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A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation

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ABSTRACT **Examination of the interactions involving transcription factor E2F activity during cell growth and terminal differentiation suggests distinct roles for Rb family members in the regulation of E2F accumulation. The major species of E2F in quiescent cells is a complex containing the E2F4 product in association with the Rb-related p130 protein. As cells enter the cell cycle, this complex disappears, and there is a concomitant accumulation of free E2F activity of which E2F4 is a major component. E2F4 then associates with the Rb-related p107 protein as cells enter S phase. Rb can be found in interactions with each E2F species, including E2F4, during G_1, but there appears to be a limited amount of Rb with respect to E2F, likely due to the maintenance of most Rb protein in an inactive state by phosphorylation. A contrasting circumstance can be found during the induction of HL60 cell differentiation. As these cells exit the cell cycle, active Rb protein appears to exceed E2F, as there is a marked accumulation of E2F–Rb interactions, involving all E2F species, including E2F4, which is paralleled by the conversion of Rb from a hyperphosphorylated state to a hypophosphorylated state. These results suggest that the specific ability of Rb protein to interact with each E2F species, dependent on concentration of active Rb relative to accumulation of E2F, may be critical in cell-growth decisions.**

Various experiments have shown that the transcription factor E2F is a relevant target of the Rb protein in its activity as a growth suppressor (1–3). The ability of Rb to interact with E2F, regulating the transcriptional activating function of E2F directly correlates with the ability of Rb to arrest cell growth in G_1. In each case, mutants that lack the ability to arrest growth also lack the ability to bind E2F and inhibit E2F-dependent transcription (4–7). Rb is now recognized as one member of a family of proteins, which includes the p107 and p130 proteins, each of which binds to and regulates E2F activity (8–11). It is also now evident that the E2F activity is complex and is composed of a family of proteins that generate various heterodimeric complexes with specific DNA-binding activity (12–14).

A variety of studies have demonstrated specificities in the interaction of E2F family members with Rb family members during cell-cycle progression (15). For instance, it is clear that phosphorylation of the Rb protein during G_1, likely mediated by G_1 cyclin-dependent kinases, abolishes the capacity of Rb to bind to E2F. It is also evident from these studies that the interactions involving the p130 protein and the p107 protein are restricted to specific periods of the cell cycle and that these proteins interact primarily with the E2F4 family member (16–20). In contrast, the precise timing of Rb–E2F interactions and the role of Rb in control of E2F activity has been less certain. For instance, it is often suggested that phosphorylation of Rb releases E2F from Rb as cells progress through G_1 stage, despite the fact that no direct evidence exists for this scenario. As such, we have sought to further investigate the role of Rb in the control E2F activity during the cell cycle. We find that Rb has the capacity to control the accumulation of each E2F species in the cell, a property unique among the Rb family members. These experiments further suggest that the balance between the levels of active Rb and E2F proteins may in part define a rate-limiting step for cell-cycle progression and exit from the cell cycle.

MATERIALS AND METHODS

Cell Culture. Human foreskin fibroblasts (HFF) (passage 2) were obtained from Clonetics and maintained in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum (FCS). All experiments used the cells between passages 10 and 25. For serum starvation, an equivalent number of the cells were plated in several 150-mm plates per each time point. After 16–20 hr, the cells were washed two to three times with DMEM without serum, starved for 48–72 hr in DMEM/0.1% FCS, and then stimulated with DMEM/10% FCS. The peak of S phase judged by [^{3}H]thymidine incorporation was 22 hr after serum stimulation.

The HL60 cell line was obtained from the American Type Culture Collection and maintained in RPMI 1640 medium/20% heat-inactivated FCS. To induce differentiation, the cells in logarithmic growth were seeded at 2×10^5/ml and treated with either 20 ng/ml of phorbol 12-myristate B-acetate (PMA) or 10^{-6} M retinoic acid.

Lymphocyte Preparation and Culture. Primary human lymphocytes were isolated from peripheral blood by Ficoll/Hypaque gradient centrifugation (400 × *g* for 35 min at room temp) and then washed twice with growth medium (RPMI 1640 medium/10% FCS). Adherent cells were removed by incubation in a nylon wool column for 45 min at 37°C, and nonadherent cells were eluted with 40 ml of warm growth medium. Purified peripheral lymphocytes were seeded at 1×10^6 cells/ml in growth medium, stimulated by the addition of phytohemagglutinin at 1 μg/ml, and then harvested at the indicated times.

Thymidine Incorporation. DNA synthesis was measured by incubating cells at 4×10^5/ml in 2 ml of medium with 10 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine for 1 hr.

Extract Preparation. Nuclear extracts from HL60 cells were prepared as described (21). For preparing HFF whole cell extracts, cells were washed twice, lysed in 10 times packed cell volume of lysis buffer [0.1% Nonidet P-40/250 mM KCl/50 mM Hepes, pH 7.9/10% (vol/vol) glycerol/4 mM NaF/4 mM sodium orthovanadate/0.2 mM EDTA/0.2 mM EGTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/1 aprotinin, pepstatin, and leupeptin each at 1 μg/ml for 30 min on ice, and then centrifuged at 14,000 × *g* for 10 min. In the case of

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Abbreviations: HFF, human foreskin fibroblasts; FCS, fetal calf serum; PMA, phorbol 12-myristate 13-acetate.

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the HFF extracts only, final volumes of supernatants of each point were adjusted by adding lysis buffer.

E2F Gel-Shift Assays. E2F DNA-binding assays were done as described (22) with minor modifications to improve the separation of free E2F species; the assays used ^{32}P-end-labeled *Hae*III–*Hin*dIII fragment, spanning the region from −103 to −23 relative to the dehydrofolate reductase (DHFR) translation start site, isolated from the DHFR–chloramphenicol acetyltransferase (CAT) plasmid (23). Cell extracts were incubated from 20 min at room temperature in 20 mM Hepes, pH 7.9/40 mM KCl/6 mM $MgCl_2$/1 mM EGTA/1 mM dithiothreitol/0.1% Nonidet P-40/10% glycerol/bovine serum albumin at 30 μg/500 ng of sonicated salmon sperm DNA/0.1 ng of ^{32}P-labeled probe, followed by electrophoresis in a 5% polyacrylamide gel (acrylamide/bisacrylamide, 75:1) in TBE (50 mM Tris-borate/1 mM EGTA] containing 5% glycerol. Competitive binding reactions included 20 ng of an unlabeled double-stranded oligonucleotide containing wild-type or mutant E2F sites as described (22).

Immunoprecipitations. Equal amounts of HFF whole-cell extracts (100 μl; adjusted by cell number) and lymphocyte or HL60 nuclear extracts (100 μg; adjusted by protein concentration) were mixed with 1 μg of antibodies linked to magnetic beads in 1× shift buffer [20 mM Hepes, pH 7.9/40 mM KCl/6 mM $MgCl_2$/1 mM EGTA/1 mM dithiothreitol/0.1% Nonidet P-40, and proteinase inhibitors for 1–2 hr at 4°C with gentle rotation. The beads were recovered using a magnetic stand, washed three times with 500 μl of ice-cold 1× shift buffer, and then treated with 16 μl of 0.8% deoxycholate (DOC) to dissociate the E2F complexes on ice. After neutralization with 4 μl of 6% Nonidet P-40, 3–4 μl of the supernatant was used for E2F gel-shift assay.

Immunoblotting. Equal amounts of HL60 cell extract (60 μg) were separated in 6% acrylamide/SDS gels and transferred to nitrocellulose. The blots were probed with a primary antibody and detected by using a horseradish peroxidase-linked secondary antibody and an enhanced chemiluminescence detection system (Amersham).

Antibodies. The p107 monoclonal antibody (SD-2, SD-4, SD-6, SD-9, and SD-15) hybridoma tissue culture supernatants (24) were provided by N. Dyson and E. Harlow (Massachusetts General Hospital Cancer Center, Charlestown). The p130 monoclonal antobody (Z11) hybridoma tissue culture supernatant (10) was from P. Whyte (McMaster University, Hamilton, ON). The p130, Rb, E2F2, and E2F4 polyclonal antibodies, the Rb monoclonal antibody (IF8), and the E2F-1 monoclonal antibody (KH95) were obtained from Santa Cruz Biotechnology. The Rb monoclonal antibody (C36) and the E1A monoclonal antibody (M73) were from Oncogene Science. The cyclin A monoclonal antibody (BF683) was obtained from PharMingen. Affinity-purified E2F-3 polyclonal antibodies were raised against carboxyl-terminal peptides corresponding to amino acid residues 499–523 (EEGISDLFDAYDLEKLP-LVEDFMCS) (12, 13).

For immunoprecipitation, the antibodies were linked to anti-mouse or anti-rabbit-coated magnetic beads (Dynal).

RESULTS

Specificities of Rb Family–E2F Family Interactions During a Cell-Growth Response. The analysis of E2F in quiescent and stimulated HFF cultures, as well as resting and stimulated peripheral lymphocytes, yields a pattern of E2F interactions similar to that seen in previous studies (Fig. 1*A*). In these assays, the majority of cells in the HFF and lymphocyte cultures enter S phase at 22 hr and 48 hr, respectively. As seen previously (18), an E2F4–p130 complex ($E2F_{G1}$) is found in quiescent cells. This complex disappears upon stimulation and gives way to an accumulation of free E2F. This result is followed by an accumulation of a complex containing p107, cyclin A, and cdk2 ($E2F_{CycA}$) (25–28), with the major E2F component being E2F4 (data not shown). The identity of these complexes has been demonstrated by various antibody supershift assays. Moreover, E2F4 is the only E2F species released from p130 or p107 immunoprecipitates (Fig. 1*B*). These results, which indicate a specific interaction of p130 and p107 with the E2F4 product, are thus consistent with other recent experiments that have documented this specificity (16, 17, 19, 20).

Two aspects of the Rb interaction with E2F are distinct from that of the other Rb family members. (*i*) An E2F–Rb complex can be observed throughout the G_1 period (Fig. 1*A*), increasing in abundance in parallel with the increase in the free E2F complexes. Thus, whereas p130 and p107 appear to cycle in their interactions with E2F, Rb can be seen in association with E2F throughout the G_1 to S-phase period. (*ii*) In contrast to the specificity of the p130 and p107 proteins for the E2F4 product, multiple DNA-binding activities can be seen in the material released from Rb immunoprecipitates (Fig. 1*B*). The slowest migrating of these species coincides with the band found in the p107 or p130 immunoprecipitates, and the E2F4 antibody selectively shifts this complex. The recognition of this complex is not due to cross-reactivity of the E2F4 antibody with other E2F family members because this antibody does not recognize complexes generated by overexpression of other E2F family members in transfected SAOS-2 cells (data not shown). Addition of an E2F1-specific antibody recognizes a distinct Rb-associated E2F complex that was not evident in the p130 or p107 immunoprecipitates. The additional complexes associated with Rb involve other E2F family members, including E2F2 and E2F3, as indicated by antibody supershift assays (Fig. 1*C*). We have not been able to assay directly for the recently described E2F5 product (20).

Control of E2F Accumulation During Cell Differentiation. We have also analyzed the interaction of E2F with Rb and other Rb family members during the time course of differentiation of the HL60 promyelocyte cell line. Various studies have characterized the behavior of HL60 cell differentiation, and a particularly attractive feature of this cell system is the fact that HL60 cells appear to completely leave the cell cycle upon induction of differentiation. As shown in Fig. 2*A*, the predominant form of E2F in undifferentiated HL60 cells is the complex containing cyclin A, cdk2, and p107, as defined by antibody supershift assays (Fig. 2*B*). This complex is characteristic of cells in S phase of the cell cycle (25–28), consistent with the fact that these cells are rapidly growing. Upon induction of differentiation with PMA, dramatic changes in the nature of the E2F complexes are observed. The cyclin A complex begins to decline by 8 hr and is completely absent by 24 hr. In parallel, there is a rise in the E2F–Rb complex (defined by antibody supershift in Fig. 2*B*) evident at 8 hr, and by 24 hr, this is the predominant state of E2F activity. An E2F–p130 complex is also seen to increase during this time, and by 48 hr after induction, there are approximately equal amounts of the two complexes. The changes in the E2F interactions involving Rb and the Rb-related p130 protein generally coincide with the exit of the cells from the cell cycle, as indicated by a measurement of [^{3}H]thymidine incorporation, which shows that DNA synthesis virtually ceases within 16 hr of PMA treatment (data not shown), a time when the E2F–Rb complex was beginning to increase. Very similar changes in E2F interactions were observed after treatment of HL60 cells with retinoic acid, which leads to induction of granulocyte/neutrophil differentiation (data not shown). In addition, recent analyses of differentiating mouse myoblast cultures reveals changes in E2F interactions that parallel those seen in HL60 cells (ref. 31 and Q. Kang, D. Taylor, W. Kraus, and J.R.N., unpublished work).

As shown in Fig. 2*C*, Rb is largely in a hyperphosphorylated state in the undifferentiated HL60 cells and then undergoes a

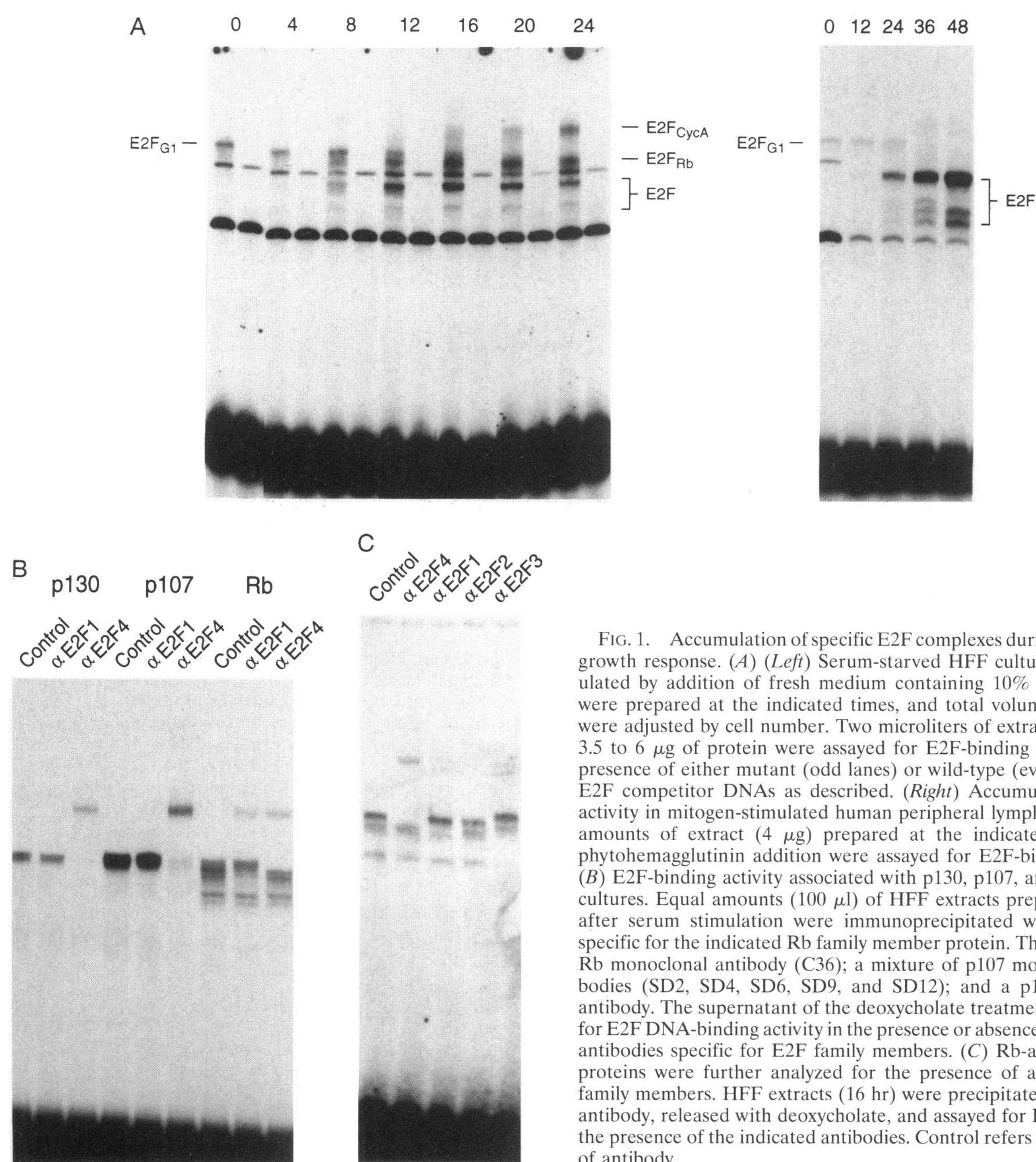

FIG. 1. Accumulation of specific E2F complexes during the cellular growth response. (*A*) (*Left*) Serum-starved HFF cultures were stimulated by addition of fresh medium containing 10% FCS. Extracts were prepared at the indicated times, and total volumes of extracts were adjusted by cell number. Two microliters of extracts containing 3.5 to 6 μg of protein were assayed for E2F-binding activity in the presence of either mutant (odd lanes) or wild-type (even lanes) cold E2F competitor DNAs as described. (*Right*) Accumulation of E2F activity in mitogen-stimulated human peripheral lymphocytes. Equal amounts of extract (4 μg) prepared at the indicated times after phytohemagglutinin addition were assayed for E2F-binding activity. (*B*) E2F-binding activity associated with p130, p107, and Rb in HFF cultures. Equal amounts (100 μl) of HFF extracts prepared at 22 hr after serum stimulation were immunoprecipitated with antibodies specific for the indicated Rb family member protein. These include an Rb monoclonal antibody (C36); a mixture of p107 monoclonal antibodies (SD2, SD4, SD6, SD9, and SD12); and a p130 polyclonal antibody. The supernatant of the deoxycholate treatment was assayed for E2F DNA-binding activity in the presence or absence of the various antibodies specific for E2F family members. (*C*) Rb-associated E2F proteins were further analyzed for the presence of additional E2F family members. HFF extracts (16 hr) were precipitated with the Rb antibody, released with deoxycholate, and assayed for E2F activity in the presence of the indicated antibodies. Control refers to the absence of antibody.

transition to a hypophosphorylated state after induction of differentiation, as shown in previous experiments (29). In particular, there is a correspondence between the transition in Rb phosphorylation and the accumulation of the E2F–Rb complex. Examination of the p130 protein in these samples revealed quite a different result because the p130 protein was virtually absent from the undifferentiated HL60 cells. Upon induction of differentiation, there was an increase in the level of the p130 protein, whereas the level of the p107 protein decreased to undetectable levels by 48 hr after induction of differentiation. Like the change in Rb phosphorylation, the appearance of the p130 protein coincides with the time of the appearance of the E2F–p130 complex and the time of exit of the cells from a proliferative state.

Rb Interacts with Each of the E2F Species, Including E2F4, During the Initial Stages of HL60 Differentiation. Using the various E2F-specific antibodies, we have also determined the identity of the E2F polypeptides present in these complexes. As shown in Fig. 3*A*, the major component of E2F complexes in proliferating HL60 cells and throughout the process of HL60 differentiation is the E2F4 product. E2F4 was mainly detected in the p107/cyclinA complex in the undifferentiated cells and then was found in the Rb complex by 16–24 hr after induction of differentiation. Indeed, at the 24-hr time point, the vast majority of the E2F activity in the cell is the complex containing E2F4 and Rb.

Moreover, there was specificity in the interactions that reflected the results seen in fibroblast and lymphocyte cultures. As seen by the analysis of E2F in immunoprecipitates of Rb family member proteins, a single E2F species is found in association with p107 and p130, which involves the E2F4 product, as shown by supershift assays (Fig. 3*B*). In contrast, multiple E2F species are found in Rb immunoprecipitates, and supershift assays demonstrate the presence of each of the E2F family members, including E2F4 (Fig. 3*B*).

From these assays we conclude that the interaction of Rb with E2F reflects the transition of HL60 cells from a rapidly proliferating state to a state of quiescence and terminal differentiation. Moreover, whereas there appeared to be a limiting amount of Rb in growing cells such that the E2F–Rb

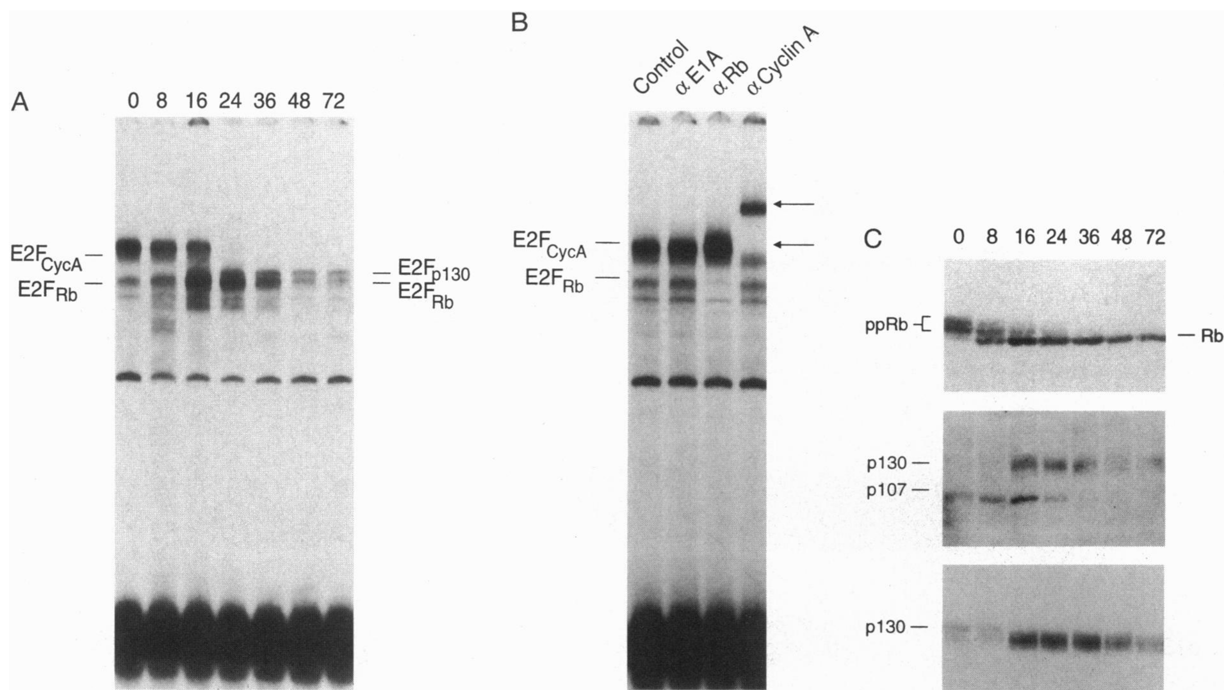

FIG. 2. E2F interactions during HL60 cell differentiation. (*A*) E2F DNA-binding assays. HL60 cells were induced to differentiate by adding PMA at 20 ng/ml. Extracts were prepared at the indicated times after adding PMA, and aliquots (2 μg) were assayed for E2F DNA binding. (*B*) Antibody supershift assays. Extracts prepared from growing HL60 cells were incubated with 1 μl of the following antibodies and assayed for E2F-binding activity: a control E1A antibody (M73) (lane 2), an Rb monoclonal antibody (IF8) (lane 3), a cyclin A monoclonal antibody (B683) (lane 4). The positions of the antibody-specific super-shifted E2F complexes are indicated by arrows. Control refers to the absence of antibody. (*C*) Levels of Rb family members and phosphorylation state during HL60 cell differentiation. Extracts prepared from HL60 cells at the indicated times after PMA addition were analyzed by SDS/gel electrophoresis. An immunoblot was probed with an Rb polyclonal antibody (*Top*), a p130 monoclonal antibody (Z11) that recognizes both p130 and p107 (10) (*Middle*), and a p130-specific polyclonal antibody (*Bottom*).

complex was only a minor component of the total E2F activity, the situation is essentially reversed in the early stages of HL60 cell differentiation, where the vast majority of the E2F activity, primarily the E2F4 product, is bound by Rb. We conclude that the conversion of hyperphosphorylated Rb to a hypophosphorylated state creates a pool of Rb sufficient to bind the majority of E2F, including E2F4, and that this process essentially inactivates E2F function.

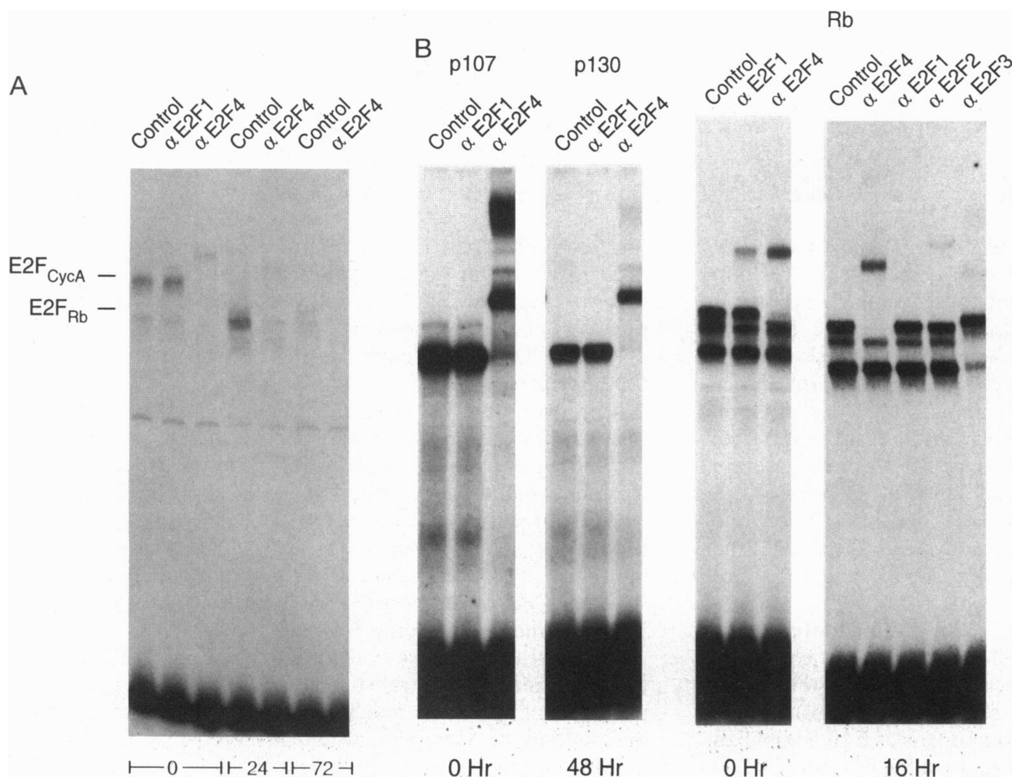

FIG. 3. Specificities in E2F complexes during HL60 differentiation. (*A*) The E2F4 product is the major E2F component in HL60 cells. Extracts prepared from HL60 cells at the indicated times (indicated at bottom of *A* and *B*) following treatment with PMA were incubated with 1 μl of the indicated antibodies and assayed for E2F DNA binding. Control refers to the absence of antibody. (*B*) Rb interacts with each E2F product, including E2F4, during differentiation. HL60 nuclear extracts prepared at the indicated times after adding PMA were immunoprecipitated with antibodies specific to the indicated Rb family members as described in Fig. 1 *B* and *C*. The supernatant of deoxycholate treatment was assayed for E2F DNA binding activity in the presence of the indicated antibodies. Control refers to the absence of antibody.

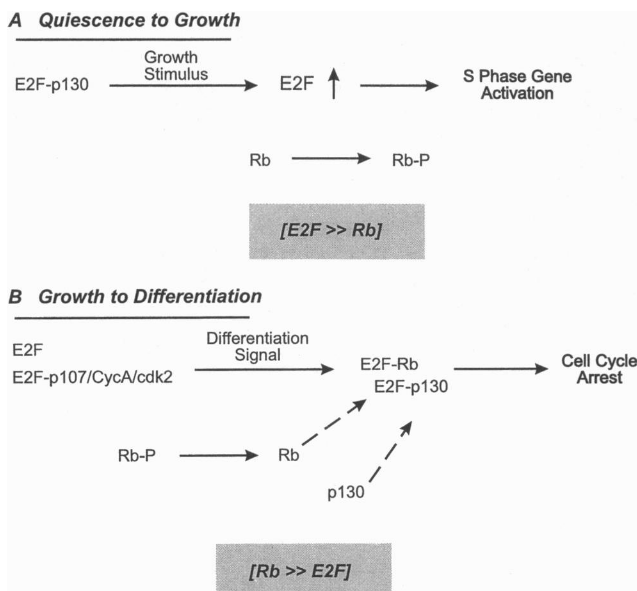

FIG. 4. Mechanisms regulating the accumulation of E2F activity during cell growth and differentiation. (*A*) In the case of quiescent cells that are stimulated to grow, active (free) E2F accumulates coincident with the disappearance of the E2F4–p130 complex. We presume that this free E2F, which is largely E2F4, can drive accumulation of additional E2F by activating the expression of the E2F1-encoding gene and possibly the E2F2-encoding gene. Because Rb becomes phosphorylated during this interval, we suggest that there is insufficient active Rb (unphosphorylated) to bind to the accumulating E2F protein. This accumulation of active E2F can then lead to the activation of target genes that allow the initiation of S phase. (*B*) Dephosphorylation of Rb during the initial stages of cell differentiation would create a pool of active Rb sufficient to capture all of the E2F4 released from the S-phase-specific complex involving p107/cyclin A/cdk2. This result would prevent E2F accumulation and, as a result, create a pool of E2F–Rb complex, as well as E2F–p130 complex, and lead to cell cycle arrest.

DISCUSSION

The cellular genes activated by E2F encode proteins critical for S-phase functions that are likely important for cells to traverse from a quiescent state to a growing state (1). Thus, the accumulation of active E2F during G_1 phase, a process controlled by Rb, could be viewed as a critical aspect of the activation of these genes and entry to S phase. Previous work has shown that the activity of Rb is regulated by phosphorylation, likely through the action of G_1 cyclin-dependent kinases, after a growth stimulus (15). As such, it has often been suggested that the phosphorylation of Rb results in the release of active E2F in G_1 that could drive the expression of S-phase genes. Yet, despite the attraction of this simple model, it has been clear from numerous studies that the E2F–Rb complex is not dissolved during G_1 but persists through this period of time when E2F-responsive genes are activated. If E2F is a target for the action of Rb, what then is the role for Rb control of E2F in the context of cell growth control?

Rather than acting as a simple switch to release E2F during G_1, we believe that the function of Rb may be to control the level of E2F accumulation during the critical point of the G_1/S transition. As summarized in Fig. 4, we suggest that the level of active Rb in proliferating cells is held in check by phosphorylation, resulting in insufficient Rb to bind all of the accumulating E2F. Thus, the E2F–Rb complex does accumulate, reflecting the accumulation of E2F, but in a productive proliferative response there is more free E2F than can be bound by Rb. As such, the levels of active Rb, as controlled by phosphorylation, are insufficient to prevent the accumulation of active E2F. A critical aspect of this model is the fact that Rb, unlike the other Rb family members, is capable of binding to each of the E2F family members, consistent with a primary role of Rb in controlling the accumulation of total E2F activity. Although this conclusion is at odds with several recent reports that have described a specificity whereby Rb interacts only poorly with E2F4 (16, 17, 19, 20), we suspect that the differences reflect the fact that the Rb interaction with E2F4 is less prominent than the interaction of E2F4 with p107 and p130, as well as the fact that this interaction may not be obvious in asynchronous populations of cells.

If the role of Rb were to titrate E2F accumulation, one would anticipate a more rapid E2F accumulation in the complete absence of Rb, leading to a shorter G_1 phase if transcription factor E2F were rate-limiting for S-phase entry. Indeed, Rb^- fibroblasts retain the capacity to arrest in a G_0 state (likely through the action of p130) but have a shortened G_1 period as they are stimulated to reenter the cell cycle (30). A contrasting situation is seen when HL60 cells are induced to differentiate, where virtually all of the E2F protein, including the abundant E2F4 product, is bound by Rb protein, likely due to the fact that the E2F levels are not increasing together with the fact that dephosphorylation of Rb leads to the conversion of the Rb pool to an active state. Just at the time that these cells are exiting the growth cycle, most E2F, which is primarily E2F4, is found complexed to Rb. Moreover, the kinetics of this Rb–E2F4 interaction coincide with the time that Rb is dephosphorylated and when the cells cease to incorporate [^{3}H]thymidine. We do not believe that this is a result peculiar to HL60 cell differentiation because very similar findings have been seen during myoblast differentiation. In short, there is a direct relationship between the interaction of Rb protein with E2F protein and the change from a proliferative state to a quiescent state. We believe it is the ratio of free E2F to Rb-complexed E2F that may be critical in the decision to continue proliferation and pass through the G_1/S transition point versus to exit from the cell cycle.

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