MOLECULAR AND CELLULAR BIOLOGY, Sept. 2000, p. 6799–6805 Vol. 20, No. 18
0270-7306/00/$04.00+0

Role of the LXCXE Binding Site in Rb Function

ANJALI DAHIYA, MARK R. GAVIN, ROBIN X. LUO, AND DOUGLAS C. DEAN*

Division of Molecular Oncology, Departments of Medicine and Cell Biology, Washington University School of Medicine, St. Louis, Missouri 63110

Received 8 March 2000/Returned for modification 10 April 2000/Accepted 7 June 2000

Oncoproteins from DNA tumor viruses such as adenovirus E1a, simian virus 40 T antigen, and human papillomavirus E7 contain an LXCXE sequence, which they use to bind the retinoblastoma protein (Rb) and inhibit its function. Cellular proteins such as histone deacetylases 1 and 2 (HDAC1 and -2) also contain an LXCXE-like sequence, which they use to interact with Rb. The LXCXE binding site in Rb was mutated to assess its role in Rb function. These mutations inhibited binding to HDAC1 and -2, which each contain an LXCXE-like sequence, but had no effect on binding to HDAC3, which lacks an LXCXE-like sequence. Mutation of the LXCXE binding site inhibited active transcriptional repression by Rb and prevented it from effectively repressing the cyclin E and A gene promoters. In contrast, mutations in the LXCXE binding site did not prevent Rb from binding and inactivating E2F. Thus, the LXCXE mutations appear to separate Rb's ability to bind and inactivate E2F from its ability to efficiently recruit HDAC1 and -2 and actively repress transcription. In transient assays, several of the LXCXE binding site mutants caused an increase in the percentage of cells in G_1 by flow cytometry, suggesting that they can arrest cells. However, this effect was transient, as none of the mutants affected cell proliferation in longer-term assays examining bromodeoxyuridine incorporation or colony formation. Our results then suggest that the LXCXE binding site is important for full Rb function. Mutation of the LXCXE binding site does not inhibit binding of the BRG1 ATPase component of the SWI/SNF nucleosome remodeling complex, which has been shown previously to be important for Rb function. Indeed, overexpression of BRG1 and Rb in cells deficient for the proteins led to stable growth inhibition, suggesting a cooperative role for SWI/SNF and the LXCXE binding site in efficient Rb function.

The retinoblastoma protein (Rb) is an important regulator of the cell cycle (42). One target of Rb is the E2F family of cell cycle transcription factors, and binding of Rb blocks transcriptional activation by E2F (1, 11, 25, 32, 34). There are conflicting reports as to whether this inactivation results simply from the binding of Rb to the transactivation domain of E2F, or whether recruitment of chromatin remodeling enzymes is required. In in vitro transcription assays Rb blocks transcriptional activation by E2F-1 in the apparent absence of chromatin remodeling complexes, suggesting that Rb may function simply by binding and masking the transactivation domain of E2F-1 (33). However, other studies have demonstrated that Rb can interact with chromatin remodeling enzymes to repress E2F activity (3, 29). One of these enzymes is histone deacetylase (HDAC), a family of at least seven different enzymes that removes acetyl groups from the tails of histone octamers. This removal of acetyl groups appears to facilitate condensation of nucleosomes into chromatin, which in turn blocks access of transcription factors, leading to gene repression (23, 24, 45). In contrast to in vitro assays, transfection assays in vivo have suggested that interaction of Rb with HDAC is required for Rb to inhibit E2F-1 (3, 29). Furthermore, the active repression by the Rb-E2F complex at the promoters of cell cycle genes is thought to be mediated at least in part by recruitment of HDAC, and HDAC activity appears to be required for Rb to repress several cellular genes (28). An IXCXE site in the C terminus of HDAC1 seems to be important in mediating association with Rb (29).

In addition to HDACs, Rb also interacts with two other chromatin remodeling enzymes, BRG1 and BRM (8, 35, 38). These proteins are ATPases which are central components of the human SWI-SNF nucleosome remodeling complex. SWI-SNF was first identified in yeast where the ATPase SWI2-SNF2 appears to be a homologue of mammalian BRG1 and BRM (reviewed in reference 23). SWI/SNF seems to function by regulating nucleosome formation and positioning around genes. Several different SWI-SNF-related remodeling complexes have now been identified, and these complexes appear to have similar activities in in vitro assays. While SWI-SNF has been thought to be involved primarily in transcriptional activation, mutation of SWI2-SNF2 led to both activation and repression of genes in yeast (more genes were activated than repressed), suggesting that SWI-SNF may also be involved in transcriptional repression (19). Additionally, SWI-SNF-related complexes have been shown more directly to be involved in transcriptional repression. For example, the Mi2β complex is associated with repression, and it is thought that the presence of HDAC1 in the complex is required for this activity (22, 40, 48).

It has been demonstrated that expression of BRG1 in SW13 cells, which are deficient for both BRG1 and BRM (30) but are Rb^+ leads to growth arrest (8). Inhibition of Rb function by expression of adenovirus E1a prevented this arrest, and mutation of E1a to selectively block its interaction with Rb significantly reduced this effect of E1a. Additionally, a dominant-negative form of BRM, containing a mutant ATPase domain but an intact Rb binding site, was able to inhibit growth suppression by Rb (8). Two additional studies also point to a role for BRG1 in Rb function: recently, it was shown that SWI-SNF activity is important for Rb repression of the c-*fos* gene (31), and earlier studies provided evidence that expression of BRM in BRG1/BRM-deficient cells was required for Rb to efficiently inhibit transcriptional activation by E2F-1 (37). Taken to-

* Corresponding author. Mailing address: Campus Box 8069, Division of Molecular Oncology, Washington University School of Medicine, 660 South Euclid Ave., St. Louis, MO 63110. Phone: (314) 362-8989. Fax: (314) 747-2797. E-mail: ddean@im.wustl.edu.

gether, the above studies point to potentially important roles for HDAC and SWI-SNF in Rb activity.

Like HDAC1, BRG1 contains an LXCXE site, and deletion of a BRG1 region containing the LXCXE site results in loss of binding to Rb (8). An LXCXE sequence is also found in adenovirus E1a, human papillomavirus (HPV) E7, and simian virus 40 T antigen (7, 10, 13, 20). These DNA tumor virus oncogene products use the LXCXE motif for high-affinity binding and inhibition of Rb. Without the LXCXE site, these oncoproteins cannot transform cells. The fact that viruses target the LXCXE binding site of Rb and that this is necessary for transformation points to the importance of this site in Rb function. The Rb pocket has been cocrystallized with an LXCXE peptide, allowing localization of the LXCXE binding site (26). A hydrophobic groove in Rb pocket domain B forms the binding site, where the four conserved amino acids Tyr 709, Lys 713, Tyr 756, and Asn 757 are involved in contacting the backbone of the LXCXE peptide. We found that mutation of these contact amino acids inhibited binding of Rb to LXCXE-like proteins such as adenovirus E1a and HDAC1 and -2 but not HDAC3, which lacks an LXCXE-like motif. The LXCXE binding site mutations inhibited HDAC-dependent active repression and efficient growth suppression by Rb, providing evidence that the LXCXE binding site is important for efficient Rb function. However, the mutations did not affect either binding of Rb to E2F or the ability of Rb to inhibit transcriptional activation by E2F. These results suggest that although the LXCXE binding site has a critical role in active transcriptional repression by Rb and is important for full Rb function, this site is not required for Rb binding and inhibition of E2F. The LXCXE binding site mutations then separate Rb functions of binding and inactivation of E2F from recruitment of LXCXE proteins. The LXCXE binding site mutations in Rb did not affect binding to the SWI-SNF ATPase, BRG1, and we found that overexpression of BRG1 with the Rb mutants led to growth arrest. These results suggest a level of cooperation between LXCXE proteins and SWI-SNF in efficient Rb function.

MATERIALS AND METHODS

Transfection assays. Cells were cultured as described elsewhere (4). One-third microgram of the adenovirus major late promoter (MLP)-chloramphenicol acetyltransferase (CAT) reporter (MLPCAT) and 1.5 μg of the E2F-CAT reporter were cotransfected into C33a or CV-1 cells on 60-mm-diameter plates along with 2 μg of Rb expression vectors (0.4 μg of E2F1 was used to activate E2F-CAT), and cells were harvested 36 h after transfection. CAT activity was determined as described elsewhere (5). For luciferase assays, 1.5 of cyclin E gene (*cycE*)- or *cycA*-luciferase gene (*luc*) reporter was transfected, along with 2 μg of Rb expression vector, into U2OS cells on 35-mm-diameter plates. Luciferase assays were performed 36 h after transfection.

Plasmids. Plasmids Gal4-Rb, E2F-CAT, and CMV-E1a have been described previously (4, 5, 43, 44). pBJ5-HDAC1-6F was kindly provided by S. L. Schreiber (16, 36), G5MLPCAT was from D. E. Ayer (2), and CMV-E2F1 was a gift from K. Helin (27). BRG1-F was constructed by inserting a Flag sequence at the C terminus of BRG1 in plasmid pBJ5-Brg1, a gift from S. Goff (8). The *cycE-luc* reporter was from R. Weinberg (15), and the *cycA-luc* reporter was provided by C. Brechot (18). Mutagenesis of Rb was performed using the QuickChange Mutagenesis system (Stratagene).

Coimmunoprecipitation assays. Coimmunoprecipitation assays were done essentially as described elsewhere (5, 28). C33a cells were transfected with 12 μg of Rb or Rb mutant expression vectors and 2 μg of HDAC1 or HDAC2 (10 μg of HDAC3) expression vector. Cells were harvested 36 h later in lysis buffer containing 250 mM NaCl (5). Lysates were precleared by 30 min of incubation with Sepharose beads (Sigma). Cleared lysates were immunoprecipitated with monoclonal anti-Gal4 antibody conjugated to agarose beads (Santa Cruz Biotechnology). Precipitates were washed three times with lysis buffer and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then immunoblotted with an anti-Flag polyclonal antibody (Santa Cruz) to detect Flag-tagged HDAC1 or an anti-E2F-1 polyclonal antibody (Santa Cruz) to detect E2F-1. Blots were then reprobed with an anti-Rb polyclonal antibody (Santa Cruz) to determine the amount of precipitated Rb. Five micrograms of the BRG1-F expression vector was cotransfected with Rb expression vectors for wild-type or mutant Rb large pocket (amino acids 379 to 928) (44) to analyze BRG1 binding, and BRG1-F was detected with the anti-Flag antibody. For E1A binding, 5 μg of an E1a expression vector was transfected along with Rb expression vectors, and E1a was immunoprecipitated with an anti-E1a monoclonal antibody (Calbiochem).

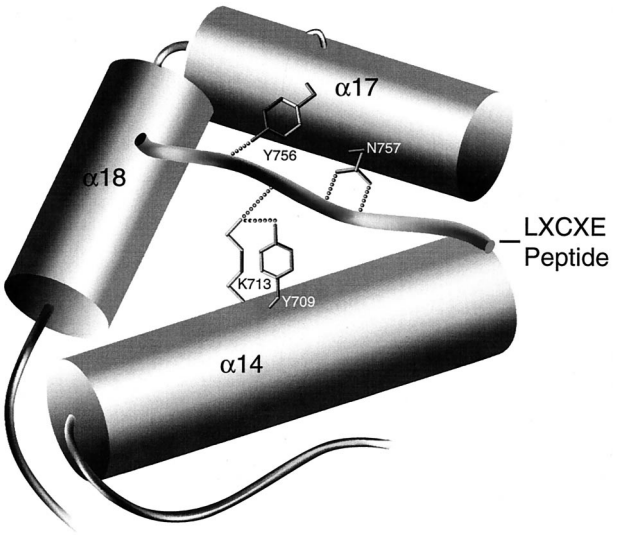

FIG. 1. Diagram of the LXCXE binding site derived from cocrystallization of the Rb pocket with an LXCXE peptide (26). Tyr 709, Lys 713, Tyr 756, and Asn 757 are conserved amino acids in the Rb pocket that appear to make important contacts with the backbone of the LXCXE peptide. Each of these amino acids was mutated to alanine either individually or in combinations.

Growth suppression assay. For colony formation assays (4), Saos-2 cells were grown to approximately 50% confluency on 100-mm-diameter plates and then cotransfected with 2 μg of an expression vector for the neomycin resistance gene and 20 μg of expression vector for wild-type or mutant Rb, using the calcium phosphate method. Cells were treated with G418 (500 μg/ml) for 3 weeks and then stained with crystal violet to assess colony formation. Colony formation assays were done in C33a cells as we have described previously (49).

BrdU incorporation and flow cytometry. Bromodeoxyuridine (BrdU) incorporation was determined essentially as described previously (49). Cells were cotransfected with 2 μg of puro-BABE and 20 μg of expression vector for Rb or Rb mutant, and cells were selected in puromycin for 72 h. For flow cytometry, cells were transfected with 2 μg of CD20 expression vector and 20 μg of Rb expression vector. Cells were harvested 48 h later, and the cell cycle profile of at least 6,000 $CD20^+$ cells was determined as described elsewhere (49, 50).

RESULTS

Mutations in the LXCXE binding site of Rb. Both HDAC1 and BRG1 contain an LXCXE-like sequence, and deletion of regions of the proteins containing this sequence prevents their association with Rb (8, 29). Therefore, we reasoned that the LXCXE binding site in Rb might have an important role in Rb function because of its recruitment of these chromatin remodeling enzymes. The central pocket region of Rb is comprised of two conserved domains, A and B. These domains interact with one another to form the LXCXE binding site located in domain B (4, 26). Crystallization of the Rb pocket bound to an LXCXE peptide revealed that Tyr 709, Lys 713, Tyr 756, and Asn 757 in Rb domain B are involved in contacting the backbone of the LXCXE peptide (reference 26 and Fig. 1). To assess the role of the LXCXE binding site in Rb function, we created mutations in these amino acids individually and in combinations.

LXCXE binding site mutations in Rb inhibit interaction with E1a. Initially, we tested the LXCXE binding site mutants for the ability to interact with the LXCXE protein E1a in coimmunoprecipitation assays. A vector expressing either wild-

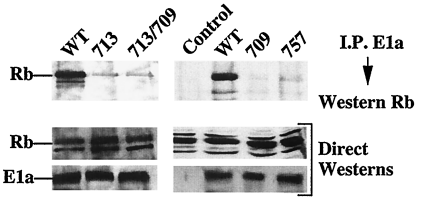

FIG. 2. Mutations in the LXCXE binding site of Rb inhibit interaction with adenovirus E1a and HDAC1. A coimmunoprecipitation assay was used to assess the effect of LXCXE binding site mutations on the binding of Rb to E1a. An expression vector for the large pocket of Rb (amino acids 379 to 928) (WT [wild type]) (44) or the indicated mutants were transfected into C33a cells along with an expression vector for E1a (43). E1a was immunoprecipitated (I.P.), and associated Rb was detected by Western blotting. "Control" indicates that an irrelevant antibody (to HPV E7) was used for immunoprecipitation.

type Rb or Rb with an LXCXE binding site mutation was cotransfected with an expression vector for E1a. Cell lysates were immunoprecipitated with an antibody to E1a and then Western blotted to detect associated Rb. Not surprisingly, the LXCXE binding site mutations inhibited binding of E1a to Rb (Fig. 2). These results provide further evidence that these contact amino acids identified in the crystal structure are important for binding to the LXCXE sequence in E1a.

Mutation of the LXCXE binding site in Rb does not affect Rb binding or inactivation of E2F. Rb appears to be targeted to a number of genes through its interaction with E2F family members bound to E2F sites on promoters. Thus, for Rb to recruit corepressors such as HDAC to promoters with E2F sites, it must bind to both E2F and HDAC simultaneously. While the binding site for E2F on Rb has not yet been defined, E2Fs do not contain an LXCXE sequence, and it has been demonstrated that Rb can bind to E2F-1 and HDAC1 simultaneously (3, 26). Therefore, we expected that the LXCXE mutations would have no effect on binding to E2F, unless they generally disrupted Rb pocket structure. Using coimmunoprecipitation assays, we found that mutations in the LXCXE binding site indeed did not affect binding of Rb to E2F-1 (Fig. 3A). Thus, we conclude that the overall structure of the Rb pocket (at least as assessed by ability to bind E2F) is not disrupted by the LXCXE binding site mutations.

To test the effect of LXCXE binding site mutations on E2F activity, a reporter plasmid containing a minimal promoter comprised of E2F sites upstream of a TATA box was cotransfected with either wild-type or mutant Rb. Each of the LXCXE mutants inhibited E2F transcriptional activity to a similar extent as wild-type Rb in these assays (Fig. 3B). In these assays, endogenous E2Fs were activating the E2F sites. However, we also found that the Rb mutants efficiently blocked transcriptional activity of the reporter when E2F-1 was overexpressed in these assays (data not shown).

Efficient binding of E1a to Rb requires the LXCXE sequence located in conserved region 2 of E1a; however, once bound, conserved region 1 of E1a can displace E2F from Rb (13, 20). Therefore, we reasoned that mutation of the LXCXE binding site in Rb should render it resistant to inhibition by E1a. Indeed, we found that the ability of E1a to block Rb inhibition of E2F was prevented with mutation of the LXCXE binding site (Fig. 3C). The LXCXE binding site mutants then appear to disrupt binding of LXCXE proteins to Rb without preventing Rb from binding and inactivating E2F. Thus, we reasoned that these mutants could be used to assess the role for the LXCXE binding site under conditions where other Rb functions (e.g., binding and inactivation of E2F) are intact.

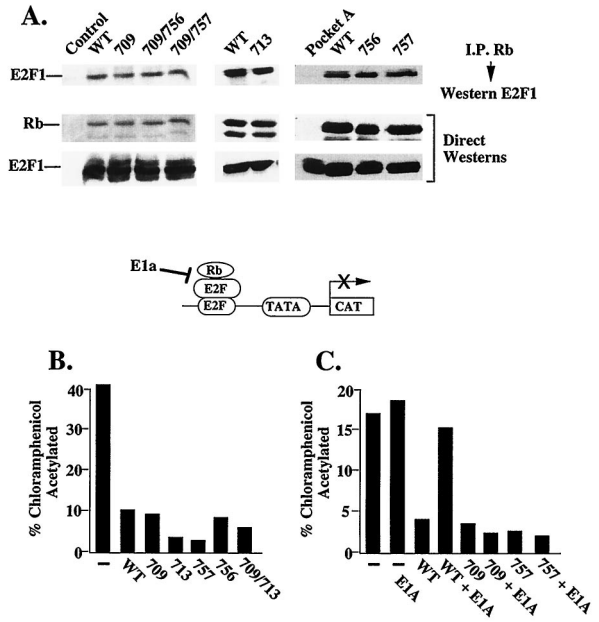

FIG. 3. Mutation of the LXCXE binding site in Rb does not affect interaction with or inactivation of E2F-1. (A) Expression vectors for E2F-1 and either wild-type (WT) or mutant Rb large pocket were cotransfected into C33a cells, and interaction was followed by coimmunoprecipitation (I.P.) as in Fig. 2. (B) The E2F-CAT reporter plasmid (44), which contains E2F sites upstream of a TATA box, was transfected into CV-1 cells. Expression vectors for wild-type or mutant Rb large pocket (4, 44) were cotransfected as indicated to determine the effect of LXCXE binding site mutations on the ability of Rb to inhibit E2F activity. (C) E1a cannot block Rb inhibition of E2F when the LXCXE binding site is mutated. Wild-type Rb and LXCXE binding site mutant expression vectors were transfected as in panel B along with an expression vector for an E1a mutant where amino acids 2 to 36 are deleted (removes the p300/CBP binding domain, leaving the Rb binding domain intact) (43). CAT activity is representative of five independent experiments, each done in duplicate.

Mutations in the LXCXE binding site of Rb inhibit binding to HDAC1 and -2 and active transcriptional repression. The interaction of HDAC1 with Rb was competed by E1a in coimmunoprecipitation assays (Fig. 4A), suggesting that the proteins may have been binding a similar sequence on Rb. HDACs are a family of seven proteins (12, 16, 21, 36, 39, 41, 46, 47). Coimmunoprecipitation assays were used to examine interaction of Rb with class I and II HDACs. We did not detect interaction between Rb and the class II HDACs, HDAC4 to -6 (results not shown); however, Rb did interact with the class I HDACs, HDAC1 to -3 (Fig. 4). It has been demonstrated previously that HDAC3 has less deacetylase activity in vitro than HDAC1 and -2 (47). Therefore, the bulk of HDAC activity associated with Rb may be derived from HDAC1 and -2. Interestingly, both HDAC1 and -2 have an LXCXE-like sequence, whereas HDAC3 lacks such a sequence (12, 47). Accordingly, mutation of the LXCXE binding site in Rb inhibited Rb interaction with HDAC1 and -2 but not HDAC3 (Fig. 4). Therefore, mutation of the LXCXE binding site only partially inhibits HDAC binding to Rb.

We wondered what consequence mutation of the LXCXE binding site and thus inhibition of HDAC1 and -2 binding might have on Rb function. We and others have demonstrated that repression of the adenovirus MLP by either Rb or Mad is dependent on HDAC (2, 17, 28). Therefore, we examined the ability of Rb mutants to collaborate with HDAC and repress the MLP. For these studies, a reporter plasmid containing Gal4 DNA binding sites upstream of the MLP was cotrans-

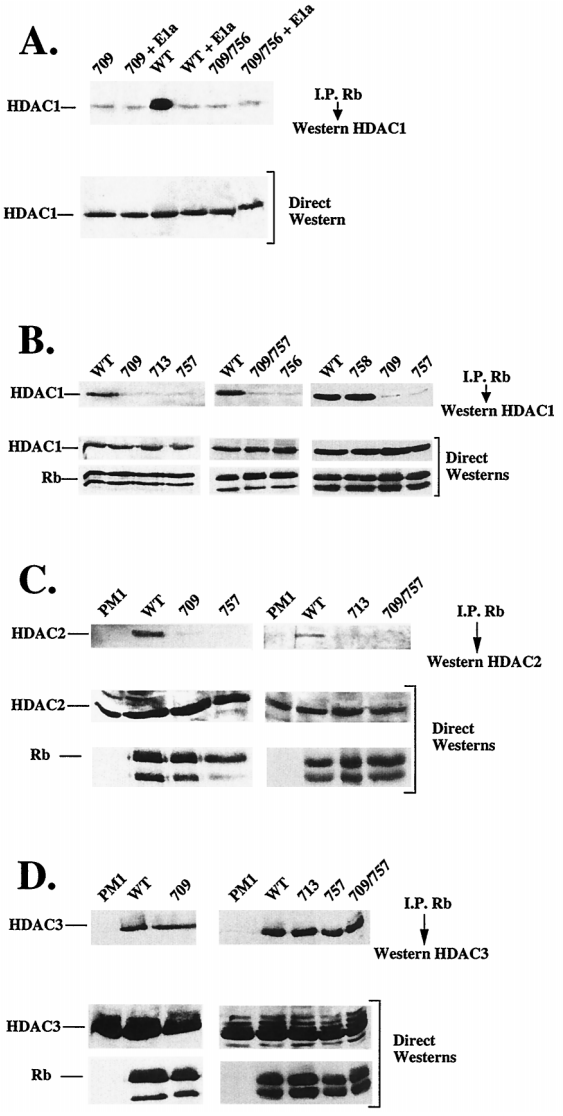

FIG. 4. Rb LXCXE binding site mutants show decreased binding to HDAC1 and -2 but retain binding to HDAC3. (A) E1a competes for binding of HDAC1 to Rb. An expression vector for wild-type (WT) or mutant Rb large pocket was cotransfected into Rb^- C33a cells along with expression vectors for Flag-tagged HDAC1 and, where indicated, E1a. Association of Rb and HDAC1 was detected by coimmunoprecipitation (I.P.) as in Fig. 2. (B) C33a cells were cotransfected with expression vectors for wild-type Rb large pocket or the indicated mutants and HDAC1 containing a Flag tag (28). Association of Rb and HDAC1 was assessed by coimmunoprecipitation as indicated. "758" indicates a control Ser-to-Leu mutation at amino acid 758; this amino acid is adjacent to the LXCXE binding site in the crystal structure, but it does not contact the LXCXE (26). (C) Wild-type Rb large pocket or Rb mutant and Flag-tagged HDAC2 expression vectors were transfected into C33a cells. Interaction between Rb and HDAC2 was determined by coimmunoprecipitation. (D) C33a cells were cotransfected with LexA-tagged HDAC3 and Rb or Rb mutant expression vectors. Binding to Rb was assessed by coimmunoprecipitation.

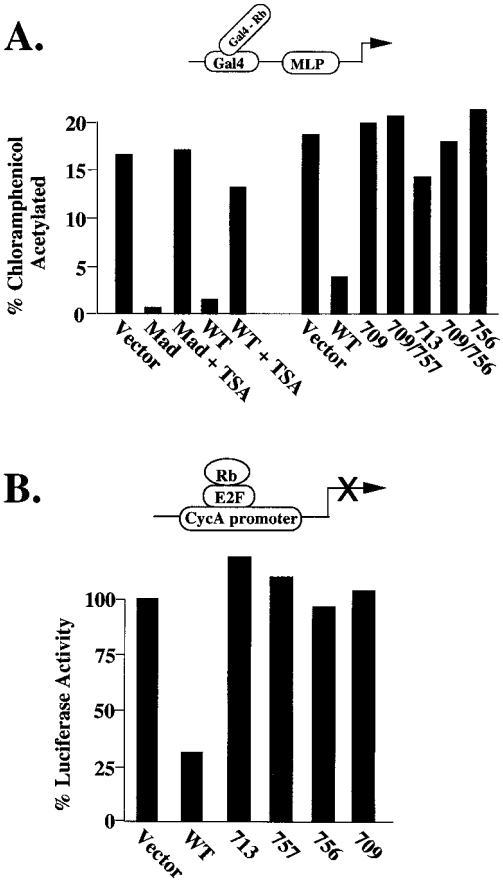

FIG. 5. Mutation of Rb's LXCXE binding site results in abrogation of Rb's ability to actively repress. (A) A reporter (MLPCAT) containing the adenovirus MLP with Gal4 DNA binding sites upstream (28) was cotransfected into CV-1 cells along with expression vectors for wild-type (WT) Rb large pocket or Rb large pocket mutants fused to the DNA binding domain of Gal4 as indicated to assess the effect of LXCXE binding site mutations on active transcriptional repression. As a control, an expression vector for Gal4-Mad (28) was cotransfected. The HDAC inhibitor trichostatin A (TSA) was added to the transfected cells as described previously (28). (B) The *cycA-luc* reporter was transfected into U2OS cells, along with wild-type Rb large pocket or Rb mutant expression vectors, and luciferase activity was measured. Luciferase activities are plotted relative to reporter alone activity, which is indicated as 100%. Transfection assays are representative of five independent experiments, each done in duplicate.

fected with expression vectors for wild-type or mutant Rb fused to the DNA binding domain of Gal4 (28, 44). When tethered directly to the promoter through Gal4, both Rb and Mad repressed MLP activity, and this repression was largely reversed by the HDAC inhibitor trichostatin A (Fig. 5A and reference 28). In contrast, the LXCXE binding site mutants were impaired in the ability to inhibit the MLP (Fig. 5A), suggesting that the LXCXE binding site is important for efficient Rb-HDAC repressor activity.

The cyclin E and A genes contain E2F sites and are examples of cellular genes repressed by Rb (15, 18). As with the MLP, we found that LXCXE binding site mutants impaired Rb repression of the cyclin A and E gene promoters in transfection assays (Fig. 5B and results not shown). These results provide evidence that the LXCXE binding site is also important for efficient Rb repression of cellular genes.

Sustained growth suppression by Rb requires the LXCXE binding site. We wondered whether mutation of the LXCXE binding site in Rb would affect its ability to suppress cell proliferation. First, we examined the effect of the LXCXE binding site mutants on the cell cycle in Rb^- Saos-2 cells. For these experiments, wild-type Rb or the mutants were coexpressed with the cell surface marker CD20 by transient transfection. $CD20^+$ cells were then analyzed for DNA content by flow cytometry 36 h following transfection. We found that several of

A.

	%G1	%S	%G2/M
Vector Control	54.2	9.0	35.8
WT	68.6	5.5	25.0
709	68.1	7.25	23.7
713	63.0	7.5	28.4
709/757	57.4	10.8	31.8
756	55.4	8.8	34.9

B.

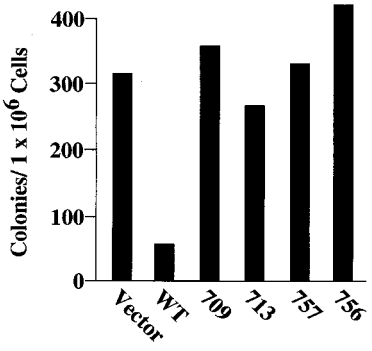

FIG. 6. Mutation of the LXCXE binding site and growth suppression by Rb. (A) Wild-type (WT) Rb large pocket or Rb mutant expression vectors were cotransfected with an expression vector for CD20 into Rb^- Saos-2 cells. Cells were harvested 36 h later, and $CD20^+$ cells were analyzed by flow cytometry for DNA content. (B) Expression vectors for wild-type Rb large pocket or large pocket mutants were cotransfected into Rb^- Saos-2 cells along with an expression vector for neomycin resistance. Cells were selected in G418 for 3 weeks; colonies were stained with crystal violet and counted.

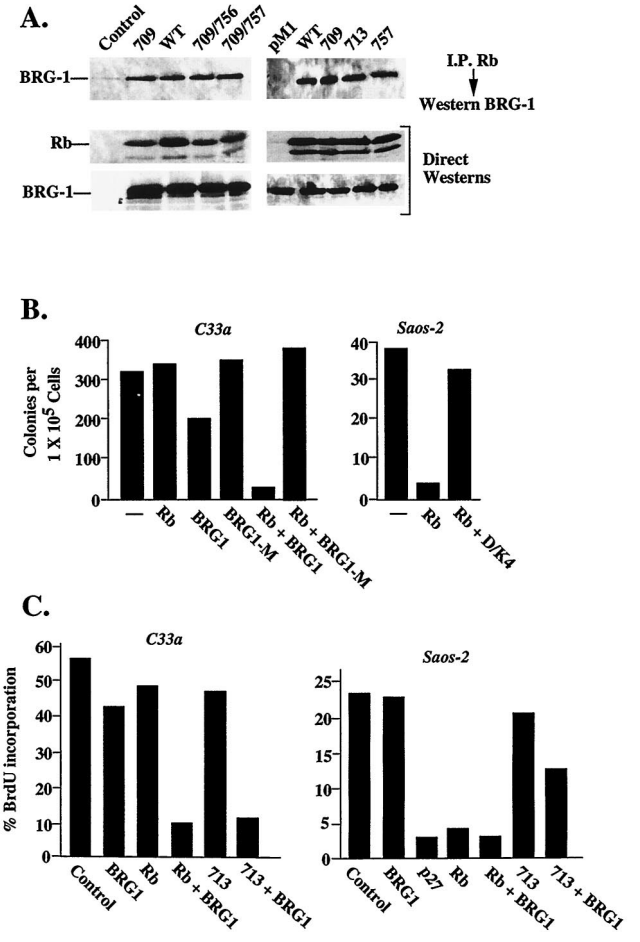

FIG. 7. BRG1 facilitates growth suppression by Rb and does not require the LXCXE binding site for interaction with Rb. (A) Rb LXCXE binding site mutants still bind to BRG1. Expression vectors for Flag-tagged BRG1 and either wild-type (WT) or mutant Rb large pocket were cotransfected into C33a cells, and association between BRG1 and Rb was assessed by coimmunoprecipitation (I.P.). (B) Wild-type Rb large pocket was transfected into Rb^-, BRG1/BRM-deficient C33a cells along with an expression vector for neomycin resistance. Cells were selected in G418 for 2 weeks; colonies were stained with crystal violet and counted. Saos-2 cells were selected in G418 for 3 weeks. (C) C33a cells or Saos-2 cells were transfected with the indicated expression vectors and a vector expressing a puromycin resistance gene. Cells resistant to puromycin were examined 5 days after transfection for BrdU incorporation.

the mutants caused an increase in G_1 (Fig. 6A), suggesting that at least some of the mutants may be able to arrest cells in G_1. However, these flow cytometry assays measure only DNA content, not cell proliferation. Therefore, we examined the mutants in colony formation assays in Saos-2 cells. For these assays, the Rb^- Saos-2 cell line was cotransfected with an expression vector for wild-type or mutant Rb and a vector expressing the neomycin resistance gene. Transfected cells were treated with the neomycin analogue G418 for 3 weeks, and colony formation was analyzed (Fig. 6B). The mutants that increased G_1 by flow cytometry did not inhibit colony formation or colony size in these assays, suggesting that the G_1 arrest seen with several of the Rb mutants by flow cytometry is only transient and is not capable of stably arresting cells. In further support of this possibility, the 713 mutant (which did lead to an increase in G_1 when cells were examined 36 h after transfection by flow cytometry [Fig. 6A]) did not inhibit incorporation of BrdU in Saos-2 cells when cells were examined 5 days following transfection (see Fig. 7C). Taken together, our results point to an important role for the LXCXE binding site in efficient (or at least sustained) growth suppression by Rb. Also, since these mutants bind and block E2F activity as efficiently as wild-type Rb, these results suggest that the ability of Rb to bind and inactivate E2F is not sufficient for efficient growth suppression—the LXCXE binding site is also required.

BRG1 can cooperate with Rb to suppress cell proliferation. Previous studies suggested that SWI-SNF activity is important for Rb growth suppression, and this is dependent on Rb binding to the ATPase, BRG1, which forms the core of SWI-SNF (8). There is an approximately 30-amino-acid region in BRG1 that contains an LXCXE and shows some similarities to HPV E7 (8). Deletion of the region of BRG1 containing the LXCXE prevented Rb binding and the ability of BRG1 to suppress cell proliferation and repress the c-*fos* gene (8, 31). We also found that deletion of this E7-like region prevented binding to Rb (data not shown). However, this deletion removes approximately 12 kDa of the protein, making it difficult to conclude that the LXCXE site alone is important for binding to Rb. In fact, we found that mutations in the LXCXE binding site of Rb had no detectable effect on Rb binding to BRG1 (Fig. 7A). These results suggest that even though BRG1 contains an LXCXE site, this sequence is not essential for binding to Rb.

The C33a cell line is both Rb^- and deficient in BRG1 and BRM (30). While expression of Rb in the $BRG1/BRM^+$ Saos-2 cells leads to growth arrest, expression of Rb in C33a cells is not sufficient for growth arrest (Fig. 7B). This raised the possibility that Rb may not be functional in these cells because

they are deficient in BRG1 and BRM. Indeed, coexpression of BRG1 with Rb led to efficient growth suppression, whereas expression of BRG1 alone did not. These results provide additional evidence that BRG1 and thus SWI-SNF activity is important for Rb to function as a growth suppressor.

Since the LXCXE binding site Rb mutants still bound to BRG1, but these mutants were unable to sustain growth suppression, it appeared that interaction with BRG1 alone (in the absence of the LXCXE binding site) was not sufficient for Rb to suppress growth. However, it was possible that the transient growth suppression with some of the mutants was due to the interaction with BRG1. In partial support of this possibility, we found that if BRG1 was overexpressed, it was then able to cooperate with Rb mutants to stably arrest cells (Fig. 7C). In these assays, cells were transfected with expression vectors for Rb proteins and BRG1 along with a vector expressing a puromycin resistance gene. Cells resistant to puromycin were then examined 5 days after transfection for BrdU incorporation as an indication of proliferation. In Rb^- and BRG1/BRM-deficient C33a cells, the Rb mutants alone were unable to inhibit proliferation, but when combined with BRG1, cell proliferation was inhibited, although not as efficiently as with wild-type Rb (Fig. 7C). In Saos-2 cells, which express BRG1 (30), overexpression of BRG1 allowed the mutants to suppress growth, although again not as efficiently as wild-type Rb. We therefore conclude that overexpression of BRG1 can restore at least partial growth suppression activity to Rb that is defective in binding to LXCXE proteins (Fig. 7B). Taken together, our results suggest roles for both LXCXE proteins and SWI/SNF in Rb activity, and they imply some level of cooperation between such proteins in Rb function.

DISCUSSION

Several DNA tumor viruses express proteins that target the LXCXE binding site in Rb. These viral proteins (E1a from adenovirus, E7 from HPV, and T antigen from SV40) block Rb's ability to suppress growth. Mutation of the LXCXE sequence in these proteins prevents their inhibitory effect on Rb and their ability to transform cells. The fact that each of these viral proteins uses an LXCXE motif to inhibit Rb function provides genetic evidence of the importance of the LXCXE binding site. Here, we have created mutations in the LXCXE binding site of Rb to address the role of LXCXE proteins in Rb function.

The LXCXE mutations appear to isolate the interaction of LXCXE proteins from the interaction of the BRG1 component of SWI-SNF. Using these mutants, we provide further evidence that both LXCXE proteins and SWI-SNF are important for efficient Rb function as a growth suppressor. The LXCXE mutations had no effect on the ability of Rb to bind and inhibit E2F, yet the mutants were unable to sustain growth arrest, suggesting that inhibition of E2F activity alone is not sufficient for sustained growth arrest. In contrast, the LXCXE binding site mutations inhibited active transcriptional repression by Rb and its binding to the corepressors HDAC1 and -2, suggesting that one role of the LXCXE binding site in Rb growth suppression is the efficient recruitment of these chromatin remodeling enzymes.

SWI-SNF has been associated previously with transcriptional activation (23). Thus, the question arises as to how this chromatin remodeling activity can be associated with transcriptional activation in some cases and repression in others. It is of note that efficient growth suppression by Rb requires the LXCXE binding site (at least in the absence of BRG1 overexpression), which is important for recruitment of HDAC1 and -2. Thus, the role of SWI-SNF in Rb growth suppression appears linked, at least in part, to the ability of Rb to efficiently recruit HDAC1 and -2. Recruitment of HDAC is also thought to be important for repression by the SWI-SNF-related complex Mi2β (48). Interestingly, in genes such as *HO* in yeast, where SWI-SNF is important for transcriptional activation (6), it is associated with activators and histone acetyltransferase (HAT) activity. Additionally, Rb is also associated with transcriptional activation in some situations. For example, Rb can enhance BRG1-dependent transcriptional activation by the glucocorticoid receptor (14). In this situation, SWI-SNF and Rb are recruited to a promoter in the presence of a transcriptional activator associated with HAT activity (the glucocorticoid receptor). Thus, the role of SWI-SNF may depend on whether it is recruited to promoters in an environment dominated by HAT or HDAC activity.

ACKNOWLEDGMENTS

We thank N. Dyson and J. Wang for communicating results prior to publication, S. Schreiber for the HDAC1 to HDAC6 expression vectors, K. Helin for the E2F-1 expression vector, D. Ayer for MLPCAT, S. Goff for the BRG1 expression vector, R. Weinberg for the *cycE-luc* reporter, C. Brechot for the *cycA-luc* reporter, S. Cotter for the Flag-tagged BRG1 expression vector, and A. Postigo for the LexA-tagged HDAC3.

This study was supported by grants from the NIH to D.C.D. A.D. was supported by training grant HL07317-22 from the National Heart Lung and Blood Institute.

REFERENCES

1. **Adams, P. D., and W. G. Kaelin, Jr.** 1996. The cellular effects of E2F overexpression. Curr. Top. Microbiol. Immunol. **208:**79–93.
2. **Ayer, D. E., Q. A. Lawrence, and R. N. Eisenman.** 1995. Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. Cell **80:**767–776.
3. **Brehm, A., E. A. Miska, D. J. McCance, J. L. Reid, A. J. Bannister, and T. Kouzarides.** 1998. Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature **391:**597–601.
4. **Chow, K. N., and D. C. Dean.** 1996. Domains A and B in the Rb pocket interact to form a transcriptional repressor motif. Mol. Cell. Biol. **16:**4862–4868.
5. **Chow, K. N., P. Starostik, and D. C. Dean.** 1996. The Rb family contains a conserved cyclin-dependent-kinase-regulated transcriptional repressor motif. Mol. Cell. Biol. **16:**7173–7181.
6. **Cosma, M. P., T. Tanaka, and K. Nasmyth.** 1999. Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle and developmentally regulated promoter. Cell **97:**299–311.
7. **DeCaprio, J. A., J. W. Ludlow, J. Figge, J. Y. Shrew, C. M. Huang, W. H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston.** 1988. SV40 large tumor antigen forms complex with the product of the retinoblastoma susceptibility gene. Cell **54:**275–283.
8. **Dunaief, J. L., B. E. Stober, 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.
9. **Dyson, N., P. M. Howley, K. Munger, and E. Harlow.** 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243:**6893–6902.
10. **Dyson, N., P. Guida, C. McCall, and E. Harlow.** 1992. Adenovirus E1A makes two distinct contacts with the retinoblastoma protein. J. Virol. **66:** 4606–4611.
11. **Dyson, N.** 1998. The regulation of E2F by pRB-family proteins. Genes Dev. **12:**2245–2262.
12. **Emiliani, S., W. Fischle, C. Van Lint, Y. Al-Abed, and E. Verdin.** 1998. Characterization of a human RPD3 ortholog, HDAC3. Proc. Natl. Acad. Sci. USA **95:**2795–2800.
13. **Fattaey, A. R., E. Harlow, and K. Helin.** 1993. Independent regions of adenovirus E1a are required for binding to and dissociation of E2F-protein complexes. Mol. Cell. Biol. **13:**7267–7277.
14. **Fryer, C. J., and T. K. Archer.** 1998. Chromatin remodeling by the glucocorticoid receptor requires the BRG1 complex. Nature **393:**88–91.
15. **Geng, Y., E. N. Eaton, M. Picon, J. M. Roberts, A. S. Lundberg, A. Gifford, C. Sardet, and R. A. Weinberg.** 1996. Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. Oncogene **12:**1173–1180.
16. **Grozinger, C. M., C. A. Hassig, and S. L. Schreiber.** 1999. Three proteins

define a class of human histone deacetylases related to yeast Hda1p. Proc. Natl. Acad. Sci. USA **96:**4868–4873.
17. **Hassig, C. A., T. C. Fleischer, A. N. Billin, S. L. Schreiber, and D. E. Ayer.** 1997. Histone deacetylase activity is required for full transcriptional repression by msin3a. Cell **89:**341–347.
18. **Henglein, B., X. Chenivesse, J. Wang, D. Eick, and C. Brechot.** 1994. Structure and cell cycle-regulated transcription of the human cyclin A gene. Proc. Natl. Acad. Sci. USA **91:**5490–5494.
19. **Holstege, F. C., E. G. Jennings, J. J. Wyrick, T. I. Lee, C. J. Hengartner, M. R. Green, T. R. Golub, E. S. Lander, and R. A. Young.** 1998. Dissecting the regulatory circuitry of a eukaryotic genome. Cell **95:**717–728.
20. **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.
21. **Kao, H.-Y., M. Downes, P. Ordentlich, and R. Evans.** 2000. Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. Genes Dev. **14:**55–66.
22. **Kim, J., B. Jones, A. Jackson, J. Koipally, S. Windandy, A. Veil, A. Sawyer, T. Ikeda, R. Kingston, and K. Georgopoulos.** 1999. Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes. Immunity **10:**345–355.
23. **Kingston, R. E., and G. J. Narlikar.** 1999. ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. Genes Dev. **13:**2339–2352.
24. **Kornberg, R. D., and Y. Lorch.** 1999. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell **98:**285–294.
25. **La Thangue, N. B.** 1994. DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. Trends Biochem. Sci. **19:**108–114.
26. **Lee, J. O., A. A. Russo, and N. P. Pavletich.** 1998. Structure of the retinoblastoma tumour-suppressor pocket domain bound to a peptide from HPV E7. Nature **391:**859–865.
27. **Lukas, J., B. O. Petersen, K. Holm, J. Bartek, and K. Helin.** 1996. Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. Mol. Cell. Biol. **16:**1047–1057.
28. **Luo, R. X., A. A. Postigo, and D. C. Dean.** 1998. Rb interacts with histone deacetylase to repress transcription. Cell **92:**463–473.
29. **Magnaghi-Jaulin, J. L., R. Groisman, I. Naguibneva, P. Robin, S. Lorain, V. J. Le, F. Troalen, D. Trouche, and B. A. Harel.** 1998. Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature **391:**601–605.
30. **Muchardt, C., and M. Yaniv.** 1993. A human homologue of *Saccharomyces cerevisiae* SNF2/SWI2 and *Drosophila* brm genes potentiates transcriptional activation by the glucocorticoid receptor. EMBO J. **12:**2497–2509.
31. **Murphy, D. J., S. Hardy, and D. A. Engel.** 1999. Human SWI-SNF component BRG1 represses transcription of the c-*fos* gene. Mol. Cell. Biol. **19:**2724–2733.
32. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science **258:**424–429.
33. **Ross, J. F., X. Liu, and B. D. Dynlacht.** 1999. Mechanism of transcriptional repression of E2F by the retinoblastoma tumor suppressor protein. Mol. Cell **3:**195–205.
34. **Slansky, J. E., and P. J. Farnham.** 1996. Introduction to the E2F family: protein structure and gene regulation. Curr. Top. Microbiol. Immunol. **208:**1–30.
35. **Strober, B. E., J. L. Dunaief, G. Sushovan, and S. P. Goff.** 1996. Functional interactions between the hBRM/hBRG1 transcriptional activators and the pRB family of proteins. Mol. Cell. Biol. **16:**1576–1583.
36. **Taunton, J., C. A. Hassig, and S. L. Schreiber.** 1996. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science **272:**408–411.
37. **Trouche, D., and T. Kouzarides.** 1996. E2F1 and E1A(12S) have a homologous activation domain regulated by RB and CBP. Proc. Natl. Acad. Sci. USA **93:**1439–1442.
38. **Trouche, D., C. Le Chalony, C. Muchardt, M. Yaniv, and T. Kouzarides.** 1997. Rb and hbrm cooperate to repress the activation functions of E2f1. Proc. Natl. Acad. Sci. USA **94:**11268–11273.
39. **Verdel, A., and S. Khochbin.** 1999. Identification of a new family of higher eukaryotic histone deacetylases. J. Biol. Chem. **274:**2440–2445.
40. **Wade, P. A., P. L. Jones, D. Vermaak, G. J. Veenstra, A. Imhof, T. Sera, C. Tse, H. Ge, Y. B. Shi, J. C. Hansen, and A. P. Wolffe.** 1998. Histone deacetylase directs the dominant silencing of transcription in chromatin: association with MeCP2 and the Mi-2 chromodomain SWI/SNF ATPase. Cold Spring Harbor Symp. Quant. Biol. **63:**435–445.
41. **Wang, A. H., N. R. Bertos, M. Vezmar, N. Pelletier, M. Crosato, H. H. Heng, J. Th'ng, J. Han, and X. J. Yang.** 1999. HDAC4, a human histone deacetylase related to yeast HDA1, is a transcriptional corepressor. Mol. Cell. Biol. **19:**7816–27.
42. **Weinberg, R. A.** 1995. The retinoblastoma and the cell cycle control. Cell **81:**323–330.
43. **Weintraub, S. J., C. A. Prater, and D. C. Dean.** 1992. Retinoblastoma protein switches the E2F sites from positive to negative element. Nature **358:**259–261.
44. **Weintraub, S. J., K. N. B. Chow, R. X. Luo, S. H. Zhang, S. He, and D. C. Dean.** 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. Nature **375:**812–815.
45. **Wolffe, A. P., and J. J. Hayes.** 1999. Chromatin disruption and modification. Nucleic Acids Res. **27:**711–720.
46. **Yang, W. M., C. Inouye, Y. Zeng, D. Bearrs, and E. Seto.** 1996. Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. Proc. Natl. Acad. Sci. USA **93:**12845–12850.
47. **Yang, W., Y. Yao, J. Sun, J. R. Davie, and E. Seto.** 1997. Isolation and characterization of cDNAs corresponding to an additional member of the human histone deacetylase gene family. J. Biol. Chem. **272:**28001–28007.
48. **Zhang, Y., G. LeRoy, H. P. Seelig, W. S. Lane, and D. Reinberg.** 1998. The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. Cell **95:**279–289.
49. **Zhang, H. S., A. A. Postigo, and D. C. Dean.** 1999. Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFbeta, and contact inhibition. Cell **97:**53–61.
50. **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.