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# Domains A and B in the Rb Pocket Interact To Form a Transcriptional Repressor Motif

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**The retinoblastoma protein (Rb) is a tumor suppressor that regulates progression from the $G_1$ phase to the S phase of the cell cycle. Previously, we found that Rb is a transcriptional repressor that is selectively targeted to promoters through an interaction with the E2F family of cell cycle transcription factors—when Rb is tethered to a promoter through E2F, it not only blocks E2F activity, it also binds surrounding transcription factors, preventing their interaction with the basal transcription complex, thus resulting in a dominant inhibitory effect on transcription of cell cycle genes. Here we examine the repressor motif of Rb. The two domains in the Rb pocket, A and B, which are conserved across species and in the Rb-related proteins p107 and p130, are both required for repressor activity. The nonconserved spacer separating A and B is not required. Although neither A nor B alone had any repressor activity, surprisingly, repressor activity was observed when the domains were coexpressed on separate proteins. Transfection assays suggest that one domain can recruit the other to the promoter to form a repressor motif that can both interact with E2F and have a dominant inhibitory effect on transcription. Using coimmunoprecipitation and in vitro binding assays, we show that A and B interact directly and that mutations which disrupt this interaction inhibit repressor activity. The Rb pocket was originally defined as the binding site for oncoproteins from DNA tumor viruses such as adenovirus E1a. We present evidence that E1a interacts with a site formed by the interaction of A and B and that this interaction with A and B induces or stabilizes the A-B interaction.**

The loss of cell growth control is a hallmark of tumors. Progression of cells from one phase of the cell cycle to the next is regulated by transformation-sensitive checkpoint genes (40). Recessive mutations in these genes have been linked to hereditary susceptibility to cancer (30, 35). The retinoblastoma susceptibility protein (Rb) regulates progression of cells past the restriction point in $G_1$ (49). The restriction point is a key regulatory checkpoint in the cell cycle—once cells pass this point, they are committed to progression into S phase (and in most cases to completion of the remainder of the cell cycle). As its name implies, Rb is absent or mutated in all retinoblastomas (14, 15, 32). In addition to retinoblastomas, mutation of Rb is also associated with several other tumors including bladder, bone, breast, prostate, cervical, and small cell lung cancers (16).

Rb shares a central motif known as the pocket with two other related proteins, p107 and p130, which like Rb also appear to regulate cell cycle progression; however, they have not yet been shown to be mutated in tumors (10, 12, 19, 33, 37). The pocket is composed of two domains, A and B, that are conserved both across species and among the related proteins. These domains are separated by a spacer region that varies in length and is not conserved. The pocket was originally identified as the minimal region in Rb sufficient for binding to oncoproteins from DNA tumor viruses such as adenovirus E1a, simian virus 40 large T antigen, and human papillomavirus E7 (23–26). The interaction of these oncoproteins with Rb disrupts the binding of Rb to the E2F family of transcription factors (referred to collectively here as E2F) (2)—binding sites for E2F are found in a number of cell cycle genes (41). Although Rb has been shown to bind to a number of other transcription factors, its interaction with E2F appears critical for function (45). We have found that Rb can be targeted to promoters though an interaction with E2F, but not through interaction with a number of other Rb-binding transcription factors (50), and studies aimed at identifying DNA sequences that are capable of associating with Rb identified E2F sites but not sites for other Rb-binding transcription factors (7, 42).

The interaction of Rb with DNA tumor virus proteins and E2F has been studied in some detail. The Rb pocket region alone is sufficient for a high-affinity interaction with the viral oncoproteins (23, 25, 26). However, the region C terminal of the pocket, in addition to the pocket, is required for high-affinity interaction with E2F (20, 43).

The binding of Rb to E2F is normally controlled during the cell cycle by alternating phosphorylation and dephosphorylation of Rb—the hypophosphorylated form binds E2F, whereas the hyperphosphorylated form does not (8, 11, 34, 38). This phosphorylation is catalyzed by $G_1$ cyclin-dependent kinases (cdks), whose activity is regulated by cytoplasmic signals that control expression of the regulatory cyclin subunits as well as specific inhibitors of the cdks (48). Additionally, the activity of at least some of these cdks is dependent upon an activating kinase and an assembly factor that facilitates formation of the activating kinase complex; however, neither the activating kinase nor the assembly factor has yet been shown to be subject to regulation (13). It is now clear that mutations in this rather elaborate pathway leading to Rb phosphorylation can result in constitutive hyperphosphorylation and thus inactivation of Rb. These results demonstrate that mutation of the Rb gene is not the only way to block the Rb pathway, and they imply that loss of the $G_1$/S checkpoint imposed by Rb may be more frequent in tumors than predicted by initial surveys of tumors looking only for mutations in the Rb gene.

Interaction of Rb inhibits transactivation by E2F, thereby blocking the transcription of genes that are dependent upon E2F sites for activation (31, 41). However, many Rb-regulated

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genes contain enhancers in addition to E2F sites, and it has been found that not only does interaction of Rb with E2F inactivate E2F, but Rb also has dominant inhibitory effect on the transcription of genes while it is tethered to a promoter through E2F (1, 5, 18, 47, 50, 52). We have examined the mechanism through which Rb represses transcription, and the results support the following model of activity (50). Once Rb is concentrated at a promoter through an interaction with E2F and its DNA-binding partner DP-1, it can interact simultaneously with surrounding transcription factors on the promoter, blocking their interaction with the basal transcription complex. On the basis of this model, Rb-binding transcription factors fall into two categories: those such as E2F and E1a that bind Rb with high affinity and can target Rb to a promoter and others such as PU.1, Elf-1, and c-myc, which bind with lower affinity and interact with Rb only after it is concentrated at a promoter through E2F (50). We found that other transcription factors that do not interact with Rb are unaffected even when Rb is tethered to a promoter through E2F.

A series of recent experiments emphasize the importance of targeting the repressor domain of Rb to promoters through an interaction with E2F (45, 47). When the repressor domain of Rb was fused directly to the DNA binding domain of E2F-1, the fusion protein efficiently blocked cell growth. However, when the E2F-1 DNA binding domain of the fusion protein was mutated, growth suppression was prevented. Therefore, targeting the repressor domain of Rb to genes with E2F sites is sufficient to suppress cell growth. When the activation and Rb-binding domain of E2F-1 was replaced by the activation domain of VP16, rendering the fusion protein and thus E2F site-containing genes resistant to Rb, there was no growth suppression by Rb. Additionally, it has been found that expression of a truncated E2F protein, in which the transactivating domain and the binding site for Rb are deleted, is sufficient to overcome the $G_1$/S block and transform cells (29). Presumably, this truncated form of E2F binds to E2F sites and, in a dominant negative fashion, inactivates the E2F site (no transactivation and no Rb binding). These results suggest that E2F-mediated tethering of the Rb repressor motif to cell cycle genes is important for the $G_1$/S block, and they imply that the release of the repressor motif of Rb from genes in mid to late $G_1$ as a result of hyperphosphorylation may be sufficient to overcome the $G_1$/S block and transform cells.

Here, we characterize the repressor domain of Rb. We show that two conserved domains in the Rb pocket are sufficient for repressor activity and that the interaction between these domains forms a motif that can bind E1a and E2F and act as a general transcriptional repressor.

## MATERIALS AND METHODS

**Transfection assays.** DNA was transfected into C33A cells by the calcium phosphate method, and chloramphenicol acetyltransferase activity was determined as described elsewhere (52). TKluc (0.5 μg) was cotransfected, and luciferase assays were done to correct for transfection efficiencies as described elsewhere (50). Results from transfections are all representative of at least five separate experiments, each in duplicate.

**Plasmids.** The Gal4 expression vectors pM1 to-3 have been described elsewhere (46). G-379-602 was constructed by deleting the two *Pst*I fragments in G-379-792 (50). G-620-792 was constructed by cloning the *Mlu*I fragment from the Rb cDNA into the *Mlu*I site in pM3. The Rb sequence in G-646-792 was obtained by PCR of Rb cDNA and cloned into the *Hin*dIII site of pM2. The 5′ oligonucleotide for amplification was 5′-ACGAATTCGAAGCT**TCTCTTTCAC TGTTTTAT**-3′, and the 3′ oligonucleotide was 5′-CCGAATTCAAGCTTA**AAA CTTGTAAGGGCTTCG**-3′ (boldfaced nucleotides are derived from the Rb cDNA; other nucleotides were added for cloning purposes). *Hin*dIII sites are underlined. G-646-792(706) was constructed in the same fashion, but G-300-928(706) (50) was used as the template; the 706 mutation was confirmed by sequencing. G-379-928 was made by cloning the *Bam*HI fragment from pGT-Rb(379-928) (27) into the *Bam*HI site of pM2. G-379-612 was constructed by cloning the *Bam*HI-*Bgl*II fragment from the Rb cDNA in pGT-Rb(379-792) (27) into the *Bam*HI site of pM2. G-379-496 was constructed by cloning the *Eco*RI-*Hae*III fragment from G-379-792 into the *Eco*RI and *Sma*I sites of pM2. G-688-792 was created by cloning the *Pst*I fragment from amino acids (aa) 688 to 792 in G-379-792 into the corresponding sites of pM3. G-612-792 was constructed by cloning the *Bgl*II-to-*Bam*HI fragment from pGT-Rb(379-792) into the *Bam*HI site of pM2. To make G-379-572/646-792, a *Bam*HI fragment of Rb from aa 379 to 572 was obtained by PCR (the 5′ primer was 5′-ACGAATTCGGAATCCG CCACCATGAACACTATCCAACAA-3′, and the 3′ primer was 5′-AGTGGA AATAAACTAGAACCTAGGATTCTTAAGCC-3′ [*Bam*HI sites are underlined]). The PCR fragment was then cloned into the corresponding site of G-646-792 and sequenced. The LexA expression vector pBXL1 has been described elsewhere (36). L-379-792 was constructed by cloning the *Eco*RI-to-*Xba*I fragment of G-379-792 into the *Sma*I site of pBXL1 by blunt-end ligation. L-379-602 was constructed by cloning the *Eco*RI-to-*Xba*I fragment of G-379-602 into the *Sma*I site of pBXL1 by blunt-end ligation. L-620-792 was constructed by cloning the *Sma*I-to-*Bam*HI fragment of G-379-602 into the *Sma*I site of pBXL1 by blunt-end ligation. pSVEC-GL is identical to pSVEC-G (50) except for the insertion of six LexA sites from $pL_6EC$ (36) into the *Xho*I site 200 bp upstream of the Gal4 sites. Orientation of inserts was determined by restriction enzyme digestion or sequencing. pGST-A was constructed by cloning the *Bam*HI-to-*Bgl*II fragment of pGT-Rb(379-792) (aa 379 to 612 of Rb) into the *Bam*HI site of pGEX2T (Pharmacia). pGST-B was constructed by cloning the *Bgl*II-to-*Bam*HI fragment of pGT-Rb(379-792) (aa 612 to 792 of Rb) into the *Bam*HI site of pGEX2T (Pharmacia). GST-612-792(706) was made by PCR. Rb aa 573 to 792 were amplified by using pM2Rb(706) (50) as a template and the following primers: 5′-TGGAATTCGTCGACATTAAACAATCAAAGGA-3′ (5′ primer) and 5′-CCGAATTCAAGCTTAAAACTTGTAAGGGCTTCG-3′ (3′ primer). The PCR product was digested with *Bgl*II and *Eco*RI (underlined) and cloned into the *Bam*HI and *Eco*RI sites of pGEX2T. G-620-928 was made by cloning a *Mlu*I fragment from G-379-928 (*Mlu*I sites are found at aa 620 of Rb and downstream of the Rb sequence in the polylinker of pM2) in frame into the *Mlu*I site of pM3. G-1-621 was made by removing the *Mlu*I fragment from G-1-928 (50) and religating. L-620-928 was made by cloning the *Sma*I-*Xba*I fragment from G-620/928 into the corresponding sites of pBXL1. L-1-621 was made by cloning the *Bam*HI-*Xba*I fragment from G-1-621 into pBXL1. All constructs made by PCR or involving blunt-end ligations were sequenced.

**Coimmunoprecipitation assays.** Five micrograms of each expression vector was cotransfected into C33A cells. After 36 h, cells were suspended in lysis buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.0], 1 M NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM EDTA, and 0.01% phenylmethylsulfonyl fluoride) and subjected to mild sonication. The cleared lysate was immunoprecipitated with monoclonal anti-Gal4 (Santa Cruz) (used at 1.5 μg/ml) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% polyacrylamide gel. Proteins were Western blotted (immunoblotted) with polyclonal anti-LexA (R. Brent) or monoclonal anti-E1a (Oncogene Science).

**In vitro binding assays.** GST-Rb fusion proteins were purified from bacteria and used in in vitro binding assays as described elsewhere (50). GST-Rb-A was digested to completion with 1 NIH unit of thrombin for 2 h, and glutathione *S*-transferase (GST) was removed with glutathione beads. Ten micrograms of Rb-A was incubated with 2 μg of GST-Rb-B, GST-Rb-B(706), or GST on glutathione beads as described elsewhere (50). Beads were washed extensively and then subjected to SDS-PAGE. The gel was then Western blotted with a domain A-specific antibody (14441A; Pharmagin).

## RESULTS

**Domains A and B in the Rb pocket are sufficient for general repressor activity.** The dominant inhibitory transcriptional activity of Rb is evident when it is fused to the DNA binding domain of the yeast transcription factor Gal4 and targeted directly to promoters containing Gal4 binding sites (Fig. 1) (1, 5, 50). To identify sequences required for repressor activity, mutations were created in the Rb sequence of Gal4-Rb fusion proteins, and the resulting expression vectors were cotransfected into the Rb-negative C33A cell line [similar results were obtained with other Rb-negative and Rb-positive cell lines (results not shown)] with the pSVEC-G reporter, which contains Gal4 sites upstream of the simian virus 40 72-bp repeats (enhancer) and E1b TATA box driving the CAT gene (Fig. 1B). As shown previously, the Rb pocket (aa 379 to 792) was sufficient for full repressor activity (50) (Fig. 1B). Mutations in either domain A or B of the pocket, which are conserved across species and in other pocket proteins (p107 and p130) (10, 19, 33, 54), blocked repressor activity; however, deletion of the nonconserved spacer separating A and B had no effect (Fig.

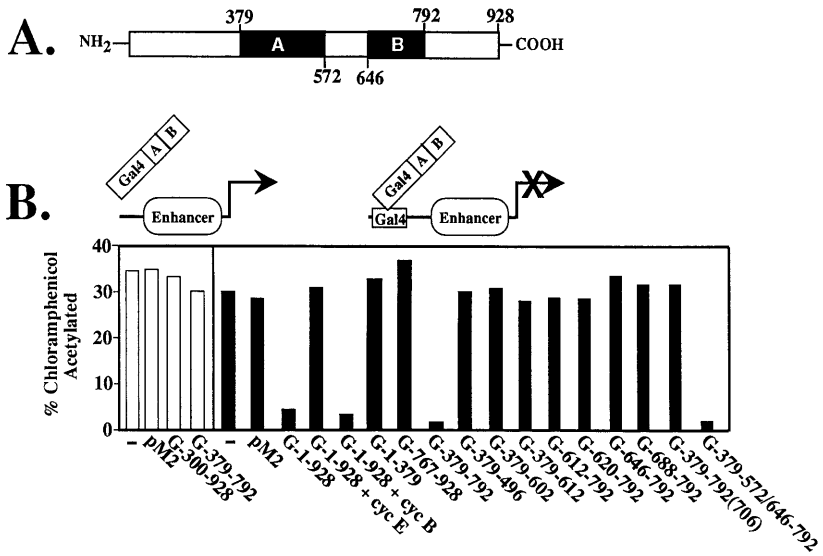


FIG. 1. Domains A and B of the Rb pocket act as a transcriptional repressor when coexpressed on separate proteins. (A) Diagram of Rb. (B) Gal4-Rb constructs were cotransfected with pSVEC-G (shown at the top) (filled bars) or the control pSVEC (parent plasmid lacking Gal4 sites) (open bars) into C33A cells. Numbers in the designations refer to amino acids in Rb. 706, a Cys-to-Phe mutation at aa 706 that blocks Rb repressor activity (28); pM2, parent Gal4 DNA binding domain expression vector; cyc E and cyc B, cyclin E and B expression vectors (21). Expression of cyc E and cyc B in transfected C33A cells is shown in reference 9. pSVEC-G or pSVEC (0.25 μg) was cotransfected with 0.5 μg of the indicated expression vector as described in Materials and Methods. A Western blot of Rb fusion proteins is shown in Fig. 2. Results are an average of duplicate assays and are representative of more than three separate experiments.

1B). We conclude that A and B are necessary and sufficient for repressor activity.

It has been demonstrated previously that overexpression of $G_1$ cyclins such as cyclin E leads to hyperphosphorylation and inactivation of Rb (1, 21, 50). Overexpression of cyclin E (but not the control cyclin B) blocked repressor activity (Fig. 1B) (50). Likewise, a Cys-to-Phe mutation at aa 706 in domain B of the Rb pocket, which is known to block Rb function (28), inhibited repressor activity.

Expression vectors for fusion proteins were transfected into C33A cells, and cell extracts were used in Western blots. Fusion proteins were of the predicted size, were expressed at similar levels, and showed similar nuclear localization by immunofluorescence (Fig. 2 and results not shown).

**A and B repress transcription when coexpressed as separate proteins.** Although neither A nor B alone had repressor activity, surprisingly, full activity was observed when the two domains were coexpressed as separate Gal4 proteins (Fig. 3). This occurred with several different A and B constructs, and it was also true when the N-terminal region of Rb was included with A and the C-terminal region was included with B. Gal4 has been shown to dimerize, and the pSVEC-G reporter used in these assays contains five adjacent Gal4 sites (similar results were also obtained with two Gal4 sites). Thus, it was possible that A and B were being forced together (in an active conformation) in these assays by Gal4 dimerization and/or the binding of fusion proteins to adjacent sites on the reporter. We then expressed one of the domains as a Gal4 protein and the other as a fusion protein with the DNA binding domain of the bacterial protein LexA. Additionally, a new reporter construct (pSVEC-GL) in which LexA and Gal4 sites are separated by 200 bp was used. As with the Gal4 proteins, coexpression of LexA-domain A and Gal4-domain B (and vice versa) resulted in full repressor activity (results not shown). Just as with the intact pocket, mutations in A or B blocked repressor activity, and the combination of controls such as LexA-domain A and Gal4-domain A, or LexA-domain B and Gal4-domain B, had no repressor activity.

To determine whether overexpression of $G_1$ cyclins would also block repressor activity when A and B were coexpressed on separate proteins, expression vectors for cyclin E or the control cyclin B were cotransfected with constructs expressing A plus the N-terminal region of Rb and B plus the C-terminal region of Rb. The N- and C-terminal regions were included in the constructs because they are thought to be important for efficient phosphorylation of Rb in vivo (21, 43). As with intact Rb, coexpression of the $G_1$ cyclins blocked repressor activity when A and B were on separate proteins, whereas the control cyclin B had no effect (Fig. 3). As a control, overexpression of $G_1$ cyclins had no effect on A and B alone (which lack the N-

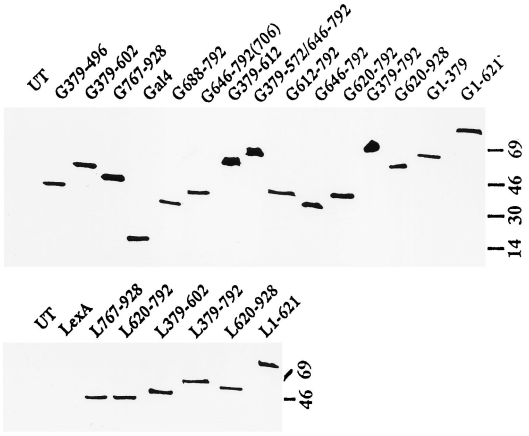


FIG. 2. Expression of Rb fusion proteins. Expression vectors for Rb fusion proteins were transfected into C33A cells as described in Materials and Methods. Five micrograms of each construct was transiently transfected into C33A cells. Thirty-six hours after transfection, cell lysates were subjected to Western blot analysis with anti-Gal4 and -LexA antibodies. The proteins were visualized by enhanced chemiluminescence as described elsewhere (50). G, DNA binding domain of Gal4; L, DNA binding domain of LexA. Size markers in kilodaltons are indicated on the right. UT, untransfected cells.

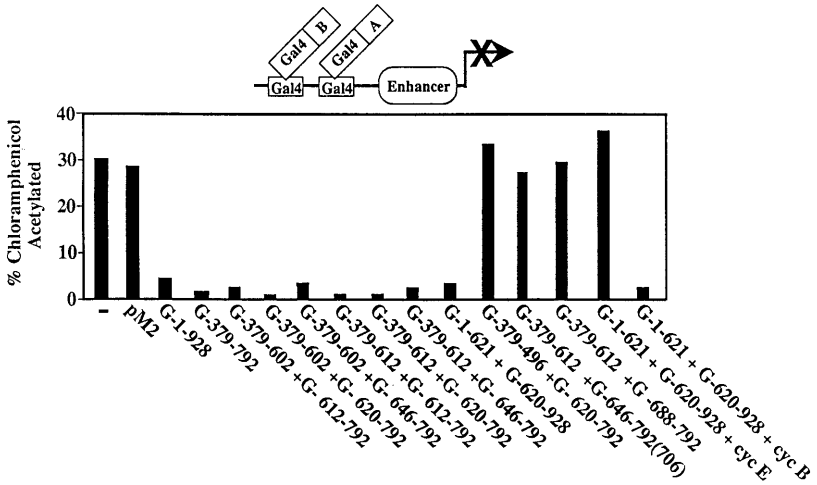


FIG. 3. A and B repress transcription when coexpressed as separate Gal4 proteins. pSVEC and pSVEC-G were cotransfected into C33A cells with the indicated expression vectors as in Fig. 1B. Numbers in the designations refer to amino acids in Rb. A Western blot of Rb fusion proteins is shown in Fig. 2.

and C-terminal regions that contain the inactivating phosphorylation sites) (results not shown).

Taken together, the above results suggest that either A and B have distinct activities (neither one of which alone is sufficient for repression) or the two domains interact to form a repressor motif.

**One pocket domain can recruit the other to the promoter to form an active repressor motif.** A and B were again coexpressed on separate proteins; however, a binding site for only one of the domains was provided to determine whether an A-B interaction could recruit the other domain to form a repressor at the promoter (Fig. 4). When a binding site was provided for only one of the domains, full repressor activity was still observed, suggesting that one domain can indeed recruit the other to the promoter to form a repressor motif. Again, mutations in A or B blocked repressor activity, and the combination of controls such as LexA-domain A and Gal4-domain A, or LexA-domain B and Gal4-domain B, showed no repressor activity.

**A and B can interact in vivo.** The above results suggested that A and B might interact to form a repressor motif. Therefore, coimmunoprecipitation assays were used to determine whether A and B can interact in vivo—under the same transfection conditions as described above. Again, expression vectors for Gal4 and LexA fusion proteins with A and B were cotransfected into C33A cells. Cell lysates were divided into three parts: 10% was used for a direct Western blot for LexA fusion proteins, another 10% was used for a direct Western blot for Gal4 fusion proteins, and the final 80% was immunoprecipitated with a monoclonal anti-Gal4 antibody (Fig. 5). The immunoprecipitated proteins were then Western blotted with polyclonal anti-LexA antisera to detect an interaction between A and B. Mutations that blocked transcriptional repressor activity when A and B were coexpressed on separate

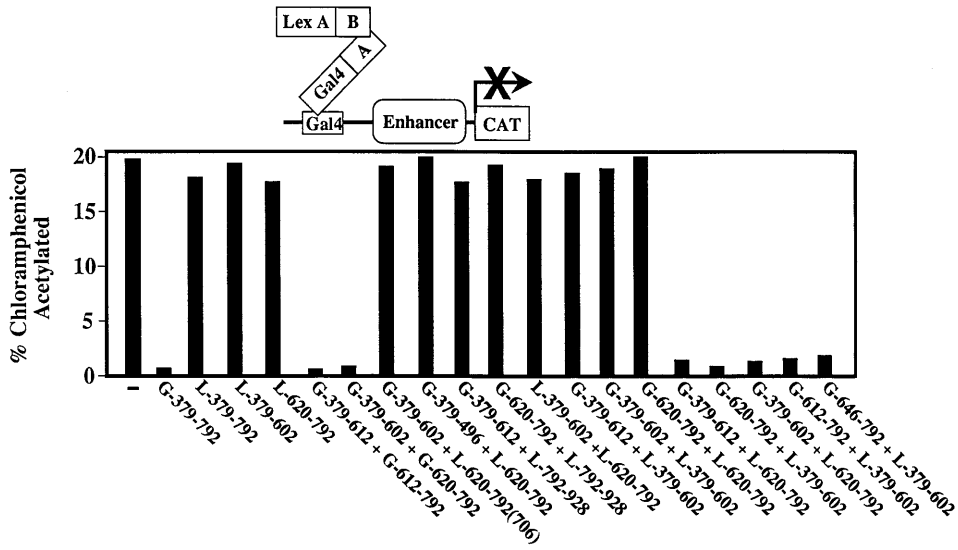


FIG. 4. A and B repress transcription when the binding site for only one of the domains is provided. Gal4- and LexA-Rb constructs were cotransfected into C33A cells as in Fig. 1B along with pSVEC-G.

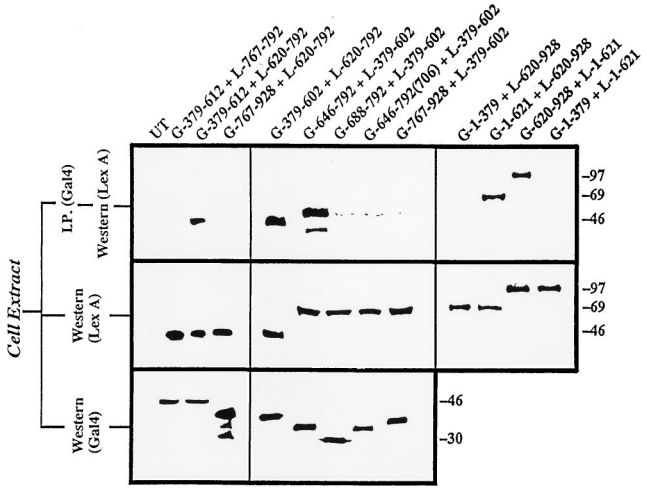


FIG. 5. A and B interact in vivo. C33A cells were transfected with 5 μg of the indicated A and B constructs, and 36 h after transfection a protein extract was made from the cells and divided into three parts. Ten percent of the extract was used for each direct Western blot (with anti-Gal4 or anti-LexA), and the remaining 80% was immunoprecipitated (I.P.) with anti-Gal4 antibody. Immunoprecipitated proteins were then subjected to a Western blot with anti-LexA antibody to detect A-B interactions. UT, untransfected cells. Size standards on the right are in kilodaltons.

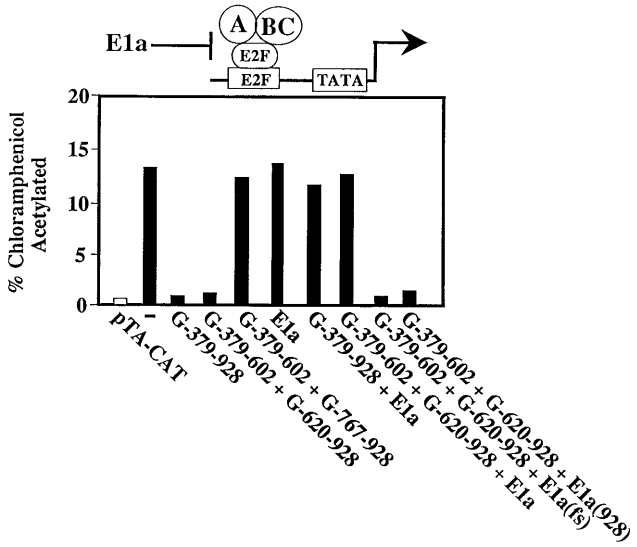


FIG. 7. The A-B interaction forms a motif that blocks E2F activity in an E1a-sensitive fashion. A minimal reporter dependent upon E2F sites for activity, pTA-E2F-CAT (52), was cotransfected with the indicated expression vectors. A, domain A; BC, domain B plus the C-terminal region of Rb (aa 646 to 928). In these experiments, the C-terminal region was included along with domain B because it is required along with the pocket for efficient E2F binding (20, 43). E1a is an aa 2 to 36 deletion of the 243-aa form of E1a, and E1a(fs) and E1a(928) are a frameshift and a point mutation in E1a at nucleotide 928, respectively, that inhibit Rb binding (39). pTA-CAT is a control reporter lacking E2F sites (51). In these experiments, 0.25 μg of pSVEC-GL and pSVEC-G or 1 μg of pTA-E2F-CAT and pTA-CAT was cotransfected with 0.5 μg of the indicated expression vectors.

proteins also inhibited the A-B interaction. The results demonstrate that A and B associate efficiently and specifically and that mutations which block repressor activity (Fig. 1B) also inhibit the association between A and B.

**A and B interact directly in in vitro binding assays.** It was unclear from the in vivo binding assays whether A and B can bind directly or whether a third bridging protein might be required to mediate their interaction. Therefore, in vitro binding assays were used to determine whether A and B can interact directly. A and B were made as GST fusion proteins in *Escherichia coli*. A bound specifically to B, and a Cys-to-Phe mutation at aa 706 blocked this binding (Fig. 6). We conclude that A and B can bind directly.

**The interaction between A and B also mediates formation of an E2F binding site.** Rb is classically targeted to promoters through an interaction with E2F, and this interaction is blocked by DNA tumor virus proteins such as adenovirus E1a that bind the pocket (2, 3, 6). The region of Rb that interacts with E2F to target Rb to a promoter is distinct from A and B in that the C-terminal region of Rb in addition to A and B is required for efficient binding and inactivation of E2F (20, 43, 44). This region is referred to collectively as the large pocket. Coexpression of separate A and B plus C-terminal region constructs inhibited transactivation by E2F as efficiently as the intact large pocket, and E1a blocked this inhibition (Fig. 7). For these experiments, the 243-aa form of E1a with an aa 2 to 36 deletion (which blocks binding of p300 and transcriptional repressor activity) was used (39). This form of E1a contains both conserved regions I and II, which are sufficient for Rb binding. As a control, a mutation in E1a at nucleotide 928 in conserved region II (39), which selectively inhibits interaction with Rb, prevented E1a from blocking. Coexpression of A and B alone or expression of the intact pocket alone had no effect on E2F activity (results not shown), demonstrating the requirement for the C-terminal region of Rb for efficient interaction with E2F. We conclude that the A-B interaction also mediates formation of the large pocket of Rb which binds to and inactivates E2F, and as with the intact large pocket, the E2F binding activity appears to be blocked by E1a.

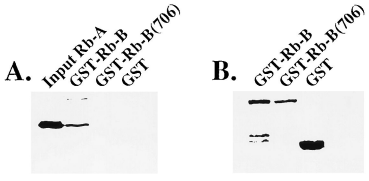


FIG. 6. A and B interact in vitro. (A) Domains A and B were expressed as GST proteins in bacteria. GST was cleaved from A, and the purified domain A was then used to bind GST-Rb-B, GST-Rb-B(706), or GST bound to glutathione beads. Binding of domain A to the GST proteins was followed by Western blot with a domain A-specific antibody. (B) A Coomassie blue-stained gel showing the input GST proteins.

**Interaction between A and B forms an E1a binding site.** The pocket was originally defined as the binding site for viral oncoproteins such as adenovirus E1a (23–26). The above experiments suggested that the A-B interaction forms an E1a binding site. To test this possibility, expression vectors for Gal4 and LexA domain A and B fusion proteins were cotransfected into C33A cells along with expression vectors for E1a and E1a mutants (39). Ten percent of the extract from the transfected cells was used for a direct Western blot for E1a (Fig. 8). The remaining extract was immunoprecipitated with anti-Gal4 antibody and Western blotted with anti-E1a antibody. The blot was then stripped and reprobed with anti-LexA antibody. The results show that, although E1a does not interact with A or B alone, it does bind to the A-B complex. In fact the interaction with E1a facilitates formation of the A-B complex. As a control, the E1a mutation at nucleotide 928, which selectively inhibits Rb binding, prevented the association of E1a with A plus B as well as its facilitation of the A-B interaction. Taken

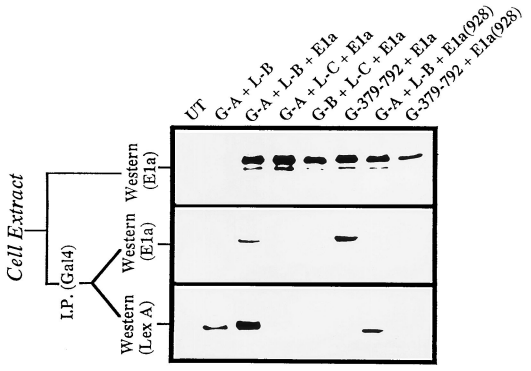


FIG. 8. A and B interact in vivo to form an E1a binding pocket. The indicated constructs were transfected into C33A cells as in Fig. 5, and Gal4- and LexA-tagged proteins were immunoprecipitated (I.P.) and Western blotted as indicated. The anti-E1a monoclonal antibody was from Oncogene Science. L-A, L-379-602; G-A, G-379-602; G-B, G-620-792; L-C, is L-767-928. UT, untransfected cells.

together, our results indicate that the interaction between A and B forms an E1a binding site, and they suggest that E1a binds to a motif composed of both domains, thereby stabilizing the A-B interaction.

## DISCUSSION

Here, we show that the pocket motif of Rb is sufficient for transcriptional repressor activity. Within the pocket are two domains known as A and B (26). These domains are conserved both across species and in the pocket domains of the Rb-related proteins p107 and p130 (10, 12, 19, 33, 37). Within the pocket, A and B are separated by a nonconserved spacer sequence. Our results indicate that both A and B are essential for repressor activity; however, most of the spacer sequence is dispensable. Unexpectedly, we also found that A and B are fully functional when they are coexpressed as separate proteins, and we present evidence that these two domains interact to form the repressor motif.

Is there a relationship between the structure of the repressor motif and the mechanism through which it represses transcription? Previously, we found that when Rb is tethered to a promoter, it inhibits transcription by binding to surrounding transcription factors and preventing their interaction with the basal transcription complex (50). Transcription factors are thought to function at least in part through interaction with components of the basal transcription complex, which serves to recruit these components to the promoter and/or to stabilize the basal transcription complex. Therefore, Rb seems to mimic the basal transcription complex by recruiting transcription factors into inactive complexes. It is thus interesting that there is some sequence similarity between domain A and the TATA-binding protein (TBP) and between domain B and TFIIB (17). This sequence similarity could identify regions of A and B that mimic transcription factor binding sites on TBP and TFIIB. Neither A nor B alone showed any repressor activity. However, it is possible that only after the A-B interaction forms an active pocket can these Rb sequences participate in transcription factor binding. The binding of TBP (and TBP-associated factors) to the promoter is thought to be the first step in the formation of the basal transcription complex, and the next step involves the binding of TFIIB to TBP (53). Therefore, this sequence similarity between A and TBP and between B and TFIIB may correspond to conserved sequences that mediate the interaction between A-B and TBP-TFIIB. Indeed, the sequences in A and B that show similarity to TBP and TFIIB do correspond to regions of TBP and TFIIB that interact (4, 22).

The A-B interaction forms a repressor motif that can bind and inactivate transcription factors such as PU.1, Elf-1, and c-myc when this motif is tethered to a promoter (this study) (50). However, this motif alone does not interact efficiently with E2F, and it is not sufficient for cell growth suppression (at least in part because it cannot be targeted to promoters through an interaction with E2F). It is clear that E2F and its binding partner DP-1 interact with a site on Rb different from other transcription factors that bind the pocket repressor motif (i.e., PU.1, Elf-1, and c-myc) (50). Such distinct binding sites appear critical for Rb function, allowing Rb to be tethered to promoters through E2F while simultaneously interacting through the repressor motif with surrounding transcription factors (50). We then conclude that the interaction between A and B results in formation of at least two distinct sites for protein binding in Rb: a binding site for E2F, requiring the C-terminal region (critical for targeting Rb to promoters), and a site(s) entirely within the pocket repressor motif that binds other transcription factors after Rb is concentrated at a promoter through interaction with E2F (dominant inhibitory activity).

The pocket domain of Rb was first identified as the binding site for oncoproteins from DNA tumor viruses; one of the best studied of these is the adenovirus E1a protein. We demonstrate here that the E1a binding site is formed by the interaction between A and B. E1a augments the binding of A and B, suggesting that it is contacting a site composed of both A and B and that this interaction is serving as a clamp to stabilize the interaction.

In another paper (9), we present evidence indicating that the A-B interaction which forms the Rb repressor motif is subject to regulation by $G_1$ cdks which hyperphosphorylate Rb and disrupt the interaction between A and B and thus the repressor motif. We also show using domain swapping experiments that the A-B repressor motif is shared by p107, suggesting that this repressor motif is conserved among Rb family members.

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