Activator Protein-1 Mediates Induced but not Basal Epidermal Growth Factor Receptor Gene Expression

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Abstract

Background: The epidermal growth factor receptor (EGFR) is expressed at different levels in many cell types and found overexpressed in many cancers. EGFR expression is increased or decreased in response to extracellular stimuli. We examined the effect of increased *c-Jun* expression on EGFR promoter activity.

Materials and Methods: We used DNAse I footprinting analysis to determine the binding of activator protein 1 (AP-1) to the promoter region. We also used cotransfection experiments and western blotting analysis to determine the effect of AP-1 family members on EGFR expression.

Results: AP-1 was able to bind to at least seven sites in the EGFR promoter region. Cotransfection of MCF-7 cells with a *c-Jun* expression vector and the EGFR promoter reporter resulted in a 7-fold increase in promoter activity. *JunB,* but not *c-fos,* also enhanced the EGFR promoter activity. An *A-Fos*–dominant negative shown to inhibit Jun-dependent transactivation was able to prevent *c-Jun* induction of the promoter activity, but only slightly decreased the basal activity of the promoter. Furthermore, the *A-Fos* dominant negative was able to inhibit phorbol ester induction of the EGFR promoter. Examination of EGFR expression of MCF-7 stable cell lines that overexpress *c-Jun* revealed an increase in EGFR expression. Additionally, a cisplatin–resistant cell line, A2780/CP70, which has an increase in AP-1 activity compared with the parental cell line, A2780, was found to have an increase in EGFR level.

Conclusions: These results indicate that AP-1 can act to increase the expression of EGFR and may play a role in upregulation of EGFR in cancer cells.

Introduction

Epidermal growth factor (EGF) has a potent mitogenic activity that can either stimulate or inhibit growth of a large variety of normal and malignant cells in vitro (1,2). The ability of EGF to activate transcription factors in a cell-specific manner and it's signaling activities are mediated through a cell surface receptor, the epidermal growth factor receptor (EGFR) (3,4). The EGFR is a 170 kDa glycoprotein that belongs to the type I growth factor receptor family (5). The cytoplasmic portion of the EGFR is homologous to the viral *erbB* oncogene and the *EGFR* is considered a proto-oncogene (6).

The expression of the *EGFR* gene is varied in tissues, tumors and cultured cell lines (7). Increasing EGFR levels through retroviral–mediated transfer results in transformation of nor-

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mal cells in the presence of ligand (8–10). Conversely, reducing the level of EGFR can lead to reversion to normal cell phenotype (11). EGFR overexpression has been correlated with poor prognosis and failure to respond to hormonal treatment in breast cancer and in malignant tissue in general (12–14). The EGFR is considered a marker for cell transformation (15,16), making it an attractive target for clinical intervention.

The regulatory region of the *EGFR* gene has been isolated and characterized (17,18). It lacks the classical TATA or CAAT boxes, but contains multiple transcriptional initiation sites and five GC boxes (17,19,20). The EGFR promoter contains binding sites for transcription factors such as Sp1 (19,21), AP2 (22), p53 (23–25), WT1 (26), and GCF (27–29). Recent studies show that the promyelocytic leukemia (PML) protein, a nuclear phosphoprotein that functions as a transcriptional regulator, interacts with Sp1 and inhibits its transactivation of the EGFR promoter (30–32). The *PML* gene is located at the breakpoint of the t(15,17) translocation in acute promyelocytic leukemia (33,34).

The activator protein-1 (AP-1) transcription factors are immediate, early-response genes involved in a variety of regulatory processes including cellular response to growth factor stimuli (35). AP-1 is a dimer formed by one protein of the *jun* family (*c-Jun, JunB, JunD*) (36–39) and one member of the *fos* family (*c-fos, FosB, Fra-1* and *Fra-2*) (40–43). AP-1 interacts with a nucleotide sequence motif known as the phorbol 12-O tetradecanoylphorbol 13 acetate (TPA) response element (TRE) (TGAC/GTCA) located in the regulatory region of responsive genes, many of which are involved in cell growth, differentiation and transformation. AP-1 can also bind to the cAMP response element (CRE) with a lower affinity (44,45). Jun proteins can homodimerize with themselves or heterodimerize with Fos proteins. The regions of *Jun* that are necessary for transcriptional activation include the DNA-binding domain, the leucine zipper and the transcriptional activation domain.

AP1 transcription factor activity has been shown to be lower in breast cancer cell lines than normal mammary epithelial cells (46). Breast cancer cell lines can possess as few as 2000 EGFR/cell and as many as two million (14). The variability of EGFR levels in breast cancer cell lines prompted us to examine whether AP1 could regulate EGFR expression. We showed previously that AP2 could bind to the EGFR promoter and partially mediate phorbol ester induction of promoter activity (22). In this study, we determined whether AP1 could bind to the EGFR promoter and examined the effect of *c-Jun* overexpression on EGFR promoter activity. We also determined the effect of a dominant negative to AP1 that abolishes DNA binding and inhibits oncogenesis. Furthermore, we analyzed the effect of overexpression of *c-Jun* on EGFR levels in MCF-7 cells. The results from this study indicate that AP1 is involved in regulating EGFR levels and participates in the phorbol ester enhancement of EGFR gene expression.

Materials and Methods

Cell Lines and Culture Conditions

NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) media supplemented with 10% (v/v) calf serum, 2 mM glutamine and penicillin/streptomycin (Life Technologies, Inc., Gaithersburg, MD). The human breast cancer cell line MCF-7 was grown in RPMI-1640 media supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine and penicillin/streptomycin. The *c-Jun*–overexpressing cell lines, 2-16, 2-31, 2-33 and the control neomycin–resistant cell lines, 7-1 and 7-2 were maintained in improved minimal Eagle's medium (IMEM) zinc optional media supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, penicillin/streptomycin and 500 μg/ml G418. Human epidermoid carcinoma KB cells and human WI-38 cells were grown in DMEM media supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine and penicillin/streptomycin. The human ovarian cell line A2780 and the cisplatin–resistant cell line A2780/CP70 were maintained in RPMI-1640 media supplemented with 2 mM glutamine, 0.2 units/ml insulin and penicillin/streptomycin.

Luciferase Assays

NIH3T3, MCF-7 and KB cells grown in triplicate in 35 mm diameter plate were transfected with the appropriate expression vector by lipofectamine (Life Technologies, Gaithersburg, MD). Luciferase reporter constructs containing the EGFR promoter were prepared by ligation

of the HinD III promoter fragments from EGFR-CAT constructs into pGL3-Basic (Promega, Madison, WI). The EGFR promoter reporter plasmids (0.1 μg) were cotransfected with the indicated amount of expression vectors and with the control vectors. DNA concentration was kept constant by addition of herring sperm DNA. Cells were harvested 24 h after transfection and cell extracts prepared according to the protocol from Analytical Luminescence Laboratory, San Diego, CA. All luciferase activities were normalized for protein concentration and transfection efficiency using RSV-β-galactosidase. All experiments were performed in triplicate or more.

DNase I Footprinting

DNase I footprinting was performed according to Dynan et al. (47). The EGFR gene promoter fragment (−1109 to −16) was labeled at the HindIII site and a 718 base pair (−734 to −16) fragment and a 375 base pair (−1109 to −734) fragment isolated after cutting with Pst I. Purified AP1, AP2, TFIID and Sp1 were purchased from Promega Corporation.

Western Blots

Whole cell extracts were prepared from cells grown until 80–90% confluent in the appropriate medium. Cells were washed three times with phosphate buffered saline without calcium and magnesium. Cells were harvested and resuspended in extraction buffer containing 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) 7.9, 0.2% NP40, 10% glycerol, 0.1 mM EDTA, 0.4 M NaCl, 1 mM DTT and protease inhibitors. Proteins were extracted while rotating at 4°C for 30 min. After extraction, samples were centrifuged at 14,000 g for 15 min. Supernatants were collected and stored at −80°C. 50 μg of protein from whole cell extracts were resolved on a 4–12% SDS-polyacrylamide gel, transferred onto PVDF membrane (Novex, Carlsbad, CA), followed by immunoblotting with the required antibodies, using the ECL detection system according to the manufacturer instructions (Amersham, Pharmacia Biotech, Uppsala, Sweden). PVDF membranes were stripped and probed with actin antibodies. Antibodies to EGFR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and actin antibodies were purchased from Boehringer-Mannheim (Mannheim, Germany).

Results

We showed previously that EGFR expression was enhanced by phorbol esters, in part, through increased binding of AP2, a member of the helix-loop-helix family of transcription factors (22). To extend these analyses, we investigated whether AP1 could also enhance EGFR promoter activity. Examination of the 1.1 kbp EGFR promoter sequence revealed 11 potential AP1 binding sites (TKAGTCA) that differed from the consensus by one or two nucleotides. Most of the potential sites were located in the 5' portion of the promoter, but three were located in the 3' proximal region that was shown to be sufficient for full promoter activity. To determine if AP1 could bind to the promoter region, we performed DNase I footprinting analysis. Using two fragments of the promoter, −1109 to −734 and −734 to −16, we detected at least seven protected regions due to AP1 binding (Fig. 1). Four of the seven footprints corresponded to potential recognition sequences, but three did not. Some of the footprints overlapped binding sites for AP2, Sp1 and TFIID (Fig. 2). The recognition sequences and nucleotide positions of the AP1 footprints are summarized in Table 1.

To examine whether AP1 could influence EGFR promoter activity, we cotransfected MCF-7 cells with a *c-Jun* expression plasmid and the EGFR promoter reporter plasmid, pER1-Luc. The activity of the EGFR reporter construct was increased in a concentration dependent manner to approximately 7-fold by *c-Jun* cotransfection (Fig. 3). As a positive control, an AP1-responsive collagenase promoter construct, AP1-Col-Luc,

Table 1. Summary of EGFR promoter AP1 footprints

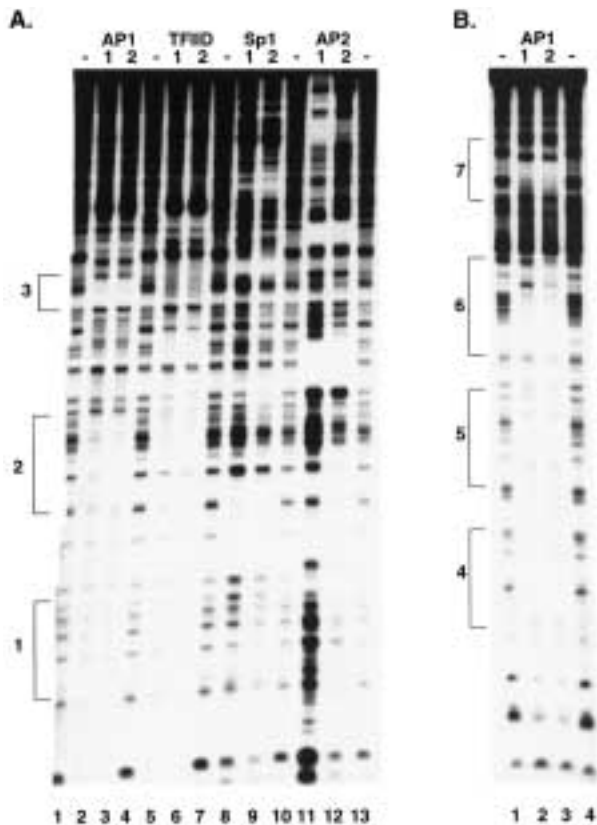| Footprint	Position	Recognition Sequence
1	−54 to −44	CTGACTCC
2	−102 to −92	CCGAGTCC
3	−170 to −160	CGGAGTCC
4	−760 to −745	TCTGATCC
5	−835 to −819	TTAGAGGC
6	−858 to −872	TTGACAAG
7	−1028 to −1010	TTGTGTCA

Fig. 1. AP1 can bind to multiple sites in the EGFR promoter. (A) DNase I footprinting was performed using 1 and 2 footprint units of purified proteins (AP1, TFIID, Sp1 and AP2 as shown above each lane) and the end-labeled EGFR promoter fragment −16 to −734. AP1 footprints are indicated on the left of the figure. (B) DNase I footprinting was performed using 1 and 2 footprint units of AP1 and the EGFR promoter fragment −1109 to −735. AP1 footprints are indicated on the left of the figure.

was used and enhanced 8- to 10-fold by *c-Jun*. As a negative control, the mutant construct, mAP1-Col-Luc, containing a mutation in the TRE was used and not enhanced by *c-Jun*. Similar results were obtained in mouse NIH3T3 cells and human KB cells in addition to the MCF-7 cells. To locate the region(s) in the promoter responsive to *c-Jun* cotransfection, experiments with deletion mutants of the EGFR promoter were performed. These experiments revealed that the promoter deletions down to nucleotide −105 were responsive to *c-Jun* (Fig. 4). The deletion constructs containing the most AP1 sites were more responsive than the ones with fewer sites. To further investigate the *c-Jun* enhancement of EGFR promoter activity, we cotransfected MCF-7 cells with pER1-Luc and

other *Jun* and *Fos* constructs. A c-Jun deletion mutant lacking the transactivation domain, *c-JunΔ1-286,* failed to enhance EGFR promoter activity (Fig. 5). Another member of the *c-Jun* family, *JunB,* was able to increase EGFR promoter activity an average of 12-fold, but a *c-fos* construct did not successfully enhance the promoter activity.

To further substantiate the role of *c-Jun* in the activation of EGFR promoter activity, we decided to use a dominant negative construct that was based on a unique structural element. The *A-Fos* dominant negative contains an amphipathic acidic extension appended to the *Fos* leucine zipper motif that inhibits *c-Jun* dependent transcription (48). Cotransfection of the *A-Fos* dominant negative and the EGFR promoter reporter resulted in a 30% decrease in promoter activity (Fig. 6). The *A-Fos* dominant negative was significantly more effective at inhibiting the activity of AP1-responsive constructs, TRE2x-Luc and AP1-Col-Luc, 80 and 90%, respectively. This result suggests that basal EGFR promoter activity is only slightly due to an effect of AP1.

To further investigate the role of AP1 in regulating EGFR promoter activity, we treated cells cotransfected with *A-Fos* and the EGFR reporter with phorbol 12-myristate 13-acetate (PMA). PMA increased promoter activity as we had previously reported (Fig. 7). However, *A-Fos* was able to inhibit the PMA induction of the promoter activity. In addition, we cotransfected cells with *c-Jun* and *A-Fos* to see if *A-Fos* could specifically inhibit *c-Jun* activation of EGFR promoter activity. *A-Fos* reduced *c-Jun* activation to 2-fold, compared with 7-fold in the absence of *A-Fos*. A dominant negative to the CAAT/enhancer binding protein (C/EBP), A-C/EBP, was used as a control. A-C/EBP has the same amphipathic acidic extension as *A-Fos* appended to the C/EBP leucine zipper (48). A-C/EBP failed to inhibit *c-jun*-dependent activation of the EGFR promoter. These results indicate that AP1 plays a role in activation of the *EGFR* gene by phorbol esters.

The RNA and protein levels of the *EGFR* gene are varied in many types of cells. To determine if the EGFR level may change in response to AP1 activity, we examined the level of the receptor in a parental cell line, A2780, and *cis*-platin resistant cell line, A2780/CP70, that has higher AP1 activity (49,50). Western blots analysis showed that EGFR level increased in the A2780/CP70 cells, compared
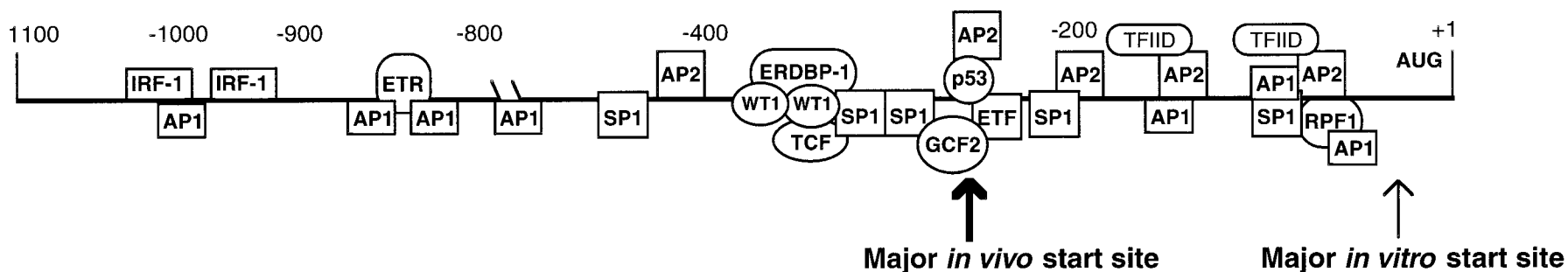
1100   -1000   -900   -800   -400   AP2   ERDBP-1   AP2   p53   -200   TFIID   TFIID   +1

IRF-1   IRF-1   ETR   SP1   WT1   WT1   SP1 SP1   ETF   AP2   AP2   AP1 AP2   AUG

AP1   AP1   AP1   AP1   TCF   GCF2   SP1   AP1   SP1 RPF1

AP1

Major *in vivo* start site   **Major *in vitro* start site**

Fig. 2.   EGFR promoter schematic with transcription factor binding sites. The schematic of the 1.1 kbp EGFR promoter region with currently known protein binding sites. The size of the symbols for each protein does not correspond to size. The positions of the binding sites on the promoter fragment are representative of location, if not exact. References for each protein binding: AP1 (this paper), AP2 (22), ERDBP-1 (56), ETF (21), ETR (57), GCF2 (58), IRF-1 (53), p53 (24), RPF-1 (59), Sp1 (19), TCF (52), TFIID (this paper), WT1 (26).
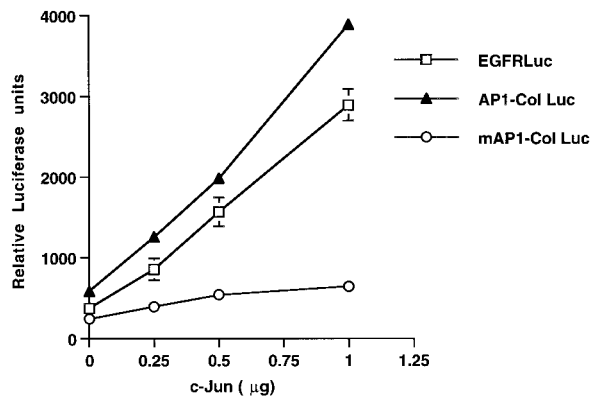
Fig. 3. EGFR promoter activity is increased by *c-Jun* **in cotransfection assays.** MCF-7 cells were transfected with 0.1 μg of reporter construct and increasing amounts of *c-Jun*. Luciferase activity was measured 24 hr post-transfection and is expressed relative to cells transfected with the empty vector. Luciferase activity was corrected for protein concentration and transfection efficiency using a RSV-β-galactosidase reporter.

Fig. 4. Enhancement of EGFR promoter activity by *c-Jun* **is not localized to a single region of the promoter.** MCF-7 cells were transfected with 0.1 μg EGFR promoter deletion constructs and 1 μg of the *c-Jun* expression vector. Luciferase activity was measured 24 hr post-transfection and is expressed relative to cells transfected with the empty vector. Luciferase activity was corrected for protein concentration and transfection efficiency using a RSV-β-galactosidase reporter. The number of AP1 binding sites in each promoter construct is shown above the error bars.

with the A2780 (Fig. 8). To more thoroughly determine if increased AP1 activity could influence EGFR levels, we examined the level of the EGFR in MCF-7 stable cell lines that overexpressed *c-Jun* (51). As shown in Figure 9, cell lines that stably expressed *c-Jun* have an increase in EGFR level. This is in agreement with the transient cotransfection experiments that showed increase EGFR promoter activity by *c-Jun*.

Discussion

We report here that AP1 plays a role in the regulation of EGFR gene expression. This finding is based on the observation that AP1 binds to at least seven sites in the EGFR promoter region. Also, *c-Jun* increases EGFR promoter activity in a concentration–dependent manner. Furthermore, an *A-Fos*–dominant negative that abolishes AP1 DNA-binding inhibits both *c-Jun* and phorbol ester activation of the EGFR. Finally, cells made to stably overexpress *c-Jun* have increased EGFR levels. The activation of EGFR promoter activity by *c-Jun* requires the transactivation domain. The effects of *c-Jun* are spread throughout the promoter region where AP1 binding sites are located. The AP1 binding sites include sites that overlap with binding sites for Sp1, AP2 and TFIID (Fig. 2). This indicates that the interplay of transcription fac-
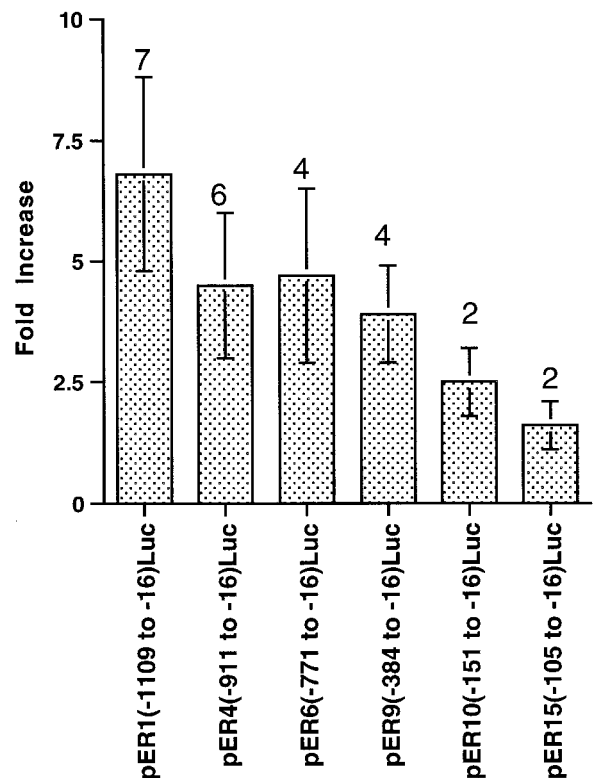
tors could be extremely important for *EGFR* gene expression. Over–expression of *c-Jun* leads to increased levels of EGFR. It is also significant that an ovarian cancer cell line selected for cisplatin resistance has increased EGFR levels, as well as increased AP1 activity. Since cells with higher levels of EGFR are more responsive to the mitogenic action of growth factors, the increase in EGFRs may provide an additional growth advantage for cancer cells that are resistant to chemotherapeutic agents.

The EGFR promoter is responsive to many agents and regulation has to be properly maintained to insure proper cell growth. Transcriptional activators such as Sp1, AP2, p53, TCF and IRF-1 have been shown to bind to the promoter region and enhance transcription (19,21,22,24,52,53). The effect of Sp1 can
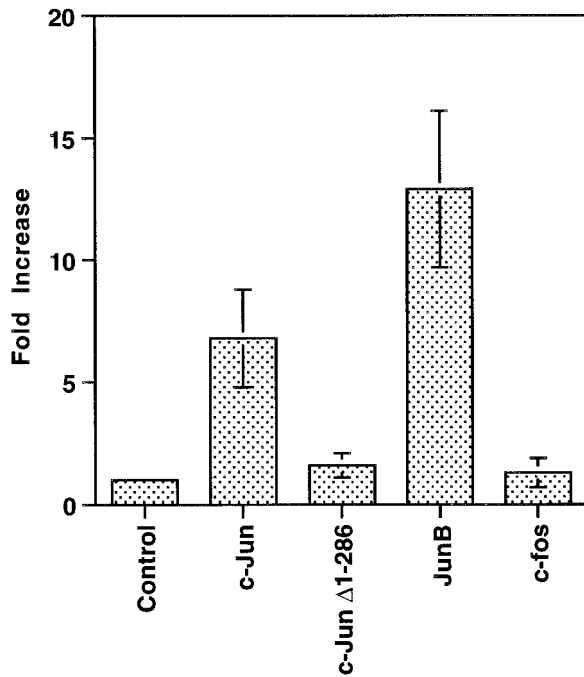
Fig. 5. *c-Jun* and *JunB,* but not *c-fos,* in EGFR promoter activity. MCF-7 cells were transfected with 0.1 μg EGFR pER1-luc and 1 μg of the indicated expression vectors. Luciferase activity was measured 24 hr post-transfection and is expressed relative to cells transfected with the control (empty vector). Luciferase activity was corrected for protein concentration and transfection efficiency using a RSV-β-galactosidase reporter.

Fig. 6. An *A-Fos* dominant-negative only slightly decreases EGFR promoter basal activity. MCF-7 cells were transfected with 0.1 μg pER1-Luc, AP1-Col-Luc or TRE2x-luc and 1 μg of the *A-Fos* expression vector. Luciferase activity was measured 24 hr post-transfection and is expressed relative to cells transfected with the empty vector. Luciferase activity was measured 24 hr post-transfection and is expressed as fold increase relative to cells transfected with the empty vector. Luciferase activity was corrected for protein concentration and transfection efficiency using a RSV-β-galactosidase reporter. The reported data is from three experiments performed in triplicates.

be inhibited by interactions with PML. The repressive effect of PML on EGFR gene transcription was mapped to the region between −150 and −16. The primary effect of *c-Jun* appears to involve two areas of activity, −150 to −44 and −1028 to −745. The −1028 to −745 region contains four AP1 binding sites. Indeed, placing a region of the EGFR promoter containing nucleotides −1109 to −569 upstream of a thymidine kinase (tk) minimal promoter makes the tk promoter responsive to *c-Jun* activation (data not shown). The −170 to −44 region of the promoter contains three AP1 binding sites and 60% of the induced activity by *c-Jun*. This region of the promoter is very important for in vitro and in vivo activities. Inhibition of *c-Jun* activation by the *A-Fos*–dominant negative allows for two conclusions: 1.) The effect of *c-Jun* is primarily an induction of the promoter activity. *A-Fos* was only able to slightly decrease the basal activity. 2.) The enhancement of EGFR expression by phorbol esters is, at least,
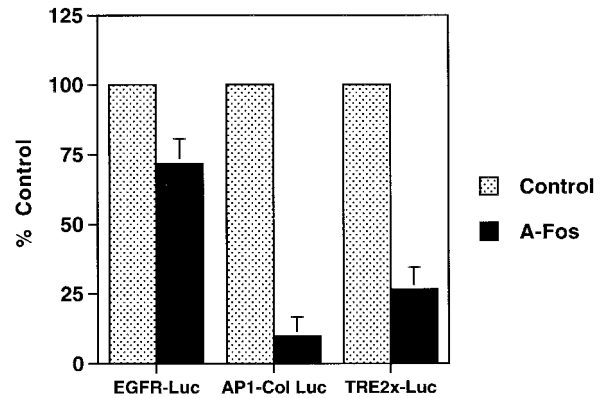
in part, due to increased AP1 activity. The *A-Fos*–dominant negative was able to almost completely inhibit the phorbol ester induction of EGFR promoter activity. Previously, we showed that AP2 was able to mediate phorbol ester induction of EGFR gene expression. In that report, we were able to partially purify a protein whose presence in the extract correlated with increased binding to the promoter upon PMA treatment. The DNase I footprint of this factor on the EGFR promoter region matched AP2, except for two protected regions. These sites matched AP1 binding sites (data not shown). In vitro transcription assays were used to show that addition of AP2 leads to an increase in EGFR promoter transcription. Taken together, the results from the previous report and the present study suggest that both AP1 and AP2 play roles in mediating the phorbol ester enhancement of EGFR gene expression.

The role of other members of the AP1 family in regulating EGFR activity remains to be examined. We showed that *JunB* was able to increase EGFR promoter activity in the co-transfection assays. Conversely, *c-fos* addition was not able to enhance EGFR promoter activ-
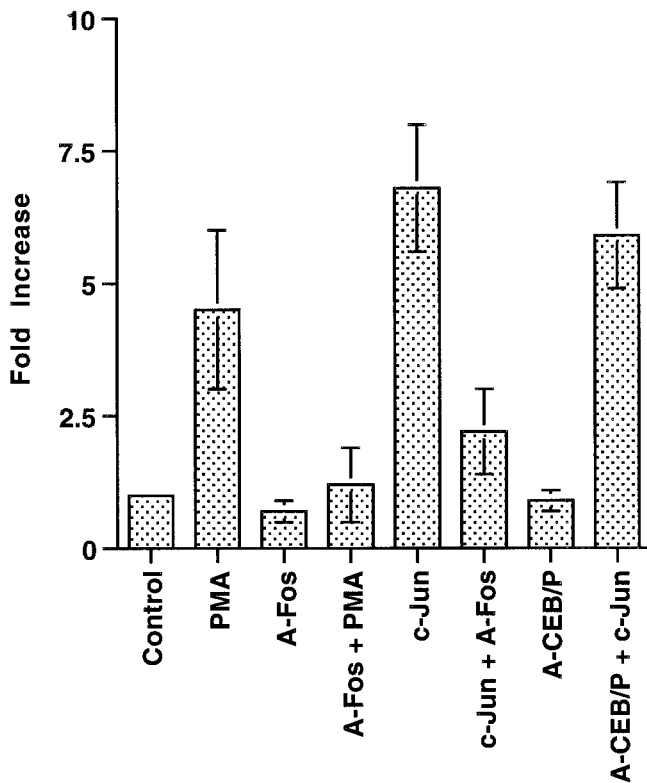
Fig. 7. An *A-Fos* dominant negative inhibits PMA and *c-Jun* activation of the EGFR promoter. MCF-7 cells were transfected with 0.1 μg pER1-Luc and treated for 19 hr with PMA (100 nM) before harvesting. In cotransfection experiments, *A-Fos,* A-CEB/P and/or *c-Jun* expression vectors (1.0 μg) were also transfected. Luciferase activity was measured 24 hr post-transfection and is expressed relative to cells transfected with the empty vector. Luciferase activity was corrected for protein concentration and transfection efficiency using a RSV-β-galactosidase reporter.

ity. These findings indicate that endogenous levels of specific factors play an important role in determining EGFR promoter activity in transient transfection assays. Indeed, we find very low levels of *c-Jun* and *JunB* in our MCF-7 cells

by western blotting (data not shown). These findings also indicate that transcription factors involved in activation of specific gene expression may not have a major role in basal expression. The identification of the additional tran-
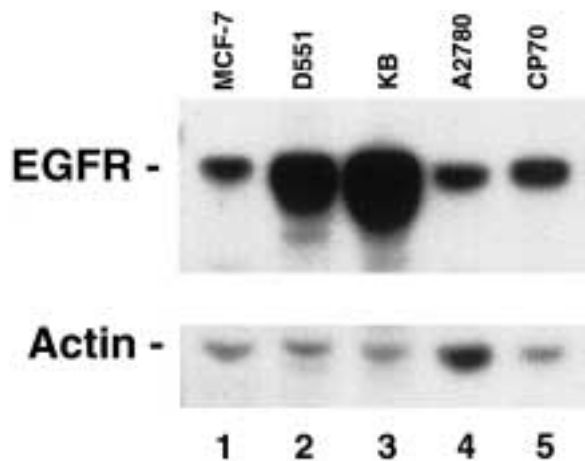

Fig. 8. EGFR is increased in *cis*-platin resistant cells that have increased AP1 activity. Protein extracts from MCF-7, D551, KB, A2780 and A2780/CP70 cells were as described in "Materials and Methods." 50 μg of each extract was fractionated on polyacrylamide gels and subjected to Western blot analysis using EGFR and actin antibodies.
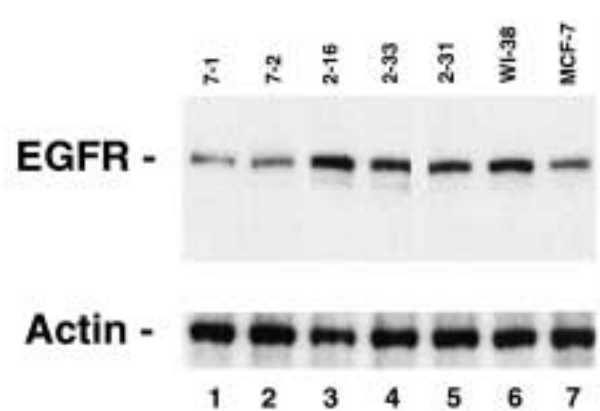

Fig. 9. Endogenous EGFR is increased in MCF-7 cells over-expressing *c-Jun*. Stable transfectants of MCF-7 expressing neomycin resistance (7-1 and 7-2) or *c-jun* (2-16, 2-33 and 2-31) were grown in selective media and protein extracts prepared. Extracts from MCF-7 and WI-38 cells were prepared in an identical fashion. 50 μg of each extract was fractionated on polyacrylamide gels and subjected to western blotting using EGFR and actin antibodies.

scription factors effecting changes in EGFR expression will aid in resolving the increased expression found in many cancers.

It is clear that EGFR gene regulation is very complex and involves the interaction of many different transcription factors with the promoter region (22,54). The *EGFR* gene responds to growth factors, tumor–promoting agents, cAMP, steroids, and retinoids (54). These agents induce the expression of transcription factors such as AP1 (35). The AP1 complex can either positively or negatively regulate transcription of a target gene, depending on the composition of the heterodimers. Some breast cancer cells have been shown to have lower AP1 activity than normal mammary epithelial cells (46). AP1 has also been implicated in the regulation of apoptosis by either inducing apoptosis or inhibiting anti-apoptotic events (55). The role of AP1 in regulating EGFR expression adds support for a role in regulating cell proliferation.

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