The ErbB3/4 ligand heregulin (HRG) profoundly affects cell growth and differentiation, but its mechanism of action is poorly understood. Ebp1, a protein isolated by its binding to ErbB3, inhibits cell growth and represses transcription of E2F-regulated cell cycle genes. Since Ebp1 shares 38% identity with a Schizosaccharomyces pombe DNA-binding protein, we postulated that Ebp1 could bind E2F consensus elements in an HRG-inducible manner leading to transcriptional repression. We show here that GST-Ebp1 bound to the DNA sequence bound by the S. pombe protein. Whereas GST-Ebp1 alone failed to bind E2F1 promoter elements, Ebp1 contained in nuclear lysates associated with E2F1 consensus sequences in the E2F1 promoter. Endogenous Ebp1 was recruited to the E2F1 promoter in vivo as demonstrated by chromatin immunoprecipitation assays. Ebp1 bound E2F consensus oligonucleotides in association with E2F1, retinoblastoma protein, and HDAC2. HRG regulated the association of Ebp1 with E2F promoter sequences and enhanced the ability of Ebp1 to repress transcription. Our findings suggest that Ebp1, by linking HRG activation of membrane receptors to E2F gene activity, may be a downstream modulator of the effects of HRG on cell cycle progression.

The ErbB3/4 ligand HRG\(^1\) has profound effects on cell proliferation and differentiation (1, 2). HRG can either inhibit (3–5) or enhance proliferation (6) depending on cell density, ErbB receptor profile, growth conditions, or HRG concentration. However, the mechanism by which HRG affects cell proliferation is still incompletely understood. Ebp1, a member of the PA2G4 gene family, was isolated as an ErbB3-binding protein in our laboratory (7). HRG regulates both the binding of Ebp1 to ErbB3 and the phosphorylation of Ebp1 (8). The ectopic expression of Ebp1 inhibits the growth of human breast and prostate cancer cells in vitro and induces cellular differentiation (9, 10). Ebp1 binds the retinoblastoma protein (Rb) (11) and histone deacetylase-2 in cultured cells (12) and inhibits transcription of both endogenous and transiently transfected E2F-regulated promoters important in cell cycle regulation such as cyclin D, cyclin E, E2F1, and c-myc. The ability of Ebp1 to repress E2F-regulated transcription has been linked to its ability to inhibit cell proliferation. However, neither the mechanism of Ebp1 transcriptional repression nor its possible regulation by HRG has been explored.

Ebp1 is a member of the SF00553 protein superfamily, the prototype of which is a 42-kDa DNA-binding protein isolated from the fission yeast Schizosaccharomyces pombe (13). Blast analysis reveals that Ebp1 and the 42-kDa protein have 38% amino acid identity and 56% similarity. Of interest, the S. pombe protein preferentially binds to a synthetic curved DNA sequence. In addition, the murine homologue p38–2G4 was isolated as a DNA-binding protein from Ehrlich ascites cells (14). P38–2G4 was also copurified with a DNA repair factor for acid-depurinated DNA (15). We were therefore interested in determining whether Ebp1 could bind DNA either by itself or as part of a multiprotein complex, providing a possible mechanism for the ability of Ebp1 to repress E2F-regulated promoters.

The E2F family of transcription factors are important in the control of cell cycle progression (16). E2F binding sites are found in the promoters of a number of genes involved in cell cycle progression such as cyclin D, cyclin E, c-myc, and cdc2. Binding of the retinoblastoma protein, Rb, and its family members p107 and p130 (17), to E2F on E2F-regulated promoters inhibits expression of many E2F-regulated genes, resulting in withdrawal from the cell cycle (18). Other proteins, such as BRG-1 and prohibitin through their binding with Rb (19), have been demonstrated to repress transcription of E2F1-regulated genes via binding to E2F1 at its target promoters. C/EBP\(\alpha\) can inhibit cell growth via direct repression of E2F-DP-mediated transcription. Whereas C/EBP\(\alpha\) alone cannot bind E2F consensus sequences, C/EBP\(\alpha\) is detected in a protein complex that binds to the E2F binding sites found in the E2F1 and dihydrofolate reductase promoters (20). Thus, we postulated that Ebp1, a repressor of E2F1 transcription with homology to DNA-binding proteins, might bind to E2F-regulated promoter sequences. In addition, we were interested in determining whether HRG could affect the ability of Ebp1 to interact with E2F-regulated promoters, thus providing one possible link between HRG-regulated ErbB signaling and cell proliferation.

In this study, we demonstrate that recombinant Ebp1 binds to the curved DNA sequence that is bound by the S. pombe protein (13). In addition, Ebp1, as part of a protein complex containing E2F1, was associated with E2F promoter elements as demonstrated by EMSA, DNA affinity precipitation, and chromatin immunoprecipitation (ChIP) assays. Finally, HRG enhanced the ability of Ebp1 to bind to E2F consensus elements and to repress transcription.
Cell Culture—All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cell lines were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Plasmids—The plasmids S10-15 and S15-12 are a kind gift of Dr. T. Mizuno (13). S10-15 (200 bp) contains a tandem repeat of (GGC/AGAAG)₆ flanked by the EcoRI-HindIII polylinker sequences of pUC19. S12-15 (233 bp) contains tandem repeats of the 15-mer (CCGGCAAAAACGGGC)₁₂. The E2F1 reporter plasmid contains a portion of pUC19. S15-12 (233 bp) contains tandem repeats of the 15-mer (CCGGCAAAAACGGGC)₁₂ flanked by the EcoRI-HindIII polylinker sequences of pUC19. The GST-E2F1 reporter plasmid was a gift from the University of Maryland Core Biopolymer Laboratory. Complementary strands were annealed by heating to 95 °C, then rapidly cooling to 4 °C over 15 min on ice. DNA was purified by phenol/chloroform extraction and precipitated in 10 mM NaCl, 0.1 M NaAc, 1 mM EDTA, 1 M NaCl, 1% formaldehyde, 1 mM dithiothreitol, 10 mM MgCl₂, and 0.1% Triton X-100. After incubation overnight, the DNA was washed repeatedly in 16 mM HEPES, pH 7.6, 100 mM NaCl, 0.4 mM EDTA, 1 mM MgCl₂, and 1% glycerol. Precipitated proteins were analyzed by SDS-PAGE and Western blotting as previously described (25). Antibodies to HDAC2 were from Zymed Laboratories Inc. (San Francisco, CA), and those to E2F1 and Rb (C-15) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

EMSA—To determine the ability of Ebp1 to bind to synthetic DNA, we used the DNA inserts cloned between the EcoRI-HindIII sites of either pUC19 S10-15 and S15-12. The DNA inserts were released by restriction enzyme digestion and gel-purified. The DNA was labeled using a 5'-end labeling kit (MBI Fermentas, Hanover, MD) and incubated with purified GST-Ebp1 as described below. In experiments determining the ability of Ebp1 to bind to an E2F oligonucleotide, an E2F consensus element derived from the human E2F-1 promoter (–35 to +1) (GGCTTTTGGCCGAAAAGGGTTGGGCGCTTAAA) and containing two overlapping E2F consensus sites (indicated in boldface type) (26). The reverse complement sequence was synthesized in the University of Maryland Core Biopolymer Laboratory. Complementary strands were mixed in equimolar ratios in 5 mM MES, 200 mM NaCl, pH 7.0, for a final concentration of 0.5 mM. Strands were annealed by incubating the mixture at 95 °C for 10 min and slowly cooling over a few hours to 20 °C. The double-stranded oligonucleotide was labeled using a 5'-end labeling kit. Where indicated, we also used a 32p-35S-labeled probe to confirm labeling efficiency. DNA-protein complexes were analyzed by gel retardation assays using a 5'-end labeling kit. DNA-protein complexes were then analyzed by gel retardation assays using a 5'-end labeling kit.

DNA Affinity Precipitation—The method of Alliston et al. (24) was used as described. Briefly, HeLa cell DNA was used to precipitate the beads with each wash. Washed beads were added to 200 μg of cell lysates and 5 μg of poly(dI-dC)·poly(dI-dC) with a final concentration of 4 mM Tris, pH 7.5, 20 mM HEPES, pH 7.5, 5% glycerol, 170 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 mM MgCl₂, and 0.1% Triton X-100. After incubation overnight, the beads were washed repeatedly in 16 mM HEPES, pH 7.6, 100 mM NaCl, 0.4 mM EDTA, 1 mM MgCl₂, and 1% glycerol. Precipitated proteins were analyzed by SDS-PAGE and Western blotting as previously described (25). Antibodies to HDAC2 were from Zymed Laboratories Inc. (San Francisco, CA), and those to E2F1 and Rb (C-15) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Results—ChIP assays were carried out using a kit from Upstate Biotechnology, Inc. (Lake Placid, NY) essentially as described by the manufacturer. Briefly, HeLa cells were grown in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 5% fetal bovine serum and cross-linked with 1% formaldehyde at 37 °C for 10 min. Cells were harvested twice with ice-cold phosphate-buffered saline, scraped into phosphate-buffered saline, and centrifuged for 5 min. The pellets were resuspended in 0.2 ml of lysis buffer (0.5% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, containing protease inhibitor mixture (Roche Applied Science). DNA was sheared on ice to the appropriate lengths (~500 base pairs) with three sets of 10-s pulses at 20% maximal output followed by sonication for 10 min at 13,000 rpm at 4 °C. Supernatants were mixed, aliquoted, and diluted in NET-N buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 1.5 mM MgCl₂, 10% glycerol, and protease inhibitor mixture) for a final volume of 1.5 ml. A portion of the diluted cell supernatant (1%) was kept to have cross-links reverse and quantitate the amount of DNA present in samples for the PCR control. After precipitation with salmon sperm DNA/protein A/agarose slurry for 30 min at 4 °C, immunoprecipitation was performed overnight on a rotary shaker at 4 °C with specific antibodies or nonspecific IgG as a control. The samples were then mixed with sonicated salmon sperm DNA (100 μg/ml) and Protein A/G-agarose (Oncogene Research Products, San Diego, CA) for another 2-h incubation. Agarose beads were washed sequentially in low salt, high salt, LiCl, and TE buffer provided with the kit and extracted two times with freshly prepared elution buffer (1% SDS, 0.1 M NaHCO₃). Eluates were pooled and incubated at 65 °C for 6 h to reverse the formaldehyde cross-links. DNA was purified by phenol/chloroform extraction and precipitated in ethanol. The DNA was dissolved in fermentation buffer (0.2 μg/ml of T·RNA in 2 volumes of ethanol at –20 °C overnight. The DNA pellets were dissolved in 50 μl of water. Nested PCR amplification of a 203-bp E2F1 promoter fragment (~65 to +137) was carried out using a 5′ primer (5′-AGCACAATTTG-GCAGGCGCT-3′) and a 3′ primer (5′-CCAAGGCCATGACGCAC-3′). The PCR products were resolved on 2.5% agarose gels and visualized with ethidium bromide.

Luciferase Assays—MCF-7 cells were plated in 12-well plates in RPMI 1640 medium with 10% fetal bovine serum. Cells were transfected using Fugene 6 (Roche Applied Science) as described (11) with the E2F1 luciferase reporter construct (0.5 μg), a Renilla thymidine kinase control luciferase plasmid (Promega), and the ebp1 expression plasmid with the concentration of 2 μg DNA/ml (12). Twenty-four hours after transfection, cells were switched to phenol-red free RPMI 1640 medium with 1% charcoal-stripped serum containing either 0, 10, or 100 ng/ml HRG β1 (R&D Systems, Minneapolis, MN). Cells lysates were collected 24 h later, and luciferase activity was assessed using a Promega dual luciferase assay kit (Madison, WI).

Statistical Analysis—Results were analyzed using a two-tailed Student’s t test. Significance was established at p < 0.05.

Results—DNA Binding Properties of Ebp1—Ebp1 is a member of the SF0553 superfamily of proteins, the prototype of which is the yeast 42-kDa DNA-binding protein. However, the ability of Ebp1 to bind DNA has not been directly tested. Therefore, we first determined the ability of recombinant GST-Ebp1 to bind to native calf thymus DNA-cellulose under conditions described for the isolation of p38–264 (26). A portion of the bacterially
synthesized GST-Ebp1 was retained on a column of native calf thymus DNA and eluted at 0.2 M NaCl. When nuclear extracts prepared from MCF 7 cells were applied to this column, a portion of the input Ebp1 protein was retained and eluted at 0.2–0.5 M NaCl (Fig. 1A). Similarly, the murine homologue p38–2G4 was initially detected in nuclear extracts by its ability to elute from DNA affinity columns between 0.1 and 0.4 M NaCl (14).

As we determined that Ebp1 bound DNA-cellulose, we tested the ability of Ebp1 to associate with the DNA sequence bound by the yeast prototype protein. The S10-15 DNA has previously been demonstrated to have an overall and strong curvature (13). On the other hand, S15-12 has nearly the same base composition, but shows no detectable signs of overall DNA curvature. Recombinant GST-Ebp1 was incubated with either the 200-bp S10-15 or the 233-bp S15-12 insert, and binding ability was assessed using EMSA analysis. Fig. 1B shows that GST-Ebp1, but not GST, bound the S10-15 DNA. The binding was specifically competed by cold S10-15 DNA. Ebp1, similar to the yeast 42-kDa protein, failed to bind to the linear S15-12 sequence. The E2F consensus element derived from the E2F1 promoter (GGCTCTTTTCGCCAAAAAGGATTGGCGCGTA AAAA) has homology to the repeat element of the S10-15 plasmid (i.e. CGGCAAAAAGG). Therefore, we tested whether E2F1 could also bind to the S10-15 element. GST-Ebp1 and GST-E2F1 were incubated with the 200-bp S10-15 insert, and their binding ability was assessed using EMSA analysis. Ebp1 bound the S10-15 element as expected. E2F1 also bound the S10-15 probe, and this binding was specifically competed by cold S10-15 (Fig. 1C).

**Purified Recombinant Ebp1 Fails to Bind E2F Promoters**—Since Ebp1 has been shown to affect E2F-mediated transcription, we examined whether Ebp1 might directly bind to either the E2F1 consensus element or the 325 bp (–324 to +1) wild type functional promoter (22). Bacterially expressed GST-Ebp1 was used in an EMSA with the E2F1 consensus oligonucleotide. GST-Ebp1, at concentrations similar to and higher than those used in the assay with S10-15 DNA, failed to bind to the E2F consensus oligonucleotide (Fig. 2A). As the ability of Ebp1 to bind to S10-15 was dependent on the curvature of the DNA, we also used the 325-bp E2F1 promoter, which has been shown to have a strong curvature (21), as a probe in EMSAs. Results indicated that recombinant GST-Ebp1 alone also failed to bind to this DNA sequence (Fig. 2B). To determine whether recombinant E2F1 could induce the binding of Ebp1 to the E2F1 consensus oligonucleotide, we added increasing amounts of Ebp1, E2F1, and DP-1 to the oligonucleotide probe. E2F1 alone did not bind to the oligonucleotide as previously reported (26). The addition of recombinant GST-E2F1 to Ebp1 even in the presence of DP-1 did not induce the binding of Ebp1 to the consensus element. Under these circumstances, nuclear extracts of HeLa cells bound the oligonucleotide as expected (Fig. 2C).

**Ebp1 Is Found in E2F Binding Complexes in HeLa Cells**—Since Ebp1 contained in nuclear extracts bound DNA-cellulose with greater affinity than GST-Ebp1, we determined whether Ebp1 contained in nuclear extracts could bind to the E2F consensus oligonucleotide. Since we did not have an Ebp1 antibody that could supershift, we transiently transfected log phase HeLa cells with a FLAG-tagged Ebp1 expression vector.
Fig. 2. Purified recombinant Ebp1 does not bind E2F consensus elements or a natural promoter. Full-length Ebp1 was expressed as a GST fusion protein and assessed for DNA binding activity in EMSAs using either an E2F1 consensus oligonucleotide (Oligo) (A) or a 325-bp natural promoter (Promoter) (B) as probes. For both A and B, lane 1 (P), probe alone; lane 2 (NE), HeLa nuclear extract; lanes 3–6, GST Ebp1 at 0.75, 1.5, 3, and 4.5 μg; lanes 7 and 8, GST at 0.75 and 4.5 μg, respectively. The arrows indicate E2F1 complexes. C, recombinant Ebp1 does not bind the E2F1 consensus oligonucleotide in the presence of E2F1. Full-length Ebp1, E2F1, and DP-1 were expressed as GST fusion proteins and assessed for DNA binding activity in EMSAs either alone or in combination as indicated. Lane 1, probe alone; lanes 2–4, GST-E2F1 at 0.45, 0.9, and 1.8 μg, respectively; lane 5, HeLa nuclear extract; lane 6, as in lane 5 plus 100× cold competitor; lanes 7–9, GST-E2F1, GST-DP-1, and GST-Ebp1 at 0.45, 0.9, and 1.8 μg each in combination.

(25). FLAG protein of the appropriate molecular weight was expressed as determined by Western blotting (data not shown). EMSAs using the consensus E2F1 oligonucleotide revealed the presence of several complexes (arrows) in keeping with previous data (27–29). The formation was specific as a 100-fold molar excess of unlabelled wild type (lanes 3 and 9) but not mutant (lane 10), DNA competed for binding (Fig. 3A). Antibody to E2F1 supershifted the lower two bands (Fig. 3A, lane 4). The FLAG antibody disrupted the lowest specific band in a dose-dependent manner (Fig. 3A, lanes 5 and 6). Nonspecific IgG had no effect (Fig. 3A, lanes 11 and 12). Ebp1 derived from HeLa cell nuclear extracts binds to DNA as a complex with E2F1. The presence of cyclin A on E2F promoters was similarly demonstrated by the disruption of a specific complex in EMSAs (30).

We next used a ChIP assay to elucidate whether Ebp1 and E2F1 could associate in vivo on the chromatin of the endogenous E2F1 promoter. Both endogenous Ebp1 and E2F1 were found to associate with a segment of genomic DNA that could be PCR-amplified using primers spanning E2F1 elements present in the E2F1 promoter (−65 to +137) (22) (Fig. 3B). These data further suggest that Ebp1 interacts with E2F1 on E2F-responsive promoters in vivo.

Recruitment of Ebp1 to the E2F1 Response Element—The foregoing data suggested that Ebp1 might bind to E2F1 response elements via its association with other nuclear proteins. We therefore used DNA affinity precipitation assays to identify proteins that associate with Ebp1 at E2F1 consensus elements. Endogenous Ebp1 from HeLa cell lysates specifically bound to a biotinylated wild type E2F consensus oligonucleotide (Fig. 4, A and B). Ebp1 failed to bind to a mutant oligonucleotide (M2) (Fig. 4A) with two GC → AT substitutions in the E2F binding site, suggesting that E2F elements are necessary for Ebp1 binding. E2F1 was detected in the complex as expected. Rb protein was also found in this complex, in keeping with our previous data that indicated that Ebp1 binds E2F1 indirectly through Rb (11). We have previously shown that Ebp1 binds to HDAC2 (12) and that the ability of Ebp1 to recruit HDAC2 is related to its ability to repress transcription. In the present study, HDAC2 was observed in the same DNA-protein complex as Ebp1, Rb, and E2F (Fig. 4B).

HRG Induces Ebp1 Association with the E2F Consensus Sequence and Enhances Ebp1 Transcriptional Repression—Both the phosphorylation of Ebp1 (8) and its ability to associate with ErbB3 are regulated by HRG (7). We were therefore interested in determining whether HRG could affect the ability of Ebp1 to bind to E2F consensus elements. We incubated HeLa cells in serum-free RPMI 1640 medium for 24 h prior to the addition of HRG (20 ng/ml) and then evaluated the ability of Ebp1 derived from nuclear lysates to bind the E2F oligonucleotide using DNA affinity precipitation. HRG induced a marked increase in Ebp1 binding at 5 and 15 min after HRG addition. Binding was decreased at 30 min but increased again 60 min after HRG treatment. There was no change in Ebp1 total protein levels at any of these time points. E2F1 protein associated with DNA was approximately equal at all time points. E2F1 protein associated with DNA was approximately equal at all time points (Fig. 5). HDAC2 was associated with the DNA at all time points tested (Fig. 5).

Finally, we reasoned that if HRG could change the affinity of Ebp1 for the E2F consensus sequence, it could affect Ebp1-induced repression of the E2F1-regulated E2F1 promoter. MCF-7 cells were transiently transfected with an E2F1 luciferase reporter plasmid and limiting amounts of an ebp1 expression construct. As expected, ebp1 at low concentrations (0.1 μg) reduced E2F1 luciferase activity 35%. Maximal inhibition of 80% was observed at 0.5 μg of ebp1 plasmid. Low concentrations of HRG (10 ng/ml), previously demonstrated to induce cell proliferation (31), did not affect Ebp1 mediated repression (Fig. 6A). In contrast, HRG at concentrations that inhibit prolifera-
tion (30) significantly (*p* ≤ 0.05) enhanced Ebp1-mediated repression at low (0.1 and 0.2 μg) amounts of the Ebp1 plasmid (Fig. 6B).

**DISCUSSION**

HRG, an ErbB3/4 ligand, inhibits proliferation, induces differentiation, or acts as a mitogen in different cell types depending on its concentration, culture conditions, and the complement of ErbB receptors. The basis for these diverse activities and the factors that mediate the effects of HRG on proliferation are poorly understood. Recently, HRG induction of leucine zip-
per transcription factors (32–34) and Ras signaling components has been demonstrated, suggesting that these proteins may be specific components of HRG signal transduction pathways. Ebp1 may also be a member of an HRG downstream pathway, since it binds the cytoplasmic unphosphorylated domain of ErbB3. Treatment with HRG induces phosphorylation of Ebp1 and causes its dissociation from ErbB3 and its translocation to the nucleus of serum-starved AU565 breast cancer cells (7). Ebp1 represses transcription of E2F-regulated genes, and its ability to inhibit cell growth is related to its ability to inhibit transcription (12). Here we demonstrate that Ebp1 can bind to E2F consensus DNA, suggesting a mechanism of transcriptional repression. In addition, Ebp1 bound an E2F1 consensus oligonucleotide in an HRG-regulated manner, and its ability to repress E2F1 transcription was enhanced by HRG. These data suggest that Ebp1 may play a role in HRG signal transduction through its effects on E2F-regulated genes.

We have previously demonstrated that Ebp1 inhibits growth of human breast and prostate cancer cells. A general slowing of the cell cycle and decrease in colony forming ability was observed. We have recently found that ectopic expression of Ebp1 inhibits the activity of both exogenous and endogenous E2F1-regulated promoters (12). Since many proteins that repress transcription do so through their interactions with transcription factors on DNA, we hypothesized that Ebp1 may bind E2F promoters. Ebp1 is classified as a member of the recently identified SF00553 superfamily of proteins, the prototype of which is the 42-kDa yeast DNA-binding protein. Other members include the mouse protein p38-2G4, which was originally isolated as a DNA-binding protein and also as a component of a DNA repair factor complex (15). Ebp1, like these other proteins, contains repetitive, charged amino acids in a putative amphipathic helix, suggesting that it may directly interact with DNA and/or other proteins. We initially found that recombinant Ebp1 bound to the same synthetically derived DNA as its yeast homologue. It is of interest that E2F1 also binds to curved DNA (22). The fact that Ebp1 can bind this DNA element supports its classification as a member of the SF00553 protein family. It also confirms that DNA binding is a functional property of this group of proteins.

However, GST-Ebp1 alone could not bind either an E2F consensus element or an E2F1 natural promoter. The reason for the greater affinity of Ebp1 for the synthetic DNA is not known. It could be simply that the synthetic DNA contains multiple copies of the consensus element, increasing the affinity of the protein for DNA. As Ebp1 bound the curved, but not linear synthetic probe, we tested the ability of Ebp1 to bind a 325-bp E2F1 natural promoter that has a strong curvature (21). GST-Ebp1 also failed to bind this DNA. It is possible that the biophysical properties of the E2F1 promoter are different from those of the synthetic curved DNA, leading to the failure of Ebp1 to bind. However, the ability of a protein to bind one DNA element, but not others, is not unprecedented. For example, C/EBPs can bind its own sequences directly but indirectly binds E2F consensus DNA sequences (20). Combining GST-Ebp1 and GST-E2F1 with DP-1 did not result Ebp1 binding, under conditions where HeLa nuclear extracts bound the E2F1 oligonucleotide. Modifications may play a role in the ability of Ebp1 and E2F1 to interact and bind DNA. For example, Ebp1 is a phosphoprotein in vivo (8), and we are currently examining whether phosphorylation of Ebp1 enhances its ability to interact with E2F1 and bind DNA. Similarly, binding of recombinant E2F1 to DNA is induced by acetylation by affecting its binding to DP-1 (26).

Nevertheless, Ebp1 interacted with E2F1 at E2F1 promoter elements. EMSAs and DNA affinity precipitation assays demonstrated that Ebp1 bound to E2F1 consensus elements as part of a complex of nuclear proteins. ChIP assays established that endogenous Ebp1 was bound in vivo to the E2F1 promoter. It is of interest that mutation of the E2F binding sites abrogated Ebp1 binding to biotinylated E2F1 oligonucleotides in DNA affinity precipitation assays. Similarly, we have found that Ebp1 does not affect E2F regulated promoters in which the E2F binding sites are mutated (12). Therefore, one other possible explanation for the fact that Ebp1 alone does not bind to E2F oligonucleotides is that Ebp1 binds the E2F1 promoter indirectly through its association with the components of E2F1 transcription complexes. Rb was also retained on the E2F consensus oligonucleotide in DNA affinity immunoprecipitation

Fig. 6. HRG enhances Ebp1-mediated repression of the E2F1 promoter.

MCF-7 cells plated in 12-well plates were transfected with an E2F1 luciferase reporter plasmid (0.5 μg) and an ebp1 expression plasmid at the indicated concentrations. HRG β1 at 10 ng/ml (A) or at 100 ng/ml (B) was added for the final 16 h of incubation, or the cells were left untreated (Control). Cell lysates were assayed for luciferase activity 48 h after transfection. In each case, repression is compared with control with no ebp1.
assays, suggesting that a pool of Rb is still functional in herpes simplex virus-infected HeLa cells. The fact that Rb was found in a complex with E2F1 and Ebp1 is consistent with our previous data suggesting that Ebp1 interacts indirectly with E2F1 via the association of Ebp1 with Rb. For example, GST pull-down assays indicate very weak interactions between E2F1 and Ebp1, whereas the overexpression of Rb stimulated complex formation between E2F1 and Ebp1 (11). The DNA affinity precipitation data support the model that Rb mediates interaction between E2F1 and Ebp1. Our previous work also suggested that binding of Ebp1 to Rb is important in transcriptional repression in Rb+ cells as Ebp1 mutants lacking the Rb binding domain could no longer repress transcription of cyclin E (11). It is of interest that Ebp1 has activity in Rb-negative cells perhaps by binding other pocket proteins that recently have been shown to be critical for binding Sin3 and HDAC to E2F1 promoters (17).

In the present study, HDAC2 was observed in the same DNA-protein complex as Ebp1, Rb, and E2F. We have previously shown that Ebp1 binds to HDAC2 (12) in vitro and in vivo and that the ability of Ebp1 to recruit HDAC is related to its ability to repress transcription. For example, the HDAC inhibitor trichostatin A significantly reduced Ebp1-mediated repression (12). Thus, the DNA binding studies further support the role of HDAC in Ebp1 transcriptional repression. Further, others have similarly reported the presence of HDAC2 in E2F-DNA complexes and that histone deacetylase activity is implicated in transcriptional repression of E2F promoters in either an Rb-dependent (36) or -independent manner (17).

Finally, we found that the ability of Ebp1 to bind to E2F consensus elements could be regulated by HRG. HRG treatment induced a rapid increase in the ability of Ebp1 to bind to the E2F consensus element. Similarly, HRG has been demonstrated to induce association of the transcription factor Sp1 with the E2F consensus element. Similarly, HRG has been demonstrated to induce association of the transcription factor Sp1 with the E2F consensus element. Similarly, HRG has been demonstrated to induce association of the transcription factor Sp1 with the E2F consensus element. Similarly, HRG has been demonstrated to induce association of the transcription factor Sp1 with the E2F consensus element. Similarly, HRG has been demonstrated to induce association of the transcription factor Sp1 with the E2F consensus element. Similarly, HRG has been demonstrated to induce association of the transcription factor Sp1 with the E2F consensus element.

It was of interest that high but not low concentrations of HRG affected the ability of Ebp1 to repress the E2F1-regulated promoter. Bacus et al. (31) have shown that HRG at low concentrations (10 ng/ml or less) has a mitogenic effect on MCF-7 cells, whereas concentrations higher than 50 ng/ml inhibit cell growth. We suggest that high concentrations of HRG enhance cell growth, whereas concentrations higher than 50 ng/ml inhibit cell growth. It is of interest that Ebp1 may be part of an HRG-stimulated signal transduction pathway that results in changes in E2F-regulated cell cycle genes and cell proliferation.

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REFERENCES
Ebp1 Binds E2F1 Promoters


Heregulin Regulates the Ability of the ErbB3-binding Protein Ebp1 to Bind E2F Promoter Elements and Repress E2F-mediated Transcription
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