

Distinct Mechanisms for Repression of RNA Polymerase III Transcription by the Retinoblastoma Tumor Suppressor Protein

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The retinoblastoma (RB) protein represses global RNA polymerase III transcription of genes that encode nontranslated RNAs, potentially to control cell growth. However, RNA polymerase III-transcribed genes exhibit diverse promoter structures and factor requirements for transcription, and a universal mechanism explaining global repression is uncertain. We show that RB represses different classes of RNA polymerase III-transcribed genes via distinct mechanisms. Repression of human U6 snRNA (class 3) gene transcription occurs through stable promoter occupancy by RB, whereas repression of adenovirus VAI (class 2) gene transcription occurs in the absence of detectable RB-promoter association. Endogenous RB binds to a human U6 snRNA gene in both normal and cancer cells that maintain functional RB but not in HeLa cells whose RB function is disrupted by the papillomavirus E7 protein. Both U6 promoter association and transcriptional repression require the A/B pocket domain and C region of RB. These regions of RB contribute to U6 promoter targeting through numerous interactions with components of the U6 general transcription machinery, including SNAP_C and TFIIB. Importantly, RB also concurrently occupies a U6 promoter with RNA polymerase III during repression. These observations suggest a novel mechanism for RB function wherein RB can repress U6 transcription at critical steps subsequent to RNA polymerase III recruitment.

The retinoblastoma (RB) protein controls the cell cycle, differentiation, apoptosis, and general growth. Typically, RB functions to repress RNA polymerase II transcription of genes that act at control points in these key cellular processes (10, 11, 14). The classical understanding of RB function is exemplified by its ability to control the cell cycle (reviewed in reference 45). For example, RB can target genes that are required to enter into the S phase of the cell cycle and whose promoters contain binding sites for the E2F family of transcriptional activator proteins (4, 17, 31, 46). RB can directly block the reactivation function of E2F (34) or alter chromatin structure to repress transcription by recruiting coregulatory proteins, such as histone deacetylases (HDACs) (2, 25, 26), ATP-dependent chromatin remodeling complexes (39, 43, 53), and histone methyltransferases (32).

RB is related to two other pocket domain proteins, p107 and p130, that have also been shown to repress E2F target genes and inhibit cell growth when overexpressed (reviewed in references 12 and 29). The different pocket domain proteins are utilized at distinct phases of the cell cycle to control transcription of E2F target genes (27, 42), suggesting that RB, p107, and p130 have specialized nonredundant functions for controlling gene expression. Interestingly, the minimal regions of RB required for RNA polymerase II transcriptional repression are different from those essential for tumor suppression. RB con-

tains 928 amino acids and can be functionally divided into at least three regions: the N-terminal region between amino acids 1 and 378, the A/B pocket domain region between amino acids 393 and 772, and the C region between amino acids 768 and 869 (47, 48). The region between amino acids 379 and 792 is sufficient to recruit HDAC activity and repress RNA polymerase II transcription (26). However, the region of RB between amino acids 379 and 928 encompassing the A, B, and C domains appears to be the minimal region necessary for tumor suppression (52). When reintroduced into RB^{-/-} mice, this same region of RB rescued the lethal phenotype and allowed mice to develop normally (52). These differences suggest that additional functions beyond RNA polymerase II repression may contribute to tumor suppression by RB.

In addition to regulating transcription of key protein-encoding genes by RNA polymerase II, RB family members can also repress transcription by RNA polymerases I (3) and III (41, 51). It was proposed that RB represses transcription of key biosynthetic genes by RNA polymerases I and III to control growth (49). Furthermore, RB may play an important role in regulating global RNA polymerase III transcription, as suggested by the observations that RB overexpression during transient transfection inhibits RNA polymerase III transcription, and transcript levels of diverse RNA polymerase III-transcribed genes are elevated in RB^{-/-} primary mouse fibroblasts relative to RB^{+/+} fibroblasts (40, 51). RNA polymerase III-regulated genes exhibit different promoter architectures and factor requirements for transcription, suggesting that RB may utilize distinct mechanisms for repression at different promoters. Genes transcribed by RNA polymerase III can be divided into three classes on the basis of their promoter architectures.

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The sole members of the class 1 group are the 5S rRNA genes, whereas class 2 genes include tRNA genes and the adenovirus (Ad) VAI gene. Members of these classes contain intragenic promoter elements. Human U6 snRNA genes are included in the class 3 group and contain extragenic promoter elements more resembling promoters of genes transcribed by RNA polymerase II (reviewed in reference 36). This diversity presents a puzzle as to how RB can recognize and regulate different RNA polymerase III-transcribed genes. The RNA polymerase III-specific factor TFIIB, which is an essential factor required for most RNA polymerase III transcription, is one proposed target for RB (7, 22, 50). TFIIB is recruited to the promoters of class 1 and 2 genes by the TFIIC complex, which directly recognizes the promoters of these genes (see reference 36). TFIIB is the initiation factor that recruits RNA polymerase III (20). RB can disrupt interactions between TFIIB and TFIIC (40) and may prevent preinitiation complex assembly at some RNA polymerase III-transcribed genes. Human TFIIB is composed of the TATA box binding protein (TBP) and at least two additional TBP-associated factors called Brf1 (28, 44) and Bdp1 (37). Within TFIIB, the Brf1 component is one target for RB (22, 40).

In contrast to most RNA polymerase III-transcribed genes, human U6 snRNA gene transcription does not require Brf1 (28) and yet is regulated by RB both in vitro (19) and in vivo (40). Thus, additional factors must be important for RB regulation of these genes. One candidate is a multiprotein complex called the snRNA-activating protein complex (SNAP_C) (35) that is also known as the proximal sequence element transcription factor (30). SNAP_C binds to the essential proximal sequence element common to human snRNA gene promoters. Endogenous RB associates with SNAP_C during both coimmunoprecipitation and chromatographic purification of SNAP_C, and RB can interact with the SNAP43 and SNAP50 subunits of SNAP_C (19). Although SNAP_C is important both for human U6 transcription and RB repression, additional data suggested that TBP or TBP-containing complexes likely contribute to RB regulation of these genes (19). In this study, we found that RB occupies a human U6 snRNA promoter simultaneously with SNAP_C and TFIIB potentially mediated by interactions with multiple components of each complex. Unexpectedly, RB and RNA polymerase III also co-occupy a U6 snRNA promoter during repression, suggesting that RB does not necessarily disrupt preinitiation complex assembly to prevent RNA polymerase III recruitment. Together, these observations suggest a novel mechanism for RB repression in which RB likely interferes with critical steps in transcription that occur subsequently to RNA polymerase recruitment.

MATERIALS AND METHODS

Recombinant protein expression and purification. Recombinant SNAP43, SNAP50, and SNAP190 (1-505) were expressed in *Escherichia coli*, assembled into mini-SNAP_C, and further purified as described previously (18). The purified mini-SNAP_C was estimated to contain approximately 5 ng of each SNAP_C protein/μl. TBP was expressed as a glutathione *S*-transferase (GST) fusion protein as previously described (18). Recombinant Brf2 and Bdp1 were expressed by using the pSBET-hBRFU and pSBET-hB' expression plasmids, respectively (37). Recombinant GST-RB (379-928) and various truncated derivatives were expressed and purified as described previously (19).

Tissue culture. Human mammary epithelial cells (184B5) were a gift from Susan Conrad (Michigan State University). Normal human foreskin cells

(NFSL83) were a gift from Justin McCormick (Michigan State University). Cells were maintained in Dulbecco's minimum essential media (Gibco) containing 10% fetal bovine serum (Gibco), 200 mM glutamine, and penicillin-streptomycin at 37°C with 5% CO₂.

Antibodies. Anti-SNAP43 (CS48) and anti-TBP (SL2) antibodies have been described previously (16, 24). The anti-Bdp1 (CS913), anti-Brf1/Brf2 (CS1043), and anti-RNA polymerase III (CS377) antibodies were gifts from Nouria Hernandez (Cold Spring Harbor Laboratory). The mouse anti-RB antibodies (G3-245) used for Western analysis were purchased from Pharmingen. The anti-RB (C-15) and control immunoglobulin G (IgG) antibodies used for chromatin immunoprecipitation (ChIP) experiments were purchased from Santa Cruz Biotechnology. The anti-RNA polymerase II antibodies (8WG16) are from Covance Research Products.

ChIP. ChIPs were performed as described previously (1). Human cells were grown to approximately 75% confluence and then cross-linked with formaldehyde for 30 min. Cells were washed sequentially in buffer I (10 mM HEPES [pH 6.5], 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100), buffer II (10 mM HEPES [pH 6.5], 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl), and then twice with ice-cold phosphate-buffered saline (10 mM NaH₂PO₄, 150 mM NaCl [pH 7.4]). The cell pellet was then suspended in lysis buffer (50 mM Tris [pH 8.0], 10 mM EDTA, 1% sodium dodecyl sulfate [SDS], 0.5 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 mM sodium bisulfite, 1 mM benzamide, 1 mM dithiothreitol [DTT]) to a density of 10⁸ cells per ml. After cell lysis and sonication, ChIPs were performed with 1 μg of each antibody overnight at 4°C and chromatin was harvested from the equivalent of 10⁷ cells per immunoprecipitation. Immunoprecipitations were performed with ChIP dilution buffer (20 mM Tris [pH 8.0], 2 mM EDTA, 1% Triton X-100, 0.5 μM phenylmethylsulfonyl fluoride, 1 mM DTT) containing 400 mM NaCl. Protein G-agarose beads were then added, and the mixture was incubated for 3 h at 4°C. The immunoprecipitated material was washed sequentially in TSE buffer (20 mM Tris [pH 8.0], 0.1% SDS, 2 mM EDTA, 1% Triton X-100), TSE buffer plus 250 mM NaCl, and TSE buffer plus 500 mM NaCl and finally in buffer III (10 mM Tris [pH 8.0], 1 mM EDTA, 0.25 M LiCl, 1% NP-40, 1% deoxycholate). Each wash step was done for 10 min at room temperature. Beads were then washed an additional three times in TE buffer (20 mM Tris [pH 8.0], 2 mM EDTA), and protein complexes were eluted in 300 μl of 0.1 M NaHCO₃-1% SDS for 30 min at room temperature. Cross-links were reversed overnight at 65°C, and the recovered chromatin was suspended in 50 μl of TE buffer. PCR analysis was performed with 5 μl of each immunoprecipitated sample or input chromatin. The primers used to amplify the genes were U6 forward (GTACAAAATACGTGACGTAGAAAG), U6 reverse (GGTGTTCGTCCTTTCCAC), U1 forward (CACGAAGGAGTTCCTCG TG), U1 reverse (CCCTGCCAGGTAAGTATG), U2 forward (AGGGCGTC AATAGCGCTGTGG), U2 reverse (TGCCTCGCCTTCGCGCCCGCCG), Cyclin A forward (CTGCTCAGTTTCCTTTGGTTTAC), Cyclin A reverse (A AAGACGCCCAGAGATGCAGC), GAPDH forward (AGGTATCCCTGA GCTGAAC), GAPDH reverse (GCAATGCCAGCCCGAGCGTC), tRNA-Lys forward (GGTTTCCTCAAGGAGGGGG), and tRNA-Lys reverse (GCCCC GATAGCTCAGTCGGTAG).

PCR products were separated by 2% Tris-borate-EDTA-agarose electrophoresis, stained with ethidium bromide, and visualized with Kodak imaging software.

Sequential ChIPs. Soluble chromatin was prepared from human 184B5 cells as described above. Primary immunoprecipitations were performed with 5 μg of anti-RB, anti-SNAP43, anti-RNAPII, and IgG antibodies with chromatin prepared from the equivalent of ~5 × 10⁷ cells per immunoprecipitation for 1 h at room temperature. Reaction mixtures were then incubated with protein G-agarose for an additional hour at room temperature. After extensive washing, precipitated protein-DNA complexes were eluted in ChIP dilution buffer containing 15 mM DTT at room temperature for 30 min. One-sixth of the recovered material was used for each secondary ChIP, and the recovered material was subsequently processed as described above for the ChIPs.

Immunoprecipitation of formaldehyde cross-linked in vitro transcription reaction mixtures. In vitro transcription reactions were performed as described previously (19, 35), except that transcription was carried out for 15 min rather than 1 h and 0.25 μg of pUC119 was added to each reaction mixture to serve as an internal negative control for DNA recovery. For each condition, 20-μl transcription reaction mixtures were prepared in triplicate and pooled. One-third was processed by T1 RNase protection to measure transcription. The remaining two-thirds was diluted to 1 ml with ChIP dilution buffer and cross-linked in 1% formaldehyde for 15 min at room temperature prior to quenching with 0.125 M glycine. Ten microliters of each cross-linked reaction mixture was used as starting material to perform pulldown experiments with glutathione-agarose beads (see Fig. 3) or sequential immunoprecipitations (see Fig. 4). Recovered DNA was

measured by PCR with the primers U6 forward (GTACAAAATACGTGACG TAGAAAAG), U6 reverse (GGTGTTCGTCCTTCCAC), VAI forward (TC CGTGGTCTGGTGG), and VAI reverse (CGGGGTTCGAACCCGG).

PCR products were separated by 2% Tris-borate-EDTA-agarose electrophoresis, stained with ethidium bromide, and visualized with Kodak imaging software.

Coimmunoprecipitation and GST pulldown experiments. Coimmunoprecipitations were performed as previously described (19) with anti-Bdp1 (CS913) or anti-BRF1/BRF2 (CS1043) antibodies (37). GST pulldown assays were performed as previously described (19), with approximately 1 μ g of each GST-tagged protein.

RT-ChIP. Soluble chromatin was prepared from human 184B5 cells as described above. For the experiment shown in Fig. 5C, the primary immunoprecipitation was performed with 30 μ l of anti-RNA polymerase III antibody (CS377; N. Hernandez) incubated with chromatin prepared from the equivalent of $\sim 5 \times 10^7$ cells per immunoprecipitation overnight at 4°C with the addition of 400 U of RNase inhibitor (Roche). Reaction mixtures were then incubated with protein G-agarose for 3 h at room temperature. After extensive washing, precipitated protein-DNA complexes were eluted in ChIP dilution buffer containing 15 mM DTT at room temperature for 30 min. One-third of the recovered material was used for each secondary ChIP, and reaction mixtures were processed as described above. Recovered chromatin and RNA were dissolved in 100 μ l of TE buffer containing 200 U of RNase inhibitor. One-twentieth of these samples was analyzed by PCR for enrichment of the U6 snRNA promoter and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) exon 2 DNA. The remaining sample was treated with 15 U of RNase-free DNase (Roche) at 37°C for 1 h. The DNase was then heat inactivated for 15 min at 70°C. Approximately 5 μ l of each immunoprecipitate and input sample was incubated in the presence and absence of murine leukemia virus reverse transcriptase (Invitrogen) and a primer corresponding to +65 of the U6 snRNA gene. Subsequently, 2 μ l of each reverse transcription (RT) reaction was analyzed by 30 to 35 cycles of PCR with +1 forward and +65 reverse U6 primers.

RESULTS

Endogenous RB occupies a human U6 snRNA promoter. We have previously shown that RB represses U6 snRNA gene transcription by RNA polymerase III (19). To determine whether RB directly occupies human U6 snRNA promoters in the cell, ChIP experiments were performed with human mammary epithelial cells (cell line 184B5) (38) and antibodies directed against RB or SNAP_C. Recovered DNA segments were analyzed by PCR with primers specific to the human U6 snRNA promoter, as well as to the U1 and U2 snRNA promoters. Primers specific to GAPDH exon 2 were used as a negative control. As shown in Fig. 1A, U6 promoter DNA was specifically immunoprecipitated with anti-RB (lane 5) and anti-SNAP_C (lane 7) antibodies. These levels are higher than that of the U6 promoter DNA recovered with IgG (lane 6). These observations suggest that RB is present at this human U6 gene promoter in these cells. In contrast, U1 and U2 promoter enrichment in the anti-RB-immunoprecipitated samples was not significantly higher than that observed for the GAPDH negative control. This observation suggests that RB does not associate with these promoters. To determine whether RB occupies this U6 promoter in different cell types, a variety of both normal and transformed cells were analyzed (Fig. 1B). As expected, RB occupies the U6 promoter but not the U2 promoter in mammary epithelial cells (cell line 184B5, top part), as demonstrated previously. These cells are immortalized but not transformed and do not initiate tumors in nude mice (38). Interestingly, RB also occupies the U6 promoter in normal foreskin fibroblast cells (cell line NFSL83, second part) and MCF-7 breast adenocarcinoma cells (third part), both of which maintain functional RB. In contrast, RB is not observed at the U6 promoter in HeLa cervical carcinoma cells (bottom

part), which exhibit dysfunctional RB activity owing to the human papillomavirus E7 protein. Thus, RB occupies this U6 promoter in a variety of different cells and U6 regulation may be lost in certain cancer cells upon loss of RB activity.

The specific association of RB with a human U6 promoter but not with the U1 or U2 snRNA promoter raised the possibility that RB occupancy of snRNA promoters correlates with the ability of RB to repress gene expression. To determine whether RB represses the expression of both RNA polymerase II- and III-transcribed snRNA genes, *in vitro* repression assays were performed. A schematic representation of the promoters tested is shown in Fig. 1C. For these *in vitro* repression experiments, HeLa cell extracts were preincubated with increasing amounts of GST-RB containing amino acids 379 to 928, hereafter referred to as GST-RB, or GST proteins. As shown in Fig. 1D, addition of GST-RB, did not repress RNA polymerase II transcription from either the U1 snRNA or the AdML promoter. In contrast, GST-RB decreased RNA polymerase III transcription from the AD VAI and U6 snRNA promoters, consistent with our previous observations (19). Thus, although the U1 and U6 snRNA genes have similar functional promoter elements, RB preferentially regulates U6 snRNA transcription by RNA polymerase III. These *in vitro* transcription results are also consistent with the observation that RB is present at an endogenous human U6 promoter but not at the U1 and U2 promoters *in vivo*.

Repression of RNA polymerase III transcription requires the RB A/B pocket domain and C region. RB regulates the cell cycle by controlling RNA polymerase II transcription of key genes that contain promoter binding sites for the E2F transcriptional activator (13). RB repression of E2F-stimulated transcription minimally requires the A/B pocket domain, which also is sufficient for HDAC recruitment (6, 26). To identify the extent of the RB pocket domain required for RNA polymerase III repression, mutant RB proteins that contain the A domain but lack regions of the carboxy terminus were analyzed by *in vitro* repression assays. A schematic representation of the truncated GST-RB proteins is shown in Fig. 2A. Each GST-RB protein was purified to near homogeneity as visualized by SDS-polyacrylamide gel electrophoresis (PAGE) analysis and subsequent staining with Coomassie blue (Fig. 2B). Purified RB proteins were then tested for the ability to repress human U6 snRNA and Ad VAI transcription *in vitro*. As shown in Fig. 2C, addition of increasing amounts of GST-RB (379-928) (lanes 2 and 3) significantly decreased the amount of transcription correctly initiated from the U6 snRNA and VAI promoters compared to the untreated extract (lane 1) or extract treated with GST (lane 10). Lower but significant repression of both genes was observed with GST-RB lacking the extreme carboxy terminus (lanes 4 and 5). However, GST-RB containing the A/B pocket but lacking both the C domain and extreme carboxy terminus (lanes 6 and 7) was unable to repress RNA polymerase III transcription of either gene, even though A/B pocket integrity should still be maintained in this mutant protein. In fact, truncated RB containing only amino acids 379 to 772 is capable of repressing RNA polymerase II transcription (6, 26). Similarly, GST-RB containing only the A domain was ineffective for repression in these assays (lanes 8 and 9). Together, these results suggest that the A/B pocket and C region of RB are necessary for repression of RNA polymerase III

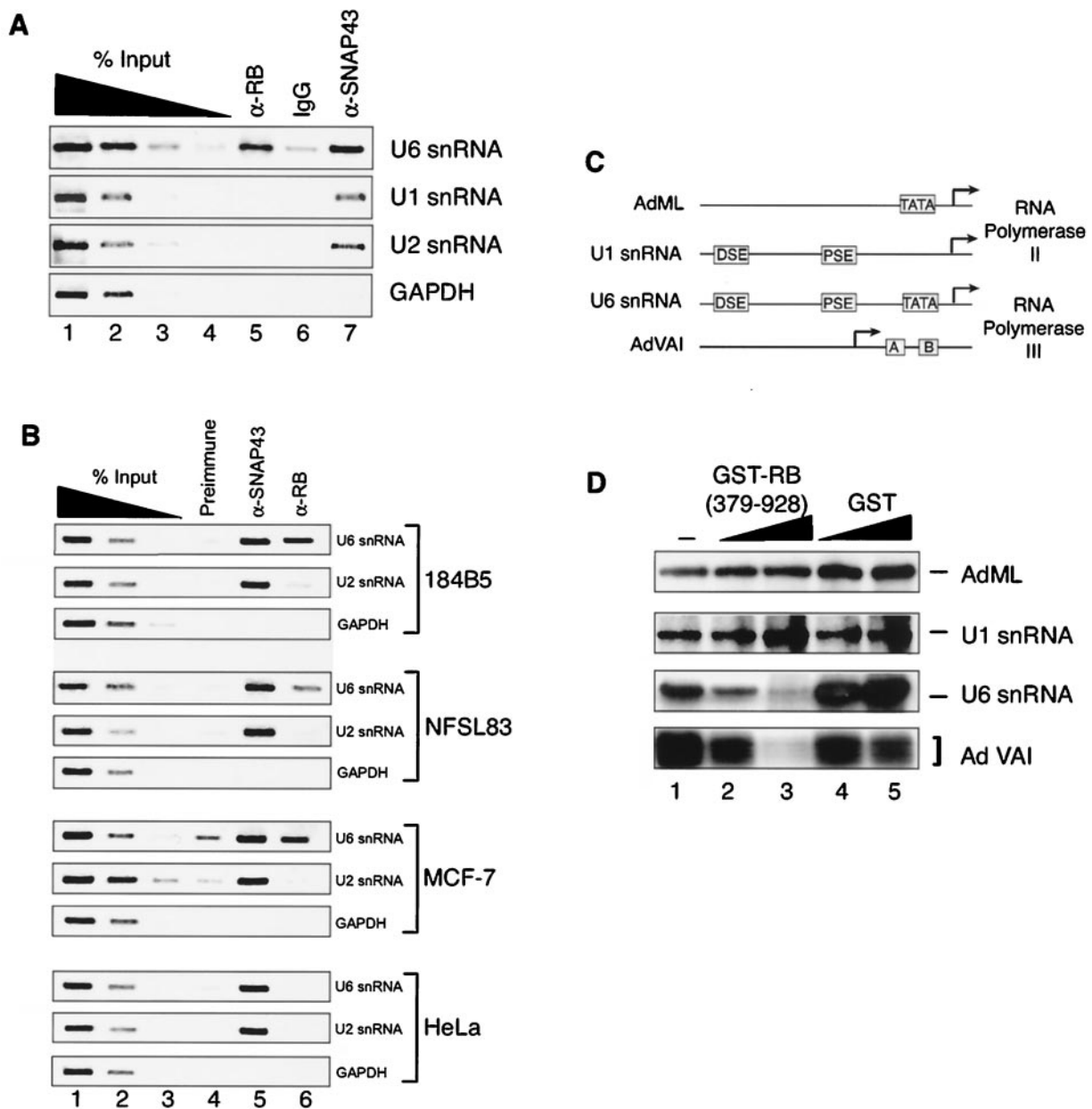


FIG. 1. RB selectively occupies U6 snRNA promoters in vivo and specifically represses U6 snRNA transcription in vitro. (A) RB occupies a human snRNA promoter in vivo. ChIP experiments from human 184B5 cells were performed with anti-RB (lane 5), anti-SNAP43 (lane 7), and nonspecific IgG (lane 6) antibodies. Precipitated DNAs were analyzed by PCR for enrichment of U6 snRNA, U1 snRNA, and U2 snRNA promoters or GAPDH exon 2 as a negative control. Lanes 1 to 4 show a 10-fold serial dilution of input chromatin from 10 to 0.01%. (B) RB occupies the U6 promoter in a variety of cell types. ChIPs from nontumorigenic human mammary epithelial cells (184B5), normal human fibroblast cells (NFSL83), human mammary epithelial adenocarcinoma cells (MCF-7), and human cervical carcinoma cells (HeLa) were performed with preimmune sera (lane 4) or anti-SNAP43 (lane 5) or anti-RB (lane 6) antibodies. Enrichment of U6 snRNA promoter, U2 snRNA promoter, or GAPDH exon 2 DNA in each immunoprecipitation reaction mixture was determined by PCR with a primer combination specific to each gene. Lanes 1 to 3 illustrate a 10-fold serial dilution (1 to 0.01%) of input chromatin. (C) Schematic representation of selected RNA polymerase II and RNA polymerase III promoters used for in vitro transcription assays. (D) RB represses U6 snRNA gene transcription by RNA polymerase III but not U1 snRNA transcription by RNA polymerase II. In vitro transcription assays for the RNA polymerase II and RNA polymerase III-transcribed genes were performed with HeLa cell nuclear extract as described previously (15, 16). Lanes 2 and 3 were treated with 200 and 800 ng of GST-RB (379-928), respectively. Lanes 4 and 5 were treated with 200 and 800 ng of GST, respectively, as a negative control.

transcription. Interestingly, this corresponds to the region of RB that is required for growth suppression (52).

One possible explanation for the lack of RNA polymerase III repression by mutant RB proteins is that interactions between RB and various components of the general transcription

machinery are disabled. It was previously observed that RB can interact with SNAP_C (19) and TFIIIB (7, 22). Therefore, to correlate the regions of RB that are important for RNA polymerase III repression with requisite regions for interaction with these transcription factors, GST pulldown experiments

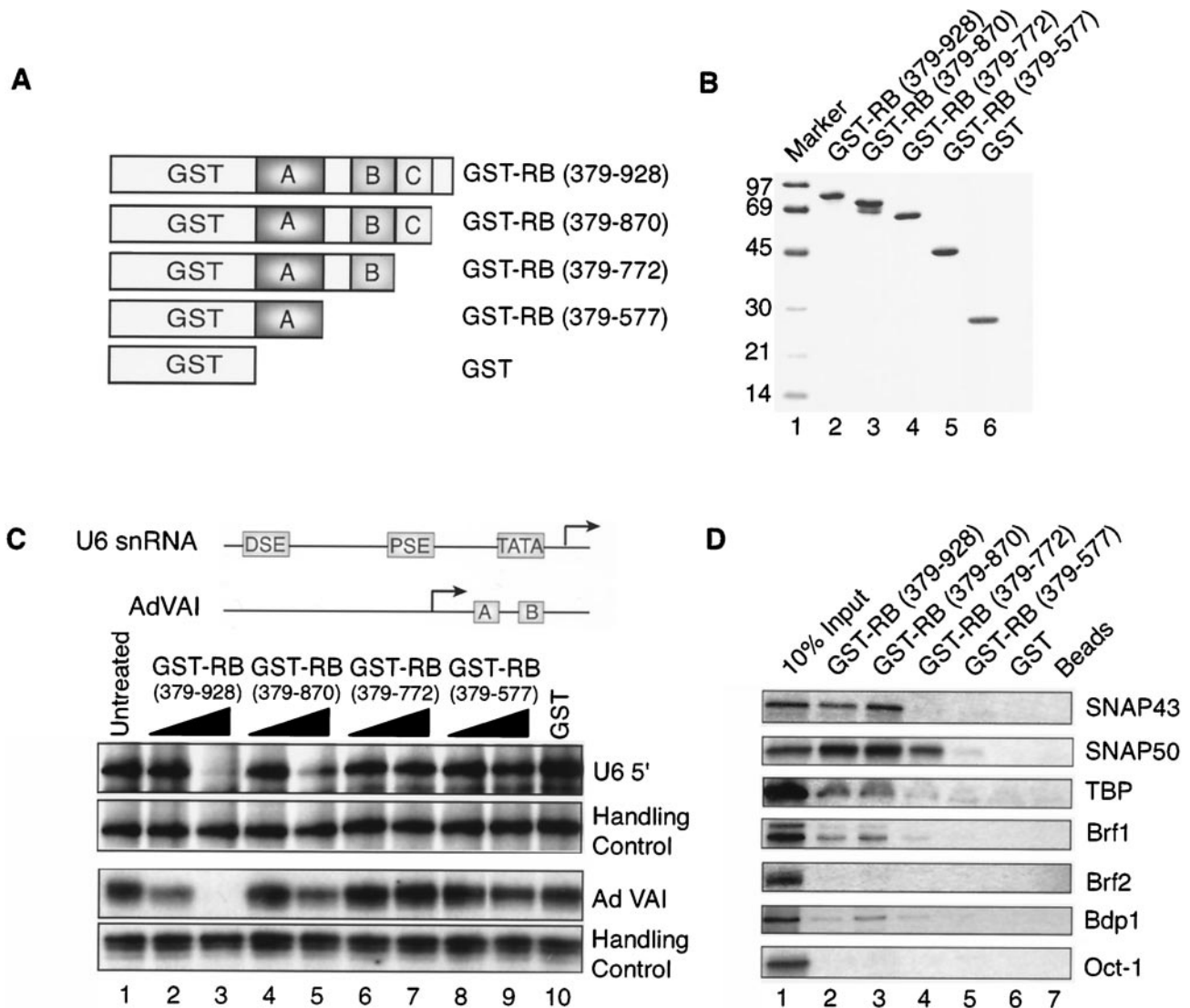


FIG. 2. The A/B pocket domain alone is insufficient for efficient repression of RNA polymerase III transcription. (A) Schematic representation of the truncated RB proteins used in these experiments. (B) Analysis of truncated GST-RB proteins. GST-RB (379-928) (lane 2), GST-RB (379-870) (lane 3), GST-RB (379-772) (lane 4), GST-RB (379-577) (lane 5), and GST (lane 6) were expressed in *E. coli* and purified by affinity chromatography with glutathione-agarose beads and competitive elution with glutathione. Purified proteins were dialyzed against Dignam buffer D, and aliquots were separated by SDS-PAGE and visualized by staining with Coomassie blue. Lane 1 contains a protein molecular size standard. (C, top) Schematic representation of the human U6 snRNA and Ad VAI promoters. (Bottom) GST-RB (379-928) and GST-RB (379-870) repress human U6 snRNA and VAI transcription by RNA polymerase III. Approximately 2 μ l of HeLa cell nuclear extract (approximately 7.5 μ g/ μ l) was incubated with 250 and 1,000 ng of GST-RB (379-928) (lanes 2 and 3), GST-RB (379-870) (lanes 4 and 5), GST-RB (379-772) (lanes 6 and 7), GST-RB (379-577) (lanes 8 and 9), or GST protein (lane 10). With separate reaction mixtures, human U6 snRNA or Ad VAI transcription in the treated extracts was performed at 30°C for 30 min. Lane 1 shows the level of transcription for each gene supported by the untreated extract. Sample handling was monitored by the presence of a nonspecific RNA handling control. (D) Characterization of the RB regions required for interactions with the RNA polymerase III basal machinery. GST pull-down analysis was performed to determine the region of RB that can interact with each component of the RNA polymerase III basal machinery. SNAP43, SNAP50, TBP, Brf1, Brf2, Bdp1, and Oct-1 were expressed in vitro and labeled with [³⁵S]methionine. Ten percent of each in vitro-expressed protein added to the reaction mixtures is shown in lane 1. Each protein was incubated with the various truncated GST-RB proteins or with GST, and stable protein complexes were purified with glutathione-Sepharose. The beads were extensively washed, and bound proteins were separated by SDS-PAGE. Associated proteins were visualized by autoradiography.

were performed. As shown in Fig. 2D, strong interactions were observed between GST-RB (379-928) and two components of SNAP_C, SNAP43 and SNAP50. Specific pairwise interactions between GST-RB (379-928) and the TBP, Brf1, and Bdp1 components of TFIIIB were also observed. However, these interactions with TFIIIB components appear to be markedly weaker than those observed with SNAP_C components. In con-

trast, GST-RB (379-928) did not interact with Brf2 or Oct-1 in these assays. Interestingly, GST-RB (379-870), which repressed RNA polymerase III transcription in the above-described repression assays, maintained the ability to interact with these SNAP_C and TFIIIB target proteins, but GST-RB (379-772), which did not repress RNA polymerase III transcription, also exhibited diminished interactions with SNAP43,

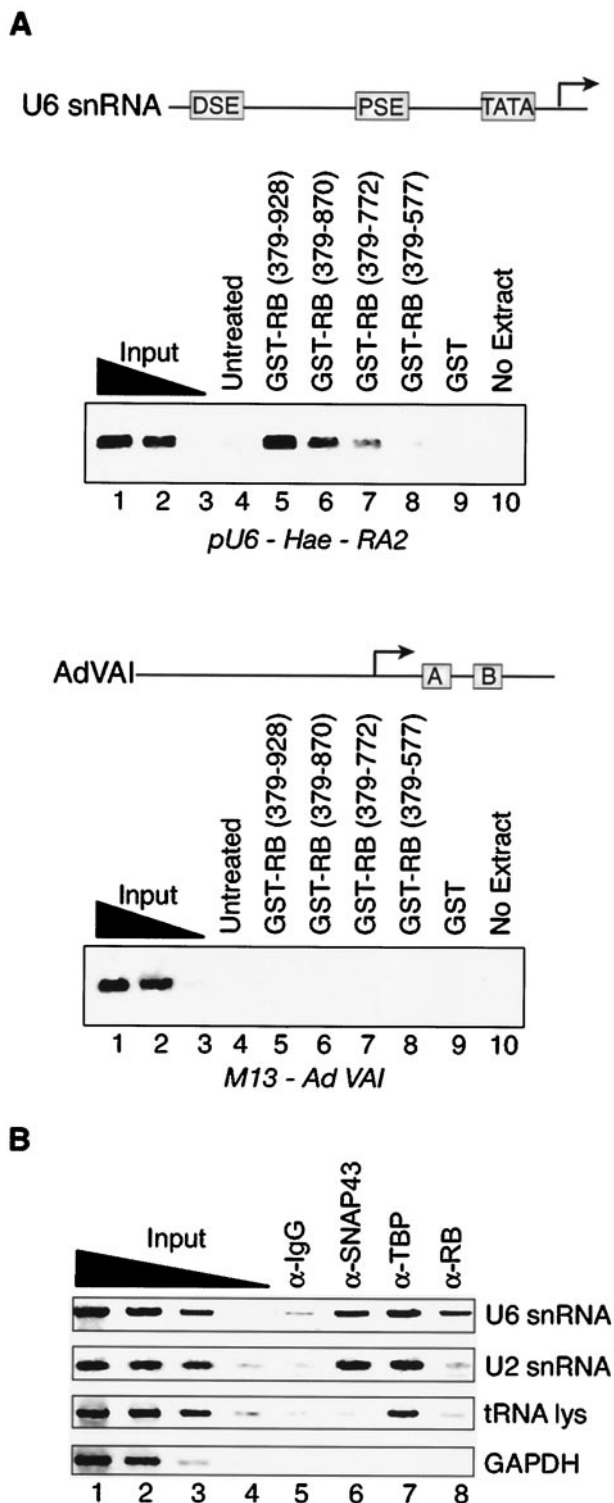


FIG. 3. RB occupies a human U6 snRNA but not Ad VAI promoter during repression. (A) Affinity purification of GST-RB proteins and associated promoter-containing DNAs from *in vitro* RB repression assays. Portions of the human U6 snRNA and Ad VAI transcription assays shown in Fig. 2C were cross-linked with formaldehyde prior to affinity purification with glutathione-Sepharose to recover the GST-RB proteins. The concomitant recovery of associated promoter plasmid DNA was then measured by PCR. The recovered samples were analyzed for the presence of the U6 reporter construct (pU6/Hae/RA.2) or the Ad VAI reporter construct (M13-Ad VAI) with primers

TBP, Bdp1, and Brf1. Thus, these interactions may be important for RB repression of RNA polymerase III transcription. In contrast, GST-RB (379-772) interacted strongly with SNAP50, suggesting that this interaction alone is insufficient to maintain RB repression. It is possible that RB interacts with SNAP50 through the SNAP50 LxCxE motif, which in other RB-interacting proteins has been shown to bind to the B domain of RB (5, 8, 21, 23). Finally, none of the SNAP_C or TFIIIB proteins interacted with GST-RB (379-577), which contains only the A domain. These observations indicate that the A/B pocket domain plus the C region is important for interactions with most RB target proteins within the RNA polymerase III-specific general transcription machinery.

RB represses different RNA polymerase III-transcribed genes by distinct mechanisms. It is possible that RB interacts with the RNA polymerase III general transcription machinery to specifically target appropriate gene promoters. To ascertain whether RB repression activity correlates with the ability to occupy a target RNA polymerase III-transcribed gene, portions of the RB repression assays shown in Fig. 2C were cross-linked with formaldehyde. Subsequently, affinity purification from these reaction mixtures was performed with glutathione-Sepharose to recover the GST-RB proteins and the concomitant recovery of promoter DNA was then measured by PCR. The recovered samples were analyzed for the presence of the U6 reporter construct (pU6/Hae/RA.2) or the Ad VAI reporter construct (M13-Ad VAI) with primers specific to each of these plasmids. As shown in Fig. 3A, significant levels of U6 promoter DNA were recovered from transcription reaction mixtures containing GST-RB (379-928) compared to the untreated or GST-treated reaction mixtures (compare lane 5 to lanes 4 and 9). Additionally, GST-RB (379-870) also associates with the U6 reporter construct (lane 6), although to a lesser extent, suggesting that the diminished ability to repress transcription may be due to a lower affinity for the U6 promoter. This result suggests that the extreme carboxy terminus of RB contributes to the regulation of U6 gene transcription. Interestingly, only relatively small amounts of GST-RB (379-772) were detected on the U6 reporter construct (lane 7), suggesting that this protein is defective for efficient U6 promoter recruitment. The mutant RB protein containing amino acids 379 to 577 did not demonstrate significant promoter occupancy, consistent with its inability to repress transcription. In contrast, none of the GST-RB proteins occupied the Ad VAI reporter constructs. The lack of detectable Ad VAI plasmid DNA is consistent with the previously suggested hypothesis that RB represses Ad VAI transcription in a distinct fashion by binding to TFIIIB and disrupting preinitiation complex assembly (40).

The results described above imply that RB may use funda-

specific to the promoters contained in these plasmids. (B) RB occupies an endogenous human U6 snRNA (class 3) promoter but not a tRNA^{Lys} (class 2) promoter *in vivo*. ChIP experiments were performed similarly to those described previously. A 10-fold serial dilution of input chromatin, from 10 to 0.01%, is shown in lanes 1 to 4. Immunoprecipitation reaction mixtures were prepared with IgG (lane 5), anti-SNAP43 (lane 6), anti-TBP (lane 7), or anti-RB (lane 8) antibodies, and any recovered DNA was analyzed with primers specific for the U6 snRNA, U2 snRNA, and tRNA^{Lys} promoters or GAPDH exon 2 as a negative control.

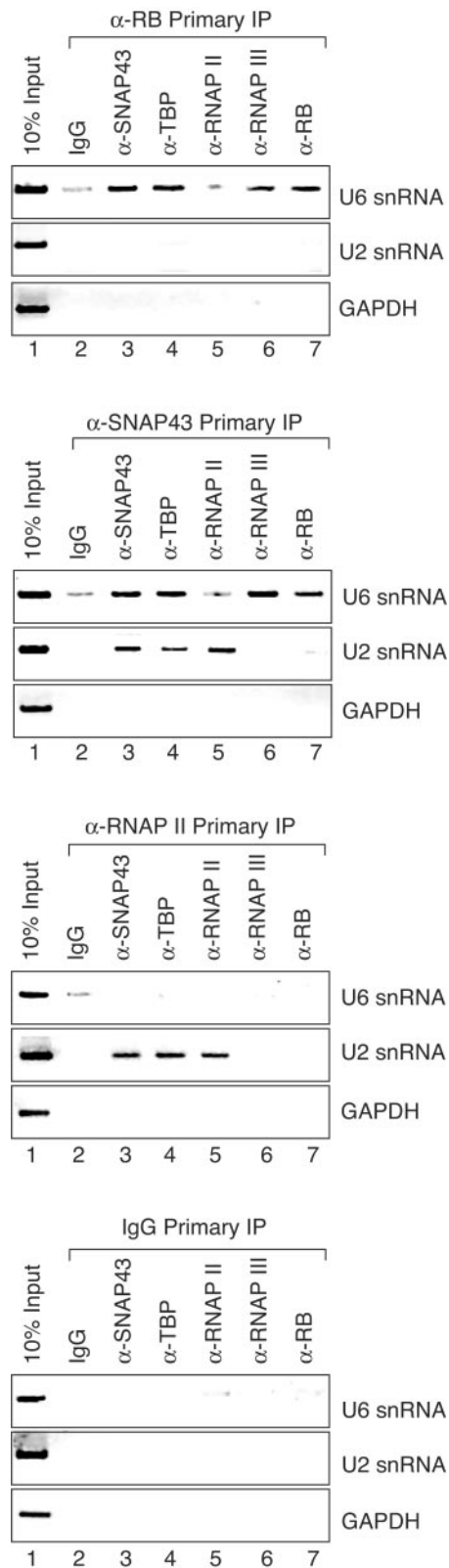


FIG. 4. RB co-occupies the same endogenous U6 snRNA promoter as SNAP_C, TFIIIB, and RNA polymerase III. Sequential ChIPs were performed from human 184B5 cells with anti-RB (top part), anti-SNAP43 (second part), anti-RNA polymerase II (third part), and IgG (bottom part) for the first round of immunoprecipitation (IP), followed by a second immunoprecipitation with the indicated antibodies.

mentally different mechanisms to regulate in vitro transcription of class 2 versus class 3 genes (e.g., Ad VAI and human U6 snRNA, respectively). To further examine the mechanisms used for repression of endogenous class 2 and class 3 genes, the in vivo RB occupancies of a tRNA^{Lys} (class 2) promoter and a U6 snRNA (class 3) promoter were analyzed by ChIP (Fig. 3B). The tRNA^{Lys} gene was selected for analysis as an example of an endogenous class 2 gene because its promoter architecture is similar to that of the Ad VAI gene and it was previously shown to be active for transcription (9). The immunoprecipitation reaction mixture prepared with anti-SNAP43 antibodies was significantly enriched for U6 snRNA and U2 snRNA promoter DNA but not for tRNA^{Lys} or GAPDH exon 2 DNA compared to IgG precipitation. This result was expected because SNAP_C functions for snRNA but not tRNA transcription. Similarly, immunoprecipitation reaction mixtures prepared with anti-TBP antibodies were enriched for U6 snRNA, U2 snRNA, and tRNA^{Lys} promoter DNA, consistent with a role for TBP in the transcription of these genes. In contrast, only U6 snRNA but not tRNA^{Lys} promoter DNA was enriched in immunoprecipitation reaction mixtures prepared with anti-RB antibodies. Detection of endogenous RB at a U6 snRNA gene but not at a tRNA gene in these ChIPs correlates with the results seen for the in vitro repression assays. Together, these results suggest that RB may regulate RNA polymerase III gene expression by two different mechanisms.

RB and RNA polymerase III co-occupy a human U6 promoter during repression. The data above imply that RB can interact with TFIIIB and SNAP_C to target a U6 snRNA promoter. To establish whether these factors co-occupy the same promoter in vivo and whether RB precludes binding of RNA polymerase III to the U6 snRNA promoter, sequential ChIPs were performed (Fig. 4). Soluble chromatin fractions were subjected to a primary anti-RB immunoprecipitation (top part), and the recovered precipitated material was subsequently re-immunoprecipitated with antibodies specific to the transcription machinery involved in snRNA gene expression, antibodies specific to RB, or control IgG antibodies. As expected, secondary immunoprecipitation reaction mixtures prepared with anti-RB antibodies (lane 7) were significantly enriched for U6 promoter DNA compared to reaction mixtures prepared with IgG (lane 2) or RNA polymerase II (lane 5). Secondary immunoprecipitation reaction mixtures prepared with anti-SNAP43 (lane 3) and anti-TBP (lane 4) antibodies were enriched for U6 promoter DNA, consistent with the idea that GST-RB co-occupies the U6 promoter with SNAP_C and TFIIIB. Of considerable interest was the enrichment of U6 promoter DNA in the anti-RNA polymerase III secondary immunoprecipitation reaction mixture. None of the secondary immunoprecipitation reaction mixtures were significantly enriched for U2 snRNA promoter DNA or GAPDH exon 2 DNA, suggesting that the observed association between RB and RNA polymerase III with this human U6 promoter is specific.

Chromatin recovered after the second immunoprecipitation was analyzed by PCR for enrichment of U6 and U2 promoter DNA or GAPDH exon 2 DNA as a negative control. Lane 1 shows 10% of the input chromatin.

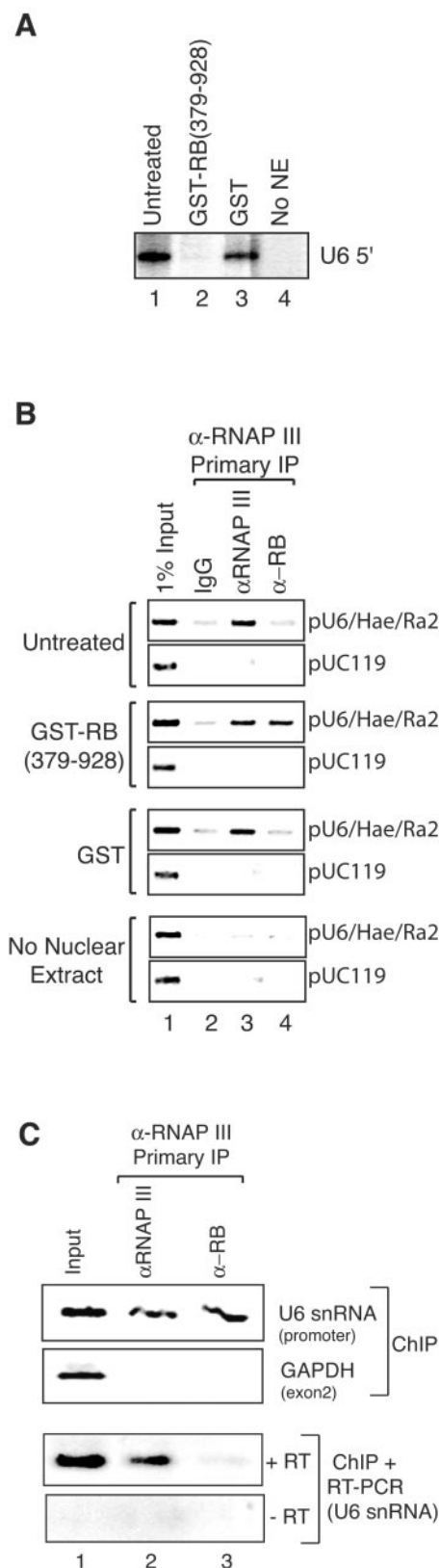


FIG. 5. RB and RNA polymerase III co-occupy the same repressed U6 snRNA promoter in vitro and in vivo. (A) In vitro U6 snRNA transcription reaction mixtures were prepared with untreated HeLa cell nuclear extracts (NE; lane 1) or extract treated with 500 ng of GST-RB (379-928) or GST (lanes 2 and 3, respectively). The reaction

Similar sequential ChIP assays were then performed with anti-SNAP43 antibodies in the first round of immunoprecipitation (second part). As expected, a secondary anti-RNA polymerase III immunoprecipitation reaction mixture was enriched for U6 but not U2 snRNA promoters (lane 6) whereas an anti-RNA polymerase II immunoprecipitation reaction mixture was enriched for U2 but not U6 snRNA promoters (lane 5). Furthermore, secondary immunoprecipitation reaction mixtures prepared with either anti-SNAP43 or anti-TBP antibodies were enriched for both U6 and U2 snRNA promoters, consistent with the suggestion that these factors function for transcription of human snRNA genes by both RNA polymerases II and III (15, 16). Importantly, secondary immunoprecipitation reaction mixtures prepared with anti-RB antibodies were enriched for U6 but not U2 snRNA promoter sequences. In contrast, none of the secondary immunoprecipitation reaction mixtures were enriched for U6 DNA when primary immunoprecipitation reaction mixtures were prepared with anti-RNA polymerase II or IgG antibodies (third and fourth parts, respectively). RNA polymerase II was found simultaneously with SNAP43 and TBP, but not with RB, on U2 promoters. These results suggest that RB does not necessarily preclude binding of SNAP_C, TFIIIB, or RNA polymerase III to an endogenous U6 snRNA gene promoter. This result is unexpected because previous models suggested that RB functions, either directly or indirectly, by preventing RNA polymerase access to promoter DNA.

To directly test whether RB precludes RNA polymerase III promoter association during repression, in vitro U6 transcription reaction mixtures were prepared with HeLa cell nuclear extracts that were left untreated or treated with either GST-RB or GST. Portions of these reaction mixtures were analyzed by RNase protection assays for full-length transcripts driven from the U6 promoter, while the remainder was subjected to formaldehyde cross-linking, followed by anti-RNA polymerase III immunoprecipitation. Secondary immunoprecipitation reaction mixtures were then prepared with IgG, anti-RNA polymerase III, or anti-RB antibodies. The precipitates were then analyzed for the presence of the U6 reporter construct (pU6/Hae/RA.2) or a negative control plasmid (pUC119) that was included in the original transcription reaction mixtures. Figure 5A shows that, as expected, GST-RB effectively repressed U6 transcription (lane 2) compared to the untreated extract (lane

mixtures shown in lane 4 contained no added extract. (B) Sequential cross-linked immunoprecipitations (IP) from the transcription reaction mixtures from part A were performed with anti-RNA polymerase III antibodies for the primary immunoprecipitation and IgG (lane 2), anti-RNA polymerase III (lane 3), and anti-RB (lane 4) antibodies for the secondary immunoprecipitation. PCR analysis of the recovered DNA was performed with primers specific to the U6/Hae/RA.2 reporter or pUC119, which was included as a negative control in these assays. (C) Sequential ChIPs from human 184B5 cells were performed with anti-RNA polymerase III antibodies for the primary immunoprecipitation and anti-RNA polymerase III (lane 2) or anti-RB (lane 3) antibodies for the secondary immunoprecipitation. A portion of chromatin recovered after the second round of immunoprecipitation was analyzed for enrichment of U6 snRNA promoter or GAPDH exon 2 DNA by PCR (top parts). The remaining portion of chromatin was treated with DNase I. Associated RNA was examined by PCR in the presence (+ RT) or absence (- RT) of reverse transcriptase with U6 snRNA-specific primers (bottom parts).

1) or the effect of GST (lane 3). The result of the sequential immunoprecipitations from these transcription reaction mixtures is shown in Fig. 5B. RNA polymerase III was detected at the U6 promoter-containing plasmid in transcription reaction mixtures prepared with untreated nuclear extract (top part) but not in reaction mixtures to which no extract was added (bottom part). Significantly, RNA polymerase III was also detected in this promoter DNA in transcription reactions that were completely repressed by GST-RB (second part). In the GST-RB-repressed transcription assays, RB also associated with the U6 promoter plasmid but not with the irrelevant plasmid. The pattern of factor association with the U6 promoter plasmid in the GST-treated samples (third part) was similar to that observed with samples containing the untreated nuclear extract.

To ascertain whether RB promoter occupancy correlates with U6 snRNA repression *in vivo*, enrichment of nascent U6 snRNA transcripts during sequential ChIPs was analyzed by RT-PCR as an indicator of relative transcriptional activity. For these experiments, the initial ChIP was performed with anti-RNA polymerase III antibodies. The precipitated material was then reprecipitated with antibodies specific to either RNA polymerase III or RB. First, a portion of the recovered material was analyzed by PCR for enrichment of the U6 snRNA promoter and GAPDH exon 2 DNAs. As shown in the top part of Fig. 5C, significant amounts of U6 promoter DNA were recovered in each precipitation compared to the GAPDH negative control, thus reiterating the previous result showing that RNA polymerase III and RB can co-occupy the same U6 snRNA promoter. Second, the remaining portion of the precipitated material was treated with DNase and associated RNA was then examined by reverse transcriptase treatment and PCR analysis with primers specific to the +1 and +65 positions of the U6 transcript (lower part of Fig. 5C). Identical reaction mixtures were also prepared without reverse transcriptase to ensure that the signal observed was in fact derived from RNA and not from contaminating DNA. As shown by the RT-PCRs in lanes 2 and 3, less nascent U6 snRNA transcript was recovered with RNA polymerase III that was simultaneously accompanied by RB, suggesting that RB represses U6 transcription. Together, these results indicate that RB does not necessarily displace RNA polymerase III during repression, but rather RB and RNA polymerase III can co-occupy the same repressed promoter both *in vivo* and *in vitro*. These data support a novel model in which RB impedes critical steps in transcription that occur subsequently to polymerase recruitment to the U6 promoter.

DISCUSSION

RB may regulate cell growth in part through regulation of RNA polymerase III gene expression because the nontranslated RNA molecules synthesized by RNA polymerase III provide metabolic building blocks that are required for growth (49). Repression of nontranslated RNA synthesis could also represent a significant obstacle to unrestricted proliferation during tumor progression. As shown herein, RB represses human U6 snRNA gene transcription *in vitro* and *in vivo* and associates with an endogenous U6 promoter in a variety of cells that maintain functional RB. However, RB does not as-

sociate with U6 promoter DNA in HeLa cervical carcinoma cells in which RB has been inactivated. HeLa cells also exhibit high levels of RNA polymerase III activity, perhaps owing to the lack of RB regulation. This correlation between RB status and RNA polymerase III activity is consistent with that previously observed in certain human osteosarcoma cells and in mouse RB^{-/-} knockout cells (51). Together, these results support the idea that RB plays a direct role in regulating RNA polymerase III transcription during normal growth, and regulation may be disrupted during progression of certain tumors.

Human RB is a versatile repressor protein capable of using distinct mechanisms to repress different RNA polymerase III-transcribed genes. A previously proposed model explaining RB repression of RNA polymerase III transcription suggests that RB disrupts preinitiation complex formation at tRNA genes by interacting with TFIIB (22). In this model, RB is not expected to be present at a tRNA promoter because it has sequestered TFIIB away from the target gene (40). The data presented in this study support this mechanism, as RB was not observed at a tRNA^{Lys} promoter *in vivo* or at a tRNA-like Ad VAI promoter *in vitro* under conditions of repression. In contrast, RB occupies a U6 snRNA promoter during repression. These results emphasize that RB represses class 2 and class 3 RNA polymerase III-transcribed genes by two different mechanisms. RB can repress class 2 genes through interactions with all three components of TFIIB, and these interactions may preclude subsequent preinitiation complex assembly and RNA polymerase III recruitment to these genes. In contrast, RB is recruited to the class 3 U6 genes potentially through cooperative interactions with SNAP_C and an snRNA-specific TFIIB complex. The requirement for multiple factors suggests that RB is recruited to the U6 promoter via a complex network of protein-protein interactions. Indeed, RB can interact with multiple components of each complex needed for preinitiation complex formation at the U6 snRNA gene. Both SNAP43 and SNAP50 of SNAP_C interacted strongly with RB. The TBP and Bdp1 proteins that are common in various TFIIB complexes also interacted well with RB. The suggestion that multiple contacts are important for RB repression is also supported by the structural and functional analyses of RB that indicate that the intact large pocket containing the A, B, and C domains is important both for protein-protein interactions and for transcriptional repression. The loss of U6 snRNA transcriptional repression by removal of the C pocket correlates with the loss of interactions with numerous components of the general machinery and with the lowered affinity for the U6 promoter by mutant RB. Interestingly, these same regions that are required for RNA polymerase III repression are similar to that required for tumor suppression (52). Once recruited to the promoter, RB may directly interfere with promoter escape by RNA polymerase III. However, we and others have not detected direct interactions between RB and RNA polymerase III (reference 40 and data not shown), and thus, the molecular mechanism of RB repression remains unclear.

Previous studies examining RB repression of RNA polymerase II transcription indicate that RB can recruit coregulatory factors, including HDACs (2, 25, 26), histone methyltransferases (32), and SWI/SNF ATP-dependent chromatin remodeling complexes (39, 43, 53), to repress transcription. It is possible that RB also uses these cofactors to regulate U6

transcription. The data shown in Fig. 4 and 5 also indicate that RB interferes with U6 transcription while occupying the promoter with RNA polymerase III. Thus, the putative coregulatory proteins that regulate RNA polymerase III transcription may interfere with promoter escape or elongation by RNA polymerase III. If so, the use of naked DNA templates in the U6 repression assays shown in the present study would suggest that these cofactors can perform other functions in repression besides influencing nucleosome behavior. A precedent for this suggestion has been observed for RB-HDAC1-mediated repression of RNA polymerase I transcription (33). In this case, repression is enacted by deacetylation of the critical UBF transcription factor required for preinitiation complex assembly at rRNA genes. Alternatively, RB repression might be a multi-step process involving establishment and maintenance of repression and the absence of nucleosomes in these U6 repression assays facilitates the establishment of RB repression without the necessity of invoking HDAC, SWI/SNF, or methyltransferase activity.

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