E2F-mediated Growth Regulation Requires Transcription Factor Cooperation*

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Paul R. van Ginkel, Kuang-Ming Hsiao‡, Hilde Schjerven, and Peggy J. Farnham§

From the McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, Wisconsin 53706

Previous studies have indicated that the presence of an E2F site is not sufficient for G1/S phase transcriptional regulation. For example, the E2F sites in the E2F1 promoter are necessary, but not sufficient, to mediate differential promoter activity in G0 and S phase. We have now utilized the E2F1 minimal promoter to test several hypotheses that could account for these observations. To test the hypothesis that G1/S phase regulation is achieved via E2F-mediated repression of a strong promoter, a variety of transactivation domains were brought to the E2F1 minimal promoter. Although many of these factors caused increased promoter activity, growth regulation was not observed, suggesting that a general repression model is incorrect. However, constructs having CCAAT or YY1 sites or certain GC boxes cloned upstream of the E2F1 minimal promoter displayed E2F site-dependent regulation. Further analysis of the promoter activity suggested that E2F requires cooperation with another factor to activate transcription in S phase. However, we found that the requirement for E2F to cooperate with additional factors to achieve growth regulation could be relieved by bringing the E2F1 activation domain to the promoter via a Gal4 DNA binding domain. Our results suggest a model that explains why some, but not all, promoters that contain E2F sites display growth regulation.

E2F plays an important role in regulating gene expression at the G1/S phase transition in the mammalian cell cycle. Promoters in which E2F sites contribute to transcriptional regulation are found in genes involved in DNA synthesis (such as dihydrofolate reductase, DNA polymerase α, and thymidine kinase) as well as in genes that are involved in cell cycle control (such as B-myb, several cyclins, and cdc2). E2F-mediated regulation of many of these genes leads to differential expression in G0 and S phase, causing low promoter activity in quiescent cells and increased activity at the G1/S phase boundary (1). However, certain promoters contain E2F sites that do not confer growth-regulated transcriptional activity (2, 3). Several models have been put forth to explain why certain E2F sites mediate growth regulation and others do not.

One such model is based on the fact that E2F is a family of transcription factors of which seven members have been characterized to date. E2F1–5 can heterodimerize with either DP1 or DP2 to create functional E2F activity (4, 5). Some of the E2Fs are present only at certain stages of the cell cycle (such as E2F1), whereas others are constitutively present (such as E2F4). Also, E2F activity is regulated by the Rb family of proteins (4–9). E2F1–3 preferentially bind Rb, whereas E2F4 and E2F5 mainly bind p107 and p130. Thus, it is possible that growth-regulated promoters have a different composition of E2F protein complexes bound to the promoter DNA than do non-growth-regulated promoters. This model, however, cannot account for results obtained from deletion analyses of growth-regulated promoters. For example, we have shown that deletion of sequences from –410 to –90 of the mouse dihydrofolate reductase promoter can eliminate growth regulation although the E2F sites and surrounding sequences remain intact (10). The fact that a particular E2F element can confer growth regulation in a given promoter, but not in a shorter version of the same promoter, suggests that subtle differences in the E2F site are not responsible for distinguishing growth-regulated versus non-growth-regulated promoters.

An alternative model of E2F-mediated growth regulation is based on the interactions of E2F-bound pocket proteins (i.e. Rb, p107, and p130) with upstream factors. Rb, p107, and p130 all contain transcription repression domains. It has been suggested that E2F-bound pocket proteins repress activity of other transcription factors bound to the promoter DNA in the G0 phase of the cell cycle. Then, as cells progress through the cell cycle, the pocket protein is phosphorylated and released from the E2F-DNA complex. This relief of repression now allows high transcriptional activity. Evidence for this model comes from mutational analysis of the E2F sites in certain promoters. For example, in the B-Myb promoter, elimination of E2F binding causes increased promoter activity in G0 phase cells (11). Since not all upstream factors can be repressed by pocket proteins (12), the presence of a repressible upstream factor may determine whether a promoter is growth-regulated.

We have previously shown that the mouse E2F1 promoter is growth-regulated and that this regulation is dependent upon two E2F sites in the promoter region (13). In this study it was further shown that growth regulation of this promoter was a result of E2F-mediated repression. However, deletion of the –176 to –45 region of the E2F1 promoter can eliminate growth regulation, although the E2F sites located between –40 and –10 are still present. We wished to determine the role of the upstream sequences in mediating this growth regulation. Therefore, we have examined the ability of a variety of transcription factors to reinstate growth regulation to an E2F1 minimal promoter. In contrast to a repression-based model of E2F-mediated regulation, we find that the growth-regulated promoter constructs display S phase-specific activation of transcription. Our results suggest that upstream factors determine whether or not an E2F site-containing promoter displays
growth-regulated transcriptional activity and that if regulation is achieved via activation or repression.

**EXPERIMENTAL PROCEDURES**

**Constructs**—pE2F1(-45/+36)-Luc and pE2F1(-84/+36)-Luc have been described previously (13). pE2F1(-45/+36)ΔE2F-Luc was made by polymerase chain reaction using pE2F1(-176/+36)ΔE2F-Luc (13) as a template and primers specific for E2F1 and the vector. For synthetic promoter reporter constructs, oligonucleotides of template and nontemplated strand containing various transcription factor binding sites were hybridized and inserted into Smal-digested pE2F1(-45/+36)-Luc or pE2F1(-45/+36)Δ-Luc. The following double-stranded oligonucleotides (the transcription factor binding sites are underlined) were inserted into the constructs: the proximal CCAAT site of the E2F1 minimal promoter reporter constructs—

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GGCGCGTAAAAGTGGCCCGGACTTTGC
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A consensus Sp1 site at 68/45:

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GGGGCGACTCGA
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A consensus YY1 site from the AAV promoter—

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GATTGGTTAAAAGTTGAGTGCT
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The 5'-tttcccgGGAGGAGGCGTTCGGCGGCGCGTcccgggttt-3' and the consensus Sp1 site at 68/45:

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GGCGAGCTCGGCCAATGGAAGCTTCCC
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A consensus Gal4 binding site, 5'-CCGGAGGC-GGGGCGGAGCTCGGCCAATGGAAGCTTCCC-3' and a consensus Sp1 site from the E2F1 promoter, 5'-GGGCGAGCTCGGCCAATGGAAGCTTCCC-3'.

**Cell Culture and Transient Transfections**—NIH 3T3 cells (American Type Culture Collection) were maintained, transfected, serum-starved, and serum-stimulated as described (13) with the following modifications. 104 NIH/3T3 cells were plated into 60-mm diameter dishes in 5 ml of maintenance medium 16–20 h prior to infection. Immediately following the glycerol shock, the cells were placed in starvation medium containing 0.5% (v/v) serum. After 40–50 h, the cells were harvested or stimulated to enter the proliferative cycle by replacing the starvation medium with medium containing 10% (v/v) serum. Cells were harvested at the indicated times, and luciferase activity was measured. For the cotransfection experiments, 5 g of reporter plasmid was transfected with a titration range of the Gal4 fusion construct and carrier DNA (15 g of total DNA/dish). For all transfection experiments, at least two DNA preparations and at least three separate experiments were averaged for each datum point (an error bar represents the S.E.; see Figs. 1–3 and 8).

**Gel Mobility Retardation Assays**—Nuclear extracts were prepared from NIH 3T3 cells essentially as described (15). Preparation of Friend cell nuclear extracts has been described (13). DNA binding activity was assayed by incubating labeled double-stranded oligonucleotides containing the Sp1 site at −77, the CCAAT site at −68, and the GC box at −50 in the E2F1 promoter with 6 g of nuclear extract prepared from Friend cells or NIH 3T3 cells using previously described assay conditions (13). The binding buffers for the GC box probe and the Eα Y box probe were the same as for the Sp1 probe and the CCAAT probe, respectively. For the NF-Y supershift assays, 400 ng of anti-Ya polyclonal antibody and 200 ng of anti-Yb antibody (16) were added to the reaction mixture. For the Sp1 and Sp3 supershift assays, 2 g of anti-Sp1 antibody (Santa Cruz Biotechnology Inc.) and 4 g of anti-Sp3 antibody (Upstate) were used. The sequences of the probes and competitor DNAs are as follows (the lowercase letters in the E2F, Sp1, CCAAT, and GC box probes represent linker sequences that are not present in the nontemplate strand containing various transcription factor binding sites).

**RESULTS**

Gal4 Fusion Proteins Did Not Confer Growth Regulation to the E2F1 Minimal Promoter—We have previously reported that the E2F sites in the E2F1 promoter (−176/+98) are necessary for growth-regulated transcription but that these same sites are not sufficient for growth regulation of a minimal (−45/+36) promoter region (13). One hypothesis that could explain the lack of growth regulation is based on transcriptional repression. Activators that normally bind upstream of the E2F sites could create a strong promoter that can be repressed in G0 by E2F-bound pocket proteins. Truncation of the promoter would remove these activators, leaving only the two E2F sites located from −40 to −10 plus an Sp1 site located downstream of the transcription start site. It is possible that these remaining factors are not sufficient to activate transcription and so promoter activity remains low even after the pocket proteins are released from E2F. To test the generality of a repression-based model of E2F-mediated growth regulation, we determined if growth regulation could be conferred to the E2F1 minimal promoter by activators brought in as Gal4 fusion proteins.

The hypothesis stated above requires the activator to confer high transcriptional activity to the minimal E2F1 promoter so that E2F can repress this activity in G0 phase cells. Therefore, we first determined if the Gal4 DNA binding domain (amino acids 1–147) fused to VP16, p53, Ets2, or MyoD could confer high activity to the minimal E2F1 promoter in S phase. Since the E2F1 promoter sequences from −84 to +36 demonstrate growth-regulated activity (11-fold; Fig. 1B), we decided that activators that provide at least as much transcriptional activity to the E2F1 minimal promoter as does the −84/+45 region would score as "potential" regulators. NIH 3T3 cells were cotransfected with Gal4-transcription factor fusion expression constructs and a luciferase reporter construct consisting of one or five Gal4 sites cloned upstream of the E2F1 minimal promoter (Fig. 1A). For each expression construct, a titration was performed to determine optimal activation of the reporter. Each of these four acidic activators was able to activate the E2F1 promoter having a single Gal4 site, resulting in an increase in luciferase activity ranging from 15-fold (MyoD) to 59-fold (p53) over that seen using the Gal4 DNA binding domain alone (Fig. 1B). Transcriptional activity mediated by each of these activators exceeded that mediated by the −84/+45 E2F1 promoter region. When the reporter plasmid having five Gal4 sites was used in cotransfection assays, activation by the same transcriptional activators ranged from 27-fold (MyoD) to 296-fold (VP16).

To determine whether VP16, p53, Ets2, or MyoD could confer growth regulation to the minimal E2F1 promoter, the Gal4 fusion constructs were analyzed in a serum starvation and stimulation assay using the reporter plasmids described above. In this assay, cells are transfected with the DNA constructs, placed in 0.5% serum for 48–60 h (which causes the cells to arrest in G0 phase), and then stimulated to synchronously enter the cell cycle by the addition of 10% serum. Reporter activity was measured in transfected cells in both G0 and S phase (12 h after serum stimulation); growth regulation is defined as the ratio of activity in S phase to that in G0 phase. As a positive control we used the −84/+36 E2F1 promoter. The activity after serum stimulation of the −84/+36 E2F1 reporter plasmid was increased 13-fold at 12 h after serum stimulation, demonstrating that cell cycle synchrony was achieved. However, there was very little change in promoter activity in G0 versus S phase using any of the Gal4 fusion proteins (as measured with either the reporter containing one or five Gal4 sites). Therefore, the presence of these strong activators at the E2F1 minimal promoter does not confer growth regulation. Since reporter activity was high in G0 and S phase, the lack of regulation resulted primarily from a failure to repress transcriptional activity in G0 phase.

Some, but Not All, Endogenous Transcription Factors Can Confer Growth Regulation to the E2F1 Minimal Promoter—Since the Gal4 fusion proteins were unable to confer regulation
to the E2F1 minimal promoter, consensus transcription factor binding sites were cloned upstream of the promoter to determine if endogenous cellular factors could confer growth regulation. Oligonucleotides containing consensus CCAAT, YY1, p53, AP2, Oct1, and NF1 sites and a Rep3A GC box were inserted upstream of the −45/+36 E2F1 promoter containing wild type or mutated E2F sites. TF, transcription factor binding site. B, some endogenous factors increase activity from the E2F1 minimal promoter. The E2F1 promoter constructs were transfected into NIH 3T3 cells and analyzed as described in the legend to Fig. 1. Reported is the promoter activity in S phase of constructs having one or five Gal4 sites when cotransfected with either the Gal4 DNA binding domain (1–147) (GalDB) or GalDB fusions to various activator proteins. The activity of the −45/+36 E2F1 promoter is shown as a reference. C, Gal4 fusion proteins do not confer growth regulation. Reported is the differential activity in G₀ versus S phase mediated by GalDB or GalDB fusion proteins from a promoter having one or five Gal4 sites.

FIG. 1. The presence of a strong activator does not ensure E2F-mediated growth regulation. A, schematic of the E2F1 minimal promoter (−45/+36) cloned upstream of the luciferase cDNA. Gal4 represents one or five Gal4 DNA binding sites (as indicated in the graphs). B, Gal4 fusion proteins increase promoter activity. NIH 3T3 cells were transfected with 5 μg of E2F1(−45/+36)Gal4-luc and 5 μg of each Gal4-transcription factor fusion construct. Transfected cells were placed in medium containing 0.5% bovine calf serum for 48 h and then stimulated with medium containing 10% serum. Cells were harvested at 0 and 12 h after stimulation, and luciferase activity was measured. Reported is the promoter activity in S phase of constructs having one or five Gal4 sites when cotransfected with either the Gal4 DNA binding domain (1–147) (GalDB) or GalDB fusions to various activator proteins. The activity of the −45/+36 E2F1 promoter is shown as a reference. C, Gal4 fusion proteins do not confer growth regulation. Reported is the differential activity in G₀ versus S phase mediated by GalDB or GalDB fusion proteins from a promoter having one or five Gal4 sites.

FIG. 2. Some, but not all, endogenous activators can confer growth regulation to the E2F1 minimal promoter. A, schematic of the E2F1 minimal promoter (−45/+36) cloned upstream of the luciferase cDNA. Consensus binding sites for CCAAT, YY1, Sp1, p53, AP2, Oct1, and NF1 sites and a Rep3A GC box were inserted upstream of the −45/+36 E2F1 promoter containing wild type or mutated E2F sites. TF, transcription factor binding site. B, some endogenous factors increase activity from the E2F1 minimal promoter. The E2F1 promoter constructs were transfected into NIH 3T3 cells and analyzed as described in the legend to Fig. 1. Reported is the promoter activity in S phase of a given construct containing a consensus transcription factor site and wild type E2F sites relative to the −45/+36 promoter construct. The right y axis represents the relative promoter activity of the reporter construct with a p53 binding site. C, some endogenous factors confer growth regulation to the E2F1 minimal promoter. The activity of reporter constructs containing wild type or mutated E2F sites with CCAAT, YY1, and p53 and three Rep3A GC boxes was measured in G₀ and S phase. Reported is the ratio of activity of a given construct in S phase to the activity of the same construct in G₀ phase cells.
E2F-mediated Growth Regulation

Fig. 3. Transcription factor cooperation can mediate activation in S phase. The activity in G₀ and S phase of E2F1 minimal promoter constructs containing wild type or mutated (ΔΔ) E2F sites plus a CCAAT site (B), GC boxes (C), or a YY1 site (D) is shown. The activity of the −45/+36 E2F1 minimal promoter by itself is also shown (A).

was independent of E2F (Fig. 2C).

E2F Cooperates with CCAAT, YY1, and GC Factors to Mediate Activation in S Phase—The mechanism by which E2F mediates transcriptional activation has been found to differ among promoters. In the B-Myb promoter, the E2F site functions solely to repress promoter activity in G₀ phase (11), whereas in the dihydrofolate reductase promoter the E2F site mediates regulation by increasing promoter activity in S phase (18, 19). A characteristic of E2F-mediated repression is that mutation of the E2F site results in increased promoter activity in G₀ phase cells, with promoter activity in S phase cells remaining high. However, in promoters regulated by E2F-mediated activation, mutation of the E2F site causes a decrease in transcriptional activity in S phase cells, and G₀ phase promoter activity remains low. To determine whether regulation of the E2F1 minimal promoter by CCAAT, YY1, and the GC element was mediated by repression in G₀ phase or activation in S phase, the DNAs of the different constructs were carefully quantitated, and then equal amounts of DNA were tested in a serum starvation and stimulation assay. Fig. 3A demonstrates the lack of growth regulation of the E2F1 minimal promoter in the presence or absence of the E2F sites. For the CCAAT constructs, a promoter having wild type or mutated E2F sites had the same luciferase activity in G₀ cells. However, luciferase activity in S phase of the promoter having mutated E2F sites was lower than activity of the wild type construct (Fig. 3B). Therefore, the CCAAT site conferred regulation to the minimal promoter by cooperating with the E2F sites to mediate activation in S phase. Mutation of the E2F sites in the Rep3A GC box-containing promoter construct also lowered S phase activity without increasing G₀ phase promoter activity (Fig. 3C). Therefore, as with the CCAAT site, the GC element regulates the minimal promoter by cooperating with E2F sites to mediate activation in S phase. We note that these results obtained using the synthetic E2F1 promoter constructs are in contrast to studies using the natural E2F1 promoter. Mutation of the E2F sites in the −176/+36 promoter results in a significant increase in transcriptional activity in G₀ phase, indicating that the −176/+36 construct is regulated by repression. For the YY1 site-containing promoter constructs, mutation of the E2F site resulted in a 3-fold increase in activity in G₀ phase and a 2-fold drop in activity in S phase (Fig. 3D). From these data we conclude that the growth regulation of the YY1 construct has two components; YY1 activity is repressed in G₀ by E2F protein complexes, and YY1 also cooperates with E2F to mediate activation in S phase. This combination of repression and activation is more similar to the results obtained with the −176/+36 E2F1 construct.

Although E2F sites have been identified in many promoters, they have not always been found to mediate G₀/S phase regulation. The data presented in this study suggest that a promoter needs specific elements in addition to the E2F site to be regulated. Our results indicate that the addition of a CCAAT site, a YY1 site, or multiple GC boxes can confer E2F-dependent activation of transcription to a simple E2F1 promoter that is not growth-regulated. We began these studies with the knowledge that the −176 to −45 region of the E2F1 promoter contained elements that were required for growth regulation. Although a sequence with homology to a YY1 site is found in the E2F1 promoter, we have not been able to detect YY1 binding to this site. The CCAAT oligonucleotide that was cloned upstream of the E2F1 minimal promoter is based on sequence from the E2F1 promoter, and we have previously shown that nuclear protein binds to this site. The Rep3A GC box with the E2F1 promoter identified an element located at −45 to −68 that has 78% identity to the Rep3A GC box, and gel shift analyses demonstrated that protein binds to this site (see below). Thus, two of the three elements that we identified as being able to cooperate with E2F factors are found in the E2F1 promoter. However, both the CCAAT and the GC elements have the potential to bind to a number of cellular proteins. To understand how these elements confer regulation to an E2F-responsive promoter, it is important to determine which proteins are cooperating with E2F.

At least four proteins with CCAAT box binding activity have

1 P. R. van Ginkel, K.-M. Hsiao, H. Schjerven, and P. J. Farnham, unpublished data.
been identified: NF-Y (also called CBF, CP1, or HAP), C/EBP, CTF/NF1, and H1TF2 (20–27). However, these proteins all prefer distinct sites, and the CCAAT site at −70 in the E2F1 promoter matches that of NFY. NFY is composed of three subunits, NFYa, NFYb, and NFYe (28, 29). To determine if NF-Y binds to the E2F1 promoter, antibodies raised against NF-Y subunits, NFYa or -Yb, but not by heat-treated antibodies, indicating that these supershifts were due to active antibody-antigen interactions (Fig. 4). In a parallel experiment, similar results were obtained using a probe containing the Y box of the major histocompatibility complex class II Ea gene, which was previously shown to bind NF-Y (30). These data indicate that NF-Y is the predominant protein in nuclear extract from mouse cells that binds to the CCAAT box from the E2F1 promoter and suggest that NF-Y may contribute to the growth regulation of the E2F1 promoter.

Using a gel mobility retardation assay, we demonstrate that protein binds to a region (−68/−45) of the E2F1 promoter that has homology to the Rep3A GC box. A competitor oligonucleotide containing a consensus Sp1 site from the −77 region of the E2F1 promoter competed for binding, while other binding sites did not (Fig. 5A). Using extracts prepared from Friend cells and antibodies to Sp1 and Sp3, we demonstrate that both Sp1 and Sp3 can bind to the −68/−45 region. We observed that neither the Sp1 nor the Sp3 antibodies alone completely supershifted the binding, but Sp1 and Sp3 antibodies together did completely supershift the band (Fig. 5B). Similar results were obtained using NIH 3T3 cells (data not shown). These findings are very similar to our data obtained from the Rep3A promoter GC element (31) and suggest that the elements from the Rep3A and the E2F1 promoters may bind the same factors, i.e. members of the Sp1 family of transcription factors.

Thus, YY1, NF-Y, and Sp1 family members can all cooperate with E2F factors to mediate growth-regulated transcription. At least two of these factors (NF-Y and Sp1) bind to the mouse E2F1 promoter. Although the binding of these factors to an E2F site-containing promoter region may not guarantee growth regulation, we note that many of the promoters known to be regulated by E2F contain at least one of these elements (1).

The E2F1 Transactivation Domain Is Sufficient to Regulate the E2F1 Minimal Promoter—Studies of Blau et al. (32) suggest that E2F1 does not require cooperation with additional factors to activate transcription in HeLa or 293 cells. Our data, that robust promoter activity in S phase requires E2F plus an additional transcription factor, are in seeming conflict with these results. One possible explanation for the apparent discrepancy is that the studies of Blau et al. employed a Gal4-E2F1 fusion protein. Therefore, we examined whether Gal4-E2F1 would be sufficient to confer growth-regulated activity or if cooperation between Gal4-E2F1 and other transcription factors would still be essential for high activity in S phase. These experiments required that we bring Gal4-E2F1 to a promoter that cannot be bound by endogenous E2Fs. Therefore, we utilized the E2F1 minimal promoter constructs having one or five Gal4 sites upstream of mutated E2F sites. Gal4-E2F1 and the reporter constructs were transfected into NIH 3T3 cells. Titration of the Gal4-E2F1 expression plasmid was performed and was critical for determining growth regulation, since excess Gal4-E2F1 protein will bind all of the Rb in the cell. This would alter cell cycle regulation of the transfected cell. Analysis of these promoter constructs demonstrates that Gal4-E2F1, but not the Gal4 DNA binding domain alone, can confer growth regulation to the E2F1 minimal promoter in the absence of additional transcription factor binding sites (Fig. 6). In the presence of Gal4-E2F1, the promoters with either one or five Gal4 sites had a 7-fold higher activity in S phase than in G0 phase.

**DISCUSSION**

An important question in the field of E2F-mediated growth regulation is why some, but not all, promoters having E2F sites display differential activity in G0 versus S phase. For example, deoxycytidine kinase has an E2F site in its promoter but does not display cell cycle-dependent regulation (2). Also, we and others have previously shown that deletion of sequences upstream of the E2F sites in the dihydrofolate reductase (18), E2F1 (13), B-Myb (11), and DNA polymerase-α (33) promoters greatly reduces growth-regulated transcriptional activity. We have now used the E2F1 minimal promoter to analyze the
promoter context necessary for E2F sites to mediate growth regulation. Since many promoters regulated by E2F sites are complex and contain multiple activator binding sites, our initial hypothesis to explain the lack of regulation of the E2F1 minimal promoter was that strong activators were needed for a high transcriptional activity. In G0 phase, this activity would then be repressed by Rb (or other pocket proteins) bound to E2F. As cells enter S phase, Rb phosphorylation would occur, relieving repression of the promoter. However, VP16, p53, Ets2, and MyoD conferred high transcriptional activity in both G0 and S phase, suggesting that simply bringing in strong activators does not ensure regulation of an E2F site-containing promoter. We did find that three different binding sites could confer regulation to the E2F1 minimal promoter. Further study of the mode of E2F-mediated regulation demonstrated that, instead of being repressed by E2F complexes in G0 phase, NF-Y, YY1, and Sp1 can cooperate with E2F to increase activity in S phase. Many cellular promoters that are regulated by E2F proteins also contain consensus Sp1 and CCAAT binding sites (34). In several cases, these sites are thought to be important for regulation. Studies of the human thymidine kinase promoter and mouse ribonucleotide reductase R2 promoters suggest that both E2F sites and CCAAT sites are critical for growth regulation (35, 36). Deletion of a region containing Sp1 sites reduces regulation of the dihydrofolate reductase and thymidine kinase.

**FIG. 5.** Sp1 and Sp3 bind the GC box in the E2F1 promoter. Gel mobility retardation analysis was performed using Friend or NIH 3T3 cell nuclear extract and an oligonucleotide containing the GC box of the E2F1 promoter. A, double-stranded oligonucleotides containing consensus binding sites for Sp1 (lane 4), CCAAT (lane 5), E2F (lane 6), Egr (lane 7), or Myc (lane 8) were used as competitors. No competitor was used in lane 2. In lane 3, cold probe was used as competitor. B, 2 μg anti-Sp1 and/or 4 μg anti-Sp3 antibody was added to the reaction mixture (lanes 3–5). The positions of supershifted or shifted bands and free probe are indicated by arrows.

**FIG. 6.** The E2F1 transactivation domain is sufficient to regulate the E2F1 minimal promoter. NIH 3T3 cells were cotransfected with 5 μg of the indicated reporter construct containing one or five Gal4 sites together with 0.1 μg of the Gal4 DNA binding domain (1–147) alone (Gal4DB) or of a Gal4-E2F1-(368–437) fusion construct and assayed in the serum starvation and stimulation assay (see Fig. 1). As shown, Gal4-E2F1 can confer a 7–8-fold regulation to the promoter having one or five Gal4 sites, whereas the Gal4DB alone does not confer growth regulation.

**FIG. 7.** Requirements for E2F-mediated growth regulation. A, E2F requires synergy with other transactivation domains. In the E2F1 minimal promoter, the E2F transactivation domain cannot activate the promoter, resulting in low activity in G0 and S phase. Brining activation domains of other transcription factors to the promoter can lead to transcriptional synergy, resulting in activation of the promoter. Our data with Gal4-E2F1 (Fig. 6) suggest that this type of synergy is not required for this E2F family member. B, E2F requires cooperation with other transcription factors for stable binding to promoter DNA. In the E2F1 minimal promoter, unstable binding of E2F to the recognition sites results in low promoter activity in G0 and S phase. Brining certain transcription factors to this promoter via their DNA binding sites can lead to cooperative binding with E2F, resulting in stable binding of E2F at the promoter. The solid arrows denote a high level of transcription, and the dashed arrows denote a low level of transcription.
promoters (18, 37). We note that the results presented in Fig. 2 suggest that consensus Sp1 sites cannot cooperate with E2F sites. While our studies were in progress, other groups found that Sp1 sites can influence E2F-mediated transcriptional regulation but that the exact spacing between the Sp1 sites and E2F site is crucial for this to occur (37, 38). Thus, it is likely that the differences observed between the ability of the Rep3A Sp1 site versus the consensus Sp1 site to cooperate with E2F are due to differences in spacing and/or orientation of the two elements. This, of course, also suggests that other factors that did not score positive in our assays could possibly cooperate with E2F if the spacing between the sites were optimized.

We propose that transcription factor cooperation explains why some, but not all, promoters that contain E2F sites are growth-regulated (Fig. 7). A number of hypotheses have been put forth to explain cooperation between transcription factors. One hypothesis is based on the observation that factors may contact different components of the basal transcription machinery. Blau et al. (32) suggest that factors that stimulate initiation versus elongation display cooperation because these two types of factors complement each other’s function. However, they found that the E2F1 transactivation domain is sufficient to stimulate both initiation and elongation. Their studies, taken in combination with our results showing that Gal4E2F1 can mediate growth regulation, suggest that the cooperation we see is not a result of a second transactivation domain complementing the function of the E2F1 transactivation domain. However, it is possible that E2F1 is not the E2F family member that is bound to the E2F sites in the E2F1 minimal promoter. Perhaps the transactivation domains of other E2Fs cannot stimulate both initiation and elongation and thus require transcriptional synergy to confer growth regulation (Fig. 7A). We note that NF-Yb, Sp1, and YY1 all have glutamine-rich regions in their activation domains. Therefore, this type of activation domain could be necessary to cooperate with the E2F to mediate activation. A second type of transcription factor cooperation is based on cooperative binding to DNA. It is possible that the ability of Gal4-E2F1 to confer regulation may be due to a property of its heterologous DNA binding domain. In the absence of certain upstream regulators, the endogenous E2F proteins may not be stably bound to the promoter DNA. Perhaps if E2F can be trapped on the DNA through interaction with upstream regulators, then growth regulation can be achieved (Fig. 7B). Recent studies (37, 38) have shown that Sp1 physically interacts with E2F family members and that binding of Sp1 in vivo to the thymidine kinase promoter can influence the occupancy of a nearby E2F site. Physical interaction between NF-Y or YY1 and E2F family members has not been reported. Experiments are in progress that will allow us to distinguish between these two alternative mechanisms of transcriptional synergy.

E2F has been postulated to confer growth regulation via repression (e.g. in the B-Myb promoter; Ref. 11) and activation (e.g. in the dihydrofolate reductase promoter; Ref. 39). These studies did not determine if the difference in the mechanism by which E2F regulates these two promoters was due to core promoter elements or subtle differences in E2F sites. We have previously shown that a longer –176/+36 E2F1 promoter construct is regulated by repression, whereas we now show that a minimal E2F1 promoter is regulated by activation. Since the same E2F sites are present in both constructs, we suggest that the distinction is due to the variety of transcription factors present in a given promoter (Fig. 8). In complex promoters having many upstream sites, E2F’s main role may be to keep transcription low in G0 phase. In such promoters, mutation of the E2F site will cause an increase in activity in G0 phase cells (Fig. 8, bottom panel). In contrast, in simple promoters that lack upstream factors, E2F may be required for activation. In these promoters, mutation of the E2F site will not result in high transcriptional activity in G0 due to an essential requirement for the E2F transactivation domain for promoter activity (Fig. 8, top panel). Thus, the main effect will be loss of activation in S phase. In support of this model, Fry et al. (19) recently showed that mutation of the E2F site in the dihydrofolate reductase promoter, the core promoter elements specify activation by E2F. If the dihydrofolate reductase E2F site is replaced by the B-Myb E2F site in the dihydrofolate reductase promoter, this promoter is still regulated by E2F-mediated activation and not repression.

In summary, we have used the E2F1 minimal promoter to further characterize the promoter requirements for E2F-mediated growth regulation. We propose that upstream factors influence E2F-mediated growth regulation in two ways: 1) they are required to confer growth-regulated transcriptional activity; and 2) the number and type of upstream regulators determines whether the main role of E2F is activation or repression.

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FIG. 8. E2F sites can regulate via activation or repression. Bringing certain transcription factors to the E2F1 minimal promoter increases promoter activity in S phase, while G0 activity remains low. This regulation is analogous to that of the dihydrofolate reductase promoter, which is also regulated by activation. Mutation of the E2F sites leads to loss of S phase induction and thus low promoter activity throughout the cell cycle. In complex promoters, however, E2F is mainly a repressor. Mutation of the E2F sites leads to increased promoter activity in G0 and thus high promoter activity throughout the cell cycle.
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