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The Accumulation of an E2F-p130 Transcriptional Repressor Distinguishes a G_0 Cell State From a G_1 Cell State

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Previous studies have demonstrated cell cycle-dependent specificities in the interactions of E2F proteins with Rb family members. We now show that the formation of an E2F-p130 complex is unique to cells in a quiescent, G_0 state. The E2F-p130 complex does not reform when cells reenter a proliferative state and cycle through G_1. The presence of an E2F-p130 complex in quiescent cells coincides with the E2F-mediated repression of transcription of the E2F1 gene, and we show that the E2F sites in the E2F1 promoter are important as cells enter quiescence but play no apparent role in cycling cells. In addition, the decay of the E2F-p130 complex as cells reenter the cell cycle requires the action of G_1 cyclin-dependent kinase activity. We conclude that the accumulation of the E2F-p130 complex in quiescent cells provides a negative control of certain key target genes and defines a functional distinction between these G_0 cells and cells that exist transiently in G_1.

The role of the E2F transcription factor in the control of cell proliferation, and as a target for the action of the retinoblastoma tumor suppressor protein (Rb) in arresting cell growth in G_1, is now well established (27, 46, 59). Rb activity is regulated by phosphorylation in G_1 (7, 9, 19, 47, 49, 55, 56, 62), through the action of the G_1 cyclin-dependent kinases (18, 26, 31, 33, 41, 81). The growth-regulatory activity of Rb directly coincides with its ability to physically interact with and regulate E2F (1, 3, 11, 28, 29, 39, 63). Indeed, overexpression of the E2F1 product, or production of an E2F1 chimera lacking sequences recognized by Rb, is sufficient to induce S phase in quiescent cell populations (2, 16, 38, 65, 67, 69). Moreover, deregulated expression of E2F1, in cooperation with an activated ras oncogene, can lead to oncogenic transformation of primary rat embryo cells (36) or an established cell line (73).

The Rb gene defines one member of a family of related genes encoding proteins that share the ability to interact with and regulate E2F transcriptional activity and to suppress cell growth. The p107 and p130 proteins have considerable sequence homology with Rb (25, 51, 54, 86, 87), and each is regulated by the action of G_1 cyclin-dependent kinases (4, 78). Nevertheless, despite these similarities, it is also true that there are distinct specificities in the interaction of the Rb family of proteins with the E2F family. A number of experiments have now shown that p130 interacts with E2F in quiescent, G_0 cells and early G_1 cells that have been stimulated to proliferate whereas the p107 protein forms an E2F complex as cells enter and progress through S phase (13, 17, 71). The Rb interaction can be seen throughout the cell cycle although there is an increase in the complex as cells move through mid- to late G_1 that likely reflects the increased amount of E2F at this time (34). Specificities can also be seen in the nature of the proteins involved in the various interactions. The p130 and p107 proteins have been shown to bind to a specific subset of the E2F family proteins that includes the E2F4 and E2F5 products (6, 21, 78). Although the Rb protein may interact preferentially with the E2F1, E2F2, and E2F3 proteins, it is also clear that unlike p130 and p107, Rb can be found to interact with each of the E2F species (34, 57).

Considerable effort has been directed at defining the nature of the E2F interactions as cells progress out of quiescence and into a cell cycle, but little is known of the events associated with a normal cell cycle. We have been particularly interested in the formation of the E2F-p130 complex in quiescent cells and the relationship of this complex to the transcriptional repression of certain genes that contain E2F recognition sequences. For instance, it is now clear that the low level of transcription in quiescent cells of certain E2F-regulated genes, including E2F1, E2F2, HsOrc1, and B-myb, is the result of E2F-mediated repression of transcription (32, 37, 45, 58, 59a, 67a). A role for E2F-mediated repression follows from other experiments that have shown an ability of Rb or Rb family members to actively repress transcription in an E2F-dependent manner (68, 82, 83). This result, together with the fact that the predominant E2F species in the quiescent cells is the E2F4-p130 complex (6, 13, 34, 70a, 78), suggests that this complex may play an important role in maintaining low levels of expression of these genes in G_0 cells. Moreover, the G_0-specific repression of B-myb coincides with in vivo footprinting experiments that define an interaction at the E2F sites that is observed in quiescent cells but that disappears as cells are stimulated to reenter the cell cycle (89).

We have further investigated the relationship of the E2F-p130 complex to the control of cell proliferation. We conclude from these experiments that the E2F-p130 complex is unique to quiescent cells and does not form as cells pass through G_1 of a normal cell cycle. Moreover, our experiments indicate that the E2F sites within the E2F1 promoter are important only in quiescent G_0 cells and play little or no role in continuously cycling cells. Finally, we also show that the loss of the E2F-p130 complex as cells enter G_1, which coincides with the relief of the E2F-mediated repression, is dependent on G_1 cyclin-dependent kinase activity.

MATERIALS AND METHODS

Cell culture. REF52, HFF, and 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The 3T3 cells were the kind gift of Charles Sherr (St. Jude Children's Research Hospital), and the HFF cells were obtained from Clonetics. HL60 cells were the kind gift of Russel Kaufman (Duke University Medical Center) and were maintained in RPMI 1640 supplemented with 20% FBS. To induce differentiation into the macrophage lineage, HL60 cells in logarithmic growth were

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seeded at 10^5 cells per ml and treated with 20 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) per ml (20). U937 cells, the gift of Brice Weinberg (Duke VA Medical Center), were seeded at 2×10^5 cells per ml in RPMI 1640 supplemented with 10% FBS and treated with 40 ng of TPA per ml to induce differentiation.

Cell synchronization. For entry into starvation time courses, REF52 cells were seeded onto plates at a density of 1,000 cells per cm^2. The cells were incubated for 36 h postplating. The cells were then washed twice with phosphate-buffered saline (PBS) and incubated in DMEM containing 0.1% FBS. For exit from starvation time courses, REF52 cells were plated at a density of 1,500 cells per cm^2. The cells were then serum starved in DMEM containing 0.1% FBS for 48 h. Following this starvation period, cells were serum stimulated by replacing the 0.1% DMEM with DMEM containing 10% FBS. For S-phase progression time courses, REF52 cells were plated at a density of 1,000 cells per cm^2 and serum starved in DMEM containing 0.1% FBS for 48 h. Cells were released into DMEM containing 10% FBS and 2 mM hydroxyurea and incubated for a period of 21 h. Following the hydroxyurea block, cells were washed twice with PBS and released into DMEM containing 10% FBS.

HFF cells were plated at a density of 1,000 cells per cm^2 and were then serum starved in DMEM containing 0.1% FBS for 48 h. The cells were then serum stimulated by the addition of DMEM containing 10% FBS. The 3T3 cells were plated at a density of 1,000 cells per cm^2 and synchronized at the G_1/S border with a double hydroxyurea block. Asynchronously growing 3T3 cells were incubated in DMEM containing 10% FBS and 2 mM hydroxyurea for 20 h. The cells were then released into DMEM containing 10% FBS for 8 h, following which time the DMEM–10% FBS–2 mM hydroxyurea medium was replaced. The cells were incubated for an additional 16 h and then released into DMEM containing 10% FBS.

HL60 cell elutriation. HL60 cells in logarithmic growth phase were seeded at 10^5 cells per ml 48 h prior to the elutriation. The cells were then collected and washed twice in PBS. The cell pellet (8×10^8 total cells) was then resuspended in RPMI 1640 without serum (at 4°C), and an aliquot of cells was removed to serve as the asynchronous sample. The HL60 cells were then fractionated on the basis of buoyant density by centrifugal elutriation in a Beckman J6B centrifuge. Cells were loaded over a period of 24 min at a flow rate of 5 ml/min and a rotor speed of 2,100 rpm. The cells were then collected at increasing flow rates (8, 11, 14, 19, 23, and 30 ml/min at 2,100 rpm) with three fractions collected at each flow rate. Two additional fractions of larger cells were collected. The first was collected at a rotor speed of 1,800 rpm and a flow rate of 25 ml/min. The second was collected as the rotor decelerated from 1,800 rpm at a flow rate of 25 ml/min. Cells were initially observed in the first two fractions removed at the 14-ml/min flow rate. Cells were pelleted and washed twice with cold PBS. An aliquot of each cell pellet was removed and prepared for flow cytometry as described below. Cell extracts were then prepared from each fraction as described below. The G_1 fraction shown in these experiments was collected from the initial two fractions taken at a flow rate of 14 ml/min. The S-phase fraction was the final fraction taken at a flow rate of 30 ml/min, which was combined with the first fraction taken at 1,800 rpm (prior to flow cytometry and extract preparation). The G_2 fraction was the final fraction taken as the rotor was decelerating.

Virus infections. The Ad-C (control) and Ad-p21 viruses were prepared as described elsewhere (15). REF52 cells were seeded onto 150-mm plates at a density of 1.5×10^3 cells per cm^2. These cells were then starved in DMEM containing 0.1% FBS for 48 h. The medium was then removed, and the cells were infected with virus at a multiplicity of 900 PFU per cell as defined by 72K assay (15). Cells were infected for 1.25 h in 5 ml of DMEM without serum containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.2. Following infection, 20 ml of DMEM containing 0.1% FBS was added to each plate and incubation was continued for 5 h. The cells were then fed with DMEM containing 10% FBS, and samples were taken at indicated times.

Preparation of cell extracts. Cells were washed twice with 10 ml of cold PBS and then harvested in cold PBS containing 0.5% bovine serum albumin (BSA). The harvested cells were spun for 5 min at $1,500 \times g$, and the supernatant was removed. The cell pellet was then washed in 1 ml of cold PBS and spun 5 min at $1,500 \times g$. Whole cell lysates were prepared as described elsewhere (34). The lysate was then spun at $60,000 \times g$ for 20 min. The pellet was discarded, and the supernatant was frozen in liquid N_2 and stored at −70°C. The protein concentration of each sample was determined by the Bradford assay (Bio-Rad). HL60 cell extracts were prepared by a previously described nuclear extract protocol (44) with the following modifications. The cell pellet was initially resuspended in a 5× volume of cytoplasmic extract buffer {10 mM HEPES [pH 7.6], 60 mM KCl, 1 mM EDTA, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.075% Nonidet P-40, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors as described elsewhere} (34). Following removal of the cytoplasm, nuclei were then lysed for 30 min on ice in a 5× volume of the whole cell lysis buffer as described previously (34). Following this lysis, the extracts were spun for 20 min at $60,000 \times g$. The pellet was then discarded, and the supernatant was frozen in liquid N_2 and stored at −70°C. As the fractions contained unequal cell numbers, the concentration of each sample was normalized to 1 mg/ml prior to use in gel shift assays.

Luciferase assays. Cells were harvested in cold PBS containing 0.5% BSA and washed with cold PBS, and the resulting cell pellet was lysed for 10 min at room temperature in 100 μl of 1× reporter lysis buffer (Promega). The lysate was spun for 5 min at $13,000 \times g$, and the supernatant was collected. Luciferase activity was determined with the Luciferase Assay System (Promega) and 20 μl of lysate on a Berthold Lumat LB 9501 luminometer. Total protein concentration was used to normalize the luciferase activity of each sample. Additionally, the absolute luciferase activity of each sample was normalized to promoter copy number as determined by Southern blot analysis.

Flow cytometry. Cells grown in 100-mm plates (luciferase experiments) or 150-mm plates (whole cell extract experiments) were processed for flow cytometry essentially as described previously (43) with the following modifications. Prior to resuspension in propidium iodide, cells were incubated in 1 ml of 2 N HCl containing 0.2 mg of pepsin per ml for 30 min at room temperature. Following this incubation, 3 ml of 0.1 M Na tetraborate (borax), pH 8.5, was added and the cells were pelleted for 5 min at $1,500 \times g$. This pellet was then washed once with PBS plus 1% BSA. The cells were then pelleted once again.

Gel mobility shift assays. The gel mobility shift assays were performed essentially as described previously (16) with the following modifications. The reaction mixtures were incubated for 20 min at room temperature, and the gel shifts displayed in Fig. 1, 2, and 6 utilized gels with a 75:1 acrylamide/bisacrylamide ratio. Antibody supershifts were performed with 1 μl (0.1 μg) of the indicated antibody: p107 (C-18), p130 (C-20) (0.3 μg was used to minimize cross-reaction with p107), or Rb (C-15) (Santa Cruz Biotechnology). Competitive binding reactions included 10 ng of an unlabeled double-stranded oligonucleotide containing wild-type or mutant E2F sites as described previously (34). The gel shift in Fig. 3 utilized a 29:1 acrylamide/bisacrylamide ratio because of the nonspecific band between the E2F-Rb and E2F-p130 complexes.

Western immunoblotting. Equal amounts of cell lysates (30 μg of total protein) were separated on a 7% acrylamide–sodium dodecyl sulfate (SDS) gel and then transferred to an Immobilon-P membrane (Millipore). The blots were blocked for 1 h in 5% lowfat milk–0.05% Tween 20 in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) and then incubated with the indicated primary antibodies. Bands were detected with a horseradish-peroxidase conjugated secondary antibody and the enhanced chemiluminescence detection system (Amersham). All antibody incubations were for 1 h at room temperature. The Rb family members were detected as follows: Rb (IF8), 1:1,000 dilution; p107 (C-18), 1:1,000 dilution; p130 (C-20), 1:1,000 dilution. Each antibody was obtained from Santa Cruz Biotechnology. Western blots were stripped and reprobed by the enhanced chemiluminescence protocol.

Generation of stable cell lines. Two 100-mm plates of REF52 cells were transfected by the calcium phosphate coprecipitation method with 10 μg of ΔSac E2F1Luc (WT) or 10 μg of ΔSac E2F1Luc (E2F−) (37). Each transfection also contained 1 μg of pCDNA3 (Invitrogen) to provide neomycin resistance and 9 μg of sonicated salmon sperm DNA as a nonspecific carrier. Cells were washed once with PBS plus 3 mM EGTA and once with PBS 16 h posttransfection, following which the DMEM–10% FBS medium was replaced. At 48 h posttransfection, the plates were split 1:2.5 and placed in selection medium (DMEM–10% FBS, 1 mg of geneticin [G418] per ml [Gibco/BRL]). The plates were incubated in selection medium for 14 days (medium was changed every 4 days), at which point the colonies on the two E2F1Luc (WT) and the two E2F1Luc (E2F−) plates were pooled. The pooled colonies were grown for an additional 6 days under selection, at which point aliquots were frozen for use in time course experiments. To control for reporter gene copy number in the cell lines, Southern blotting was performed with genomic DNA from the E2F1Luc (WT) and E2F1Luc (E2F−) cells. The blots were probed with the *Eco*RI-*Eco*RV fragment of the luciferase gene, and the bands were quantitated on a Phosphoimager to determine the relative copy number of integrated luciferase constructs. A second probing of the blots with an E2F4 genomic probe was used to normalize the results of the luciferase probe. The E2F1Luc (WT) cell line was found to have 1.9 times the luciferase copy number of the E2F1Luc Mut cell line. This factor was used to correct the luciferase activities of the experiments in Fig. 5B.

RESULTS

Cell cycle-dependent E2F complex formation. Although previous experiments have explored the nature of E2F interactions with Rb family member proteins as cells progress from G_0/G_1 to S phase (8, 10, 13, 21, 34, 48, 50, 64, 71, 78, 84), very little emphasis has been placed on the control of E2F as cells progress through S phase and into a new cell cycle. For instance, it is not clear whether the various fluctuations in E2F-Rb family member interactions are specifically related to the stimulation of cell growth or whether these interactions also reflect cell cycle-dependent events. To approach these questions, we have chosen to study the interactions of E2F species with Rb family member proteins in cycling cells as well as growth-stimulated cells.

An example of the E2F complexes formed during a cell growth response, as seen in many previous such experiments, is

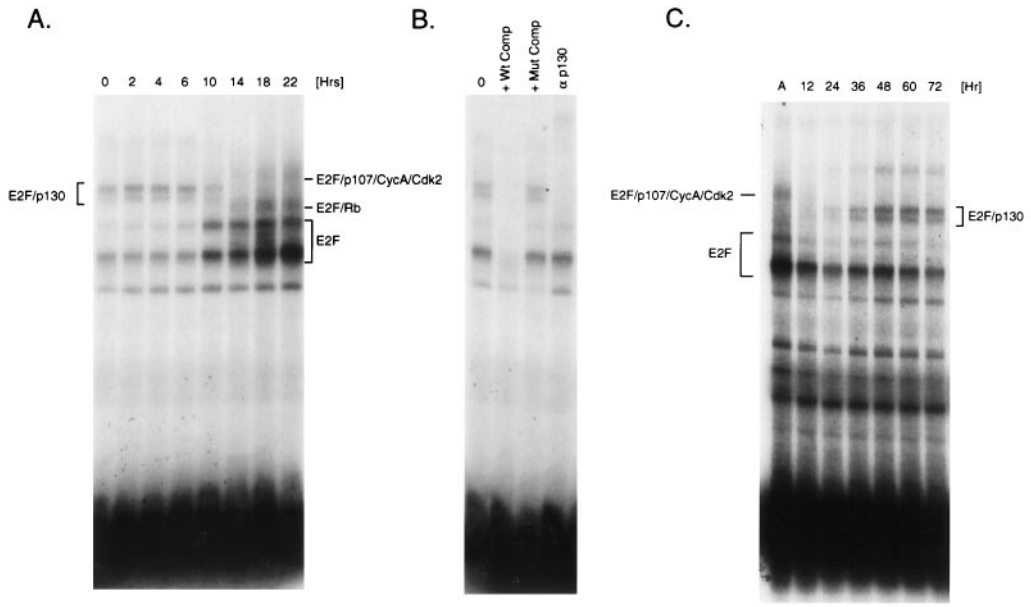


FIG. 1. E2F complexes in growing and quiescent cells. (A) REF52 cells were synchronized in G_0 via starvation for 48 h in 0.1% serum. In each lane, 1 μl of an extract prepared at the indicated times post-starvation release was assayed as described in Materials and Methods. The positions of the E2F-p130 complex, E2F-Rb complex, E2F-p107-CycA-cdk2 complex, and free E2F species are marked. (B) One microliter of extract from quiescent cells ($T = 0$) was assayed in each lane. Lanes 2 and 3 depict competition with either wild-type or mutant E2F binding sites. Lane 4 is an antibody supershift assay using 1 μl of a polyclonal p130 antibody. (C) An E2F-p130 complex accumulates in serum-starved cells. Asynchronously growing REF52 cells (A) were placed into medium containing 0.1% serum, and extracts were prepared at the indicated times. One microliter of extract was assayed as described in Materials and Methods. The E2F-p107-CycA-cdk2 complex, the E2F-p130 complex, and free E2F species are marked.

depicted in Fig. 1A. In this assay, REF52 cells were synchronized in G_0 via serum starvation and released into the cell cycle by the addition of fresh medium containing 10% serum. The predominant form of E2F in G_0 cells is a complex that contains the E2F4 protein associated with p130 (6, 13, 34, 78), although it is clear that the E2F5 product can also associate with the p130 protein (30, 66). Since we are unable to distinguish between E2F4 and E2F5 in these assays, we will refer to this complex as the E2F4/5-p130 complex. As cells are stimulated to grow by serum addition, the E2F4/5-p130 complex declines and disappears and is replaced by an accumulation of an E2F-Rb complex as well as free E2F. Subsequently, as cells begin to enter S phase, there is an accumulation of the E2F complex containing the p107 protein and the cyclin A-cdk2 kinase. Specificity controls for this experiment can be seen in Fig. 1B.

We have also analyzed the effects of induced quiescence on E2F interactions. As shown in Fig. 1C, asynchronously growing REF52 cells contain several different free E2F species, along with an E2F-Rb complex and the E2F-p107-cyclin A-cdk2 complex, largely reflecting the activities found when quiescent cells are stimulated to grow. In contrast, there was no evidence of the E2F4/5-p130 complex in the asynchronously growing cells, but as these cells are forced to exit the cell cycle following serum removal, the complexes found in the growing cells decline or disappear and are replaced by the p130-containing complex. From these analyses, we conclude that the presence of the E2F4/5-p130 complex may be largely confined to quiescent, G_0 cells. We do note that this conclusion is at odds with a recent report that detected E2F-p130 interactions in both quiescent and growing cells (78). Although the basis for the difference is not fully clear, it is true that the assays of Vairo and colleagues employed single samples for quiescent or proliferating cells. Given the fact that the decline in p130 is not immediate, the presence of a p130-E2F interaction in proliferating cells may reflect the kinetics of cell cycle reentry. In addition, the extent to which a fraction of the T-cell population may not have reentered the cell cycle would also contribute to the presence of the E2F4/5-p130 complex. Alternatively, as Vairo et al. measured E2F and p130 proteins and not E2F DNA binding activity, it is possible that an E2F-p130 interaction persists but is inactive in DNA binding.

The E2F-p130 complex accumulates in G_0 cells. The assays shown in Fig. 1 suggest a correlation between the presence of the E2F4/5-p130 complex and the quiescent state of the cell culture. Nevertheless, it is also possible that the complex might be induced transiently in G_1 and thus not represent a significant contribution to that seen in the population of asynchronous cells. To more closely examine the nature of E2F complexes as cells move through a cell cycle, growing REF52 cells were synchronized at the G_1/S boundary with a hydroxyurea block and then released from this arrest and allowed to progress through S, G_2, M, and back into G_1. Cell synchrony was monitored through the use of flow cytometry. As shown in Fig. 2A, the arrested cells possessed a 2N DNA content indicating cells in G_1. Three hours after release from the block, the majority of cells had entered S phase, exhibiting a >2N DNA content. Cells are in late S by 6 h postrelease and are in G_2 by 8 h postrelease (as indicated by their 4N DNA content). At 10 h, the cells had passed through mitosis and were once again in G_1.

Cell extracts were prepared from the synchronized cells, and E2F activity was measured by gel shift assay. Although there were changes evident as the cells progressed through S, G_2, and into the next G_1, including a moderate decline in the E2F-p107-cyclin A-cdk2 complex as cells entered G_1, the most notable feature of these analyses was the relatively constant nature of the pattern of E2F complexes throughout the cell cycle (Fig. 2B). The E2F-Rb complex also showed a moderate decline as cells entered G_1. In contrast to the near-constant

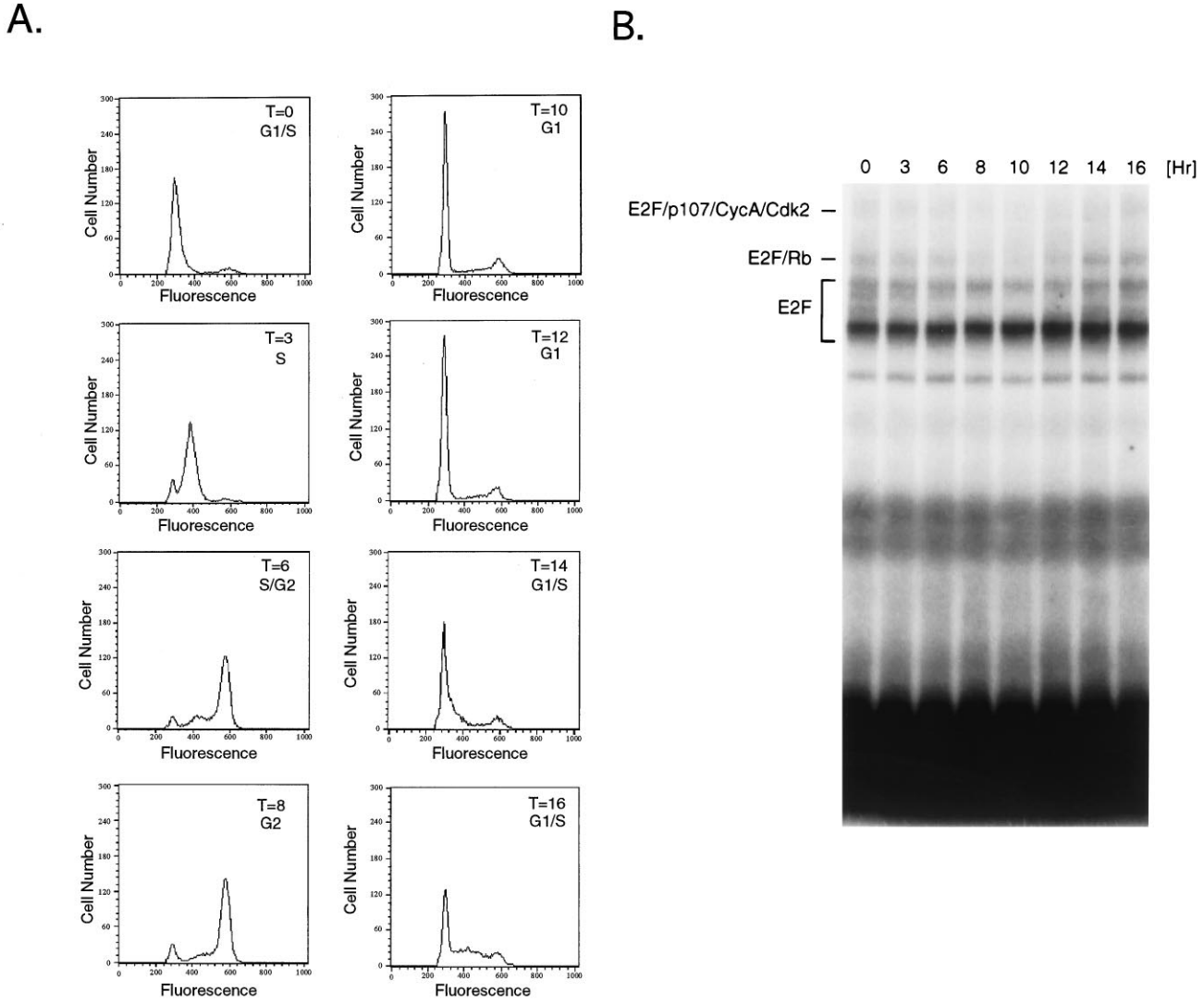


FIG. 2. E2F complexes in cycling cells. (A) REF52 cells were synchronized at the G_1/S boundary via a hydroxyurea block as described in Materials and Methods. Samples were taken at the indicated times postrelease and prepared for flow cytometry with a propidium iodide stain (Materials and Methods). Cells were then sorted for relative DNA content. The histograms for a representative time course are shown in the figure. As can be seen, arrested cells have a 2 N DNA content. The cells then progress into S phase at 3 h postrelease and into G_2 (DNA content = 4 N) at 6 and 8 h postrelease. Entry into another cell cycle is marked by a return to a 2 N DNA content at 10 h postrelease. (B) E2F complexes during the S-to-G_1 transition. Whole cell extracts of REF52 cells synchronized as described in Fig. 2A were prepared. One microliter of these extracts was assayed as described in Materials and Methods. The E2F-p107-CycA-cdk2, E2F-Rb, and free E2F species are marked. As can be seen, the E2F-p130 complex is not formed as cells enter G_1.

presence of the E2F-p107-cyclin A-cdk2 complex and the E2F-Rb complex, there was no evidence for the appearance of the E2F4/5-p130 complex as cells progressed through the cell cycle. This includes each of the samples that represent enriched, early G_1-specific populations of cells.

As an alternative approach, we have also assayed for the presence of the E2F4/5-p130 complex in cell extracts from G_1 cell populations that have been isolated by elutriation to enrich for cells in G_1 from asynchronously growing cultures. For this experiment, we made use of the HL60 cell line in which we have previously identified and characterized the various E2F complexes (34). In addition, these cells offer the added advantage of linking these events to the induction of differentiation and exit from the cell cycle. Extracts were prepared from growing HL60 cells as well as elutriated fractions of the growing population enriched for cells in the G_1, S, and G_2/M stages of the cell cycle. Extracts were also prepared from HL60 cells which had been induced to differentiate into macrophages via the addition of TPA. The elutriated fractions chosen for analysis represent the most highly enriched fractions for G_1, S, and G_2 cells, respectively, as determined by flow cytometry (Fig. 3A). As seen from the data in Fig. 3B, there are changes in the pattern of E2F DNA binding complexes present in the different stages of the cell cycle although the overall pattern is relatively constant. Importantly, the E2F-p130 complex that is seen to accumulate in the differentiated HL60 cells (TPA plus 72 h) is absent from the G_1 enriched HL60 fraction.

Based on all of these results, we conclude that the E2F4/5-p130 complex is not found in G_1 of a cycling population of cells but rather is unique to cells that have entered a G_0, quiescent state such as the growth factor-deprived fibroblasts or the terminally differentiated HL60 cells.

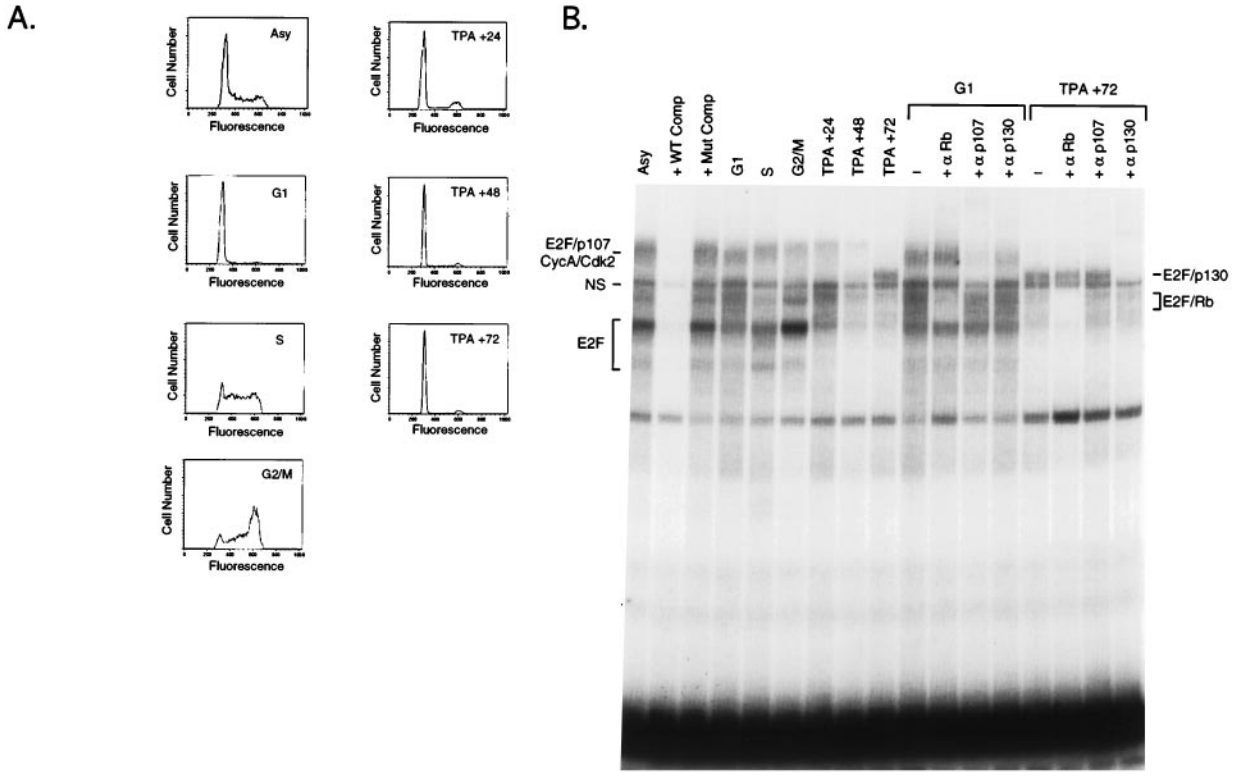


FIG. 3. The E2F-p130 complex is absent from HL60 cells in G_1 but accumulates as cells enter quiescence. (A) HL60 cells were fractionated by centrifugal elutriation as described in Materials and Methods. Shown are the flow cytometry profiles from asychronously growing cells (Asy) and the elutriated fractions enriched for G_1, S, and G_2/M cells. Also shown are the flow cytometry profiles for HL60 cells treated with TPA to induce differentiation. (B) An E2F-p130 complex is not detected in G_1-enriched HL60 cells. Cell extracts were prepared from HL60 cells as described in Materials and Methods. These extracts were adjusted to equal protein concentrations, and 1 μl of each was assayed. Lane 1 depicts the assay of the asynchronous HL60 extract, and specificity is indicated by competition with wild-type or mutant E2F site oligonucleotides (lanes 2 and 3). Assays of the elutriated fractions enriched in G_1, S, and G_2/M populations are shown in lanes 4 to 6, respectively. Extracts of cells induced to differentiate by TPA treatment for 24, 48, and 72 h are shown in lanes 7 to 9, respectively. The G_1-enriched extracts (lanes 10 to 13) or the TPA-induced extracts (lanes 14 to 17) were further assayed by antibody supershift with Rb, p107, and p130 antibodies. The band marked as NS represents a nonspecific binding activity.

The p130 protein accumulates in quiescent, G_0 cells. To explore the basis for control of the E2F4/5-p130 complex, Western blotting was performed with cell extracts from synchronized REF52 cells and then blots were probed with antibodies to the three known Rb family members. Consistent with previous work (13, 34, 78), the p130 protein is abundant in quiescent cells (Fig. 4A). As these cells reenter the cell cycle, the p130 protein is seen to initially undergo phosphorylation (data not shown), as indicated by the change in mobility of the protein, and then declines to undetectable levels as cells enter S phase. The levels of the p107 protein behave in a fashion the inverse of that of the p130 levels. The p107 protein is not detected in starved cells but then accumulates as cells reenter the cell cycle. Finally, Rb levels are relatively constant in the cell during the progression into S phase, although much of the protein becomes hyperphosphorylated in mid- to late G_1 as shown in numerous previous experiments (81).

We have also examined the levels of the Rb family member proteins as cells progress into a quiescent state. The p130 protein is virtually undetectable in the asynchronously growing cells. As cells begin to enter quiescence, the amount of p130 protein in the cell rises dramatically starting approximately 24 h poststarvation. The levels of p107 protein decay in a manner the inverse of that of the observed rise in p130 levels, with the majority of p107 disappearing by 24 h following the removal of serum. The levels of Rb protein in the cell stay relatively constant compared with the changes in p107 and p130.

Additionally, we have examined the levels of the Rb family proteins in cycling cells that progress from G_1/S through G_2 and back into G_1 (Fig. 4C). Consistent with the previous assays, very low p130 levels are observed in the growing cells, compared with a quiescent cell population, and this does not change as the cells pass through the cell cycle. What little p130 protein is found in growing cells is largely hyperphosphorylated in comparison with the p130 found in G_0 cells. The levels of p107 and Rb remain relatively constant in the cycling cells. Both of these proteins show some dephosphorylation upon entry into G_1.

Finally, we have examined the levels of p130 protein in several additional cell lines to assess the extent to which the accumulation of p130 is a general feature of cell cycle exit. Mouse 3T3 and human (HFF) fibroblasts were assayed for p130 levels in both quiescent and asynchronously cycling cell populations. HFF cells were brought to quiescence by incubation in DMEM containing 0.1% FBS for 48 h. The cycling HFF population represents cells 22 h after release into 10% FBS. The quiescent mouse 3T3 fibroblasts represent density-arrested cells while the cycling population represents cells which have passed into G_1 following synchronization at the G_1/S

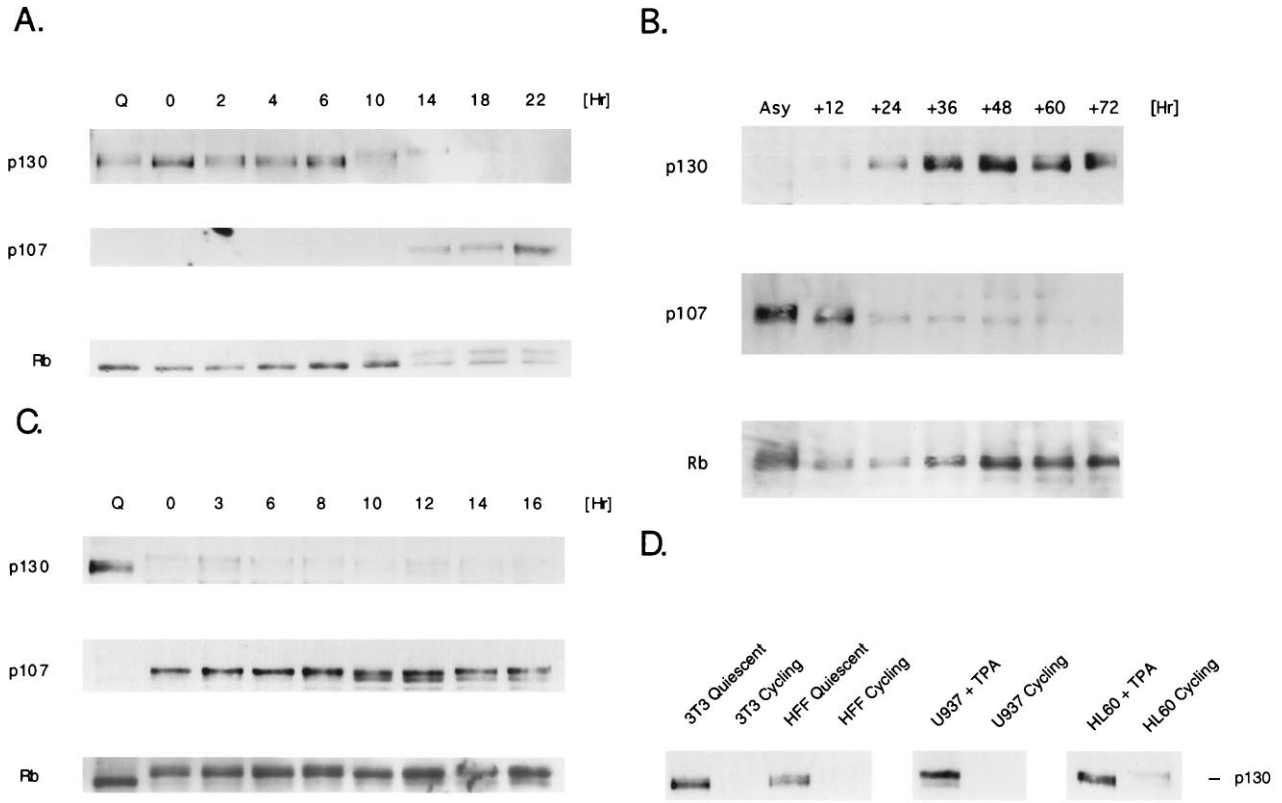


FIG. 4. The p130 protein accumulates in G_0 cells. (A) REF52 cells were released from starvation as described previously (Fig. 1A). Thirty micrograms of whole cell extract was separated on a 7% acrylamide-SDS gel, and Western blotting with a polyclonal anti-p130 antibody was performed as described in Materials and Methods. The blots were then stripped and reprobed with a polyclonal anti-p107 antibody or a monoclonal anti-Rb antibody (as indicated). The "Q" lane represents the 48-h-into-starvation sample from Fig. 1C. (B) Asynchronously growing REF52 cells were allowed to enter starvation as described previously (Fig. 1C). Thirty micrograms of whole cell extract was separated on a 7% acrylamide-SDS gel and Western blotted as described above. (C) Hydroxyurea-blocked REF52 cells were released from arrest and allowed to progress into S phase as described elsewhere (Fig. 2A). Thirty micrograms of whole cell extract was separated on a 7% acrylamide-SDS gel and Western blotted as detailed above. The "Q" lane again represents the 48-h-into-starvation sample from Fig. 1C. (D) Analysis of p130 levels in quiescent and growing cells. Growing and quiescent 3T3, HFF, U937, and HL60 cells were analyzed for p130 levels. A sample (30 μg) of whole cell extract from each cell type was separated on a 7% acrylamide-SDS gel and Western blotted with a polyclonal anti-p130 antibody. The U937 + TPA and HL60 + TPA lanes represent the differentiated samples of these cell types. 3T3 and HFF cells were made quiescent as described above (Materials and Methods).

boundary via hydroxyurea block. In both of these cell lines, a substantial amount of p130 protein was observed in the quiescent cell populations whereas little or no p130 was detected in the cycling cells. We have also examined p130 protein levels in the HL60 samples described in Fig. 3. The cycling HL60 sample represents the asynchronous sample from Fig. 3B whereas the quiescent sample represents the terminally differentiated TPA plus 72-h sample from the same figure. In these cells, the p130 band is readily detectable in the quiescent sample. A small amount of phosphorylated p130 is also detected in the asynchronous sample, although this protein does not result in the formation of an E2F-p130 DNA binding complex as was shown in Fig. 3B. In addition, in view of previous work (78), we have examined asynchronously growing and differentiated U937 cells. As shown in Fig. 4D, although we could readily detect p130 in the TPA-induced U937 cultures, we could not detect the p130 protein in the growing cell population.

Based on all of these results, we conclude that the accumulation of the p130 protein reflects the quiescent nature of various types of cell cultures. Thus, it is likely that the lack of an E2F-p130 complex in actively growing cells reflects the absence of the p130 protein in those cells. Moreover, whereas levels of the Rb protein remain relatively constant in both cycling and quiescent cells, the p107 and p130 proteins show an inverse regulation in which p107 is found in actively growing cells whereas the p130 protein accumulates in quiescent cells.

E2F elements in the E2F1 promoter are important in quiescent but not cycling cells. The results described in the preceding experiments demonstrate that the E2F4/5-p130 complex is unique to quiescent cells. This pattern of accumulation reflects the G_0 cell-specific repression of transcription of various target genes including B-Myb (45), E2F1 (32, 37, 58), and E2F2 (67a), suggesting that the role for E2F in the control of these genes may be specific to quiescent cells rather than cycling cells. To directly address the functional significance of the E2F4/5-p130 complex in quiescent cells, we have used the E2F1 promoter as a model system for study of the regulation of E2F site-containing promoters in cycling cells. Although previous assays have employed transient transfections followed by serum withdrawal to synchronize cells in a G_0 state (37), the study of normally cycling cells is difficult if not impossible with transient transfections. As an alternative approach, we have generated stable cell lines containing luciferase reporter genes under the control of the E2F1 promoter constructs as depicted in Fig. 5A. The E2F1Luc (WT) plasmid contains a 728-bp fragment of the human E2F1 promoter upstream of the luciferase reporter gene. The E2F1Luc (E2F−) plasmid contains the same promoter fragment in which the E2F sites have been

mutated (37). These plasmids were transfected into the REF52 cell line along with a second plasmid containing the neomycin resistance gene, and cells were then placed under G418 selection. The resulting colonies were pooled and used in the experiments described below. Luciferase-promoter copy number was determined for each pooled cell line and used to normalize the results of luciferase activity as described in Materials and Methods.

When quiescent REF52 cells were induced to enter the cell cycle by serum addition, the activity of the wild-type E2F1 promoter, as indicated by the luciferase assays, rose approximately 10- to 15-fold and with kinetics that reflect the activation of the endogenous E2F1 gene. In contrast, the activity of the E2F1 promoter bearing mutations in the E2F sites was already elevated in the quiescent cells, approximately 10-fold over the wild-type promoter. This result thus corresponds closely to the results obtained through transient transfection experiments and provides further indication for the E2F-dependent repression of E2F1 expression in quiescent cells. Likewise, the assays shown in the middle panel, which depict the activity of the two promoters when asynchronously growing cells are forced to enter quiescence by serum starvation, are also consistent with a role for the E2F elements in mediated repression of E2F1 transcription. In particular, whereas the activity of the wild-type E2F1 promoter declined approximately 8- to 10-fold, the activity of the E2F1 mutant promoter remained high with little decline (<2-fold) as cells entered quiescence. Clearly, both analyses emphasize the role of E2F-mediated repression of the E2F1 promoter in quiescent cells.

A quite different result was obtained with growing cells that synchronously progressed through a cell cycle. As shown in the bottom panel of Fig. 5B, there was little difference in the activity of the wild-type versus the mutant promoter during the course of this experiment. It thus appears that the role of the E2F sites in the E2F1 promoter is restricted to cells in a quiescent state and that the elements play little or no role in growing cells. The fact that the predominant E2F species in quiescent cells is the E2F4/5-p130 complex suggests that this complex is a likely candidate for mediating the active repression of the E2F1 promoter.

Decay of the E2F-p130 complex following growth stimulation requires G_1 cyclin-dependent kinase activity. The unique presence of the E2F4/5-p130 complex in quiescent cells, coupled with the fact that the transcription of various E2F-regulated genes is repressed in quiescent cells, suggests that the activity of this complex may be critical to the control of cell proliferation. As such, the events controlling the presence and then loss of the complex would represent an important aspect of the initial growth response. A variety of studies have suggested that the G_1 cyclin-dependent kinases, both cdk2 and cdk4, are likely responsible for the phosphorylation of Rb family member proteins during G_1 (4, 18, 26, 31, 33, 41, 81). To determine if the decline in the E2F4/5-p130 complex may be the result of p130 phosphorylation by G_1 cyclin kinases, we have examined the effect of inhibiting G_1 cyclin kinase activity on the decay of the E2F4/5-p130 complex.

To inhibit kinase activation, we made use of a recombinant adenovirus containing the p21 cDNA which encodes a cyclin kinase inhibitor of broad specificity (70). Following infection of REF52 cells with the virus, there is an efficient inhibition of G_1 cyclin kinase activity, as measured by in vitro kinase assays following immunoprecipitation with various G_1 cyclin antibodies (data not shown). In addition, expression of p21 prevents the phosphorylation of Rb that would normally occur following serum stimulation of quiescent cells. We have thus prepared extracts from cells stimulated for various periods of time by serum addition that were infected with the p21-expressing virus or a control virus lacking an insert. As seen in Fig. 6, whereas the E2F4/5-p130 complex had essentially disappeared by 14 h following stimulation in the control cells, there was no loss of this complex through 22 h in the cells infected with the p21-expressing virus. Additionally, there is no loss of p130 protein, nor change in its mobility, as seen by Western blot assays of extracts from the p21-infected cells (data not shown). We thus conclude that the decline and loss of the E2F4/5-p130 complex following the stimulation of cell growth is dependent on G_1 cyclin-dependent kinase activity.

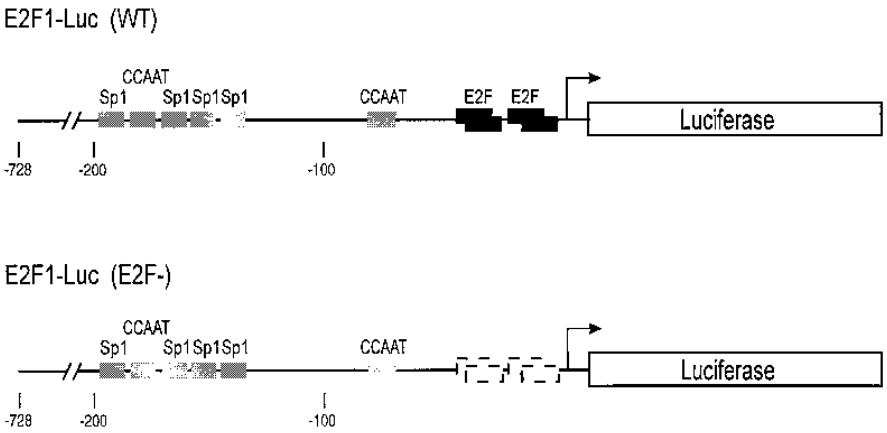
DISCUSSION

We believe that the experiments described here provide additional insight into the nature of the E2F interactions that are regulated during the cell proliferation process. In particular, we believe that these experiments demonstrate that an E2F-p130 complex, previously shown to accumulate in quiescent cells, is in fact unique to quiescent cells and distinguishes a G_0 cell state from a cell merely passing through G_1. The presence of the complex in quiescent cells coincides with the repression of transcription of the E2F1 gene (32, 37, 58), the E2F2 gene (67a), B-myb (45), and the human homolog of the yeast Orc1 gene (59a). Moreover, the decay of the complex, and thus the decay of repression of these genes, is dependent on the action of G_1 cyclin-dependent kinase activity, previously shown to be critical for the entry of cells into a cell cycle (53, 60, 61, 77, 79). As such, the phosphorylation-dependent loss of the E2F-p130 complex may represent a critical biochemical event associated with the initial action of these kinases in promoting cell cycle entry, such as when resting lymphocytes receive a mitogenic signal. Conversely, the accumulation of the p130 protein and formation of the E2F-p130 complex may represent a critical event associated with cell cycle exit, such as during the process of terminal differentiation.

Distinct roles for E2F and Rb family proteins in transcriptional control. Previous experiments have suggested a role for the E2F transcription factor in the activation of transcription of cell growth-regulated genes. Targets include the genes encoding dihydrofolate reductase (DHFR), DNA polymerase α, thymidine kinase, thymidylate synthetase, cyclin A, and proliferating cell nuclear antigen (PCNA) (59). This activation is presumed to reflect the accumulation of free, active E2F as cells progress through G_1 and is consistent with other experiments demonstrating an ability of E2F1 overexpression to induce S phase in otherwise quiescent cells (38, 65, 69, 85). Recent work suggests that one role for Rb may be in regulating this accumulation of free E2F as cells pass through G_1 (34). Other experiments have suggested quite a different scenario whereby the E2F-Rb family protein complex functions as a transcriptional repressor, inhibiting the activity of an otherwise functional promoter (Fig. 7). Thus, rather than simply negating the transcriptional activity of E2F, the association of Rb or Rb family members with E2F creates a dominant-acting repressor of transcription (82, 83). This has been most clearly shown as the elevation of promoter activity upon elimination of E2F binding sites, such as that seen with the E2F1 (32, 37, 58) and B-myb (45) promoters. Moreover, in vivo footprinting experiments have provided evidence for an interaction involving the B-myb E2F elements in quiescent but not proliferating cells (89). This result, together with the fact that the E2F4/5-p130 complex predominates in quiescent cells, provides compelling evidence for an active role for this complex in the control of transcription of key cell cycle regulatory genes.

The characteristics that determine which cellular promoters

A.


E2F1-Luc (WT)
CCAAT
Sp1
Sp1Sp1Sp1
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E2F
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-728
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E2F1-Luc (E2F-)
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B.

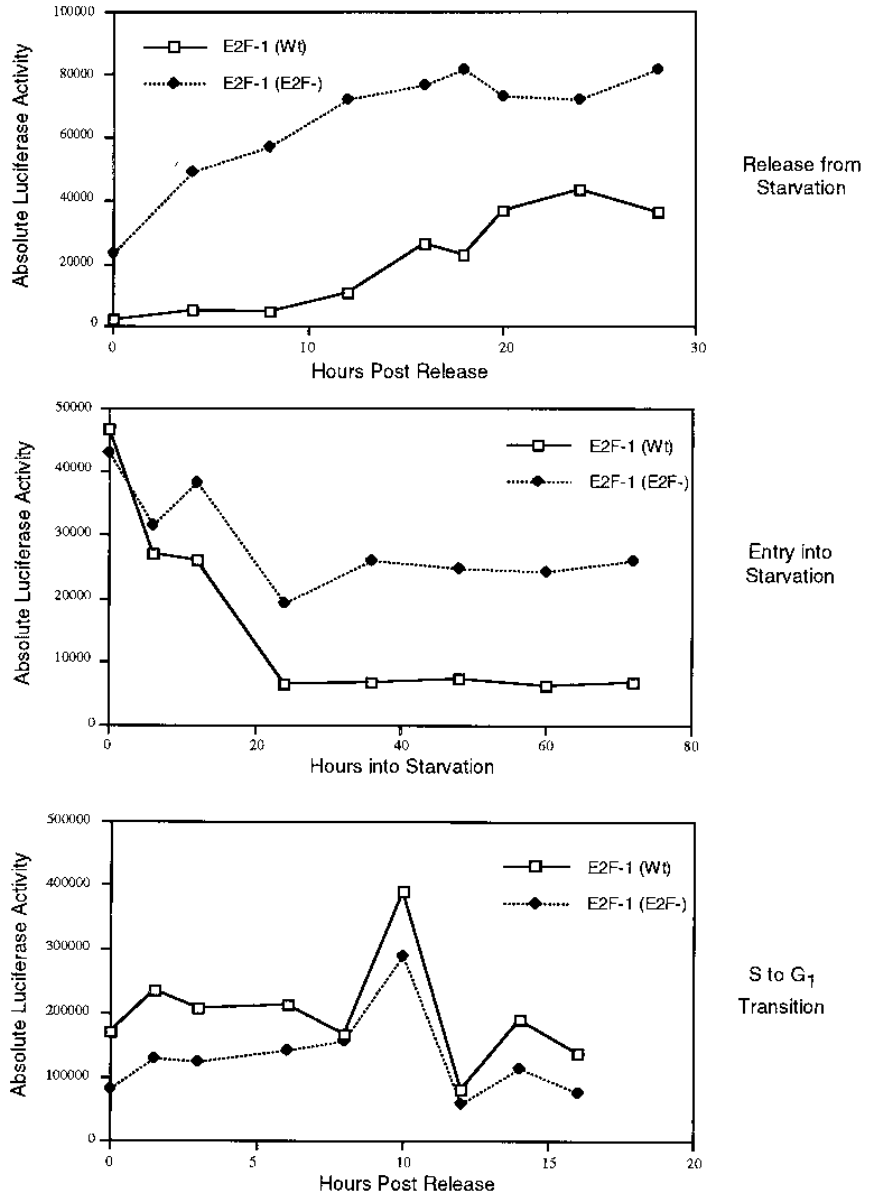
Absolute Luciferase Activity
E2F-1 (Wt)
E2F-1 (E2F-)
Hours Post Release
Release from Starvation
Absolute Luciferase Activity
E2F-1 (Wt)
E2F-1 (E2F-)
Hours into Starvation
Entry into Starvation
Absolute Luciferase Activity
E2F-1 (Wt)
E2F-1 (E2F-)
Hours Post Release
S to G1 Transition

FIG. 5. E2F-dependent transcription control in cycling and quiescent cells. (A) REF52 cells were stably transfected with plasmids containing the E2F1Luc (WT) plasmid which contains the native E2F1 promoter upstream of the luciferase reporter gene. Neomycin resistance was conferred via the cotransfection of a second plasmid. The E2F1Luc (E2F−) plasmid, in which the E2F sites in the E2F1 promoter have been mutated, was used to construct stable cell lines in a similar manner. (B) Analysis of E2F1 promoter activity in the cell cycle. Stably transformed REF52 cell lines were synchronized as described previously, and duplicate cell extracts were prepared at the indicated time points. Cells were lysed in reporter assay buffer (Promega), and the lysate luciferase activity was assayed. The luciferase values of each sample were normalized to protein concentration, and the duplicate time points were averaged for the graphs shown. The graphs are from representative experiments (each experiment was performed at least three independent times). (Top) Cells were starved 48 h in 0.1% serum and released. (Middle) Asynchronous cells were placed into 0.1% serum and allowed to enter starvation. (Bottom) Cells were blocked at G_1/S and allowed to progress through S, G_2/M, and back into G_1.

are targeted by the E2F-p130 complex, and thus subject to E2F-dependent repression, remain to be determined. However, the observation that the Rb family of proteins can interact with various cellular transcription factors (5, 14, 22–24, 80, 82) suggests the possibility that these interactions could underlie the specificity in repression of transcription. For instance, if the binding of E2F4 or E2F5 with its DNA recognition sequence is a relatively weak interaction, it is possible that the ability of p130 to associate with other promoter-bound factors not only defines the basis for the repression, by interfering with the ability of these factors to activate transcription, but also determines the specificity of repression. That is, the stabilization of the E2F4/5-p130 complex through interaction with other transcription factors would depend on the transcription factors bound to other sites in the promoter. This could then explain the presence of an E2F-specific footprint at the B-myb promoter in quiescent cells but not in growth-stimulated cells (89), despite the abundance of free E2F at this time.

Alternatively, it is also possible that subtle variations in the sequence of the E2F consensus site could result in preferential binding by different members of the E2F family. This would in turn lead to differential promoter regulation as sites favored by E2F4 or E2F5 would be able to recruit the p130 complex during G_0 whereas sites favored by E2F1 or E2F2 would be unoccupied in a G_0 cell. Thus, E2F-p130 mediated promoter repression may actually be a complex mixture of the structure of the E2F sites present as well as the overall promoter architecture.

If promoter context, either through DNA sequence specificity or protein factor specificity, determines which genes are subject to E2F-p130-mediated repression, how then does the accumulating free E2F result in the activation of genes during G_1? For instance, mutation of the E2F sites in the DHFR promoter does not elevate activity in quiescent cells but rather eliminates the activation in mid- to late G_1 (74). Moreover, although cyclin D-cdk4 can activate the E2F1 promoter in quiescent cells, consistent with the elimination of repressor activity by phosphorylation of p130 or Rb, cyclin D-cdk4 does not activate the DHFR promoter under these circumstances (35). Thus, in this case it is the accumulation of free, active E2F that would drive the transcription of the DHFR promoter (Fig. 7). Whether this will also be the case for other genes encoding DNA synthesis activities, such as thymidine kinase, PCNA, DNA polymerase α, and others, is yet to be determined. The E2F activity associated with this activation remains to be determined, but the E2F1 or E2F2 protein appears to be a likely candidate. Clearly, E2F1 and E2F2 cannot be responsible for the repression of transcription in G_0 cells because of their absence at this time. In contrast, both E2F1 and E2F2 accumulate at precisely the time when genes such as DHFR are activated. Perhaps the specificity is inherent in the particular E2F family member which possesses a unique ability to

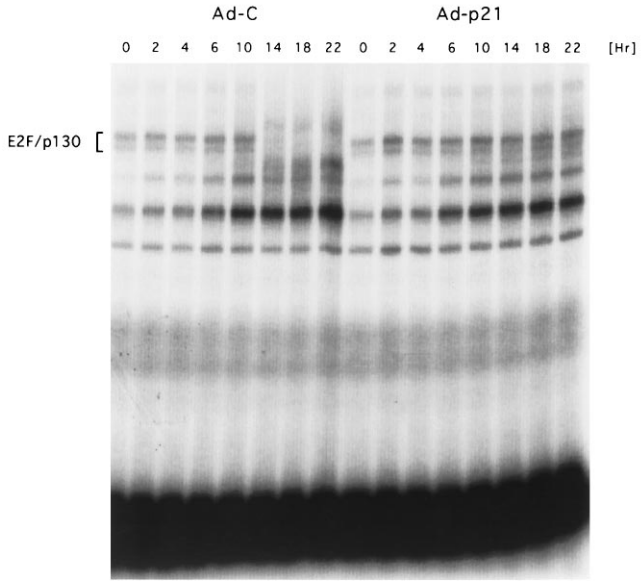

FIG. 6. Inhibition of G_1 cyclin-dependent kinase activity prevents loss of the E2F4-p130 complex. REF52 cells were brought to quiescence and infected with either the control adenovirus lacking an insert (Ad-C) or the p21-containing virus (Ad-p21). One microliter of cell extract was assayed in each lane. The times indicated represent hours post-serum addition. The position of the E2F-p130 complex is indicated.

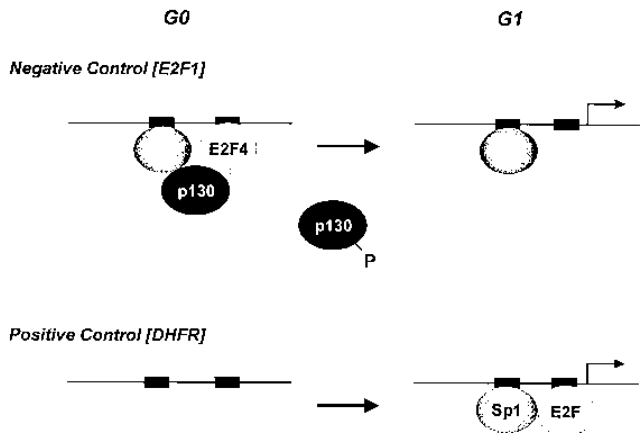

FIG. 7. Positive and negative control of transcription by E2F. Depicted are two roles for transcriptional control by E2F. Based on the data presented here and elsewhere, E2F elements in promoters typified by E2F1 facilitate a negative control of transcription in quiescent cells, coincident with the presence of the E2F4/5-p130 complex. In this context, p130 functions as a corepressor brought to the promoter by E2F4/5, blocking the transcriptional activation capacity of other promoter-bound factors. Specificity in the repression, such as seen for the E2F1 promoter, might result from specificity in these interactions or specificity in sequence recognition by the E2F4/5-p130 complex. An alternative circumstance can be seen with the DHFR promoter, in which the E2F elements appear to facilitate positive activation of transcription in mid- to late G_1, coincident with the accumulation of free E2F. Again, one possible mechanism could involve specific interactions with other factors binding to promoter elements.

interact with these promoters, either due to subtle differences in DNA recognition specificity or due to specific protein interactions involving other promoter-bound factors. Indeed, the latter possibility is supported by recent work that demonstrates a synergy between E2F1 and Sp1 in transcriptional activation (52) that correlates with a physical interaction between the two proteins (40, 52).

Although the work presented here along with that described elsewhere (13, 34, 35, 42, 57, 72, 76, 78, 81) has helped to further define the role of the Rb and p130 proteins in cell growth control, a cellular role of the p107 protein is far from clear. Although p107 specifically interacts with E2F4 and E2F5, as does p130, the pattern of expression of p107 clearly suggests a role in growing rather than quiescent cells. The assembly of an E2F4/5-p107 complex also includes cyclin A and cdk2, and while this association has been shown not to be required for the ability of p107 to repress cell growth or alter E2F function (75, 86), the persistent presence of the complex suggests that the p107-cyclin association is important for the physiological role of the p107 protein.

A unique role for the E2F-p130 complex in quiescent cells. The experiments described here and elsewhere strongly suggest that an E2F-p130 complex is uniquely found in quiescent cells, either serum-starved fibroblasts, resting lymphocytes, or terminally differentiated HL60 cultures (34). As such, the presence of this complex provides a molecular identity to a G_0, quiescent cell that distinguishes it from a cell passing through the G_1 phase of the cell cycle. Furthermore, the presence of the complex can be seen as a functional activity that may play a role in the creation of a quiescent state through the inhibition of genes that encode activities important for cell proliferation. This distinguishes the p130 protein from the related p107 protein that is found only in proliferating cells. In fact, it is possible that E2F4-p130 also controls the expression of the p107 gene, given the presence of E2F sites in the p107 promoter (88) and the inverse relationship of p130 and p107 expression. An additional consequence of this view of the E2F-p130 complex is that E2F4 or E2F5, in conjunction with p130, would take on the role of a growth suppressor by providing a means by which the repressor complex is targeted to critical promoters. Recent work has analyzed the consequence of targeted disruption of the p130 gene for mouse development. Although the disruption of the p130 gene had no apparent consequence on mouse development, there clearly was compensation of p130 function by the p107 gene product and likely the Rb protein (12). Whether there will be circumstances in which p130 plays a uniquely important role in controlling cell growth is yet to be seen.

Finally, the fact that the p130 protein is found predominantly in quiescent and terminally differentiated cells, together with the role of the E2F-p130 complex in repressing transcription of growth-regulatory genes, suggests that the mechanisms regulating the accumulation of p130 will be a critical aspect of cell cycle withdrawal. Possibly, the p130 promoter may be regulated by transcription factors that are activated as cells are induced to leave the cell cycle. Alternatively, the rise in p130 protein could be a posttranscriptional event. Regardless of the underlying mechanism, an understanding of this process may well contribute important insights into the early events associated with cell cycle exit and terminal differentiation.

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REFERENCES

1. **Arroyo, M., and P. Raychaudhuri.** 1992. Retinoblastoma-repression of E2F-dependent transcription depends on the ability of the retinoblastoma protein to interact with E2F and is abrogated by the adenovirus E1A oncoprotein. Nucleic Acids Res. **20:**5947–5954.
2. **Asano, M., J. R. Nevins, and R. P. Wharton.** 1996. Ectopic E2F expression induces S phase and apoptosis in Drosophila imaginal discs. Genes Dev. **10:**1422–1432.
3. **Bagchi, S., R. Weinmann, and P. Raychaudhuri.** 1991. The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. Cell **65:**1063–1072.
4. **Beijersbergen, R. L., L. Carlee, R. M. Kerkhoven, and R. Bernards.** 1995. Regulation of the retinoblastoma protein-related p107 by G1 cyclin complexes. Genes Dev. **9:**1340–1353.
5. **Beijersbergen, R. L., E. M. Hijmans, L. Zhu, and R. Bernards.** 1994. Interaction of c-myc with the pRb-related protein p107 results in inhibition of c-myc-mediated transactivation. EMBO J. **13:**4080–4086.
6. **Beijersbergen, R. L., R. M. Kerkhoven, L. Zhu, L. Carlee, P. M. Voorhoeve, and R. Bernards.** 1994. E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. Genes Dev. **8:**2680–2690.
7. **Buchkovich, K., L. A. Duffy, and E. Harlow.** 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell **58:**1097–1105.
8. **Cao, L., B. Faha, M. Dembski, L. H. Tsai, E. Harlow, and N. Dyson.** 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature (London) **355:**176–179.
9. **Chen, P.-L., P. Scully, J.-Y. Shew, J. Y. J. Wang, and W.-H. Lee.** 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell **58:**1192–1198.
10. **Chittenden, T., D. M. Livingston, and J. A. DeCaprio.** 1993. Cell cycle analysis of E2F in primary human T cells reveals novel E2F complexes and biochemically distinct forms of free E2F. Mol. Cell. Biol. **13:**3975–3983.
11. **Chittenden, T., D. M. Livingston, and W. G. Kaelin, Jr.** 1991. The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. Cell **65:**1073–1082.
12. **Cobrinik, D., M.-H. Lee, G. Hannon, G. Mulligan, R. T. Bronson, N. Dyson, E. Harlow, D. Beach, R. A. Weinberg, and T. Jacks.** 1996. Shared role of the pRB-related p130 and p107 proteins in limb development. Genes Dev. **10:**1633–1644.
13. **Cobrinik, D., P. Whyte, D. S. Peeper, T. Jacks, and R. A. Weinberg.** 1993. Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes Dev. **7:**2392–2404.
14. **Datta, P. K., P. Raychaudhuri, and S. Bagchi.** 1995. Association of p107 with Sp1: genetically separable regions of p107 are involved in regulation of E2F- and Sp1-dependent transcription. Mol. Cell. Biol. **15:**5444–5452.
15. **DeGregori, J., T. Kowalik, and J. R. Nevins.** 1995. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis and G_1/S regulatory genes. Mol. Cell. Biol. **15:**4215–4224.
16. **DeGregori, J., G. Leone, K. Ohtani, A. Miron, and J. R. Nevins.** 1995. E2F1 accumulation bypasses a G1 arrest resulting from the inhibition of G1 cyclin-dependent kinase activity. Genes Dev. **9:**2873–2887.
17. **Devoto, S. H., M. Mudryj, J. Pines, T. Hunter, and J. R. Nevins.** 1992. A cyclin A-protein kinase complex possesses sequence-specific DNA binding activity: p33cdk2 is a component of the E2F-cyclin A complex. Cell **68:**167–176.
18. **Ewen, M. E., H. K. Sluss, C. J. Sherr, H. Matsushime, J. Kato, and D. M. Livingston.** 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell **73:**487–497.
19. **Furukawa, Y., J. A. DeCaprio, A. Freedman, X, M. Nakamura, T. J. Ernst, D. M. Livingston, and J. D. Griffin.** 1990. Expression and state of phosphorylation of the retinoblastoma susceptibility gene product in cycling and noncycling human hematopoietic cells. Proc. Natl. Acad. Sci. USA **87:**2770–2774.
20. **Gaynor, R., K. Simon, and P. Koeffler.** 1991. Expression of c-jun during macrophage differentiation of HL-60 cells. Blood **77:**2618–2623.
21. **Ginsberg, D., G. Vairo, T. Chittenden, Z. Xiao, G. Xu, K. L. Wydner, J. A. DeCaprio, J. B. Lawrence, and D. Livingston.** 1994. E2F-4, a new member of the E2F transcription factor family, interacts with p107. Genes Dev. **8:**2665–2679.
22. **Gu, W., K. Bhatia, I. T. Magrath, C. V. Dang, and R. Dalla-Favera.** 1994. Binding and suppression of the Myc transcriptional activation domain by p107. Science **264:**251–254.
23. **Gu, W., J. Schneider, G. Condorelli, S. Kaushal, V. Mahdavi, and B. Nadal Ginard.** 1993. Interaction of myogenic factors and the retinoblastoma pro-

tein mediates muscle cell commitment and differentiation. Cell **72:**309–324.
24. **Hagemeier, C., A. J. Bannister, A. Cook, and T. Kouzarides.** 1993. The activation domain of transcription factor PU.1 binds the retinoblastoma (RB) protein and the transcription factor TFIID *in vitro*: RB shows sequence similarity to TFIID and TFIIB. Proc. Natl. Acad. Sci. USA **90:**1580–1584.
25. **Hannon, G. J., D. Demetrick, and D. Beach.** 1993. Isolation of the Rb-related p130 through its interaction with cdk2 and cyclins. Genes Dev. **7:**2378–2391.
26. **Hatakeyama, M., J. A. Brill, G. R. Fink, and R. A. Weinberg.** 1994. Collaboration of G_1 cyclins in the functional inactivation of the retinoblastoma protein. Genes Dev. **8:**1759–1771.
27. **Helin, K., and E. Harlow.** 1993. The retinoblastoma protein as a transcriptional repressor. Trends Cell Biol. **3:**43–46.
28. **Hiebert, S. W.** 1993. Regions of the retinoblastoma gene product required for its interaction with the E2F transcription factor are necessary for E2 promoter repression and pRb-mediated growth suppression. Mol. Cell. Biol. **13:**3384–3391.
29. **Hiebert, S. W., S. P. Chellappan, J. M. Horowitz, and J. R. Nevins.** 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev. **6:**177–185.
30. **Hijmans, E. M., P. M. Voorhoeve, R. L. Beijersbergen, L. J. van'T Veer, and R. Bernards.** 1995. E2F-5, a new E2F family member that interacts with p130 in vivo. Mol. Cell. Biol. **15:**3082–3089.
31. **Hinds, P. W., S. Mittnacht, V. Dulic, A. Arnold, S. I. Reed, and R. A. Weinberg.** 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell **70:**993–1006.
32. **Hsiao, K., S. L. McMahon, and P. J. Farnham.** 1994. Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter. Genes Dev. **8:**1526–1537.
33. **Hu, Q. J., J. A. Lees, K. J. Buchkovich, and E. Harlow.** 1992. The retinoblastoma protein physically associates with the human cdc2 kinase. Mol. Cell. Biol. **12:**971–980.
34. **Ikeda, M.-A., L. Jakoi, and J. R. Nevins.** 1996. A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation. Proc. Natl. Acad. Sci. USA **93:**3215–3220.
35. **Johnson, D. G.** 1995. Regulation of E2F-1 gene expression by p130 (Rb2) and D-type cyclin kinase activity. Oncogene **11:**1685–1692.
36. **Johnson, D. G., W. D. Cress, L. Jakoi, and J. R. Nevins.** 1994. Oncogenic capacity of the E2F1 gene. Proc. Natl. Acad. Sci. USA **91:**12823–12827.
37. **Johnson, D. G., K. Ohtani, and J. R. Nevins.** 1994. Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. Genes Dev. **8:**1514–1525.
38. **Johnson, D. G., J. K. Schwarz, W. D. Cress, and J. R. Nevins.** 1993. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature (London) **365:**349–352.
39. **Kaelin, W. G., D. C. Pallas, J. A. DeCaprio, F. J. Kaye, and D. M. Livingston.** 1991. Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. Cell **64:**521–532.
40. **Karlseder, J., H. Rotheneder, and E. Wintersberger.** 1996. Interaction of Sp1 with the growth- and cell cycle-regulated transcription factor E2F. Mol. Cell. Biol. **16:**1659–1667.
41. **Kato, J., H. Matsushime, S. W. Hiebert, M. E. Ewen, and C. J. Sherr.** 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev. **7:**331–342.
42. **Kiess, M., R. Gill, and P. A. Hamel.** 1995. Expression and activity of the retinoblastoma protein (pRB)-family proteins, p107 and p130, during L_6 myoblast differentiation. Cell Growth Differ. **6:**1287–1298.
43. **Kowalik, T. F., J. DeGregori, J. K. Schwarz, and J. R. Nevins.** 1995. E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. J. Virol. **69:**2491–2500.
44. **Kowalik, T. F., B. Wing, J. S. Haskill, J. C. Azizkhan, A. S. Baldwin, and E. S. Huang.** 1993. Multiple mechanisms are implicated in the regulation of NF-kappa B activity during human cytomegalovirus infection. Proc. Natl. Acad. Sci. USA **90:**1107–1111.
45. **Lam, E. W., and R. J. Watson.** 1993. An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. EMBO J. **12:**2705–2713.
46. **La Thangue, N. B.** 1994. DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. Trends Biochem. Sci. **19:**108–114.
47. **Lee, W.-H., J.-Y. Shew, F. D. Hong, T. W. Sery, L. A. Donoso, L.-J. Young, R. Bookstein, and E. Y.-H. P. Lee.** 1987. The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. Nature (London) **329:**642–645.
48. **Lees, E., B. Faha, V. Dulic, S. I. Reed, and E. Harlow.** 1992. Cyclin E/cdk2 and cyclin A/cdk2 kinases associated with p107 and E2F in a temporally distinct manner. Genes Dev. **6:**1874–1885.
49. **Lees, J. A., K. J. Buchkovich, D. R. Marshak, C. W. Anderson, and E. Harlow.** 1991. The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. EMBO J. **10:**4279–4290.
50. **Lees, J. A., M. Saito, M. Valentine, T. Look, E. Harlow, N. Dyson, and K. Helin.** 1993. The retinoblastoma protein binds to a family of E2F transcription factors. Mol. Cell. Biol. **13:**7813–7825.
51. **Li, Y., C. Graham, S. Lacy, A. M. V. Duncan, and P. Whyte.** 1993. The adenovirus E1A-associated 130-kd protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. Genes Dev. **7:**2366–2377.
52. **Lin, S.-Y., A. R. Black, D. Kostic, S. Pajovic, C. N. Hoover, and J. C. Azizkhan.** 1996. Cell cycle-regulated association of E2F1 and Sp1 is related to their functional interaction. Mol. Cell. Biol. **16:**1668–1675.
53. **Lukas, J., J. Bartkova, M. Rohde, M. Strauss, and J. Bartek.** 1995. Cyclin D1 is dispensable for G_1 control in retinoblastoma gene-deficient cells independently of cdk4 activity. Mol. Cell. Biol. **15:**2600–2611.
54. **Mayol, X., X. Grana, A. Baldi, N. Sang, Q. Hu, and A. Giordano.** 1993. Cloning of a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. Oncogene **8:**2561–2566.
55. **Mihara, K., X.-R. Cao, A. Yen, S. Chandler, B. Driscoll, A. L. Murphree, A. T'Ang, and Y.-K. T. Fung.** 1989. Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. Science **246:**1300–1303.
56. **Mittnacht, S., J. A. Lees, D. Desai, E. Harlow, D. O. Morgan, and R. A. Weinberg.** 1994. Distinct sub-populations of the retinoblastoma protein show a distinct pattern of phosphorylation. EMBO **13:**118–127.
57. **Moberg, K., M. A. Starz, and J. A. Lees.** 1996. E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. Mol. Cell. Biol. **16:**1436–1449.
58. **Neuman, E., E. K. Flemington, W. R. Sellers, and W. G. Kaelin, Jr.** 1994. Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. Mol. Cell. Biol. **14:**6607–6615.
59. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science **258:**424–429.
59a. **Ohtani, K., J. DeGregori, G. Leone, D. R. Herendeen, T. J. Kelly, and J. R. Nevins.** Expression of the *HsOrc1* gene, a human *ORC1* homolog, is regulated by cell proliferation via the E2F transcription factor. Mol. Cell. Biol. **16:**6977–6984.
60. **Ohtsubo, M., and J. M. Roberts.** 1993. Cyclin-dependent regulation of G1 in mammalian fibroblasts. Science **259:**1908–1912.
61. **Ohtsubo, M., A. M. Theodoras, J. Schumacher, J. M. Roberts, and M. Pagano.** 1995. Human cyclin E, a nuclear protein essential for the G_1-to-S phase transition. Mol. Cell. Biol. **15:**2612–2624.
62. **Qian, Y., C. Luckey, L. Horton, M. Esser, and D. J. Templeton.** 1992. Biological function of the retinoblastoma protein requires distinct domains for hyperphosphorylation and transcription factor binding. Mol. Cell. Biol. **12:**5363–5372.
63. **Qin, X.-Q., T. Chittenden, D. M. Livingston, and W. G. Kaelin, Jr.** 1992. Identification of a growth suppression domain within the retinoblastoma gene product. Genes Dev. **6:**953–964.
64. **Qin, X.-Q., D. M. Livingston, M. Ewen, W. R. Sellers, Z. Arany, and W. G. Kaelin, Jr.** 1995. The transcription factor E2F-1 is a downstream target of RB action. Mol. Cell. Biol. **15:**742–755.
65. **Qin, X.-Q., D. M. Livingston, W. G. Kaelin, and P. D. Adams.** 1994. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc. Natl. Acad. Sci. USA **91:**10918–10922.
66. **Sardet, C., M. Vidal, D. Cobrinik, Y. Geng, C. Onufryk, A. Chen, and R. A. Weinberg.** 1995. E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. Proc. Natl. Acad. Sci. USA **92:**2403–2407.
67. **Schwarz, J. K., C. H. Bassing, I. Kovesdi, M. B. Datto, M. Blazing, S. George, X. Wang, and J. R. Nevins.** 1995. Expression of the E2F1 transcription factor overcomes type β transforming growth factor-mediated growth suppression. Proc. Natl. Acad. Sci. USA **92:**483–487.
67a. **Sears, R., K. Ohtani, and J. Nevins.** Submitted for publication.
68. **Sellers, W. R., J. W. Rodgers, and W. G. Kaelin, Jr.** 1995. A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. Proc. Natl. Acad. Sci. USA **92:**11544–11548.
69. **Shan, B., and W. Lee.** 1994. Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol. Cell. Biol. **14:**8166–8173.
70. **Sherr, C. J., and J. M. Roberts.** 1995. Inhibitors of mammalian G_1 cyclin-dependent kinases. Genes Dev. **9:**1149–1163.
70a. **Shin, E. K., A. Shin, C. Paulding, B. Schaffhausen, and A. S. Yee.** 1995. Multiple changes in E2F function and regulation occur upon muscle differentiation. Mol. Cell. Biol. **15:**2252–2262.
71. **Shirodkar, S., M. Ewen, J. A. DeCaprio, J. Morgan, D. M. Livingston, and T. Chittenden.** 1992. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell **68:**157–166.
72. **Shiyanov, P., S. Bagchi, G. Adami, J. Kokontis, N. Hay, M. Arroyo, A. Morozov, and P. Raychaudhuri.** 1996. p21 disrupts the interaction between cdk2 and the E2F-p130 complex. Mol. Cell. Biol. **16:**737–744.
73. **Singh, P., S. W. Wong, and W. Hong.** 1994. Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. EMBO J. **13:**3329–3338.
74. **Slansky, J. E., and P. J. Farnham.** 1996. Introduction to the E2F family: protein structure and gene regulation. Curr. Top. Microbiol. Immunol. **208:** 1–30.

75. **Smith, E. J., and J. R. Nevins.** 1995. The Rb-related p107 protein can suppress E2F function independently of binding to cyclin A/cdk2. Mol. Cell. Biol. **15:**338–344.
76. **Tommasi, S., and G. P. Pfeifer.** 1995. In vivo structure of the human *cdc2* promoter: release of a p130-E2F-4 complex from sequences immediately upstream of the transcription initiation site coincides with induction of *cdc2* expression. Mol. Cell. Biol. **15:**6901–6913.
77. **Tsai, L., E. Lees, B. Faha, E. Harlow, and K. Raibowol.** 1993. The cdk2 kinase is required for the G1-to-S transition in mammalian cells. Oncogene **8:**1593–1602.
78. **Vairo, G., D. M. Livingston, and D. Ginsberg.** 1995. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes Dev. **9:**869–881.
79. **van den Heuvel, S., and E. Harlow.** 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. Science **262:**2050–2054.
80. **Wang, C. Y., B. Petryniak, C. B. Thompson, W. G. Kaelin, and J. M. Leiden.** Regulation of the Ets-related transcription factor Elf-1 by binding to the retinoblastoma protein. Science **260:**1330–1335.
81. **Weinberg, R. A.** 1995. The retinoblastoma protein and cell cycle control. Cell **81:**323–330.
82. **Weintraub, S. J., K. N. B. Chow, R. X. Luo, S. H. Zhang, S. He, and D. C. Dean.** 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. Nature (London) **375:**812–815.
83. **Weintraub, S. J., C. A. Prater, and D. C. Dean.** 1992. Retinoblastoma protein switches the E2F site from positive to negative element. Nature (London) **358:**259–261.
84. **Wu, C.-L., L. R. Zukerberg, C. Ngwu, E. Harlow, and J. A. Lees.** 1995. In vivo association of E2F and DP family proteins. Mol. Cell. Biol. **15:**2536–2546.
85. **Wu, X., and A. J. Levine.** 1994. p53 and E2F-1 cooperate to mediate apoptosis. Proc. Natl. Acad. Sci. USA **91:**3602–3606.
86. **Zhu, L., G. Enders, J. A. Lees, R. Beijersbergen, R. Bernards, and E. Harlow.** 1995. The pRB-related protein p107 contains two growth suppression domains: independent interactions with E2F and cyclin/cdk complexes. EMBO J. **14:**1904–1913.
87. **Zhu, L., S. van den Heuvel, K. Helin, A. Fattaey, M. Ewen, D. Livingston, N. Dyson, and E. Harlow.** 1993. Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. Genes Dev. **7:**1111–1125.
88. **Zhu, L., E. Xie, and L.-S. Chang.** 1995. Differential roles of two tandem E2F sites in repression of the human p107 promoter by retinoblastoma and p107 proteins. Mol. Cell. Biol. **15:**3552–3562.
89. **Zwicker, J., N. Liu, K. Engeland, F. C. Lucibello, and R. Muller.** 1996. Cell cycle regulation of E2F site occupation in vivo. Science **271:**1595–1596.