Autoregulatory control of *E2F1* expression in response to positive and negative regulators of cell cycle progression

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Both positive and negative signals govern the progression of cells from G_1 into S phase, and a variety of data implicate the E2F transcription factor as a target for the action of one class of negative regulators, the Rb family of growth suppressors. We now find that the *E2F1* gene, which encodes one of the components of E2F activity, is subject to autoregulatory control during progression from G_0 to S phase and that this primarily reflects a negative control in G_0 and early G_1, a time when the majority of E2F activity exits as a complex with Rb family members. In addition, we find that deregulated expression of G_1 cyclins in quiescent cells stimulates the *E2F1* promoter and that this is augmented by coexpression of cyclin-dependent kinases in an E2F-dependent manner. We conclude that the *E2F1* gene is a downstream target for G_1 cyclin-dependent kinase activity, most likely as a consequence of phosphorylation of Rb family members, and that the autoregulation of *E2F1* transcription may provide a sensitive switch for regulating the accumulation of E2F activity during the transition from G_1 to S phase.

[*Key Words*: Autoregulatory control; *E2F1* expression; positive/negative regulators; cell cycle progression; G_1 cyclins]

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The control of cell proliferation is central to the differentiation of specific cell types, the maintenance of tissue homeostasis, and the ability of certain cell types to expand rapidly in number. Moreover, the loss of cell growth control, as it occurs in the development of tumors, can have disastrous consequences for the organism. Although much of our current understanding of the underlying mechanisms responsible for the regulated transition of cells through a cell cycle stems from the combined genetic and biochemical studies available with yeast, additional critical information relative to the control of mammalian cell growth has been found in studies of oncogenic viruses. Each of the small DNA tumor viruses encodes proteins that inactivate the function of two key cellular growth regulatory proteins, the retinoblastoma gene product and the p53 protein, presumably to facilitate a productive viral infection in an otherwise quiescent cell (Levine 1993). In so doing, normal cell growth control is disrupted and in those circumstances where a productive viral infection cannot take place, this action leads eventually to an oncogenic transformation.

A series of recent observations have provided a more detailed picture of the action of the Rb protein in growth suppression. These experiments have shown that the E2F transcription factor, originally identified in the context of early adenovirus transcription control (Kovesdi et al. 1986), is a target for the action of the Rb tumor suppressor protein (Bagchi et al. 1991; Bandara et al. 1991; Chellappan et al. 1991; Chittenden et al. 1991). These experiments have shown that the Rb protein, as well as other members of the Rb family, form specific complexes with E2F and, in so doing, inhibit the capacity of the factor to activate transcription (Hiebert et al. 1992; Hiebert 1993; Schwarz et al. 1993; Zamanian and La Thangue 1993). The adenovirus E1A protein (Bagchi et al. 1990), as well as the SV40 T antigen and the HPV E7 product (Chellappan et al. 1992), possess the ability to disrupt these complexes, releasing E2F in a transcriptionally active form. The strategy of the viral proteins in targeting E2F would appear to be the activation of a group of cellular genes that encode proteins involved in DNA synthesis, such as dihydrofolate reductase (DHFR), thymidylate synthase, and DNA polymerase α (Nevins 1992). Previous experiments have shown that these genes are induced when quiescent cells are infected by SV40 or adenovirus, concomitant with the induction of S phase, presumably to create an appropriate environment for viral DNA replication (Tooze 1981). Each of these genes contain E2F sites within critical promoter sequences, each is activated in late G_1 as cells normally progress toward S phase, and recent analyses of the

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DHFR gene have provided strong evidence that the E2F elements are critical for activation in late G_1 (Slansky et al. 1993). This finding, together with the fact that the active form of E2F normally accumulates at this time (Mudryj et al. 1991; Shirodkar et la. 1992; Chittenden et al. 1993; Schwarz et al. 1993), supports the view that E2F is an important element of control in the progression to S phase.

It is now clear that the previously described E2F activity is actually a family of polypeptides encoded by distinct genes. The initial gene isolated encoding an E2F polypeptide, termed E2F1, was cloned through its ability to bind directly to Rb (Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992). Expression of the *E2F1* cDNA can specifically activate the adenovirus E2 gene in a transient transfection assay and cotransfection of an Rb-expressing vector inhibits E2F1-dependent activation (Cress et al. 1993; Flemington et al. 1993; Helin et al. 1993a). In addition, p107 can inhibit E2F1 transcriptional activity, whereas the adenovirus E4 19-kD protein can cooperate with E2F1 to activate the E2 promoter synergistically (Cress et al. 1993). Thus, E2F1 appears to display all the known properties and regulatory events shown for cellular E2F activity.

Recent experiments have provided a direct demonstration of a role for the *E2F1* cDNA product in regulating cell proliferation. Using a transient transfection assay, it was shown that overexpression of the *E2F1* product could prevent cells from entering quiescence when deprived of serum (Johnson et al. 1993). Moreover, expression of the *E2F1* product in already quiescent cells was found to induce S phase, coincident with the ability of E2F1 to activate the promoters of two S phase-regulated genes, DHFR and thymidine kinase. These findings suggest that regulation of E2F1 activity in the cell may be critical in maintaining normal proliferation control.

Much of the recent focus on the regulation of E2F1 activity has concentrated on post-translational regulation by E2F-binding proteins such as Rb and p107. However, it is also clear that transcription of the *E2F1* gene is cell cycle regulated. After serum stimulation of starved cells, *E2F1* mRNA is induced with delayed early kinetics, dependent on new protein synthesis (Slansky et al. 1993). To study the factors involved in the transcriptional regulation of the *E2F1* gene, we have isolated a human genomic clone containing the sequence found at the 5′ end of the *E2F1* cDNA and we have identified sequences within this region that possess promoter activity. We find that the E2F1 product can autoregulate transcription directed from its own promoter, E2F-mediated negative regulation appears to play a significant role in controlling E2F1 activity during the transition from G_0 into the cell cycle, and the *E2F1* gene is a target for regulation by the G_1 cyclins.

Results

Isolation of E2F1 *genomic clones containing putative promoter sequences*

To identify sequences containing the *E2F1* promoter, a full-length E2F1 cDNA clone (Helin et al. 1992) was used to screen a human placenta genomic library. Initially, 10 positive clones were isolated from a screen of 600,000 plaques. One of these clones hybridized to a 280-bp fragment containing the 5′-untranslated region and the first 144 bp of coding sequence of the E2F1 cDNA. DNA from this clone was digested with a variety of restriction enzymes and analyzed by Southern blot using either the 5′ *E2F1* cDNA fragment or an oligonucleotide corresponding to a 20-nucleotide sequence in the 5′-untranslated region of the E2F1 cDNA as the probe. Two restriction fragments that hybridized to these probes, a 1.5-kb *Apa*I fragment and a 2-kb *Sac*I fragment, were subcloned for further analysis. Sequence determination of the subclones confirmed that these DNA fragments did contain the 5′ sequence of the *E2F1* cDNA (Fig. 1A). The sequence of the genomic clone matches the cDNA sequence to a point 262 nucleotides 3′ of the ATG initiation codon and then diverges in sequence. Immediately preceding this point of divergence is the sequence CGGTACG, a close match to a consensus splice donor sequence suggesting that this is the 3′ end of an exon.

To determine the position of transcription initiation, we used RNase protection to identify the sequences corresponding to the 5′ end of the *E2F1* mRNA. As shown in Figure 1B, a protection assay using an RNA probe that spanned the 5′ sequence of the *E2F1* cDNA yielded a major protected fragment of 67 nucleotides in length, as well as several additional protected fragments. The size of the major protection product corresponds to a position 115 nucleotides 5′ to the E2F1 ATG sequence (Fig. 1). The 5′ end of the cDNA isolated by Helin et al. (1992) corresponds to a site mapping 12 nucleotides upstream of this site, whereas the 5′ ends in the two other *E2F1* cDNAs that have been published (Kaelin et al. 1992; Shan et al. 1992) map at or downstream from this site. Whether the sites downstream of the longest *E2F1* cDNA, including the major site, are actually additional start sites or result from internal RNase cleavage is not clear. Thus, we have used the most upstream site, which corresponds to the 5′ end of the cDNA isolated by Helin et al. (1992), as the +1 reference point.

E2F1 *upstream sequences provide cell cycle-regulated promoter activity*

Previous experiments have shown that the expression of the E2F1 mRNA is tightly regulated during the cell growth response. E2F1 mRNA is present at only very low levels in quiescent cells and then accumulates in late G_1 after restimulation (Kaelin et al. 1992; Slansky et al. 1993). Thus, sequences that provide promoter activity for the *E2F1* gene should do so in a cell cycle-dependent manner. A series of DNA fragments containing 5′-flanking sequence from the *E2F1* genomic clone were placed upstream of a luciferase reporter gene and then transfected into the rat embryo fibroblast cell line REF-52, along with a cytomegalovirus (CMV)-driven β-galactosidase reporter plasmid as an internal control. After transfection, cells were washed, starved of serum for ≥24 hr

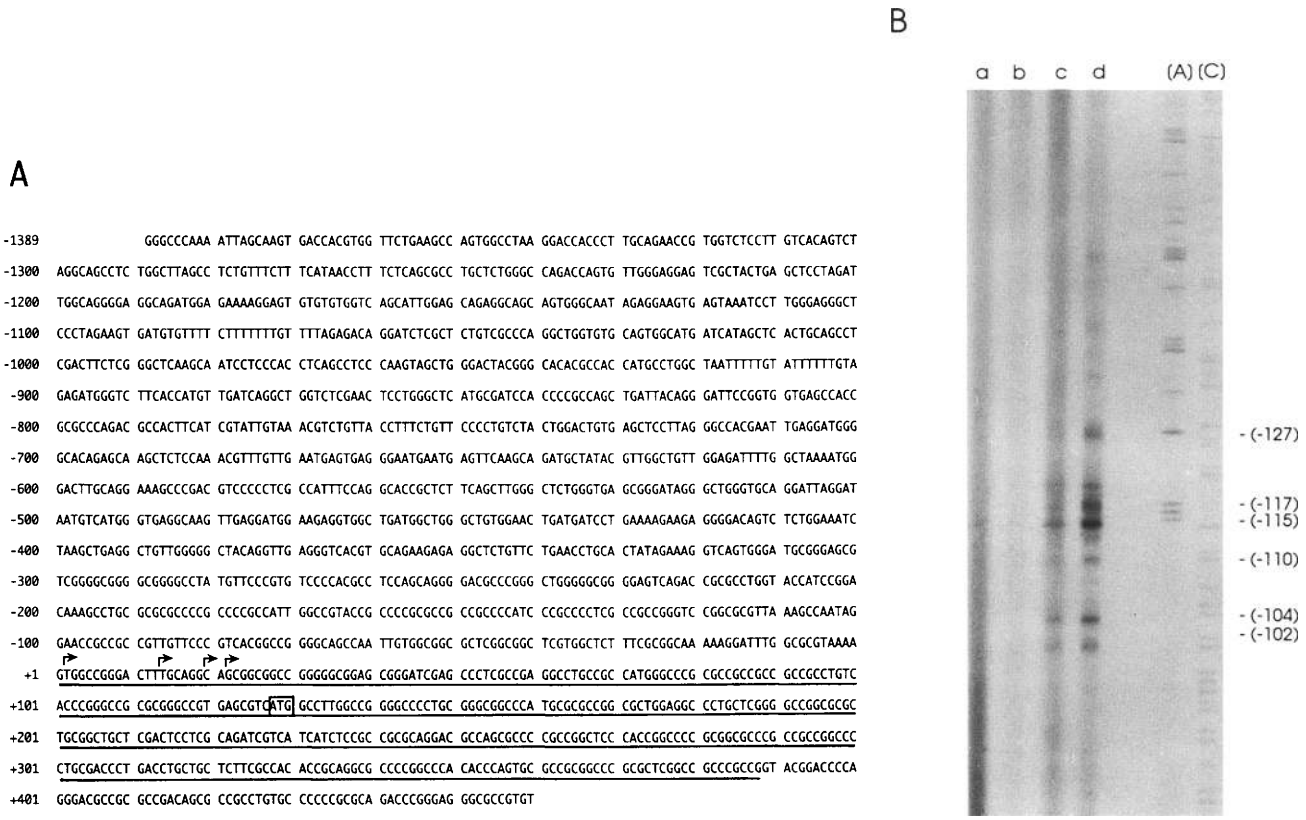

Figure 1. Genomic sequence and characterization of the E2F1 5′-flanking region. (*A*) DNA sequence of the human *E2F1* gene that includes the 5′ end of the E2F1 cDNA. The ATG translation initiation codon is indicated by a box. The region of identity of the cDNA sequence with the genomic sequence is underlined. Potential mRNA 5′ ends, as determined by RNase protection assays, are indicated by arrows. (*B*) Mapping of the 5′ end of *E2F1* mRNA by RNase protection. The RNase protection probe begins at the *Apa*I site in the 5′-untranslated region and ends at the *Sma*I site 323 bases upstream. Protected fragments were run on a 6% polyacrylamide sequencing gel next to a sequencing reaction as a marker. (Lane *a*) No RNA; (lane *b*) 50 μg of *Drosophila* total RNA; (lane *c*) 50 μg of HeLa total RNA; (lane *d*) 5 μg of HeLa poly(A)$^+$ RNA. [(A), (C)] A and C sequencing reactions, respectively. The positions indicated are relative to the ATG codon.

to allow the majority of cells to enter quiescence, and serum was then added. Cells were harvested every 4 hr after serum addition, extracts were prepared, and then assayed for luciferase activity and β-galactosidase activity. The luciferase assay results were adjusted for transfection efficiency as well as any fluctuations in general transcriptional activity by using the β-galactosidase activity as an internal control. The CMV promoter (β-galactosidase activity) was used as an internal control because its activity was relatively constant throughout the cell cycle, increasing only about twofold from G_0 into S phase.

As shown in Figure 2A, luciferase activity was detected only at low levels in the unstimulated cells and the early G_1 cells but then increased approximately six- to nine-fold after serum stimulation, with a peak at 24 hr. This timing coincides with the kinetics of accumulation of the endogenous *E2F1* mRNA (Fig. 2B; Slansky et al. 1993) and the time when free E2F-binding activity accumulates (Schwarz et al. 1993). Assays of luciferase constructs containing varying amounts of *E2F1* 5′ flanking sequences revealed that cell cycle control was retained with 204 nucleotides of sequence 5′ to the mRNA start site, whereas further deletion to −122 nearly abolished promoter activity.

An examination of the DNA sequence that conferred cell cycle-dependent promoter activity revealed the presence of two potential E2F-binding elements adjacent to the transcription start site (Fig. 2C). Each of the putative E2F sites exhibits an organization similar to that found in the *DHFR* promoter (Hiebert et al. 1991), consisting of overlapping E2F recognition motifs. Upstream of the E2F sites are several canonical CCAAT boxes and seven Sp1-binding sites. The loss of promoter activity by deletion to −122 coincides with the removal of a cluster of Sp1-binding sites.

E2F binds to the E2F1 *promoter*

Gel mobility-retardation assays were used to test whether the sequences in the *E2F1* promoter were functional E2F-binding sites. A 101-bp *Eag*I fragment from

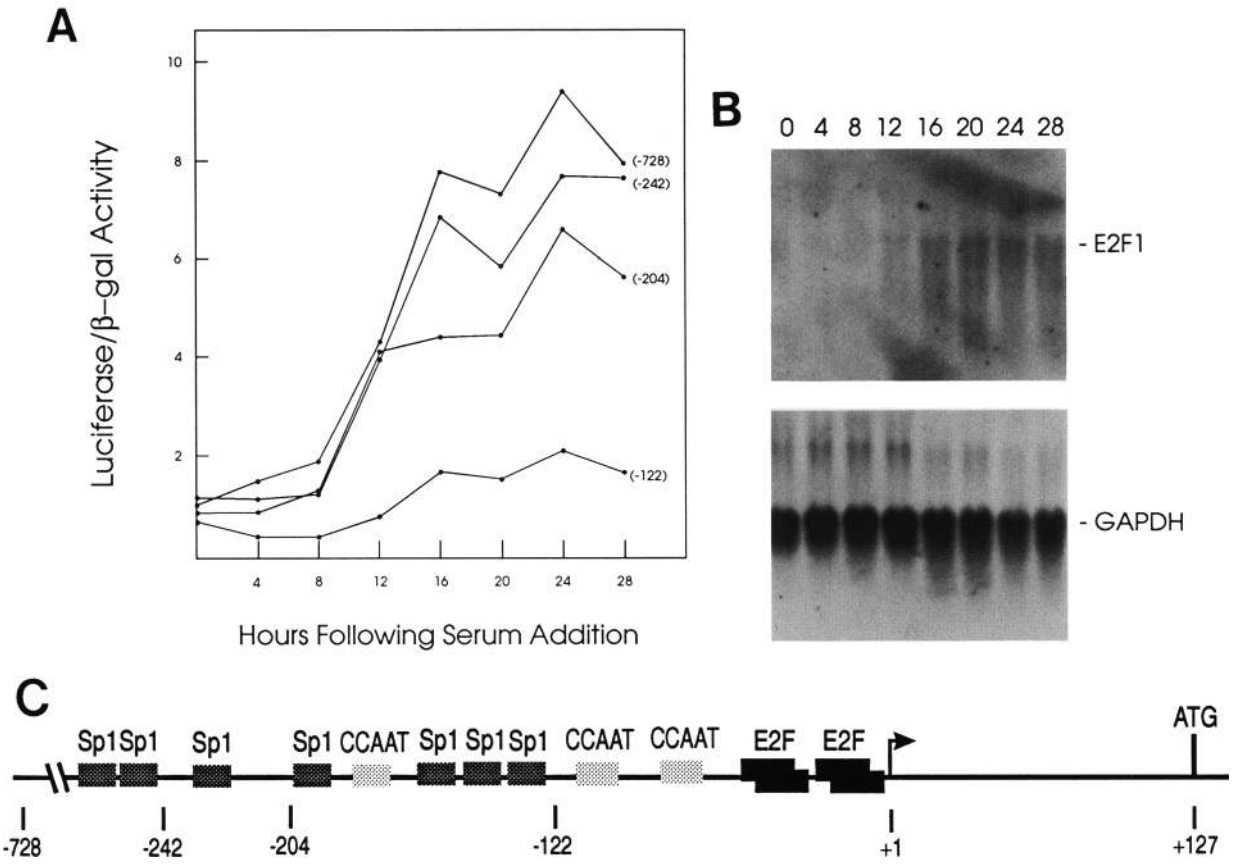

Figure 2. Cell cycle promoter activity of the *E2F1* 5′-flanking sequences. (*A*) Serum induction of E2F1 promoter–luciferase constructs. REF-52 cells were transfected with 5 μg of E2F1–LUC constructs, 3 μg of CMV β-galactosidase, and 12 μg of sheared salmon sperm DNA as carrier in 100-mm plates. Calcium–phosphate precipitates were applied to the cells overnight and then cells were washed and placed in Dulbecco's modified Eagle medium (DMEM) supplemented with 0.1% serum for 24–28 hr. Serum was added to the plates to a final concentration of 20%, and the cells were harvested at the indicated times. Cell extracts were assayed for luciferase and β-galactosidase activity and the data presented as luciferase activity (*E2F1* promoter) relative to β-galactosidase activity (CMV promoter). (*B*) Northern blot analysis of *E2F1* mRNA during serum induction. REF-52 cells were serum starved (0.1% serum) for 48 hr, and fetal calf serum was added to a final concentration of 20%. Cells were harvested every 4 hr and RNA prepared. Samples of whole cell RNA (19 μg) were analyzed in a 1% agarose gel, transferred to a nitrocellulose membrane (Schleicher & Schuell), and hybridized to a probe containing the 5′ half of the *E2F1* cDNA including the DNA-binding domain. After hybridization and washing, the filter was exposed on film and then analyzed on a PhosphorImager. This filter was then stripped and reprobed with a GAPDH probe. (*C*) Schematic representation of putative promoter elements in the E2F1 5′-flanking region. Consensus Sp1-binding sites (CCGCCC) and CCAAT boxes are indicated as well as E2F-binding sites. The translational start codon (ATG) is shown, and the first transcriptional start site is indicated by an arrow. The site indicated as +1 corresponds to the 5′ end of the E2F1 cDNA isolated by Helin et al. (1992). The endpoints of the deletion mutants are identified relative to the +1 site.

the *E2F1* gene, containing the putative E2F-binding sites, was used as a probe to test for E2F binding with a partially purified E2F preparation from HeLa cells (Fig. 3A). An oligonucleotide containing the E2F-binding sites found in the adenovirus E2 promoter could specifically compete the complex whose mobility was typical of an E2F–DNA complex, whereas a mutated form of this oligonucleotide could not. Conversely, using the adenovirus E2 promoter as a probe and an extract from human diploid fibroblasts, the *E2F1* promoter fragment could compete efficiently for E2F-binding activity (Fig. 3B). From these results we conclude that the *E2F1* promoter does contain sequences that bind E2F, although we cannot say unequivocally that each of the potential sites is an E2F-binding site.

E2F-dependent regulation of E2F1 *promoter activity*

The finding of E2F-binding sites in the *E2F1* promoter, coupled with previous observations concerning the role of such elements in transcriptional activation, suggested the potential for autoregulation of the *E2F1* promoter. As a test for this possibility, a plasmid containing the *E2F1* 1.5-kb *Apa*I fragment cloned upstream of a chloramphenicol acetyltransferase (CAT) reporter gene was transfected into REF-52 cells together with an E1A or an

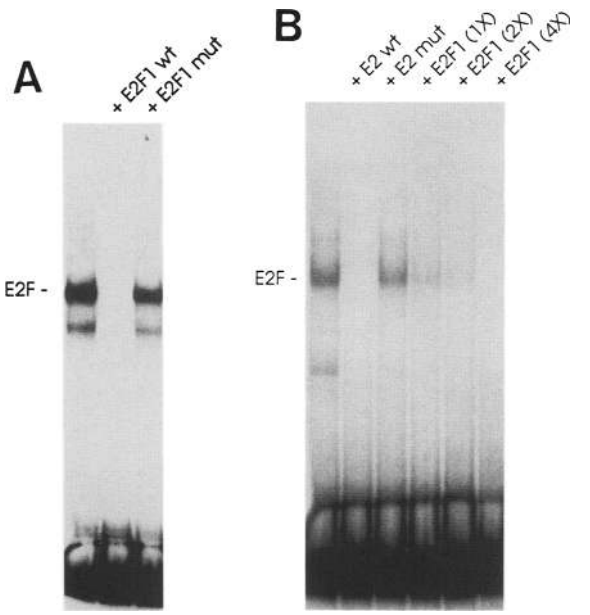

Figure 3. E2F binding to the *E2F1* promoter. (*A*) Gel mobility retardation analysis with the 101-bp *Eag*I fragment from the *E2F1* promoter as a probe with partially purified E2F protein (a heparin–agarose fraction) from HeLa cells. Competitions were performed using the wild-type E2 promoter oligonucleotide (wt, lane 2), or the mutant form of the E2 oligonucleotide (lane 3). (*B*) Gel mobility retardation assay using an adenovirus E2 promoter fragment as a probe and whole cell extract from human foreskin fibroblasts. Competitions were performed using the E2 promoter oligonucleotide (wt, lane 2), the mutated E2 promoter oligonucleotide (lane 3), or increasing amounts of the 101-bp *Eag*I fragment from the *E2F1* promoter (1×, 2×, 4×).

E2F1 expression plasmid. Indirect evidence for E2F autoregulation was provided by the data shown in Figure 4A in which *E2F1* promoter activity was stimulated about eightfold upon cotransfection with the $E1A_{12S}$ plasmid. In contrast, no activation was observed when the *E2F1* promoter was transfected together with an E1A point mutant which does not bind to Rb and is unable to activate E2F (Raychaudhuri et al. 1991).

A more direct demonstration of a role for the E2F1 product in regulating activity of the *E2F1* promoter was shown by transfection of an E2F1 expression vector. As seen in Figure 4A, the E2F1 product resulted in a 3.3-fold activation of the *E2F1* promoter in asynchronously growing cells. Recent experiments have shown that the E2F1 product forms a heterodimer with the DP1 product and this heterodimer possesses the enhanced DNA binding and transcriptional activation properties characteristic of E2F (Bandara et al. 1993; Helin et al. 1993b; Krek et al. 1993). Whereas the DP1 product alone had little effect on *E2F1* promoter activity, cotransfection of the DP1-expressing plasmid with the E2F1 expression vector resulted in a further 2.5-fold stimulation over that with E2F1 alone, a level of activation similar to that seen with E1A.

An assay for E2F1 autoregulatory properties in serum-starved cells demonstrated an even more dramatic activation of the *E2F1* promoter by E2F1, yielding nearly a 100-fold increase in activity (Fig. 4B). In addition, two other recently identified members of the E2F gene family, E2F2 and E2F3 (Ivey-Hoyle et al. 1993; Lees et al. 1993), were also able to activate transcription from the *E2F1* promoter, again achieving substantial activation in the quiescent cells (Fig. 4B). The activation by E2F2 and E2F3 is significant given the fact that only very low levels of E2F1 are found in quiescent cells. Finally, a variety of experiments have shown that E2F-dependent transcription is inhibited by the action of the Rb protein as well as the Rb-related p107 protein (Hiebert et al. 1992; Schwarz et al. 1993; Zamanian and La Thangue, 1993). As shown in Figure 4C, expression of Rb as well as p107 led to a near complete inhibition of *E2F1* promoter activity, similar to the effects on an E2F-dependent test promoter and consistent with a role for the E2F transcription factor in the control of *E2F1* promoter activity. We conclude from these results that the *E2F1* promoter does respond to E2F, either as a result of the action of E1A or as a direct consequence of the expression of the E2F1, E2F2, or E2F3 products and that the negative regulatory activity of the Rb family members can repress the promoter as well.

Mutation of the E2F-binding sites relieves negative control of the E2F1 *promoter in G_0 and early G_1*

The observation that the E2F sites in the *E2F1* promoter might contribute to transcriptional control of the gene, together with the past experiments that have demonstrated the changing nature of E2F during the cell cycle, prompted us to examine the role of the E2F sites in growth-regulated activity of the promoter. To test directly the role of the E2F-binding sites in cell cycle regulation, the E2F-binding sites located between −31 and −3, as well as a third potential site at −59/−52, were mutated (Fig. 5A). DNA-binding assays demonstrated that these alterations did eliminate E2F binding to the promoter fragment (Fig. 5B). The *E2F1* E2F(−) mutant promoter was then assayed for cell cycle regulation by transfection into REF-52 cells followed by serum starvation and then restimulation.

As shown in Figure 5, C and D, elimination of the E2F-binding sites created a promoter with an ~3.5- to 5-fold greater activity than the wild-type promoter in quiescent cells. A kinetic analysis of the two promoters revealed that as cells progressed through G_1, the activity of the mutant promoter increased slightly, whereas the wild-type promoter was induced five- to six-fold, reaching a final level equivalent to the activity of the mutant promoter. We conclude that a major element of E2F1 control during the cell growth response is an E2F-mediated negative control of transcription in G_0 and early G_1 cells. Because the *E2F1* promoter lacking E2F-binding sites attains an activity nearly equal to that of the wild-type promoter, the role of the free form of E2F may be to exclude the E2F inhibitory complexes or simply titrate the available Rb or Rb family member proteins.

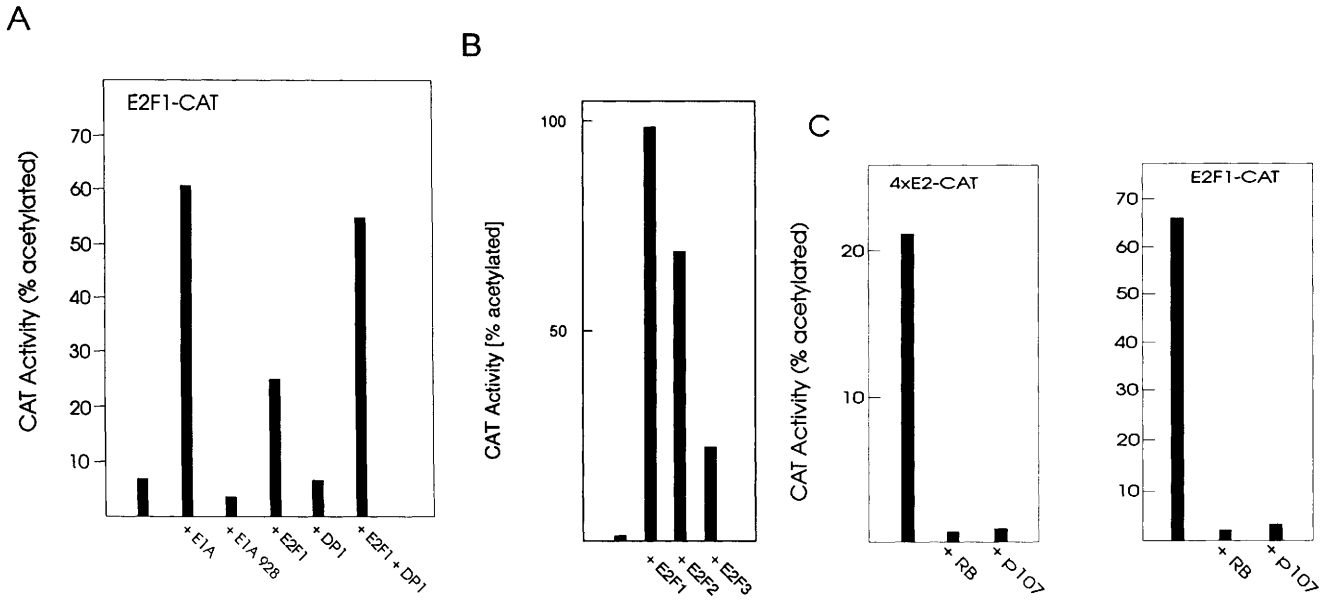

Figure 4. Autoregulation of the *E2F1* promoter. (*A*) E2F1-mediated activation of the *E2F1* promoter. REF-52 cells were transfected with 5 μg of E2F1–CAT, 3 μg of CMV–β-galactosidase, and 12 μg of sheared salmon sperm DNA as carrier. $E1A_{12S}$, $E1A_{928}$, E2F1, or DP1 expression plasmids (100 ng) were included in the transfections where indicated. After transfection, cells were placed in DMEM supplemented with 10% serum and incubated for a total of 48 hr. Cells were harvested, extracts prepared, and CAT and β-galactosidase assays performed. CAT activity was then corrected for transfection efficiency using β-galactosidase activity as an internal control. (*B*) Activation of the *E2F1* promoter by E2F family members in serum-starved cells. REF-52 cells were transfected with 5 μg of E2F1–CAT, 3 μg of CMV-β-galactosidase, and 10 μg of sheared salmon sperm DNA as carrier. E2F1, E2F2, or E2F3 expression vector (or vector alone) (2 μg) was included in the transfections where indicated. After transfection, cells were washed and placed in DMEM supplemented with 0.1% serum for 48 hr before harvesting. CAT and β-galactosidase assays were performed and CAT assay results corrected using β-galactosidase activity. (*C*) Repression of *E2F1* promoter by Rb and p107. SAOS-2 cells (Rb − / −) were transfected with 5 μg of 4×E2–CAT or E2F1–CAT, 1 μg of CMV–β-galactosidase, 4 μg sheared salmon sperm DNA, and 10 μg of CMV vector alone, CMV–Rb, or CMV–p107 plasmid where indicated. After transfection, cells were washed and placed in DMEM supplemented with 15% FCS for a total of 48 hr. Cells were then harvested, extracts prepared, and CAT and β-galactosidase assays performed. CAT assay results were corrected using β-galactosidase activity as an internal control.

G_1 cyclins activate E2F1 *transcription in quiescent cells*

Given the finding that the *E2F1* promoter is subject to negative control in G_0 and early G_1 cells, the events that initiate and trigger the release from this control are clearly of some considerable importance. G_1 cyclin-dependent kinase (cdk) activity has been shown to be critical for appropriate G_1 progression into S phase, and overexpression of both cyclin E- and D-type cyclins shorten the G_1 interval and reduce the requirement for serum (Baldin et al. 1993; Ohtsubo and Roberts 1993; Quelle et al. 1993). Deregulated expression of cyclins, particularly cyclin D1, has been associated with the development of several human cancer types (Motokura et al. 1991; Rosenberg et al. 1991; Seto et al. 1992), and recent experiments have shown that the cyclin D1 gene, together with an activated *ras* gene, can participate in the transformation of cells to an oncogenic state by complementing an E1A mutant (Hinds et al. 1994). Previous experiments have shown that phosphorylation of the Rb protein regulates its ability to bind to and control E2F, and recent experiments have implicated the G_1 cyclins, together with the cdk2 or cdk4 kinase, as being at least partially responsible for this control of Rb (Hinds et al. 1992; Dowdy et al. 1993; Ewen et al. 1993b; Kato et al. 1993).

In light of these findings, we have addressed the possibility that the action of G_1 cyclins and associated kinases might trigger the accumulation of E2F1 activity. Specifically, we have assayed for the ability of the D-type cyclins and cyclin E to activate the *E2F1* promoter in serum-starved cells. REF-52 cells were transfected with E2F1–CAT expression vectors together with expression plasmids encoding the D1, D2, and D3 cyclins. After transfection, cells were deprived of serum for 36 hr and then assayed for CAT activity. As shown in Figure 6A, cDNAs expressing the D-type cyclins mediated a 3.5- to 9-fold activation of the *E2F1* promoter when assayed in serum-starved cells. This activation was dependent on the presence of E2F-binding sites in the *E2F1* promoter as the activity of a mutant promoter was not increased in the cells cotransfected with the D-type cyclins. These results thus demonstrate that the *E2F1* promoter is a downstream target for the action of D-type cyclins, dependent on the presence of intact E2F-binding sites, con-

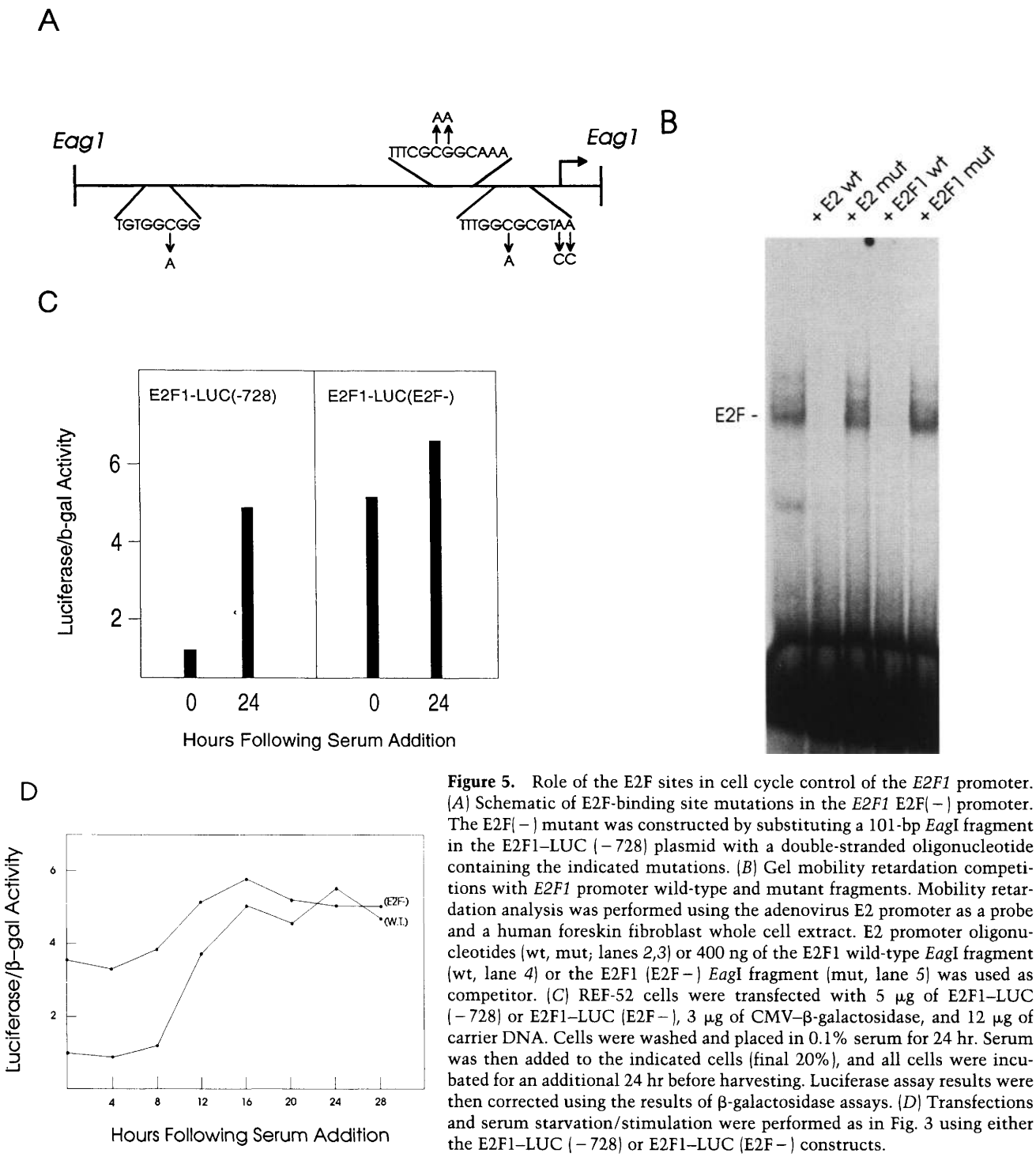

Figure 5. Role of the E2F sites in cell cycle control of the *E2F1* promoter. (*A*) Schematic of E2F-binding site mutations in the *E2F1* E2F(−) promoter. The E2F(−) mutant was constructed by substituting a 101-bp *Eag*I fragment in the E2F1–LUC (−728) plasmid with a double-stranded oligonucleotide containing the indicated mutations. (*B*) Gel mobility retardation competitions with *E2F1* promoter wild-type and mutant fragments. Mobility retardation analysis was performed using the adenovirus E2 promoter as a probe and a human foreskin fibroblast whole cell extract. E2 promoter oligonucleotides (wt, mut; lanes *2,3*) or 400 ng of the E2F1 wild-type *Eag*I fragment (wt, lane *4*) or the E2F1 (E2F−) *Eag*I fragment (mut, lane *5*) was used as competitor. (*C*) REF-52 cells were transfected with 5 μg of E2F1–LUC (−728) or E2F1–LUC (E2F−), 3 μg of CMV–β-galactosidase, and 12 μg of carrier DNA. Cells were washed and placed in 0.1% serum for 24 hr. Serum was then added to the indicated cells (final 20%), and all cells were incubated for an additional 24 hr before harvesting. Luciferase assay results were then corrected using the results of β-galactosidase assays. (*D*) Transfections and serum starvation/stimulation were performed as in Fig. 3 using either the E2F1–LUC (−728) or E2F1–LUC (E2F−) constructs.

sistent with the role of these proteins in mediating a phosphorylation of the Rb protein, an inhibitor of E2F activity.

The most straightforward interpretation of the results of Figure 6A is an activation of the *E2F1* promoter as a result of increased cyclin-dependent kinase activity. As further evidence in support of this possibility, we have assayed the ability of two cyclin-dependent kinases to augment the activation. Plasmids expressing the cdk2 and cdk4 protein kinases were cotransfected with the D-type cyclin plasmids to measure effects on *E2F1* promoter activity. Transfection of either the cdk2- or cdk4-expressing plasmid had little effect on *E2F1* promoter activity (Fig. 6B). In contrast, cotransfection of either the cdk2 or the cdk4 plasmid augmented the activation achieved with cyclin D1 or cyclin D2. In addition, cotransfection of a cyclin E-expressing plasmid also led to an activation of the *E2F1* promoter and this was in-

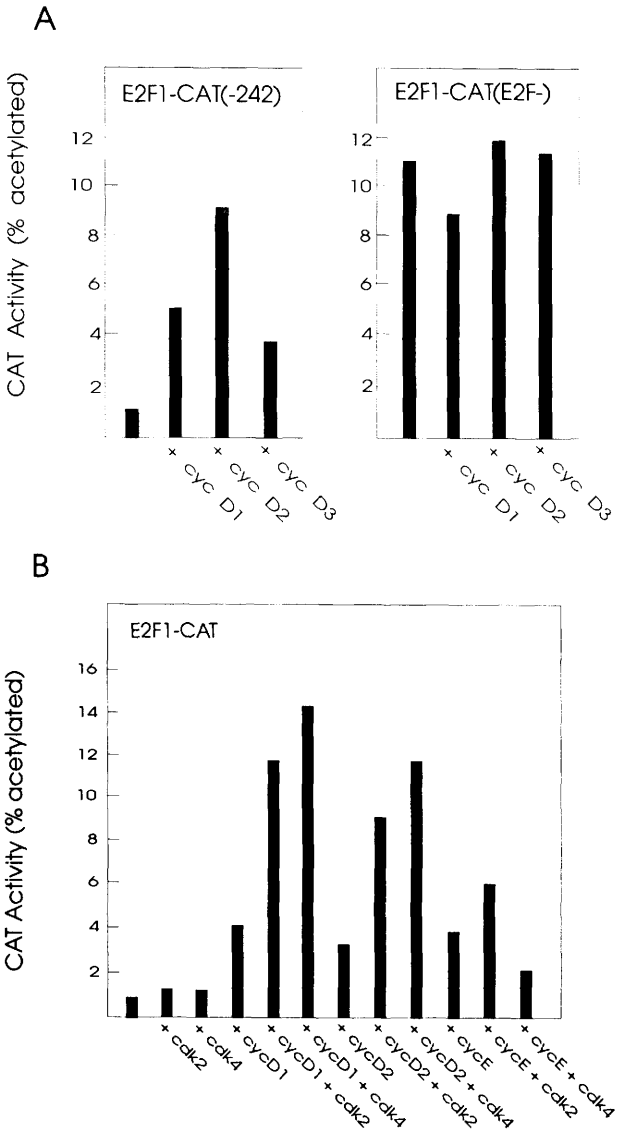

Figure 6. Activation of the *E2F1* promoter by G_1 cyclins. (*A*) E2F-dependent activation of the *E2F1* promoter by cyclin D1, D2, and D3. REF-52 cells were transfected with 5 μg of E2F1–CAT (−242) or 5 μg of E2F1–CAT (E2F−), 3 μg of CMV–β-galactosidase, and 12 μg of Rc/RSV (InVitrogen), Rc/D1, Rc/D2, or Rc/D3. After transfection, cells were washed and placed in DMEM supplemented with 0.1% serum for 48 hr before harvesting. CAT and β-galactosidase assays were performed, and CAT assay results were corrected using β-galactosidase activity. (*B*) Cyclin-dependent kinase expression augments the activation of the *E2F1* promoter by G_1 cyclins. REF-52 cells were transfected as in *A* using 5 μg of E2F1–CAT and 3 μg of CMV–β-galactosidase. CMV vector alone or 10 μg of CMV cycD1, CMV cycD2, or CMV cycE and/or 2 μg of CMV cdk2 or CMV cdk4 was included as indicated.

creased slightly by cotransfection of the cdk2 plasmid but not the cdk4 plasmid. We conclude from these results that the ability of the G_1 cyclins to stimulate the *E2F1* promoter is most likely a function of their ability to activate cyclin-dependent kinase activity as would normally occur during G_1 progression.

Discussion

The majority of E2F activity in most cell types is in association with the *RB1* gene (Rb) product and other members of the Rb family, and many studies have focused on the ability of these proteins to control the activity of E2F. A variety of experiments have shown that the interaction of Rb and the Rb-related p107 protein with E2F results in an inhibition of E2F transcription capacity (Hiebert et al. 1992; Flemington et al. 1993; Helin et al. 1993a; Schwarz et al. 1993; Zamanian and La Thangue 1993), consistent with the observation that the Rb-binding domain overlaps with the transcription activation domain of the E2F1 product (Helin et al. 1992; Kaelin et al. 1992). The disruption of this control by the DNA tumor virus proteins, such as adenovirus E1A, SV40 T antigen, and human papillomavirus (HPV) E7, appears to facilitate the entry to S phase as a consequence of the E2F-mediated activation of a group of cellular genes that encode proteins important for DNA replication (Nevins 1992). Although the control of E2F by protein interactions is clearly important, it is also evident that activation of *E2F1* gene transcription is a significant component in the regulation of E2F1 activity during the cell growth response (Slansky et al. 1993). The experiments presented here demonstrate that this transcriptional control is primarily the result of E2F-mediated negative regulation in G_0 and the early stages of G_1.

Although E2F sites are generally associated with positive transcriptional control, and there are numerous reports documenting the fact that E2F does act in a positive manner, there have also been previous reports of E2F sites acting as negative elements. For instance, recent experiments have shown that E2F sites can mediate an Rb-dependent repression of transcription driven by the early SV40 promoter/enhancer (Weintraub et al. 1992), thus suggesting that the interaction of Rb with E2F does not simply negate its activity but, rather, can create a dominant-acting repressor. In addition, studies of the cell cycle control of the B-*myb* gene have shown that an E2F site acts as a negative promoter element, as mutation of the site results in constitutive activation, relieving repression in G_0 and early G_1 (Lam and Watson 1993), a result very similar to that for the *E2F1* promoter described here. In short, evidence suggests that E2F can play both a positive as well as a negative role in transcription control, dependent on the state of the E2F factor.

A series of observations suggest an important role for the E2F transcription factor in cell growth control and the regulation of G_1 progression. These observations include the fact that the ability of Rb, as well as the Rb-related p107 protein, to arrest cell growth in G_1 is dependent on the ability to interact with and inhibit the function of E2F (Qian et al. 1992; Qin et al. 1992). In addition, the Rb-mediated G_1 arrest can be overcome by coexpression of the *E2F1* cDNA product (Zhu et al. 1993). Late G_1 expression of the DHFR gene, and likely an additional group of S-phase regulated genes, is dependent on the action of E2F (Slansky et al. 1993), which is

coincident with the accumulation of free, active E2F in late G_1 (Schwarz et al. 1993). Finally, recent experiments have shown that the expression of the *E2F1* cDNA product prevents quiescence upon serum deprivation and expression of E2F1 in quiescent cells stimulates S phase (Johnson et al. 1993).

These findings, taken together with additional recent studies of G_1 regulatory activities, implicate E2F as a critical downstream target of G_1 control events that lead to the induction of cellular DNA replication (Fig. 7). In particular, previous work has shown that the Rb protein is regulated by phosphorylation and that this is likely mediated by various G_1 cyclin/cdk complexes (Hinds et al. 1992; Dowdy et al. 1993; Ewen et al. 1993a; Kato et al. 1993). E2F control could thus be viewed as a downstream target for G_1 cyclin/kinase action, as the phosphorylation of Rb, and perhaps other Rb family members, affects its ability to bind to and regulate E2F (Chellappan et al. 1991; Kato et al. 1993; Schwarz et al. 1993). In addition, the negative growth regulatory activity of transforming growth factor-β (TGF-β) has been shown to coincide with the inhibition of cyclin E/cdk2 activity (Koff et al. 1993), as well as cyclin D/cdk4 activity (Ewen et al. 1993b), and recent experiments demonstrate that TGF-β-mediated growth inhibition can be overcome by expression of the E2F1 product (J. Schwarz, C. Bassing, X.-F. Wang, and J.R. Nevins, in prep.). In short, many of these G_1 control events can be seen to converge on E2F, consistent with the ability of E2F1 to stimulate quiescent cells to enter S phase (Johnson et al. 1993).

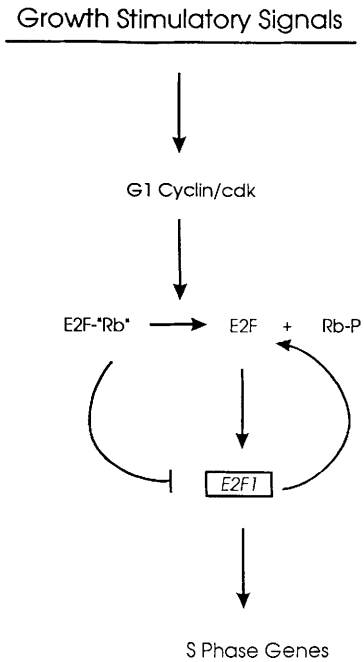

Figure 7. *E2F1* control as a downstream target for G_1 regulatory events. Growth stimulatory signals would include various growth factors that lead to the activation and accumulation of G_1 cyclins. One target for the action of G_1 cyclins, in conjunction with cyclin-dependent kinases (cdk), is the Rb protein and possibly other members of the Rb family. In this regard, "Rb" not only refers to the retinoblastoma tumor-suppressor protein but also to other Rb family members such as p107 and p130. Phosphorylation of "Rb" prevents its interaction with E2F, which apparently relieves negative control of the *E2F1* gene.

Cyclin E and cyclin D1 were cloned through their ability to rescue a G_1 cyclin-deficient yeast strain (Koff et al. 1991; Xiong et al. 1991). Cyclin D1 was also cloned as a delayed early responsive gene induced by colony-stimulating factor 1 (CSF-1) (Matsushime et al. 1991) and as a gene activated by a human chromosome 11 inversion in parathyroid adenomas (Motokura et al. 1991). Cyclin D2 and D3 were cloned through their homology to cyclin D1 (Matsushime et al. 1991). The cyclin D1 gene has been found to be deregulated in B-cell lymphomas, squamous cell tumors, and breast carcinomas (Sherr 1993) and is a likely candidate for the *Bcl*I oncogene. Blocking cyclin D1 function arrests fibroblasts in G_1 and overexpression of cyclin E, D1, or D2 shortens the G_1 interval of the cell cycle and reduces dependency on serum (Ohtsubo and Roberts 1993; Quelle et al. 1993). Taken together, these data suggest an important role for the E- and D-type cyclins in G_1 progression and in the regulation of normal cell proliferation. This role appears, at least in part, to involve the phosphorylation of Rb (Hinds et al. 1992; Dowdy et al. 1993; Ewen et al. 1993a; Kato et al. 1993). This G_1 cyclin/cdk-mediated phosphorylation destabilizes the interaction of Rb with E2F, suggesting a role for the G_1 cyclins in regulating the transcriptional activity of E2F. Our finding that E- and D-type cyclins can activate the *E2F1* promoter, dependent on the E2F sites within the promoter, provides a direct demonstration that this is the case.

Although the Rb protein can repress transcription from the *E2F1* promoter, recent experiments suggest that Rb may not be the major regulator of E2F in G_0 or G_1 cells. Rather, the major E2F-binding activity at this time appears to contain the Rb-related proteins p107 and p130 (Lees et al. 1992; Shirodkar et al. 1992; Cobrinik et al. 1993; Schwarz et al. 1993). Therefore, the E2F–p107 and/or the E2F–p130 complexes are likely candidates for an active transcriptional repressor in quiescent cells. As cells enter late G_1 and S phase the E2F–p107 and E2F–p130 complexes disappear and free E2F accumulates. A simple model for the role of E2F in the cell cycle regulation of *E2F1* gene expression would be that the E2F–p107 and E2F–p130 complexes bind to the promoter and block transcription initiation in G_0/early G_1. As these complexes disappear and free E2F appears in late G_1, transcription driven by other factors, such as Sp1, can occur. This would lead to the synthesis of additional E2F1, increased activation of the *E2F1* gene and, thus, further increases in the levels of E2F1. Finally, although these experiments point to the importance of E2F in regulating the *E2F1* gene, it remains possible that other regulatory activities also impinge on the expression of the gene. For instance, we have noted the presence of potential binding sites for the c-Myb and c-Myc proteins in the *E2F1* upstream sequences. The functional relevance of these sites is yet to be determined, but it is possible that these factors may also contribute to *E2F1* regulation during G_1 progression.

Autoregulation provides a very sensitive means to control the accumulation of a rate-limiting product, and the experiments presented here suggest that the *E2F1* gene is under autoregulatory control as cells progress from G_0 to S phase. In this regard, we use the term autoregulation in a general sense to include regulation not only mediated by the E2F1 product but also other E2F family members. It would appear that the E2F1 product is not a major constituent of the E2F activity found in G_0 or early G_1 cells, and it is likely that different E2F species participate in the regulation of the *E2F1* promoter at this time. This autoregulatory control would allow an initial modest accumulation of E2F, possibly as a result of the release of E2F from complexes containing p130 or p107, to rapidly amplify expression of the *E2F1* gene resulting in the further accumulation of E2F activity. Moreover, the phosphorylation of Rb that occurs in mid- to late G_1 (Buchkovich et al. 1989; Chen et al. 1989; Ludlow et al. 1990) would allow this accumulation to take place, as phosphorylation of Rb prevents its interaction with E2F (Chellappan et al. 1991; Schwarz et al. 1993). Given the likely possibility that E2F1 activity is important in the transition from G_1 to S phase, as demonstrated by recent transfection and microinjection experiments (Johnson et al. 1993), this dramatic control could certainly represent a switch used by the cell to make the transition from G_1 into S.

In many respects, the control and action of E2F exhibits properties similar to the G_1/S checkpoint Start, defined genetically in yeast (Hartwell et al. 1974), and likely coincides with the restriction point in mammalian cells (Pardee 1989). In *Saccharomyces cerevisiae*, the *cdc28* gene and one of the *CLN* genes are required for Start. Two genes have been associated with Start in fission yeast, *cdc2*, the homolog of the *S. cerevisiae cdc28* gene, and *cdc10* (Nurse and Bissett 1981). The cdc10 gene product and related proteins found in *S. cerevisiae* encode transcription factor activity that shares some characteristics with E2F, notably the activation of S-phase genes in late G_1 of the cell cycle (Lowndes et al. 1991, 1992). The cdc2 kinase, in conjunction with G_1 cyclins (the *CLN* genes), is functionally analogous to the mammalian G_1 cyclin-dependent cdk2 and cdk4 kinases that are known to target the E2F regulatory protein Rb (Dowdy et al. 1993; Ewen et al. 1993a; Kato et al. 1993). All of these results point to the possibility that the accumulation of E2F activity in late G_1 may represent the functional analog of Start, or at least one aspect of the critical G_1/S control point.

Materials and methods

Isolation of E2F1 *promoter sequences*

A human placenta genomic phage library (a gift from R. Lefkowitz, Duke University Medical Center, Durham, NC) was plated on twelve 150-mm L-plates at a density of 50,000 plaques per plate. Plaques were lifted in duplicate and screened using a full-length E2F1 cDNA (Helin et al. 1992) as a probe. Ten plaques scoring positive on duplicate filters were picked, replated, and rescreened. In the third round of screening, a 280-bp fragment from the 5′ end of the E2F1 cDNA was used as a probe and a single positive clone was isolated. Phage DNA was digested with various restriction enzymes, transferred to nitrocellulose and probed using either the 5′ E2F1 fragment or a 20-bp oligonucleotide corresponding to the 5′-untranslated E2F1 sequence. Two fragments that hybridized to these probes, a 1.5-kb *Apa*I fragment and a 2.0-kb *Sac*I fragment, were subcloned for further analysis. Sequencing of these fragments confirmed that this clone contained the 5′ end of the *E2F1* gene.

RNase protection assay

RNase protection was performed as described previously (Johnson et al. 1990). Briefly, a 323-base antisense RNA probe beginning at the *Apa*I site in the E2F1 5′-untranslated region was hybridized to 50 μg of *Drosophila* total RNA, 50 μg of HeLa total RNA, or 5 μg of HeLa poly(A)$^+$ RNA for 16 hr at 65°C. RNA hybrids were RNase digested, and protected fragments were analyzed on a 6% polyacrylamide sequencing gel. Sequencing reactions using a primer corresponding to the end of the RNase protection probe were used as markers to identify the E2F1 transcriptional start site.

E2F1 promoter constructs and expression plasmids

The 1.5-kb *Apa*I fragment from the *E2F1* genomic λ phage was subcloned into pGEM11 (Promega) to generate pGEM7A1. This plasmid was then used for sequencing, synthesis of the antisense RNA probe, and construction of *E2F1* promoter reporter plasmids. The entire 1.5-kb *Apa*I fragment was subcloned into pCAT basic (Promega) to make pE2F1CAT. Luciferase reporter deletion mutants were created by subcloning restriction fragments (−728, *Sac*I; −242, *Sma*I; −204, *Bsp*E1; −122, *Ava*II) into the pGL2 vector (Promega). The E2F1–LUC (E2F−) construct was created by substituting a 101-bp *Eag*I fragment in the E2F1–LUC(−740) plasmid with a double-stranded oligonucleotide containing appropriate base changes. The E2F1–CAT (E2F−) plasmid was generated by first constructing E2F1–CAT (−242) by deleting an *Apa*I–*Sma*I fragment from E2F1–CAT followed by replacement of the *Eag*I fragment with the *Eag*I fragment from E2F1–LUC (E2F−). The E1A, E2F1, and DP1 expression plasmids were described previously (Kraus et al. 1992; Cress et al. 1993). The E2F2 and E2F3 expression plasmids have been described (Lees et al. 1993). Cyclin D1, D2, and D3, as well as cdk4 expression plasmids, have been described previously (Quelle et al. 1993). The cyclin E expression plasmid (Hinds et al. 1992) and the cdk2 expression plasmid (Tsai et al. 1991) have been described previously. The 4×E2CAT reporter plasmid (Ohtani and Nevins 1994) and the pRB and p107 expression plasmids (Cress et al. 1993) have been described elsewhere.

CAT, β-galactosidase, and luciferase assays

CAT and β-galactosidase assays were performed as described (Johnson et al. 1993). Luciferase assays were performed using a luciferase reporter assay system (Promega).

Northern blot analysis

Total RNA (19 μg) was run on a 1% agarose gel containing MOPS and 2.2 M formaldehyde. RNA was transferred to a nitrocellulose membrane and prehybridized in 5× SSC, 0.1% SDS, 50 mM Tris-HCl (pH 7.5), 0.1% Ficoll, 0.1% polyvinylpyrolidone, 0.1% BSA, 100 μg/ml denatured sheared salmon sperm DNA, and 45% formamide at 42°C overnight. The filter was then hybridized under similar conditions with either an E2F1 or GAPDH probe. The filter was then washed once in 0.5× SSC, 0.1% SDS, at 50°C, once at 55°C, and once at 60°C. The

filter was then exposed to X-ray film followed by PhosphorImager analysis.

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