The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F

Scott W. Hiebert,[1] Srikumar P. Chellappan,[1] Jonathan M. Horowitz,[2] and Joseph R. Nevins[1]

[1]Howard Hughes Medical Institute, Section of Genetics; [2]Section of Cell Growth, Regulation, and Oncogenesis, Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710 USA

Recent experiments have shown that the E2F transcription factor is in a complex with the *RB1* gene product. The E2F–pRB complex can be reconstituted in an in vitro assay using a GST–RB fusion protein isolated from *Escherichia coli*. This interaction is dependent on pRB sequences involved in E1A/T-antigen binding as well as carboxy-terminal pRB sequences that are not necessary for E1A/T binding. Moreover, reconstitution assays reveal a requirement for an accessory factor, in addition to E2F and pRB, for formation of the E2F–pRB complex. Assays of transcription from the adenovirus E2 promoter in transfection experiments demonstrate that formation of the complex containing pRB and E2F coincides with an inhibition of E2F-dependent transcriptional activity. A mutant pRB protein that does not associate with E2F does not inhibit transcription. We conclude that as a consequence of its interaction with E2F, pRB may regulate the transcriptional function of the E2F factor.

Inactivation of the retinoblastoma susceptibility gene (*RB1*), as a result of deletion or mutation, is associated with the development of a variety of human cancers (Friend et al. 1987; Fung et al. 1987; Harbour et al. 1988; Huang et al. 1988; Lee et al. 1988; T'Ang et al. 1988; Templeton et al. 1991; Weichselbaum et al. 1988; Yokata et al. 1988; Varley et al. 1989; Xu et al. 1989; Bookstein et al. 1990; Horowitz et al. 1990; Shew et al. 1990). Inactivation of *RB1* function in human cancers can apparently also occur through the action of viral proteins. The majority of human cervical carcinomas harbor human papillomavirus (HPV) sequences and express the HPV E7 protein, which is known to complex with the *RB1* gene product pRB (Dyson et al. 1989). The *RB1* gene in these cells encodes a normal, wild-type protein, whereas in the minority of cervical carcinomas that do not express HPV sequences, the *RB1* gene has suffered a mutation (Scheffner et al. 1991). It would thus appear that inactivation of *RB1* function, either through mutation or through the action of a viral transforming protein, is an important event in the genesis of some human cancers.

The *RB1* gene product is a 110-kD nuclear protein whose state of phosphorylation varies with the cell cycle. The retinoblastoma (pRB) protein is underphosphorylated during G_1, becomes hyperphosphorylated near the end of G_1, and remains in this state until the cell reenters G_1 (Buchkovitch et al. 1989; Chen et al. 1989;

DeCaprio et al. 1989; Mihara et al. 1989). The fact that the underphosphorylated form of pRB is the target of the transforming protein of SV40 (Ludlow et al. 1989) suggests that this G_1-specific form of pRB plays a role in negative growth control. Thus, the inactivation of pRB, either through mutation or by interaction with tumor viral antigens, likely mimics the phosphorylation event leading to the functional inactivation of pRB.

A series of recent experiments have suggested that at least a part of the antiproliferative activity of pRB is mediated through an interaction with the E2F transcription factor (Bagchi et al. 1991; Chellappan et al. 1991; Chittenden et al. 1991) or a related factor termed DRTF1 (Bandara and LaThangue 1991). In particular, various conditions that are presumed to lead to an inactivation of pRB function coincide with the loss of the E2F–pRB complex and the release of a free E2F factor (Chellappan et al. 1991). For instance, in normal growing cells, the release of free E2F may occur at the G_1/S transition, prior to the formation of a complex of E2F with the cyclin A protein. In cells transformed by viral oncogene products such as E1A, T antigen, and E7, there appears to be constitutively free E2F (Mudryj et al. 1991; S. Chellappan, V. Kraus, B. Kroger, K. Munger, P. Howley, W. Phelps, and J. Nevins, in prep.).

Given these observations, a determination of the functional consequence of the E2F interaction is critical. With this in mind, we have explored the nature of the

E2F–pRB interaction, including the pRB sequence requirements. We have then assayed for the transcriptional activation capacity of E2F as a function of this interaction. Our results suggest that the free form of E2F, which is likely generated at G_1/S or constitutively in transformed cells, is the form that is active in transcription.

Results

In vitro reconstitution of the E2F–pRB complex

An example of the pattern of E2F-containing complexes revealed in gel mobility-shift assays of extracts of U937 cells is shown in Figure 1A. Two distinct E2F complexes are evident in these assays, one involving the cyclin A protein and the other involving the pRB protein, as de-

termined by recognition with appropriate antibodies. As shown in this experiment, a similar assay of an extract from the C-33A cervical carcinoma cell line, which contains a mutant *RB1* gene (Scheffner et al. 1991), revealed the E2F–cyclin A complex, whereas the E2F–pRB complex was absent. As shown in Figure 1B, the addition of an *Escherichia coli*-produced glutathione S-transferase (GST)–RB protein to the C-33A extract produced a DNA–protein complex of the expected mobility for the E2F–pRB complex. Moreover, the same E2F–pRB complex could be reconstituted using a partially purified preparation of E2F derived from HeLa cell extracts (Fig. 1C). This experiment also demonstrates that the reconstitution of the complex is prevented by the addition of the adenovirus E1A protein.
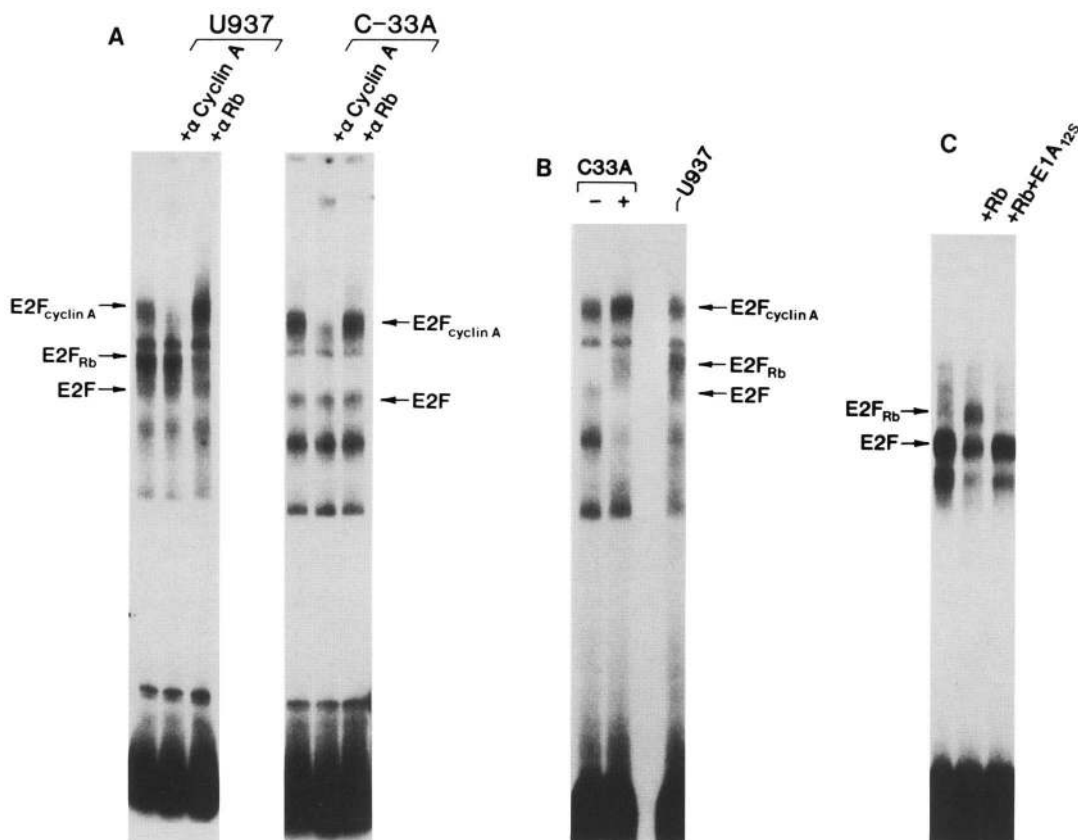

Figure 1. Reconstitution of the E2F–pRB complex. (*A*) Absence of the E2F–pRB complex in C-33A cervical carcinoma cells. Whole-cell extracts were prepared from the U937 cell line and the C-33A cell line and assayed for E2F-binding activity as described previously (Chellappan et al. 1991). Detection of the $E2F_{cyclin\ A}$ complex and the $E2F_{RB}$ complex, through the use of specific antisera, has been described previously (Chellappan et al. 1991). The additional bands in the gel are the result of nonspecific interactions, based on competition assays. The exception is the complex migrating slightly faster than the E2F complex, which appears to result from the interaction of an E2F breakdown product with the DNA (Yee et al. 1989). (*B*) Reconstitution of the E2F–RB complex with a purified RB protein. Whole-cell extracts prepared from C-33A cells were incubated with 500 ng of the wild-type glutathione transferase (GT)–RB fusion protein [pGT–RB(379–928)] for 15 min on ice in buffer containing 20 mM Tris (pH 7.5), 50 mM KCl, and 0.5 mM DTT in a total volume of 10 μl. Following incubation, E2F binding was assayed by gel retardation. The prominent band migrating faster than the band marked E2F appears to be a degradation product of E2F. (Lane −) No added pGT–pRB (379–928); (lane +) addition of pGT–pRB (379–928). A U937 whole-cell extract was used to demonstrate the mobility of the E2F–cyclin A and E2F–pRB complexes. (*C*) Reconstitution of the E2F–pRB complex with purified RB and partially purified E2F. The pGT–pRB(379–928) protein (500 ng) was incubated with or without a GST–E1A(12S) protein (500 ng) on ice for 45 min. At that time, a preparation of partially purified E2F (through the Mono Q column step, as described in Materials and methods), was added and incubated for an additional 15 min. E2F-binding activity was then measured by gel retardation. Mobility of free E2F and the E2F–pRB complex is as indicated.

RB sequence requirement for formation of the E2F–pRB complex

To assess the requirements for the formation of the E2F–pRB complex, we have made use of this in vitro reconstitution assay to determine the regions of pRB necessary to form a specific interaction with E2F. For these experiments we have made use of a series of GST–RB proteins, described previously (Kaelin et al. 1991), that contain various alterations in *RB1*-encoded sequences, as detailed in Figure 2A. Each of the proteins was isolated in pure form by glutathione affinity chromatography and then added to partially purified E2F prepared from HeLa cells. As shown in Figure 2B, only the GST–RB (379–928) protein, containing wild-type sequence, was able to reconstitute the E2F–pRB complex. Each of the mutant pRB proteins, altered in the region shown previously to be involved in binding to E1A or T antigen (Hu et al. 1990; Kaelin et al. 1990), failed to generate the E2F–pRB complex. Moreover, the pRB protein deleted of the carboxy-terminal 136 amino acids also failed to interact with E2F. These sequences have not been shown to be important for the interaction of E1A or T antigen with pRB (Hu et al. 1990; Kaelin et al. 1990), thus indicating that the interactions of E2F with pRB, at least those that generate a complex that can bind to DNA, are overlapping but distinct from the interactions of E1A and T antigen with pRB.

Formation of the E2F–pRB complex requires an additional component

The assays shown in Figure 2 demonstrate that the E2F–pRB complex can be reconstituted with pure pRB protein and partially purified E2F. We have purified the E2F factor further by DNA affinity chromatography to determine whether these were the only two components required for complex formation. Although the affinity column purification does not yield homogeneous E2F, it is nevertheless considerably purified; generally, there is a 500- to 1000-fold purification by this step (Yee et al. 1989). In contrast to the results observed in Figure 2, the affinity-purified E2F was incapable of forming the E2F–pRB complex upon addition of pRB (Fig. 3). The affinity column eluate clearly contained E2F, but there is no indication that this material could participate in the formation of the E2F–RB complex. In contrast, the load material, the small amount of E2F found in the flowthrough, and the wash fractions did combine with pRB to generate the complex. It would thus appear that
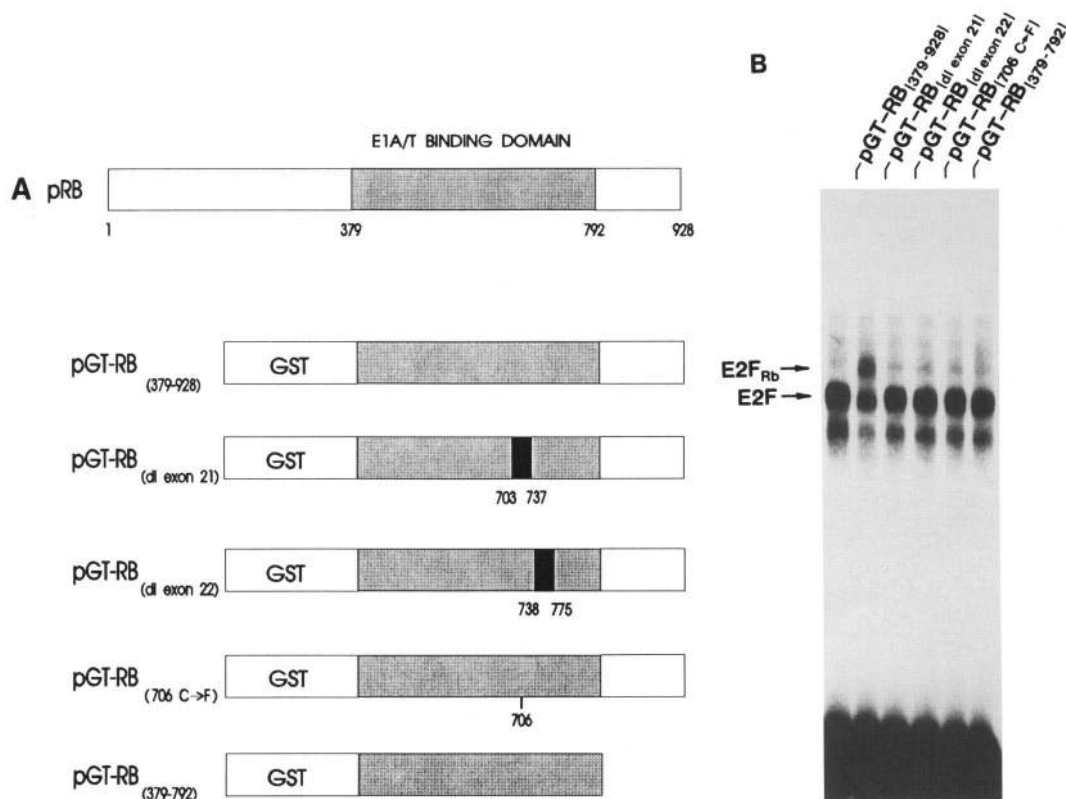

Figure 2. RB1 sequence requirements for the formation of the E2F–pRB complex. (*A*) Structure of the GST–RB proteins. The construction of the GST–RB fusion plasmids has been described (Kaelin et al. 1991). The shaded boxes indicate the regions required for interaction with SV40 large T antigen and adenovirus E1A. Deletions are indicated by solid boxes. (*B*) Reconstitution of the E2F–pRB complex. A preparation of partially purified HeLa E2F (as described in Fig. 1) was incubated with the indicated pGT–RB fusion proteins. E2F binding was then assayed by gel retardation. The positions of the E2F$_{RB}$ complex and the free E2F DNA complex are indicated.
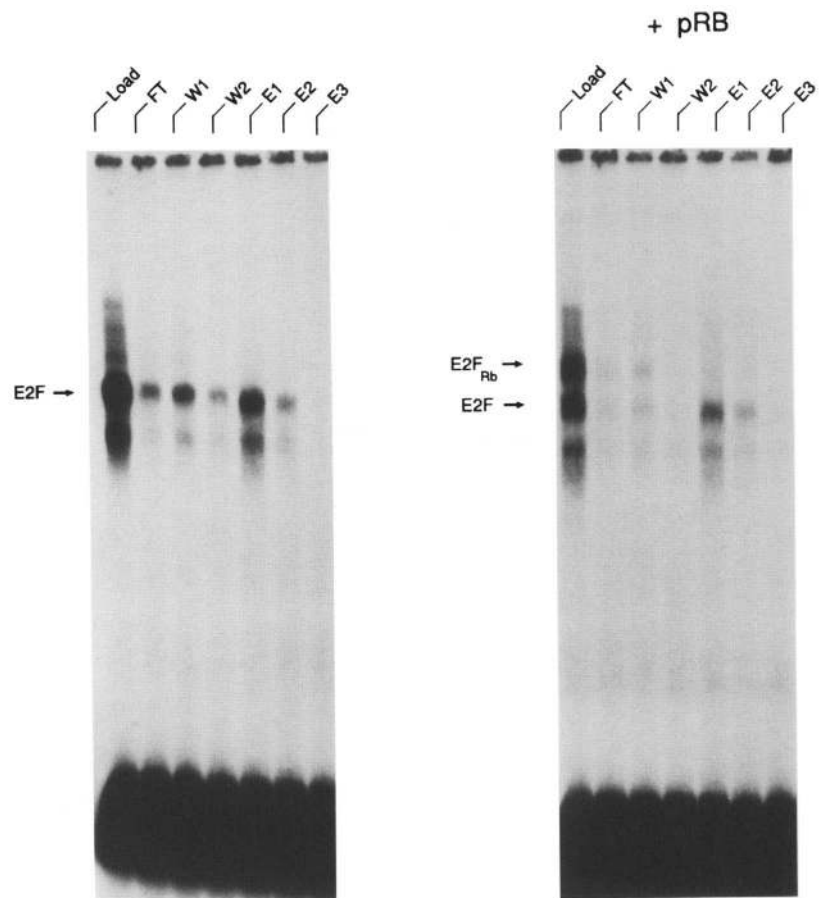
Figure 3. An accessory factor is required for formation of the E2F–RB complex. A partially purified preparation of E2F from HeLa cells was purified further through an E2F-specific DNA affinity column. The load material as well as the column fractions were assayed for E2F-binding activity in the absence or presence of the GST–RB protein. (Load) E2F purified from HeLa cells through the Mono Q step (Yee et al. 1989); (FT) flowthrough; (W1) first wash; (W2) second wash; (E1–E3) elution fractions 1–3.

an additional component is necessary for E2F–pRB complex formation and that this accessory factor has been lost during the affinity column purification.

The interaction of pRB with E2F inhibits E2F transcriptional activity

To assess the functional significance of the interaction of pRB with E2F, we have made use of the C-33A cell line, which contains a mutant *RB1* gene and, as shown in Figure 1, lacks the E2F–pRB complex. In Figure 1 a portion of the E2F found in C-33A extracts is in a complex with cyclin A and a portion is found as free E2F. On the basis of past experiments (Mudryj et al. 1991), we anticipate that the E2F–cyclin A complex is functionally inert with respect to the E2 promoter, whereas the free E2F

might be active in the stimulation of transcription. Our strategy was to introduce a pRB-expressing plasmid into the C-33A cells so as to measure a potential effect of pRB interaction with the free E2F. Previous experiments have shown that the adenovirus E2 promoter is dependent on the two E2F sites for both basal and E1A-dependent transcription, providing a direct assay for E2F transcription-activating function (Loeken and Brady 1989).

Transfection of the E2 promoter–chloramphenicol acetyltransferase (CAT) construct into the C-33A cells resulted in a readily measurable level of expression (Fig. 4B). With this assay system, we then asked whether production of a functional pRB protein in the transfected cells would affect E2 transcription. Cotransfection of a plasmid expressing the wild-type p105-RB protein re-

Figure 4. Functional consequence of the interaction of RB with E2F. (A) Schematic of the E2 promoter–CAT constructs depicting the wild-type E2 promoter (E2–CAT) and the promoter in which the two E2F sites have been mutated [E2–CAT (E2F-)]. The construction of the E2–CAT fusion plasmids has been described (Loeken and Brady 1989). Arrows indicate the transcriptional start site. (B) Effect of RB expression on E2F-dependent transcription. E2 promoter-dependent transcription, as assayed by the level of CAT activity produced in transient transfection assays, was measured in the absence or the presence of the wild-type pRB protein (+RB), a mutant RB protein (+RB$_{J82}$), or the wild-type pRB protein plus the E1A$_{12S}$ product (+RB+E1A$_{12S}$). (Left) Assays using the wild-type E2 promoter; (center) assays of the E2F-promoter; (right) assays of the effect of HPV E7 coexpression on the RB-mediated inactivation of E2F. Transient transfection analysis of the E2–CAT construct alone, with wild-type RB, or with wild-type RB plus an E6, E7 expression plasmid. The values for CAT activity were corrected for the activity derived from the RSV–SEAP plasmid as described in C. (C) Effect of RB expression on the RSV long terminal repeat promoter. The transfection assays depicted in B included an internal control of the RSV–SEAP plasmid as described in Materials and methods. The assays for SEAP activity are depicted. These values were used to correct for the CAT activities presented in B.

sulted in a reproducible four- to fivefold reduction in expression of the E2 promoter (Fig. 4B).

A series of controls demonstrate that the pRB-depen-

dent inhibition of E2 promoter activity is a specific event. First, the effect was E2F dependent because a promoter deleted of E2F sites but retaining an ATF site [E2–
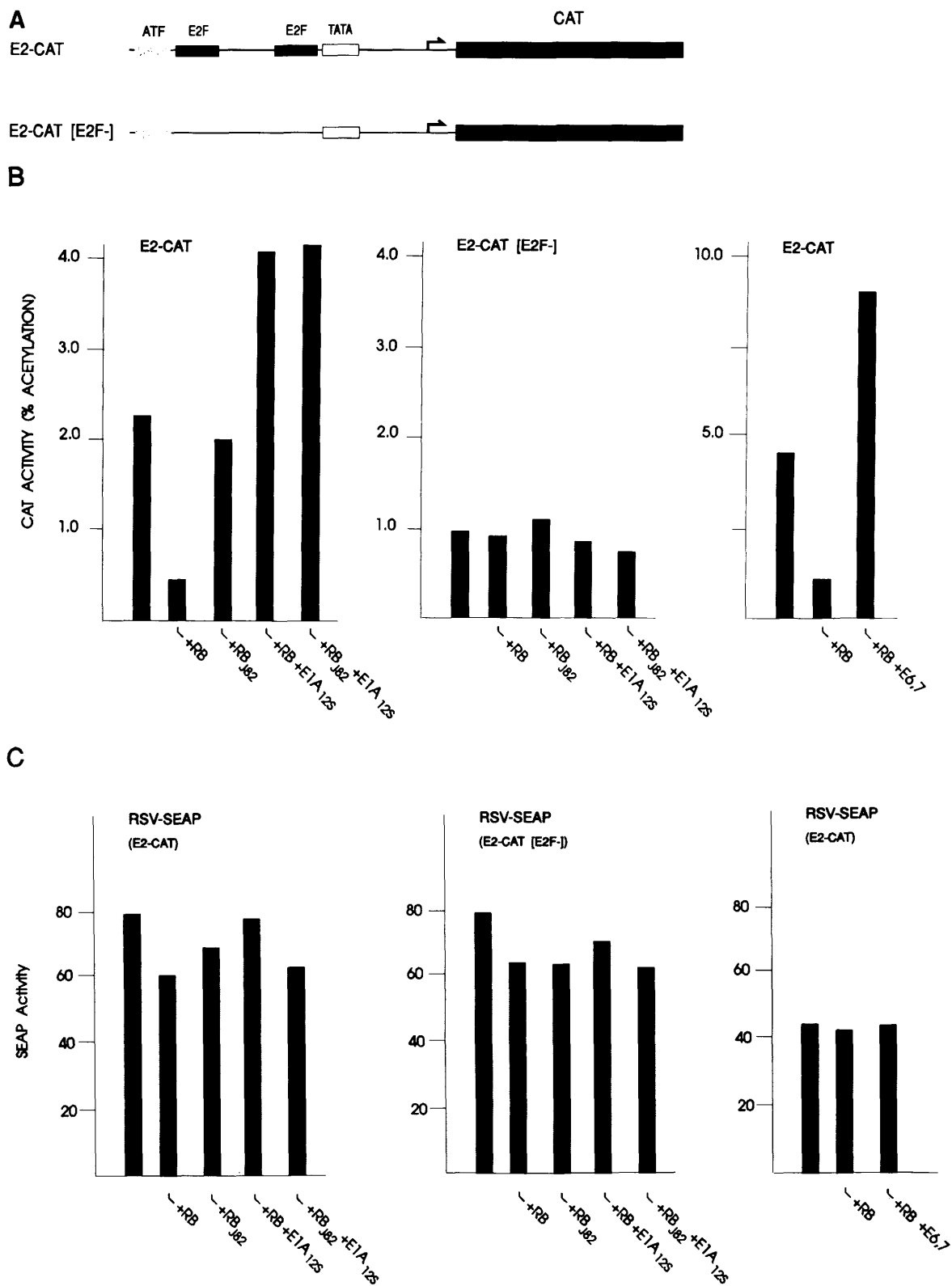

Figure 4. (See facing page for legend.)

CAT (E2F-)], was not affected by the cotransfection of pRB (Fig. 4B). Second, cotransfection of a mutant pRB protein, in this case one derived from the J82 cell line, had no effect on the transcription of the E2 promoter. The J82 mutation results in the deletion of exon 21; and as shown in Figure 2, this alteration abolishes the capacity of pRB to interact with E2F in the in vitro reconstitution assays. Thus, the inhibition of E2F transcriptional activity requires a functional pRB protein that can complex with E2F. Third, cotransfection of a plasmid expressing the $E1A_{12S}$ protein, a protein that can interact with wild-type pRB and dissociates the E2F–pRB complex, blocks the pRB-mediated repression of E2 transcription (Fig. 4B). Cotransfection of the $E1A_{12S}$ protein results in a stimulation of E2 transcription above that observed in the absence of any other addition. Moreover, the same effect was observed in cotransfections with the mutant J82 pRB. Previous experiments have shown that release of E2F from the E2F–cyclin A complex coincides with a stimulation of E2 transcription (Mudryj et al. 1991). Thus, it is likely that the stimulation observed in this experiment is the result of the release of E2F from the cyclin A interaction in the C-33A cells. Moreover, coexpression of the HPV E7 protein in these transfections also reverses the pRB-mediated inhibition (Fig. 4B). E7 is known to bind to pRB, and recent experiments have shown that E7 can dissociate the E2F–pRB complex in vitro (S. Chellappan et al., in prep.). Finally, each transfection assay included a Rous sarcoma virus—secreted alkaline phosphatase (RSV–SEAP) plasmid as an internal control. In each case, there was less than a 10% variation in RSV promoter activity as a function of pRB or E1A (Fig. 4C). Thus, the inhibition was specific for the E2 promoter and required the presence of the E2F sites within the promoter.

The assays shown in Figure 5 demonstrate that the pRB-dependent reduction in E2 promoter activity coincides with the formation of the E2F–pRB complex in the cells transfected with the wild-type pRB-expressing plasmid. Extracts prepared from cells transfected with wild-type RB, mutant RB from the J82 cell line, or wild-type RB together with E1A were immunoprecipitated with an RB monoclonal antibody. Coimmunoprecipitation of E2F-binding activity was measured as described previously (Chellappan et al. 1991). Formation of an E2F–pRB complex was evident in the RB-expressing cells, as indicated by the coprecipitation of E2F with the RB antibody, but not in the J82 RB mutant-expressing cells or in the cells coexpressing wild-type pRB and E1A. These data therefore indicate that the E2F–pRB complex does form in the transfected cells and coincides with the inhibition of the transcriptional activity of E2F.

Discussion

A variety of previous experiments suggest that the *RB1* gene is involved in regulating the growth of cells during the G_1 period of the cell cycle, likely dependent on the phosphorylation state of the pRB protein (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989; Mihara et al. 1989; Xu et al. 1989). Importantly, each of the con-
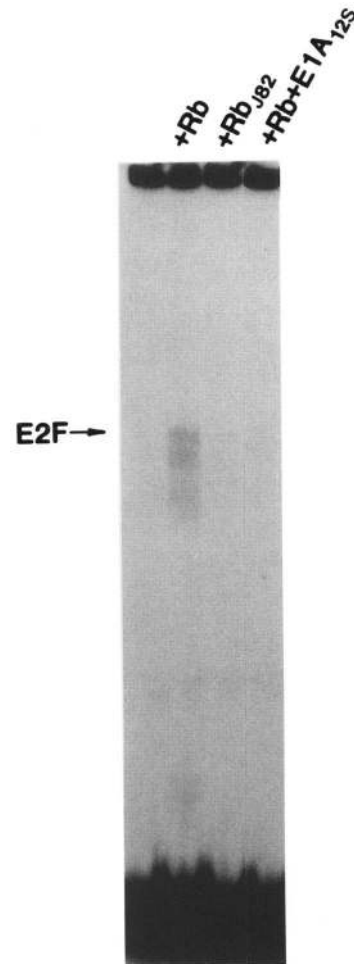

Figure 5. Detection of an E2F–RB complex in transfected cells. Extracts were prepared from cells transfected with the E2 promoter construct alone (lane 1), or the E2 construct plus either the wild-type RB (+RB), the J82 mutant RB (+RB_{J82}), or wild-type RB plus E1A (RB+$E1A_{12S}$). Immunoprecipitation of E2F DNA-binding activity from extracts of C-33A cells using a RB monoclonal antibody has been described (Chellappan et al. 1991).

ditions that appear to inactivate pRB, including the normal cell cycle-regulated phosphorylation of the protein, also results in the loss of the E2F–pRB interaction (Chellappan et al. 1991). In short, the interaction of pRB with E2F appears to be a functionally important event in relation to the cell cycle.

The data presented here demonstrate that the domain of pRB that interacts with E1A or T antigen is also involved in the interaction with E2F. However, our data also demonstrate that this domain is not sufficient because a mutant pRB that retains the E1A/T domain but lacks the carboxyl terminus is unable to form the E2F–pRB complex. This region of pRB may be a target for phosphorylation by the *CDC2* kinase (Taya et al. 1989). Thus, one might imagine that the phosphorylation of the carboxy-terminal domain of pRB is the functional equivalent (with respect to E2F) of a carboxy-terminal deletion, preventing the association of pRB with E2F.

These experiments also demonstrate that the forma-

tion of the E2F–pRB complex requires at least one other component (Fig. 3). Although the identity of this factor is not yet known, a role for an additional component is not altogether unexpected. Recent experiments have shown that the E2F–cyclin A complex contains additional proteins, including the p107 protein and a *cdc2* kinase (Devoto et al. 1992; Cao et al. 1992; Shirodkar et al. 1992). Furthermore, the observation that the E2F–pRB interaction requires at least one additional component may provide an explanation for previous, apparently contradictory findings. Several lines of evidence have now shown that the E2F–pRB complex has DNA-binding capacity (Bandara and La Thangue 1991; Chellappan et al. 1991; Chittenden et al. 1991). In contrast, a recent report suggested that a previously identified activity that would inhibit E2F DNA binding (Raychaudhuri et al. 1991) also contained the pRB protein (Bagchi et al. 1991). Perhaps a resolution of this apparent paradox is to suggest that different accessory proteins are involved. In one case, the complex may bind to DNA, whereas in the presence of a different factor, the complex is prevented from binding to DNA.

Finally, and most importantly, the experiments presented here demonstrate that the interaction of pRB with E2F very likely abrogates the ability of E2F to stimulate transcription. Although we cannot rule out the possibility that the effect of RB on E2 transcription is quite indirect, perhaps resulting from an arrest of the transfected cells in G_1, we view this extreme case to be unlikely. The fact that RB inhibits E2 transcription in an E2F-dependent fashion, together with the fact that RB also complexes with E2F, dependent on RB sequence that is also important for inhibition of E2 transcription, argues strongly that the two events are linked. It is possible that RB expression does cause a G_1 arrest but that this is a result of the interaction of RB with E2F.

Clearly, the E2F–pRB complex is still competent in DNA binding. Perhaps, as suggested in Figure 6, the interaction of the pRB protein with E2F blocks a transcriptional activation domain of E2F. Regardless of the actual mechanism by which pRB inhibits E2F transcriptional activity, these observations suggest that at least one role of pRB in controlling cell proliferation may be to control the activity of E2F as a transcription factor. What, then, might be the consequence of such control? Analysis of the phosphorylation state of pRB suggests that the E2F–pRB interaction may be lost at the end of G_1 (Chellappan et al. 1991). We note with interest that a group of genes that contain E2F sites, including dihydrofolate reductase (DHFR) (Blake and Azizkhan 1989), DNA polymerase α (Pearson et al. 1991), and thymidylate synthetase (Jolliff et al. 1991), encode proteins that are required for S phase. As such, it is possible that the control of E2F transcriptional activity, as a consequence of the pRB interaction, may influence the transcription of these genes, in part, controlling the entry into S phase. In pRB-negative cells or cells transformed by E1A, this control would be lost by creating a constitutive pool of free E2F available for transcription.

Materials and methods

Cells and preparation of extracts

The C-33A cervical carcinoma cell line and U937 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. HeLa cell cultures were maintained in minimal essential medium (S-MEM, Joklik modified) supplemented with 5% bovine calf serum. Extractions of U937, C-33A, and HeLa cells were performed as described (Yee et al. 1989).

Reconstitution of the E2F–pRB complex

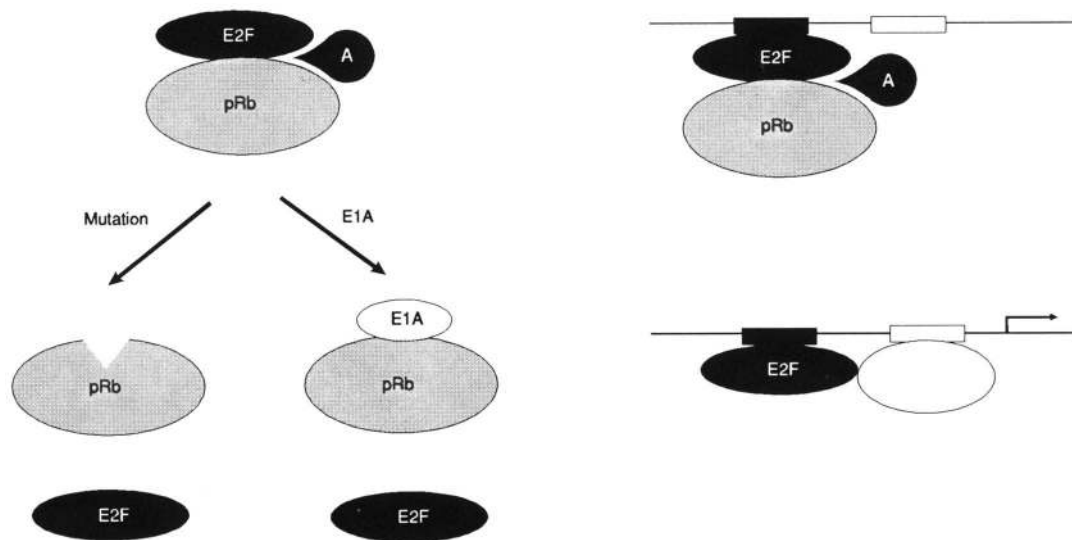The construction, expression, and purification of the GST–pRB

Figure 6. Functional consequence of the interaction of pRB with E2F. The schematic diagram depicts the interaction of E2F with pRB and an accessory factor (A). This complex, although it can bind to DNA, cannot stimulate transcription. As a result of E1A action or mutation of pRB, E2F is released from the interaction with pRB and can then function to stimulate transcription, perhaps by making contact with an adjacent transcription factor.

fusion proteins has been described (Smith and Johnson 1988; Kaelin et al. 1991). The GST–E1A$_{12S}$ fusion protein was constructed by using the polymerase chain reaction to produce full-length E1A$_{12S}$ cDNA, which was cloned into the pGEX2T vector.

E2F was purified from HeLa cell whole-cell extracts, essentially as described previously, for the purification of E2F from adenovirus-infected cells (Yee et al. 1989). Whole-cell extracts were fractionated on a heparin–agarose column as described previously, and the fractions containing E2F activity were identified by gel-retardation assays. Fractions containing E2F were pooled, dialyzed, and applied to a FPLC Mono Q column. The column was eluted as described previously (Yee et al. 1989), and fractions containing E2F activity were pooled and dialyzed. These fractions were then incubated in ice with salmon sperm DNA and applied to a specific DNA affinity column as described (Chellappan et al. 1991).

For reconstitution of the E2F–pRB complex, the pGT–pRB proteins (500 ng) were incubated with or without a GST–E1A$_{12S}$ protein (500 ng) on ice for 45 min in a buffer containing 20 mM Tris (pH 7.5), 50 mM KCl, and 0.5 mM DTT in a total volume of 10 µl. At that time, an aliquot of the E2F preparation was added and incubated for an additional 15 min. E2F DNA-binding activity was then measured by gel retardation of a ^{32}P-end-labeled probe from the adenovirus type 5 early region 2 promoter containing two E2F sites as described (Hiebert et al. 1991).

Detection of the E2F–pRB complex

The immunoprecipitation of E2F DNA-binding activity from extracts of C-33A cells transfected with pRB-expressing plasmids, using a pRB monoclonal antibody (Oncogene Science RB Ab1), has been described (Chellappan et al. 1991). Extracts of transfected cells were prepared by the method of Scholer et al. (1989) and diluted 1 : 4 with gel mobility-shift binding buffer containing 2 mg/ml of BSA prior to the addition of the anti-RB monoclonal antibody. Immunoprecipitation and gel mobility-shift analysis were performed as described (Chellappan et al. 1991).

Inhibition of complex formation, using either the pRB monoclonal antibody (1 µl) or a cyclin A polyclonal antibody (1 µl of a 1 : 5 dilution) (a kind gift of J. Pines and T. Hunter, Salk Institute, CA), was performed by preincubating the cell extract with the antibody prior to addition of the labeled probe DNA (Chellappan et al. 1991).

Transient transfection assays

Calcium phosphate precipitates of plasmid DNA (2.5 µg of E2–CAT plasmids, 10 µg of RB1 or pRB1 mutant plasmids, 10 µg of E1A plasmid, and 5 µg of RSV–SEAP) were applied to cells as described (Gorman et al. 1982). Construction of the E2–CAT or the E2–CAT (E2F-) DNA has been described (Loeken and Brady 1989). The constructions of the wild-type RB1 plasmid pJ3hRB and the RB1 mutant from the J82 cell line (pJ3hRbJ82) have been described (Robbins et al. 1990). The natures of the E1A-expressing construct (pBC12E1A12S) and the HPV E6, E7 coexpressing construct have been described (Phelps et al. 1991; Raychaudhuri et al. 1991). A plasmid expressing RSV–SEAP was used as an internal control (Berger et al. 1988). The CAT activity and SEAP activity produced in each transfection were measured as described previously (Gorman et al. 1982; Berger et al. 1988). Values given for SEAP activity are the average rate of change of the A405 in 5 min × 10^{-3}. Following the determination of SEAP activity in each sample, the value for the +RB sample was set at 1.0. The CAT activities in the other samples were then corrected for the relative SEAP activities in these samples, which did not vary by more than 10%.

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