The epidermal growth factor receptor (EGFR) is a cell surface glycoprotein that mediates the actions of EGF (1, 2). The binding of EGF to the receptor results in receptor endocytosis. Ultimately the receptor is transferred to lysosomes where it is degraded (3-6). This process accounts for the loss of cell surface receptors. Ligand binding also activates the receptors' tyrosine kinase activity, which phosphorylates various cellular proteins and the receptor itself (7, 8). Treatment of cells with EGF results in increased EGFR mRNA and EGFR synthesis (9-11). EGFR was shown to increase the half-life of EGFR mRNA (12). In addition, agents such as estrogen (13), retinoic acid (14), and thyroid hormone (15) have been shown to influence the number of EGFR binding sites. EGFR mRNA levels increase in regenerating rat liver (16) and also by treatment of various cell lines with PMA (9). The mechanisms by which the various agents modulate EGFR gene expression are most likely quite diverse and probably will include both transcriptional and post-transcriptional regulation.

The regulation of the EGFR is of considerable importance. The receptor is the cellular homolog of the avian erythroblastosis virus erbB oncogene product (17, 18). Overexpression of the EGFR has been detected in some tumors and cell lines derived from tumors (19-21). Overexpression is often associated with gene amplification with or without gene rearrangement (22-27). Furthermore, retroviral mediated overexpression of the EGFR in NIH 3T3 cells results in cell transformation and EGFR-induced malignancy and tumorigenicity (28, 29). Thus, regulation of the EGFR appears to play a vital role in cellular growth and transformation.

To understand how the EGFR gene is regulated, we have located and isolated the EGFR gene promoter (30). The promoter is GC-rich, contains multiple transcriptional start sites, and lacks both CAAT and TATA boxes (30). The promoter binds the transcription factor Sp1 at multiple sites and has binding sites for additional nuclear proteins (31). One of those additional DNA binding proteins, termed ETF (EGFR transcription factor), has been obtained in a highly purified form. Both Sp1 and ETF promote EGFR transcription in cell-free extracts (32, 33). The promoter contains an S1 nuclease-sensitive region that binds ERDP-1, EGF responsive DNA binding protein (34, 35). Wild type p53 can also activate the EGFR promoter (36). Two repressor proteins have been identified that bind to the EGFR promoter, ETR (EGFR transcriptional repressor) and GCF (GC factor). ETR is a 128-kDa protein that binds to a site located between -889 and -870 (37). A GCF cDNA was cloned, and GCF was shown to repress EGFR promoter activity by binding to three sites in the promoter (38).

The regulation of the EGFR gene by various agents is likely to be mediated by trans-acting factors that bind to the promoter region of the gene. The T3 receptor has been shown to suppress Sp1-dependent transcription from the EGFR promoter via overlapping binding sites (39). Transcriptional responses to phorbol esters, cAMP, and retinoic acid have also been reported for the EGFR promoter (40). Phorbol esters and cAMP responses are reported to be mediated via the transcription factors AP1 and AP2 (41-44). The EGFR gene promoter contains sequences similar to the binding sites for AP1 and AP2. To investigate whether the response of the EGFR gene to PMA treatment was transcriptional and perhaps mediated by AP2, I have performed transient transfection assays, nuclear run-off experiments, in vitro transcription analyses, and binding studies. The results indicate that PMA increases EGF receptor gene expression and may mediate the PMA response of this gene.

Mediated by Activator Protein 2

The response of the epidermal growth factor (EGF) receptor gene to phorbol 12-myristate 13-acetate (PMA) was analyzed using nuclei and nuclear extracts prepared from PMA-treated KB cells. Transient transfection assays and nuclear run-off experiments showed that PMA increased EGF receptor gene transcription. Cell-free transcription with promoter mutants revealed that the region of the promoter containing nucleotides -150 to -16 was sufficient for PMA inducibility. A promoter fragment containing nucleotides -167 to -105 showed increased binding of a factor present in extracts prepared from PMA-treated cells. When this factor was partially purified by column chromatography, it showed specific PMA-dependent binding to an EGF receptor promoter fragment. This binding was competed by an SV40 fragment containing binding sites for Sp1, AP1, and AP2. Purified AP2 was used in DNase I footprinting experiments to show that this factor can bind to the EGF receptor promoter. Oligonucleotides corresponding to the AP2 binding sites found in the EGF receptor promoter showed the ability to bind AP2 and compete for the binding of a factor induced by PMA treatment. The addition of AP2 to nuclear extract resulted in increased transcription from the EGF receptor promoter. These results demonstrate that AP2 can activate EGF receptor gene expression and may mediate the PMA response of this gene.
transcription and that the transcription may be mediated through AP2.

**MATERIALS AND METHODS**

Cell Lines—KB and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Cells were treated with 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma) in Me2SO (Aldrich) for 24 h in the above medium.

Nuclear Run-off Transcription—Nuclei were isolated from KB cells treated with 100 nM PMA or Me2SO (control cells) for 1.5 and 3 h by lysing the cells in 0.25% Nonidet P-40 as described (45). Transcription took place under standard reaction conditions in the presence of 200 mM KCl and [32P] GTP (45, 46). RNA samples of equal radioactivity were hybridized to immobilized DNA for 3–4 days at 42°C in 50% (v/v) formamide. Filters were washed in 30 mM NaCl, 3 mM sodium citrate, pH 7.0, 0.1% SDS at 50°C for 1 h, treated with RNase A and subjected to autoradiography (45).

In Vitro Transcription—Crude nuclear extracts were prepared from control and PMA-treated cells by modifications of Wilderman et al. (47) and Dignam et al. (48) as described previously (32). RNAs were synthesized by promoter-CAT constructs and analyzed by hybridizing a CAT-specific primer to the RNA and reverse transcribing (32). The primer-extended products were subjected to electrophoresis on a 5% polyacrylamide, 7M urea gel. After electrophoresis, the gels were transferred to Whatman 3 MM paper and exposed to Kodak XAR film at −70°C with an intensifying screen. Films were developed and scanned by a laser densitometer for quantitation.

Gel Mobility Shift Assays—Mobility shift assays were performed as described previously (31). Briefly, end-labeled DNA fragments or oligonucleotides were incubated with crude nuclear extract, fractionated extract, or purified AP2 at room temperature (23°C) for 15 min in the presence of 20 mM Hepes, pH 7.9, 72 mM KCl, 10 mM EDTA, 12.5 mM glycerol, 100 mM poly-dI-dC, 250 μg/ml bovine serum albumin, and 5% glycerol. Samples (20 μl) were loaded onto a 5% polyacrylamide gel, and electrophoresis was performed at 150 V for 2 h using 0.5 TBE (45, 46) as running buffer.

DNase I Footprinting—DNase I footprinting was performed according to Dynan et al. (49). For the SV40 promoter, pSV2CAT was labeled at the HindIII site and then cut with PvuII to obtain the 346-bp probe fragment. The EGFR gene promoter fragment (−771 to −16) was labeled at the HindIII site, and a 553-base pair (−569 to −16) fragment was isolated after cutting with TaqI. Purified AP2 and Sp1 were obtained from Promega Corp.

Transfection—KB cells or HeLa cells were seeded at 5 × 10^5 cells/100 mm dish 24 h prior to the addition of CaPO4-DNA precipitate (50). One microliter of the precipitate containing 10 μg of pERCAT plasmid DNA was added to the medium cells were incubated for 4 h at 37°C and then exposed to glycerol for 30 s. Cells were incubated overnight at 37°C, and new medium containing PMA or Me2SO was added the following day. Twenty-four hours after PMA addition, cells were harvested, extracts were prepared, and CAT activity was assayed as previously reported (51).

**RESULTS**

EGFR mRNA levels and synthesis of the receptor itself are increased by EGFR and phorbol ester tumor promoters (9–11). To determine if the PMA was acting at the level of transcription, nuclear run-off assays were performed. Samples of radiolabeled RNA isolated from the nuclei of KB cells treated with or without 100 nM PMA were hybridized to EGFR DNA to see if there were any changes in the transcription of this gene (Fig. 1). The RNA hybridized at increased levels of 3.5- and 4.3-fold to the EGFR DNA (pE7) after PMA treatment of cells for 1.5 and 3.0 h, respectively. No significant increase in hybridization of labeled RNA with ribosomal DNA or β-actin DNA was detected (data not shown). No hybridization with pBR322 occurred (Fig. 1). These results indicate that PMA is acting to influence the level of EGFR gene transcription.

To localize the region of the promoter that may be involved in the enhanced transcription, deletion mutants of the promoter fused to the CAT gene were used for transient transfection assays (Table I) and transcription assays in vitro with nuclear extracts from control and PMA-treated KB cells (Fig. 2). Transient transfection assays show that EGFR promoter CAT constructs deleted to −105 respond to PMA treatment of KB cells. As indicated in Fig. 2, a band corresponding to the transcription product from the major in vitro start site was detected and shown to be PMA-inducible (Fig. 2A). Deletion mutants of the promoter (−771 to −16, −384 to −16, −150 to −16, and −105 to −16) all showed an increased response to PMA. The largest deletion (−105 to −16) showed the smallest response, 1.8-fold (Table I). As a positive control for these experiments, pSV2CAT was used in these reactions. The SV40 promoter contains elements that are stimulated by phorbol esters (33, 34). When pSV2CAT is used in these experiments, we can also detect an increase of 3- to 4-fold in response to PMA (Fig. 2A). As a negative control, a CAT construct containing an SV40 minimal promoter (NcoI-HindIII), in which PMA-responsive elements and GC boxes have been removed, was used. This construct exhibited similar amounts of expression in control and PMA-treated extracts (Fig. 2B). Thus, the EGFR and SV40 promoters respond to PMA treatment in KB cells. Deletion mutants of the EGFR promoter down to −105 show similar amounts of increased expression and therefore probably contain a DNA element through which the action of PMA is mediated.

Transcriptional enhancement of SV40 by PMA is mediated by trans-acting factors that bind to the promoter region (33, 34). To investigate whether a similar type of factor(s) mediated the EGFR promoter response, DNA binding studies were performed. Since the deletion mutants indicated that the −150 to −16 region contained PMA-responsive elements and the −105 to −16 region had lost part of the response, an Aval fragment containing nucleotides −167 to −105 was used in gel retardation assays. When crude extracts from control and PMA-treated cells were used with this fragment, two retarded bands were observed (Fig. 3A, lanes 2 and 3). The lower retarded band (B2) was substantially increased when the PMA

TABLE I

<table>
<thead>
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<th>Plasmid Relative increase</th>
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<tr>
<td>pERCAT 6 (−771 to −16)</td>
<td>4.4 ± 0.3</td>
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<tr>
<td>pERCAT 9 (−384 to −16)</td>
<td>3.8 ± 0.5</td>
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<tr>
<td>pERCAT 10 (−150 to −16)</td>
<td>3.2 ± 0.3</td>
<td></td>
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<tr>
<td>pERCAT 15 (−105 to −16)</td>
<td>1.8 ± 0.2</td>
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* Results are expressed ± the standard deviation compared with cells treated with Me2SO. All results are obtained from four independent experiments.
To partially purify and characterize this factor, the crude extract was subjected to column chromatography on heparin-agarose followed by DEAE-Sepharose. Fractions from each column were tested for the presence of this trans-acting factor by gel retardation (Fig. 3). This factor appears primarily in the 0.4 M KCl heparin-agarose fraction (Fig. 3A) and the 0.25 M KCl DEAE-Sepharose fraction (Fig. 3B). The difference in the amount of this factor binding to the EGF receptor promoter fragment in control versus PMA-treated extracts is somewhat reduced during the purification. However, the difference remains significant after both columns.

To determine the DNA sequences in the promoter to which this factor may be binding, DNase I footprinting was performed using the enriched DEAE-Sepharose fraction from PMA-treated cells. DNase I footprints were observed at six sites within the EGFR fragment (Fig. 4). These sites contain recognition sequences similar to the AP2 binding site. The DEAE-Sepharose fraction also protected the AP2 binding region of the SV40 promoter from DNase I digestion (data not shown). When purified AP2 was used in DNase I footprinting experiments with the EGFR promoter fragment, strong footprints were observed that corresponded to sites 2, 3, 5, and 6, and a weak AP2 footprint was observed at site 4 (Fig. 5). Some of these footprints also overlap DNase I footprints found when Sp1 was used with the EGFR promoter fragment (data not shown). To further examine whether AP2 is involved in the increased binding of nuclear protein to the EGFR promoter region after PMA treatment, gel mobility shift assays were performed. Oligonucleotides corresponding to sites 2 and 3 were labeled and used with the fractionated extract from heparin agarose. Both oligonucleotides showed increased binding using the PMA-induced sample (Fig. 6). Also, both oligonucleotides bound purified AP2 (data not shown). The EGF promoter fragment containing nucleotides -167 to -105 was also end-labeled and used in gel mobility shift assays with competition. When excess amounts of this fragment in an unlabeled form were used to compete against the labeled fragment in a gel retardation assay, bands B1 and B2 were diminished in intensity (Fig. 7A, 7B).
These bands were also competed with the SV40 enhancer fragment (Fig. 7A, lane 5). When excess amounts oligonucleotides containing AP2 binding sites in the EGFR promoter or consensus AP2 oligonucleotide were used as competitors, retarded band B1 remained the same intensity, while B2 was reduced in intensity (Fig. 7B, lanes 4–6). Neither retarded band was diminished in intensity when nonspecific (φX174) DNA was used as a competitor (Fig. 7B, lane 7).

To examine the effect of AP2 on EGFR promoter activity, in vitro transcription experiments were performed using HeLa nuclear extract. Upon addition of AP2 to the extract, an increase in both SV40 and EGFR promoter activity was detected (Fig. 8). Thus, AP2 can bind to specific sequences in the EGFR promoter and stimulate EGFR gene transcription in vitro.

**DISCUSSION**

In this study, I report that the mechanism by which PMA increases EGFR mRNA levels is at least in part through an enhancement of transcription. Nuclear run-off assays, transfection assays, and cell-free transcription analyses showed increased production of EGFR RNA in the nuclei and in nuclear extracts prepared from PMA-treated cells. To localize the region of the EGFR gene promoter involved in PMA-enhanced transcription, mutants with various portions of the EGFR promoter deleted were used for in vitro transcription. The results from these experiments indicate that the region from −150 to −16 is sufficient for PMA inducibility, although at a level less than that of the full promoter. Gel mobility shift assays with a promoter fragment that contains this region showed two bands with altered mobility, one of which was more prevalent in extracts from PMA-treated cells. DNase I footprinting of the EGFR gene promoter showed distinct regions of binding with fractionated extracts from PMA-treated cells. The binding to this promoter by the fractionated extract was identical to that of purified AP2. AP2 was able to bind to oligonucleotides containing EGFR promoter sequences similar to the consensus AP2 binding site. AP2 was also able to stimulate EGFR transcription in vitro. These results indicate that induction of
EGFR promoter activity by PMA can be modulated by binding of AP2 to the promoter.

The EGFR gene promoter contains elements that bind a variety of trans-acting factors. Sp1 binds to at least four sites in the promoter (31). ETF binds to TCCTCCTC repeats in the promoter region that contains an S1 nucleosome-sensitive site (34). In addition to these three factors, other nuclear factor binding sites have been located using A431 nuclear extracts and exonuclease III protection analyses. Another factor termed TCF (TCF) binds to the promoter and exerts a positive influence on EGFR gene tran-
scription (33). Another factor termed TCF (TCF) binds to the promoter region that contains an S1 nucleosome-sensitive site (34). In addition to these three factors, other nuclear factor binding sites have been located using A431 nuclear extracts and exonuclease III protection analyses.

The promoter region of the EGFR gene contains two sites and a DNA structural element may play a role in the recognition of the EGFR promoter by AP2.

A sequence similar to the AP1 binding sequence is located between –56 and –48. This eight-base pair sequence differs by only one base pair from the consensus AP1 sequence (51). In vitro transcription analysis with the –105 to –16 region of the promoter constructs resulted in a 50% higher activity using extract prepared from PMA-treated cells compared with extracts from control cells (Fig. 2, Table I). This suggests that the DNA sequence surrounding the recognition sequence or a DNA structural element may play a role in the recognition of the EGFR promoter by AP2.

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