E2F-1 accumulation bypasses a G₁ arrest resulting from the inhibition of G₁ cyclin-dependent kinase activity

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Numerous experiments have defined a critical role for the G₁ cyclins and associated kinases in allowing a normal progression of cells from a quiescent state, through G₁, and into S phase. We now demonstrate that G₁ cyclin-dependent kinase activity is critical for the accumulation of E2F activity late in G₁. Moreover, E2F-1 overexpression can overcome a G₁ arrest caused by the inhibition of G₁ cyclin-dependent kinase activity, consistent with E2F activation being an important consequence of the action of G₁ cyclins. E2F-1 also overcomes a G₁ block caused by γ irradiation and leads to an apparent complete replication of the cellular genome and entry into mitosis. This E2F-1-mediated induction of S phase and mitosis is not accompanied by the rise in either cyclin D-associated kinase activity or cdk2 activity that is normally observed during the G₁ phase of the cell cycle. We conclude that one key function for G₁ cyclin-dependent kinase activity is the activation of E2F-1, that the accumulation of E2F activity may be sufficient to allow initiation and completion of S phase, but that additional events, including G₁ cyclin kinase activity, are likely necessary for a normal proliferative event.

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The decisions that dictate cell growth, quiescence, and terminal differentiation are governed by a variety of extra- and intracellular cues that impinge on the regulatory mechanisms governing the cell cycle. Particularly critical are the cues that regulate the passage of cells through the G₁ phase of the cell cycle and into S phase. Mammalian cells remain responsive to these cues for most of G₁, until they pass through a checkpoint referred to as the restriction point, after which they become committed to enter and complete S phase (Pardee 1989). Considerable attention has focused on the various G₁ cyclins and partner cyclin-dependent kinases (cdks) as key regulatory activities that govern the transition of the cell through G₁ and that allow entry into S phase. These include the D-type cyclins (D1, D2, and D3), which activate the cdk4 and cdk6, as well as cyclin E, which activates cdk2 (Sherr 1994). Cyclin D-dependent kinase activity arises in mid-G₁ and is required through late G₁ for progression into S phase (Baldin et al. 1993; Quelle et al. 1993). Cyclin E-dependent kinase activity also oscillates during the cell cycle, accumulating somewhat later in G₁, near the beginning of S phase, and is also required for S-phase entry (Ohtsubo et al. 1995).

In addition to the G₁ cyclins, the cyclin A protein accumulates near the beginning of S phase and associates

with and activates cdk2 (Pines and Hunter 1992). Inhibition of either cyclin A (Girard et al. 1991; Pagano et al. 1992; Zindy et al. 1992) or cdk2 function (Pagano et al. 1993; Tsai et al. 1993; van den Heuvel and Harlow 1993) prevents entry into S phase. A direct role for cyclin A and cdk2 in S phase has been suggested on the basis of their colocalization at sites of DNA replication (Cardoso et al. 1993). Moreover, *Xenopus* extracts depleted of the cdk2 protein are incapable of initiating DNA replication, whereas extracts depleted of cdc2 kinase do not enter mitosis (Blow and Nurse 1990; Fang and Newport 1991).

In addition to cyclin association, cdk activity during cell-cycle progression can be regulated by the association of the recently identified cdk inhibitors (CKIs) (Peter and Herskowitz 1994). The binding of these proteins to the kinases can inhibit cdk activity either by preventing the activating phosphorylation of the kinase subunit or by directly abrogating the kinase activity of the catalytic subunit. This group of inhibitors includes p15 and p16, which appear to specifically target D-type cyclin kinases, and p21^{CIP1/WAF1/SDI1} and p27^{KIP1} (henceforth termed p21 and p27), which can inhibit all of the G₁ cdks yet assayed. The importance of these CKIs in cell-cycle control has been inferred from the observations that the inhibitory protein transforming growth factor- β (TGF- β) induces the expression of various CKIs (Elbendary et al. 1994; Hannon and Beach 1994; Reed et al. 1994; Data et

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al. 1995; Li et al. 1995), that the anti-mitogenic effect of rapamycin in T-cells prevents the interleukin-2 (IL-2)-stimulated down-regulation of p27 (Nourse et al. 1994), and that cAMP-mediated G_1 arrest appears to be mediated by the induction of p27, which prevents the activation of cdk4 (Kato et al. 1994). Furthermore, the DNA damage-mediated G_1 arrest can be partially attributable to the p53-dependent induction of the p21 CKI (Dulic et al. 1994; El-Deiry et al. 1994). Overexpression of either p16, p21, or p27 arrests cell growth (Harper et al. 1993; Noda et al. 1994; Polyak et al. 1994; Toyoshima and Hunter 1994; Nakanishi et al. 1995; Serrano et al. 1995).

Considerable attention has been focused on the retinoblastoma gene product Rb as a key substrate for the action of the G_1 cdk. In particular, it appears that the phosphorylation of Rb that occurs as quiescent cells enter the cell cycle, which is believed to inactivate its capacity to function as a growth suppressor, is mediated by the D-type cdk (Dowdy et al. 1993; Ewen et al. 1993a; Kato et al. 1993). One consequence of this phosphorylation is the incapacitation of Rb to bind to and regulate the E2F transcription factor (Chellappan et al. 1991). Convincing evidence has accumulated suggesting that the E2F transcription factor is a relevant target of the action of Rb as a growth suppressor. E2F activity controls the transcription of a group of genes that are normally regulated at the G_1/S transition and that encode proteins important for S-phase events including dihydrofolate reductase (DHFR), thymidine kinase, and DNA polymerase α (Nevins 1992; Helin and Harlow 1993; La Thangue 1994). The interaction of Rb with E2F results in an inhibition of E2F transcriptional activity (Cress et al. 1993; Flemington et al. 1993; Helin et al. 1993; Dynlacht et al. 1994), and the ability of Rb to interact with E2F directly correlates with the ability of Rb to arrest cell growth in G_1 (Qian et al. 1992; Qin et al. 1992; Hiebert 1993). In each case, Rb mutants isolated from human tumors or constructed in vitro that lack the ability to cause a growth arrest also lack the ability to bind E2F and inhibit E2F-dependent transcription. Overexpression of the E2F-1 product drives quiescent cells into S phase (Johnson et al. 1993; Qin et al. 1994; Shan and Lee 1994), can transform an established cell line (Singh et al. 1994; Xu et al. 1995), or in cooperation with an activated *ras* oncogene leads to oncogenic transformation of primary rat embryo cells (Johnson et al. 1994a).

All of these results suggest a central role for E2F in cell-cycle control, functioning as an Rb-regulated transcriptional activity important for the transition from G_1 into S phase. To explore the role of E2F in the progression through the G_1/S control point, particularly as it relates to the role of E2F as a target for G_1 cyclin-dependent kinases, we have assayed for the ability of these kinases to activate E2F function. We have also measured the ability of the E2F-1 product to overcome a G_1 arrest resulting from the inhibition of G_1 cdk activity. These experiments demonstrate that E2F-1 can overcome the G_1 arrest and show that S phase can proceed without the normal accumulation of G_1 cdk activity.

Results

Activation of the E2F transcription factor by G_1 cdk

A role for G_1 cdk activity in E2F transcriptional activation was suggested previously by the ability of overexpressed cyclin/cdk proteins to activate the E2F-1 promoter in serum-starved fibroblasts, depending on the presence of E2F binding sites in the E2F-1 promoter (Johnson et al. 1994b). To test further the possibility that G_1 cdk might regulate E2F-dependent transcription, two CKIs (p21 and p27) known to inhibit a broad spectrum of cdk in both in vitro and in vivo assays (Peter and Herskowitz 1994; Morgan 1995) were assayed for their effects on E2F transcription activity. The established rat embryo fibroblast line REF52 was transfected with cdk2- or cdk4-encoding plasmids together with cyclin E or various D-type cyclin-encoding plasmids, with or without the CKI-expressing plasmids, and assayed for E2F-1 promoter activity in quiescent cells using an E2F-1 promoter-CAT fusion. As shown in Figure 1A, the expression of G_1 cyclins, together with cdk, led to a stimulation of E2F-1 promoter activity. In addition, the results demonstrate clearly that each of the CKIs tested was capable of abrogating the cyclin/cdk-dependent activation of the E2F-1 promoter.

Transcription of the E2F-1 gene is cell-cycle regulated, detectable only at very low levels in serum-starved cells but at severalfold higher levels late in G_1 , and at a maximum level during S phase (Slansky et al. 1993; Johnson et al. 1994b; Hsiao et al. 1994). The cdk are key regulators of, and are themselves tightly regulated by, the cell cycle. Given the fact that exogenously expressed kinase activity can modulate E2F-1 promoter activity, we have sought to determine whether endogenous G_1 cdk activity might be responsible for the E2F-dependent increase in E2F-1 transcription observed late in G_1 . To this end, REF52 cells were transfected with plasmids encoding p21 or p27 along with an E2F-1-promoter-luciferase construct, starved for 48 hr, and then stimulated by serum addition. Cells were harvested at 4 hr intervals and assayed for luciferase activity. As shown in Figure 1B, transfection of either p21 or p27 abrogated the serum-induced activation of the E2F-1 promoter, indicating that cdk activity during G_1 is required for late G_1 E2F activation.

Whereas p21 and p27 can inhibit each of the G_1 cyclin-associated kinases as well as cyclin A-dependent kinase activity, p16 has been shown to be a specific inhibitor of D-type cdk activity (Serrano et al. 1993; Chan et al. 1995). D-type cyclin kinases are thought to be responsible for the phosphorylation of Rb family members during mid- to late G_1 (Hinds et al. 1992; Dowdy et al. 1993; Kato et al. 1993), preventing Rb interaction with E2F, thus allowing an accumulation of E2F transcriptional activity. We have therefore determined whether the more specific kinase inhibitor, p16, could also abrogate the accumulation of E2F activity found late in G_1 . In a similar serum-stimulation transfection assay to that described above, cotransfection of a p16-expressing plasmid completely eliminated the serum-dependent accu-

mulation of E2F activity late in G₁ (Fig. 1C). Taken together, these experiments provide direct evidence for a role of G₁ cdk activity in the cell cycle-dependent accumulation of E2F activity.

E2F-1 expression overcomes a G₁ arrest induced by CKIs

Various experiments suggest that the accumulation of D-type cyclin-associated kinase activity is critical for a cellular growth response and coincides with the phosphorylation of the Rb protein, as well as other Rb family members, during the transition from a quiescent state, through G₁, and into S phase (Sherr 1994). The inhibition of cyclin D1 function has been shown to arrest cells in G₁ (Baldin et al. 1993; Quelle et al. 1993), whereas over-

expression of cyclin D1 was shown to accelerate progression through G₁ (Jiang et al. 1993; Quelle et al. 1993; Resnitzky et al. 1994). Likewise, the inhibition of cdk2 activity also blocks the entry of cells into S phase (Pagano et al. 1993; Tsai et al. 1993; van den Heuvel and Harlow 1993) as does the inhibition of cyclin E (Ohtsubo et al. 1995) and cyclin A (Girard et al. 1991; Pagano et al. 1992; Zindy et al. 1992). To assess the role of these kinases in relation to E2F accumulation, we sought to inhibit either D-type cdk activity or cdk2 activity and then measure the ability of E2F-1 to override the growth-suppressing effect resulting from this inhibition.

REF52 cells were transfected with the plasmids expressing p16, p21, or p27 alone or together with the E2F-1-expressing plasmid. In addition, we also employed an inactive cdk2 mutant (cdk2dn), shown previously to arrest cells in G₁ presumably as a result of the inhibition of cdk2 activity (van den Heuvel and Harlow 1993). BrdU was added 42 or 48 hr later, the cells were incubated 18 hr more, and then fixed and processed for β -galactosidase and BrdU double immunofluorescence staining. The percent BrdU labeling from three different experiments is presented in Figure 2A. These results demonstrate clearly that each of the inhibitors effectively blocked cell proliferation as measured by BrdU incorporation and that E2F-1 expression abrogates the growth arrest allowing DNA replication to ensue.

We have also determined whether E2F-1 could overcome a G₁ arrest that was already established by expression of each of the inhibitors. REF52 cells were first transfected with plasmids encoding the inhibitors, then infected with an E2F-1-expressing adenovirus (AdE2F-1) or a control cytomegalovirus (AdCMV), and subsequently incubated with BrdU for 24 hr more. As seen from the data in Figure 2B, E2F-1 overexpression was able to overcome the induced G₁ block and allowed cells

Figure 1. Activation of the E2F-1 promoter by G₁ cyclin-dependent kinases. (A) Expression of G₁ cdk activate the E2F-1 promoter. REF52 cells were transfected with E2F-1-CAT, CMV- β -gal, together with CMV-cycD1, CMV-cycD2, CMV-cycD3, or CMV-cycE, and CMV-cdk2 or CMV-cdk4 as indicated. In addition, transfections included either the CMV vector (open bar), CMV-p21 (solid bar), or CMV-p27 (hatched bar). 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 by use of β -galactosidase activity. (B) Cyclin kinase-dependent activation of the E2F-1 promoter by serum stimulation. REF52 cells were transfected with E2F-1-LUC (luciferase), CMV- β -gal, and CMV-p21 (○), CMV-p27 (◇), or CMV control vector (□). After transfection, cells were washed and starved in DMEM with 0.1% serum, and subsequently stimulated with DMEM containing 20% serum. Cells were harvested at 4-hr intervals and assays for luciferase and β -galactosidase were performed. Luciferase results were corrected by use of β -galactosidase activity. (C) D-type cyclin kinase-dependent activation of the E2F-1 promoter by serum stimulation. REF52 cells were transfected with E2F-1-LUC, CMV- β -galactosidase, and either CMV-p16 (◇) or CMV control vector (□). Cells were then treated, harvested, and assays were performed as in B.

Figure 2. E2F-1 overcomes a G_1 arrest by CKIs. (A) E2F-1 expression prevents a G_1 arrest by G_1 CKIs. REF52 cells were transfected with CMV- β -gal with CMV5, CMV-p16, CMV-p21, CMV-p27, or neo-Bam-cdk2dn, and with CMV-E2F-1 as indicated. Following transfections, cells were trypsinized and replated on dishes containing glass coverslips. BrdU was added after 42 hr, or in the case of neo-Bam-cdk2dn after 48 hr, and incubated an additional 18 hr. Coverslips were then fixed and processed as described in Materials and methods and examined by use of fluorescence microscopy. The data plotted represents the average of three independent experiments in which ~250 cells were counted per sample. (B) E2F-1 expression can induce S phase in cells arrested by G_1 CKIs. REF52 cells were transfected with CMV- β -gal with CMV5, CMV-p16, CMV-p21, CMV-p27, or neo-Bam-cdk2dn. Following transfections, cells were trypsinized, replated, and, after 48 hr, infected with Ad-CMV or AdE2F-1, or mock-infected as described in Materials and methods. BrdU was subsequently added and 24 hr later, cells were fixed, processed and examined as in A. The data plotted represent the average of three independent experiments in which 250 cells were counted per sample. (C) E2F-1 transcriptional activity is required to overcome G_1 arrest. REF52 cells were transfected with CMV- β -gal with CMV5, CMV-p21, CMV-p27, or neo-Bam-cdk2dn and with CMV vector, CMV-E1A₁₂₅, CMV-E2F1, CMV-E2F1-VP16, or CMV-E2F1₁₃₂. Cells were subsequently treated as in A.

associated kinases involved in the regulation of S-phase entry.

Although it seemed most likely that the ability of E2F-1 to overcome the CKI-mediated arrest was attributable to the activation of E2F-regulated target genes, an alternative possibility was that the overexpression of E2F-1 bypasses the block by simply sequestering Rb. We have addressed this issue through the use of an E2F-1-VP16 chimera, in which sequences involved in transcriptional activation as well as Rb binding have been replaced with the transcription activation domain of VP16. This chimera activates transcription in an E2F-dependent manner but does not bind to and is not repressed by Rb (Johnson et al. 1994a). As shown in Figure 2C, the E2F-1-VP16 fusion protein can also overcome a G_1 arrest mediated by p21, p27, or the cdk2dn mutant. Furthermore, an E2F-1 mutant (E132), which does not bind DNA and does not activate transcription but does retain the ability to bind to Rb (Cress et al. 1993), fails to overcome the G_1 arrest. We conclude that the ability of E2F-1 to override the G_1 arrest is not dependent on Rb sequestration but, rather, the ability of E2F-1 to activate transcription.

E2F-1-mediated bypass of a γ -irradiation arrest leads to replication of the cellular genome

Irradiation of normal cells by γ rays results in a cell-cycle arrest, either in G_1 or G_2 , presumably to allow for the repair of damaged DNA. At least part of the G_1 arrest is attributable to a p53-mediated activation of the p21 gene, resulting in an inhibition of G_1 cdk (Dulic et al. 1994; El-Deiry et al. 1994). The fact that this G_1 arrest can be overcome by E2F-1 expression (DeGregori et al.

to enter S phase. The above results suggest that the accumulation of E2F activity is a key end-target of cyclin-

1995), together with the ability of the AdE2F-1 virus to express the E2F-1 protein in the entire population of cells, affords the opportunity to examine the events associated with this E2F-1-mediated bypass. Although the assay of BrdU incorporation provides clear evidence for a role of E2F-1 in the induction of DNA synthesis (Fig. 3A) (DeGregori et al. 1995), it provides little information as to the nature of the event and whether a normal S phase is occurring. To better analyze the induction of S phase following E2F-1 expression, we have measured DNA content in the cells by flow cytometry. Density-arrested REF52 cells were trypsinized, γ -irradiated (+ γ) or mock treated (–), infected with the AdE2F-1 or the control AdCMV virus, and then replated at subconfluent densities for analysis. As seen in Figure 3B, density-arrested cells exhibited a G₁ content of DNA. Replating of the unirradiated cells (CMV–) resulted in an induction of S phase by 18 hr and an accumulation of cells with a G₂ content of DNA by 21–24 hr, indicating the completion of DNA replication. The γ -irradiated cells (CMV + γ) maintained the G₁ DNA content throughout the experiment, with minimal progression into S phase. In sharp contrast, the γ -irradiated cells that were expressing the E2F-1 product (E2F + γ) exhibited a profile virtually identical to the unirradiated cells. Clearly, the expression of E2F-1 does not merely induce DNA synthesis but appears to induce a complete, or essentially complete, round of cellular DNA replication.

Given the accumulation of cells with a G₂ DNA content as a consequence of E2F-1 expression, we have also examined the cultures for evidence of mitotic figures at a time point 24 hr after replating (Fig. 3C). Cells that were γ -irradiated and infected with the control virus exhibited very few mitotic cells ($0.25 \pm 0.25\%$), in agreement with the inhibition of DNA synthesis under these conditions. In contrast, the γ -irradiated cells that were infected with the AdE2F-1 virus exhibited numerous mitotic cells ($6.23 \pm 0.07\%$), similar in number to the cultures of the nonirradiated cells ($4.49 \pm 0.39\%$). Therefore, cells overexpressing E2F-1 can progress through S phase and into mitosis following a γ -irradiation-induced G₁ block. It is also clear, however, that the mitotic cells resulting from E2F-1 expression following γ -irradiation were not normal (Fig. 3C). Chromosomes did not segregate properly and nuclear blebbing was observable, possibly as a consequence of mitosis in the presence of irradiation-induced DNA strand breaks.

Western blot assays for E2F-1 production demonstrated that the level of E2F-1 in the infected cells was ~10-fold above that in unirradiated cells (Fig. 4A). Examination of functional E2F DNA-binding activity in extracts of these cells (Fig. 4B) revealed an increase in free E2F-1 activity (indicated by arrow) in the virus-infected cells over that seen in the control, γ -irradiated cells (cf. lanes 1 and 5). This accumulation of free E2F coincides with the activation of endogenous E2F target genes (DeGregori et al. 1995). It is also clear from this assay that although E2F-1 is overexpressed relative to the endogenous levels found in these cells (Fig. 4A), there is not a gross overproduction of total E2F DNA-binding activity

under these conditions. Finally, it is also true that the endogenous E2F_{G1} complex, which contains the Rb-related p130 protein in association with E2F-4 and is characteristic of G₀ and early G₁ cells (Vairo et al. 1995), is maintained in the AdE2F-1-infected cells (cf. lanes 1 and 5). Thus, although these cells do enter S phase as a consequence of E2F-1 expression, events associated with the normal progression of cells through G₁ and into S phase are not occurring.

E2F-1-mediated bypass of γ -irradiation arrest is not accompanied by the normal accumulation of G₁ cdk activity

Numerous experiments have shown that G₁ cdk activity is required for the onset of S phase in mammalian fibroblasts. The fact that E2F-1 expression could overcome cell-cycle blocks resulting from the inhibition of G₁ cdk activity raised the possibility that these E2F-1-mediated bypasses might simply be attributable to the restoration of G₁ cdk activity. Alternatively, E2F-1 expression might obviate the need to restore the activation of G₁ cdk, as might be expected if a major role of G₁ cdk activity is to activate E2F. We have thus investigated this question directly by examining kinase activity in extracts of cells treated as described above. Cyclin D1-associated kinase activity was low in density-arrested cells, increased substantially 12 hr after replating of the cells, and then rose further as the cells progressed through G₁ and into S phase (Fig. 5A, CMV– samples). In contrast, γ -irradiated cells exhibited a minimal induction of cyclin D1-associated kinase activity (Fig. 5A, CMV + γ) likely accounted for by the small percentage of cells that enter S phase under these conditions (Fig. 3A). Importantly, irradiated cells that were infected with the AdE2F-1 virus exhibited no increase in cyclin D1-associated kinase activity over the levels seen in the control irradiated cells (Fig. 5A, E2F + γ). Similarly, there was no restoration of cyclin D2- or D3-associated kinase activity in the E2F-1-expressing cells (Fig. 5B).

Similar to the results of D-type cdk activity, cdk2 activity was also inhibited following γ irradiation. As shown in Figure 6, A and B, cdk2 activity was very low in density-arrested cells and then rose to peak levels 18 to 24 hr after the replating of the unirradiated cells. Subsequently, this activity decreased as cells progressed through M phase. Cyclin E and cyclin A-associated kinase activity also increased during the G₁/S transition. As expected from previous work (Dulic et al. 1994; El-Deiry et al. 1994), γ irradiation prevented the G₁ accumulation of cdk2, and cyclin E- and cyclin A-specific kinase activities. The inhibition of kinase activity and cell-cycle progression in γ -irradiated REF52 cells is p53-dependent (data not shown). Similar to the assays for D cyclin-dependent kinase activity, irradiated cells that were infected with the AdE2F-1 virus revealed only a minimal increase in either cdk2 activity or cyclin E- or cyclin A-associated kinase activity that was comparable to the levels seen in the γ -irradiated, arrested cells. The small increase in cyclin A-associated kinase activity that

Figure 3. E2F-1-mediated bypass of a γ -irradiation G_1 arrest leads to a completion of S phase and entry into mitosis. (A) Density-arrested REF52 cells were γ irradiated (+ γ) or mock treated (–), infected with the indicated virus, and then replated at subconfluent densities. Cells were labeled with BrdU for the 6-hr period prior to fixation, and BrdU incorporation was detected by indirect immunofluorescence. At least 300 DAPI-stained nuclei were scored. (B) Cells were harvested at the indicated times after plating and examined for DNA content by propidium iodide staining and flow cytometry. Each plot represents the analysis of 10,000 gated events. (C) Cells were γ irradiated (+ γ Irrad) or mock treated (– γ Irrad), infected with either the AdE2F-1 (+E2F-1) or the AdCMV virus (other panels), fixed 24 hr after plating in 1:1 methanol/acetone and the DNA stained with DAPI. Representative mitoses are shown.

was observed in AdE2F-1-infected, irradiated cells likely represents cdc2 kinase activity because we reproducibly observed no increase in total cdk2 activity, whereas cdc2

kinase activity was restored in the AdE2F-1-infected, irradiated cells (Fig. 6A; data not shown). In addition, a recombinant adenovirus that overexpresses the E2F-1–

Figure 4. E2F-1 activity following infection with AdE2F-1. (A) Western blot assay. Density-arrested REF52 cells were γ irradiated (+ γ) or mock treated (–), infected with the indicated virus, replated at subconfluent densities, and harvested 18 hr later. Approximately 10^5 cells were resuspended in SDS loading buffer, and E2F-1 protein levels analyzed by Western blotting. (B) DNA-binding activity. An electrophoretic mobility shift assay with lysates harvested 24 hr after replating was performed with a DHFR E2F binding site as a probe. The indicated antibodies (0.1 μ g of purified antibody or 0.25 μ l of immune or preimmune antiserum) were added after 20 min of binding, and the reaction continued for an additional 20 min. Where indicated, mutant (mut) or wild-type (wt) E2F binding site oligonucleotide (10 ng) was added as a competitor to the E2F-1 + γ extract prior to probe addition. The bracket labeled E2F (right) depicts the position of free E2F complexes. The positions of the free E2F-1 and the E2F-1 and the E2F1Rb complexes are indicated at left.

VP16 chimera was also able to induce S phase, indicating that the E2F-1-mediated bypass of the G₁ block is not dependent on Rb sequestration. Importantly, this γ -irradiation bypass did not result in the restoration of cdk2 activity (Fig. 6C).

In contrast to the above results, E2F-1 rescue of DNA synthesis in γ -irradiated cells did result in cyclin B1-associated kinase activation with kinetics similar to control, nonirradiated cells (Fig. 6A,B), that coincided

with entry into mitosis (Fig. 3C). This observation raises the interesting possibility that the activation of the M-phase kinase does not depend on the prior activation of cdk2s. The activation of cyclin B1-dependent cdc2 kinase, however, is dependent on progression through S phase, because the inhibition of DNA synthesis by aphidicolin eliminated M-phase kinase activation (data not shown). The activation of cyclin B1-dependent cdc2 kinase may partially result from the AdE2F-1-dependent restoration of cdc2 mRNA levels, which are down-regulated by γ -irradiation (DeGregori et al. 1995).

In light of the assays showing minimal induction of D-type cyclin and cdk2-associated kinases, we examined

Figure 5. E2F-1-mediated S-phase induction does not lead to accumulation of D-type cdk activity. (A) Density-arrested REF52 cells were γ irradiated (+ γ) or sham treated (–), infected with the indicated virus, and then replated at subconfluent densities. Cells were lysed in IP buffer at the indicated times (in hours) after plating, immunoprecipitated with antibodies against either the LacZ protein (control lane) or cyclin D1 (other lanes). The immunoprecipitates were then tested for kinase activity with GST-Rb as a substrate (top). The 0-hr lysate was from the density-arrested cells. The control reaction contained AdCMV-infected, mock-treated cell lysates harvested at 12 hr. The relative kinase activity was determined by quantitation of the signal from the individual GST-Rb bands, subtraction of the signal obtained for the control reaction, and determination of the values relative to the maximal signal. (□) CMV–; (◇) CMV+ γ ; (○) E2F+ γ . (B) Cells were lysed in IP buffer at 18 hr after plating, immunoprecipitated with antibodies against cyclin D1, D2, D3, or the LacZ protein (Z), and the immunoprecipitates tested for kinase activity with GST-Rb as substrate.

Figure 6. E2F-1-mediated S-phase induction does not lead to the accumulation of cdk2 activity. (A) Density-arrested REF52 cells were γ irradiated (+ γ) or sham treated (–), infected with the indicated virus, and then replated at subconfluent densities. Cells were lysed in RIPA buffer at the indicated times (in hours) after plating, immunoprecipitated with antibodies against the indicated cyclin or cdk, and the immunoprecipitate tested for kinase activity against histone H1. The 0-hr lysates were from the density-arrested cells. The control reactions are AdCMV-infected, mock-treated cell lysates harvested either 18 hr (first lane in first and second rows) or 24 hr (first lane in third row) after plating and immunoprecipitated with an antibody against the LacZ protein. (B) The relative histone kinase activity in A was determined by quantitation of the signal from the individual histone bands, subtraction of the signal obtained for the control at 24 hr (first lane in third row, A), and normalization of the values relative to the maximal signal for a particular antibody used. (□) C–, AdCMV–, (◇) C+ γ , AdCMV+ γ ; (○) E+ γ , AdE2F-1+ γ . (C) Bypass of the irradiation-induced G₁ block by E2F-1 does not require Rb binding. Density-arrested REF52 cells were γ irradiated (+ γ) or mock treated (–), infected with the indicated virus (VP16, AdE2F-1–VP16), and then replated at subconfluent densities. Cells were labeled with BrdU from 0 to 24 hr, and BrdU incorporation was detected by indirect immunofluorescence (not shown). At least 300 DAPI-stained nuclei were scored and the percentage of nuclei that incorporated BrdU was as follows: AdCMV + γ , 6.3%; AdE2F-1 + γ , 47.4%; AdE2F-1–VP16 + γ , 30.0%; AdCMV – γ , 88.3%. Cells were lysed in RIPA buffer at the indicated times after plating, immunoprecipitated either with an antibody against the indicated cyclin or cdk, or with rabbit IgG, and the immunoprecipitate tested for kinase activity against histone H1 as shown. (D) Assays of Rb and p107 phosphorylation. Cells were harvested in lysis buffer at the indicated times and immunoprecipitated with antibodies against pRb or p107. pRb and p107 in the immunoprecipitates were detected by Western blotting.

the phosphorylation state of the Rb and related p107 proteins, two suspected targets of these kinases (Beijersbergen et al. 1995; Weinberg 1995). Rb and p107 were immunoprecipitated from cells at the indicated times after replating and detected by Western blotting. Both Rb and p107 migrate in polyacrylamide gels as multiple species, with faster migrating bands corresponding to hypophosphorylated forms and slower bands to hyperphosphorylated forms (Laiho et al. 1990; Beijersbergen et al. 1995). As expected, both Rb and p107 were found primarily in a hypophosphorylated state in the control, irradiated cells, while they both became hyperphosphorylated in the control, nonirradiated cells that progressed through the cell cycle (Fig. 6D). In agreement with the lack of D-type kinase activity in the E2F-1-expressing irradiated cells, and with the fact that p107 phosphorylation can be blocked by a cdk4 dominant-negative mutant (Beijersbergen et al. 1995), p107 remained hypophosphorylated in these cells, displaying a pattern similar to the control, irradiated cells. Although Rb was also hypophosphorylated in the 12-hr sample of E2F-1-expressing cells, it did become phosphorylated at 24-hr, similar to the unirradiated cells. Given the greatly reduced level of cyclin D- and cdk2-associated kinase activities in these cells, it is possible that a different kinase may be responsible for this phosphorylation, particularly given the contrasting p107 result.

From these results we conclude that E2F-1 overexpression can overcome a G₁ arrest mediated by γ -irradiation, that this coincides with the apparent complete replication of the cellular genome, and that such cells are capable of entering into mitosis. All of this takes place without the normal accumulation of G₁ cdk activity. This result, together with the fact that E2F-1 expression can bypass a cell-cycle arrest imposed by either p16, p21, p27 or the cdk2 inactive mutant (Fig. 2), suggests that E2F-1 overexpression can alleviate, at least in part, the requirement for the normal G₁ cdk accumulation necessary for S-phase entry.

Discussion

Role of cyclin-dependent kinases in the control of cell-cycle progression and the accumulation of E2F activity

The evidence in support of a role for the G₁ cdk in controlling cell growth is substantial and underscores the importance of the identification of downstream targets (substrates) for these activities. Phosphorylation of Rb family member proteins during G₁ inactivates the ability of these proteins to control cell growth, and mounting evidence now implicates these proteins as relevant targets of G₁ cdk activity (Cobrinik et al. 1992; Ewen 1994; Weinberg 1995). Several lines of study have now revealed a link between Rb and p107 phosphorylation and E2F activation. First, only the hypophosphorylated form of Rb can interact with E2F; phosphorylation of Rb or p107 would thus lead to the release of E2F and the activation of E2F target genes (Chellappan et al.

1991; Beijersbergen et al. 1995). Second, previous experiments have shown that expression of G₁ cyclins, together with their cognate kinases, can lead to an E2F-dependent activation of the E2F-1 promoter (Johnson et al. 1994b). Similarly, cyclin D1 expression results in an E2F site-dependent activation of the *c-myc* promoter, which can be prevented by Rb expression (Oswald et al. 1994). Likewise, cyclin D1 overexpression results in an E2F-dependent activation of the DHFR and adenovirus E2 promoters that can be blocked by the expression of the p16 CKI (Schulze et al. 1994). Thus, E2F accumulation, as a consequence of inactivation of Rb family members by phosphorylation, is likely to be a downstream event of G₁ cdk action.

Additional experiments indirectly support a role for E2F as a downstream target of the D-type cdk. First, p16 expression has been shown to inhibit the ability of oncogenic Ha-ras to either induce DNA synthesis or transform primary rat embryo fibroblasts in cooperation with *c-myc*, whereas transformation by E1A and oncogenic Ha-ras was not affected by p16 expression, suggesting that E1A may bypass the p16 block (Serrano et al. 1995). Also, the ability of p16 expression to induce cell-cycle arrest is lost in cells lacking functional Rb (Koh et al. 1995; Lukas et al. 1995b). Moreover, whereas the inhibition of cyclin D1 has been shown to prevent S phase in rodent fibroblasts (Baldin et al. 1993; Quelle et al. 1993), cyclin D1 function has been demonstrated not to be required for G₁/S progression in cells expressing the DNA tumor virus oncoproteins, large T antigen, E1A, or E7 (Lukas et al. 1994; Tam et al. 1994), or in Rb^{-/-} fibroblasts (Lukas et al. 1995a). In addition, other experiments have demonstrated that growth inhibition by TGF- β and γ -irradiation, which appears to result from the inhibition of G₁ cdk activity by CKIs, can be overcome by expression of the E2F-1 gene product (DeGregori et al. 1995; Schwartz et al. 1995). These results suggest that activation of E2F may be a consequence of the action of the G₁ cdk, particularly the D-type cyclin kinases. The experiments presented in this paper highlight the ability of the E2F-1 transcription factor, one component of the cellular E2F activity, to induce S phase and mitosis in cells that would otherwise be arrested in G₁ with low levels of G₁ cdk activity.

S-phase induction without the normal accumulation of G₁ cdk activity

The ability of E2F-1 to stimulate the onset of S phase has now been demonstrated in a variety of circumstances including the ability to overcome a serum starvation (Johnson et al. 1993), TGF- β -mediated G₁ arrest (Schwarz et al. 1995), and as shown here, a CKI-mediated arrest. Moreover, the evidence that this represents a normal replication of cellular DNA is best illustrated by the fact that γ -irradiated cells accumulate a G₂ content of DNA following expression of E2F-1, with kinetics similar to that of restimulated cells (Fig. 3B). These E2F-1-expressing cells also proceed into mitosis. Strikingly, E2F-1 overcomes a G₁ block and stimulates DNA repli-

cation without the normal accumulation of G₁ cdk activity.

Previous studies have suggested a direct role for these G₁ cdks in the initiation of S phase. For instance, the inhibition of various G₁ cyclins or kinases with antibodies, antisense RNAs, or dominant-negative mutants has been shown to block S phase. The inhibition of S phase by inactivation of D-type cdks can be explained as a loss of Rb control and thus a loss of E2F control, particularly given the fact that cyclin D1 is no longer required in Rb-deficient cells (Lukas et al. 1995a). Similar experiments show that cyclin E is still essential in Rb-deficient cells (Ohtsubo et al. 1995), suggesting an additional role for cyclin E in the initiation of S phase. These cells, however, do express other Rb family members, which are also capable of controlling E2F and are also likely targets for the action of G₁ cdks. In addition, a variety of experiments have shown that the cyclin A protein is required for G₁/S progression in mammalian fibroblasts (Girard et al. 1991; Pagano et al. 1992; Zindy et al. 1992). In support of this, cyclin A and cdk2 colocalize to sites of DNA replication (Cardoso et al. 1993), suggesting a direct role in replication.

Studies in *Drosophila* and *Xenopus* also highlight the importance of cdk2 in DNA replication. For instance, recent experiments employing *Drosophila* strains bearing mutations in cyclin E and E2F-1, while demonstrating a critical role for both in S-phase entry, also point to a role for cyclin E/cdk2 in activating S-phase independent of its role in activating E2F (Duronio and O'Farrell 1995; Duronio et al. 1995). Moreover, cdk2 is required for in vitro DNA replication in *Xenopus* egg extracts (Fang and Newport 1991) and the addition of cyclin A or cyclin E restores replication in p21-inhibited *Xenopus* extracts (Strausfeld et al. 1994). Also, cdk activity can activate in vitro replication of SV40 DNA in extracts from G₁ cells, and addition of cyclin A to a G₁ cellular extract is sufficient to start DNA replication (D'Urso et al. 1990; Dutta and Stillman 1992), although it is unclear whether cyclin A is the cyclin that performs this function in vivo. In contrast, *Drosophila* embryos that are deficient in cyclin A arrest in G₂ but are competent for DNA replication (Lehner et al. 1991). Moreover, *Xenopus* egg extracts depleted of cyclin A initiate S phase and undergo mitosis but lack the checkpoint that ensures the completion of DNA replication prior to mitosis (Walker and Maller 1991). Under these circumstances, cyclin A does not appear to be required for DNA synthesis.

Although E2F-1 overexpression can clearly overcome a G₁ block without the normal activation of D-type kinases and cdk2, and as such E2F would appear to represent a critical target of these G₁ cdk activities, it is possible that the residual kinase activity remaining in these cells may be sufficient for phosphorylation of certain key targets and may therefore still be required for S-phase entry. The fact that Rb does become hyperphosphorylated, albeit with kinetics that are significantly delayed relative to controls, under conditions where D-type cdk and cdk2 activities remain low, raises the possibility that an alternative E2F-1-induced kinase activity could

result in the phosphorylation of Rb and could potentially be important for the E2F-1-mediated S-phase induction observed here. Nevertheless, the fact that p107 remains hypophosphorylated during an E2F-1-induced S phase, together with recent evidence that p107 phosphorylation is mediated by cyclin-D-kinase (Beijersbergen et al. 1995), once again underscores the fact that a normal G₁ cdk activation has not taken place. In this regard, we also note that the p130-E2F-4 complex persists in cells infected with AdE2F-1. The disappearance of this complex is normally a reflection of the progression of cells out of an arrested state and into the cell cycle, and coincides with phosphorylation of the p130 protein (M.-A. Ikeda, L. Jakoi, and J.R. Nevins, in prep.). The fact that this does not occur in the E2F-1-expressing cells provides yet another indication, in this case functional, that E2F-1 is obviating the need for normal G₁-related events.

Pathways controlling cell proliferation

Finally, we wish to emphasize that although E2F-1 expression can induce an apparently complete round of DNA replication and allow cells to pass through mitosis without the normal G₁ cdk accumulation, we do not believe that this represents a normal proliferative event. This conclusion is based on several observations. First, although these E2F-1-expressing cells do proceed through S phase and into mitosis, we have not seen evidence of a second round of S phase in these cells. This is in contrast to the nonirradiated control cells, which clearly do enter a second cell cycle. Second, there is also evidence that E2F-1 overexpression in γ -irradiated REF52 cells results in apoptosis (data not shown). This observation is consistent with several recent reports that describe an E2F-1-mediated apoptosis that is dependent on p53 function (Qin et al. 1994; Shan and Lee 1994; Wu and Levine 1994; Kowalik et al. 1995). Although the details of this process remain obscure other than the involvement of E2F-1 and p53, analogy to other such events suggests a scenario as generally outlined in Figure 7. Specifically, we suggest that a normal growth stimulus may result in the activation of a series of signal transduction pathways, one of which involves the G₁ cyclins. On the basis of our results and others, we suggest that the activation of E2F is one critical aspect of the action of G₁ cdks and that the activation of E2F may be a key event in triggering S phase. It is apparent, however, that under some circumstances G₁ cdk activity plays a role in addition to the activation of E2F, as illustrated in the analysis of cyclin E in *Drosophila* as described above. Finally, it is also likely that even under the conditions in which the activation of E2F is sufficient for S-phase entry as described here, the activation of E2F may not be sufficient for a complete proliferative event. Additional events regulated by G₁ cdk action could be important for preparing cells for normal mitosis, competency for multiple cell cycles, and prevention of programmed cell death pathways that might otherwise occur.

Growth Stimulatory Signals

Figure 7. E2F activation during the cellular growth response. An E2F activation pathway resulting from growth factor-dependent stimulation of the G₁ cdk activity is depicted. It is assumed that additional signal transduction events take place following growth-factor stimulation involving both additional G₁ cdk targets as well as additional pathways that are independent of G₁ cdk.

Materials and methods

Cells and virus

Viral stocks were created as described previously (Schwarz et al. 1995). Viral titers were determined by an indirect immunofluorescent assay specific for the viral 72-kD E2 gene product as described (DeGregori et al. 1995). The construction of the AdE2F-1 and AdCMV viruses has been described (Schwarz et al. 1995). The AdE2F-1-VP16 adenovirus was similarly created by ligation of the *Bam*HI-*Xba*I fragment containing E2F-1-VP16 from pRSV E2F-1-VP16 (Johnson et al. 1994a) into the *Bam*HI and *Xba*I digested recombination plasmid pGEM-CMV. REF52 cells were a gift from E. Ruley (Vanderbilt University, Nashville, TN) and were grown in Dulbecco's modified Eagle medium (DMEM) containing 5% fetal bovine serum (FBS) and 5% calf serum (CS). REF52 cells were infected with 500 pfu/ml.

γ -Irradiation

Density-arrested REF52 cells (24 hr postconfluent) were trypsinized, resuspended in DMEM containing 10% serum (5% CS and 5% FBS), centrifuged, and resuspended in the same media at 1.5×10^6 cells per ml. The cells were then γ irradiated or mock treated in polypropylene tubes with 12.5 Grays (Gy), centrifuged, washed once in DMEM, and then resuspended in DMEM at 1×10^7 cells/ml. Virus was then added, and the cells

were infected in suspension for 20 min with constant, gentle swirling. Following infection, the cells were replated at subconfluent densities (1.5×10^6 cells/150-mm plate, 4×10^5 /100-mm plate, or 5×10^4 cells/35-mm plate) in 10% serum in DMEM. Prior to cell plating, tissue culture dishes were treated with 0.5% gelatin for ~30 min and then washed with PBS.

BrdU incorporation and DAPI staining

Cells grown on coverslips in 35-mm plates were labeled with 50 μ M BrdU and then fixed and processed for BrdU incorporation as described (Pagano et al. 1994), except that the secondary antibody was an anti-mouse rhodamine-conjugated antibody (Pierce Chemical) at a 1:50 dilution. Counterstaining for DNA was performed by incubation for 10 min in 1 μ g/ml of DAPI [4', 6-diamidino-2-phenylindole-dihydrochloride, Boehringer Mannheim (BMB)] in PBS. In two independent experiments (one experiment shown in Fig. 3C), >200 DAPI-stained nuclei per sample were scored for mitotic figures.

Histone H1 kinase assays

Cells in 150-mm plates were washed twice with Tris-buffered saline and then lysed on the plate (2 ml/150-mm plate) for 30 min at 4°C in RIPA [50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.5% NP-40 containing 1 mM dithiothreitol (DTT), 50 μ g/ml of phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml aprotinin, 20 mM NaF, 0.1 mM sodium orthovanadate]. Total protein in each sample was normalized by use of the Bradford assay (Bio-Rad). Lysates were microcentrifuged for 10 min, and the indicated primary antibody (1 μ g of a purified antibody or 2 μ l of the anti-cyclin A antiserum) added and incubated for 1 hr at 4°C. Twenty microliters of 50% protein A/G-agarose (Oncogene Sciences) was added and incubation was continued for 2 hr more with rocking. Immune complexes on the beads were washed twice in wash A [20 mM Tris-HCl (pH 8.0), 250 mM NaCl, 1 mM EDTA, 0.5% NP-40], twice in the same buffer containing 100 mM NaCl (wash B), and then once in kinase buffer [50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 1 mM DTT]. Affinity-purified rabbit polyclonal antibodies anti-cdk2 (M2), anti-cyclin B1 (GNS1) and anti-cyclin E (M-20) were purchased from Santa Cruz Biotechnology. Control antibodies were anti-LacZ (DC14C7 from Santa Cruz Biotechnology) and purified rabbit IgG (Cappel). Polyclonal antiserum to cyclin A was raised in rabbits by use of glutathione S-transferase (GST)-cyclin A fusion protein as described (Schwarz et al. 1993).

Kinase activity in the washed immunoprecipitate was assayed (Ewen et al. 1993b) by addition of kinase buffer, 5 μ g of histone H1 (BMB), 1 μ M ATP, and 5 μ Ci of [γ -³²P]ATP (3000 Ci/mmol) (DuPont NEN) to a final volume of 50 μ l, and incubation for 30 min at 30°C. The reaction was stopped by addition of SDS sample buffer. The samples were then boiled for 5 min, electrophoresed through a 12% SDS-polyacrylamide gel, and exposed to X-ray film. Dried gels were also exposed and analyzed with a Phosphorimager to quantitate histone H1 kinase activity.

Rb kinase assays

Kinase assays with GST-Rb as the substrate were performed as described (Matsushime et al. 1994), with the following modifications. Cells in 150-mm plates were trypsinized, resuspended in DMEM with 10% serum, washed once with PBS, and resuspended in 1 ml per three 150-mm plates in IP buffer [50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) at pH 8.0], 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 1 mM DTT, 0.1% Tween 20] containing 10% glycerol, 0.1 mM PMSF, 10 μ g/ml of aprotinin, 1 mM NaF, and 0.1 mM sodium orthovana-

date. Cell lysates were frozen in liquid nitrogen. Lysates were thawed and incubated on ice for 2 hr and then microcentrifuged for 10 min. Total protein in each sample was normalized by use of the Bradford assay ($\sim 5 \times 10^6$ cells/immune precipitation). The clarified lysates were then precipitated for 2–4 hr at 4°C with 30 μ l of 50% protein A/G-agarose precoated with 3 μ g of the indicated antibodies. Immune complexes on the beads were washed four times with 1 ml of base IP buffer and then twice with Rb kinase buffer [50 mM HEPES (pH 8.0), 10 mM MgCl_2 , 1 mM DTT]. Affinity-purified rabbit polyclonal antibody anti-cyclin D2 (M20) and monoclonal antibodies anti-cyclin D1 (72-13G), anti-cyclin D3 (18B6-10), and anti-LacZ (DC14C7) were purchased from Santa Cruz Biotechnology. The anti-cyclin D2 antibody has some cross-reactivity with the cyclin D1 protein. The beads containing the immune complexes were then suspended in 30 μ l of Rb kinase buffer containing 2 μ g of fresh GST-Rb and 2.5 mM EGTA, 10 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM NaF, 20 μ M ATP, 2 mM glutathione (from the GST-Rb elution), and 5 μ Ci of [γ - ^{32} P]ATP and incubated for 30 min at 30°C with occasional mixing. The reactions were stopped by addition of SDS sample buffer, and Rb kinase activity was determined as described for the histone H1 assays, except that 7.5% gels were used.

For preparation of Rb substrate, an overnight culture in LB broth plus 50 μ g/ml of ampicillin (LBamp) of *Escherichia coli* transformed with pGEX-Rb [379–926] [Ewen et al. 1993a] was diluted 1:10 in LBamp, grown at room temperature until an OD_{600} of 0.6–0.8 was obtained, and then induced overnight at room temperature following addition of 0.5 mM isopropylthioglycoside. Cells were then recovered by centrifugation at 4000 rpm for 15 min, and the pellet resuspended in 20 ml of TSE [50 mM Tris at pH 8.0, 25% sucrose, 1 mM EDTA, 0.3 mM PMSF] for a 500-ml culture. Sixty milligrams of lysozyme was added and the lysate incubated at room temperature for 5 min. Then 1/200 volume of 2 M MgCl_2 , 1/1000 volume of 1 M MnCl_2 and 1/100 volume of DNase I (1 mg/ml in water) were added, and the lysate incubated at 37°C for 15 min. Then 1/8 volume of 10 $\times$ PBS, 1/16 volume each of 20% (vol/vol) Tween 20 and 20% (vol/vol) Triton X-100, and 1/80 volume of 1 M DTT was added, the lysate mixed well, and centrifuged at 15,000g for 20 min at 4°C. The cleared lysate was then mixed with 400 μ l of 50% glutathione-Sepharose 4B (Pharmacia) and incubated for 2 hr at 4°C. Beads were washed three times with NETN buffer [50 mM Tris at pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40], twice with Rb kinase buffer, and the GST-Rb protein was eluted at 4°C for 2–4 hr by incubation in 500 μ l of kinase buffer containing 2 mM reduced glutathione.

Western immunoblotting

Cell lysates were prepared as described for histone kinase assays. Total protein in each sample was normalized by use of the Bradford assay (Bio-Rad). Lysates were microcentrifuged for 10 min, precleared by incubation with 50 μ l (packed) of Pansorbin (Cal Biochem) (first washed three times in lysis buffer) for 1 hr, and then microcentrifuged twice to remove Pansorbin. The indicated primary antibody (2 μ l of the anti-Rb#207 antiserum or preimmune, or 10 μ l of a mixture of anti-p107 monoclonal antibodies) was then added to the cleared lysates and incubated for 1 hr at 4°C. Twenty-five microliters of 50% protein A/G-agarose was added, and incubation was continued for 2 hr more with rocking. Immune complexes on the beads were washed twice in 50 mM Tris-HCl (pH 8.0), 1000 mM NaCl, 0.5% NP-40 containing 1 mM DTT, 0.1 mM PMSF, and 0.1 mM sodium orthovanadate, twice in the same buffer containing 200 mM NaCl, and then twice in the same buffer containing no NaCl. Immunopre-

cipitates were then electrophoresed through a 7% SDS-polyacrylamide gel, and transferred to an Immobilon membrane (Millipore). Rb was detected by use of 0.2 μ g/ml of IF8 (Santa Cruz Biotechnology) and 1 μ g/ml PharMingen anti-Rb (14001A) monoclonals and standard chemiluminescence protocols (Amersham). p107 was detected by use of 0.03 μ g/ml of anti-p107 polyclonal (C-19; Santa Cruz Biotechnology). The anti-Rb#207 rabbit polyclonal antiserum (the kind gift of Dr. Michael Imperiale, University of Michigan Medical School, Ann Arbor) was raised against a mouse Rb-TrpE fusion protein. The p107 monoclonal mix consists of SD-2, -4, -6, -9, and -15 hybridoma tissue culture supernatants [Dyson et al. 1993]. E2F-1 protein was detected by Western blotting with the anti-E2F-1 monoclonal KH95 (Santa Cruz Biotechnology) as described [Kowalik et al. 1995].

Flow cytometry

REF52 cells on 100-mm dishes were processed for flow cytometry as described [Kowalik et al. 1995].

E2F DNA-binding assays

E2F assays were performed as described previously [Ikeda and Nevins 1993] with the following modifications. The assays utilized a ^{32} P-end-labeled *Hae*III–*Hind*III fragment that spans the region from –13 to –23 relative to the DHFR gene translation initiation site, isolated from the DHFR–chloramphenicol acetyltransferase (CAT) plasmid [Blake and Azizkhan 1989]. Cell extracts were incubated for 40 min at room temperature in reaction mixtures containing 20 mM HEPES (pH 7.9), 40 mM KCl, 6 mM MgCl_2 , 1 mM EGTA, 1 mM DTT, 0.1% NP-40, 10% glycerol, 30 μ g of BSA, 500 ng of sonicated salmon sperm DNA, and 0.1 ng of the ^{32} P-labeled probe. For antibody supershift assays, 0.1 μ g of purified polyclonal antibody (anti-p130 C20, or anti-E2F-1 KH95 from Santa Cruz) or 0.25 μ l of serum (preimmune or anti-Rb#207) was added 20 min into the incubation. The samples were then electrophoresed through a 5% polyacrylamide gel in TBE containing 5% glycerol. Competitive binding reactions included 10 ng of an unlabeled double-strand oligonucleotide containing wild-type or mutant E2F sites as described previously [Ikeda and Nevins 1993].

Expression plasmids

Bluescript vectors containing the coding sequences for the human p21 and p27 products (BSp21 and BSp27, respectively) were obtained from Xiao-Fan Wang (Duke University Medical Center, Durham, NC). A *Cla*I–*Xba*I fragment from BSp21 was subcloned into CMV4 to generate the CMV–p21 construct. Similarly, a *Hind*III–*Xba*I fragment from BSp27 was subcloned into CMV4 to generate the CMV–p27 construct. A Bluescript vector containing the coding sequence for the human p16 product (BSp16) was obtained from D. Beach (Cold Spring Harbor Laboratory). An *Eco*RI–*Xho*I fragment from BSp16 was subcloned into the *Eco*RI and *Sal*I sites of CMV5 (CMVp16). All constructs were confirmed by restriction analysis. The E2F-1–CAT, CMV- β -galactosidase, CMV–E1A_{12S}, CMV–E2F1, CMV–E2F1–VP16, and CMV–E2F1_{E132} plasmids have been described [Johnson et al. 1994a,b].

CAT, β -galactosidase, and luciferase assays

REF52 cells were transfected by the calcium phosphate coprecipitation method with 2 μ g of CMV- β -gal and either 3 μ g of E2F1–LUC or E2F1–CAT, and the indicated plasmids; in addi-

tion CMV4 or CMV5 was added as nonspecific carrier DNA to a total of 20 µg/1 ml of transfection solution. Cell monolayers were washed once with DMEM and then incubated in DMEM containing 0.1% serum. After 48 hr of starvation, cells were either harvested, or for the serum-stimulation experiments, the media were replaced with DMEM containing 20% serum and then harvested at 4-hr intervals. β-Galactosidase, CAT, and luciferase assays have been described previously [Johnson et al. 1994b].

Detection of β-galactosidase and BrdU by immunofluorescence

REF52 cells in 100-mm dishes were transfected as described above with the indicated plasmids. After removal of the transfection solution from cell monolayers, cells were allowed to recover for 4 hr in DMEM containing 10% serum and subsequently trypsinized and replated on 35-mm dishes with gelatin-treated coverslips, and incubated in DMEM with 10% serum. After 42 or 48 hr, as indicated, BrdU was added to a final concentration of 50 µM and cells were incubated for an additional 18 hr. Alternatively, 48 hr after replating, cells were infected with either AdCMV or AdE2F-1, or mock-infected, and BrdU was added 6 hr later and cells incubated for an additional 24 hr. Coverslips were fixed and processed as described previously [Johnson et al. 1993].

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