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Cellular Targets for Activation by the E2F1 Transcription Factor Include DNA Synthesis- and G_1/S-Regulatory Genes

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Although a number of transfection experiments have suggested potential targets for the action of the E2F1 transcription factor, as is the case for many transcriptional regulatory proteins, the actual targets in their normal chromosomal environment have not been demonstrated. We have made use of a recombinant adenovirus containing the E2F1 cDNA to infect quiescent cells and then measure the activation of endogenous cellular genes as a consequence of E2F1 production. We find that many of the genes encoding S-phase-acting proteins previously suspected to be E2F targets, including DNA polymerase α, thymidylate synthase, proliferating cell nuclear antigen, and ribonucleotide reductase, are indeed induced by E2F1. Several other candidates, including the dihydrofolate reductase and thymidine kinase genes, were only minimally induced by E2F1. In addition to the S-phase genes, we also find that several genes believed to play regulatory roles in cell cycle progression, such as the *cdc2*, cyclin A, and B-*myb* genes, are also induced by E2F1. Moreover, the cyclin E gene is strongly induced by E2F1, thus defining an autoregulatory circuit since cyclin E-dependent kinase activity can stimulate E2F1 transcription, likely through the phosphorylation and inactivation of Rb and Rb family members. Finally, we also demonstrate that a G_1 arrest brought about by γ irradiation is overcome by the overexpression of E2F1 and that this coincides with the enhanced activation of key target genes, including the cyclin A and cyclin E genes.

Through a combination of genetic and biochemical approaches, extraordinary progress has been made over the past several years in defining the regulatory activities that govern the initial commitment to cell growth. These studies have revealed a series of positive-acting components, including the G_1 cyclins and associated kinases, as well as negative-acting components that include a family of proteins that control the activity of the G_1 cyclin kinases as well as the p53 and Rb tumor suppressor proteins (for reviews, see references 23 and 65). The growth-regulatory function of the Rb tumor suppressor protein, a likely target for the action of G_1 cyclin-dependent kinase activity, appears to be dependent on its ability to bind to and inhibit the activity of the cellular transcription factor E2F (20, 56, 57). Various experiments have now shown that the E2F transcription factor appears to be an integral part of the growth-regulatory network which controls the progression of cells from G_0 and early G_1 into the S phase of the cell cycle (34, 48). Moreover, it is also now clear that the disruption of various components of this control pathway, which include Rb and Rb family members, G_1 cyclins and associated kinases, and various activities that regulate these kinases, can lead to the development of cancer (24).

A series of recent studies using the E2F1 gene, which encodes a component of cellular E2F activity, have directly demonstrated a role for E2F in both cell cycle regulation and oncogenesis. Expression of E2F1 can block cells from entering quiescence and can induce already quiescent cells to enter S phase, dependent on its ability to bind DNA and activate transcription (28, 58). Moreover, deregulated expression of E2F1 can lead to transformation of an established rat embryo fibroblast cell line (67) or, in cooperation with an activated *ras* oncogene, can lead to oncogenic transformation of primary rat embryo cells (26). An E2F1-VP16 chimera, in which sequences involved in transcriptional activation as well as Rb binding have been replaced with the activation domain of the herpesvirus VP16 protein, exhibits increased transformation ability (26). This E2F1-VP16 chimera activates transcription in an E2F-dependent manner but has a much decreased affinity for Rb, suggesting that transformation by E2F1 or E2F1-VP16 does not simply involve sequestering Rb but likely involves the transcriptional activation of E2F-regulated genes.

E2F binding sites are found in the promoters of several genes that encode proteins involved in DNA synthesis such as dihydrofolate reductase (DHFR), thymidine kinase (TK), and DNA polymerase (pol) α (48). These genes are coordinately regulated during the cell cycle, being induced in mid- to late G_1 at a time when active E2F accumulates (41, 44, 68). For at least the DHFR gene, the E2F sites have been shown to be critical for the cell cycle-dependent activation of transcription (5, 42). E2F sites are also found in the promoters of several cellular proto-oncogenes, including c-*myc*, N-*myc*, *PRAD1*/*Bcl1* (cyclin D1), *erb-B* (epidermal growth factor receptor), c-*myb*, and B-*myb* (17, 21, 33, 43–45, 48, 61), as well as genes encoding cell cycle-regulatory proteins such as cyclin E (49a) and cyclin A (19). Given the role of these genes in both normal cell proliferation and tumorigenesis, it is appealing to speculate that one or more of these cellular proto-oncogenes may represent an important target gene for E2F-mediated S-phase induction and oncogenic transformation.

Experiments to date have generally used transient-transfection assays to measure the induction of suspected target genes by a transcriptional activator such as E2F1. As such, these assays provide evidence for the activity of the transcriptional activator but fail to reproduce the normal in vivo circumstance of gene control that might be influenced by the chromosomal environment of the target gene. In the ideal, one might hope to merely delete or inactivate the transcriptional activator and then determine which target genes are affected. This is difficult in mammalian cells and is particularly difficult if the transcrip-

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tional activator is essential for cell cycle progression and cell viability. An alternative approach is simply to express the activator independently of its normal control and determine which potential target genes are activated. We have chosen to approach this problem for the E2F transcription factor through the use of AdE2F1, an adenovirus vector in which the E2F1 gene has been recombined into the viral genome. This vector allows the production of high levels of the E2F1 product upon infection of susceptible cells. In addition, adenovirus has the important property of being able to infect quiescent, nondividing cells. Thus, we have used this system as a means to measure the activation of E2F target genes that would normally be expressed when quiescent or arrested cells progress through G_1 and into S phase.

MATERIALS AND METHODS

Cells and virus. Viral stocks were created as described previously (62). Virus titers were determined by an indirect immunofluorescence assay specific for the viral 72-kDa E2 gene product. Target 293 cells were grown to approximately 70% confluence in 35-mm-diameter plates and then infected with dilutions of the viral stocks. Virus was absorbed to the cells for 1 h in 0.5 ml of Dulbecco modified Eagle medium (DMEM), and then the infection medium was replaced with DMEM containing 4% fetal bovine serum (FBS). After 16 h of incubation at 37°C, the cells were washed once with phosphate-buffered saline (PBS), fixed for 5 min in 3.7% formaldehyde, washed with PBS, fixed for 2 min in 90% methanol–PBS, and washed twice in PBS. The fixed cells were then incubated for 45 min in a 1:100 dilution of a rabbit polyclonal antiserum raised against the viral 72-kDa protein (diluted in 1% bovine serum albumin [BSA]–PBS), washed three times with PBS, incubated for 45 min in fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G antiserum (Boehringer Mannheim) at 1:500 in 1% BSA–PBS, and then washed four times with PBS. Cells positive for 72-kDa protein expression were scored by fluorescein isothiocyanate immunofluorescence; titers were determined by counting positive cells within several square millimeter fields and extrapolating to the number of infected cells on the entire plate and were expressed as focus-forming units per milliliter.

REF52 cells were a gift from E. Ruley (Vanderbilt University) and were grown in DMEM containing 5% FBS and 5% calf serum. To bring cells to quiescence, REF52 cells were plated at approximately 3,500/cm^2 and incubated overnight. For 35-mm-diameter plates, cells were plated on coverslips. The next day, the cells were washed once with PBS, and the culture medium was replaced with DMEM containing 0.2% fetal calf serum. Cells were further incubated for 48 h prior to virus infection or serum stimulation by replacement with culture medium containing 15% fetal calf serum. Cells on plates were infected in DMEM for 30 min at 37°C in 1 ml/150-mm-diameter plate or 0.08 ml/35-mm-diameter plate (layered on the coverslip). REF52 cells were infected with 500 focus-forming units/ml unless otherwise stated.

γ irradiation. Density-arrested REF52 cells (24 h postconfluence) were trypsinized, resuspended in DMEM containing 10% serum (5% calf serum and 5% FBS), centrifuged, and resuspended in the same medium at 1.5×10^6 cells per ml. The cells were then γ irradiated or sham treated in polypropylene tubes at 25 Gy, centrifuged, washed once in DMEM, and then resuspended in DMEM at 10^7 cells per ml. Virus was then added to a multiplicity of infection of 500, and the cells were infected in suspension for 20 min with constant, gentle swirling. Following infection, the cells were replated at subconfluent densities (2×10^6 cells per 150-mm-diameter plate or 10^5 cells per 35-mm-diameter plate) in 10% serum in DMEM. Cells in 35-mm-diameter plates were labeled with 50 μM bromodeoxyuridine (BrdU) for the 12-h period prior to fixation. Cells were either harvested for poly(A)$^+$ RNA isolation (150-mm-diameter plates) or processed for BrdU incorporation (35-mm-diameter plates).

BrdU incorporation. Cells grown on coverslips in 35-mm-diameter plates were labeled with 50 μM BrdU and then fixed and processed for BrdU incorporation as described previously (52) except that the secondary antibody was an anti-mouse rhodamine-conjugated antibody (Pierce Chemical) at a 1:50 dilution. Following the secondary-antibody incubation, counterstaining for DNA was performed by incubation for 10 min in 1 μg of DAPI (4′,6-diamidine-2-phenylindole dihydrochloride) (Boehringer Mannheim) per ml in PBS.

Northern (RNA) analysis. Total RNA was isolated by the RNAzol method (Biotec Laboratories, Inc.), and poly(A)$^+$ RNA was isolated with a Polytract isolation system, using the protocols supplied by Promega except that bound RNA was washed once with 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then twice with 0.2× SSC prior to elution with water. RNA yield was determined prior to poly(A)$^+$ RNA isolation and used to equalize the amount of poly(A)$^+$ RNA loaded. RNAs were electrophoresed in a 1% agarose formaldehyde gel and transferred to GeneScreen (NEN) as recommended by the manufacturer. Blots were probed with ^{32}P-labeled random-primed cDNA probes (NEN) or an [α-^{32}P]dCTP terminal transferase (Boehringer Mannheim)-labeled E2F1 oligonucleotide at 42°C overnight in 45% formamide–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–5× Denhardt's solution–1% sodium dodecyl sulfate (SDS) according to GeneScreen protocols. The filters were washed twice at room temperature in 0.2× SSC–0.2% SDS, once at 65°C in 1× SSC–1% SDS, and then twice at 65°C in 0.5× SSC–0.2% SDS. An additional wash at 65°C in 0.2× SSC–0.2% SDS was performed following hybridization with human E2F1, cyclins D1, D2, and D3, cdk2, cdk4, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pol α, Rb, or ribonucleotide reductase 1 (RR1). Except where indicated, all exposures of the radioactive filters were to Kodak X-Omat film. Blots were stripped by boiling for 10 min in 0.5% SDS prior to reprobing.

The cDNAs used as probes for Northern analysis were kindly provided by the following investigators: human c-*myc* by D. Bently, mouse RR1 and RR2 by L. Thelander, mouse cyclins D1, D2, and D3 and cdk4 by C. Sherr, mouse DNA pol α by H.-P. Nasheuer, mouse thymidylate synthase (TS) by L. Johnson, human E2F1, human DP1, and human cdk2 by E. Harlow, human E2F2 by from J. Lees, mouse proliferating cell nuclear antigen (PCNA) by M. Prystowsky, human cyclin A by J. Pines, human cyclin E by X.-F. Wang, human B-*myb* by B. Calabretta, and human Rb by J. Horowitz.

The cDNAs for mouse TK, mouse cdc2, mouse DHFR, and human GAPDH were obtained from the American Type Culture Collection. The oligonucleotide used for the specific detection of endogenous E2F1 was 5′-CAT GCA CAC ATG TGG ACA CAC CAG GAT GTG TCT GCA GCA CAC ATA CAG A -3′. This sequence is antisense to a region in the mouse E2F1 3′ untranslated region that is not present in AdE2F1.

RESULTS

Use of an adenovirus vector to induce E2F activity in quiescent cells. Previous experiments have shown that expression of the E2F1 product can induce quiescent cells to enter S phase as measured by BrdU incorporation (28). However, since these assays used a transfected or microinjected E2F1 gene, it has not been possible to determine the nature of changes in gene expression during this induction. In particular, although a number of genes that encode S-phase activities have been implicated as targets for the action of E2F, no direct experiments lending support to this hypothesis have been described. For instance, transfection assays have shown that the E2F1 product can activate a cotransfected DHFR promoter (68), but whether this is actually the case for the endogenous gene is not established. To further understand the role of E2F1 in the induction of DNA synthesis, and to identify endogenous targets that are transactivated by E2F1, we have constructed an adenovirus vector that contains the E2F1 cDNA under the control of the cytomegalovirus promoter (AdE2F1; Fig. 1A).

As shown in Fig. 1B, infection of REF52 cells with the recombinant virus leads to the production of virus-encoded E2F1 mRNA. That this also leads to the production of functional E2F activity is shown by the analysis depicted in Fig. 1C. REF52 cells were starved for 48 h in 0.2% FBS and then either stimulated with 15% FBS or infected with the E2F1-expressing virus (AdE2F1) or a control virus (an adenovirus-cytomegalovirus recombinant lacking the E2F1 cDNA [AdCMV]). Cells were labeled with BrdU for a 24-h period prior to fixation, and then BrdU incorporation was determined by indirect immunofluorescence. Infection of the quiescent cells with AdE2F1 induced approximately 60% of the quiescent REF52 cells to enter S phase (Fig. 1C), similar to the results of previous transfection and microinjection experiments (28), although the efficiency was higher with the virus infection. Although the assays of BrdU incorporation would not discriminate between induced cellular DNA replication and any viral DNA replication that might occur under these conditions, other experiments have shown by CsCl gradient analysis that there is little if any viral DNA synthesis under these conditions (31, 62). By 48 h after infection with AdE2F1, nearly half of the infected cells had died, and by 72 h, most of the cells were dead as a consequence of apoptosis, consistent with recent reports concerning the role of E2F1 in apoptosis (31, 58, 72).

Activation of S-phase genes by E2F1. Most discussions concerning the role of E2F as an element of cell growth control

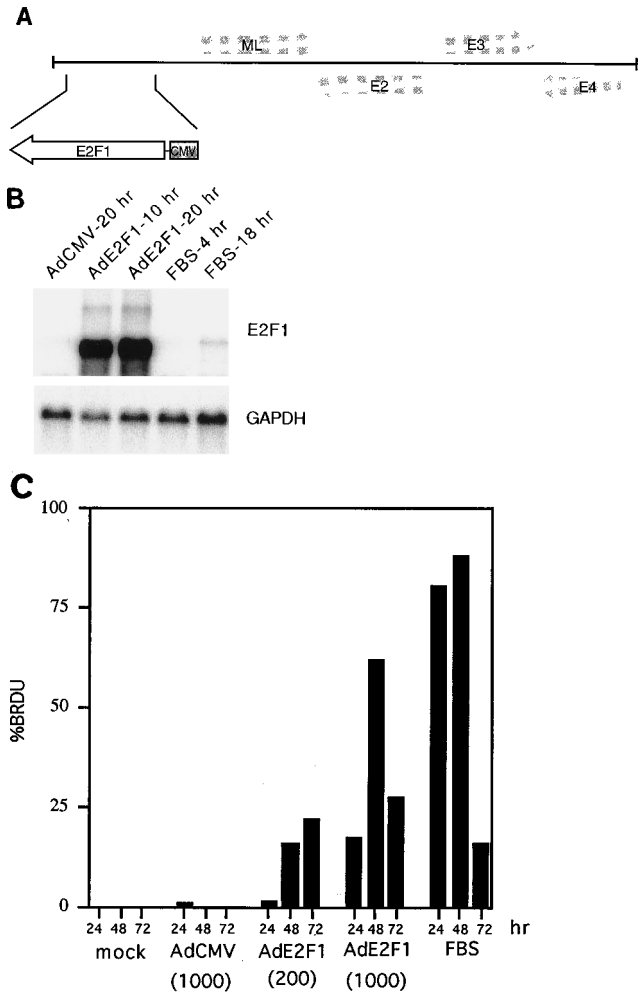


FIG. 1. Expression of E2F activity by an adenovirus recombinant vector. (A) Structure of the recombinant vector AdE2F1. Adenovirus sequences from 191 to 325 have been replaced with an expression cassette containing the E2F1 cDNA under the control of the cytomegalovirus (CMV) promoter. ML, major late promoter. (B) Synthesis of E2F1 mRNA by AdE2F1. Serum-starved REF52 cells were infected or serum stimulated and then harvested at the indicated times, and poly(A)$^+$ RNA was isolated. The AdCMV-infected cells were harvested at 20 h postinfection. Poly(A)$^+$ RNA (derived from 120 μg of total RNA per lane) was then separated by agarose gel electrophoresis, transferred to nitrocellulose, and probed with the human E2F1 cDNA as described in Materials and Methods. Equivalent loading of RNA has been confirmed for this and all subsequent blots by probing with the GAPDH cDNA, and a representative blot is shown. After hybridization and washing, the filters were exposed and analyzed with a PhosphorImager. The figure was generated from the PhosphorImager computer file. (C) Induction of cellular DNA synthesis. Serum-starved REF52 cells were infected or serum stimulated and then labeled with BrdU for a 24-h period prior to fixation, and BrdU incorporation was determined by indirect immunofluorescence. At least 200 nuclei (stained with DAPI) were scored for BrdU immunofluorescence.

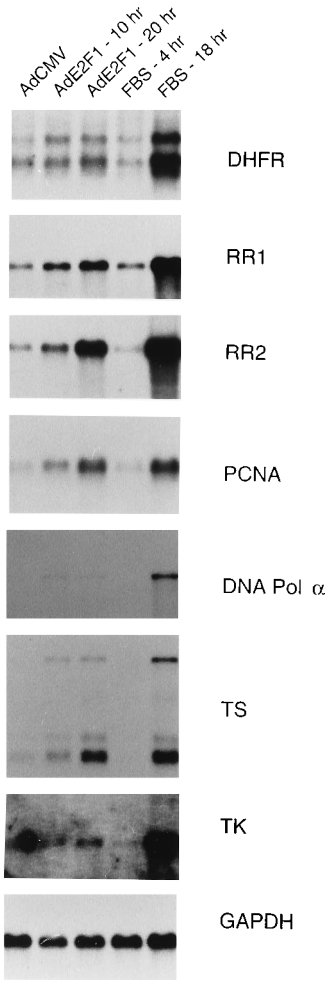


FIG. 2. Activation of cellular transcription by E2F1 expression. Northern blots were prepared and probed with cDNAs for DHFR, RR1, RR2, PCNA, DNA pol α, TS, TK, and GAPDH as described in the legend to Fig. 1 and Materials and Methods. After hybridization and washing, the filters were exposed to Kodak X-Omat film.

and as a target for the growth-suppressing activity of Rb family members have focused on the activation of a group of genes that encode proteins directly involved in S phase. These proteins include various activities that generate deoxynucleotides such as DHFR, TK, TS, and RR as well as DNA pol α and the polymerase accessory factor PCNA. Using AdE2F1, we have sought to determine if these target genes are indeed induced as a consequence of E2F1 action and may thus account for the ability of E2F1 to induce S phase in quiescent cells.

REF52 cells were starved for 48 h and either serum stimulated or infected with AdE2F1 or the control virus AdCMV. The cells were then harvested at the indicated times, poly(A)$^+$ RNA was isolated, and the RNA was fractionated by agarose gel electrophoresis and then transferred to nitrocellulose. The resulting Northern blots were probed with a variety of cDNAs specific to the various S-phase genes. The relative levels of mRNAs in these and all following Northern blots were compared with the levels of GAPDH mRNA in the samples. It is clear from the data shown in Fig. 2 that infection of quiescent cells with the E2F1-expressing virus does lead to an activation of many of the genes previously suspected to be induced by E2F. In particular, the genes encoding TS, PCNA, RR, and DNA pol α were clearly induced in response to expression of E2F1. In some cases, the increase following E2F1 virus infection was comparable to that seen when cells were stimulated by serum addition.

For at least two reasons, we believe that the activation of these genes is a direct consequence of E2F1 rather than an event secondary to the induction of S phase. First, all of these genes contain E2F binding sites within promoter sequences, and in many cases, these sites have been shown to be important for promoter activity. Second, there is already a substantial

increase in the RNA levels of the various genes by 10 h postinfection, a time when there is no evidence of DNA synthesis (data not shown).

In contrast to the group of genes that are clearly activated by E2F1, levels of expression of other genes suspected to be induced by E2F were only mildly increased following infection with AdE2F1. For example, the DHFR and TK genes, both of which contain E2F sites in their promoters and both of which have been shown in transient-transfection assays to be induced by E2F (36, 68), were only slightly increased in activity. On one level, these results clearly demonstrate the specificity in the effects of AdE2F1 in that there is not a generalized induction of gene expression and indeed even genes that could respond to E2F1 do not. The basis for the specificity is certainly of considerable interest, with the possibility that other determinants are important or the possibility that other E2F family members are responsible for the induction of these genes.

A role for E2F1 in the activation of G_1- and S-phase-regulatory genes. In addition to the genes that encode products that function during S phase, a variety of other genes encoding proteins that regulate G_1 as well as S-phase progression are also potential targets for E2F1 activation. In some cases, this inference is based on the presence of E2F sites in promoter sequences of genes such as the cyclin D1, Rb, and c-*myc* genes. However, as seen in the analysis shown in Fig. 3, there was no evidence for the induction of any of these genes. Moreover, assays of several additional genes that also participate in G_1 control, including the genes encoding cyclin D2 and D3 and the cyclin-dependent kinases cdk2 and cdk4, also showed no evidence for activation by E2F1.

In sharp contrast, the gene encoding cyclin E was markedly induced by E2F1 expression. In fact, the increase in cyclin E expression as a function of E2F1 was greater than that seen in serum-stimulated cells. This finding is consistent with our recent observations that the cyclin E promoter contains multiple E2F binding sites which are essential for activation by E2F1 and for activation during the cell cycle (49a). Once again, these results demonstrate a specificity to the activation by E2F1 and point to a potential importance of the cyclin E gene in E2F1 action.

In addition to the various G_1-regulatory genes, we have also assayed for the ability of E2F1 to activate a series of genes that likely function during S phase and indeed are likely critical for the induction of S phase. For instance, the function of the cyclin A gene product has been shown to be essential for S phase (14, 51), and recent experiments have demonstrated a role for the B-*myb* product in overcoming a G_1 arrest (37, 60). Previous experiments have shown that all of these genes contain E2F sites within their promoter sequences (7, 19, 33). As shown in Fig. 3, infection of quiescent cells with AdE2F1 demonstrated that each of these genes was induced by E2F1. Finally, an assay for the *cdc2* gene revealed a moderate induction by E2F1, consistent with results of previous transfection assays (7, 8).

E2F autoregulation. Recent experiments have shown that the E2F1 gene is subject to autoregulatory control during the progression of cells through G_1 (27). We therefore wished to determine the effect of E2F1 overexpression on the expression of different E2F family members. Using an oligonucleotide probe that is specific for the endogenous E2F1 gene, we observe an increase in E2F1 mRNA levels upon infection by AdE2F1 (Fig. 4A). Moreover, E2F1 overexpression similarly results in increased levels of E2F2 mRNA (Fig. 4B). Although E2F2 levels are low in REF52 cells, we do observe an increase in the E2F2 mRNA at 18 h following serum stimulation (Fig. 4), suggesting that, like the E2F1 gene, this gene is subject to

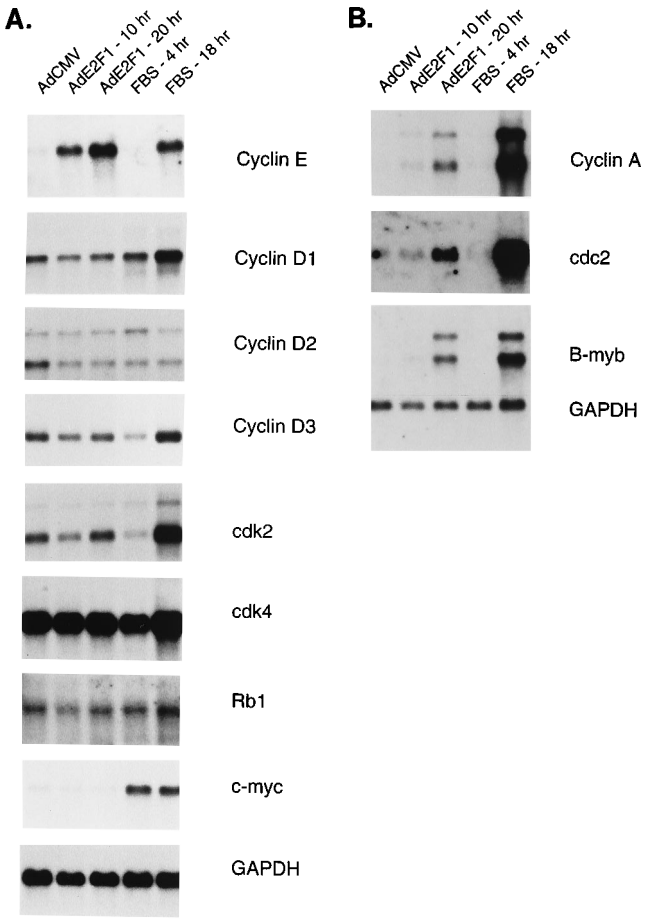


FIG. 3. Activation of G_1- and S-Phase-Regulatory genes by E2F1. Northern blots were prepared and analyzed as described in the legend to Fig. 1 and Materials and Methods and were probed with cDNAs for the indicated G_1-regulatory genes (A) and S-phase-regulatory genes (B). After hybridization and washing, the filters were exposed to Kodak X-Omat film.

growth control during the G_1-to-S phase transition. As previously reported (36), the expression of the *DP1* gene, which encodes a heterodimer partner for E2F1, -2, and -3, increases at 18 h following serum stimulation. This increased *DP1* expression as cells progress into S phase may result from the generated E2F activity, since infection with AdE2F1 similarly increases *DP1* transcript levels. The transcriptional activation of the components of E2F activity by E2F1 may serve to amplify the accumulation of E2F activity during the G_1-to-S phase transition. Furthermore, this finding indicates that some of the AdE2F1-dependent increase in target gene expression may have resulted from an E2F1-dependent transactivation of other components of E2F activity.

E2F1 overexpression overcomes a γ-irradiation-induced G_1 arrest. The results presented thus far demonstrate that the ability of E2F1 overexpression to induce S phase in serum-arrested cells coincides with the induction of many of the genes encoding critical S-phase activities as well as other regulatory genes that operate in G_1 and S phases. Obviously, this system provides an opportunity to explore other circumstances of G_1 arrest to understand the general role of the E2F1-mediated activation of target genes that results in the abrogation of cell growth arrest. A variety of experiments have shown that irradiation of normal cells by γ rays results in their arrest in the G_1

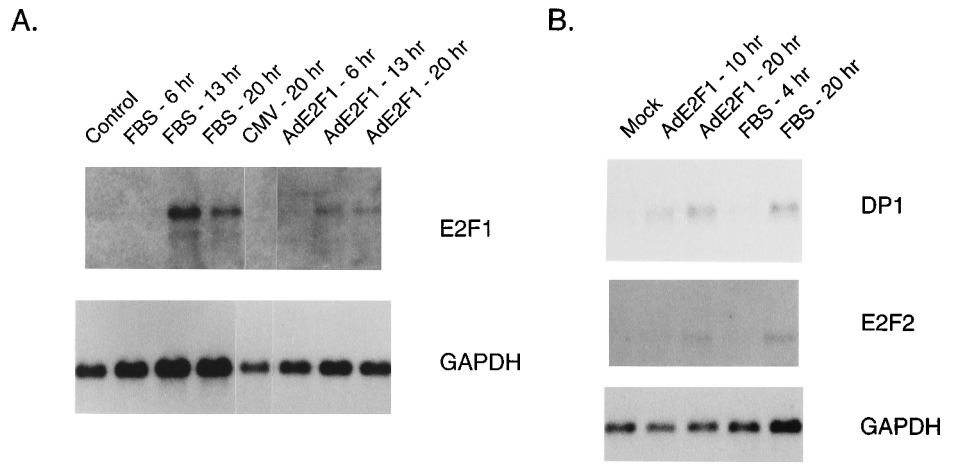


FIG. 4. Autoregulation of E2F genes. (A) Northern blots were prepared as described in the legend to Fig. 1 and Materials and Methods, although from a separate experiment, and probed with either an oligonucleotide specific to the endogenous E2F1 transcript or with the GAPDH cDNA. Control cells were mock infected and harvested at 20 h postinfection. After hybridization and washing, the filters were exposed to Kodak X-Omat film. (B) Northern blots were prepared and analyzed as described in the legend to Fig. 1 and Materials and Methods and were probed with the indicated cDNAs. Mock RNA was prepared from cells infected with AdCMV and harvested at 20 h postinfection. After hybridization and washing, filters were exposed to Kodak X-Omat film.

or G2 phase of the cell cycle, presumably to allow for the repair of damaged DNA. At least part of the G_1 arrest is due to a p53-mediated activation of the *p21/Waf1/Cip1* gene (henceforth referred to as p21), the product of which inactivates the cyclin E-dependent kinases (10, 11) and possibly other cyclin-dependent kinases. We have sought to determine whether E2F1 might bypass this block.

Density-arrested REF52 cells were trypsinized, γ irradiated, infected with AdE2F1, and then replated at subconfluent densities. Cells were labeled with BrdU for a 12-h period prior to fixation, and then BrdU incorporation was detected by indirect immunofluorescence. As shown in Fig. 5A and B, irradiation inhibited S-phase entry by greater than 90% in infections with AdCMV or in mock-infected cells (data not shown). In contrast, infection of irradiated cells with AdE2F1 overcame the irradiation block. Flow cytometric analysis indicated that the AdE2F1-infected, irradiated cells indeed progressed through an apparently normal S phase and into G_2 (data not shown). Moreover, this bypass appears to be independent of Rb binding by E2F1, as we have recently demonstrated that an E2F1-VP16 chimera, which has a much decreased affinity for Rb (26), similarly bypassed the γ-irradiation block (data not shown).

To explore the events associated with the ability of E2F1 to overcome an irradiation-induced block, we examined the expression of various genes following the replating of irradiated, control infected cells as well as following infection with AdE2F1. Density-arrested REF52 cells were irradiated as before, and poly(A)$^+$ RNA was isolated from cells at either 12 or 24 h postinfection. As shown in Fig. 5C, irradiation prevented the accumulation of cyclin A, cdc2, DHFR, and endogenous E2F1 mRNAs following replating. TS is only mildly induced following replating without irradiation, and irradiation prevents this increase. Cyclin E mRNA levels are also only moderately affected by irradiation, in agreement with a previous report showing no change in protein levels following irradiation (10). Infection of the irradiated cells with AdE2F1 restored cyclin A, cdc2, DHFR, and E2F1 mRNA levels. The cyclin E gene was induced over 60-fold by E2F1 in irradiated cells, and TS mRNA is increased over 8-fold. As these transcripts are induced by 12 h postinfection, by which point the cells have not yet entered S phase, we conclude that the activation by E2F1 must be direct rather than an indirect consequence of the cells entering S phase. In contrast, cdk4 mRNA levels are not affected by either irradiation or E2F1 infection.

From these results, we conclude that the transcriptional activation of genes such as the cyclin A, cyclin E, and DHFR genes may account for the ability of AdE2F1 to bypass an irradiation-induced block. These results also suggest that E2F may be a critical target of the irradiation-induced block.

DISCUSSION

The ability to understand the overall importance and role of a gene-regulatory pathway is most often limited by the capacity to define relevant target genes. Thus, despite enormous effort, the precise targets for control by proteins such as c-Myc and Fos/Jun, to name but a few examples, are not known. Transfection assays have the capacity to demonstrate that a given transcriptional activator is able to stimulate a particular promoter, but these assays do not prove that the activator does stimulate the gene in its normal chromosomal environment. The approach that we describe, making use of an adenovirus vector to express the E2F1 protein in quiescent cells, represents a viable approach to this difficult problem. In particular, these experiments have allowed us to determine if suspected targets for E2F activation are indeed induced within the context of the stimulation of cell proliferation.

Prior experiments have provided considerable evidence for candidate E2F target genes. In particular, the progression from G_1 to S phase is accompanied by the activation of a variety of genes involved in DNA synthesis and many of these genes, including those encoding DHFR, TK, pol α, RR1, RR2, PCNA, and cyclin A, contain E2F binding sites (4, 19, 48, 69, 73). It has been known for some 20 years that these very genes are activated upon infection of quiescent cells by the DNA tumor viruses, an action to facilitate the replication of viral DNA in the natural host for the virus, a postmitotic cell. This provides a further link to E2F, since the ability of these viruses to replicate in quiescent cells and activate these genes is dependent on the ability of viral oncoproteins to inactivate members of the Rb family of proteins (71), which results in the liberation of E2F activity (2).

Sequences spanning the E2F sites in the DHFR gene are

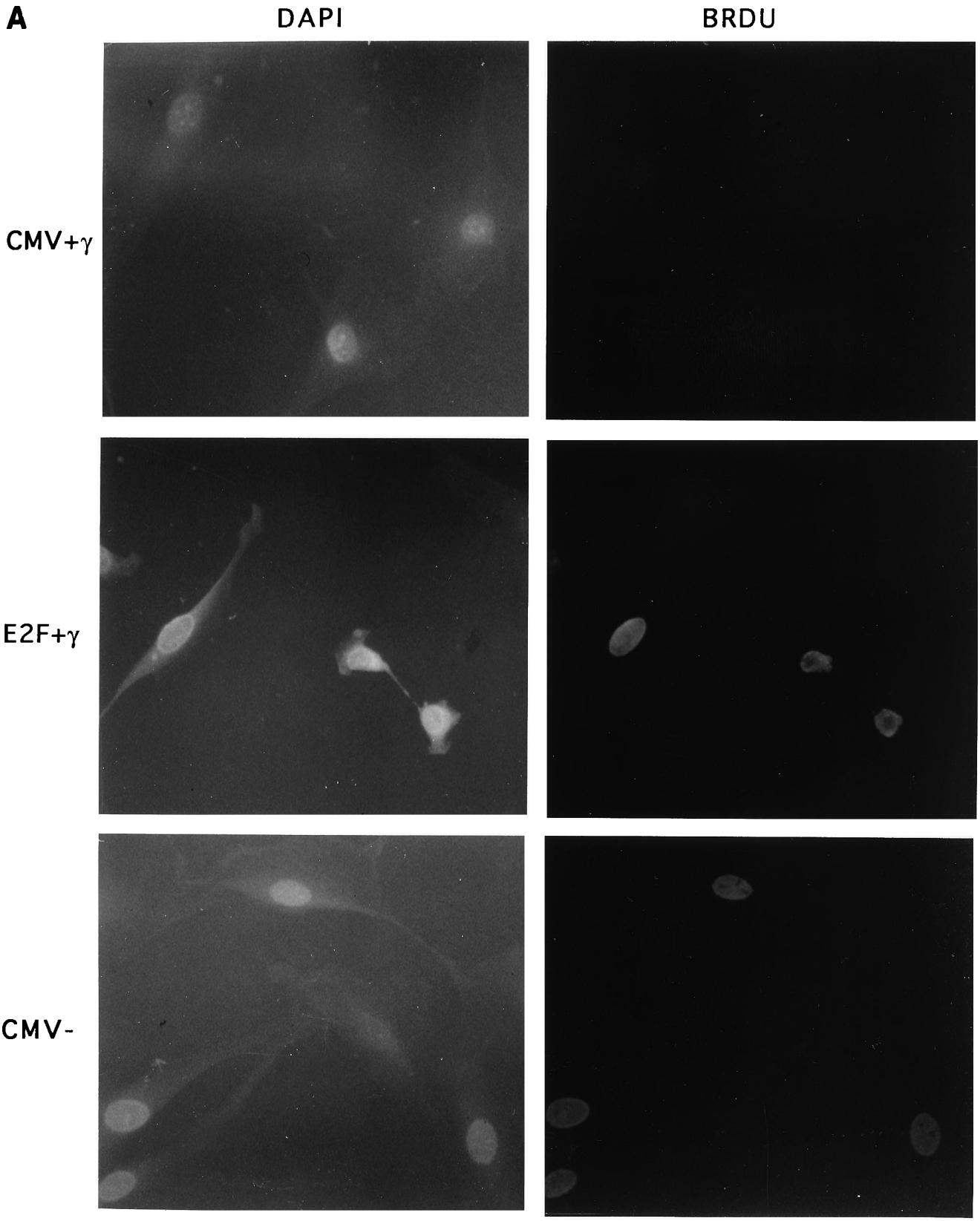
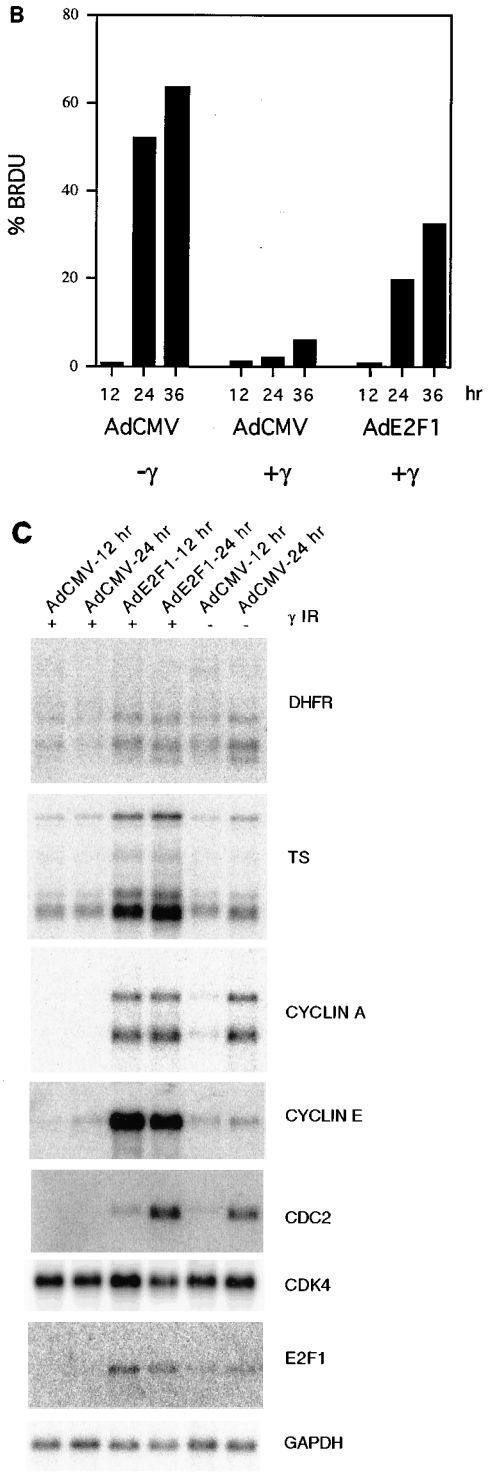


FIG. 5. E2F1 overcomes an irradiation-induced G_1 arrest. (A) Density-arrested REF52 cells were γ irradiated (+) or sham treated (−), infected with the indicated virus (the prefix "Ad" is omitted), and then replated at subconfluent densities. Cells were labeled with BrdU for the 12-h period prior to fixation, and BrdU incorporation was detected by indirect immunofluorescence. Counterstaining for DNA was performed with DAPI. (B) Quantitation of the results presented in panel A, each scored with at least 165 DAPI-stained nuclei. IR, irradiation. (C) Poly(A)$^+$ RNA was isolated either 12 or 24 h postinfection. Poly(A)$^+$ RNA (derived from 160 μg of total RNA per lane) was separated by agarose gel electrophoresis, transferred to nitrocellulose, and probed with either cDNA for DHFR, TS, cyclin A, cyclin E, cdc2, cdk4, or GAPDH or the endogenous specific E2F1 oligonucleotide as described in Materials and Methods. After hybridization and washing, the filters were exposed and analyzed on a PhosphorImager. The figure was generated from the PhosphorImager computer file.



both necessary (5, 42) and sufficient (68) for G_1/S-phase activation of DHFR. The regulation of the TK (12) and *cdc2* (7) promoters by serum has similarly been shown to be dependent on their E2F binding sites. Additionally, the E2F1 gene product can activate the promoters of the DHFR (68), TK, and DNA pol α (36) genes when assayed in cotransfection with promoter-reporter constructs. Thus, E2F appears to be an important element of control in the progression to S phase. Indeed, transient transfection of the E2F1 gene prevented cells from entering quiescence, and expression of E2F1 in already quiescent cells induced S phase (28). The experiments reported here demonstrate that many of these previously suspected targets for the action of E2F are indeed induced by the overexpression of the E2F1 gene product.

Activation of DNA synthesis genes by E2F1. Many of the genes expected to be targets for E2F control, whose products are directly involved in nucleotide metabolism and DNA replication, are indeed activated by AdE2F1 in quiescent cells. Thus, with this finding, we can now firmly establish a link between E2F activation by the DNA tumor virus oncoproteins and the ability of these viral proteins to create the S-phase environment upon infection of a quiescent cell. This is not to say that this is the only relevant activity for these viral proteins in the activation of S phase. Indeed, it is clear that the N-terminal domain of adenovirus E1A also contributes to the activation of cell proliferation but activates transcription in an E2F-independent manner (32, 70).

Despite the clear activation of many of the genes previously suspected to be E2F targets, some genes were only mildly induced by E2F1 overexpression and others were not induced at all, despite previous experiments that have shown binding of the E2F1 product to the respective promoters and transactivation by E2F1 in transient-transfection assays (50, 59, 63, 68). The basis for these differences is not yet clear, but several possibilities can be envisioned. The lack of activation could be a reflection of the complexity of the transcriptional regulation of these genes. Certainly, it is possible that although E2F1 is a component of the control of these genes, other factors may also be critical but not produced in the cells overexpressing E2F1. Indeed, mutational analysis of the DNA pol α promoter indicates that multiple factors, including E2F1, contribute to the induction of pol α transcription by serum stimulation of quiescent cells (53). In support of this possibility, we observe a much better activation of the DHFR, *cdc2*, and cyclin A genes by AdE2F1 in irradiated cells than in serum-starved cells. Perhaps the cooperative action of other transcription factors, together with E2F1, dictates promoter responsiveness. Alternatively, it is also possible that genes such as the DHFR and TK genes are regulated by other members of the E2F family rather than the E2F1 product. Various experiments have now clearly documented a complex array of gene products that contribute to the E2F activity within the cell (3, 13, 15, 18, 25, 29, 35, 64). Although the pattern of accumulation of these additional E2F gene products is not yet clear, particularly with respect to cell cycle accumulation, it remains possible that other E2F family members also participate in G_1/S control but with different promoter specificities.

The involvement of E2F1 in a cell growth-regulatory loop. The fact that the S-phase target genes for E2F1 action include not only genes encoding proteins directly involved in DNA replication but also regulatory genes such as the cyclin A gene and B-*myb* places E2F in a broader context of cell growth regulation. Various experiments have documented an essential role for cyclin A in the progression of cells into and through S phase (14, 51). Likewise, antisense inhibition of B-Myb expression in mouse 3T3 cells inhibits cell growth (60), and the overexpression of B-Myb reduces the serum requirement for these cells and allows anchorage-independent growth (60). Moreover, constitutive expression of the B-*myb* gene has been shown to bypass a p53-induced G_1 arrest (37). Although the precise biochemical function of either cyclin A or B-Myb is yet to be defined, these previous experiments clearly depict an essential role for these proteins in cell growth. The results

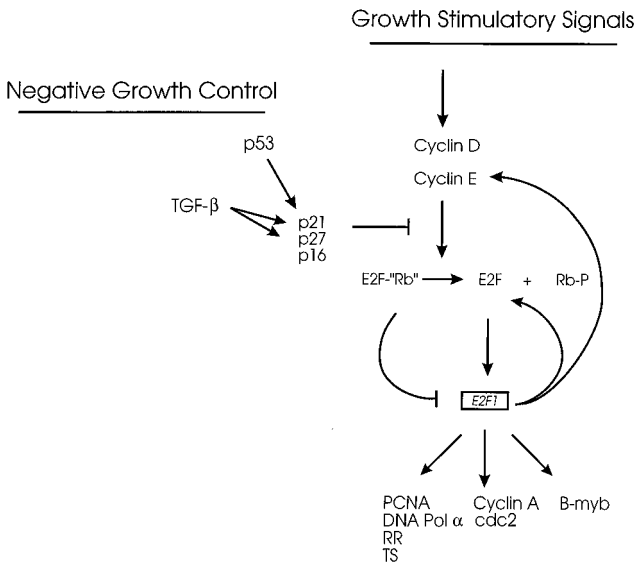


FIG. 6. Role of the E2F1 gene product in G_1 growth control. TGF-β, transforming growth factor β.

presented here suggest that the accumulation of these proteins during the cell growth response is dependent on E2F activity.

The progression of cells through G_1 and into S phase involves a complex series of regulatory events that operate in both a positive and a negative manner. These activities have been identified through a convergence of studies directed at basic cell cycle events in yeast systems as well as in studies of mammalian oncogenesis. What has emerged from this work is a complex regulatory cascade schematically depicted in Fig. 6. Growth-stimulatory signals activate G_1 cyclins and associated kinases. These kinases are subject to negative control by a family of inhibitory proteins including p16, p21, and p27, some of which mediate the growth regulatory actions of the p53 tumor suppressor as well as transforming growth factor β (54). At least one of the targets for G1 cyclin/kinase activity is the Rb protein and likely other Rb family members. Given the results of past experiments that have shown that only the unphosphorylated form of Rb can interact with E2F (6), this phosphorylation would lead to the release of E2F and the activation of E2F target genes.

The movement of cells out of G_0 and through G_1 is dependent on the accumulation of the G_1 cyclins, including cyclin E and the D-type cyclins (66). The results presented here clearly show a role for the E2F1 product in the control of accumulation of the cyclin E gene product. In addition, our previous experiments have shown that activation of the E2F1 gene is a relevant downstream event of the action of the G_1 cyclins and associated kinases, as indicated by the fact that elevation of either D-type cyclin/kinase activity or cyclin E/kinase activity leads to an activation of the E2F1 promoter (27). In addition, our experiments also demonstrate that the E2F1 gene product regulates its own transcription, consistent with recent experiments investigating the function of the E2F1 promoter (22, 27, 47). Moreover, two other components of E2F activity, E2F2 and DP1, are also induced by E2F1 overexpression. It is thus evident that a complex feedback control, serving to amplify the accumulation of E2F activity, can be seen to operate during the progression of cells through G_1.

In many respects, the relationship of E2F activity and G_1 cyclin/kinase activity is much the same as the components identified in yeast cells that define the G_1/S checkpoint known as Start. The product of the *Saccharomyces cerevisiae SWI6* gene is a component of two related transcription activities termed SBF and MBF. MBF, which is a heterodimeric complex containing the SWI6 product and a 120-kDa protein, activates the expression of S-phase genes such as the DHFR, TK, and RR genes (9, 16, 38, 40, 55), whereas SBF, which also contains the *SWI6* product but together with the *SWI4* product rather than p120, is responsible for the activation of G_1 cyclin gene transcription (46, 49). In turn, the G_1 cyclins, together with cdc28, may stimulate the accumulation of active SWI4 and SWI6 (1). In the fission yeast *Schizosaccharomyes pombe*, the *cdc10* gene product, which has homology to the *SWI4* and *SWI6* products, is a component of a heteromeric complex that activates the transcription of the RR gene (39) and *cdc18* (30), the only genes demonstrated to be cell cycle regulated in *S. pombe*. The fact that these yeast transcriptional regulatory activities define part of the Start checkpoint, together with the fact that the targets for activation are the same genes that we show here to be activated by E2F1, suggests that E2F1 activation may constitute the mammalian cell equivalent of Start, otherwise known as the restriction point. This realization, together with previous experiments that demonstrate an ability of E2F1 to induce quiescent cells to enter S phase, clearly implicates E2F as a critical regulator of cell growth during G_1.

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