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A Protein Synthesis-Dependent Increase in E2F1 mRNA Correlates with Growth Regulation of the Dihydrofolate Reductase Promoter

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Enhanced expression of genes involved in nucleotide biosynthesis, such as dihydrofolate reductase (DHFR), is a hallmark of entrance into the DNA synthesis (S) phase of the mammalian cell cycle. To investigate the regulated expression of the DHFR gene, we stimulated serum-starved NIH 3T3 cells to synchronously reenter the cell cycle. Our previous results show that a *cis*-acting element at the site of DHFR transcription initiation is necessary for serum regulation. Recently, this element has been demonstrated to bind the cloned transcription factor E2F. In this study, we focused on the role of E2F in the growth regulation of DHFR. We demonstrated that a single E2F site, in the absence or presence of other promoter elements, was sufficient for growth-regulated promoter activity. Next, we showed that the increase in DHFR mRNA at the G_1/S-phase boundary required protein synthesis, raising the possibility that a protein(s) lacking in serum-starved cells is required for DHFR transcription. We found that, similar to DHFR mRNA expression, levels of murine E2F1 mRNA were low in serum-starved cells and increased at the G_1/S-phase boundary in a protein synthesis-dependent manner. Furthermore, in a cotransfection experiment, expression of human E2F1 stimulated the DHFR promoter 22-fold in serum-starved cells. We suggest that E2F1 may be the key protein required for DHFR transcription that is absent in serum-starved cells. Expression of E2F also abolished the serum-stimulated regulation of the DHFR promoter and resulted in transcription patterns similar to those seen with expression of the adenoviral oncoprotein E1A. In summary, we provide evidence for the importance of E2F in the growth regulation of DHFR and suggest that alterations in the levels of E2F may have severe consequences in the control of cellular proliferation.

A commonly employed model system for studying reentry into the proliferative state is serum starvation and subsequent stimulation of fibroblasts in culture. Addition of high concentrations of serum or growth factors to quiescent cells leads to a synchronously growing population in which transient activation and repression of growth-responsive genes can be monitored. Between 15 min and 4 h after serum stimulation, the immediate-early genes are expressed. These genes are transcribed in the presence of protein synthesis inhibitors, indicating that preexisting factors in quiescent cells are sufficient for activation of transcription (45). The early-response gene products include transcription factors such as Fos and EGR2. Several hours later (8 to 12 h after serum stimulation), a wave of gene expression requiring protein synthesis occurs at the transition from G_1 to DNA synthesis (S) phase. This protein synthesis requirement suggests that factors other than those present in quiescent cells are necessary for activation of transcription. Many late-serum-response gene products, such as DNA polymerase alpha, thymidine kinase, thymidylate synthase, carbamoyl phosphate synthase-aspartate carbamoyltransferase-dihydroorotase (CAD), and dihydrofolate reductase (DHFR), are required for DNA synthesis. Protein synthesis is also required for DNA synthesis (7), suggesting that activation of some of these genes may be required for entry into S phase.

We are using the murine DHFR promoter as a model to study the regulation of late-serum-response genes. This gene encodes an enzyme involved in de novo synthesis of purines, thymidylate, and glycine. DHFR mRNA and protein concentrations increase at the G_1/S-phase boundary of the cell cycle in mouse, human, and hamster cells (16, 19, 26, 38, 46). DHFR promoter sequences extending from −270 to +20 (relative to the transcription initiation site) are sufficient to confer similar regulation on a reporter gene (36). We have previously demonstrated that DHFR promoter sequences from the initiation region are critical both in selection of the start site of transcription and for increased transcription at the G_1/S boundary (35, 36). When an oligonucleotide spanning this region is used in DNA affinity chromatography of HeLa nuclear extract, the predominant protein in the purified fraction is a 180-kDa protein termed HIP1 (housekeeping initiator protein 1) (36). However, more-recent experiments have shown that DNA binding activity corresponding to a protein of about 60 kDa is also detected in the column fraction (33). It has been noted previously that the DHFR promoter harbors two overlapping consensus binding sites for the 60-kDa transcription factor E2F (6) which are located in the −8 to +1 region of the mouse DHFR promoter. Moreover, purified cloned human E2F1 can bind to these sequences (28). In consideration of the new data, the function of the HIP1 protein is unclear. Because cloned E2F1 can bind to this sequence, we now refer to this region of the DHFR promoter as the E2F element.

E2F was originally identified as a DNA-binding protein that activates the adenovirus E2 promoter (56). The E2F site has been shown to activate (6) and repress (24, 36, 50, 54) cellular promoters in proliferating cells. One proposed mechanism is that E2F regulates transcription by interacting with the retinoblastoma gene product (RB), a tumor suppressor

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protein (9, 10, 23, 28, 49). The interaction of RB and E2F was confirmed when E2F1 (also called RBAP1 [RB-associated protein 1] [28] or RBP3 [RB-binding protein 3] [23]) was cloned by probing a cDNA library with radiolabeled RB. In this report, E2F1 refers to the cloned human or mouse gene used in our assays. E2F refers to the protein with general activities possessed by E2F1 or any related members of an E2F family. The adenoviral oncoprotein, E1A, can mediate activation via an E2F element (3, 24, 56) and is thought to confer some of its transforming effects by interacting with RB and dissociating cellular proteins such as E2F from an RB complex (1, 10, 25, 49, 55). The finding that E2F associates with a tumor suppressor protein which may be a key mediator of the regulated cellular events leading to DNA synthesis (8, 12, 20, 21) indicates that tight regulation of E2F may be critical for normal cell growth.

The goal of our work is to understand the mechanism by which DHFR expression at the G_1/S boundary is increased. We have shown previously that the E2F site is required for serum-regulated expression from the DHFR promoter (36). Here we employ a serum starvation and stimulation assay to examine the role of the E2F binding site and the E2F protein in regulating expression from the DHFR promoter. First, we show that the E2F element is sufficient to create a growth-regulated promoter and that the regulated expression of DHFR mRNA requires protein synthesis. Next, we present evidence that murine E2F1 is also a late-serum-response gene. Finally, we show that constitutive expression of human E2F1 increases the activity of the DHFR promoter in serum-starved cells. Taken together, our data suggest that regulation of DHFR transcription is a direct result of levels of E2F.

MATERIALS AND METHODS

Plasmids. The pST410 plasmid contains DHFR sequences from −365 to +61 in pUC9 (16). To create E2F-luc, the oligonucleotide 5′-CTAGCAGCTGCTGCGATTTCGCGC CAAACTTGACG-3′, which contains −20 to +9 from the DHFR promoter plus a *Pvu*II site for screening and *Xho*I and *Nhe*I sites at the 5′ and 3′ ends, was inserted into the vector pGLBasic (Promega Biotec), digested with *Xho*I and *Nhe*I. To create E2F-SV40e-luc, the same sequence was inserted into the *Xho*I- and *Nhe*I-digested pGLPromoter (Promega Biotec), which contains the simian virus 40 (SV40) early promoter upstream of the luciferase cDNA. To create Sp1-luc, the oligonucleotide 5′-CGCGTGGGCGGAACTGGGC GGAGTTAGGGGCGGGA-3′, which contains three consensus Sp1 binding sites from the SV40 promoter, was inserted at the *Sma*I site of pGLBasic. DHFR-luc (originally called pWTluc) and the DHFR promoter with a mutant E2F site, pNWluc, were previously described (36). Fos-luc contains sequences from −356 to +109 of the human c-*fos* promoter (11, 44). EGR2-luc contains sequences from −840 to +6 of the human EGR2 promoter (11). The human E2F1 expression construct, pCMV-RBAP1 (CMV-E2F1), and the control vector, pCMV (28), were previously described. The 12S E1A expression construct, pCMV-E1A, was a gift from Joe Nevins. The control vector was created by deletion of the *Bam*HI fragment of the E1A cDNA. pBSM13 (Stratagene) was added as a DNA carrier in transfection experiments. pBSM13i contains a partial cDNA of mouse E2F1 (33) inserted into the *Eco*RI site of pBSM13+. pBSGAPDH contains the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA inserted in the *Pst*I site of pBSM13− (11, 53).

Cell culture and transfections. NIH 3T3 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 5% (vol/vol) defined-supplemented bovine calf serum (HyClone)–100 U of penicillin per ml–100 μg of streptomycin per ml (GIBCO) in 5% CO_2 at 37°C. Cells were passaged when subconfluent with 0.05% trypsin-EDTA (GIBCO) in phosphate-buffered saline (PBS). Cells to be harvested for RNA analysis were placed in starvation medium (0.5% [vol/vol] serum) for 45 h. At 30 min before stimulation with 10% (vol/vol) serum, half the samples received cycloheximide to a final concentration of 10 μg/ml. The cells were harvested at the indicated time points. Cell pellets were frozen in liquid nitrogen and stored at −70°C.

Calcium phosphate transfections were performed as described previously (36) with the following alterations. A total of 2×10^5 NIH 3T3 cells per 60-mm dish was plated 15 to 20 h before transfection. Each plate received a precipitate of 15 μg of DNA in 450 μl of transfection buffer (36)–50 μl of 1.25 M $CaCl_2$. A total of 5 μg of E2F-SV40e-luc reporter plus 10 μg of sonicated salmon sperm DNA, 15 μg of E2F-luc, 15 μg of Sp1-luc, 5 μg of DHFR-luc, 5 μg of pNWluc, 1 μg of Fos-luc, or 2 μg of EGR2-luc was used per transfection. Plasmids pCMV-E2F and the vector control, pCMV, were used at a 1:1 ratio with the reporter plasmids. pCMV-E1A and the control vector plasmid were used at a 1:4 ratio with the reporter plasmids. pBSM13+ was added to transfections to bring the total DNA to 15 μg. After 6 h, the cells were rinsed with maintenance medium, shocked for 4 min with 1 ml of 15% (vol/vol) glycerol in transfection buffer, and allowed to incubate for 1 to 3 h in maintenance medium. The medium was then replaced with starvation medium. After 45 to 60 h, the cells were harvested or the medium was replaced with stimulation medium. Cells were harvested at the indicated times, and luciferase activity of total cell lysates was assayed with a luminometer (Analytical Luminescence Laboratory). At least two DNA preparations were tested, and at least three separate experiments were averaged for each datum point. Error bars represent the standard errors of the mean. Data are represented as ratios between the indicated populations of cells, not raw numbers, because the variation in transfection efficiency (due to cell passage number and different DNA preparations and other reagents) makes an averaged raw number uninterpretable. The activity of each luciferase reporter construct was approximately 20- to 100-fold above that of the background unless otherwise stated.

The progression of cells through the cell cycle was monitored by flow cytometry as described previously (36). Briefly, cells fixed in 70% ethanol were stained with 50 μg of propidium iodide per ml in PBS and analyzed on an Epics Profile II flow cytometer (Coulter Electronics, Inc.) to generate histograms of cell number versus DNA content.

RNA preparation and analysis. To prepare cytoplasmic RNA, 10^8 cells were resuspended in lysis buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-Cl [pH 8.6], 0.5% [vol/vol] Nonidet P-40, 10 mM vanadyl ribonucleoside complex), incubated on ice for 5 min, and spun in the microcentrifuge for 20 min. The supernatant was collected, and an equivalent volume of PK buffer (0.2 M Tris-Cl [pH 7.5], 25 mM EDTA, 0.3 M NaCl, 2% [wt/vol] sodium dodecyl sulfate [SDS], 0.5 μg of proteinase K per μl) was added and incubated for 30 min at 42°C. After phenol and chloroform extraction, the samples were ethanol precipitated. RNA was quantitated spectrophotometrically, and the concentrations were verified on a 1% agarose gel by ethidium bromide staining.

RNase protection assays were carried out as described previously with modifications (52). An RNA probe complementary to the 5′ ends of the DHFR mRNAs was transcribed from the ST410 plasmid linearized with *Eco*RI by using Sp6 RNA polymerase. A total of 20 μg of cytoplasmic NIH 3T3 RNA was incubated with 10^5 cpm of probe in 8 μl of formamide (pH 8)–2 μl of hybridization buffer [200 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) disodium salt (PIPES), 2 M NaCl, 5 mM EDTA] at 85°C for 10 min and then at 52°C for 3 h. The RNA was then digested with 20 μg of RNase A in 300 μl of digestion buffer (10 mM Tris-Cl [pH 7.5], 5 mM EDTA, 300 mM NaCl) for 30 min at 30°C. The reaction mixtures were treated with 0.65% (wt/vol) SDS and 0.167 μg of proteinase K per μl for 15 min at 37°C and then phenol and chloroform extracted before being ethanol precipitated. The products were resolved by gel electrophoresis and visualized by autoradiography.

Northern (RNA) analysis was performed with 5 μg of RNA resolved on 6% formaldehyde–0.8% agarose gels, in running buffer (20 mM morpholinepropanesulfonic acid [MOPS] [pH 7.0], 5 mM sodium acetate, 1 mM EDTA). After the gel was stained with ethidium bromide to confirm equal loading, the RNA was transferred to a GeneScreen membrane (Dupont, NEN) as specified by the manufacturer. The *Eco*RI fragment of pBSM13i and the *Pst*I fragment of pBSGAPDH were isolated to make probes for E2F and GAPDH mRNAs, respectively. A total of 200 ng of each fragment was labeled by nick translation (43) and denatured with 0.15 M NaOH before being neutralized with 0.15 M HCl. The membrane was prehybridized with 50% formamide–10% dextran sulfate–10× Denhardt solution–1% [wt/vol] SDS–0.1% [wt/vol] sodium pyrophosphate–50 mM Tris-Cl [pH 7.5]–1 M NaCl–100 μg of denatured salmon sperm DNA per ml for 1 h at 42°C. The radiolabeled probe was then added, and hybridization continued for approximately 40 h for the E2F probe or 12 h for the GAPDH probe. Filters were washed in 2× SSC (0.30 M NaCl, 0.015 M sodium acetate)–0.5% (wt/vol) SDS for 30 min at room temperature and then for 30 min at 55 to 60°C. The signals were visualized by autoradiography.

RESULTS

The E2F site is necessary and sufficient for the increase in DHFR promoter activity at the G_1/S boundary. In previous work, we demonstrated that the DHFR promoter fragment from −270 to +20 confers late-serum-response regulation on the luciferase cDNA in a serum starvation and stimulation assay. Selected point mutations in the E2F binding site abolish protein binding and prevent an increase in luciferase activity at the G_1/S boundary. We concluded that protein binding to the E2F element is necessary for regulation of the DHFR promoter (36). However, these experiments did not determine whether other elements in the DHFR promoter were also required for growth regulation. To determine whether the E2F element could confer growth regulation on a constitutive promoter, we inserted the E2F oligonucleotide upstream of the SV40 early promoter to create E2F-SV40e-luc. After transfection with SV40e-luc or E2F-SV40e-luc, the cells were forced into quiescence in starvation medium. The starvation medium was then replaced with stimulation medium for synchronous reentry into the cell cycle. Samples were harvested at indicated times and analyzed for DNA content (50) and luciferase activity. The addition of the E2F element did not alter the activity of the SV40 early promoter in proliferating cells more than twofold (50). Without the E2F site, the activity of SV40e-luc increased about threefold after serum stimulation. However, the activity of E2F-SV40e-luc peaked during S phase (Fig. 1A) at the same time as did activity from DHFR-luc (36). These results indicated that the E2F element can confer late serum responsiveness on a constitutive promoter. However, the SV40 early promoter contains many transcription factor binding sites, including six Sp1 binding sites. It remained possible that growth regulation by the E2F element required other protein binding sites.

To determine whether a single E2F site was sufficient to confer growth regulation on a heterologous reporter gene, we constructed E2F-luc. This plasmid contains sequences from −20 to +9 of the DHFR promoter inserted 5′ of the luciferase cDNA. Because this small region contains both a transcription initiation element (30, 35) and E2F sites, it can function alone as a promoter. In quiescent cells, luciferase activity from the E2F-luc construct was about threefold above the background activity from the luciferase vector. Twelve hours after serum stimulation, when the cells were in mid-S phase, luciferase activity from E2F-luc peaked at a level 35-fold higher than that in starved cells (Fig. 1B). Luciferase activity of the vector did not increase above that of the background at any time after serum stimulation (50). This result indicated that the E2F element is sufficient to mediate a transcriptional increase at the G_1/S boundary in the absence of other protein binding sites.

To confirm that the regulation of E2F-luc was not a result of vector sequences, we tested a construct containing three Sp1 consensus binding sites. Sp1-luc had approximately 100-fold higher activity in quiescent cells than did the E2F construct because of the strong activation properties of Sp1 (27). However, activity from Sp1-luc did not change after serum stimulation (Fig. 1B). This result confirms our previous finding of constitutive activity from the SV40 early promoter and from a DHFR promoter with a mutant E2F site (36), both of which contain several Sp1 sites. If, as others have suggested, Sp1 is a direct or indirect target for transcriptional control by RB (10, 29, 41), our results indicate that not all RB-responsive elements are sufficient to confer serum response regulation on a heterologous gene.

The increase in DHFR mRNA at the G_1/S-phase boundary requires protein synthesis. To determine whether the increase in DHFR mRNA requires de novo protein synthesis, a characteristic of late-serum-response genes, DHFR mRNA was examined in NIH 3T3 cells that were serum starved and then stimulated in either the presence or the absence of the protein synthesis inhibitor, cycloheximide (Fig. 2). Analysis of DNA content by flow cytometry demonstrated that the cells entered quiescence during the starvation period and that S phase began approximately 10 h after release from serum starvation in the absence of cycloheximide. The inhibition of protein synthesis has been shown by others to prevent entry into S phase (7). As expected, cells did not enter S phase after serum stimulation in the presence of cycloheximide (Fig. 2B).

To monitor DHFR mRNA levels as cells reentered the cell cycle, RNase protection assays were performed with an RNA probe that would produce 66- and 126-nucleotide bands corresponding to the major and minor DHFR transcription initiation sites, respectively (47). In the absence of cycloheximide, DHFR mRNA levels increased at about the same time as the cells entered S phase (Fig. 2A). However, there was no appreciable increase in DHFR mRNA by 16 h in the cells treated with cycloheximide. Ornithine decarboxylase (50) and GAPDH (Fig. 3B) mRNAs were easily de-

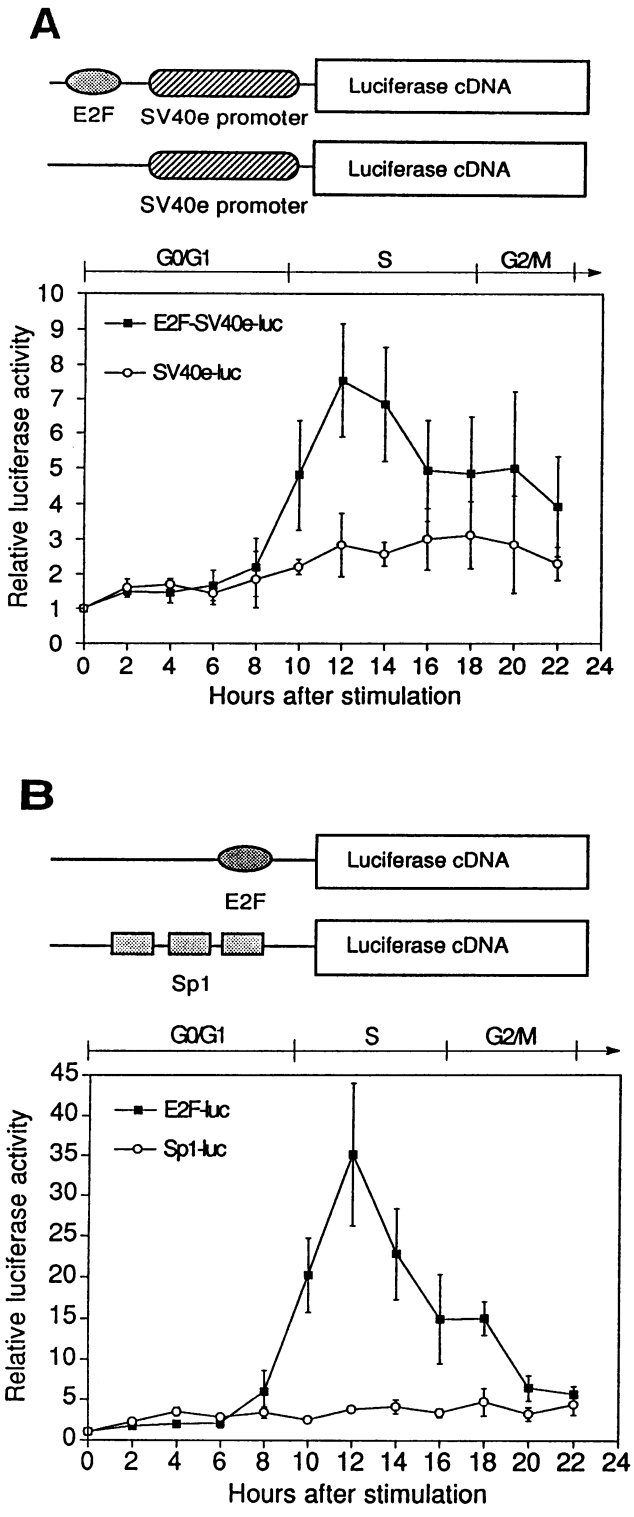

FIG. 1. The E2F element from the DHFR promoter is sufficient to confer growth regulation on a heterologous reporter gene in either the presence or the absence of other promoter elements. The activities of SV40e-luc versus E2F-SV40e-luc (A) or E2F-luc versus Sp1-luc (B) were examined in transiently transfected 3T3 cells in serum starvation and stimulation experiments. Luciferase constructs are represented schematically at the tops of the figures. The boundaries of the different stages of the cell cycle were previously determined by flow cytometry. Luciferase values are reported as a ratio of the activity of each promoter at different times in the cell cycle relative to the activity of the same promoter in starved cells. Bars represent standard errors of the mean.

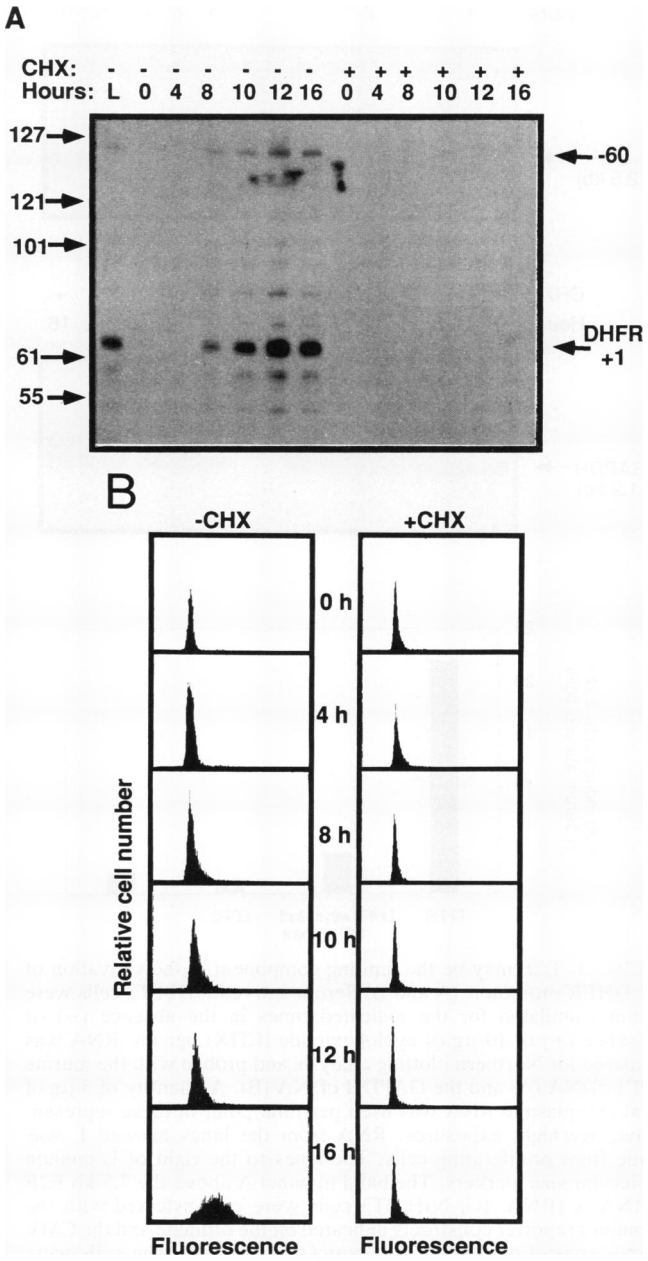

FIG. 2. The increase in DHFR mRNA at the G_1/S boundary requires protein synthesis. NIH 3T3 cells were serum starved for 45 h and serum stimulated in the absence (−) or presence (+) of 10 μg of cycloheximide (CHX) per ml for the indicated times for RNase protection assays (A) and determination of DNA content by flow cytometry (B). In panel A, the arrows indicate the positions of bands representing the major (+1) and minor (−60) DHFR start sites. Molecular size markers are indicated at the left of the figure in base pairs. RNA from the lane marked L was made from proliferating cells. In panel B, G_0 and G_1 cells contain a 2 N DNA content and therefore fluoresce half as much as G_2 and M cells, which have a 4 N DNA content. Cells in S phase were designated as the cells between the G_0 and G_1 peak and the G_2 and M peak. In the absence of cycloheximide, the G_1/S boundary occurs approximately 10 h after serum stimulation when approximately 20% of the cells have entered S phase (see Fig. 5 for graph).

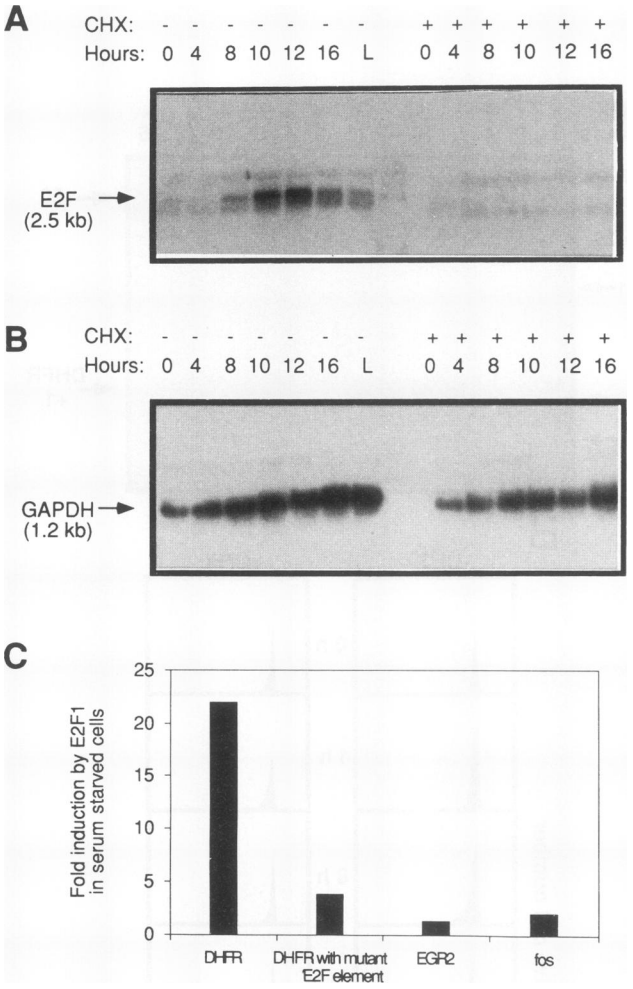

FIG. 3. E2F may be the limiting component in the activation of the DHFR promoter. (A and B) Serum-starved NIH 3T3 cells were serum stimulated for the indicated times in the absence (−) or presence (+) of 10 μg of cycloheximide (CHX) per ml. RNA was prepared for Northern blotting analysis and probed with the murine E2F1 cDNA (A) and the GAPDH cDNA (B). A quantity of 5 μg of total cytoplasmic RNA was used per time point in these representative overnight exposures. RNA from the lanes marked L was made from proliferating cells. The lanes to the right of L contain molecular size markers. The band in panel A above the 2.5-kb E2F mRNA is rRNA. (C) NIH 3T3 cells were cotransfected with the promoter reporter constructs indicated on the ordinate and the CMV vector control or CMV-E2F1 expression plasmid. The cells were incubated in starvation medium and then harvested for luciferase assays. Luciferase values are reported as a ratio of the activity of each promoter transfected with CMV-E2F1 relative to that of the same promoter transfected with CMV-vector control alone.

tected in the cycloheximide-treated samples, indicating that intact RNA was present. These data confirm that DHFR mRNA levels increase at the G_1/S-phase boundary as previously observed (46) and show that, unlike with early-response genes, production of the DHFR mRNA is abolished by protein synthesis inhibitors. The lack of DHFR mRNA after serum stimulation of cycloheximide-treated cells either may be due to the accumulation of a repressor (15) of DHFR transcription or may indicate that one or more proteins required for DHFR transcription are missing in quiescent cells.

E2F1 is a late-serum-response gene, suggesting that E2F1 protein may be the limiting component in the activation of the DHFR promoter. Since the levels of DHFR mRNA do not increase in the presence of cycloheximide (Fig. 2A), one prediction is that the cell cycle-specific increase in DHFR expression requires de novo synthesis of an activator protein. Since the E2F site is required for the increase in DHFR transcription at the G_1/S boundary, E2F1 may be limiting in serum-starved cells (17, 36). In support of this idea, E2F1 mRNA is present at low levels in quiescent human T cells and increases in S phase after mitogen treatment (28). In contrast, others have suggested that E2F is present throughout the proliferative cell cycle (9, 40, 49). To examine the pattern of E2F1 expression after growth stimulation of quiescent 3T3 cells, we probed a Northern blot of RNA from serum-starved and -stimulated cells with a mouse E2F1 cDNA probe. The abundance of the E2F1 (Fig. 3A) mRNA reached a maximum during S phase. The increase in E2F1 mRNA required protein synthesis, as there was no detectable transcript in serum-stimulated cells treated with cycloheximide (Fig. 3A). The delayed increase in E2F1 mRNA after addition of serum (8 to 10 h) and the protein synthesis requirement for this increase indicate that E2F1 is also a late-serum-response gene. GAPDH message was present in RNA samples prepared from serum-stimulated cells treated with cycloheximide, indicating that the absence of E2F1 mRNA in these samples was not due to general mRNA degradation (Fig. 3B). The correlation between the abundance of E2F1 mRNA and activation of the DHFR promoter suggests that the low DHFR promoter activity in G_0 and G_1 cells may be due to limiting amounts of E2F1 protein. Once an antibody against mouse E2F1 is available, it will be possible to determine whether E2F1 protein concentrations correlate with E2F1 mRNA concentrations.

If low E2F levels are responsible for low DHFR promoter activity in quiescent cells, we predict that increasing the amount of E2F1 protein present during G_0 and G_1 would result in higher DHFR promoter activity. To examine this hypothesis, we performed transient cotransfection experiments with DHFR-luc and either the constitutively expressed human E2F1 construct, CMV-E2F1, or the cytomegalovirus (CMV) promoter-vector control. One hour after transfection, the cells were placed in starvation medium for 50 to 60 h and then harvested without serum stimulation. DHFR promoter activity was approximately 22-fold higher in serum-starved cells constitutively expressing E2F1 (Fig. 3C) than in cells transfected with the same plasmid lacking the E2F1 cDNA. The same experiment was performed with a DHFR promoter with a mutant E2F site. In this case, luciferase activity increased less than fourfold in the presence of E2F1 relative to the vector control. We have shown that upstream DHFR promoter sequences can contribute to E2F1 activation of the DHFR promoter (18). The fourfold increase in activity of the DHFR promoter with a mutant E2F site is likely due to the upstream sequences. E2F1 did not increase the activity of all growth-regulated promoters. For example, the activities of the early-response EGR2 and Fos promoters (Fig. 3C) and of the late-response CAD promoter (50) were not increased by expression of E2F1. These results indicate that specific activation of the DHFR promoter by E2F1 occurs primarily through the E2F element. Because an increase in E2F1 mRNA correlates with an increase in DHFR transcription (both at the G_1/S-phase boundary of serum-stimulated cells and in serum-starved cells constitutively expressing E2F1), we propose that E2F1 is a limiting factor for DHFR transcription.

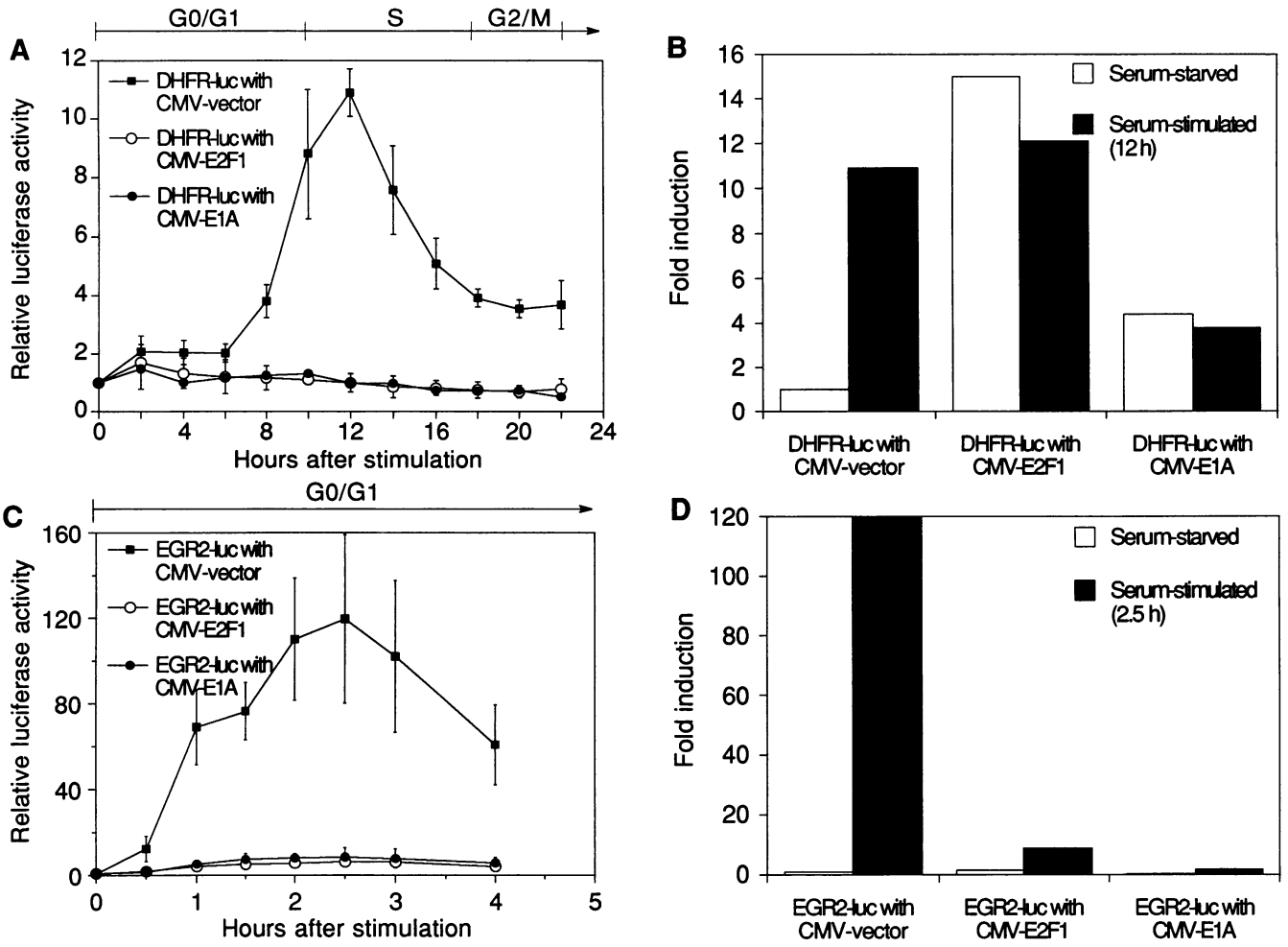

FIG. 4. Constitutive expression of E2F1 or E1A eliminates the serum-induced transcriptional increase from the DHFR and EGR2 promoters. (A) NIH 3T3 cells were transfected with DHFR-luc and either CMV-E2F1, CMV-E1A, or a CMV-vector control. The cells were harvested at the indicated times after serum stimulation. Luciferase values are reported as a ratio of the activity of DHFR-luc in the presence of an expression plasmid relative to the promoter activity with the same expression plasmid in starved cells. Cell cycle phase boundaries are given for the cells transfected with the CMV-vector control. (B) Luciferase values are reported as a ratio of the activity of DHFR-luc in the presence of an expression plasmid relative to the promoter activity in the presence of CMV-vector in starved cells. (C) The same experiment as in panel A was performed, but EGR2-luc was used in the place of DHFR-luc. (D) The same experiment as in panel B was performed, but EGR2-luc was used in the place of DHFR-luc. For panels A and C, bars represent standard errors of the mean.

Overexpression of E2F abolishes the serum-induced transcriptional increase from early- and late-serum-response promoters. The effects of constitutive, high-level expression of E2F1 on DHFR growth regulation were tested in the serum starvation and stimulation assay after cells were cotransfected with DHFR-luc and CMV-E2F1. In the absence of overexpressed E2F1 (CMV-vector), DHFR-luc activity increased 10-fold 12 h after serum addition, as previously reported (36). In the presence of the CMV-E2F1 construct, the luciferase activity was 15-fold higher in the serum-starved cells than in the presence of the vector control, but no further increase was observed for the following 22 h (Fig. 4A and B). Thus, serum regulation of the DHFR promoter could not be detected in the presence of constitutively expressed E2F1. As shown in Fig. 4B, loss of regulation from the DHFR promoter was due to high levels of transcription in both serum-starved and serum-stimulated cells. It was possible that overexpression of E2F1 increased transcription from the DHFR promoter to a level at which another component of the cellular transcriptional or translational machinery was limiting and no further increase in luciferase activity was possible. Therefore, we performed the same experiment with E2F-luc. Expression from CMV-E2F also led to constitutive expression of E2F-luc (50). Compared with that of the DHFR promoter, the activity of E2F-luc is low throughout the assay. Therefore, it is unlikely that general transcription or translation factors are limiting in this experiment. Cotransfection of late-serum-response promoters with other cDNAs (such as Sp1, EGR2, and c-Raf-1) does not abrogate all growth response (37, 50).

The viral oncoprotein E1A can transform mammalian cells (48). It is thought to do this at least in part by binding to RB and preventing it from inhibiting other cellular proteins (such as E2F) that stimulate cell proliferation. To determine whether loss of cell growth control due to E1A expression leads to deregulation of cell cycle-specific expression from the DHFR promoter, we cotransfected NIH 3T3 cells with E1A and DHFR-luc. The cells were serum starved and then

harvested at different times after serum stimulation. In the presence of CMV-E1A, DHFR promoter activity was fourfold higher in serum-starved cells than in the presence of the vector control (50). As seen in Fig. 4A, expression of E1A abolished the serum-induced transcriptional increase from the DHFR promoter. Similar to the results with E2F1, expression of E1A resulted in constitutive levels of transcription from the DHFR promoter in serum-starved and -stimulated cells (Fig. 4B). This could be due to specific deregulation of E2F-activated genes or to a global deregulation of cell cycle control. To distinguish these possibilities, we also tested the effect of E1A expression on the immediate-early-serum-response EGR2 promoter. Fig. 4C demonstrates that serum-regulated expression from the EGR2 promoter was also abolished by E1A. In contrast to the DHFR promoter, activity from the EGR2 promoter is low in both serum-starved and serum-stimulated cells (Fig. 4D). These results are best explained if the overexpression of E1A keeps the cells from entering quiescence after serum removal, possibly because of a functional loss of RB protein. An alternative explanation, that the cells are irreversibly blocked in G_0, is incompatible with the known effects of E1A on cellular proliferation (4, 34).

As shown in Fig. 4A, overexpression of E2F1 resulted in loss of growth regulation from the DHFR promoter. This deregulation could be specific to E2F-driven genes or could be a result of a more general disruption of the serum response pathway. To distinguish these possibilities, we examined the effects of E2F1 overexpression on the serum regulation of the EGR2 promoter, which neither contains consensus E2F sites nor is activated by E2F1 in quiescent cells (Fig. 3C). EGR2-luc activity increased 120-fold 2.5 h after serum stimulation (Fig. 4C and D). However, in the presence of constitutively expressed E2F1, EGR2 promoter activity increased only ninefold at this same time point. Thus, expression of E2F1 resulted in low EGR2 promoter activity in both serum-starved and serum-stimulated cells. Similar results (i.e., the loss of a serum-induced transcriptional increase) were also obtained after cotransfection of E2F1 with the early-serum-response Fos and late-serum-response CAD promoters (50). These results suggest that, similar to E1A expression, a general disruption of cell cycle-regulated transcription occurs in the presence of constitutively expressed E2F1. In conclusion, we have demonstrated that E2F1 can influence transcription in two ways. First, E2F1 can act as a transcriptional activator in serum-starved cells via an E2F element. Second, constitutive expression of E2F1 can disrupt the serum-induced transcriptional increase from both early- and late-serum-response promoters, including promoters that lack an E2F site. Perhaps overexpression of E2F1, like that of E1A, prevents cells from entering a quiescent state by complexing all the RB protein in the cell. Alternatively, overexpression of E2F1 may prevent cells from exiting G_0 after serum stimulation, perhaps by activating the RB promoter via its E2F sites and increasing the amount of RB in a cell. The production of cell lines stably expressing E2F1 will allow these possibilities to be distinguished. Although the mechanism is unclear, our results suggest that altered expression of E2F1 may have a global effect on the cell cycle.

DISCUSSION

We propose a model in which changes in the concentration of E2F1 protein in the cell are responsible for a transient increase in E2F-driven promoter activity at the G_1/S boundary after stimulation of serum-starved cells (Fig. 5). The model is based on the following observations. First, the DHFR element is both necessary and sufficient to create a promoter that is activated at the G_1/S-phase boundary. Second, the increase in DHFR mRNA requires de novo synthesis of a protein that is lacking in serum-starved cells. Third, the levels of E2F1 mRNA are very low in serum-starved and early-G_1 cells but increase at the G_1/S-phase boundary in a protein synthesis-dependent manner. Fourth, if E2F1 is expressed in serum-starved cells from a constitutive promoter, DHFR promoter activity is greatly increased. A possible sequence of events for G_1/S-phase gene activation could be as follows. Serum added to quiescent cells initiates various signal transduction pathways resulting in the synthesis of immediate-early genes. An early-response transcription factor initiates events which lead to the activation of the E2F1 promoter, and E2F1 mRNA levels begin to increase. As the cells approach S phase, the amount of E2F1 protein reaches the level needed to activate the DHFR promoter. E2F1 mRNA levels then decrease in late S phase, and DHFR transcription drops. Various aspects of this model can be tested. For example, cloning of the E2F1 promoter will allow activation of E2F1 transcription by early-response factors to be examined. Also, once an antibody against mouse E2F1 is available, the levels of E2F1 protein can be measured.

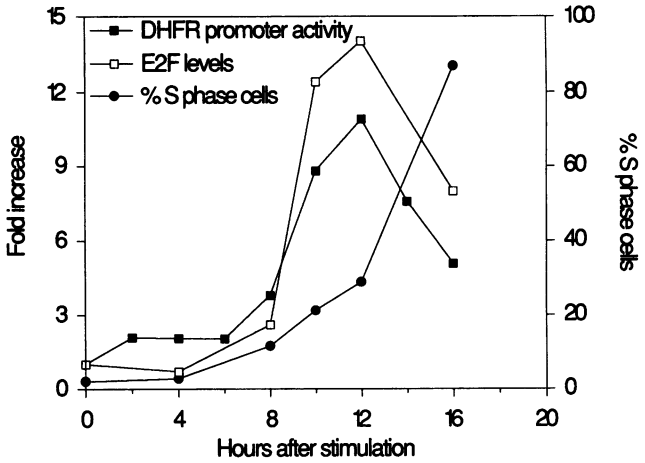

FIG. 5. A comparison of DHFR promoter activity (from Fig. 4A), E2F mRNA levels (from Fig. 3A), and the percentage of cells in S phase (from Fig. 2B) is shown. See Discussion for details.

Cyclin A, DNA polymerase alpha, thymidine kinase, thymidine synthase, and CAD are other serum response genes that are transcriptionally activated prior to the G_1/S-phase boundary. The promoter region of each of these genes contains putative E2F sites (18, 32), and some of these sites have been implicated in growth regulation (14, 32). Further investigation of these will indicate whether they are indeed regulated by E2F1 in a manner similar to that of the DHFR promoter. Since the early-response promoters c-*fos* and c-*myc* also contain E2F binding sites (22, 29, 39, 51), low levels of E2F may be sufficient for activation of these promoters, or it is possible that a family of E2F-related DNA-binding proteins exist. If so, different members may activate different sets of growth-regulated genes. In fact, cDNAs different from E2F1 that contain similar DNA binding domains have recently been cloned (31, 33).

It has been suggested that the interaction of E2F with RB

is responsible for repressing a constitutive E2F activity (2, 10, 22, 25). A number of different model systems have been employed to investigate E2F activity throughout the cell cycle. Very little E2F1 mRNA (Fig. 3) or E2F DNA binding activity (39) can be detected in serum-starved cells. RB would not be required to repress E2F activity in these cells. However, gel shift analysis using extracts prepared after size separation of proliferating cells detects E2F DNA binding activity throughout the cell cycle (13, 42, 49). Perhaps E2F activity is controlled differently in proliferating versus growth-arrested cells. In quiescent cells, such as serum-starved NIH 3T3 cells, resting T cells, and differentiated liver cells, very little E2F is made; when these cells are stimulated to enter the proliferative cell cycle by serum (Fig. 3A), mitogen addition (28), or partial hepatectomy (5), E2F expression is increased. In the G_1 phase of the subsequent cell cycles, RB would then regulate E2F activity by binding to the E2F protein. RB may also inactivate any residual protein that remains in G_0 after the cells have entered quiescence.

Our results suggest that tight control of the levels of E2F may be critical to maintain normal cell cycle control. We have shown that overexpression of E2F1 in a transient transfection assay can mimic certain effects of the oncoprotein E1A. Perhaps one explanation for these results is that overexpression of the E2F1 protein may complex all the RB in the cell and disrupt control typically exerted by RB in the regulation of many growth-specific genes. Alternatively, high levels of E2F1 may activate the RB promoter and result in too much RB protein for cell growth. If overexpression of E2F1 does indeed disrupt RB-controlled pathways, we would predict severe cellular consequences of altered E2F expression.

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