MOLECULAR AND CELLULAR BIOLOGY, June 1995, p. 3256–3265 Vol. 15, No. 6
0270-7306/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Direct Transcriptional Repression by pRB and Its Reversal by Specific Cyclins

ROD BREMNER,[1, 2]* BRENDA L. COHEN,[1] MARY SOPTA,[3] PAUL A. HAMEL,[4] C. JAMES INGLES,[3, 5] BRENDA L. GALLIE,[1, 5] AND ROBERT A. PHILLIPS[1, 5]

Division of Immunology and Cancer Research, Hospital for Sick Children, Toronto, Canada M5G 1X8[1]; Eye Research Institute of Canada, Toronto Western Hospital, Toronto, Canada M5T 2S8[2]; and Banting and Best Department of Medical Research,[3] Department of Cellular and Molecular Pathology,[4] and Department of Molecular and Medical Genetics,[5] University of Toronto, Toronto, Canada M5G 1L6

Received 4 November 1994/Returned for modification 18 January 1995/Accepted 9 March 1995

It was recently shown that the E2F-pRB complex is a negative transcriptional regulator. However, it was not determined whether the whole complex or pRB alone is required for repression. Here we show that pRB and the related protein p107 are capable of direct transcriptional repression independent of E2F. When fused to the DNA binding domain of GAL4, pRB or p107 represses transcription of promoters with GAL4 binding sites. Thus, E2F acts as a tether for pRB or p107 but is not actively involved in repression of other enhancers. This function of pRB maps to the pocket and is abrogated by mutation of this domain. This result suggests an intriguing model in which the pocket has a dual function, first to bind E2F and second to repress transcription directly, possibly through interaction with other proteins. We also show that direct transcriptional repression by pRB is regulated by phosphorylation. Mutations which render pRB constitutively hypophosphorylated potentiate repression, while phosphorylation induced by cyclin A or E reduces repression ninefold.

The childhood eye cancer retinoblastoma (RB) results from loss of function of the protein expressed by the *RB1* locus (72). Consistent with its role as a tumor suppressor, the RB protein (pRB) is able to block the growth of some but not all cell types (72). A close relative of pRB, p107, is also capable of growth suppression (74), although its involvement in tumor growth has not been documented. Three members of the RB family have now been isolated: pRB, p107 (16), and pRB2/p130 (26, 43, 48). Homology is greatest in the so-called pocket region, which consists of A and B domains separated by a spacer (26, 43, 48). The pocket was originally identified as the minimal region of pRB required to bind the adenovirus E1A and simian virus 40 (SV40) large T oncoproteins (32) and has also been shown to be essential for the interaction of pRB with a variety of cellular proteins (10, 11, 15, 21, 33, 37, 54, 56, 57, 66).

The function of pRB is tightly regulated by phosphorylation. It is hypophosphorylated in the G_1 phase of the cell cycle but becomes progressively more phosphorylated upon entry into S phase (3, 7, 9). Phosphorylation appears to disable pRB in several functional assays. Thus, hypo- but not hyperphosphorylated pRB binds to the viral proteins E7 and large T (14, 47), various cellular proteins (11, 20, 28, 36, 60, 66, 69), and components of the cell which allow nuclear tethering of pRB (52, 62). In addition, overexpression of pRB blocks the RB^- cell line SAOS-2 in G_1, where pRB is hypophosphorylated (18, 63), and this inhibition of growth is overcome by cotransfection of cyclins which mediate the phosphorylation of pRB through cyclin-dependent kinases (cdks) (30). Finally, transcriptional activation by E2F or Elf-1 is more sensitive to repression by a mutant pRB molecule which is constitutively hypophosphorylated than to wild-type pRB (25, 66).

The molecular mechanism behind the phenotypic effects of pRB presumably lies in its ability to modulate the expression of various genes. However, exactly how pRB regulates promoter activity is poorly understood. Transcriptional activation by enhancer-binding proteins appears to involve direct or indirect contact between one or more transcriptional activation domains (TADs) and one or more of the general transcription factors responsible for basal transcription (64). E2F contains a binding site for pRB within its TAD (36, 60). pRB may therefore repress E2F activity by blocking its interaction with the basal machinery. Consistent with this hypothesis is the finding that E2F binds to the TATA-box binding protein (TBP) and that the site in E2F required for this interaction overlaps with its pRB binding site (22). Furthermore, pRB competes with TBP for binding to E2F (53). However, this effect may only partly explain the function of the E2F-pRB complex. Weintraub et al. (68) have shown that the E2F-pRB complex is a negative transcriptional regulator, capable of inhibiting the activity of other enhancers. It is possible that both components of this complex are required for this property or, alternatively, that E2F functions simply as a tether, directing the repressive activity of pRB to specific promoters. To investigate this matter, we have fused pRB to the DNA binding domain of the yeast transcription factor GAL4 and monitored the effect of this fusion protein on promoters bearing GAL4 binding sites which drive expression of the reporter gene chloramphenicol acetyltransferase (CAT). A similar approach has led to the identification of the repressive element in other transcription complexes (4, 39, 67, 71). Using this assay, we have found that pRB and its relative p107 repress transcription directly in a phosphorylation-sensitive manner.

MATERIALS AND METHODS

Cell culture, transfection, and CAT assay. C33A cells were grown in Iscove's medium supplemented with 10% Fetal Clone II. Cells were transfected at 60 to 80% confluency in 60-mm-diameter dishes by the calcium phosphate method. CAT assays were performed 24 h posttransfection by the method of Sleigh (61). Experiments were performed in duplicate and repeated at least once. The reactions were allowed to proceed for 3 h when pG5EC or pE2(-80/-70)CAT was the reporter plasmid and for 1 h when pLD85 was the reporter plasmid. One microgram of plasmid RSVβgal was included in each transfection so that CAT

* Corresponding author. Permanent address: Eye Research Institute of Canada, Toronto Western Hospital, 399 Bathurst St., Toronto, Ontario, Canada M5T 2S8. Phone: (416) 603-5800, ext. 2504. Fax: (416) 603-5126. Electronic mail address: rbremner@camtwh.eric.on.ca.

values could be normalized to β-galactosidase activity. The typical levels of normalized CAT activity, which are referred to as 100% in the figure legends, were as follows: pG5EC, 1,500 cpm; pLD85, 10,000 cpm; and pE2(-80/-70)CAT, 8,000 cpm. Expression of all of the effector genes relied on the SV40 promoter, with the exception of the cyclin B1 and B2 genes, which were driven by the cytomegalovirus promoter. To prevent competition effects by the promoters driving the effector genes on the reporter CAT plasmids, we used SVLUC as a filler plasmid where necessary. This plasmid contains the luciferase gene driven by the SV40 promoter. When pG5EC was the reporter, we found it sufficient to use the same total amount (i.e., micrograms) of plasmid. However, when pLD85 was the reporter, it was important use the same molar quantity of each plasmid, as this reporter was much more sensitive to competition from effector plasmids.

Plasmids. The reporter construct pE2(-80/-70)CAT has been described previously (45). pG5EC was a gift from M. Ptashne. pLD85 was derived from pLD83. The latter was constructed by insertion of the 115-bp *Hpa*II-*Hae*III fragment of the *GAL1-10* promoter (which contains a single GAL4 binding site; $UASg^{tv}$) into pBLCAT2 (46) cut with *Bam*HI and *Hin*dIII and blunt ended with Klenow enzyme. To create pLD85, the SV40 enhancer, containing two 72-bp repeats, was exised from pBEL2 as a *Pvu*II-*Bam*HI fragment (70), blunt ended, and inserted into *Sma*I-cut pLD83.

The plasmid expressing amino acids 1 to 147 of GAL4 (referred to as GAL in the plasmid designations) is pBXG1 and was from M. Ptashne. GALhRB was built by Z. Jiang by insertion of a 4-kb acyl/filled *Nsi*I RB fragment into *Eco*RI/filled *Pst*I-cut pBXGI. The *Acy*I site is immediately upstream of the initiating ATG in human RB; the *Nsi*I site was from the pGEM7 vector which contained the RB cDNA. Only the *Eco*RI site is regenerated. GALhRB301-928 was built by removing a 900-bp *Eco*RI fragment from GALhRB, followed by Klenow treatment and insertion of a 10-bp *Sal*I linker (CGGTCGACCG). *Eco*RI is regenerated on both sides of the linker. GALmRB was built by insertion of an *Eco*RI-*Sca*I 3.4-kb fragment from RB^K (23) into *Eco*RI-*Sma*I-digested pBXGI. The *Eco*RI-*Sac*I fragment was then replaced by the *Eco*RI-*Sac*I fragment of pECEΔPvu (23) to generate GALmRB236-921. The 1.3-kb *Dra*III-*Xba*I fragment in this plasmid was replaced by a 2.4-kb *Dra*III-*Xba*I fragment of pECEΔ22 (23) to generate GALmRB236-921Δ22. GAL107Δ133 was built by Z. Jiang by insertion of the 2.9-kb *Bam*HI fragment of pBSHp107c (a gift of M. Ewen) into pBXG1 cut with *Bam*HI and treated with calf intestinal phosphatase. The plasmid used to express wild-type mouse pRB was ΔBX. This plasmid and the Δp34 expression vector have been described before (24). GALΔp34 was built by replacing the *Eco*RI-*Xba*I insert of GALmRB with that of Δp34. SVhRB, used to express human pRB, consists of human RB cDNA in the pECE vector, with the backbone replaced by pGEM7 (*Apa*I-*Bam*HI fragment of pGEM7 replaces *Pvu*II-*Bam*HI of pECE). The expression vectors for cyclin A (pECEcycA), cyclin D1 (pECEhD1), cyclin E (pECEhE), and p107 (pECEp107) were constructed by insertion of the appropriate cDNA into the pECE vector. The cyclin B1 and B2 expression vectors have been described before (30). Plasmid pCE, which expresses E1A, was obtained from S. Benchimol.

Western blot (immunoblot) analysis. Transfected C33A cells from 60-mm-diameter dishes were lysed in 100 μl of reporter lysis buffer (Promega). One-tenth of the lysate was used to determine β-galactosidase activity generated by the 1 μg of plasmid RSVβgal included in each transfection. The volume of lysate run on a gel corresponded to an equal level of β-galactosidase activity for each lysate. This volume was usually approximately one-fifth of the original lysate volume. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose (Bio-Rad) and detected by using the light-sensitive enhanced chemiluminescence system (Amersham). The anti-RB antibody used for Fig. 4B was from Pharmingen (catalog no. 14001A). Expression of GALmRB and/or GALhRB (collectively referred to as GALRB) and GAL107Δ133 proteins was confirmed by using an anti-GAL4 rabbit polyclonal antibody obtained from M. Ptashne and I. Sadowski. Note that the expression of GALmRB236-921 was identical to that of GALmRB, as judged by using the anti-GAL4 antibody. However, the anti-RB antibody described above shows lower affinity for GALmRB236-921 than for GALmRB. The same deletion in a construct lacking the GAL4 tag (pECEΔPvu) was previously shown to be poorly detected by this antibody (23).

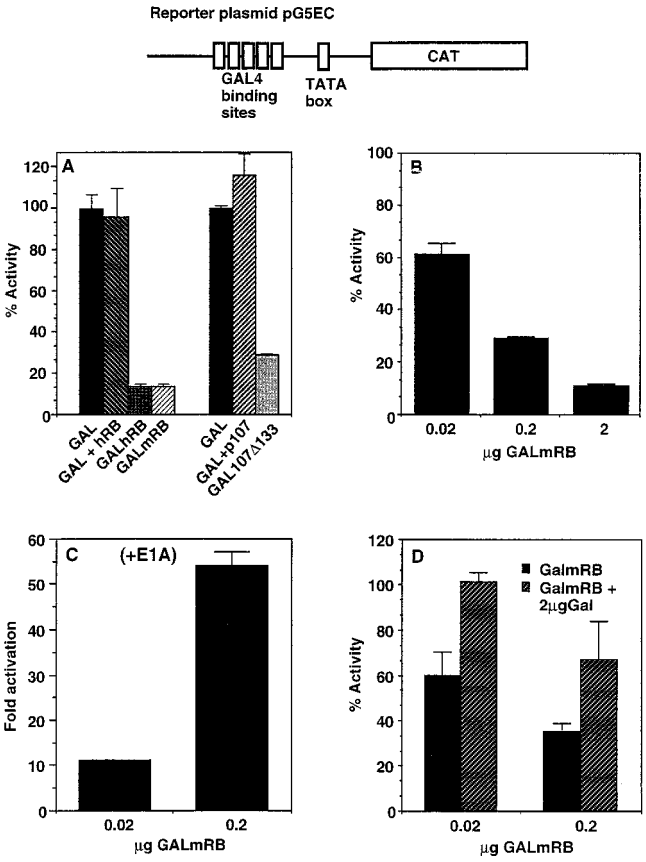

FIG. 1. GALRB and GAL107 repress transcription of an enhancerless promoter. (A) Repression by GALhRB, GALmRB, and GAL107Δ133. Two micrograms of each plasmid was cotransfected with the reporter construct. The GAL plasmid, pBXG1, expresses the DNA binding domain of GAL4 (amino acids 1 to 147). Plasmids GALhRB and GALmRB were full-length human and mouse RB, respectively, fused in frame to the DNA binding domain of GAL4 in pBXG1. GAL107Δ133 expresses the DNA binding domain of GAL4 fused to a version of p107 lacking the first 133 amino acids. The human RB (hRB) and p107 plasmids were SVhRB and pECEp107, respectively. (B) Titration of repression by GALmRB. The indicated amounts of GALmRB were cotransfected with the reporter construct. (C) Activation by 13S E1A bound to GALmRB. One microgram of an E1A plasmid (pCE) was cotransfected with the indicated amounts of GALmRB and the reporter plasmid. The fold activation above the level obtained with GALmRB alone is plotted. (D) Competition for promoter binding by DNA binding domain of GAL4. The indicated amounts of GALmRB were transfected alone or together with 2 μg of GAL plasmid. In panels A to D, 3 μg of the reporter construct pG5EC (top) was transfected. In panels A, B, and D, 100% activity is that obtained by transfection of 2 μg of GAL plasmid. This value was 1.2- to 1.5-fold higher than that obtained with reporter plus 2 μg of a control plasmid (SVLUC; see Materials and Methods).

RESULTS

pRB and p107 repress transcription directly. When a reporter plasmid consisting of five GAL4 binding sites upstream of a TATA box and the CAT gene was transfected into the human cervical carcinoma line C33A, together with full-length human or mouse RB fused to the DNA binding domain (amino acids 1 to 147) of GAL4, potent inhibition of transcription was observed (Fig. 1A). Repression was dependent on covalent attachment of GAL4 to RB and on interaction of GALRB with GAL4 sites in the promoter, since coexpression of human pRB with a plasmid expressing the DNA binding domain of GAL4 (GAL plasmid) gave no repression (Fig. 1A). Mouse RB, like human RB, also had no effect on promoter activity when cotransfected with GAL plasmid (data not shown). These data indicate that transcriptional repression by the E2F-pRB complex is mediated by pRB and that E2F is required only as a DNA tether for pRB.

pRB is a member of a family of proteins, three of which have been isolated: pRB, p107 (16), and pRb2/p130 (26, 43, 48). We therefore tested the ability of another member of this family, p107, to directly repress transcription. Using the original version of p107 cDNA, which lacks the region encoding the N terminus (16), we constructed GAL107Δ133. This plasmid lacks the first 133 amino acids of p107, as determined from the recently published complete sequence of p107 (74). Like GALhRB and GALmRB, GAL107Δ133 repressed the activity of pG5EC (Fig. 1A). The result demonstrates conservation of this function in the RB family. Cotransfection of plasmids express-

ing p107 and the DNA binding domain of GAL4 did not cause repression (Fig. 1A). Thus, as with pRB, promoter contact is a prerequisite for direct repression by p107. This requirement also proves that transcriptional repression is not an indirect effect of the ability of p107 to arrest C33A cell growth (74).

The promoter construct used in these assays lacks known enhancer binding sites, so it is possible that GALRB represses basal transcription. However, it may be that there are weak cryptic enhancers in the plasmid and that repression is dependent on their presence. A similar effect is observed in CHO cells (data not shown), indicating that the effect is not limited to one cell line. In the following experiments we used only C33A cells, which express a mutant pRB (59).

We detected repression by GALmRB even when very low amounts of plasmid were transfected (Fig. 1B). Since we detected protein by Western analysis with 2 μg of plasmid (see Fig. 4B) but not when 0.2 or 0.02 μg of plasmid was used (data not shown), we used another approach to verify the expression of transfected GALmRB in these experiments. When a plasmid expressing GALRB is cotransfected with E1A, the interaction between GALRB and E1A brings the transcriptional activation domain of 13S E1A into contact with the transcriptional machinery, resulting in strong stimulation of transcription (44). In our experiments, even transfection of only 0.2 or 0.02 μg of GALmRB gave detectable activation in the presence of E1A (Fig. 1C). Cotransfection of E1A with 2 μg of GAL plasmid did not activate transcription (not shown). This result confirmed the presence and activity of GALmRB protein in cells transfected with 0.2 or 0.02 μg of plasmid DNA. We also tested whether repression by GALmRB was sensitive to competition for the GAL4 binding sites in the reporter construct. The repression observed with 0.2 or 0.02 μg of GALmRB was considerably reduced when this plasmid was cotransfected with 2 μg of a plasmid expressing just the DNA binding domain of GAL4 (Fig. 1D). This result confirmed the specificity of repression by low amounts of GALmRB and the requirement for an interaction between GALmRB and the promoter.

Involvement of the pRB pocket in direct transcriptional repression. To provide evidence that direct repression of transcription by pRB is physiologically relevant, we sought to identify a mutation in pRB which abrogates or severely compromises its ability to directly repress transcription. Deletion of the first 235 amino acids of mouse pRB or the first 300 amino acids of human pRB did not reduce the capacity of the protein to repress pG5EC promoter activity (Fig. 2). The finding that the C-terminal portion of pRB, which includes the AB pocket, is able to repress transcription is consistent with the well-characterized role of this domain in protein-protein interactions (10, 11, 15, 21, 33, 37, 54, 56, 57, 66) and repression of activated transcription (25, 29, 73) or cell growth (54, 56, 63). Further confirmation of a role for the pocket in direct transcriptional repression was obtained from the observation that a deletion in the B domain severely compromised repression (Fig. 2, GALmRB236-921Δ22). Transfection of 2 μg of this mutant plasmid gave approximately the same level of repression as that detected by transfection of 0.02 μg of wild-type GALmRB, a 100-fold difference (cf. Fig. 1B and 2B). The Δ22 mutation removes 36 of the 38 amino acids encoded by exon 22 of mouse RB. A mutation resulting in deletion of exon 22 has been observed in small cell lung carcinoma (31) and in retinoblastoma (13). The dramatic effect of this mutation on repression was not due to a decrease in protein stability, since by Western blot analysis with an anti-GAL4 antibody, we detected equivalent amounts of all of the proteins tested in Fig. 2A (data not shown).

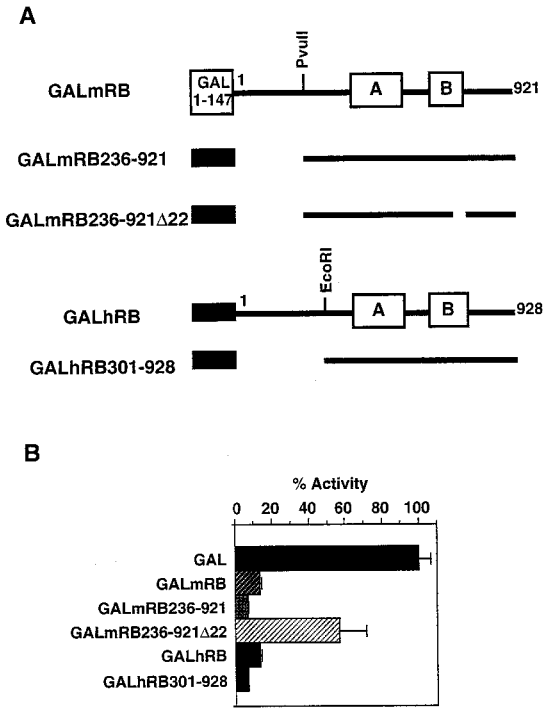

FIG. 2. Involvement of the pocket domain in repression. (A) GALmRB and GALhRB mutants. The portion containing amino acids 1 to 147 of GAL4 (GAL1-147) is shown as a black box in all but the top line. The first and last amino acid numbers of mouse and human pRB are indicated. The *Pvu*II and *Eco*RI sites used to construct the N-terminal deletion mutants in mouse and human RB, respectively, are shown. The A and B domains of the pocket are shown as boxes. The position of the Δ22 mutation in the B domain is indicated. (B) Transcriptional effects of the fusion proteins in depicted in panel A. Two micrograms of each plasmid was transfected with 3 μg of pG5EC; 100% activity is that obtained with GAL plasmid.

As discussed above, removal of the N-terminal portion of mouse or human pRB did not impair the ability to repress transcription directly. On the contrary, these mutants actually repressed better than wild-type GALRB fusion proteins (Fig. 2B). One explanation for this result is that the N-terminal portion of pRB negatively regulates repression by the pocket domain. However, it has previously been documented that the N terminus is required for phosphorylation of pRB (23, 54). It is therefore possible that GALmRB236-921 and GALhRB301-928 show enhanced repression because they are resistant to negative regulation by phosphorylation. Below (Fig. 4 to 6 and Table 1), we demonstrate that phosphorylation of pRB can indeed alleviate its ability to repress transcription directly.

pRB represses enhancer activity. While bound to E2F, pRB blocks not only E2F activity (17, 27) but also transcriptional activation by adjacent enhancer elements (68). To determine if pRB, when tethered to DNA by GAL4 rather than E2F, would also interfere with enhancer function, we assayed the ability of GALRB fusion proteins to repress a promoter containing one GAL4 binding site and driven by the powerful SV40 enhancer (Fig. 3A, pLD85). As shown in Fig. 3B, both GALmRB and GALhRB blocked enhancer activity. Repression required promoter contact since coexpression of pRB with the DNA binding domain of GAL4 resulted in the same level of activity from the reporter construct as seen when the latter was expressed alone (data not shown). Removal of the N-terminal portion of pRB enhanced repression of pLD85, and a deletion in the B domain of the pocket abrogated the effect (Fig. 3B). These

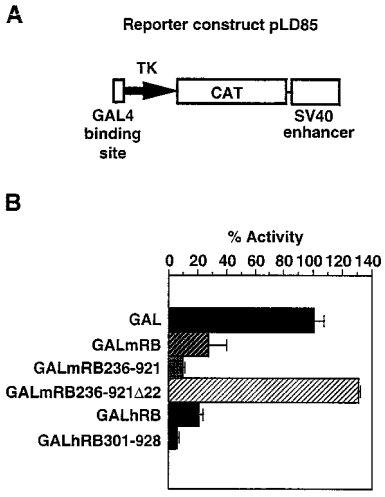

FIG. 3. GALRB can repress enhancer activity. (A) Diagram of pLD85. A single GAL4 binding site lies upstream of the thymidine kinase (TK) promoter, the CAT gene, and the SV40 enhancer. (B) Effects of GALRB and mutants on pLD85 activity. Three-tenths microgram of pLD85 was transfected with the indicated GAL plasmids. This amount of pLD85 was in the linear range of activity for this reporter construct (not shown). The effect of 4 μg of GALmRB was compared with the effect of molar equivalents of the other plasmids. One hundred percent activity refers to that obtained with the GAL plasmid alone; this value was the same as that obtained with reporter plus a molar equivalent of SVLUC.

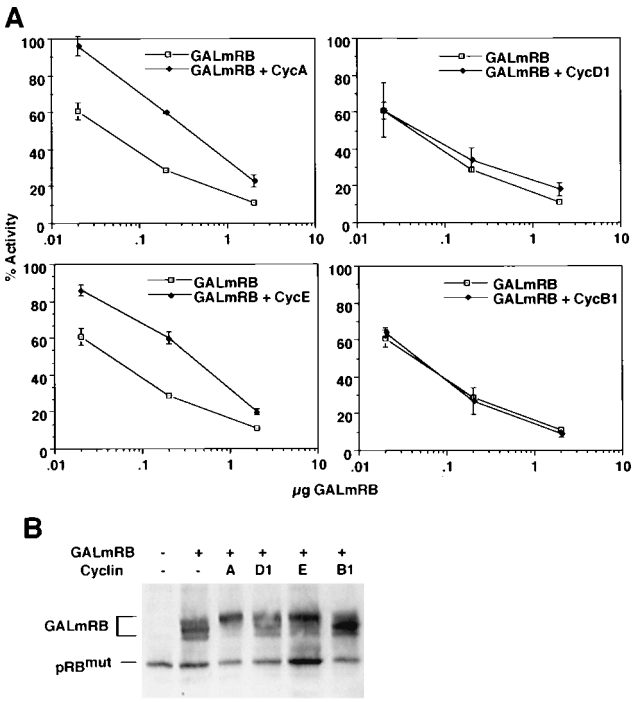

FIG. 4. Cyclins A and E (CycA and CycE) alleviate repression by GALmRB. (A) Effects of cyclins on repression by GALmRB. Three micrograms of pG5EC was transfected with 0.02, 0.2, or 2 μg of GALmRB together with 2 μg of a control plasmid (SVLUC) or 2 μg of the indicated cyclin. Additional SVLUC was used as a filler in the transfections with 0.02 and 0.2 μg of GALmRB; 100% activity is that obtained with 2 μg of GAL plasmid. The cyclins did not affect the activity of pG5EC in the absence of GALmRB (not shown). (B) Phosphorylation of GALmRB by cyclins. Lysates of cells transfected with the indicated plasmids were run on an SDS–7% polyacrylamide gel and transferred to nitrocellulose, and GALmRB was detected with an anti-GAL4 polyclonal rabbit serum. The position of bands corresponding to GALmRB and endogenous C33A mutant pRB (pRB^{mut}) are indicated.

mutations had a similar effect on repression of the enhancerless promoter in pG5EC (Fig. 2), suggesting that a similar mechanism mediates the effect in both cases.

Cyclins A and E reverse direct repression by pRB. The data in Fig. 2 and 3 are consistent with a model in which repression of transcription by pRB occurs through interaction between the RB pocket and another protein (or proteins) which can influence transcription. Given that phosphorylation disrupts pRB's ability to interact with other proteins (11, 20, 28, 36, 60, 66, 69), it is likely that phosphorylation also disrupts an interaction required for direct repression of transcription. To investigate this possibility, we tested the effect on transcription of coexpressing a variety of cyclins with GALmRB and the pG5EC reporter plasmid. Some of these cyclins mediate the phosphorylation of pRB (15, 30, 38). As shown in Fig. 4A, cotransfection with cyclin A or E reduced the ability of GALmRB to repress transcription. Cylin D1 had no effect on repression. Cylin B1 did not alter promoter activity (Fig. 4A), and neither did cyclin B2 (data not shown). At any single dose of GALmRB plasmid, cyclins A and E caused a 1.5- to 2-fold increase in promoter activity (Fig. 4A). However, titration of GALmRB activity demonstrated that to achieve a 2-fold increase in pG5EC promoter activity, one must reduce the amount of transfected GALmRB plasmid approximately 10-fold (Fig. 1B and 4A). Thus, cyclins A and E reduce the potency of GALmRB by up to 10-fold. This point is illustrated clearly in Table 1, where the repressive activity of GALmRB is calculated on the basis of the amount of plasmid required to achieve 50% pG5EC activity. In the presence of cyclin A or E, ninefold more GALmRB is required to repress the pG5EC promoter to 50% activity. Determination of the dose of GALmRB required to give a certain activity is a more reliable indication of the effect of cyclins than a comparison of activities at the same dose. Values obtained by the latter (but not the former) approach differ depending on the responsiveness of the promoter to changes in dose. We demonstrate this point below, using a promoter which is more responsive to changes in GALmRB concentration (see Fig. 6).

Our results are consistent with the observation that cyclins A and E can reverse suppression of SAOS-2 cell growth by pRB, while cyclins B1 and B2 cannot (30, 74). Expression of the B cyclins from the plasmids used in these experiments has been

TABLE 1. Effects of cyclins on pRB repressive activity

Cyclin[a]	Relative repressive activity of RB (%)[b]	
	pG5EC assay[c]	pE2(-80/-70)CAT assay[d]
None	100	100
A	11	5.9
D1	90	67
E	11	4.0
B1	100	100

[a] The cyclin cotransfected with GALmRB (pG5EC assay) or the mouse RB plasmid (pE2(-80/-70)CAT assay) is indicated.

[b] The amount of RB plasmid required for repression is inversely proportional to the repressive activity. Thus, 100% activity represents the reciprocal of the amount of RB plasmid required in the absence of any cyclin to repress promoter activity to 50%.

[c] Relative activity of GALmRB in the pG5EC assay was calculated from the graphs in Fig. 4.

[d] Relative activity of the mouse RB plasmid in the pE2(-80/-70)CAT assay was calculated from the graphs in Fig. 7.

confirmed (30). Although direct interaction between cyclin D1 and pRB has been reported (11, 15), the ability of cyclin D1 to reverse the growth-suppressive properties of pRB is much weaker than that of cyclin A or E (30, 74), a result which is consistent with our observation that cyclin D1 does not reverse repression of transcription by pRB. To confirm that cyclin D1 does not reverse transcriptional repression in these cells, we tested in the assay a plasmid which expressed mouse cyclin D1 tagged with the hemagglutinin epitope. Expression of this version of cyclin D1, which also failed to reverse repression, was confirmed by using an antibody against the epitope tag (data not shown).

The ability of cyclins A and E to reverse the growth-suppressive properties of pRB correlates with the phosphorylation of pRB, presumably by cdks (30). To determine if transfection of these cyclins led to alterations in the phosphorylation state of GALmRB in C33A cells, we carried out a Western blot analysis. pRB exhibits a characteristic mobility shift upon phosphorylation such that the hyperphosphorylated species migrate more slowly than the hypophosphorylated species when analyzed by SDS-PAGE. As expected, we detected a high proportion of phosphorylated GALmRB protein in cells transfected with 2 μg of GALmRB and 2 μg of cyclin A or E expression vector (Fig. 4B).

As shown in Fig. 4B, none of the cyclins altered the level of GALmRB protein. In any case, it is important to emphasize that the observed changes in promoter activity could not be due to small fluctuations in protein amounts; as stated above, the amount of GALmRB must be reduced by an order of magnitude to raise promoter activity by twofold.

Below, we provide confirmatory evidence for the physiological relevance of the effect of cyclins A and E on direct repression by pRB. First, we demonstrate that constitutively hypophosphorylated mutants of pRB are more active in direct repression assays. Second, using the pLD85 promoter, which is more sensitive to changes in the concentration of GALmRB, we show that cyclins A and E cause a greater (threefold) increase in promoter activity at a given dose of GALmRB. Finally, we demonstrate that the effects of cyclins A and E on regulation of E2F activity by pRB are of the same magnitude as those observed in the direct repression assay.

Enhanced repression by constitutively hypophosphorylated pRB. Another way to determine the effect of phosphorylation on direct repression by pRB is to test in the assay a mutant which is constitutively hypophosphorylated. To do this experiment, we constructed plasmid GALΔp34, which expresses the GAL4 DNA binding domain fused to a version of pRB in which eight $p34^{cdc2}$ consensus sites have been mutated. These modifications render pRB superactive in assays which measure repression of activated transcription (25, 66). Although GALmRB and GALΔp34 were expressed at the same level (data not shown), the latter was significantly more potent in repressing transcription than the wild-type molecule (Fig. 5A). While 0.03 μg of plasmid GALΔp34 repressed promoter activity to 30% of that obtained with GAL plasmid alone, 0.11 μg of GALmRB was required to repress to the same degree, three- to fourfold difference (Fig. 5A).

Previously, it has been shown that the Δp34 mutant is approximately 30-fold more potent than wild-type pRB in repressing activated transcription mediated by E2F (25). The latter experiment was carried out in retinoic acid-treated P19 embryocarcinoma cells, using pE2(-80/-70)CAT as the reporter plasmid (Fig. 5B). The larger effect observed in these cells compared with direct repression by GAL-RB in C33A cells may be due to a greater sensitivity of the E2F-pRB interaction to phosphorylation or to different intrinsic properties of

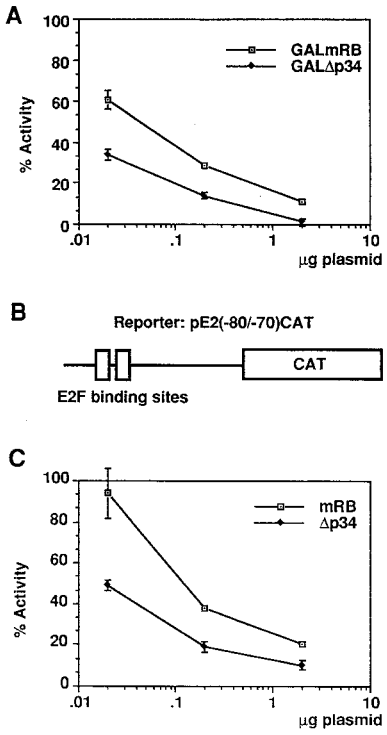

FIG. 5. A constitutively hypophosphorylated pRB mutant shows enhanced repression. (A) Three micrograms of pG5EC was transfected with 0.02, 0.2, or 2 μg of GALmRB or GALΔp34; 100% activity is that obtained with 2 μg of GAL plasmid. (B) Structure of the reporter plasmid pE2(-80/-70)CAT. It consists of 160 bp of the adenovirus EIIaE promoter. Transcription is dependent on two E2F binding sites upstream of the TATA box, as is repression by pRB in C33A cells (74). (C) Three micrograms of pE2(-80/-70)CAT was transfected with 0.02, 0.2, or 2 μg of mouse RB or Δp34; 100% activity is that obtained with 2 μg of a control plasmid (SVLUC).

C33A and P19 cells. When we measured repression of E2F-stimulated transcription in C33A cells, we found that the difference in repression of E2F activity by wild-type pRB and Δp34 was much less in C33A cells than in P19 cells. The amounts of pRB and Δp34 required to repress the E2 enhancer to 50% of its activity in the absence of pRB were 0.1 and 0.02 μg, respectively, a fivefold difference (Fig. 5C). Thus, in C33A cells, mutation of phosphorylation sites in pRB potentiated direct repression or repression of E2F activity to similar degrees, a finding which supports the conclusion that regulation of direct repression by phosphorylation is physiologically relevant. The larger difference between wild-type pRB and Δp34 in P19 cells may reflect a greater capacity to phosphorylate pRB in P19 cells than in C33A cells.

Phosphorylation negatively regulates direct repression of enhancers by pRB. To further characterize the role of phosphorylation in the regulation of direct repression by pRB, we tested the effects of cyclins on the ability of GALmRB to repress transcription from the enhancer-driven promoter in pLD85. As was observed with the enhancerless promoter, cyclins A and E were able to alleviate transcriptional repression by GALmRB (Fig. 6A). Once again the effect was specific, since cyclin D1 or B1 had no effect. At a single dose of GALmRB (4 μg), cyclins A and E increased promoter activity threefold. In the pG5EC assay (Fig. 4A), the effect of the same amount of these cyclins on a smaller amount of GALmRB (2 μg) was lower, 1.5- to 2-fold. The explanation for this result lies in the greater sensitivity of pLD85 to changes in the concen-

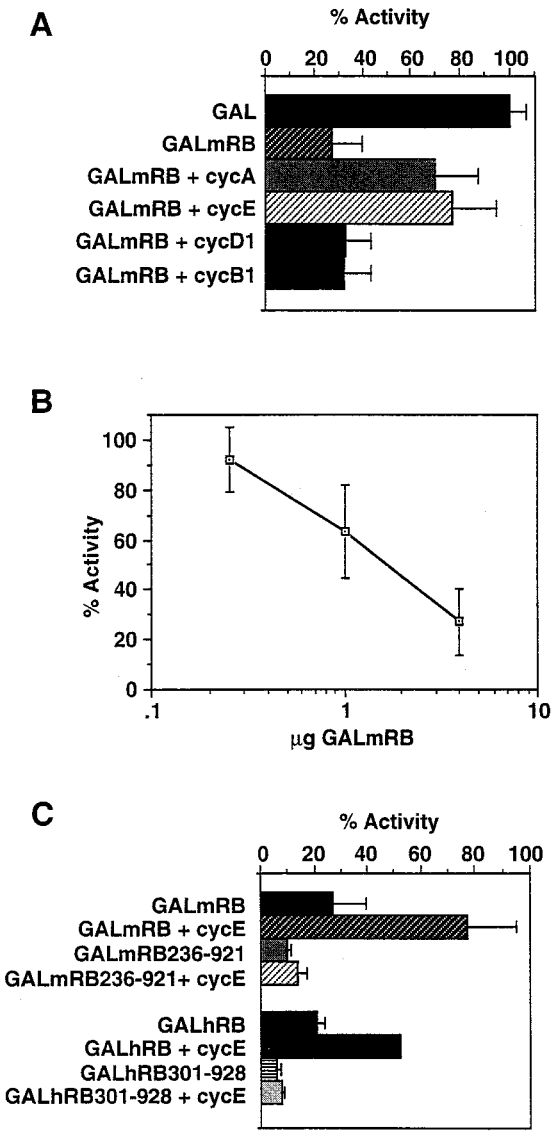

FIG. 6. Repression of an enhancer by GALRB is alleviated by cyclins A and E (cycA and cycE). (A) Effects of cyclins on repression of the SV40 enhancer by GALmRB. The reporter construct pLD85 (Fig. 3A) was transfected with GAL plasmid or GALmRB together with vector alone (pECE) or the indicated cyclin. (B) Titration of repression of the SV40 enhancer by GALmRB. The indicated amounts of GALmRB were cotransfected with pLD85. An appropriate amount of SVLUC was included in the transfections containing 1 and 0.25 μg of GALmRB to equalize the total number of moles transfected. An additional amount of pECE vector, equivalent to that used in panel A, was also transfected so that the data in panels A and B could be compared. (C) Cyclin E does not alleviate repression by constitutively hypophosphorylated GALRB. pLD85 was transfected with the indicated GAL fusion plasmid together with pECE vector or cyclin E. In panels A to C, 0.3 μg of pLD85 was used. In panels A and C, 4 μg of GALmRB and molar equivalents of the other GAL plasmids were used. Two micrograms of cyclin E and molar equivalents of the other cyclins or pECE vector were used. One hundred percent activity is that obtained with GAL plasmid plus pECE; this value was not different when GAL plasmid was transfected with any of the cyclins (data not shown).

tration of active GALmRB. As shown previously, a 10-fold drop in the amount of GALmRB results in a 2-fold increase in the activity of the enhancerless promoter in pG5EC (Fig. 1B or 4A). However, a 10-fold drop in the amount of GALmRB (from 4 to 0.4 μg) increases the activity of pLD85 from 27 to 80%, a 3-fold effect (Fig. 6B). Thus, the 3-fold effect of cyclins A and E observed in Fig. 6A represents a 10-fold reduction in the potency of GALmRB. The effects of these cyclins are therefore the same in the pG5EC assay and the pLD85 assay, but the greater responsiveness of the latter to changes in the amount of active GALmRB protein results in a greater apparent effect of cyclins A and E.

If cyclin-induced alleviation of repression is a specific effect, operating through phosphorylation of pRB, then constitutively hypophosphoryated pRB should be resistant to these effects. To investigate this hypothesis, we tested the effect of cyclin E on mouse and human versions of GALRB which lack the N-terminal portion of pRB and are therefore constitutively hypophosphorylated (GALmRB236-921 and GALhRB301-928; Fig. 2A). While cyclin E alleviated repression by wild-type GALmRB and GALhRB, it had no effect on the enhanced repression seen with the N-terminal mutants (Fig. 6C).

Specificity and magnitude of cyclin effect on repression of E2F activity by pRB. Further support for the role of the cyclins in regulating direct repression by pRB came from analysis of their effect on repression of E2F activity by pRB. In an assay using pE2(-80/-70)CAT, the activity of which depends on two functional E2F binding sites, we detected the same trends previously observed with the pG5EC repression assay. Thus, cyclins A and E, but not D1 or B1, alleviated repression of E2F activity by pRB (Fig. 7 and Table 1). Furthermore, the magnitude of the effect was very similar to that observed in the direct repression assay (Table 1). We conclude that the effects of cyclins A and E on pRB are not peculiar to the assay for direct repression.

We observed some activation of the E2 promoter when cyclin E was transfected with 0.02 μg of pRB (Fig. 7). The effect was specific to cyclin E, since although cyclin A was able to lift repression of E2 by pRB, no such activation was detected. However, given the relatively limited conditions under which the phenomenon was observed, we are uncertain as to its significance.

DISCUSSION

pRB and p107 are direct transcriptional repressors. Previously, it was shown that the E2F-pRB complex is a negative transcriptional regulator (68). Our work advances understanding of this observation by demonstrating that when provided with a DNA binding domain, pRB and its relative p107 act as transcriptional repressors. This result suggests that E2F serves as a DNA tether for pRB and p107 but is not actively involved in transcriptional repression. In this way, pRB resembles repressors which were initially identified as being part of a complex and subsequently, by techniques similar to those described here, shown to be the repressive component in the complex; these include the yeast repressors SIN3 (67), SSN6, and TUP1 (39) as well as the adenovirus 55K E1B protein (71). The proteins which tether SIN3 to DNA are unknown, but SSN6 and TUP1 are known to interact with the MCM1-a2 complex (39), and the E1B 55K protein is tethered to DNA through interaction with p53 (71).

Interaction of pRB or p107 with other transcription factors, such as c-Myc (19, 57) Elf-1 (66), MyoD (20), and PU.1 (21), may also tether these repressors to DNA. pRB could also contact promoters through its interaction with the DNA-binding protein c-Abl (69) or through various other pRB-binding proteins, the functions of which are unclear (10, 37).

In certain contexts, pRB is able to positively influence promoter activity. pRB potentiates SP1-mediated activation of a variety of promoters (40, 65) and activates the transforming growth factor β2 promoter through ATF-2 (41). Our data

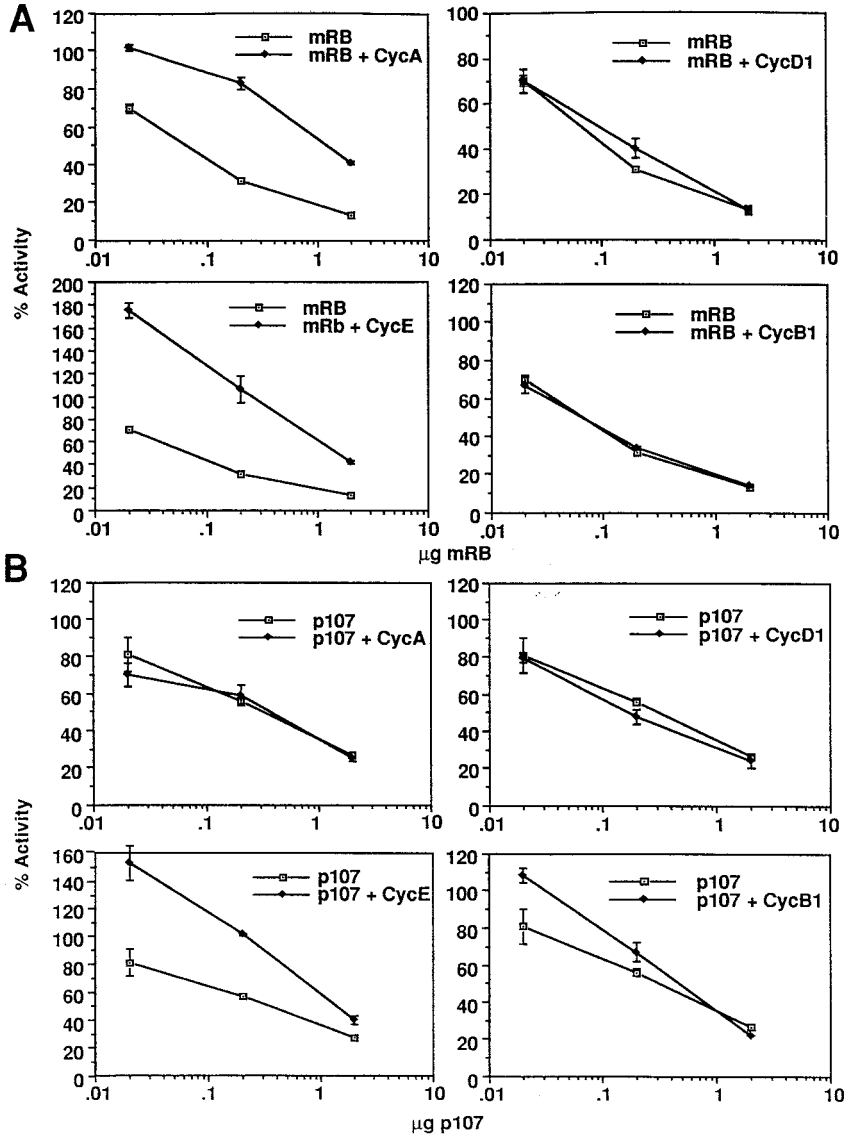

FIG. 7. Effects of cyclins on repression of E2F activity by pRB. Three micrograms of the reporter plasmid pE2(-80/-70)CAT was transfected with 2 μg of mouse RB (mRB) plasmid ΔBX, together with 2 μg of a control plasmid (SVLUC) or the indicated cyclin; 100% activity is that obtained from transfection of the reporter with 4 μg of the control plasmid. In the absence of mRB, the cyclins had only marginal effects on E2CAT activity: an 8% drop in activity in the case of cyclin A (CycA), a 21% drop with cyclin D1 (CycD1), a 24% increase with cyclin E (CycE), and a 25% increase with cyclin B1 (CycB1).

indicate that in the simplest context (a promoter containing a TATA box but no enhancers), pRB is a transcriptional repressor. Clearly, transcriptional activation by pRB is a more complex phenomenon, requiring the presence of one or more additional elements. In addition, the E2F-pRB transcriptional repression complex is targeted to promoters by its association with the E2 enhancer (1, 2, 5, 8), and as we have shown, direct repression by pRB requires association with the promoter. In contrast, stimulation of SP1 activity by pRB does not involve tethering of pRB to the promoter. Instead, pRB appears to remove a negative regulator bound to SP1 (6).

A dual role for the pRB pocket. We have found that an intact pRB pocket is required for direct transcriptional repression. Many of the protein-protein interactions involving pRB, including its association with E2F, are abrogated by mutations in the pocket (11, 15, 37, 54–57, 66). Our finding is important because it suggests that the pocket performs a dual function: tethering of pRB to DNA through interaction with E2F, and direct transcriptional repression, probably by interaction with another protein(s). That the pocket is capable of multiple simultaneous interactions is supported by the observation that pRB can bind both E2F and the CR2 region of E1A at the same time (34). In view of these data, it may be more appropriate to think of the RB pocket as a combination of several minipockets, capable of contact with multiple proteins.

pRB contacts a region within the TAD of E2F (36, 60). The interaction blocks transcriptional activation by E2F, apparently by interfering with the ability of E2F to contact TBP (17, 22, 27, 53). Thus, pRB appears to repress E2F activity by specifically targeting its TAD and to repress the activity of other enhancers in the same promoter by a more general mechanism. This dual activity may extend to interaction of pRB with other transcription factors. For example, pRB also binds to the TADs of c-Myc and PU.1 in vitro (21, 57), although the effect

of this interaction on their activity in vivo is unclear. It has been demonstrated, however, that p107 binds the TAD of c-Myc in vivo, resulting in repression of transcription induced by c-Myc (19). The RB family may be part of a class of repressors which operate in this manner, since the interaction of E1B with p53 also occurs through the TAD of the latter (71).

Mechanism of repression by pRB. Our results are entirely consistent with the possibility that direct repression by pRB is mediated by protein-protein interactions. The property requires an intact pocket, which, as described above, is essential for the interaction of pRB with many proteins. Many of these interactions are abrogated by phosphorylation of pRB. Significantly, direct repression by pRB was weakened ninefold by cyclin-induced phosphorylation. In addition, the Δp34 pRB mutant, which is constitutively hypophosphorylated (25), was three- to fourfold more potent than wild-type pRB in repressing transcription. In fact, the degrees to which Δp34 was a better repressor than wild-type pRB of either an enhancerless promoter or an E2F-dependent promoter were the same. Deletion of the N-terminal portion of pRB also enhanced repression, which is consistent with the observation that removal of this region also gives rise to constitutively hypophosphorylated pRB (23, 54). Taken together, these observations support the proposal that direct repression of transcription by pRB (or p107) is mediated by specific protein-protein interactions which are tightly regulated by phosphorylation.

What protein-protein contacts might pRB require to repress transcription directly? Saha et al. have identified a class of repressors which contain a highly basic region and have proposed that they act by binding to and inhibiting activators (58). Given the large and growing number of activators with which pRB has been shown to bind, it is possible that pRB represses transcription in a similar manner. If so, then interactions between pRB and other factors which are not detectable in stringent immunoprecipitation assays may be relevant on promoters for which, because of local concentration and proximity effects, low-affinity interactions are favorable. pRB may also contact directly one or more of the basal transcription factors. The proteins NC1 and NC2 (49, 50), Dr1, and Dr2 (35, 51) all repress basal transcription by competing with TFIIA for association with TBP. It is also possible that there are corepressors which mediate contact between pRB and the basal transcription factors. As has been suggested for transcriptional activators, connections to the basal apparatus may occur through mediators, adaptors, or coactivators (42). In this regard, it is of note that the A and B domains of the pRB pocket share significant homology with TBP and TFIIB, respectively (21). Thus, it is conceivable that pRB competes for factors which bind TBP and TFIIB. An alternative view is that pRB represses transcription by modifying chromatin structure. Such a mechanism has been proposed for repression by the Polycomb group proteins in *Drosophila melanogaster* (reference 4 and references therein). Significantly, it has recently been shown that pRB binds BRG1, which is a member of a family of proteins involved in altering chromatin structure (12).

Regulation of transcriptional repression by specific cyclins. We carried out several experiments which demonstrate that phosphorylation negatively regulates direct repression of transcription by pRB. First, we showed that cyclin-mediated phosphorylation of GALmRB reduces the repressive potency of this molecule ninefold. On an enhancer-containing promoter, which was very responsive to changes in the amount of active GALmRB, this ninefold reduction in potency translated into a threefold increase in promoter activity. On an enhancerless promoter, which was less sensitive to changes in the amount of active GALmRB, promoter activity was increased 1.5- to 2-fold in response to cyclin-mediated phosphorylation of GALmRB. Second, we complemented these data by showing that two mutant versions of pRB which are constitutively hypophosphorylated (Δp34 and N-terminal deletion mutants) are more effective repressors than wild-type pRB. Finally, we showed that the enhanced repression by constitutively hypophosphorylated N-terminal pRB mutants is unaffected by cyclins.

The physiological relevance of these findings is supported in two ways. First, we found that the effect was specific; only cyclins A and E, not cyclin D1 or the B cyclins, were able to alleviate direct repression by pRB. Significantly, these results match the finding that cyclins A and E, but not cyclin D1 or the B cyclins, are effective at relieving growth suppression by pRB (30). Second, we showed that the magnitude of the effect of cyclins A and E on direct repression by GALmRB was similar to the effect of these cyclins on repression of E2F activity by pRB. Our results suggest that the abilities of cyclins A and E to reverse growth suppression by pRB correlate with their abilities to reverse transcriptional repression by pRB.

Cyclin D1 binds directly to pRB (11) and is capable of mediating phosphorylation of pRB by cdk4 in insect cells (15, 38). The inability of cyclin D1 to phosphorylate pRB in our assay, or in other assays (15, 30), may be a cell-specific effect relating, perhaps, to the availability of an appropriate kinase. However, it is also possible that cyclin D1 has a separate role in mammalian cells. In this regard, it has been shown that while cyclin D1 only partially reverses growth suppression of SAOS-2 cells by pRB, a mutant D1 which is unable to bind pRB alleviates growth suppression effectively (11). pRB may therefore regulate D1 rather than vice versa. We have also shown that cyclin D1 is unable to alleviate repression of E2F activity by pRB in C33A cells. This finding is consistent with the inability of cyclin D1 to reverse growth suppression of SAOS-2 cells by pRB (30). However, using extracts of SAOS-2 cells transfected with RB and cyclin D1 plasmids, Ewen et al. (15) have shown that cyclin D1 is able to dissociate the E2F-pRB complex. Further work is required to determine why dissociation of this complex in SAOS-2 cells is insufficient to alleviate growth suppression by pRB, what effect this dissociation has on promoters which bind E2F in SAOS-2 cells, and whether D1 can dissociate the complex in C33A cells in which it has no effect on E2F activity.

In conclusion, we have shown that p107 and pRB directly repress transcription independent of E2F. This activity likely involves protein-protein interactions, since direct repression by pRB is alleviated by phosphorylation and abrogated by mutation of the pocket domain. The next critical step will be to determine what protein-protein interactions mediate this effect.

ACKNOWLEDGMENTS

We thank J. Greenblatt for critical evaluation of the manuscript. We also thank M. Ewen, P. Hinds, and M. Ptashne for kindly providing plasmid constructs, Z. Jiang for construction of GALhRB and GAL107Δ133, L. Demirjian for construction of pLD85, and M. Ptashne and I. Sadowski for the anti-GAL4 antibody.

This research was supported by a Terry Fox Program Project Grant from the National Cancer Institute of Canada (NCIC) with funds from the Terry Fox Run. M.S. was supported by a fellowship from the NCIC with funds from the Canadian Cancer Society. B.L.G. is a research associate of the Ontario Cancer Treatment and Research Foundation.

REFERENCES

1. **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.
2. **Bandara, L. R., and N. B. La Thangue.** 1991. Adenovirus E1a prevents the

retinoblastoma gene product from complexing with a cellular transcription factor. Nature (London) **351:**494–497.
3. **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.
4. **Bunker, C. A., and R. E. Kingston.** 1994. Transcriptional repression by *Drosophila* and mammalian Polycomb group proteins in transfected mammalian cells. Mol. Cell. Biol. **14:**1721–1732.
5. **Chellappan, S. P., S. Hiebert, M. Mudryj, J. M. Horowitz, and J. R. Nevins.** 1991. The E2F transcription factor is a cellular target for the RB protein. Cell **65:**1053–1061.
6. **Chen, L. I., T. Nishinaka, K. Kwan, I. Kitabayashi, K. Yokoyama, Y.-H. F. Fu, S. Grunwald, and R. Chiu.** 1994. The retinoblastoma gene product RB stimulates Sp1-mediated transcription by liberating Sp1 from a negative regulator. Mol. Cell. Biol. **14:**4380–4389.
7. **Chen, P. L., P. Scully, J. Y. Shew, J. Y. Wang, and W.-H. Lee.** 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell **58:**1193–1198.
8. **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.
9. **DeCaprio, J. A., J. W. Ludlow, D. Lynch, Y. Furukawa, J. Griffin, H. Piwnica-Worms, C. M. Huang, and D. M. Livingston.** 1989. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell **58:**1085–1095.
10. **Defeo-Jones, D., P. S. Hunang, R. E. Jones, K. M. Haskell, G. A. Vuocolo, M. G. Hanobik, H. E. Huber, and A. Oliff.** 1991. Cloning of cDNAs for cellular proteins that bind to the retinoblastoma gene product. Nature (London) **352:**251–254.
11. **Dowdy, S. F., P. W. Hinds, K. Louie, S. I. Reed, A. Arnold, and R. A. Weinberg.** 1993. Physical interaction of the retinoblastoma protein with human D cyclins. Cell **73:**499–511.
12. **Dunaief, J. L., B. E. Strober, S. Guha, P. A. Khavari, K. Alin, J. Luban, M. Begemann, G. R. Crabtree, and S. P. Goff.** 1994. The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. Cell **79:**119–130.
13. **Dunn, J. M., R. A. Phillips, X. Zhu, A. J. Becker, and B. L. Gallie.** 1989. Mutations in the RB1 gene and their effects on transcription. Mol. Cell. Biol. **9:**4596–4604.
14. **Dyson, N., P. M. Howley, K. Münger, and E. Harlow.** 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243:**934–937.
15. **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.
16. **Ewen, M. E., Y. Xing, J. B. Lawrence, and D. M. Livingston.** 1991. Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. Cell **66:**1155–1164.
17. **Flemington, E. K., S. H. Speck, and W. Kaelin, Jr.** 1993. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc. Natl. Acad. Sci. USA **90:**6914–6918.
18. **Goodrich, D. W., N. P. Wang, Y. W. Qian, E. Y. H. P. Lee, and W. H. Lee.** 1991. The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. Cell **67:**293–302.
19. **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.
20. **Gu, W., J. W. Schneider, G. Condorelli, S. Kaushal, V. Mahdavi, and B. Nadal-Ginard.** 1993. Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell **72:**309–324.
21. **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.
22. **Hagemeier, C., A. Cook, and T. Kouzarides.** 1993. The retinoblastoma protein binds E2F residues required for activation *in vivo* and TBP binding *in vitro*. Nucleic Acids Res. **21:**4998–5004.
23. **Hamel, P. A., B. L. Cohen, L. M. Sorce, B. L. Gallie, and R. A. Phillips.** 1990. Hyperphosphorylation of the retinoblastoma gene product is determined by domains outside the simian virus 40 large-T-binding regions. Mol. Cell. Biol. **10:**6586–6595.
24. **Hamel, P. A., R. M. Gill, R. A. Phillips, and B. L. Gallie.** 1992. Regions controlling hyperphosphorylation and conformation of the retinoblastoma gene product are independent of domains required for transcriptional repression. Oncogene **7:**693–701.
25. **Hamel, P. A., R. M. Gill, R. A. Phillips, and B. L. Gallie.** 1992. Transcriptional repression of the E2-containing promoters EIIaE, c-*myc*, and *RB1* by the product of the *RB1* gene. Mol. Cell. Biol. **12:**3431–3438.
26. **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.
27. **Helin, K., E. Harlow, and A. Fattaey.** 1993. Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol. Cell. Biol. **13:**6501–6508.
28. **Helin, K., J. A. Lees, M. Vidal, N. Dyson, E. Harlow, and A. Fattaey.** 1992. A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell **70:**337–350.
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. **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.
31. **Horowitz, J. M., S.-H. Park, E. Bogenmann, J.-C. Cheng, D. W. Yandell, F. J. Kaye, J. D. Minna, T. P. Dryja, and R. A. Weinberg.** 1990. Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells. Proc. Natl. Acad. Sci. USA **87:**2775–2779.
32. **Hu, Q. J., N. Dyson, and E. Harlow.** 1990. The regions of the retinoblastoma protein needed for binding to adenovirus E1A or SV40 large T antigen are common sites for mutations. EMBO J. **9:**1147–1155.
33. **Huang, S., W.-H. Lee, and E. Y.-H. P. Lee.** 1991. A cellular protein that completes with SV40 T antigen for binding to the retinoblastoma gene product. Nature (London) **350:**160–162.
34. **Ikeda, M.-A., and J. R. Nevins.** 1993. Identification of distinct roles for separate E1A domains in disruption of E2F complexes. Mol. Cell. Biol. **13:**7029–7035.
35. **Inostroza, J. A., F. H. Mermelstein, I. Ha, W. S. Lane, and D. Reinberg.** 1992. Dr1, a TATA-binding protein-associated phosphoprotein and inhibitor of class II gene transcription. Cell **70:**477–489.
36. **Kaelin, W. G., Jr., W. Krek, W. R. Sellers, J. A. DeCaprio, R. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar, D. M. Livingston, and E. K. Flemington.** 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell **70:**351–364.
37. **Kaelin, W. G., Jr., 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.
38. **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.
39. **Keleher, C. A., M. J. Redd, M. C. Schultz, and A. D. Johnson.** 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. Cell **68:**709–719.
40. **Kim, S. J., U. S. Onwuta, Y. I. Lee, R. Li, M. R. Botchan, and P. D. Robbins.** 1992. The retinoblastoma gene product regulates Sp1-mediated transcription. Mol. Cell. Biol. **12:**2455–2463.
41. **Kim, S. J., S. Wagner, F. Liu, M. A. O'Reilly, P. D. Robbins, and M. R. Green.** 1992. Retinoblastoma gene product activates expression of the human *TGF-b2* gene through transcription factor ATF-2. Nature (London) **358:**331–334.
42. **Lewin, B.** 1990. Commitment and activation at Pol II promoters: a tail of protein-protein interactions. Cell **61:**1161–1164.
43. **Li, Y., C. Graham, S. Lacy, A. M. V. Duncan, and P. Whyte.** 1993. The adenovirus E1A-associated 130kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. Genes Dev. **7:**2366–2377.
44. **Liu, F., and M. R. Green.** 1990. A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1a protein. Cell **61:**1217–1224.
45. **Loeken, M. R., and J. Brady.** 1989. The adenovirus EIIA enhancer. J. Biol. Chem. **264:**6572–6579.
46. **Luckow, B., and G. Schutz.** 1987. CAT constructs with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nucleic Acids. Res. **15:**5490.
47. **Ludlow, J. W., J. A. DeCaprio, C.-M. Huang, W.-H. Lee, E. Paucha, and D. M. Livingston.** 1989. SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. Cell **56:**57–65.
48. **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.
49. **Meisterernst, M., and R. G. Roeder.** 1991. Family of proteins that interact with TFIID and regulate promoter activity. Cell **67:**557–567.
50. **Meisterernst, M., A. L. Roy, H. M. Lieu, and R. G. Roeder.** 1991. Activation of class II gene transcription by regulatory factors is potentiated by a novel activity. Cell **66:**981–993.
51. **Merino, A., K. R. Madden, W. S. Lane, J. J. Champoux, and D. Reinberg.** 1993. DNA topoisomerase I is involved in both repression and activation of transcription. Nature (London) **365:**227–232.
52. **Mittnacht, S., and R. A. Weinberg.** 1991. G1/S phosphorylation of the retinoblastoma protein is associated with an altered affinity for the nuclear compartment. Cell **65:**381–393.

53. **Pearson, A., and J. Greenblatt.** 1994. Personal communication.
54. **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.
55. **Qian, Y.-W., Y.-C. J. Wang, R. E. Hollingsworth, Jr., D. Jones, N. Ling, and E. Y.-H. P. Lee.** 1993. A retinoblastoma-binding protein related to a negative regulator of Ras in yeast. Nature (London) **364:**648–652.
56. **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.
57. **Rustgi, A. K., N. Dyson, and R. Bernards.** 1991. Amino-terminal domains of c-*myc* and N-*myc* proteins mediate binding to the retinoblastoma gene product. Nature (London) **352:**541–544.
58. **Saha, S., J. M. Brickman, N. Lehming, and M. Ptashne.** 1993. New eukaryotic transcriptional repressors. Nature (London) **363:**648–652.
59. **Scheffner, M., K. Munger, J. C. Byrne, and P. M. Howley.** 1991. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc. Natl. Acad. Sci. USA **88:**5523–5527.
60. **Shan, B., X. Zhu, P. L. Chen, T. Durfee, Y. Yang, D. Sharp, and W. H. Lee.** 1992. Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F. Mol. Cell. Biol. **12:**5620–5631.
61. **Sleigh, M. J.** 1986. A nonchromatographic assay for expression of the chloramphenicol acetyltransferase gene in eukaryotic cells. Anal. Biochem. **156:** 251–256.
62. **Templeton, D. J.** 1992. Nuclear binding of purified retinoblastoma gene product is determined by cell cycle-regulated phosphorylation. Mol. Cell. Biol. **12:**435–443.
63. **Templeton, D. J., S. H. Park, L. Lanier, and R. A. Weinberg.** 1991. Nonfunctional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering. Proc. Natl. Acad. Sci. USA **88:**3033–3037.
64. **Tjian, R., and T. Maniatis.** 1994. Transcriptional activation: a complex puzzle with few easy pieces. Cell **77:**5–8.
65. **Udvadia, A. J., K. T. Rogers, P. Higgins, Y. Murata, K. H. Martin, P. A. Humphrey, and J. M. Horowitz.** 1993. Sp-1 binds promoter elements regulated by the RB protein and Sp-1-mediated transcription is stimulated by RB coexpression. Proc. Natl. Acad. Sci. USA **90:**3265–3269.
66. **Wang, C. Y., B. Petryniak, C. B. Thompson, W. G. Kaelin, and J. M. Leiden.** 1993. Regulation of the Ets-related transcription factor Elf-1 by binding to the retinoblastoma protein. Science **260:**1330–1335.
67. **Wang, H., and D. J. Stillman.** 1993. Transcriptional repression in *Saccharomyces cerevisiae* by a SIN3-LexA fusion protein. Mol. Cell. Biol. **13:**1805–1814.
68. **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.
69. **Welch, P. J., and J. Y. J. Wang.** 1993. A c-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-abl tyrosine kinase in the cell cycle. Cell **75:**779–790.
70. **Wildeman, A. G.** 1989. Transactivation of both early and late simian virus 40 promoters by large tumor antigen does not require nuclear localization of the protein. Proc. Natl. Acad. Sci. USA **86:**2123–2127.
71. **Yew, P. R., X. Lui, and A. J. Berk.** 1994. Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. Genes Dev. **8:**190–202.
72. **Zacksenhaus, E., R. Bremner, Z. Jiang, R. M. Gill, M. Muncaster, M. Sopta, R. A. Phillips, and B. L. Gallie.** 1993. Unraveling the function of the retinoblastoma gene. Adv. Cancer Res. **61:**115–141.
73. **Zamanian, M., and N. B. La Thangue.** 1992. Adenovirus E1a prevents the retinoblastoma gene product from repressing the activity of a cellular transcription factor. EMBO J. **11:**2603–2610.
74. **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.