. There were no significant differences, however, in the transfection efficiencies between plasmid constructs as determined by *Renilla* luciferase assay. Protein content of cell extracts was determined by Bradford Assay (Bio-Rad).

Gel Shift Assays. ME-180 cells were grown to 80% confluency on 150 mm dishes, serum-starved in IMEM for 16 hours and treated with or without 5 ng/mL EGF for 1 hour. Nuclear extracts were prepared as previously described [10]. Binding reactions with -70/-51 and -80/-63 probes were carried out as previously described [10] with 5 μg or 1 μg, respectively, of ME-180 nuclear extracts, binding buffer (20mM Tris pH7.5, 60mM KCl, 5% Glycerol, 0.5mM DTT, 2.0mM EDTA), and 250 ng poly dIdC. Binding reaction with -55/-30 probe was carried out with 5μg ME-180 nuclear extracts and 500 ng poly dIdC. Supershift antibodies (2 μg) were added to binding reaction for 10 min on ice before adding 20 fmoles of labeled probe. Reactions were carried out 45 min on ice and analyzed by 6% polyacrylamide gel electrophoresis. Supershift antibodies purchased from Santa Cruz were the following: Fos-specific antibodies c-Fos (K-25)-x, c-Fos (4)-x, Fos B (102)-x, Fra-1 (R-20)-x, and Fra-2 (Q-20)-x; Jun-specific antibodies c-Jun/AP-1 (D)-x, c-Jun/AP-1 (N)-x, Jun B (N-17)-x, and Jun D (329)-x; Sp1-specific antibody

Sp1 (PEP 2)-x; and C/EBP-specific antibodies C/EBP α (14AA)-x, C/EBP β (C-19)-x, C/EBP β (Δ 198)-x, C/EBP γ (C-20)-x, C/EBP δ (M-17)-x, and C/EBP ε (C-22)-x. For oligonucleotide competition, a 50-fold molar excess of unlabeled double-stranded competitor was added to the binding reaction and incubated 10 min on ice before addition of the labeled probe. Sequence of the C/EBP consensus fragment was 5'-TGCAGATTGCGCAATCTGCA-3' (Santa Cruz Biotech.).

RESULTS

EGF treatment increases FGF-BP mRNA in SCC cells. We have shown previously that phorbol ester (TPA) treatment of the human cervical SCC cell line ME-180 results in a time- and dose-dependent increase of FGF-BP mRNA which is mediated via the PKC signal transduction pathway [10]. Similarly, treatment of ME-180 cells with EGF resulted in a rapid increase in the steady-state levels of FGF-BP mRNA with no effect on GAPDH mRNA levels (Fig. 1A). FGF-BP mRNA induction was detectable after 1 hour of treatment and was maximal after 6 hours with an average increase of 4.5 fold (Fig 1B). The rapid and transient nature of EGF induction of FGF-BP mRNA is identical to that seen after TPA treatment [10], suggesting that these agents might induce FGF-BP through a similar transcriptional mechanism.

To determine whether FGF-BP is upregulated by EGF at the transcriptional level, we tested the effect of the transcription inhibitor Actinomycin D on the EGF induction of FGF-BP mRNA. Pre-treatment with Actinomycin D completely blocked the induction of FGF-BP by EGF (Fig. 4). Combined treatment with EGF and cycloheximide had no effect on induction of FGF-BP mRNA (data not shown), indicating that *de novo* protein synthesis is not required for the EGF response. Furthermore, transient transfection of the full-length FGF-BP promoter from –1060 to +62 into ME-180 cells resulted in a 4.5-fold increase in luciferase activity upon EGF treatment (Fig 2). These data demonstrate that EGF, like TPA, can directly increase the rate of FGF-BP gene transcription.

Identification of EGF response elements within the FGF-BP promoter. Functional analysis of the FGF-BP promoter has shown that TPA-induced transcription involves a combination of both positive and negative regulatory elements located within the first 118 base pairs of the proximal promoter [10]. Full TPA induction was mediated by a C/EBP consensus site between -48 and -40, as well as through a juxtaposed Sp1/AP-1 element between -76 and -59 (depicted in Fig. 2 as Sp1(b)). The Sp1(b) site between -76 and -58 was previously described as an Ets element based on its homology to other Ets consensus binding sites [10]. However, we have now determined through supershift analysis that this site is bound by the Sp1 transcription factor (Fig.

3D). To investigate whether these same regulatory elements play a role in EGF induction of the FGF-BP promoter, we transiently transfected a series of mutated promoter constructs into ME-180 cells and tested their ability to be induced after treatment with EGF. Deletion of promoter sequences from -1060 to -118 had no effect on the level of promoter activity and resulted in a similar 5-fold EGF induction (Fig. 2), demonstrating that the EGF regulatory region of the promoter is located within the first 118 base pairs of the promoter. Although the upstream Sp1 site (Sp1(a)) drives a significant portion of basal promoter activity [10], deletion of this site had no effect on EGF induction (data not shown). Deletion of the Sp1(b) site within the context of the -118/+62 promoter fragment also had no effect on EGF induction (Fig. 2). In contrast, mutation of the AP-1 site resulted in a significant 40% decrease in EGF induction as compared to the wild-type -118/+62 promoter. Similarly, complete deletion of the juxtaposed Sp1(b)/AP-1 site resulted in a 46% decrease in EGF response (Fig. 2). This result shows that unlike TPA induction, which requires the entire Sp1(b)/AP-1 element, EGF induction is driven mainly through the AP-1 site in the promoter. This reflects possible differences in the mechanisms by which each of these agents regulate the transcription of FGF-BP.

To test the contribution of the C/EBP element to EGF induction, we introduced an internal deletion of this site within the context of the -118/+62 promoter. Removal of the C/EBP site significantly reduced the EGF effect by 46% (Fig 2), demonstrating that an intact C/EBP site is necessary for full EGF induction of the FGF-BP promoter.

In addition to these positive regulatory elements, we recently showed that TPA regulation of the FGF-BP promoter involves a negative regulatory element, lying just downstream of the AP-1 site, which shows similarity to an E-box factor binding site [10]. We wanted to determine whether this E-box repressor site also played a role in regulation by EGF. Point mutation of this site at position -58, or deletion of this site from -57 to -47 resulted in a dramatic increase in EGF induction of the promoter from about 5-fold in the wild-type promoter to about 8-10 fold in the repressor mutants (Fig. 2), indicating loss of repressor activity. Therefore, the repressor element

between -58 and -47 also plays a regulatory role in EGF induction by limiting the transcriptional response to growth factor stimulation.

AP-1, C/EBP, and Sp1 binding to the FGF-BP promoter. Because the AP-1 site in the FGF-BP promoter appears to be important during TPA and growth factor regulation of FGF-BP, we determined which members of the Fos and Jun family might be binding to the FGF-BP AP-1 site. To identify transcription factor binding to the AP-1 site, we carried out gel shift analysis using labeled promoter sequence from -70 to -51 (Fig 3A) which was incubated in the presence of nuclear extracts from control or EGF-treated ME-180 cells. Protein binding in the uppermost complex, which has previously been shown to represent AP-1 [10], is highly induced by EGF treatment (Fig 3B, lanes 1 and 2). To determine the composition of the AP-1 complex, we used Fos and Jun-specific antibodies for supershift analysis. As shown in Fig 3B, addition of a Fos antibody, which recognizes all Fos family members (lane 3) or which specifically recognizes c-Fos (lane 4), resulted in a supershifted complex. Antibodies against FosB, Fra-1, or Fra-2 had no effect. Furthermore, incubation with a general Jun family antibody (Fig 3B, lane 8) or with a JunD-specific antibody (lane 11) either blocks or supershifts the AP-1 complex, respectively. The c-Jun- or Jun B-specific antibodies had no effect on AP-1 binding. These results demonstrate that c-Fos and Jun D are the major components of AP-1 binding to the FGF-BP promoter.

The binding of C/EBP to the FGF-BP promoter was investigated using a promoter fragment spanning the C/EBP site from -55 to -30 (Fig 3A). Gel shift analysis with this fragment in the presence of nuclear extracts from untreated or EGF-treated ME-180 cells showed no significant change in the binding of C/EBP (Fig 3C, lanes 1-2). Activation of C/EBP, however, can occur through post-translational modifications such as phosphorylation, which would not necessarily be reflected at the level of DNA binding. The specificity of C/EBP binding in the upper complex of the gel shift (Fig 3C, arrow) is demonstrated through competition with molar excess of unlabeled -55/-30 fragment (lane 9), as well as a C/EBP consensus element (lane 10). Incubation with an antibody against C/EBPβ, which also cross-reacts with other members of the C/EBP family, resulted in one prominently supershifted band (Fig 3C, lane 3). In addition, antibodies

specific for C/EBPβ (lane 4) and C/EBPδ (lane 7) also supershifted the complex, whereas antibodies for C/EBPα (lane 5), C/EBPγ (lane 6) and C/EBPε (lane 8) had less of an effect on C/EBP binding. Therefore, EGF induction through C/EBP site most likely involves activation of C/EBPβ and C/EBPδ family members.

As mentioned previously, the promoter element between –80 and –63 shows some homology to an Ets factor binding site [25]. Closer analysis of this site also revealed homology to a variant Sp1 site which has shown to be induced by EGF treatment [26]. In fact, supershift analysis of this element showed specific binding of Sp1 (Fig 3D). This element has been denoted Sp1(b) in order to distinguish from the Sp1 site further upstream (Sp1(a)). Interestingly, the binding of Sp1 to the Sp1(b) site increased in the presence of nuclear extracts from EGF-treated cells (Fig 3D, lanes 1-2) despite the fact the removal of the Sp1(b) site alone has no impact on the overall EGF induction of the FGF-BP promoter (Fig. 2).

Role of PKC and MEK2 signal transduction pathways in FGF-BP regulation. In order to differentiate between the possible signaling pathways involved in EGF induction of FGF-BP, we chose to test pharmacological inhibitors of signal transduction components for their effect on FGF-BP regulation. We found that treatment with the EGFR tyrosine kinase inhibitor Tyrphostin AG1478 reduced EGF induction of FGF-BP to 30% (Fig 4), which was not significantly different from the untreated (without EGF or drug treatment) control (data not shown). Therefore, as expected, EGFR tyrosine kinase activity is essential for the EGF effect on the FGF-BP gene. In addition, we have shown previously that TPA induction of FGF-BP transcription was mediated through a PKC-dependent pathway [10]. To establish whether PKC activation was also required for the EGF effects on FGF-BP, we treated ME-180 cells with the specific PKC inhibitor Calphostin C [27] and found that this completely blocked EGF induction of FGF-BP mRNA (Fig 4). Therefore, PKC activation is central in mediating FGF-BP transcriptional activation upon either EGF or TPA stimulation.

To determine the contribution of the MAP kinase kinases (MEK1 and MEK2) to FGF-BP regulation, we tested the effects of pharmacological inhibitors of MEK1 and MEK2 on EGF

signaling. PD98059 inhibits MEK1 with an IC_{50} of 5-10 μM and MEK2 with an IC_{50} of 50 μM. Pretreatment with PD98059 at a concentration which fully inhibits MEK1 but not MEK2 had no effect on EGF induction of FGF-BP mRNA (Fig 4) indicating that MEK1 activation does not play a significant role in the regulation of FGF-BP. In contrast, pretreatment with the drug U1026, which is a potent inhibitor of both MEK1 and MEK2, could effectively block EGF induction of FGF-BP mRNA (Fig 4). Consistent with the selective role of MEK2 in FGF-BP induction, higher concentrations of PD98059 (100 μM), which inhibit MEK2, also blocked the EGF effect on FGF-BP (data not shown). Since MEK1 inhibition alone had no effect on the EGF response, these results indicate that selective activation of MEK2 is necessary for FGF-BP gene regulation.

EGF is also known to stimulate intracellular signaling via the PI3 kinase pathway [18]. In order to test the contribution of PI3 kinase to FGF-BP regulation, we used the PI3 kinase specific inhibitor Wortmannin which we have used previously in our laboratory to effectively block the PI3 kinase pathway [28]. Pretreatment with Wortmannin had no effect on EGF induction of FGF-BP mRNA (Fig 4), ruling out a role for PI3 kinase in the regulation of FGF-BP.

Induction of FGF-BP by p38 activation. Stimulation of the JNK/p38 MAP kinase pathway has also been shown to regulate AP-1 activity in response to mitogens and stress [17]. We therefore tested whether JNK or p38 activation could induce FGF-BP gene expression by treating with the antibiotic anisomycin. Anisomycin treatment at concentrations below 200 nM is known to be an effective stimulator of both JNK and p38 [29]. Treatment of ME-180 cells with anisomycin alone resulted in a significant and dose-dependent increase of FGF-BP mRNA levels up to 2.3-fold (Fig 5A), showing that activation of the JNK/p38 pathway can induce FGF-BP in ME-180 cells.

Because anisomycin is capable of activating both p38 and JNK pathways, we tested the contribution of p38 to FGF-BP induction using the p38-specific inhibitor SB203580, which has no inhibitory activity for JNK or ERK1/2 [30]. Treatment with SB203580 reduced EGF induction of FGF-BP mRNA by 60% in a dose-dependent manner (Fig. 5B). Additionally, p38 inhibition

completely blocked anisomycin induction of FGF-BP (Fig. 5B), demonstrating that both anisomycin and EGF induction of FGF-BP mRNA require p38 activation.

Effect of MEK, ERK, JNK, and p38 dominant-negatives on FGF-BP promoter activity. Inhibition of MEK2 and p38 with pharmacological agents demonstrated the importance of these kinases in mediating the EGF response on FGF-BP. To support the role of each of these pathways in the regulation of FGF-BP, we used dominant-negative expression constructs which specifically suppress the enzymatic activity of their endogenous counterparts. Co-transfection of a dominant-negative for MEK2 with the FGF-BP promoter luciferase construct (-118/+62) into ME-180 cells resulted in a significant decrease in EGF induction, whereas the dominant-negative for MEK1 had no significant effect (Fig. 6A). These results support the conclusions from Figure 5A that inhibition of MEK1 alone (PD98059) had no effect on EGF induction, and strongly point to a role for MEK2 activation in the regulation of FGF-BP. To identify which targets of MEK2 might be involved, we co-transfected kinase-deficient dominant negative mutants of the MAP kinases ERK1 and ERK2. We found that inhibition of ERK2 activity resulted in a significant decrease in EGF induction, whereas ERK1 inhibition had no effect (Fig 6A). These results clearly suggest that selective activation of MEK2 and ERK2 by EGF are necessary in the induction of the FGF-BP gene.

To test the contribution of p38 and JNK to EGF induction of FGF-BP, we co-transfected dominant-negative expression vectors for JNK and p38 along with the FGF-BP promoter into ME-180 cells. Each dominant negative mutant contains an alanine and phenylalanine in place of the activating threonine and tyrosine phosphorylation sites [23,24]. Expression of the dominant-negative JNK had no significant effect on EGF induction compared to the empty vector. On the other hand, expression of the dominant-negative p38 consistently reduced EGF induction of FGF-BP transcription (Fig 6B). Although inhibition of p38 activity blocked the EGF effect by only 23%, this difference was statistically significant. Overall these data indicate that two important MAP kinase pathways, MEK2/ERK2 and p38, are necessary for full induction of FGF-BP transcription by EGF.

DISCUSSION

This study shows for the first time that transcription of the FGF-BP gene is directly induced by EGF. The EGF family of growth factors, which include EGF, transforming growth factor-alpha (TGFα), and other structurally related peptides, cause cellular signaling through the EGFR pathway, regulating proliferation and differentiation of many tissue types. Deregulation of the EGF-induced signaling network is known to play an important role in the tumorigenesis for several human cancers, including neoplasms of the brain, lung, breast, ovary, pancreas, prostate, and colon, as well as in squamous cell carcinoma (SCC) of the skin and cervix [11,12,31]. Here, we have shown that EGF regulates FGF-BP gene expression suggesting a link between activated EGF signaling in a cell and subsequent FGF activation, ultimately leading to activation of FGF-mediated processes, such as angiogenesis, during development and tumor growth.

The up-regulation of FGF-BP by EGF was found to show characteristics similar to TPA induction of this gene, including a rapid and transient upregulation of the FGF-BP mRNA, and the requirement of transcriptional elements within the TPA regulatory region (-118 to +62) of the promoter. Transcriptional regulation of FGF-BP by EGF requires the AP-1 site between -65 and -61 which contains c-Fos and JunD family members whose binding is highly induced upon EGF induction (Fig 2 and Fig 3B). The finding that c-Fos contributes to FGF-BP gene expression is significant since an important role for c-Fos during SCC formation has already been described. Studies with c-*fos* knockout mice demonstrated that c-*fos* is required for malignant transformation of skin papillomas into malignant carcinomas since c-*fos* (-/-) papillomas became desiccated, hyperkeratinized, lacked vascularization, and remained benign [32]. Since the FGF-BP gene was previously found to be highly induced during skin carcinogenesis [8], it seems possible that this gene could be a target of c-Fos activation during skin SCC tumor formation, providing at least one mechanism for the recruitment of new blood vessels to the tumor.

The other required EGF response element in the FGF-BP promoter is the C/EBP site between –47 and –33, which is predominantly bound by the C/EBPβ and C/EBPδ family members (Fig 2 and Fig 3C). C/EBP proteins are a family of leucine zipper transcription factors which play

a central role in the acute phase response and in a number of cell differentiation pathways (reviewed in [33]). C/EBP family members recognize similar DNA elements in their target genes where they bind either as homodimers or heterodimers with other C/EBP family members or with other leucine zipper factors [34]. Whereas C/EBPα is generally associated with growth arrest and cellular differentiation, C/EBPβ and C/EBPδ are often correlated with gene activation during cellular proliferation, inflammation, and tumorigenesis [35-37]. Analysis of different C/EBP target gene promoters has demonstrated a finely tuned regulation of gene expression through the interplay of different C/EBP factors. During the acute phase response, for example, the amount of C/EBPα homodimers or heterodimers is reduced and replaced by C/EBPβ and C/EBPδ complexes [38-40]. Interestingly, study of cyclooxygenase-2 (COX-2) promoter regulation by C/EBP during skin carcinogenesis showed a change in C/EBP complexes from C/EBPα/C/EBPβ in normal skin to predominantly C/EBPβ/C/EBPδ complexes in skin SCC [41]. The presence of C/EBPβ/C/EBPδ complexes on the FGF-BP promoter in ME-180 SCC cells suggests the possibility that FGF-BP upregulation during tumor formation may be in part due to an interplay between the different C/EBP family members.

In trying to delineate the signal transduction pathway mediating EGF induction of FGF-BP, we found that activation of PKC plays a central role, since inhibition of PKC with Calphostin C blocks both the EGF (Fig 4) and TPA [10] effects on this gene. PKC activation in response to growth factor stimulus can lead to stimulation of the classical MAP kinase pathway Raf/MEK/ERK and subsequent activation of AP-1. PKC-mediated signaling through this pathway can be achieved through either Ras-dependent [15] or Ras-independent [16] mechanisms. The involvement of Ras in FGF-BP regulation is suspected since FGF-BP expression is increased after DMBA treatment and in ras-transformed keratinocytes [8]. A direct role for Ras in the activation of FGF-BP expression, however, hasn't yet been determined.

This study has shown that activity of the MAP kinase kinase MEK2 is required for FGF-BP gene regulation in response to EGF. The requirement for MEK2 activation was shown both by pharmacological inhibition (Fig. 4) as well as by expression with a MEK2 dominant negative (Fig

6A). The mechanism of MEK2 selectivity in the regulation of FGF-BP remains unknown at this point. MEK1 and MEK2 can be phosphorylated by common upstream dual specificity kinase kinases, such as Raf-1 [42,43], cMos [44,45], or MEKK1 [46,47]. Specific regulation of individual MEK family members can also be attributed to substrate-specific upstream regulators. A-Raf, for example, selectively activates MEK1 but not MEK2 [48]. In addition, MEK1 and MEK2 have been found to be differentially regulated depending on the cell setting [49], or on the kinetics of activation by serum growth factors [50]. Furthermore, it has been suggested that MEK2 activation may require additional tissue-specific factors which integrate MEK2 specific signaling [51].

MEK2 can phosphorylate both ERK1 and ERK2, which in turn leads to phosphorylation of a number of cellular and nuclear targets. In this study, inhibition of ERK2 but not ERK1 results in a significant inhibition of FGF-BP induction by EGF, demonstrating a specific requirement for ERK2 activation in the regulation of FGF-BP by EGF. Although the mechanism for this selectivity is also unclear, it is presumably due to ERK2 target specificity of factors which act on the AP-1 and/or C/EBP sites of the FGF-BP promoter. The MEK2/ERK2 selectivity for EGF regulation of FGF-BP presents an example of how activation of the MEK/ERK pathway can be selective and non-redundant, raising the therapeutic potential for pathway-specific targeting in treating cancer angiogenesis.

p38 is a JNK-related MAP kinase which is activated in response to a variety of stimuli including growth factors, phorbol esters, cytokines and environmental stress [17]. In this study, we have shown that in addition to ERK2, p38 activation contributes to the EGF induction of FGF-BP. EGF induction of FGF-BP was inhibited both by a p38 dominant negative and by the p38 inhibitor SB203580 (Fig. 5B). SB203580 is a pyridinyl imidazole compound which selectively inhibits p38α and p38β2 isoforms but has no effect on the activity on other p38 isoforms, JNK, or ERK [30,52,53]. Transcription factor targets of p38 include CREB and ATF1 [54,55], ATF2 [56,57], MEF-2C [58] and the C/EBP family members CHOP [59] and C/EBPβ [60,61],

suggesting that C/EBPβ and/or C/EBPδ could be targets of p38 activation on the FGF-BP promoter.

p38 is phosphorylated and activated by the dual specificity protein kinases MKK3, MKK4 and MKK6 [24,56,62-65]. However, the upstream signal transduction cascades which connect EGFR activation to phosphorylation of p38 have not yet been determined. One possible mechanism which is currently under investigation is the involvement of two members of the Rho family of GTPases, Rac and CDC42, which are known to regulate the activity of both JNK and p38 [66-69]. These studies have indicated that Rac and CDC42 are downstream of Ras activation [66], thereby connecting p38 and JNK activation to growth factor effects on cell growth and proliferation.

In conclusion, this study demonstrates that the growth factor EGF induces FGF-BP gene transcription and characterizes the mechanisms by which this effect is accomplished. The EGF-mediated pathways leading to FGF-BP transcription and subsequent angiogenic activation include the selective activation of MEK2/ERK2 and p38 MAP kinase pathways. This study highlights several targets for potential anti-angiogenic therapy of human cancers which utilize FGF-BP's angiogenic properties for tumor growth.

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FOOTNOTES

[1]The abbreviations used are: aFGF and bFGF, acidic and basic fibroblast growth factor; ECM, extracellular matrix; FGF-BP, fibroblast growth factor-binding protein; SCC, squamous cell carcinoma; DMBA, 7,12-dimethylbenz[*a*]anthracene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; C/EBP, CCAATT/enhancer binding protein; EGF, epidermal growth factor; ERK, extracellular signal regulated kinase; MEK, mitogen-activated ERK kinase; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; JNK, Jun kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

[2]Aigner, A., Wellstein, A., manuscript in preparation.

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FIGURE LEGENDS

Fig. 1. Induction of FGF-BP mRNA by EGF in ME-180 cells. **(A)** Northern analysis of FGF-BP mRNA levels in ME-180 cells which were either untreated or treated with 5 ng/ml EGF for the indicated amounts of time. Bands corresponding to FGF-BP mRNA (1.2 kb) and the control GAPDH mRNA (1.3 kb) are indicated. **(B)** Quantitation of northern blot signal intensities and normalization to GAPDH. Open circles represent control (untreated) levels and closed circles represent EGF treatment. The mean and standard deviation of at least two separate experiments are given.

Fig. 2. Effect of FGF-BP promoter mutations on the transcriptional induction by EGF. The histogram (*right*) shows the impact of each promoter deletion (*left*) on EGF induction of luciferase activity. ME-180 cells were transiently transfected with the indicated FGF-BP promoter luciferase constructs and were either untreated or treated with 5 ng/ml of EGF for 18 hours. Promoter constructs are described in text and in ref. [10]. Luciferase activity is expressed as fold induction of EGF-treated over untreated for each construct. Values represent the mean and standard error from at least three separate experiments, each done in triplicate wells. Statistically significant differences relative to the –118/+62 promoter construct are indicated (*, $p<0.05$ and **, $p<0.01$, student's t test).

Fig. 3. Characterization of transcription factor binding to FGF-BP promoter elements. **(A)** double-stranded oligonucleotide sequences of FGF-BP promoter elements used for gel shift analysis. Supershift analysis of transcription factor binding to the **(B)** AP-1 site, **(C)** C/EBP site, or **(D)** Sp1(b) site of the FGF-BP promoter. Gel shift assay with labeled FGF-BP promoter sequences as indicated were incubated in the presence of nuclear extracts from untreated or EGF-treated ME-180 cells. Binding reactions were incubated in the presence of supershift antibodies as indicated in each figure. Specific binding of AP-1, C/EBP and Sp1 are indicated by an arrow at the left of each panel. Supershift complexes are labeled by an asterix on either side of the gel. Competition for C/EBP binding was carried out in the presence of 50-fold molar excess of unlabeled oligonucleotides as indicated.

Fig. 4. Effect of signal transduction inhibitors on EGF induction of FGF-BP mRNA. FGF-BP mRNA levels from ME-180 cells treated with 5 ng/ml EGF for 6 hours. Cells were pretreated for 1 hour with vehicle alone (Me_2SO 0.1%), 5 μg/ml Actinomycin D (transcription inhibitor), 100 μM Tyrphostin AG1478 (EGFR tyrosine kinase inhibitor), 10 μM Calphostin C (PKC inhibitor), 50 μM PD98059 (MEK1 inhibitor), 10 μM U1026 (MEK1/2 inhibitor), or 100 nM Wortmannin PI3 kinase inhibitor). Northern blot signal intensities of FGF-BP were quantitated and normalized to GAPDH. The mean and standard deviation of at least two separate experiments are given. FGF-BP levels from untreated cells were approximately 25% of EGF treated.

Fig. 5. Involvement of p38 MAP kinase in the anisomycin and EGF induction of FGF-BP mRNA. **(A)** Anisomycin induction of FGF-BP mRNA. ME-180 cells were treated for 6 hours with the indicated concentrations of Anisomycin. The mean and standard error of at least three separate experiments are given. Statistically significant differences relative to the control (untreated) group are indicated (*, $p<0.05$ and **, $p<0.01$, student's t test). **(B)** p38 inhibition with SB203580 decreases anisomycin (open circles) and EGF (closed circles) induction of FGF-BP mRNA. ME-180 cells were pretreated for 1 hour with the indicated concentration of SB203580, followed by 6 hour treatment with 200 nM anisomycin or 5 ng/ml EGF. The mean and standard deviation of at least two separate experiments are given. Northern blot signal intensities of FGF-BP mRNA were quantitated and normalized to GAPDH.

Fig. 6. Effect of dominant negatives on EGF induction of FGF-BP promoter activity. **(A)** ME-180 cells were transiently co-transfected with the –118/+62 FGF-BP promoter construct along with either empty vector or with dominant negative expression constructs for MEK1, MEK2, ERK1, or ERK2. **(B)** ME-180 cells co-transfected with –118/+62 FGF-BP promoter construct and either empty vector or dominant negative for JNK or p38. Transfected cells were either untreated or treated with 5 ng/ml of EGF for 18 hours. Luciferase activity is expressed as fold induction of EGF-treated over untreated for each construct, and the fold EGF induction with the empty vector is set at 100%. Values represent the mean and standard error from at least three separate experiments, each done in triplicate wells. Statistically significant differences relative to the empty vector control are indicated (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, student's t test).

Figure 1A

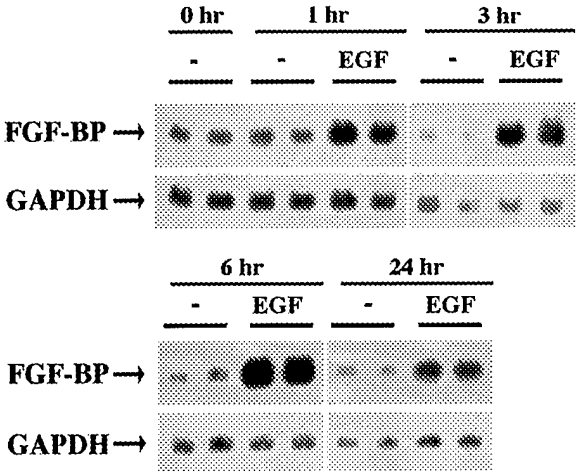

Figure 1B

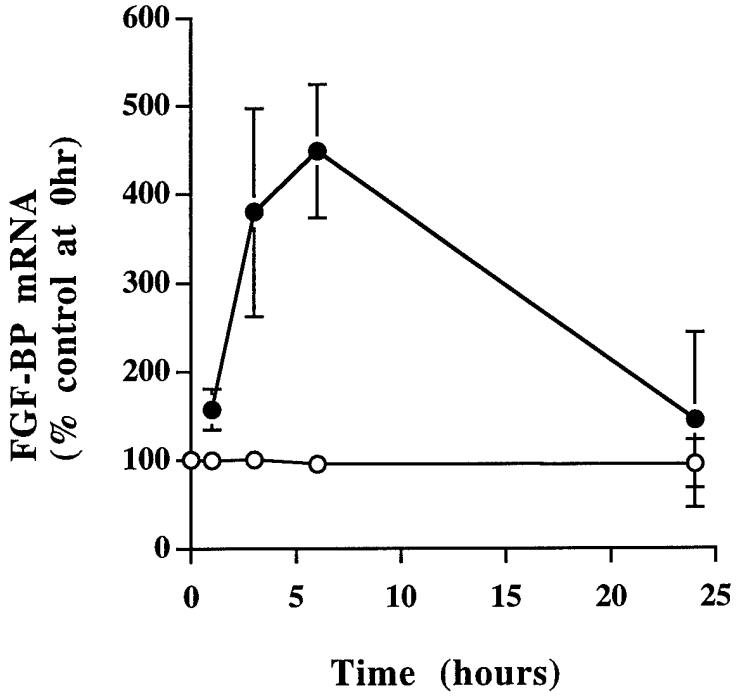
FGF-BP mRNA
(% control at 0hr)
600
500
400
300
200
100
0
0
5
10
15
20
25
Time (hours)

Figure 2

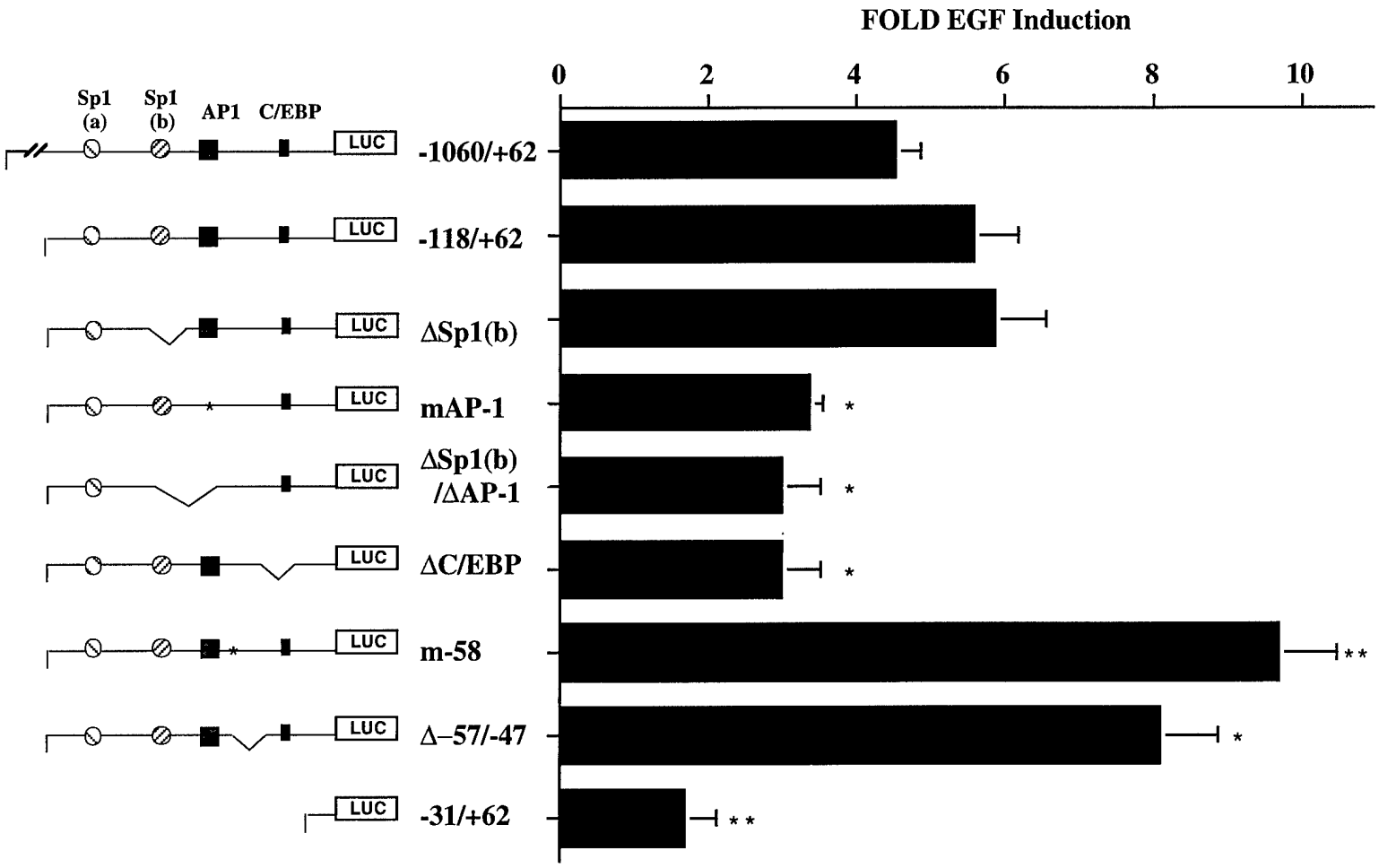
FOLD EGF Induction
0
2
4
6
8
10
Sp1 (a)
Sp1 (b)
AP1
C/EBP
LUC
-1060/+62
-118/+62
ΔSp1(b)
mAP-1
ΔSp1(b) /ΔAP-1
ΔC/EBP
m-58
Δ–57/-47
-31/+62
*
**

Figure 3A

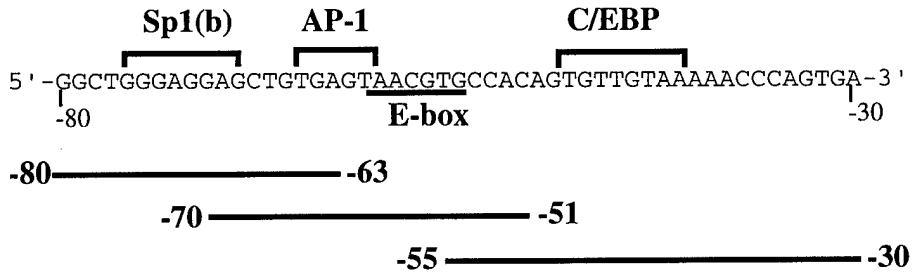
Sp1(b)
AP-1
C/EBP
5'-GGCTGGGAGGAGCTGTGAGTAACGTGCCACAGTGTTGTAAAAACCCAGTGA-3'
-80
E-box
-30
-80
-63
-70
-51
-55
-30

Figure 3B

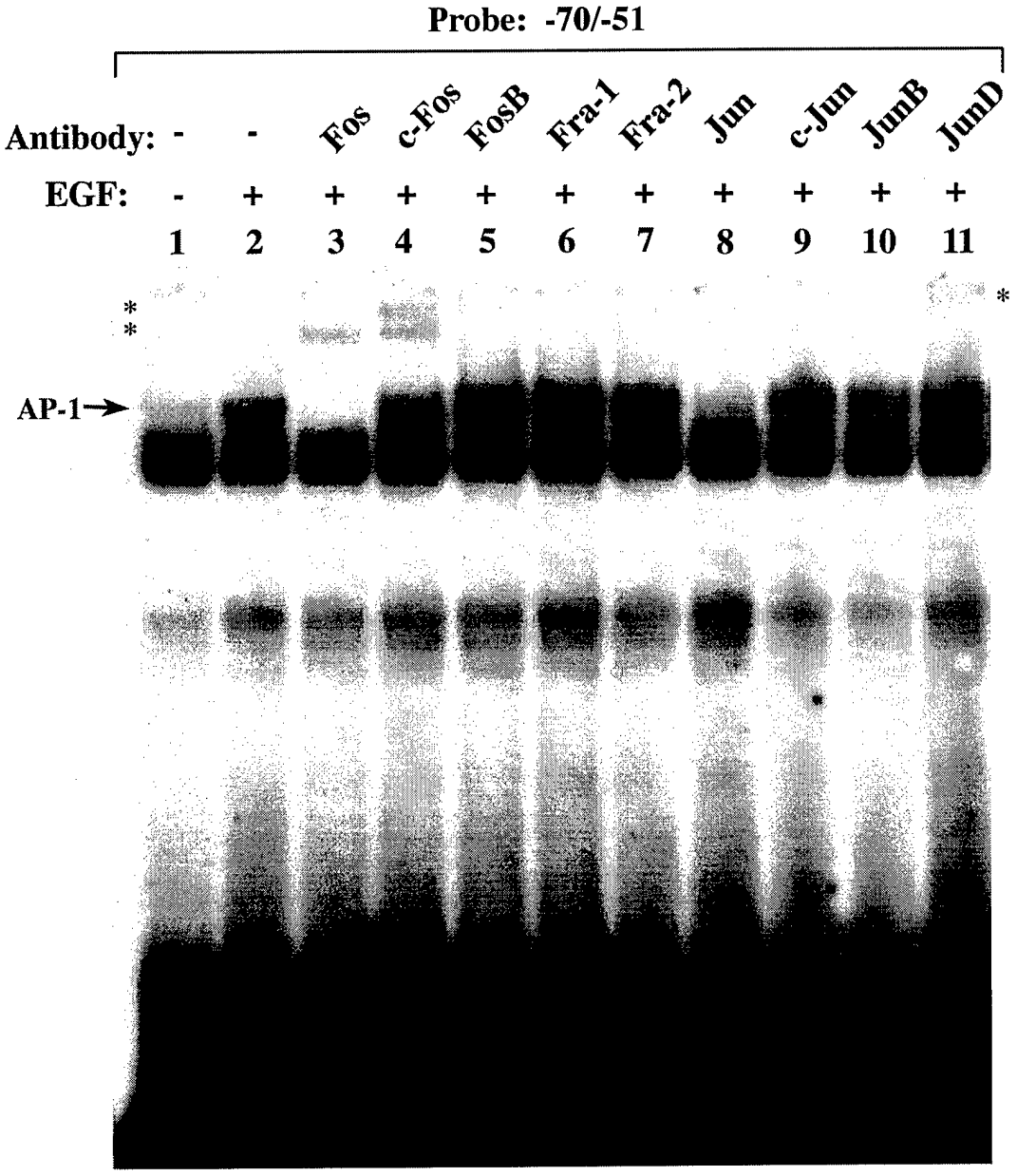
Probe: -70/-51
Antibody: - - Fos c-Fos FosB Fra-1 Fra-2 Jun c-Jun JunB JunD
EGF: - + + + + + + + + + +
1 2 3 4 5 6 7 8 9 10 11
AP-1

Figure 3C

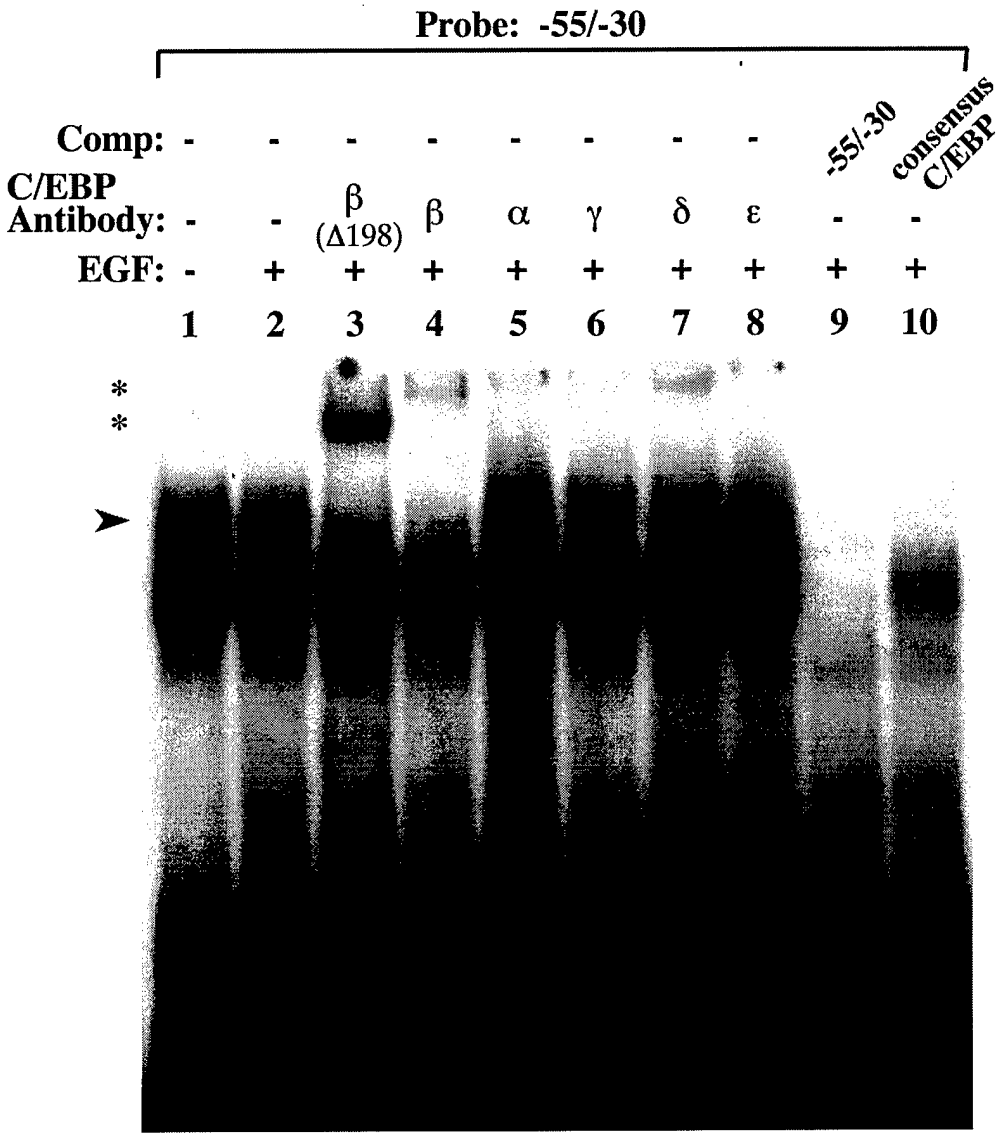
Probe: -55/-30
Comp: - - - - - - - - -55/-30 consensus C/EBP
C/EBP Antibody: - - β (Δ198) β α γ δ ε - -
EGF: - + + + + + + + + +
1 2 3 4 5 6 7 8 9 10
*
*

Figure 3D

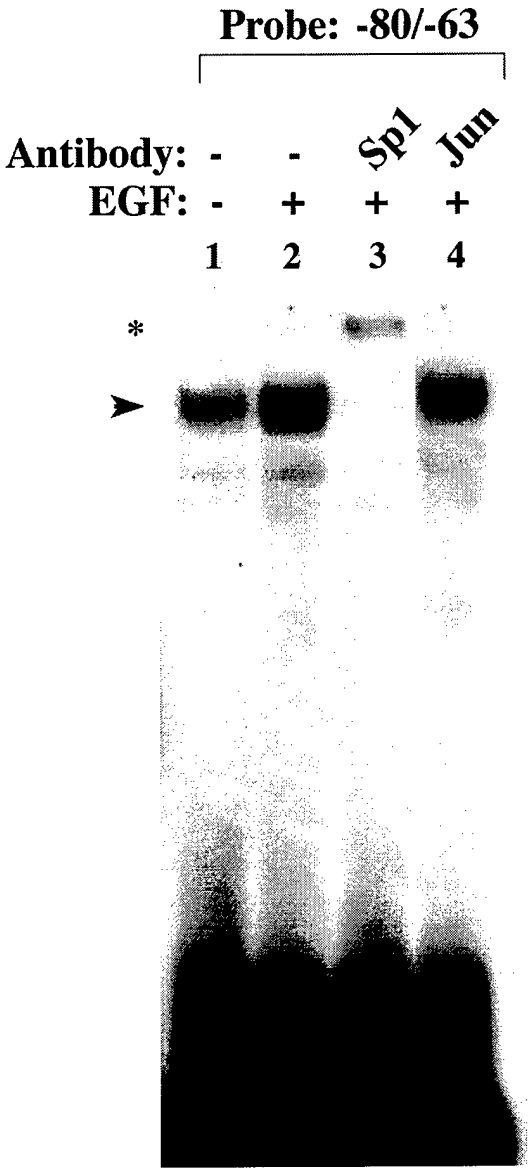

Figure 4

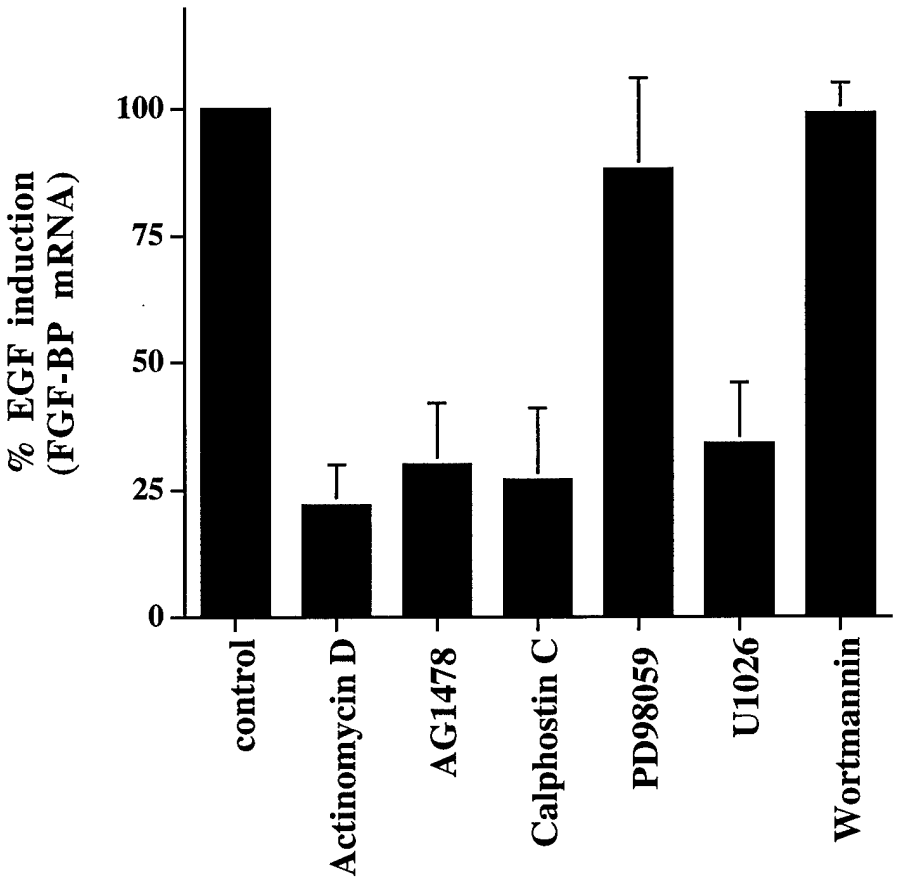
% EGF induction
(FGF-BP mRNA)
100
75
50
25
0
control
Actinomycin D
AG1478
Calphostin C
PD98059
U1026
Wortmannin

Figure 5A

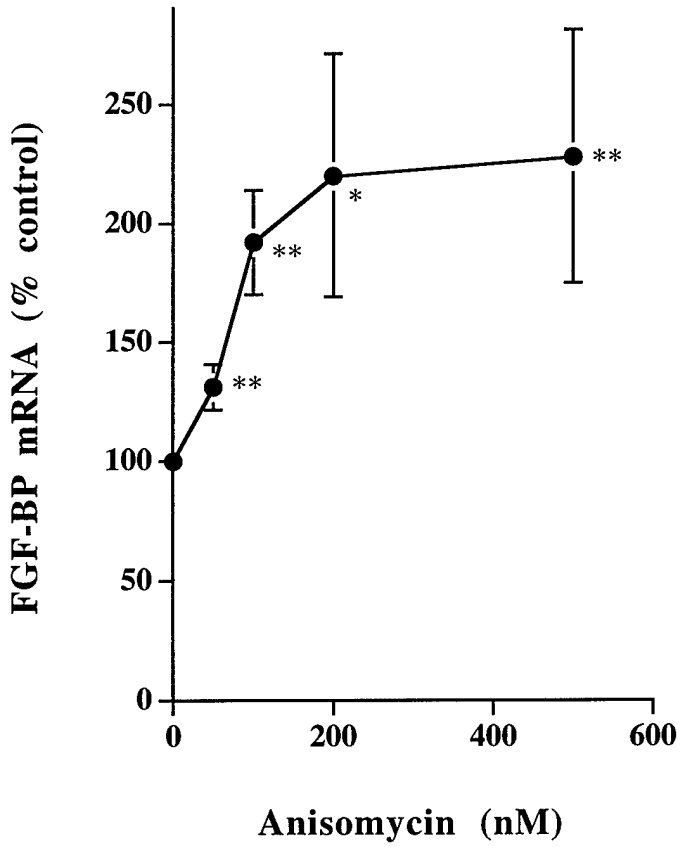
FGF-BP mRNA (% control)
250
200
150
100
50
0
0
200
400
600
Anisomycin (nM)
**
**
*
**

Figure 5B

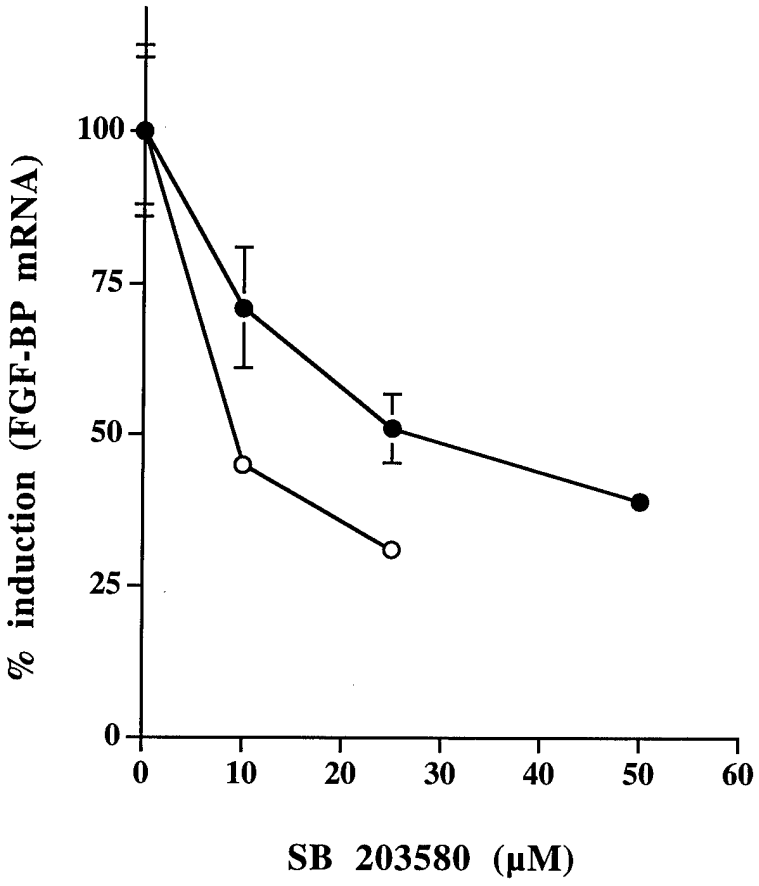
% induction (FGF-BP mRNA)
100
75
50
25
0
0
10
20
30
40
50
60
SB 203580 (μM)

Figure 6A

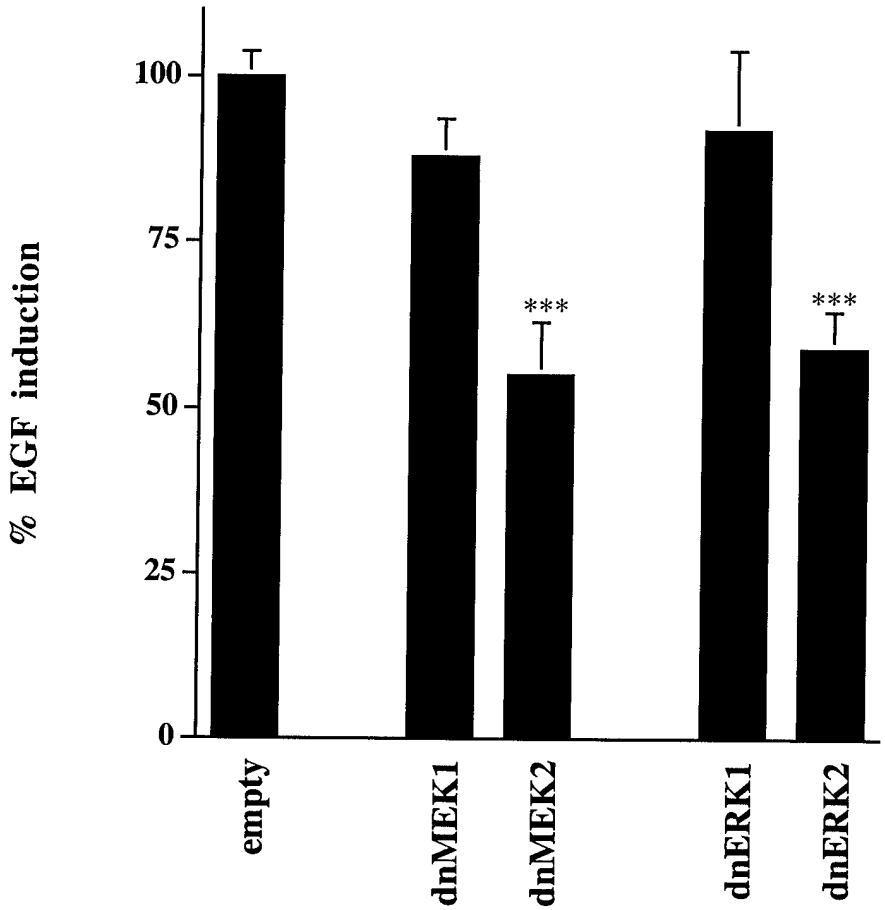
% EGF induction
100
75
50
25
0
***
***
empty
dnMEK1
dnMEK2
dnERK1
dnERK2

Figure 6B