TRANSCRIPTIONAL REGULATION OF RNA POLYMERASE III-TRANSCRIBED GENES BY THE RETINOBLASTOMA TUMOR SUPPRESSOR PROTEIN

By

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ABSTRACT

TRANSCRIPTIONAL REGULATION OF RNA POLYMERASE III-TRANSCRIBED GENES BY THE RETINOBLASTOMA TUMOR SUPPRESSOR PROTEIN

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The Retinoblastoma Tumor Suppressor Protein (Rb) is a critical regulator of cellular proliferation. In the canonical model, Rb regulates the G1-to-S phase transition by repressing RNA polymerase II transcription of genes necessary for DNA replication and cell cycle progression. Rb also represses RNA polymerase III-transcribed genes, including those of the U6 snRNA family; however, the contribution of this regulatory pathway to cellular physiology is not well understood. Saos2-tet-Rb osteosarcoma cells harboring an inducible Rb transgene exhibited diminished U6 snRNA levels in early G1 upon RB expression. Interestingly, Rb occupancy of the RNU6-1 locus was distributed around a promoter proximal nucleosome, suggesting that chromatin states are altered during Rb repression. Indeed, HDAC1 and HDAC2 histone deacetylases associated with the endogenous RNU6-1 locus in an Rb-dependent manner. Consistently, histone deacetylation was important for Rb repression of U6-1 in vitro transcription from chromatin templates but not templates lacking histones. Rb also enhanced DNMT1 and DNMT3a DNA methyltransferase recruitment to the RNU6-1 locus, although methylation was not required for repression. These data show that Rb represses RNA polymerase III transcription through the cell cycle by altering the chromatin architecture of the U6 promoter through recruitment of histone deacetylases and chromatin remodeling complexes.

ACKNOWLEDGEMENTS

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I leave the rest of my thanks for my family; my husband, daughter and son, for giving me the motivation to work hard.

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Abbreviations

ATP	Adenosine triphosphate
Brf2	TFIIIB related factor 2
Brg1	Brahma-related gene 1
Brm	Brahma
ChIP	Chromatin Immunoprecipitation
CSB	Cockayne syndrome group B protein
DNMT	DNA methyltransferase
DSE	Distal sequence element
GTF	General transcription factor
HDAC	Histone deacetylase
IC	Internal control
Oct1	Octomer 1
PCR	Polymerase chain reaction
PNC	Perinucleolar compartment
Pol	RNA Polymerase
PSE	Proximal sequence element
Rb	Retinoblastoma tumor suppressor
RPC4	RNA polymerase C protein 4
rRNA	Ribosomal RNA
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
SNAPc	Small nuclear RNA activating protein complex
snRNA	Small nuclear RNA
SWI/SNF	Switch/sucrose non-fermentable
TBP	TATA-binding protein
TFIIIX	Transcription factor III X
tRNA	Transfer RNA
TSA	Trichostatin A

Chapter 1

Introduction

The Retinoblastoma tumor suppressor protein is inactive in most human cancers. As cancer is characterized by uncontrolled cellular proliferation, this demonstrates the importance of Rb in regulating proper cell cycle control. Because of this, Rb has been the target of many studies to identify its role in cell cycle regulation. The traditional role of Rb is in the regulation of cell cycle progression in response to growth signals, during which time it is active in the G1 phase of growing cells to regulate the transition to S phase (Mancini, Shan et al. 1994). The canonical role for Rb activity during G1 is inhibition of E2F factors, which are necessary for transcription of various RNA polymerase II-transcribed protein-coding genes involved in DNA replication and cell cycle progression. However, evidence is pointing to Rb taking on a much more diverse role in the cell, including involvement in differentiation, apoptosis, DNA replication and DNA repair (Classon and Harlow 2002). In addition, work from Dr. David Arnosti's lab has shown that *Drosophila* Rbf1, a homologue of Rb, targets many genes involved in multiple signaling pathways, such as those that regulate *Drosophila* body development. Also, multiple binding motifs were found, in addition to E2F sites. This suggests that Rb associates with factors other than E2F and is playing many roles in the cell, most of which are poorly understood.

Rb is a 928 amino acid protein and is approximately 110 kDa; however the molecular weight varies based on the phosphorylation status. The activity of Rb is

mediated by cyclin E and D/CDK complexes that phosphorylate Rb at key points during the cell cycle (Chen, Scully et al. 1989; Mittnacht 1998). Rb is hypophosphorylated during late M phase to early/mid G1 phase. Upon hyperphosphorylation in late G1 phase Rb becomes inactive and loses it repressive ability. In the canonical model, hypophosphorylated Rb binds to E2F at the promoters of RNA polymerase II-transcribed genes and inhibits the ability of E2F to recruit general transcription factors and co-activators (Figure 1-1) (Harbour and Dean 2000). In a second mechanism, Rb is proposed to recruit corepressors complexes to repress transcription of the gene (Zhang, Gavin et al. 2000; Zhang and Dean 2001). During late G1 Rb becomes phosphorylated and disassociates from E2F, allowing transcription of the genes necessary for cell cycle progression into S phase.

The repressive activity of Rb has been linked to its C-terminal half, which consists of the A and B domains. These domains form the “small pocket”, and in conjunction with the C-terminus, the “large pocket”, which is responsible for interacting with many corepressors, including those harboring LXCXE motifs (Figure 1-2). These corepressors include the histone deacetylases (HDAC) 1 and 2, the ATP-dependent chromatin remodeling machine; SWI/SNF, and the DNA methyltransferases; DNMT1, 3A and 3B, many of which have been shown to be important factors in Rb-mediated repression of RNA polymerase II-transcribed genes (Zhang and Dean 2001). In addition to RNA polymerase II transcription regulation, Rb also acts as a global repressor of RNA polymerase I and III transcription for non-coding RNAs (Cavanaugh, Hempel et al. 1995; White, Trouche et al. 1996), although the involvement of corepressors in this process has not been determined.

RNA polymerase III transcripts have been shown to be up-regulated in tumor cell lines (White 2004), suggesting Rb-mediated regulation of RNA polymerase III transcription is an important component of maintaining cellular homeostasis. There are three types of RNA polymerase III genes (Figure 1-3). Type 1 genes (5S rRNA) contain the intragenic promoter elements; A box, intermediate element and C box (Hernandez 1992; Schramm and Hernandez 2002). The core promoter is recognized by the transcription factor TFIIIA, which recruits TFIIIC and TFIIIB. The type 2 genes (tRNAs, 7SL) contain the intragenic promoter elements; A box and B box, which are recognized by TFIIIC and recruits TFIIIB. The type 3 genes (U6, Y, MRP, 7SK) contain external promoter elements; a TATA box, proximal sequence element (PSE) and distal sequence element (DSE). This promoter is bound by the core transcription machinery TFIIIB-Brf2, the snRNA-activating protein complex (SNAPc) and an activator, Oct1. The mechanism of Rb repression of these various types of RNA polymerase III-transcribed genes differs. Rb has been shown to disrupt pre-initiation complex assembly at type 1 and type 2 genes by binding to TFIIIB (Larminie, Cairns et al. 1997). At type 3 genes (such as the U6 snRNA gene), our lab has shown that Rb interacts with SNAPc and TBP, part of the core transcription machinery, and stably associates at the promoter along with RNA polymerase III, as determined by chromatin immunoprecipitation (ChIP) (Hirsch, Gu et al. 2000; Hirsch, Jawdekar et al. 2004). In addition, The U6-1 gene has a powerful, well characterized promoter (Zhao, Pendergrast et al. 2001; Domitrovich and Kunkel 2003). For these reasons, we use the U6 snRNA gene as a model for Rb repression mechanism studies.

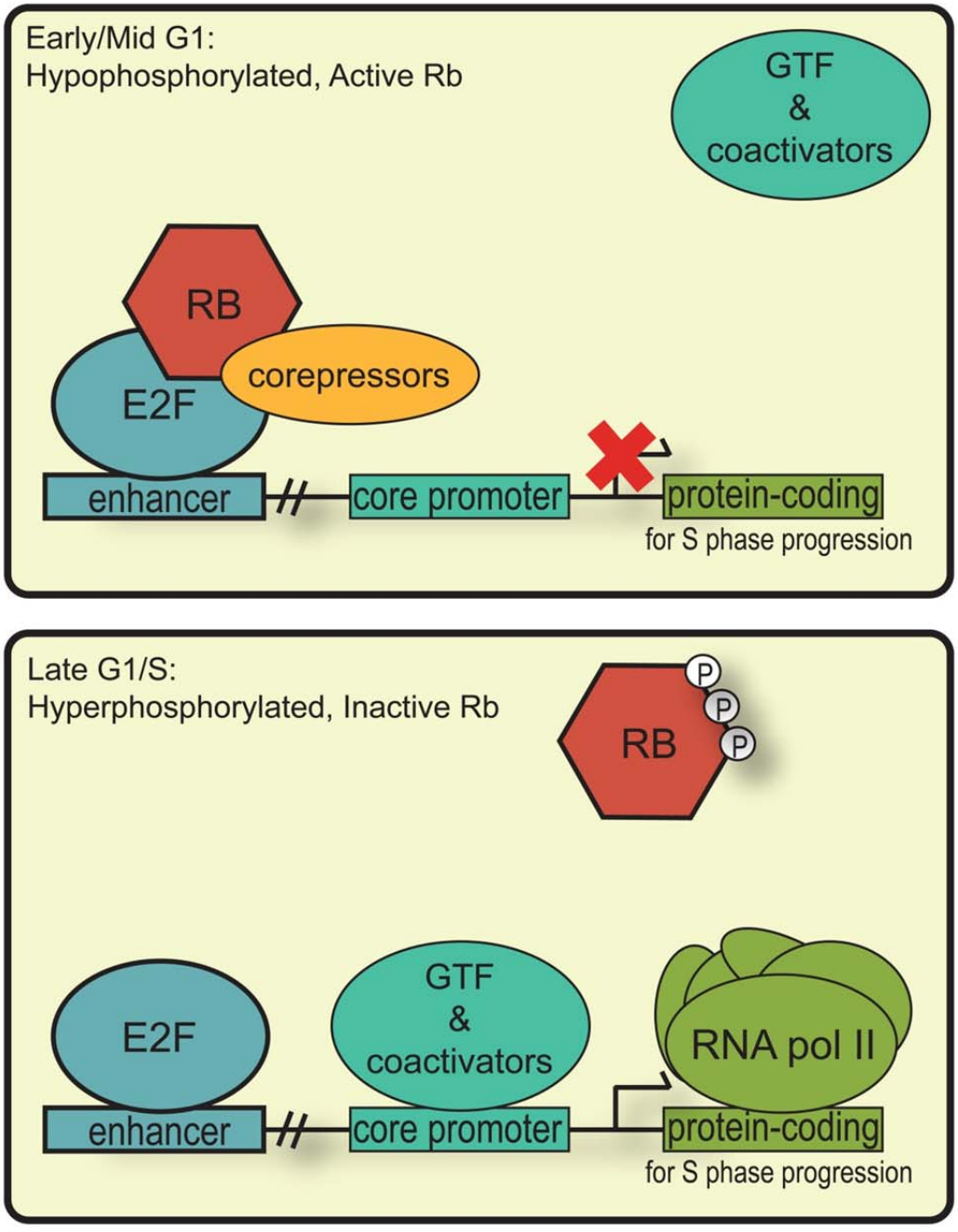
The U6 snRNA gene encodes the RNA component of the U6 spliceosomal complex which is required for mRNA processing. As U6 snRNA plays a critical role in protein synthesis, regulation of this gene by Rb could potentially limit the biosynthetic capacity of the cell. Here I will discuss my work at uncovering the mechanism of Rb repression of U6 transcription during the cell cycle.

Figure 1-1. The canonical model of Rb function at RNA polymerase II-transcribed genes

Top panel: During early/mid G1 Rb is in its hypophosphorylated form and active. It represses transcription of RNA polymerase II-transcribed genes regulated by E2F transcription factors that are required for entry into S-phase. The mechanism involves inhibiting recruitment of the general transcription factors (GTF) and coactivators and recruitment of corepressors complexes.

Bottom panel: During late G1 Rb becomes Hyperphosphorylated by cyclin/CDK complexes and can no longer bind to E2F. This frees E2F to recruit the general transcription factors and coactivators and the gene is transcribed.

Figure 1-1. The canonical model of Rb function at RNA polymerase II-transcribed genes



For interpretation of the references to color in this and all other figures, the reader is reffered to the electronic version of this thesis.

Figure 1-2. The structure of Rb

Rb is a 928 amino acid protein. The C-terminal half is required for repression and contains the A and B domains, which together, with the C terminus, constitutes the large binding pocket. There are 15 phosphorylation sites (x) along Rb which regulate its activity.

Figure 1-2. The structure of RB

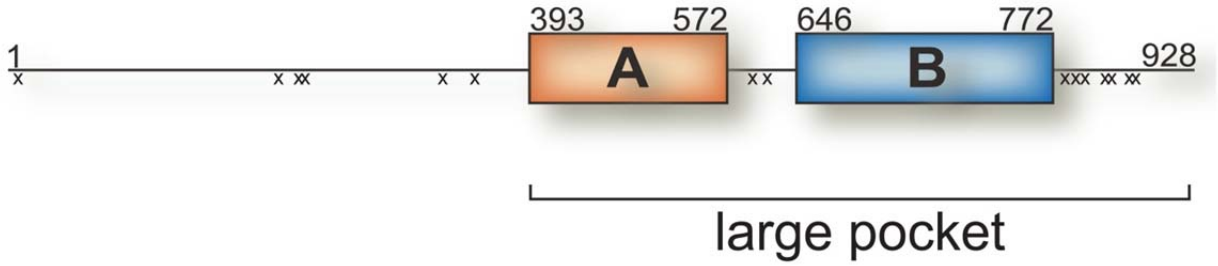
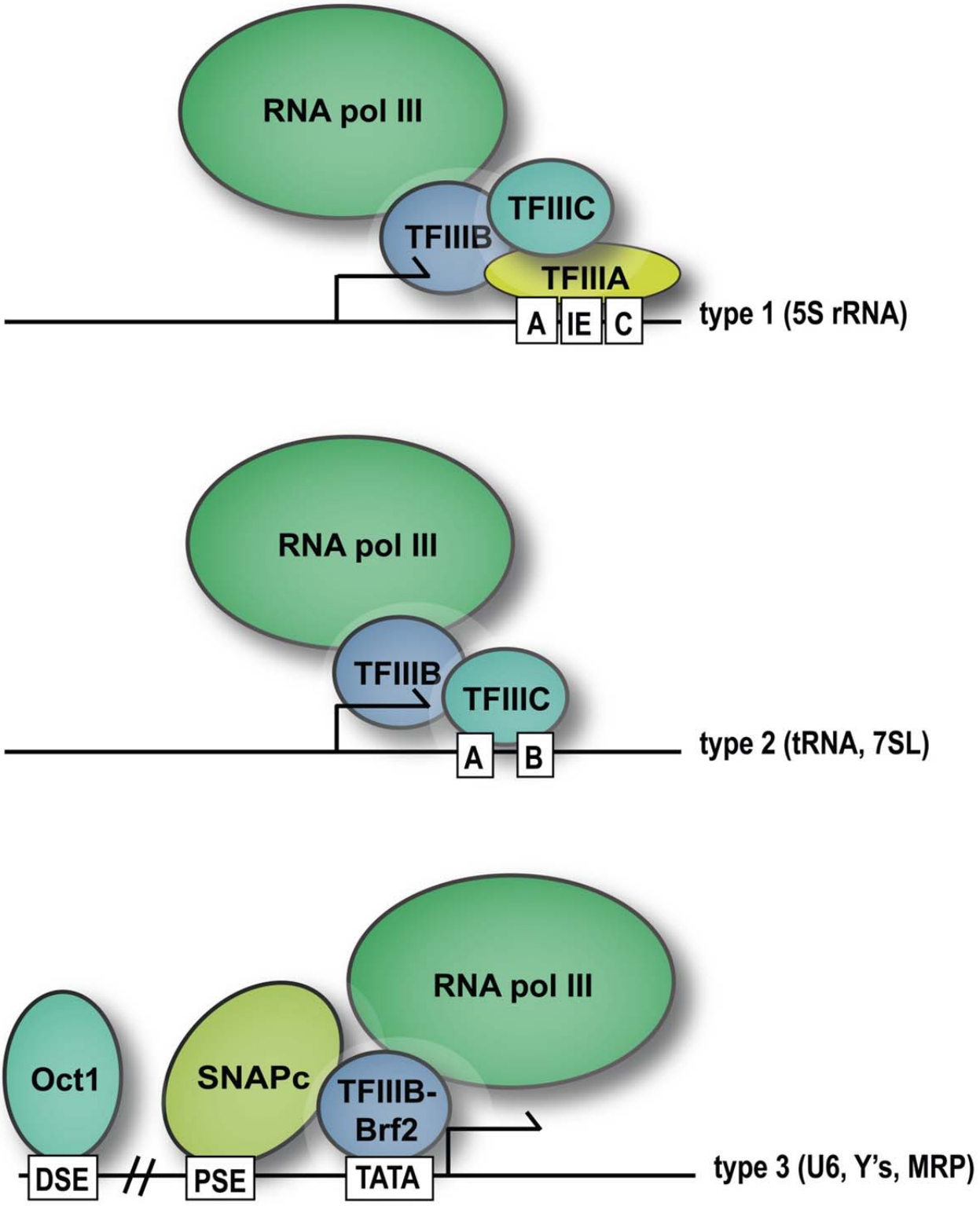


Figure 1-3. The structure of RNA polymerase III-transcribed genes

There are three types of RNA polymerase III genes (Figure X). Type 1 genes (5S) contain the intragenic promoter elements; A box, intermediate element and C box. The core promoter is recognized by the transcription factor TFIIIA, which recruits TFIIIC and TFIIIB. The type 2 genes (tRNAs, 7SL) contain the intragenic promoter elements; A box and B box, which is recognized by TFIIIC and recruits TFIIIB. The type 3 genes (U6, Y, MRP, 7SK) contain external promoter elements; a TATA box, proximal sequence element and distal sequence element. This promoter is bound by the core transcription machinery TFIIIB-Brf2, the snRNA-activating protein complex (SNAPc) and an enhancer, Oct1.

Figure 1-3. The structure of RNA polymerase III-transcribed genes



Chapter 2

Rb represses U6 RNA expression during G1 and requires the chromatin modifying enzymes, HDAC1, HDAC2 and Brg1/Brm, to enact repression.

Abstract

The canonical model of Rb function is in the regulation of E2F-controlled RNA polymerase II-transcribed genes during the G1-to-S phase transition. However, Rb is also known to regulate RNA polymerase III-transcribed genes and in doing so, could potentially limit the biosynthetic capacity of the cell. However, the timing and mechanism of this regulation remains a mystery. Here I show that Rb represses the U6 snRNA steady-state levels during early/mid G1 of the cell cycle, as determined by staging of Saos2tetRb cells, qPCR and flow cytometry. Mining of published ChIP-seq data revealed that Rb localizes preferentially at type 3 genes and that Rb crosslinked at multiple sites flanking the positioned nucleosome in the U6-1 promoter. Additionally, the chromatin modifying machinery, HDAC's and SWI/SNF, were found to localize to the U6-1 promoter in an Rb-dependent manner, as determined by chromatin immunoprecipitation. Rb-mediated repression of a chromatinized U6-1 reporter was abrogated by Trichostatin A and Rb-mediated repression of a U6-1 reporter was lost in a Brg1/Brm null cell line. This demonstrates the importance of these machines in Rb-mediated repression of U6-1.

Introduction

In the canonical model of Rb repression, in addition to blocking the recruitment of activators, Rb has been shown to recruit corepressors to the promoters of RNA polymerase II-transcribed genes in order to enact repression. These corepressors include histone deacetylases (HDACs) and chromatin remodeling complexes (such as SWI/SNF) (Zhang and Dean 2001).

Acetylation of histone tails by histone acetyltransferases, the mark of an active gene, is thought to loosen DNA/histone interactions by shielding the positive charge on histone lysines. HDACs remove these acetyl groups, causing electrostatic attraction between the DNA and histones and a repressed gene state. Rb has been shown to bind HDAC 1 through the large pocket, and this interaction is important for the repression of a chromosomally-integrated E2F reporter gene (Brehm, Miska et al. 1998), directly implicating HDAC1 in Rb-mediated repression.

Rb has also been shown to associate with Brg1 (Dunaief, Strober et al. 1994), the ATPase subunit of the chromatin remodeling machinery, hSWI/SNF. hSWI/SNF uses the energy of ATP hydrolysis to reposition or remove nucleosomes and has been implicated in both transcriptional activation and repression. The ability of Brg1 to interact with Rb through its binding pocket was shown to be critical for Rb-mediated growth arrest (Dunaief, Strober et al. 1994), suggesting Brg1 is a major component of the Rb pathway.

The U6-1 gene has a nucleosome positioned between the DSE and PSE of the promoter that is required for activated levels of transcription (Zhao, Pendergrast et al.

2001). This positioned nucleosome places the enhancer element (DSE) in close proximity to the PSE, allowing Oct1 to stabilize binding of SNAPc to the core promoter (Zhao, Pendergrast et al. 2001). The ability of Rb to recruit chromatin modifying enzymes, such as Brg1/Brm and HDACs, to the promoter could cause a shift or removal of this positioned nucleosome and repressed transcription (Figure 2-1). In addition, an Rb-hSWI/SNF-HDAC repressor complex was shown to be necessary for a G1 arrest (Zhang, Gavin et al. 2000), leading to the hypothesis that Rb recruits the chromatin modifying enzymes, HDACs and hSWI/SNF, to the U6-1 promoter to reposition the nucleosome during G1 of the cell cycle.

Here I will discuss my work demonstrating that Rb represses U6 RNA steady-state levels during G1 of the cell cycle, and that repression of RNA steady-state levels during G1 is specific to U6 and not all type 3 RNA polymerase III-transcribed genes. In addition, at times when Rb resides at the U6-1 promoter it is not at other type 3 promoters. However, Rb is able to repress the transcription of various type 3 genes, suggesting that Rb uses different mechanisms to repress the transcription of these genes.

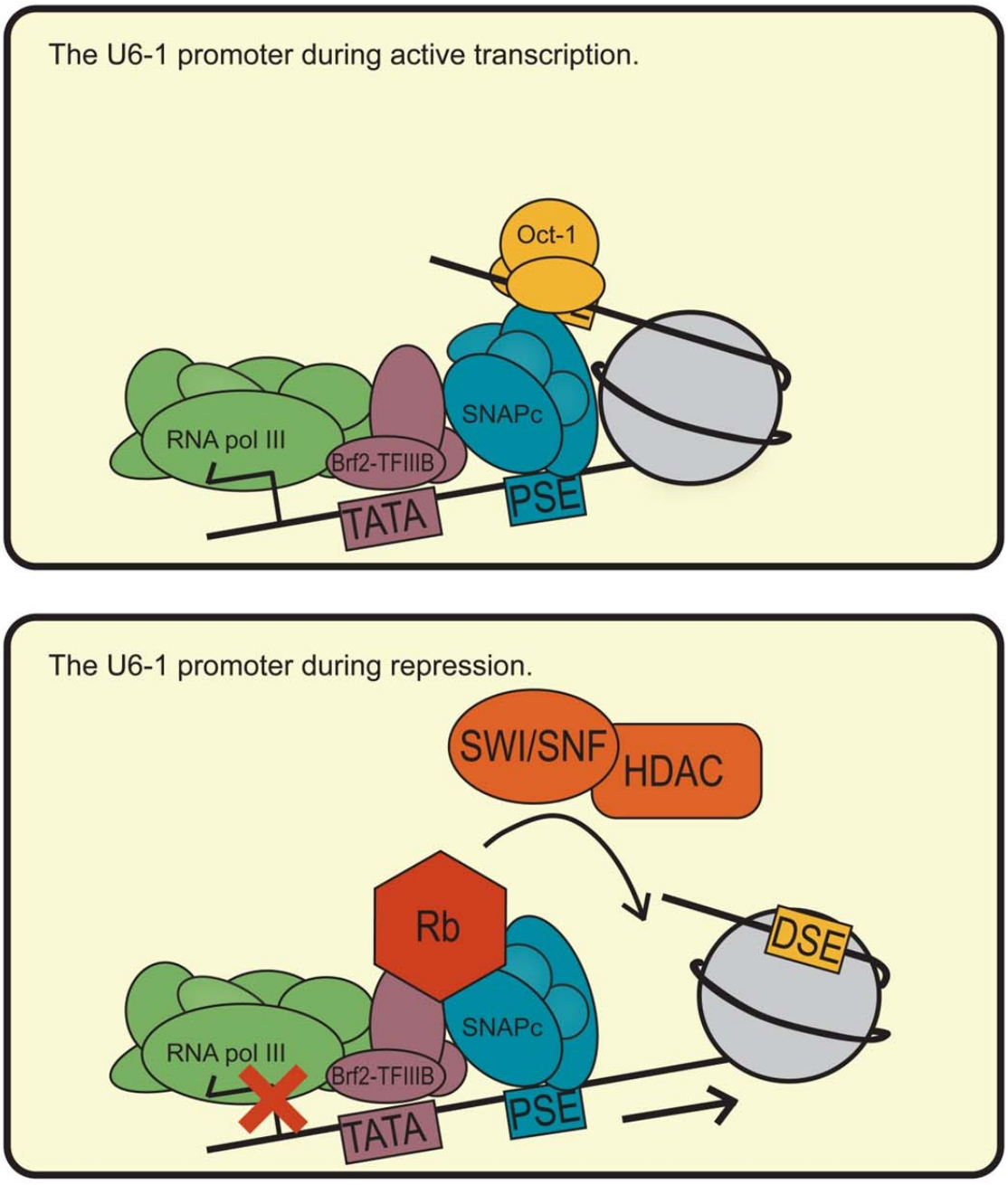
In-depth analysis of published ChIP-seq data was used to determine that Rb is distributed at the U6-1 promoter around a positioned nucleosome, suggesting the positioned nucleosome is a critical target of the regulation mechanism and that chromatin modifying machinery is likely involved. In addition, we have shown that Rb directs HDAC1 and HDAC2, as well as Brg1, to the U6-1 promoter and their function is required for Rb-mediated repression.

Figure 2-1. A model for the role of the U6-1 positioned nucleosome

Upper panel: A nucleosome positioned between the DSE and PSE of the U6-1 gene allows Oct1 to contact SNAPc and stabilize the core transcription machinery, activating transcription.

Lower panel: Rb is recruited to the promoter by SNAPc and TFIIIB and, in turn, recruits the chromatin remodeling machinery; HDACs and SWI/SNF. These enzymes remodel chromatin, possibly via nucleosome sliding (arrow), disrupting contacts between Oct1 and SNAPc, and thereby, repressing transcription.

Figure 2-1. A model for the role of the U6-1 positioned nucleosome



Materials and Methods

Tissue Culture

HeLa, U2OS and Saos2tetRb cells were grown at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium with 5% Fetal Bovine Serum. Saos2tetRb cells (Jiang, Karnezis et al. 2000) were grown in 5 µg/mL Puromycin and 1 µg/mL Doxycycline. To express Rb in the Saos2tetRb cell line the cells were washed 2X in PBS and re-plated at the required density. After attachment, the cells were washed once in PBS.

Cell Synchronization

Cells were seeded on 150 mm plates ($3x10^6$ U2OS or $5x10^6$ Saos2tetRb cells per plate). After 24 hours the media was replaced with media containing 600 ng/µL Nocodazole (Sigma #M1404) and the cells were incubated for 24 additional hours. The media was removed gently and the rounded, mitotic cells were collected via mitotic shake-off by pipetting the media up and down and knocking the plates. The mitotic cells were pelleted by centrifugation at 1200xg for 10 minutes and washed 1X in PBS to remove the drug. Cells were then either re-plated in DMEM and grown further or collected immediately for analysis. After release from the cell cycle block, growing cells were collected at various time points. For each time point, the total RNA was collected with 1 mL TRIzol and processed according to the manufactures protocol. Total cellular protein was extracted with Tween Lysis Buffer (0.05 M HEPES, pH 7.9, 0.15 M NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% TWEEN-20, 10% glycerol) and normalized to cell number for Western Blot analysis. Protein from $4x10^5$ cells was analyzed by 12.5%

SDS-PAGE and an Rb Western blot (BD Pharmingen # 554136) was performed. The cell cycle progression was monitored with ethanol fixation, propidium iodide staining and flow cytometry.

Quantitative Reverse Transcription PCR

Total RNA was collected with TRIzol and processed according to the manufacturer's instructions. The cDNA was made with random primers using the High Capacity cDNA Synthesis Kit (Applied Biosystems) from 60 ng total RNA (as determined by Nanodrop) per 20 µL reaction. Two µL of the cDNA was used for quantitative PCR with 2X Sybr Green Master Mix (Applied Biosystems) and primers specific for the following transcripts:

U6 F 5'-GTGCTCGCTTCGGCAGCACA-3'
R 5'-AATATGGAACGCTTCACGAA-3'
MRP F 5'-GTTCGTGCTGAAGGCCTGTA-3'
R 5'-TGCACGTGGCACTCTCTGCC-3'
Y1 F 5'-AGACTAGTCAAGTGCAGTAG-3'
R 5'-GGCTGGTCCGAAGGTAGTGA-3'
Y3 F 5'-GGCTGGTCCGAGTGCAGTGG-3'
R 5'-GAAGCAGTGGGAGTGGAGAA-3'
18S F 5'-GGCCCTGTAATTGGAATGAGTC-3'
R 5'-CCAAGATCCAACTACGAGCTT-3'

In Vivo Transcription Assays

Reporters: pBS-Y1-997: The Y1 gene was amplified from 184B5 gDNA from -997 bp to +38 bp with primers containing BamHI and HindIII sites, respectively. The amplicon was ligated into the pBS-U1-β-globin vector (digested with BamHI and HindIII to remove the U1 promoter but retain the β-globin insert, U1 3' box and T run) to make pBS-Y1-997. pBS-MRP-228: The MRP gene was amplified from 184B5 gDNA from -228 bp to +1

bp with primers containing EcoRI and HindIII sites, respectively. The amplicon was ligated into the pBS-U1-β-globin vector (digested with EcoRI and HindIII to remove the U1 promoter but retain the β-globin insert, U1 3' box and T run) to make pBS-MRP-228. pU6/Hae/RA.2 and the pU6 5' probe plasmid have been described previously (Lobo and Hernandez 1989).

Rb U6-1 Repression in HeLa: HeLa cells were seeded in 6-well plates at $2x10^5$ cells/well. Twenty four hours after seeding, cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with the following combinations:

250 ng pU6/Hae/RA.2 and 1000-1500 ng pCMV-RB/ pCMV-EV
500 ng pBS-Y1 and 375-750 ng pCMV-RB/ pCMV-EV
250 ng pBS-MRP and 1-2 µg pCMV-RB/ pCMV-EV.

After 24 hours the total RNA was collected with TRIzol and processed according to the manufactures directions.

Rb U6-1 Repression HeLa/ SW13 Comparison: HeLa was seeded at $10x10^5$ cells per 100 mm plate. SW13 was seeded at $15x10^5$ cells per 100 mm plate. After 24 hours the cells were transiently transfected by Lipofectamine 2000 with 250 ng pU6/Hae/RA.2 and 3 µg pCMV-Rb or EV.

RNase T_1 protection assay: The reporter-specific probes were transcribed from their respective plasmids (pU6 5' probe, pBS-Y1 or pBS-MRP) and labeled with αP^{32} CTP. One µg total RNA was hybridized to the appropriate probe overnight at 61°C. The single stranded DNA was digested with RNase T_1 for 30 minutes at 30°C and the purified

double stranded RNA was analyzed on a 6% urea sequencing gel at 3000V for 2.5 hours. The radioactivity was analyzed with PhosphorImager (Molecular Dynamics) and quantified.

Chromatin Immunoprecipitations

184B5 cells were seeded at $1.25x10^6$, $2.5x10^6$ and $5x10^6$ cells per 150 mm plate. ChIPs were performed as described (Hirsch, Jawdekar et al. 2004). Polyclonal antibodies (Rb-MI-172, SNAP43-CS48) were used to precipitate Rb and SNAP43, respectively. Nonspecific IgG (Santa Cruz, sc-2025) was used as a negative control. The precipitated DNA (5 µL out of 50 µl) was amplified by PCR with the following primer pairs:

U6 F 5'-GTACAAAATACGTGACGTAGAAAG-3'
R 5'-GGTGTTTCGTCCTTTCCAC-3'
Y1 F 5'-GCTGAGCCACTCAAGACAAAC-3'
R 5'-AGCCCCCAAATTTGTTCATT-3'
Y3 F 5'-GTGGGAGTCTTTGATGGATG-3'
R 5'-CGGTGACTATTCACAGGAGC-3'
MRP F 5'-GCATAAGATAGTGTCATTCTAGA-3'
R 5'-TAGGCGGAAAGGGGAGGAAC-3'

Conditions:
94°C- 1.5'- 1 cycle
94°C- 30s
55°C- 1' 31 cycles
72°C- 1'
72°C-4'-1 cycle
The PCR products were separated by 2% TBE agarose gel electrophoresis, stained with ethidium bromide and visualized with Fuji imaging software.

Bioinformatic Analysis of ChIP-Seq Data

To determine where Rb localized at RNA polymerase III genes, I compared to ChIP-seq data sets. The distribution of RNA polymerase III across the human genome was

determined by Nouria Hernandez (Canella, Praz et al. 2010). The distribution of Rb under three different growth conditions (actively growing, quiescent and senescent) was determined by Scott Lowe (Chicas, Wang et al. 2010). The following ChIP-seq data sets (fastq files) were downloaded from the NCBI Gene Ontology Omnibus site:

RNA polymerase III (GSE18184)
RPC4 (GSM454595)
Input (GSM454598)

Rb (GSE19898)
Growing cells (GSM497489)
Quiescent cells (GSM497490 and GSM497491)
Senescent cells (GSM497493 and GSM497494)
Mock (GSM497500)

The read tags (fastq files) were aligned to the human genome (GRCh37) with Bowtie on the GEDD3 server with the following parameters: bowtie –p 2 –m 1000 –v 0. The tag enrichment (over input or mock) was determined with QuEST on the GEDD3 server with the recommended settings (sharp peaks for the Rb ChIP and broad peaks for the pol III ChIP). The co-localization of RPC4 and Rb (under each growth condition) was determined with the help of John Johnston from the Institute of Cyber Enabled Research (iCER) using a script (stored on the GEDD3 server) which looked for overlap of the middle of the RPC4 and Rb peaks within 1000 bp of each other. The distribution of Rb at the U6-1 locus (Ch 15 65,919,000- 65,920,000) was determined by loading the files noted above (GSM454595 and GSM497493 downloaded from the NCBI Gene Ontology Omnibus site) against the human genome (hg18) on the Human Genome Browser.

Results

U6 RNA steady-state levels are repressed during early-mid G1.

Rb has previously been shown to induce a G1 block in Nocodazole-synchronized cells (Goodrich, Wang et al. 1991), demonstrating the activity of Rb under these conditions. I therefore used Nocodazole to synchronize an Rb^+ osteosarcoma cell line, U2OS, and measured the changes in U6 steady-state RNA levels through the cell cycle. Cell cycle progression was monitored by propidium iodide staining and flow cytometry. As shown in Figure 2-2A, the cells were blocked in G2, progressed together into early G1 (2 hours after removal of the Nocodazole), mid G1 (5 hours), late G1 (8 hours) and into S phase (15 hours) where they began to lose synchrony. Quantitative reverse-transcription PCR was used with primers specific for the U6 coding region to measure the RNA levels at each time point, which was normalized to 18S RNA (Figures 2-2B). The U6 RNA decreased during early to mid G1, increased significantly in late G1, and remained at that level into S phase. This demonstrates that the U6 RNA levels do fluctuate through the cell cycle in a pattern consistent with Rb-regulation (low levels in early G1 and high levels in late G1/S).

Interestingly, the expression profile of other type 3 RNA polymerase III-transcribed genes did not fluctuate through the cell cycle to the extremes of that seen in U6. The RNA steady-state levels of Y1, Y3 and MRP remained relatively constant relative to U6 (Figure 2-2C), demonstrating that the mechanism of Rb-mediated regulation of U6 transcription is unique relative to other type-3 RNA polymerase III-transcribed genes.

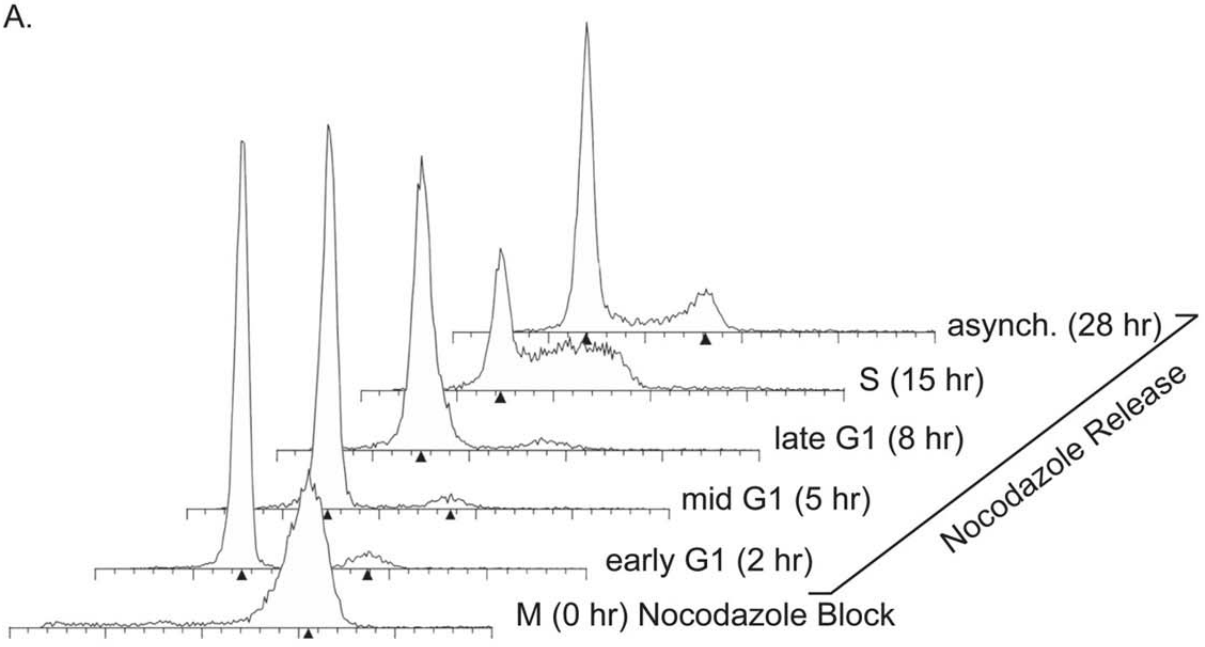
Figure 2-2. RNA Polymerase III transcript steady-state RNA levels fluctuate through the cell cycle

A. U2OS cells were blocked in G2 with Nocodazole. Upon release the cells entered G1 and remained in synchrony until S phase (15 hrs). The cell cycle progression was analyzed by propidium iodide staining and flow cytometry.
B. The total RNA was collected with TRIzol and was converted into cDNA by reverse transcription with random primers. Quantitative PCR with U6 and 18S specific primers was used to measure the levels of cDNA. The U6 cDNA levels were normalized to 18S cDNA levels.
C. U6, Y1, Y3 and MRP steady-state RNA levels were measured quantitatively by conversion into cDNA and utilizing qPCR with target-specific primers. All cDNA levels were normalized to 18S cDNA.

*statistically significant relative to M phase ($p<0.05$)

Figure 2-2. RNA Polymerase III transcript steady-state RNA levels fluctuate through the cell cycle.

A.



B.

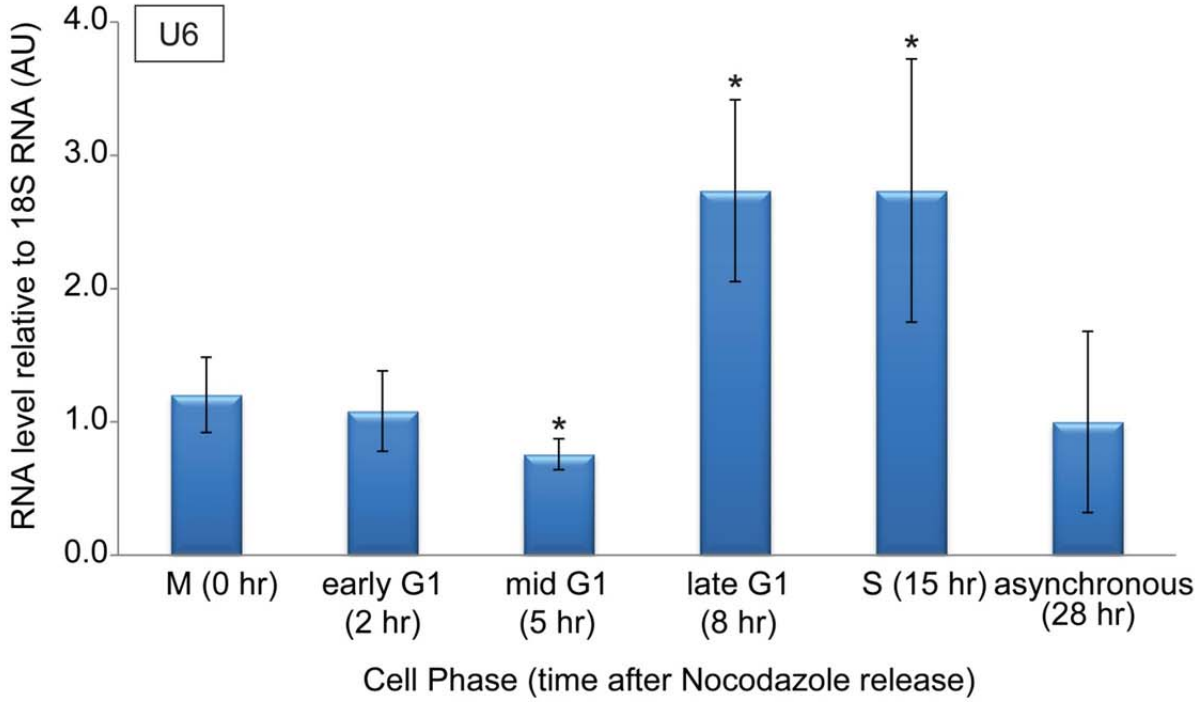
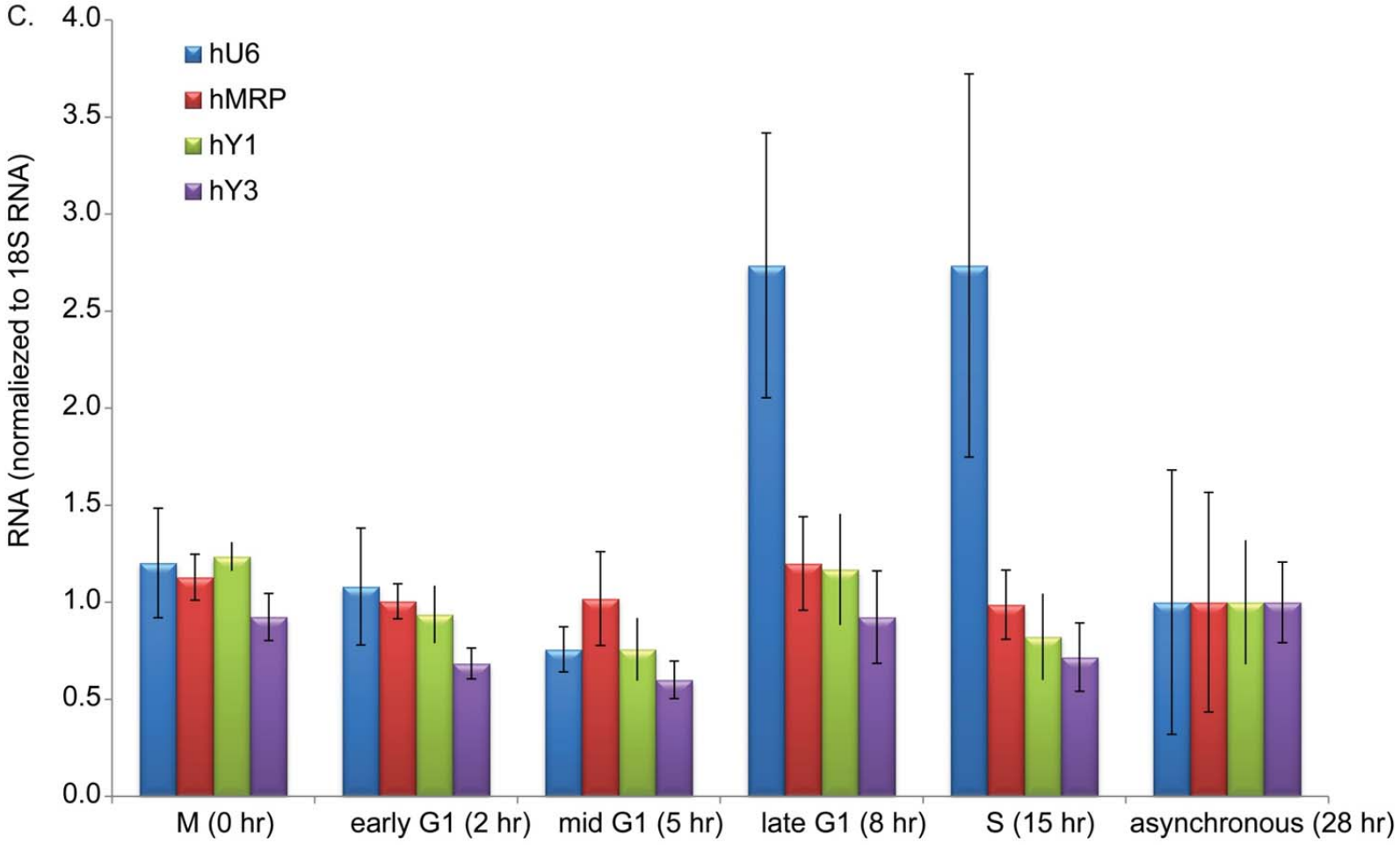


Figure 2-2 (cont'd)



Rb is necessary for repression of U6 RNA steady-state levels during early-mid G1.

To determine if the decrease in U6 RNA levels during G1 is due to Rb, Saos2tetRb cells harboring an inducible Rb transgene were staged with Nocodazole. We have previously shown that Rb localizes to the U6-1 promoter in this cell line upon Rb expression in an asynchronous cell population (Hirsch, Jawdekar et al. 2004). In addition, induction of Rb in Soas2tetRb has been shown to inhibit BrdU incorporation and therefore, DNA replication, demonstrating the activity of expressed Rb in this system (Jiang, Karnezis et al. 2000). After Nocodazole synchronization and release, cell cycle progression was monitored by FACS for comparison to Rb expression and steady-state U6 RNA levels. As shown in Figure 2-3A, Rb had no effect on the profile of asynchronous cells, the G2 block, or early after release (6 hours). However, 30 hours after removal of Nocodazole, the majority of Rb-deficient (Rb^-) cells (84%) entered into S/G2 while Rb-expressing (Rb^+) cells (50%) were blocked in G1; demonstrating Rb has a functional relevance at this time.

The total protein was isolated and normalized to cell number to monitor the Rb expression through the course of the experiment. In the presence of doxycycline, no Rb expression is observed (Figure 2-3B, top panel). Rb was expressed 24 hours after induction, as can be seen in the asynchronous population (Figure 2-3B, lower panel, lane 3) and in the G2-arrested population (lane 4). Expression of Rb continued throughout the course of the cell cycle (lanes 5-6). Interestingly, Rb became increasingly hypophosphorylated by 30 hours, suggesting the activity peaked at 30 hours when the G1 block was observed.

To determine the effect of Rb expression on U6 steady-state RNA levels, the total RNA was collected, DNase-treated and the U6 RNA level was normalized to cell number and measured by qRT-PCR. In the Rb^- cells, the U6 RNA increased 2.4-fold between G2 (0 hours) and G1 (6 hours), when the cell mass would have decreased by 50% after mitosis, suggesting U6 was being actively expressed during this time (Figure 2-3C). By 30 hours, the majority of the Rb^- cells are in G2, the cell mass has doubled and the U6 RNA level has increased another 1.6-fold from G1. Demonstrating that without Rb the levels of U6 increase constantly through the cell cycle.

The U6 RNA levels from the Rb^+ cells responded much differently. From G2 to G1 the RNA level decreased 2-fold, suggesting there was no U6 expression during this time when the cell divided. From 6 to 30 hours the RNA level increased 2.2-fold, but remained 3.4-fold lower than the level of U6 expression observed in the Rb^- cells at the same time point. This demonstrates that Rb represses U6 RNA steady-state levels during Rb-induced G1 arrest. However, whether this repression is direct or in-direct is not addressed by this experiment.

Rb represses transcription of U6-1, and other type 3 genes, in vivo

To determine whether the Rb-mediated repression of U6 was direct or in-direct, I measured the effect of Rb on U6 transcription *in vivo*. I transiently transfected the U6-1 reporter, or other type 3 RNA polymerase III reporters harboring the Y1, Y3 and MRP promoters, and an Rb expression vector into HeLa, a cervical carcinoma cells. HeLa lacks functional Rb due to expression of the HPV E7 protein

Figure 2-3. Rb represses U6 steady-state RNA levels during G1

A. Saos2tetRb cells were blocked in G2 with Nocodazole and the doxycycline was removed from the media (+Rb) or not (-Rb). The Nocodazole was removed from the media and the cells were released from the block. Rb expression had no measurable effect on the cell cycle in asynchronous cells, the ability of the cells to block in G2, or to progress into G1 (6 hrs). At 30 hours after release, Rb induced a G1 block.

B. An Rb western blot. In the presence of doxycycline, no Rb was expressed (top panel). After removal of doxycycline, Rb was expressed by 24 hours in asynchronous and G2 blocked cells (lane 3 and 4). 6 hours and 30 hours after induction Rb appears to become more hypophosphorylated.

C. U6 RNA steady-state levels were measured for each time point by qRT-PCR. Without Rb, U6 RNA levels increase after G1 and into G2. With Rb, the U6 RNA levels decrease during G1 (6 hr) and remain low (30 hr).

Figure 2-3. Rb represses U6 steady-state RNA levels during G1

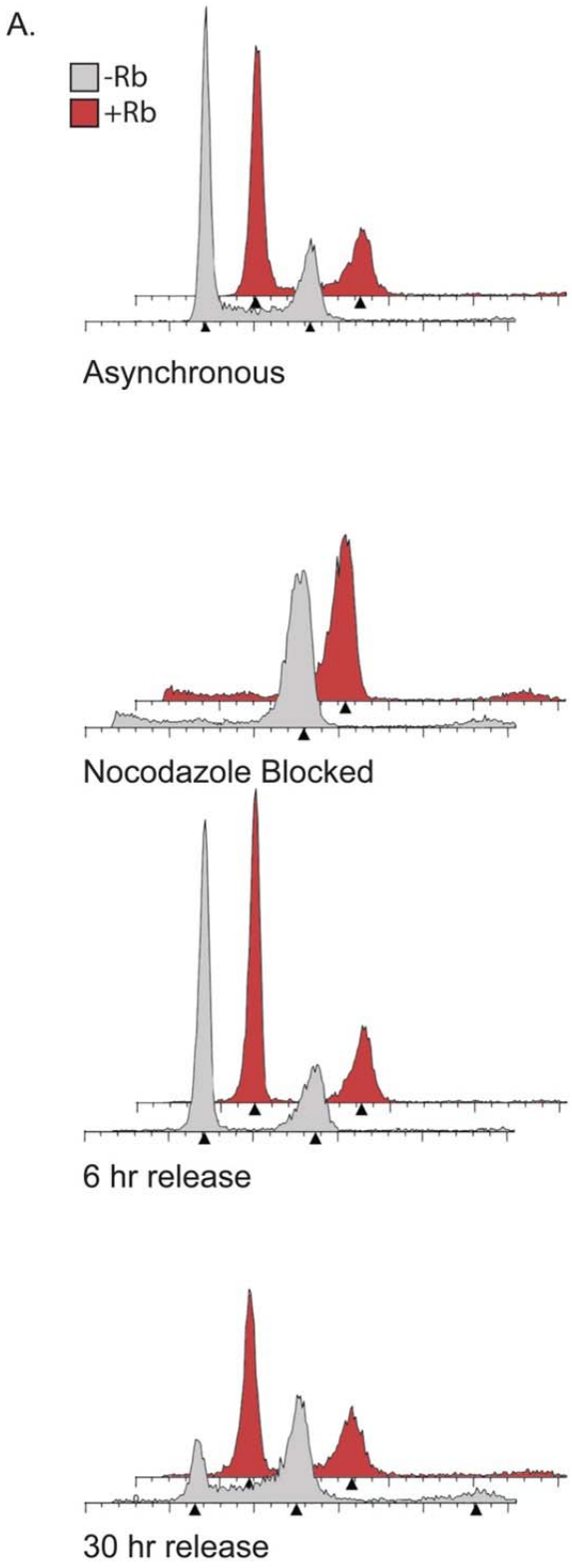
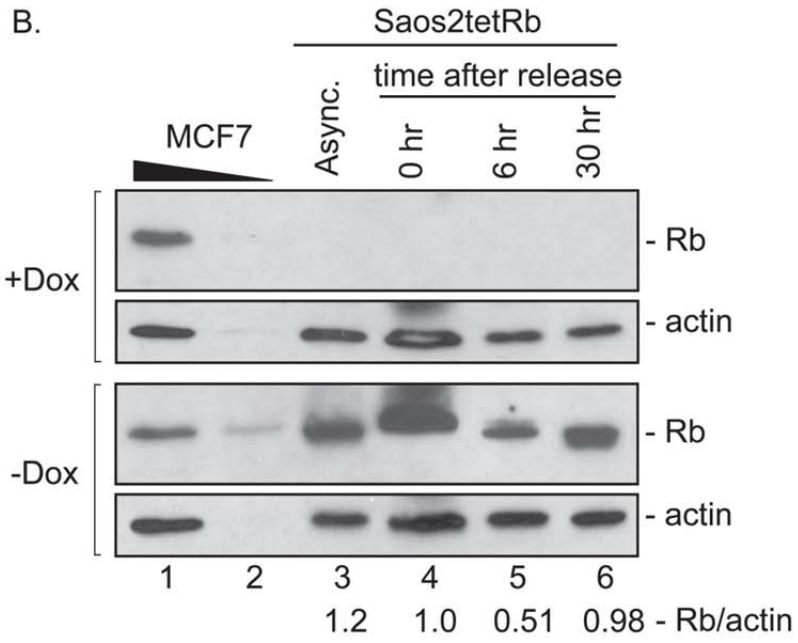
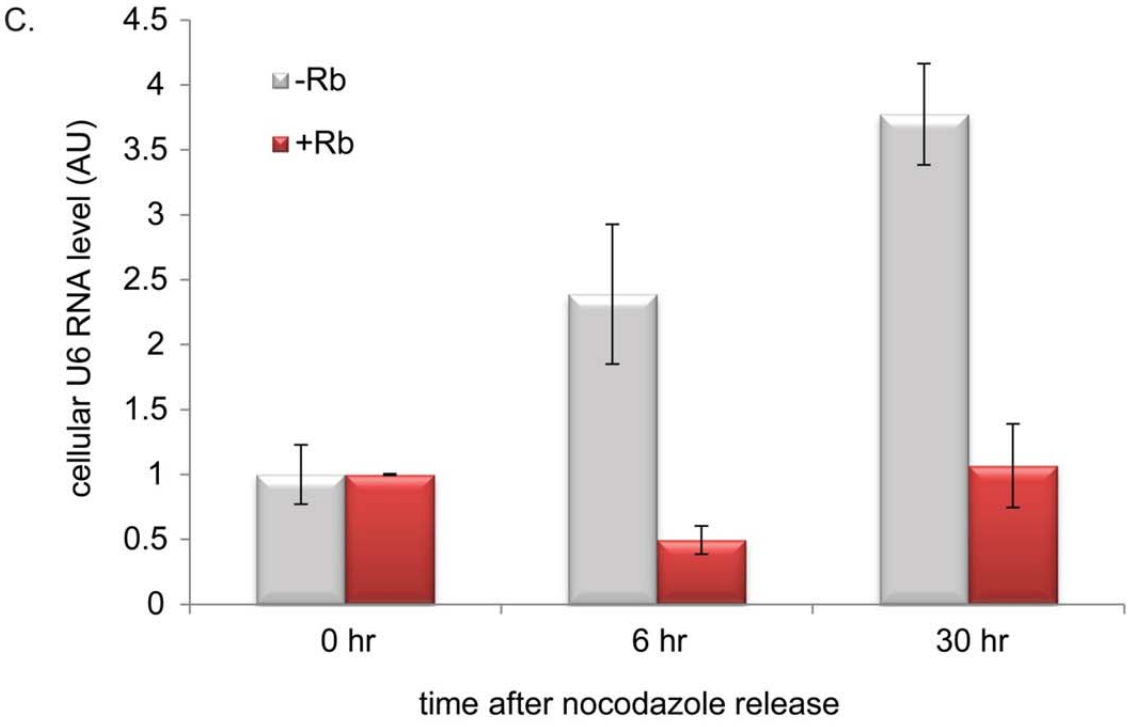


Figure 2-3 (con't)





(Munger, Basile et al. 2001), which binds Rb in the large pocket. Twenty-four hours after transfection, total RNA was collected and measured to determine the RNA transcripts derived from the transfected reporter genes by an RNase T_1 protection assay. As shown in Figure 2-4, Rb repressed transcription of U6-1 10-fold, while the control (EV) was not able to repress U6-1. Interestingly, although the RNA expression profiles of Y1, Y3 and MRP were considerably different than that of U6, Rb was still able to repress transcription significantly from these reporters.

Rb preferentially localizes to the U6-1 promoter

Although the expression profiles of the various type 3 genes were different during the cell cycle (U6 was repressed during G1, Y1, Y3 and MRP are relatively constant), Rb could repress transcription of all of them. However, whether or not Rb could localize to all of these responsive genes was unknown. We have previously demonstrated that Rb can reside at U6-1; however the condition under which this occurs is unclear. In our lab, we have found that cell density impacted the ability of Rb to reside at U6-1. To determine if Rb residency changes with cell density at U6-1, Y1, Y3 and MRP, I performed a chromatin immunoprecipitation in immortal, non-transformed, mammary fibroblasts (184B5) grown at various cell densities. The cells were harvested after 48 hours and had a low (~25%), medium (~50%) and high (>75%) confluency. As shown in Figure 2-5, Rb was found at the U6-1 promoter only when the cells were harvested at a medium confluency, and not when the confluency was too low or too high. SNAP43 was found at the promoter under all conditions. This suggests that Rb residency at U6-1 is

dependent upon growth conditions and may only localize to U6-1 when the cells are actively cycling.

Figure 2-4. Rb represses RNA polymerase III-transcribed genes

HeLa cells were transiently transfected with and Rb expression vector, or an empty vector, and a reporter (U6, Y1, or MRP) for 24 hours. The total RNA was collected and the reporter transcripts were measured using an RNase T1 protection assay. A portion of the total RNA was also analyzed by agarose gel electrophoresis and ethidium bromide staining for normalization.

Figure 2-4. Rb represses RNA polymerase III-transcribed genes

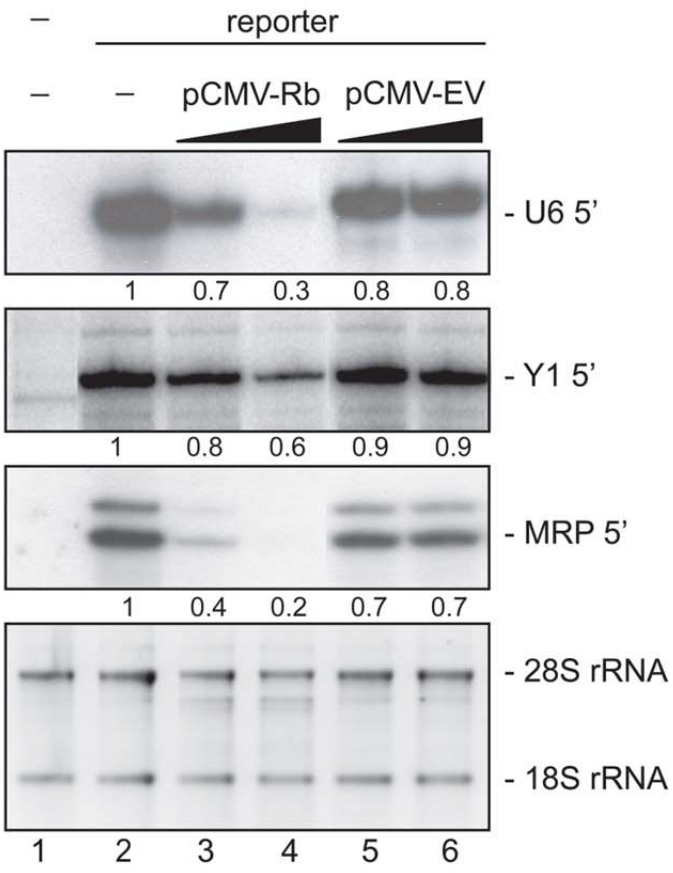
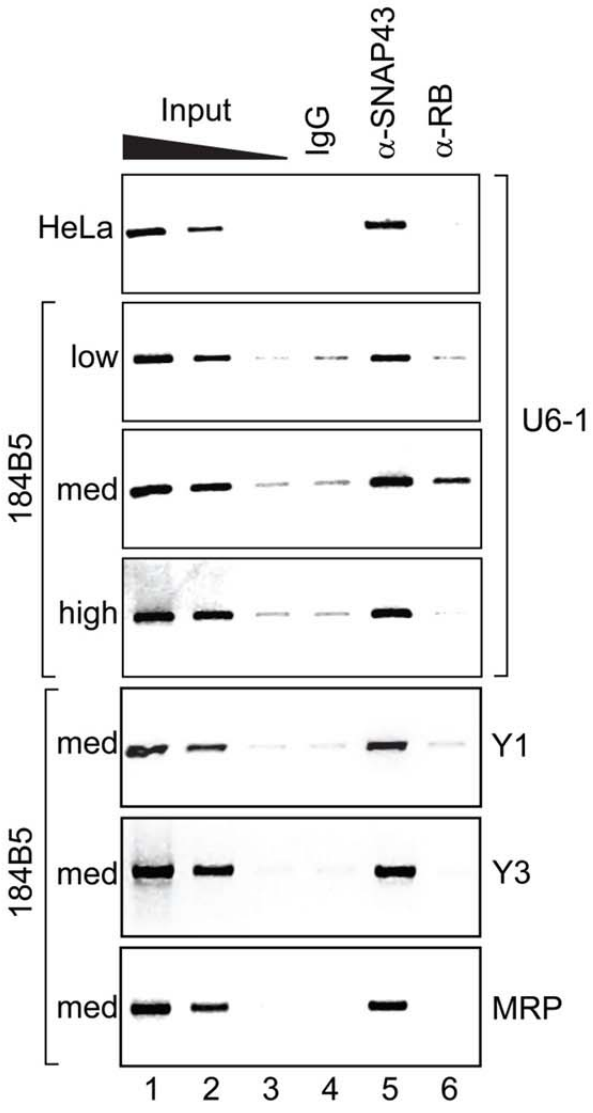


Figure 2-5. Rb localization to RNA polymerase III-transcribed genes is based on cell density

184B5 cells (Rb^+) were grown at three different densities (low, medium and high) for the same period of time and used for a ChIP with antibodies against SNAP43 and RB, or nonspecific IgG as a negative control. HeLa cells (Rb^-) were also examined as a negative control. The U6-1, Y1, Y3 and MRP promoters were amplified from the immunoprecipitated DNA by PCR. Lanes 1-3 are the input DNA (1%, 0.1% and 0.01% respectively). Lane 4 is the DNA immunoprecipitated with IgG, lane 5 with SNAP43, and lane 6 with Rb.

Figure 2-5. Rb localization to RNA polymerase III-transcribed genes is based on cell density



In addition, Rb localization at other type 3 genes, Y1, Y3 and MRP, under the conditions in which Rb was seen at U6-1 was measured. Rb was not found at the promoters of any of these other type 3 genes even though Rb is known as a global repressor of RNA polymerase III-transcribed genes (White, Trouche et al. 1996). This suggests the mechanism of repression of these various type 3 genes is different. Rb is thought to repress type 1 and type 2 genes by sequestering TFIIIB and inhibiting its ability to localize to the promoters (Chu, Wang et al. 1997). Therefore, it is possible that repression of some type 3 genes follows this same mechanism.

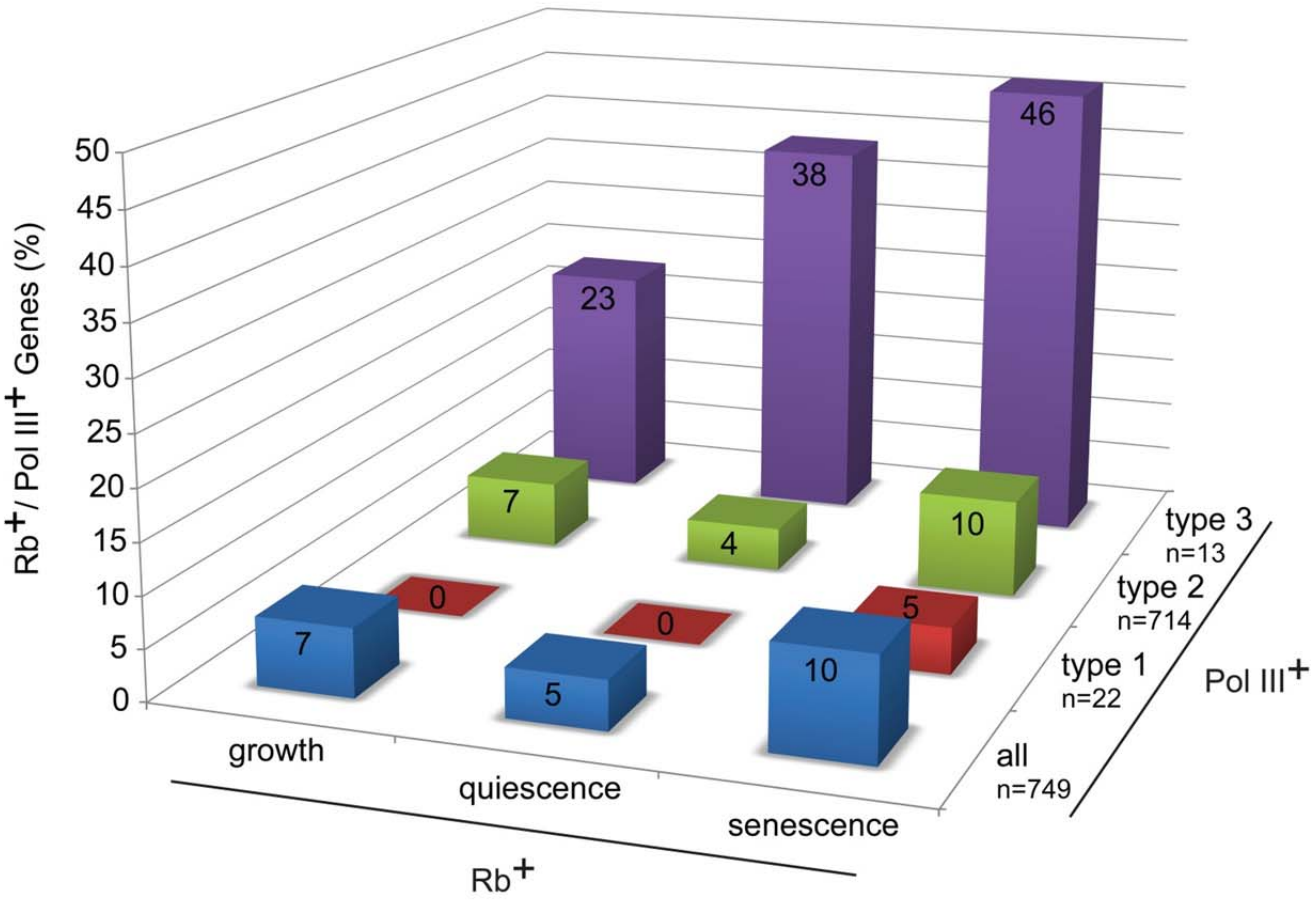
During quiescence and senescence, Rb preferentially localizes to type 3 RNA polymerase III-transcribed genes.

To determine the Rb localization at RNA polymerase III genes, I mined the data from two published ChIP-seq experiments. Nouria Hernandez identified the localization of RNA polymerase III in the genome of an actively growing human lung fibroblast (IMR90) cell line (Canella, Praz et al. 2010). Scott Lowe identified the localization of Rb *in the same cell line* during various growth conditions, including active growth, quiescence and senescence (Chicas, Wang et al. 2010). Alignment of these two data sets allowed me to determine where Rb resides at RNA polymerase III genes under these different growth conditions. As shown in Figure 2-6 (front row), Rb resides at RNA polymerase III-transcribed genes under all growth conditions, with a slight increase in Rb localization during senescence. However, when I analyzed Rb localization by the *type* of RNA polymerase III promoter (back three rows of Figure 2-6) it became clear that Rb prefers type 3 genes and preferentially localizes to these genes during senescence and quiescence. Rb resides at 10% of all pol III-transcribed genes during senescence,

Figure 2-6. Rb localization at genome-wide RNA polymerase III-transcribed genes

The RNA polymerase III ChIP-seq data from N. Hernandez (2010) and the Rb ChIP-seq data from S. Lowe (2010), both performed in human IMR90 cells, were aligned and the Rb^{+}/Pol III^{+} genes were identified. The Pol III genes are separated out into the various type (1, 2 or 3).

Figure 2-6. Rb localization at genome-wide RNA polymerase III-transcribed genes



7% during active growth and 5% during quiescence. However, 46% of type 3 genes contained Rb during senescence, compared to the 10% of type 2 and 5% of type 1, demonstrating that Rb preferentially localizes to type 3 genes.

However, Rb localization has proven to be complex. Analysis of each of the RNA polymerase III^+ type 3 genes illustrated that Rb occupancy varied during the growth conditions (Table 2-1). In addition, Rb occupied only some alleles, and not others. Rb was seen at the 7SK, RNaseP and U6ATAC genes under all growth conditions. The MRP and U6-1 genes had Rb only during quiescence and senescence. U6-2 contained Rb during senescence only. U6-7, U6-8, U6-9 and all 4 of the Y RNA genes did not have Rb under any growth conditions. This illustrates the complexity of identifying Rb^+ genes and the conditions under which they occur.

The distribution of Rb around the U6-1 promoter suggests a repression mechanism involving remodeling of the chromatin architecture.

We have focused our study of the Rb repression mechanism using the U6-1 gene as our model because we have localized Rb directly to the promoter and we have shown that Rb can repress transcription of a U6-1 reporter *in vivo*. However, the mechanism of repression is not well understood. Previous work from our lab has demonstrated that Rb co-localizes to the U6-1 promoter with RNA polymerase III and GST-pulldowns have shown that Rb interacts with various subunits of the core transcription machinery, including SNAPc and TFIIIB (data not shown, work performed by G. Jawdekar and X. Zhou). To better understand the repression mechanism of Rb, I studied the distribution of Rb (under various growth conditions) around the U6-1

Table 2-1. The localization of Rb at type 3 RNA polymerase III-transcribed genes

The RNA polymerase III ChIP-seq data from N. Hernandez (2010) and the Rb ChIP-seq data from S. Lowe (2010), both performed in human IMR90 cells, were aligned and the Rb^+/type 3 Pol III^+ genes were identified.

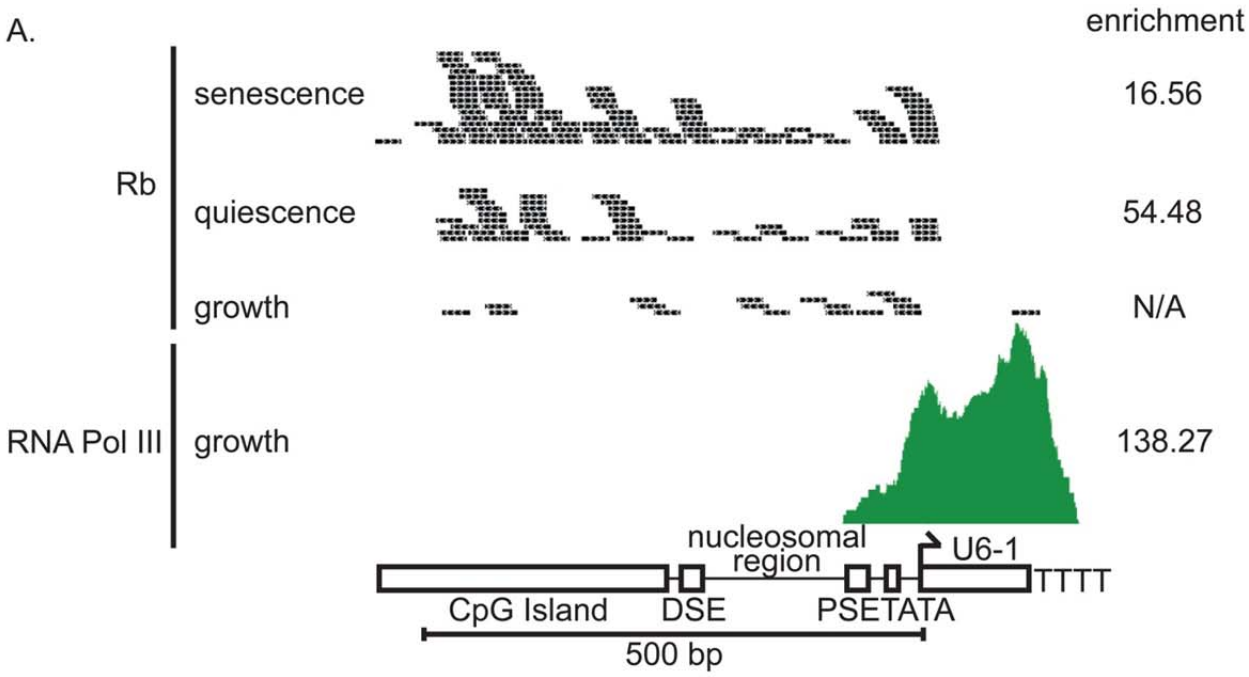
Table 2-1. The localization of RB at type 3 RNA polymerase III-transcribed genes

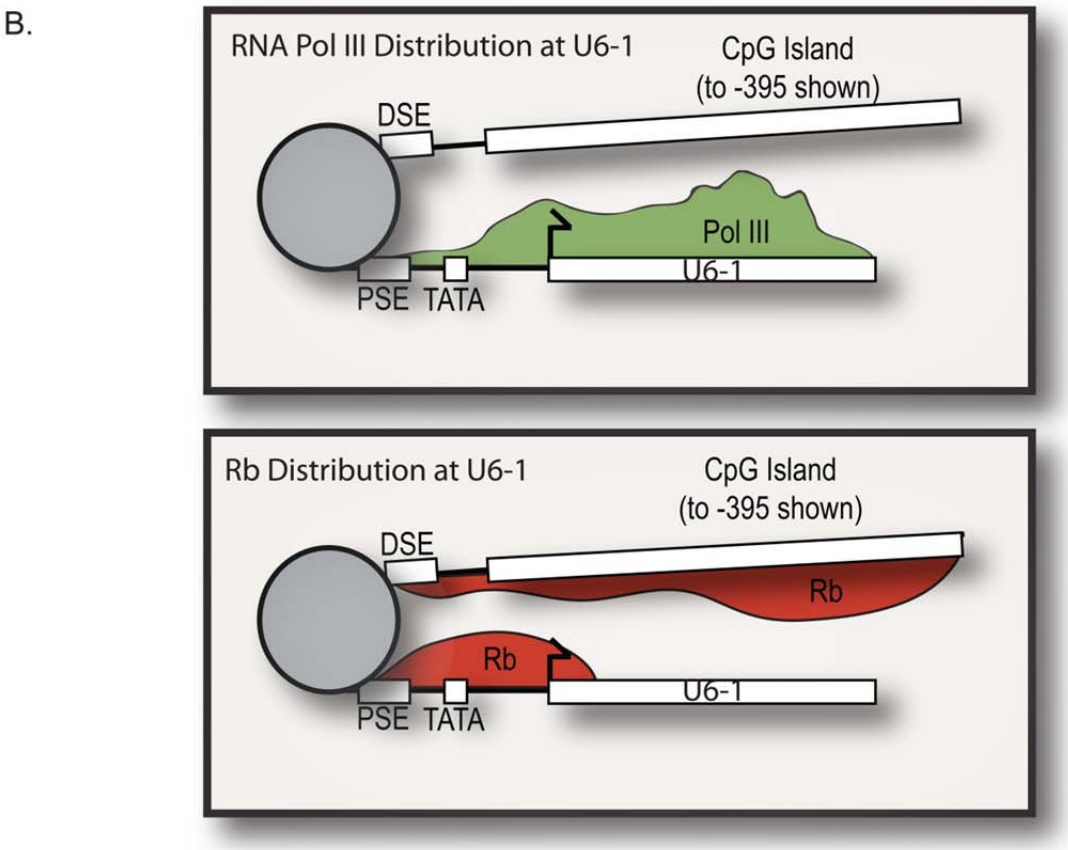
Type 3 Pol III$^+$ Genes	Rb^+ Genes		
	growth	quiescence	senescence
7SK	+	+	+
MRP	-	+	+
RNase P	+	+	+
U6-1	-	+	+
U6-2	-	-	+
U6-7	-	-	-
U6-8	-	-	-
U6-9	-	-	-
U6ATAC	+	+	+
Y1	-	-	-
Y3	-	-	-
Y4	-	-	-
Y5	-	-	-

Figure 2-7. Rb distribution surrounds the positioned nucleosome at U6-1

A. Bioinformatic analysis of the RNA polymerase III ChIP-seq data (Hernandez N., 2010) and Rb ChIP-seq data (Lowe SW., 2010) at the U6-1 locus was analyzed using the Human Genome Browser to determine the distribution of Rb around the gene under different growth conditions.
B. The distribution of RNA polymerase III (top panel) and Rb (bottom panel) across the U6-1 gene is modeled around the positioned nucleosome.

Figure 2-7. Rb distribution surrounds the positioned nucleosome at U6-1





promoter by aligning the ChIP-seq data sets from Nouria Hernandez (pol III) and Scott Lowe (Rb) using the Human Genome Browser. As shown in Figure 2-7A, Rb is seen at the core promoter, as expected, during times of cellular silence (quiescence and senescence). Interestingly, Rb is also seen about 400 bp upstream over the CpG island and was *not* observed where there is known to be a positioned nucleosome. Rb was also not seen extending into the coding region. This data demonstrates that Rb surrounds the positioned nucleosome. In Figure 2-7B, I have depicted a model for the distribution of RNA polymerase III and Rb along the U6 promoter based on the location of the positioned nucleosome, whereby Rb is located at the core promoter, enhancer and the CpG island due to the close proximity of the DNA strands due to wrapping of the DNA around the nucleosome. The localization of Rb around the nucleosome, in conjunction with the fact the nucleosome is required for activated levels of transcription (Zhao, Pendergrast et al. 2001), suggests chromatin remodeling machinery plays a role in the Rb-mediated repression mechanism. Rb may recruit chromatin remodelers to disrupt contacts between the transcriptional activator, Oct1, and the core machinery, SNAPc.

Rb directs corepressors Brg1, Brm, HDAC1 and HDAC2 to the U6 promoter to enact repression.

It has previously been shown that an Rb/HDAC/SWI/SNF complex is necessary for progression into and through the S phase (Zhang, Gavin et al. 2000) and that mutations in the Brg1 component of human SWI/SNF inhibited Rb-mediated G1 arrest (Bartlett, Orvis et al. 2011), suggesting Rb may direct these corepressors to the U6 promoter to enact cell cycle repression programs. To determine if these corepressors

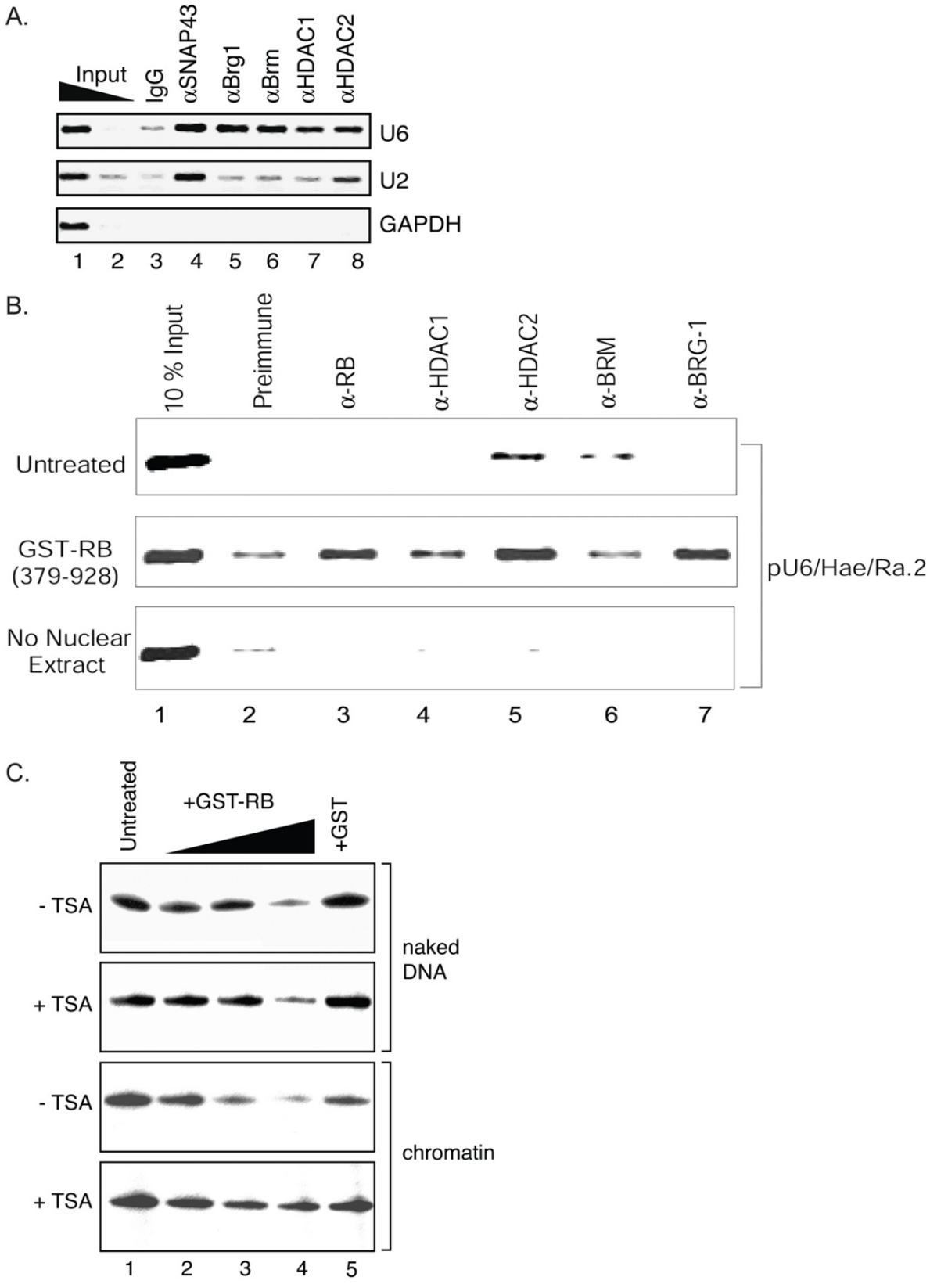
Figure 2-8. Rb directs chromatin modifying corepressors required for U6-1 repression to the promoter

A. 184B5 (Rb^+) cells were used in a ChIP with antibodies against SNAP43, Brm, Brg1, HDAC1 and HDAC2. The promoters of U6-1, U2 and GAPDH were amplified from the immunoprecipitated DNA by PCR. Lanes 1-4 contain 10%, 1%, 0.1% and 0.01% of input DNA. Lanes 5 is from nonspecific IgG. Lanes 6 is from α-SNAP43, lane 7 from α-Brm, lane 8 from α-Brg1, lane 9 from α-HDAC1 and lane 10 from α-HDAC2 antibodies. Experiment performed by Gauri Jawdekar, as described in her thesis.

B. Cross-linked U6-1 *in vitro* transcription reactions were subjected to immunoprecipitation reactions with α-Rb (lane 3), α-HDAC1 (lane 4), α-HDAC2 (lane 5), α-Brm (lane 6) and α-Brg1 (lane 7) antibodies or nonspecific serum (lane 2). The immunoprecipitated DNA was analyzed for the U6-1 reporter with specific primers by PCR. Experiment performed by Gauri Jawdekar, as described in her thesis.

C. HeLa cell nuclear extract was incubated with the U6-1 reporter (naked, or assembled into chromatin). Increasing amounts; 300 ng (lane 2), 600 ng (lane 3), 1000 ng (lane 4) of GST-Rb (379-928) were tested for U6 repression in the presence of 500 μM Trichostatin A (TSA) or in a control reaction without any added TSA. 1000 ng GST (lane 5) was used as a negative control. Lane 1 shows the level of transcription supported by untreated extracts. Experiment performed by Gauri Jawdekar, as described in her thesis (Jawdekar 2006).

Figure 2-8. Rb directs chromatin modifying corepressors required for U6-1 repression to the promoter



reside at the U6-1 locus, chromatin immunoprecipitation assays were performed with antibodies for Brg1, Brm, HDAC1 and HDAC2. As shown in Figure 2-8A, all four corepressors were at the U6-1 promoter, but only HDAC2 was found at the U2 promoter, a gene transcribed by RNA polymerase II and not thought to be regulated by Rb. SNAP43 was at both loci, as expected. GAPDH served as a negative control.

To determine the role of Rb in the occupancy of these corepressors at the U6-1 loci, an *in vitro* immunoprecipitation was performed. The U6-1 reporter and GST-RB were incubated in HeLa nuclear extract and RB, HDAC1, HDAC2, Brg1 and Brm were immunoprecipitated. As shown in Figure 2-8B, with GST-Rb HDAC1, HDAC2, Brm and Brg1 were found at the U6 reporter. Without Rb, HDAC2 and Brm were found at the reporter. Without nuclear extract, nothing was found at the reporter. This demonstrates that Rb has the ability to recruit HDAC1, HDAC2 and Brg1 to the U6 promoter.

To measure the involvement of HDAC activity in Rb-mediated repression of U6-1, trichostatin A (TSA) was used as a HDAC inhibitor during an *in vitro* Rb repression assay with a chromatinized U6 reporter or a naked U6 reporter. Rb was able to repress U6 transcription with or without TSA when the reporter was not assembled into chromatin (Figure 2-8C). However, once the U6 reporter was chromatinized the ability of Rb to repress transcription was dependent on active HDACs. The addition of TSA hindered Rb in enacting repression by over 5-fold. This demonstrates that HDACs are a critical component of Rb-mediated repression of U6-1 when the template is chromatinized, and that the mechanism of Rb repression does not require HDAC activity when the DNA is naked.

Until now, we have demonstrated that the chromatin remodeling ATPase subunits Brg1and Brm localize to the U6 promoter and that Rb can recruit Brg1 to the U6-1 promoter. To determine if Rb can interact with Brg1, a GST-pulldown was performed in HeLa nuclear extract. As shown in Figure 2-9A, recombinant GST-Rb (379-928) copurified with Brg1. In addition, a truncation involving deletion of the A domain in the pocket region of Rb ablated this interaction. This demonstrates that Rb can interact with Brg1 through it's A domain.

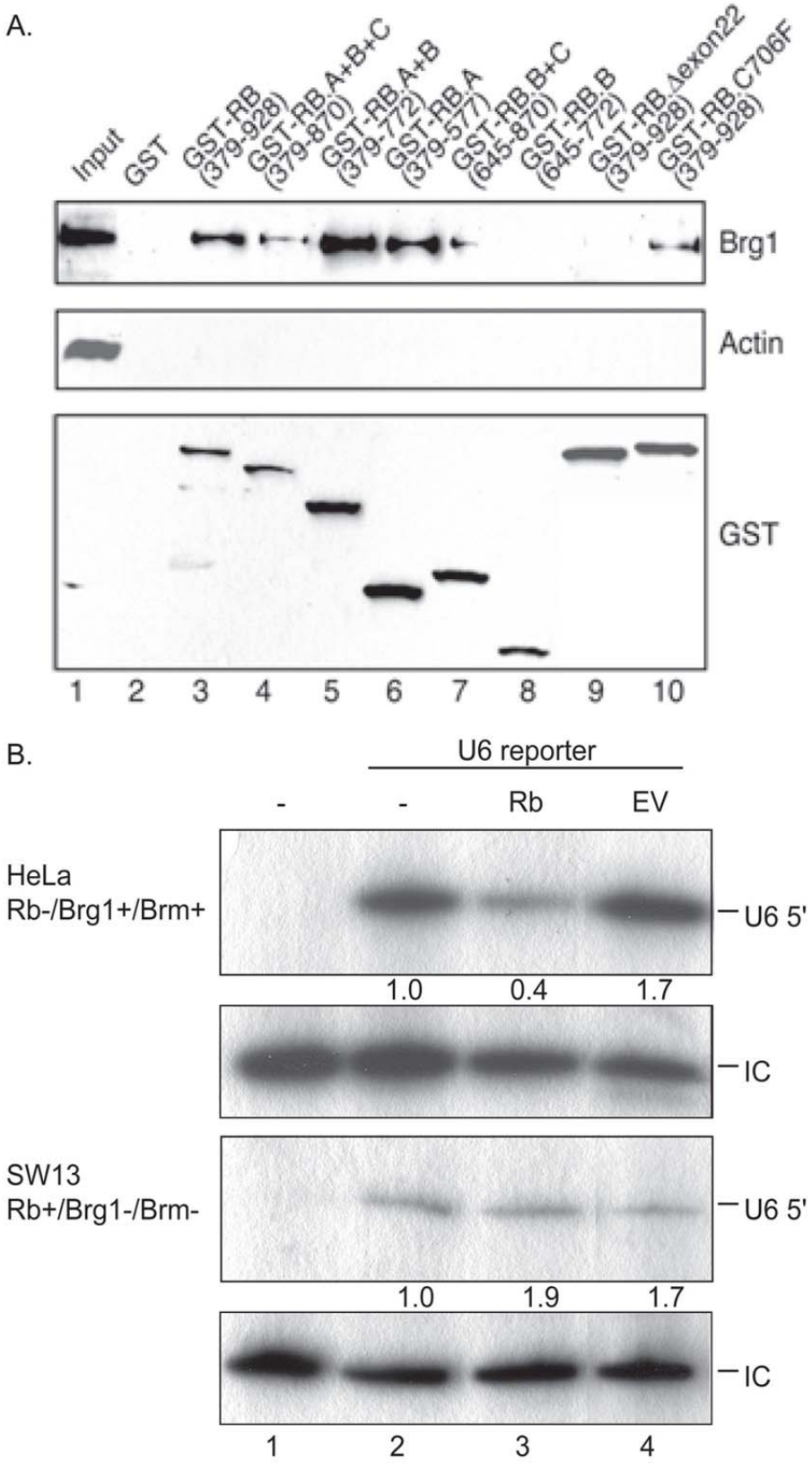
To determine if Rb-mediated repression of U6-1 requires Brg1 or Brm, I performed an *in vivo* repression assay with the U6-1 reporter and an Rb expression vector in a Brg1/Brm$^+$ cell line (HeLa) and a Brg1/Brm$^-$ cell line (SW13). Total RNA was collected and analyzed with an RNase T_1 protection assay. As shown in Figure 2-9B, Rb repressed the U6 reporter 60% in the Brg1/Brm$^+$ cell line but was not able to repress the reporter at all in the Brg1/Brm$^-$ cell line. This demonstrates that the Brg1/Brm corepressors are an important aspect of Rb-mediated repression of the U6-1 gene.

Figure 2-9. Brg1 is a component of the Rb repression mechanism at U6-1

A. Characterization of the Rb domains required for its association with Brg1. GST pulldown analysis was performed by incubating 300 µL of HeLa cell nuclear extract with 500 ng GST (lane 2), GST-Rb (379-928) (lane 3), GST-Rb A/B/C domains (379-870) (lane 4), GST-Rb A/B domains (379-772) (lane 5), GST-Rb A domain (379-577) (lane 6), GST-Rb B/C domains (645-870) (lane 7), GST-Rb B domain (645-772) (lane 8), GST-Rb ∆exon22 (379-928) (lane 9) and GST-Rb C706F (379-928) (lane 10). Precipitated proteins were analyzed by SDS-PAGE. The associated proteins were assessed by Western Blot with an α-Brg1 antibody. Western blot analysis using GST antibody indicated the relative amount of GST tagged Rb proteins recovered after processing. Experiment performed by Xianzhou Song, as described in his thesis.

B. Rb-mediated repression of U6-1 is lost in a Brg1/Brm$^-$ cell line. The U6-1 reporter was transiently-transfected into HeLa (Brg1/Brm$^+$) and SW13 (Brg1/Brm$^-$), along with an Rb expression vector (Rb, lane 3) or an empty vector (EV, lane 4), or nothing as a control (lane 2). Lane 1 is the control signal from mock (no plasmid DNA) transfected cells. The total RNA was collect after 24 hours and analyzed with an RNase T_1 protection assay. The P32-labeled probe was imaged using a Phosphoimager.

Figure 2-9. Brg1 is a component of the Rb repression mechanism at U6-1



Discussion

Rb is a known tumor suppressor gene and is thought to act, in part, by regulating the G1/S phase transition. In our study, I have found that Rb represses U6 steady-state RNA levels during G1. From the literature we know that RNA polymerase III transcription is repressed during late S phase due to phosphorylation of TFIIIB by CK2, explaining the decrease in U6 RNA levels during this time (Hu, Samudre et al. 2004). Since we know that Rb stably associates with the U6 promoter, and that Rb is hypophosphorylated and active in early G1, I favor the idea that the decrease in U6 steady state levels in G1 is due to Rb repression. The number of U6 transcripts per cell has been estimated at $3x10^5$ (Weinberg and Penman 1968). This suggests that between mid and late G1, U6 is de-repressed and the powerful promoter drives expression of roughly 20 transcripts per second! In addition, I have shown that Rb causes repressed U6 levels in conjunction with a G1-block, demonstrating that Rb represses U6 levels during its canonical function during G1.

However, the timing of Rb localization at U6-1 still remains unclear. In my experiments, Rb was localized to U6-1 only when the cells were grown to an intermediate confluency, but not when the cell density was too low or too high. This observation suggests Rb may be involved in establishing a repressed cell state and is not required once this state is set. However, my analysis of ChIP-seq data from Scott Lowe's lab revealed that Rb was at U6-1 during quiescence and senescence, and not during active growth. This demonstrates that Rb localization can be variable and is dependent on multiple factors. However, Rb does preferentially localize to type 3 genes, and for the U6-1 gene we

have previously shown that Rb colocalizes with the core transcription machinery; SNAPc, TBP and RNA polymerase III (Hirsch, Jawdekar et al. 2004). In my studies, Rb was not observed at other type 3 genes (Y1, Y3 and MRP) under the conditions it was observed at U6, suggesting the mechanism of Rb recruitment may vary between the different type 3 genes.

The mechanism of Rb-mediated repression has been suggested to be centered on modification of the local chromatin architecture (Frolov and Dyson 2004). Rb has previously been described as a molecular adapter due to its ability to be recruited to different genes by various DNA-binding proteins and then, in turn, to recruit different corepressors to the sites. In this way, Rb is recruited to the U6 promoter by SNAPc and TFIIIB and recruits the corepressors HDAC1 and HDAC2, and hSWI/SNF. The distribution of Rb around the positioned nucleosome suggests these enzymes may function by repositioning, or removing, it. Although we have not yet been able to determine if Rb leads to changes in the position of this nucleosome, we have shown that Rb-mediated repression at U6-1 is dependent on both HDAC1/2 and Brg1/Brm.

Chapter 3

Rb recruits DNA methyltransferases to the U6-1 promoter and can direct low-level methylation.

Abstract

CpG methylation of gene promoters is a repressive mark and is therefore a possible component of the Rb-mediated repression mechanism. All active U6 alleles have maintained high-density CpG/GpC promoters, suggesting these sites play an important regulatory role in U6 expression. Rb has been shown to interact with DNA methyltransferase (DNMT) 1, suggesting that DNMT-directed methylation of the U6-1 gene may be an important mechanism of Rb-mediated repression. Here we show that loss of DNMTs results in increased levels of U6 RNA. In addition, Rb recruits DNMT1 and DNMT3A to the U6-1 promoter and Rb can direct low-level methylation of the gene. However, methylation of the U6-1 gene is not required for Rb-mediated repression of U6 RNA steady-state levels in cycling cells.

Introduction

My focus for the mechanism of Rb repression has been on the U6-1 gene because we have shown that Rb can directly repress transcription by associating with the promoter, and that Rb represses U6 RNA levels during G1 of the cell cycle. In addition to U6-1, there are eight other U6 genes. All nine U6 genes in the human genome contain identical coding regions. Of these U6 genes, five are actively transcribed and the others are not (Domitrovich and Kunkel 2003). The actively transcribed genes have a high CpG/GpC ratio relative to the inactive genes, suggesting the CpG sites play a regulatory role in U6 gene expression (Figure 3-1).

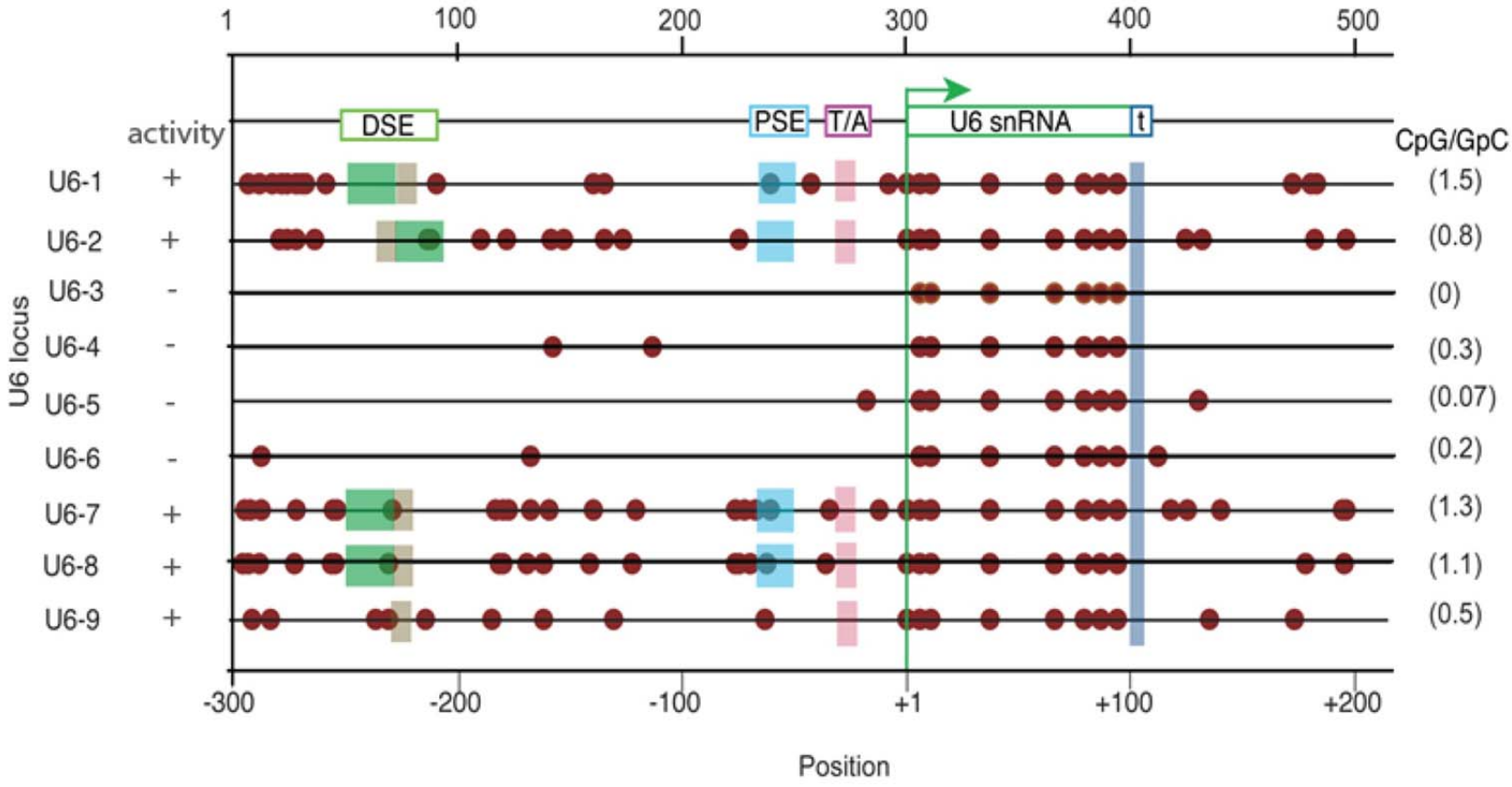
The cytosine of CpG dinucleotides is a target of methylation and this methylation has been shown to lead to gene repression (Tate and Bird 1993; Merlo, Herman et al. 1995). In addition, aberrant methylation of tumor suppressor genes, such as Rb, and oncogenes has been correlated with tumorigenesis (Merlo, Herman et al. 1995; Stirzaker, Millar et al. 1997; Maruyama, Toyooka et al. 2001), demonstrating the importance of proper CpG methylation maintenance.

There are three DNA methyltransferases in the mammalian system; DNMT1, DNMT3A and DNMT3B. DNMT1 is considered the maintenance methyltransferase, adding methyl groups from the methyl donor, *S*-adenosylmethionine, to the cytosine of CpG dinucleotides in hemimethylated DNA. DNMT3A and 3B are considered the *de novo* methyltransferases, adding methylation marks to CpG dinucleotides in unmethylated DNA, making hemimethylated DNA.

Figure 3-1. The CpG density of the nine U6 loci correlates with transcriptional activity.

A schematic representation of the nine U6 loci, with identical coding regions, in the human genome. The CpG sites are indicated by a red dot. The transcriptionally active loci (U6-1, 2, 7, 8, 9) contain the TATA box, PSE and DSE promoter elements, with exception of U6-9, which has lost the consensus PSE but still recruits SNAPc. The inactive loci (U6-3, 4, 5, 6) have lost the essential promoter elements. The transcription start site is denoted by the green line. The termination signal is denoted by the *t*.

Figure 3-1. The CpG density of the nine U6 loci correlates with transcriptional activity



Rb has been shown to copurify with DNMT1 and HDAC1, and this interaction was shown to be important for the repression of E2F-regulated RNA polymerase II-transcribed genes (Robertson, Ait-Si-Ali et al. 2000). Our lab has previously shown that Rb co-localizes to the U6-1 promoter with DNMT1, DNMT3A, HDAC1 and HDAC 2, and that Rb can recruit DNMT1 and DNMT3A to the promoter (data not shown). In addition, U6 genes contain a conserved CpG at the start site. Dr. Thara Selvakumar from our lab has demonstrated that Rb can direct low-level methylation of this CpG site (data not shown).

Here I will discuss my work in determining the role of DNA methylation in Rb-mediated repression of the U6-1 gene. U6 RNA expression levels are activated by inhibition of the DNMTs, demonstrating the role of these enzymes in repressing RNA levels. In addition, gene-wide analysis of U6-1 methylation demonstrated that Rb can direct low-level methylation of the U6-1 start site, although not to a significant level, and this methylation is cell line specific. Rb directed significant low-level methylation just downstream of the start site in an osteosarcoma cell line with inducible Rb expression. However, no significant methylation of the U6-1 gene was observed under conditions when Rb was actively repressing U6 steady-state RNA levels during G1 of the cell cycle. This suggests that DNA methylation is not playing a critical role in Rb-mediated repression of U6-1, at least under the conditions I have tested.

Materials and Methods

5'-Aza-2'-deoxycytidine (AdC) Inhibition of DNMTs

U2OS cells were seeded in 6-well plates at $1x10^4$ cells per well. After 24 hours the cells were treated with 10 μM or 20 μM AdC (Sigma #A3656) and grown for 2 to 5 more days. Each day the media was changed and replaced with media containing fresh AdC. The cells were collected with TRIzol and stored at -80°C. The RNA was harvested according to the manufactures protocol. The U6 RNA (50 ng in a 40 μl total volume) was reverse transcribed into cDNA with the High-Capacity cDNA kit using random primers according to the manufacturers protocol. 2 μl of the cDNA was used for quantitative PCR with 2X Sybr Green Master Mix and the U6 specific primers described previously (see Chapter 2 Materials and Methods).

Bisulfite Sequencing

HeLa cells (approximately $1x10^6$) were co-transfected with 2 μg pCMV-RB expression plasmid or empty vector DNA along with 250 ng of the expression plasmid pEGFP, encoding the enhanced green fluorescent protein. 48 hrs after transfection, approximately 10^5 EGFP-expressing cells from each condition were collected by fluorescence activated cell sorting (FACS) to enrich for transfected cells, and these cells were then subjected to bisulfite conversion using the EZ DNA Methylation-Direct Kit (Zymo Research) using the procedure described below. The effect of RB on the methylation of the U6-1 locus in Saos2-tet-Rb cells was determined as above, except RB expression was induced for 48 hours by removal of doxycycline. The methylation

status of U6-1 through the cell cycle was determined using Nocodazole-synchronized Saos2-tet-Rb cells described in the Chapter 2: Material and Methods section.

The genomic DNA from $3x10^4$ cells was collected for methylation analysis by bisulfite sequencing with the Zymo Research EZ DNA Methylation-Direct Kit (#D5020). The DNA was eluted in a 15 µl of elution buffer and 2 µl was used for the first round of PCR with the following primers and conditions:

U6-1 Top Strand-
F -519: ATGGAAGCTTTAGGGAAGGGTTTAATTAG
R +328: CCACTCTAGAAAAACCAAAAACAAAATACA
U6-1 Bottom Strand-
F -584: CCATAAGCTTACCCCTTCCAACCCACAATA
R +328: TCCGTCTAGAAGGGTTAGAGGTAAAATGTA

Conditions:
94°C- 4'- 1 cycle
94°C- 30s
52°C- 1' 40 cycles
72°C- 1.5'
72°C-4'-1 cycle

A second round of PCR was performed with 4 µl of the original PCR product using the following primers and conditions:

U6-1 Top Strand-
F -461: GTGTAAGCTTTGGGAATTTTATGGGTATTA
R +266: CCACTCTAGAATCTCCTAAAACTACTACAT
U6-1 Bottom Strand-
F -519: CCATAAGCTTCAAAAAAAAATCCAACCAA
R +62: TCCGTCTAGAGGGTTATGTTAATTTTTTTTG

Conditions:
94°C- 4'- 1 cycle
94°C- 30s
52°C- 1' 18 cycles
72°C- 1.5'
72°C-4'-1 cycle

The amplicons were gel purified (Promega kit #A9281) and double digested with HindIII and XbaI (NEB) and cloned into pUC119 for sequencing.

Bisulfite Sequence Analysis

The sequenced clones were analyzed with BISMA software (Rhode et al., BMC Bioinformatics 2010). The clones were aligned pair-wise to a U6-1 template sequence. The cutoff parameters for analysis were set at a 95% minimum conversion rate, 90% minimum sequence identity, 20% maximum of N-sites at cytosine positions and 20% maximum for insertions/deletions. The analyzed sequences were compared and the methylation at each CpG site was calculated. The statistical significance of each methylation event was analyzed using the T-test.

Results

Rb directs low level methylation of the U6-1 promoter.

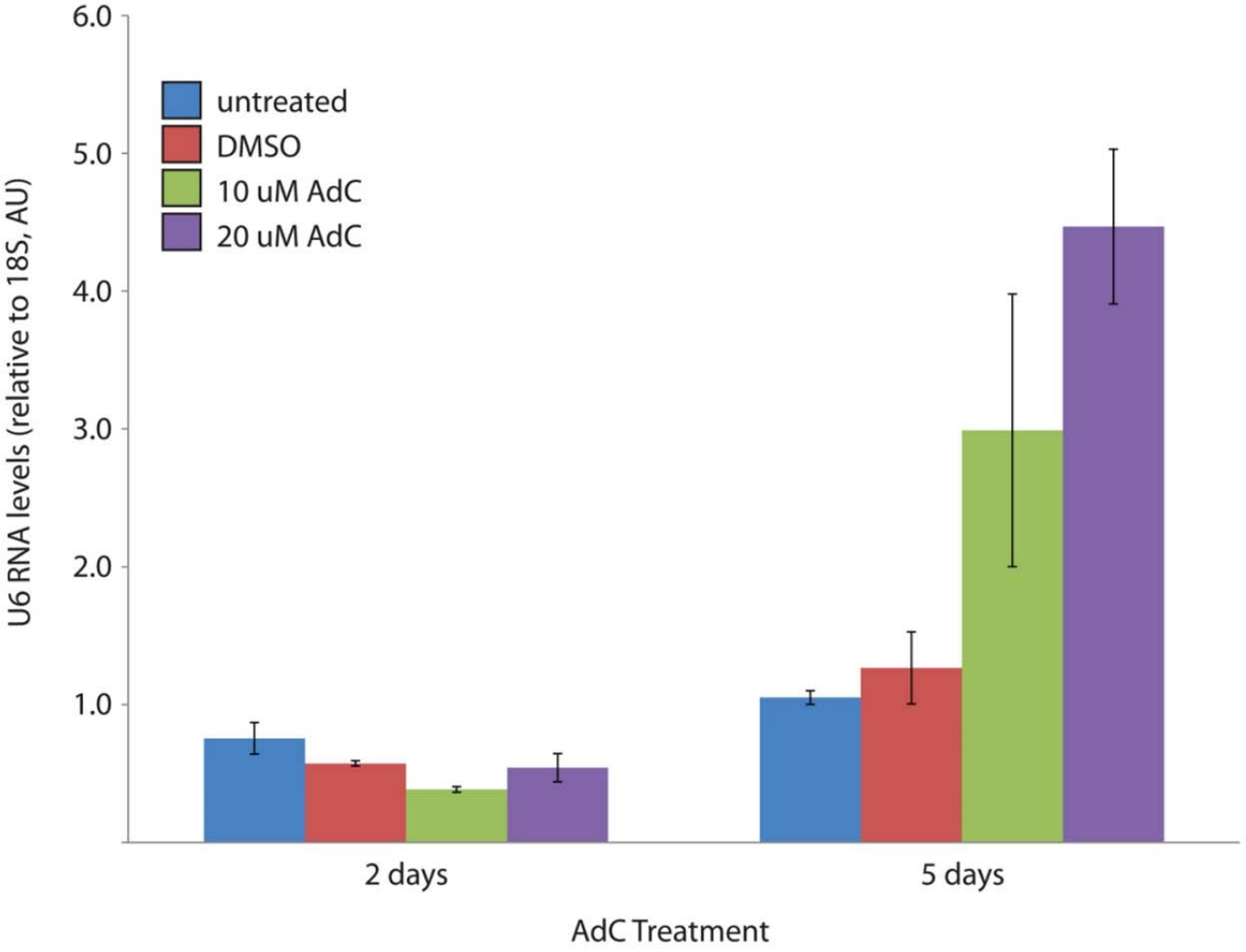
To determine if DNMT's are necessary for repression of U6 steady-state levels, the DNMT's were inhibited with 5'-aza-2'-deoxycytidine (AdC), a cytidine analog which becomes incorporated into the DNA and forms an irreversible adduct with the DNMT enzymes (Christman 2002). An Rb^+ osteosarcoma cell line, U2OS, was treated with 10 or 20 μM AdC for 2 or 5 days. As shown in Figure 3-2, inhibition of the DNMT's for 2 days had no effect. However, after 5 days of AdC treatment the U6 RNA levels increased over 4-fold, demonstrating that the DNMT's are important for repressing U6 RNA levels.

Preliminary results have shown that Rb has the ability to direct low-level methylation of the U6 reporter transcription start site when transiently transfected in HeLa, as measured by methylation sensitive restriction enzymes and PCR amplification across the start site (data not shown). To measure the ability of Rb to methylate the entire U6 promoter I utilized bisulfite sequencing. An Rb expression vector (or empty vector) and a GFP expression vector were transiently transfected into HeLa cells. The GFP^+ cells were collected and the DNA was analyzed for methylation across the U6-1 loci. Interestingly, small, low-level methylation (4%) was observed in the Rb^+ cells at the transcription start site on the template strand (Figure 3-3A). However, the methylation level did not meet statistical significance ($p>0.05$) and low-level methylation was found sporadically across the U6-1 promoter, on both the template and non-template strand

Figure 3-2. U6 RNA steady-state levels increase upon DNMT inhibition.

U2OS cells were treated with 10 μM or 20 μM AdC for 2 to 5 days. The total RNA was collected with TRIzol and converted into cDNA. The U6 cDNA levels were measured by qPCR and normalized to 18S rRNA.

Figure 3-2. U6 RNA steady-state levels increase upon DNMT inhibition



and with or without Rb. Due to this, it is hard to say whether the start site methylation is meaningful. No start site methylation was observed in the empty vector treated cells (Figure 3-3B). Importantly, low-level start site methylation has been observed by two different people using two different analysis methods, suggesting the start site methylation may be meaningful.

To determine if Rb may direct more significant levels of DNA methylation of the U6-1 loci in a different system, I induced Rb expression for 48 hours in asynchronously growing Saos2tetRb cells and analyzed the DNA by bisulfite sequencing. Interestingly, Rb induced significant methylation (12%) at the +6 CpG on the nontemplate strand (Figure 3-4A). No methylation was seen on the template strand. Analysis of the Rb^- sample revealed no significant DNA methylation on either strand, anywhere, from the upstream DSE through the coding region (Figure 3-4B). No methylation was seen at the start site CpG under any condition.

I have previously shown that Rb can induce a G1 block and repress U6 steady-state RNA levels in Nocodazole-synchronized Saos2tetRb cells (Chapter 2, Figure 2-3). To determine if Rb was also directing DNA methylation of the U6-1 locus during this time I analyzed the cellular DNA from each time point with bisulfite sequencing. As shown in Figure 3-5, no significant methylation was observed from the CpG island through the coding region. This demonstrates that DNA methylation is not a component of Rb-mediated U6 repression in Nocodazole-synchronized cells.

Figure 3-3. Low-level methylation of the -1 CpG at U6-1 is reproducible.

The gene-wide methylation status of the U6-1 locus was measured by bisulfite sequencing. An Rb expression vector or EV were co-transfected with GFP into HeLa cells (A=+Rb, B=+EV). The GFP^+ cells were collected, the gDNA was bisulfite converted and both strands of the U6-1 locus was amplified. The amplicon was cloned into pUC119 and sequenced. The sequenced clones were analyzed by BISMA. The number of clones sequenced is denoted by *n*. The dotted line denotes the level of methylation necessary to reach statistical significance (p=0.05).

Figure 3-3. Low-level methylation of the -1 CpG at U6-1 is reproducible

A.

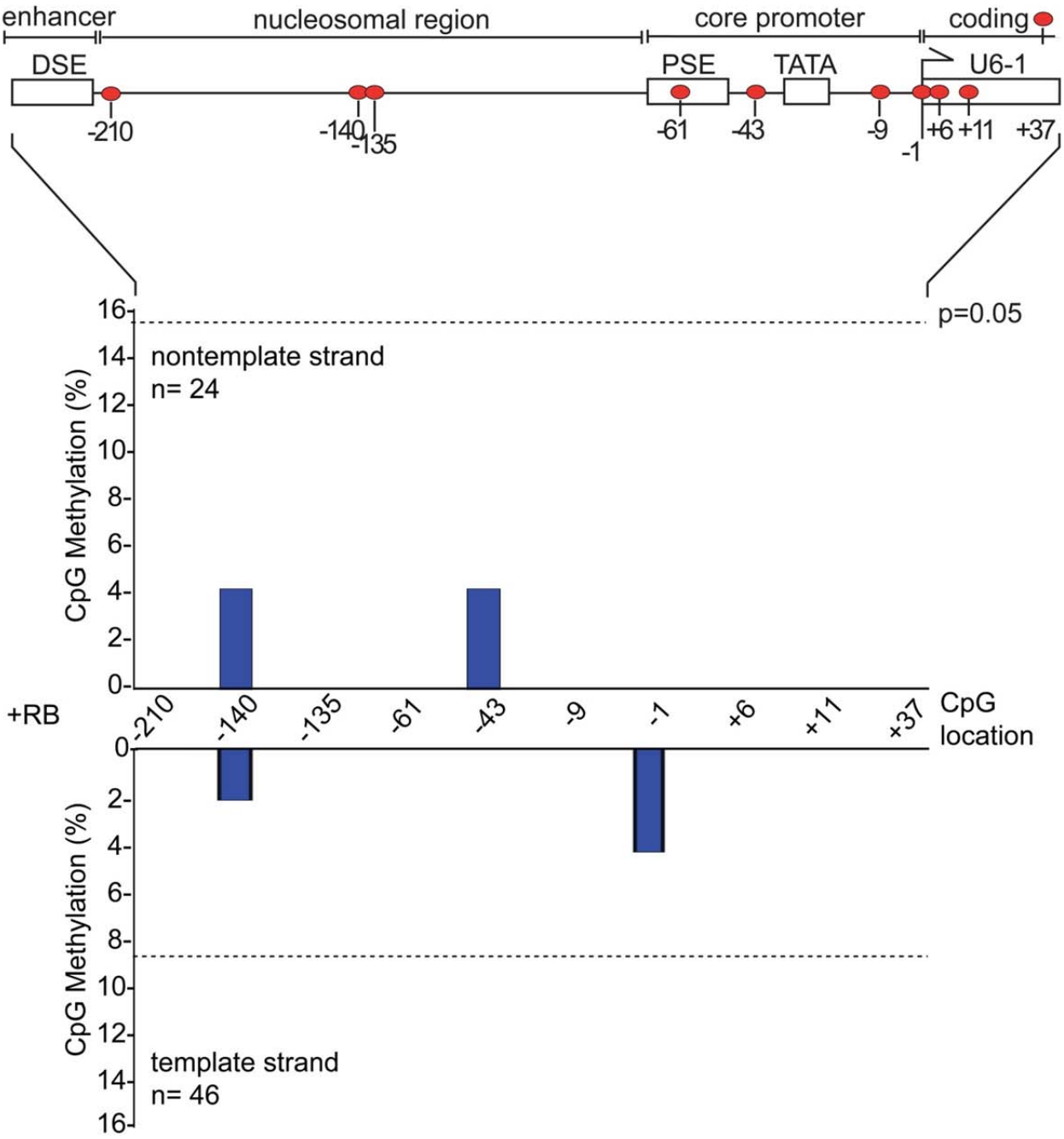


Figure 3-3 (cont'd)

B.

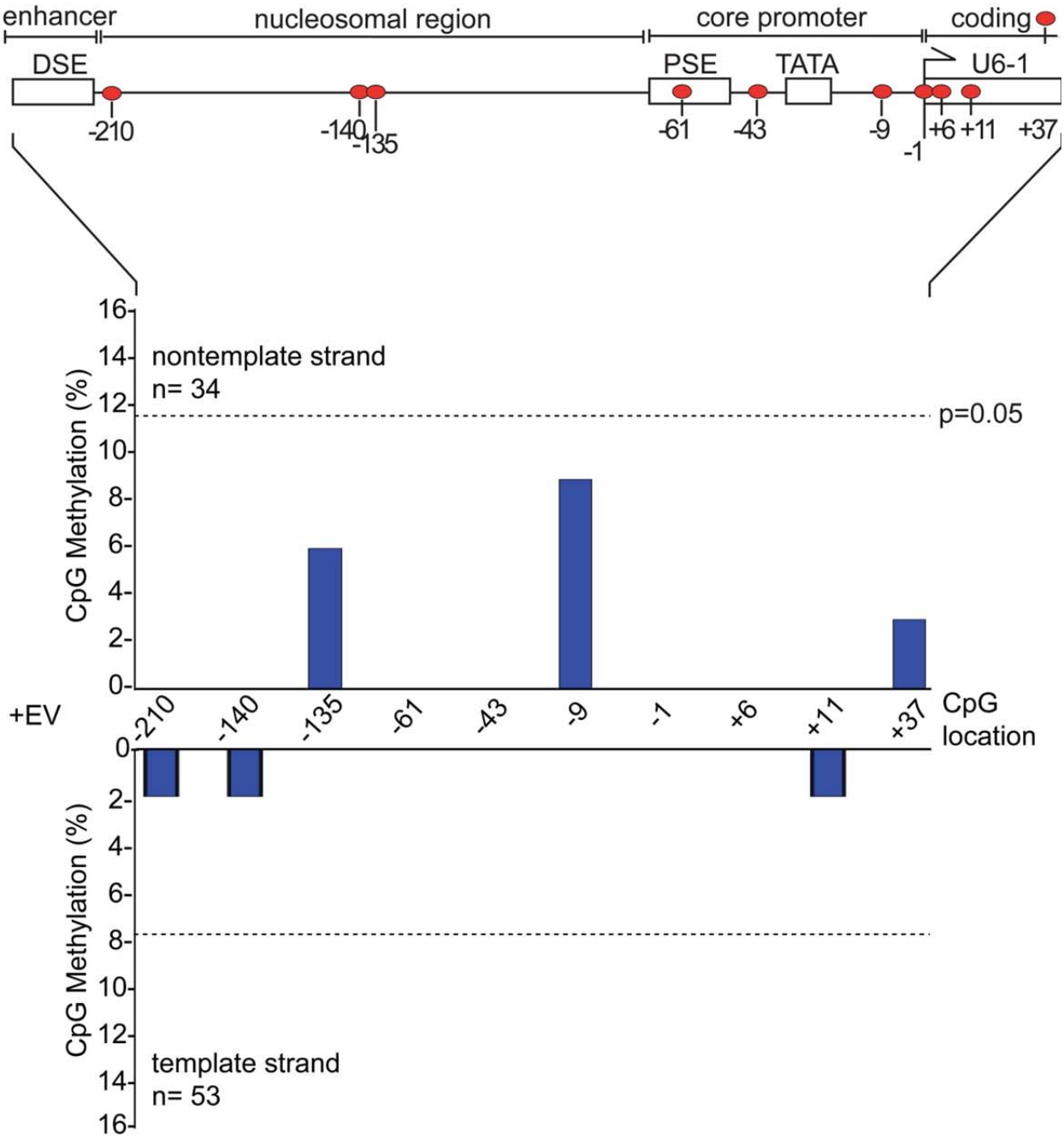


Figure 3-4. Rb directs significant low-level methylation of the +6 CpG

The methylation of the U6-1 locus was measured by bisulfite sequencing. Rb was expressed for 48 hours in Saos2tetRb by removal of doxycycline from the media (A= +Rb, B=-Rb). The gDNA was bisulfite converted and both strands of the U6-1 locus was amplified by PCR. The amplicon was cloned into pUC119 and sequenced. The sequenced clones were analyzed by BISMA. The number of clones sequenced is denoted by *n*. The dotted line denotes the level of methylation necessary to reach statistical significance (p=0.05).

Figure 3-4. Rb directs significant low-level methylation of the +6 CpG

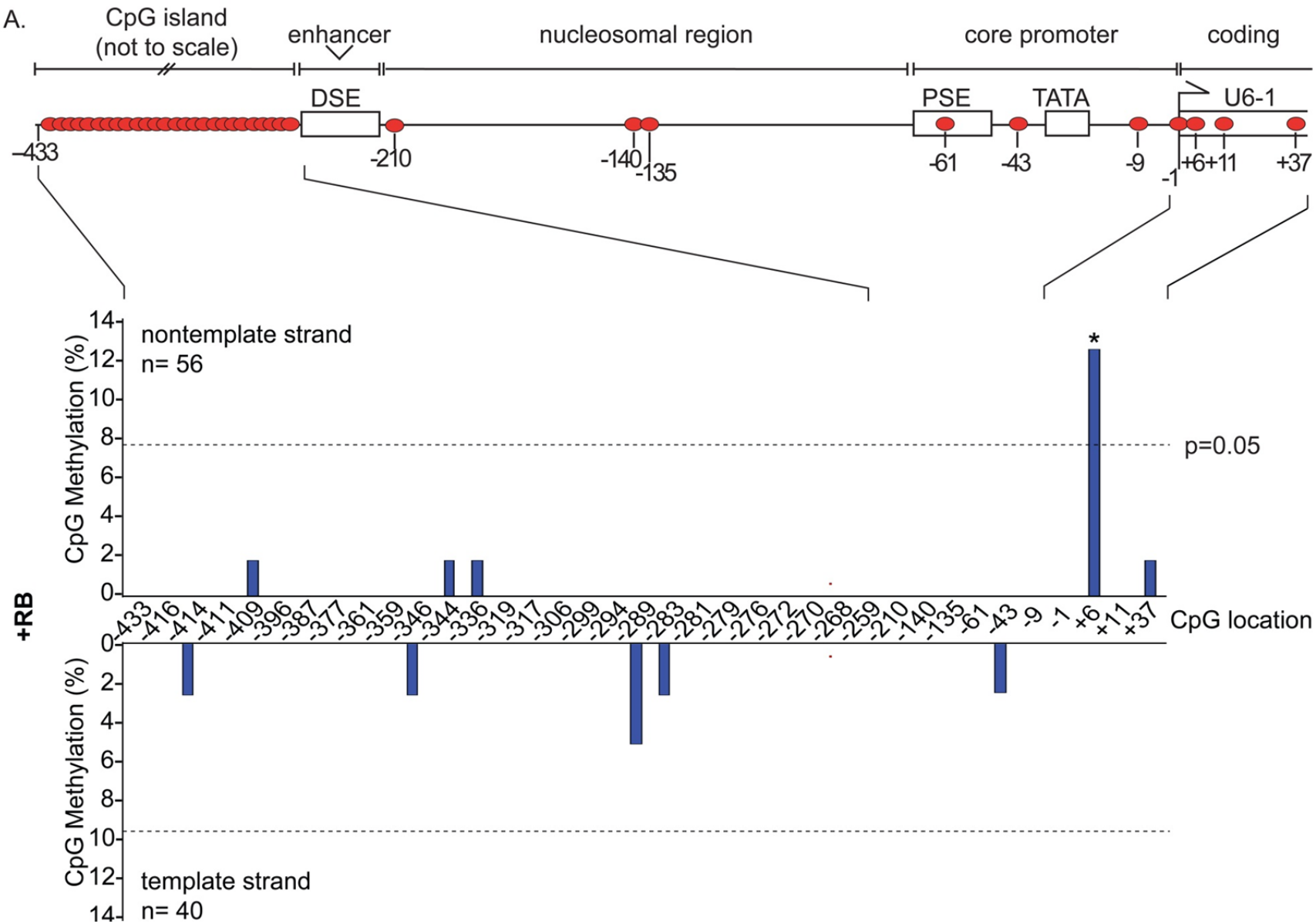


Figure 3-4 (cont'd)

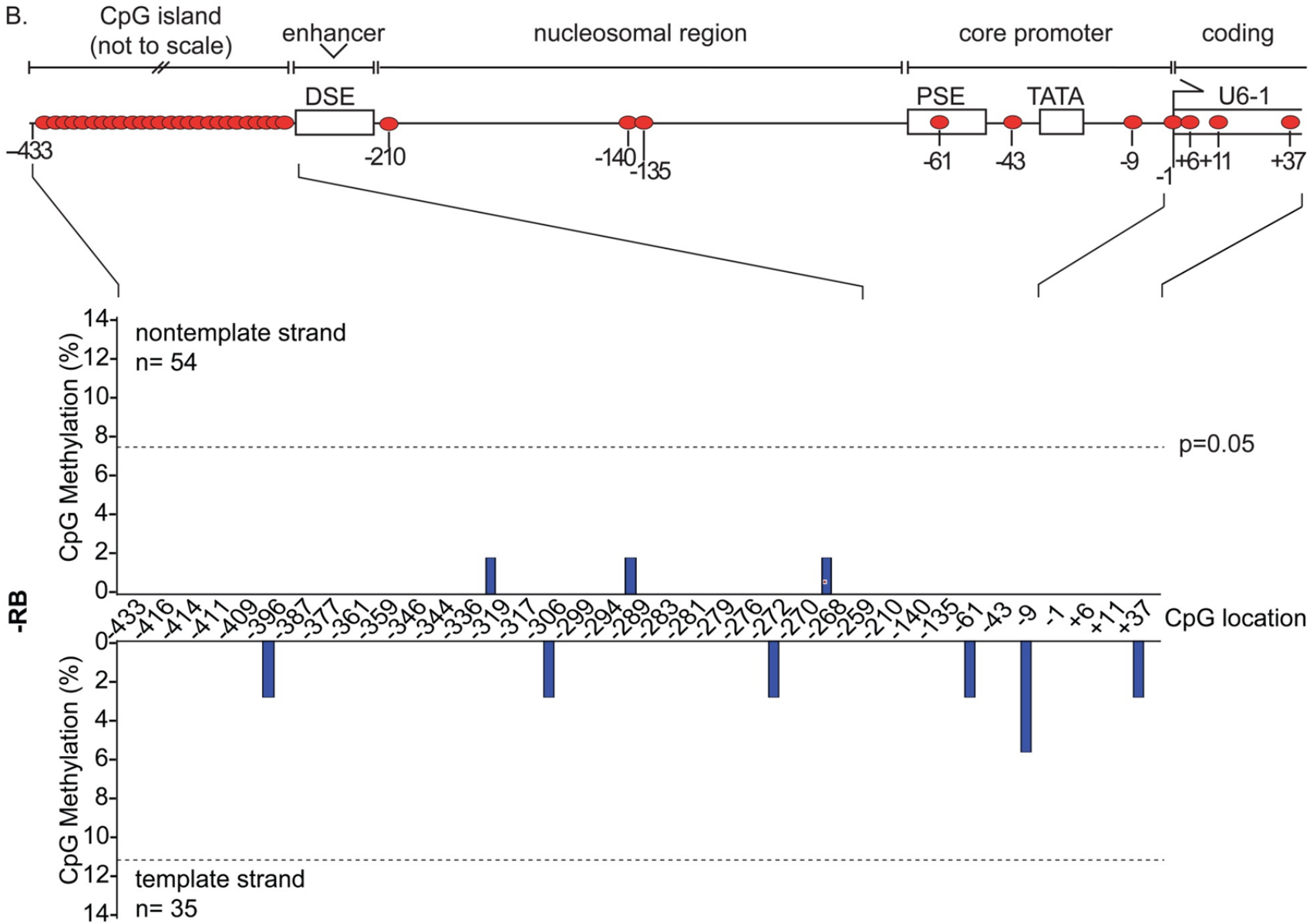
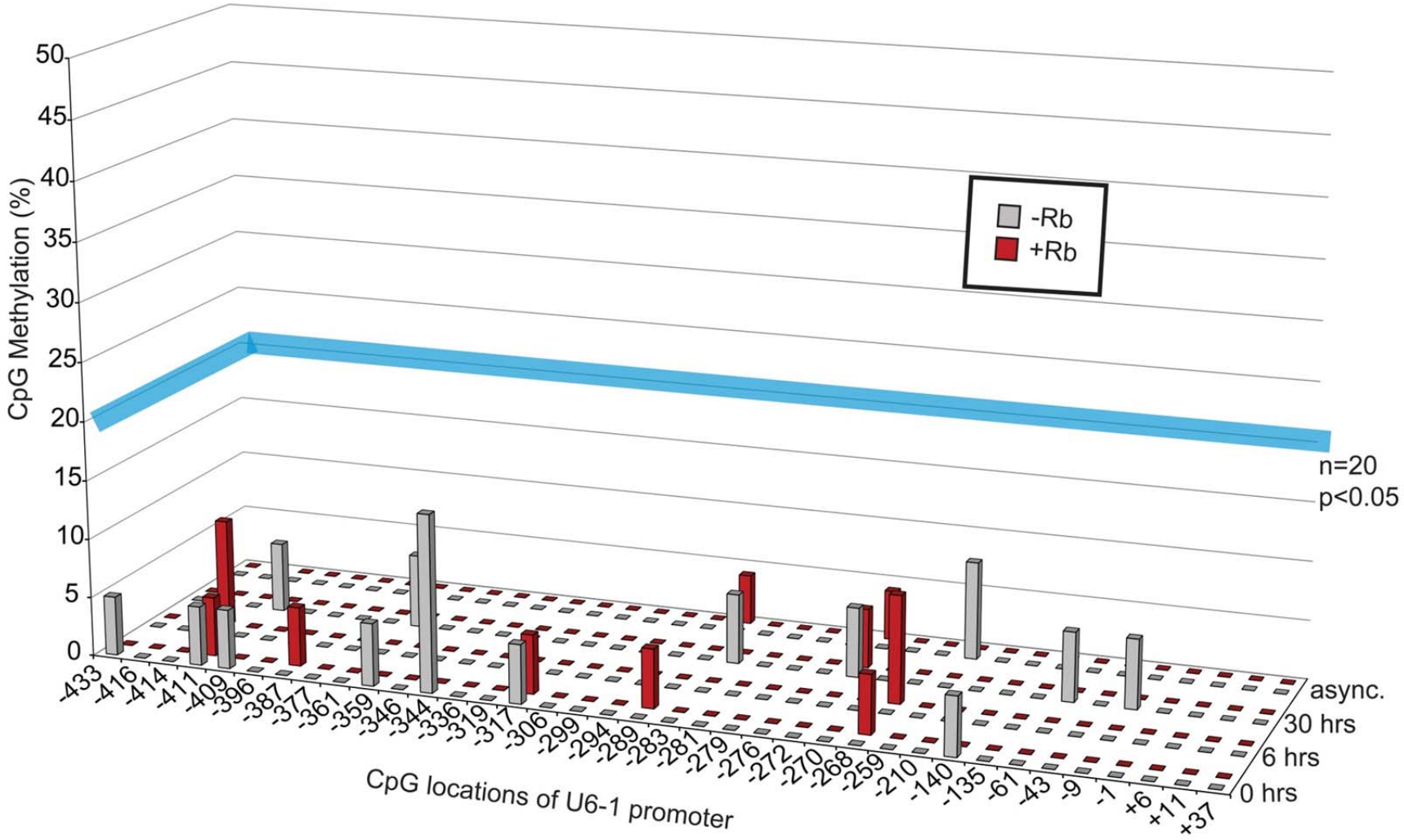


Figure 3-5. Rb activity during G1 does not correlate with promoter methylation.

The methylation of the U6-1 locus was measured by bisulfite sequencing. Rb was induced in Saos2tetRb by removal of doxycycline from the media in Nocodazole synchronized cells. Cells were collected at various time points after release from the Nocodazole block and Rb induction. The gDNA was bisulfite converted and the nontemplate strand of the U6-1 locus was amplified by PCR. The amplicon was cloned into pUC119 and sequenced. The sequenced clones were analyzed by BISMA. The number of clones sequenced is denoted by *n*. The dotted line denotes the level of methylation necessary to reach statistical significance (p=0.05).

Figure 3-5. Rb repression of U6 during G1 does not correlate with promoter methylation



Discussion

Of the nine conserved U6 genes in the human genome, the five active genes contain a high density of CpG sites relative to the inactive U6 genes, suggesting these sites play an important regulatory role in gene expression. I have shown here that inhibition of the DNMTs activated U6 RNA expression 4-fold after 5 days. In comparison, after only 2 days of AdC treatment, p16 levels increased much more dramatically. This suggests that DNMTs play a less critical role in the regulation of U6 genes at this time. In addition, we have previously shown that Rb can direct low-level methylation of the U6-1 start site, and I was able to reproduce these results in a different system. However the level of methylation observed is not considered statistically significant and is not reproducible in other cell types. Significant low-level methylation was observed in a second cell type, osteosarcoma cells, just downstream of the start site, however the significance of this methylation is not known. It may act to inhibit RNA polymerase III elongation or to recruit additional corepressors, such as SWI/SNF and HDACs. Analysis of the U6-1 start site under conditions in which Rb is known to be inducing a G1 block and repressing U6 RNA steady-state levels did not reveal any significant methylation, suggesting that DNA methylation is not an important aspect of Rb-mediated U6 repression in cycling cells.

APPENDIX

Appendix

CSB positively regulates U6 transcription.

Cockayne syndrome symptoms include pre-mature aging and unusually high sensitivity to UV light. The disease has been linked to mutations in the Cockayne syndrome group B (CSB) protein, which has been found to be involved in transcription-coupled DNA repair and global DNA repair (Licht, Stevnsner et al. 2003). Our lab has shown a correlation between CSB and transcription of RNA polymerase III-transcribed genes in response to UV light. To determine the effect of CSB on U6 transcription in vivo, I knocked down CSB expression with siRNA in HeLa cells and measured transcription from the U6 reporter by an RNase T_1 protection assay. In addition, I monitored the transcription of a Y1 reporter. As shown in Figure A1, CSB siRNA knocked down the CSB mRNA levels by 60% relative to the control siRNA. Diminution of CSB caused a 50% reduction in U6 transcription relative to the level of transcription in the control. Y1 transcription was not effected by CSB knockdown. This suggests CSB plays a role in positively regulating U6 transcription and that the mechanism of regulation differs between the type 3 RNA polymerase III-transcribed genes.

Rb co-purifies with rRNAs

Our lab has previously shown that recombinant Rb co-purifies with bacterial rRNAs. I confirmed this finding, as shown in Figure A2. GST-Rb was expressed in *E. coli* DH5α and purified with glutathione-agarose beads. The purified extract was proteinase K treated and either DNase-treated, RNase-treated or left untreated, and

analyzed by agarose gel electrophoresis and ethidium bromide staining. Total RNA from E. coli was analyzed as a comparison. As can be seen, the purifying nucleic acids were sensitive to RNase-treatment and not DNase-treatment. They also aligned with the 23S and 16S bacterial rRNAs. No small RNAs co-purified with the GST-Rb. GST alone did not purify with any nucleic acids. This demonstrates that Rb can interact with bacterial rRNAs. However, it could easily be argued that this interaction is only due to the overexpression of Rb. I therefore wanted to determine if endogenous Rb can interact with rRNAs in mammalian tissue culture. I purified Rb from two Rb^{+} cell lines, a mammary adenocarcinoma cell line (MCF7) and normal fibroblasts (NF). After Rb immunoprecipitation I DNase-treated the co-purifying nucleic acids and used reverse transcription qPCR to measure the amount of 18S and 5S rRNA in the sample. In MCF7 cells, Rb purified with 4X more 18S rRNA than pre-immune alone and over 2X more 5S rRNA than pre-immune (Figure A3 A). In NFs, Rb purified with over 2X more 18S and 5S rRNA than pre-immune. I analyzed the Rb levels in these two cell lines and, as can be seen in Figure A3 B, MCF7 has a much higher level of Rb than NFs.

To determine if the Rb association was similar with all rRNAs I repeated the experiment in MCF7 cells and measured the amount of all rRNAs (Figure A3 C). Rb associated primarily with 18S rRNA and 28S rRNA. Very little association was seen with 5S, 5.8S rRNA or vault1-1 RNA. This data suggests that Rb has some capability to bind either RNA directly or to ribosomal proteins. Although the data is preliminary, it does highlight a possible role for RNA binding in the Rb repression mechanism.

RNA polymerase III transcription in relation to perinucleolar compartment formation

The perinucleolar compartment is a non-membrane bound structure associated with the nucleolus and it has been shown to be highly enriched in RNA polymerase III transcripts (Huang, Deerinck et al. 1998; Slusarczyk and Huang 2008). In addition, PNC prevalence increases with the progression of cancer, demonstrating its use as a prognostic marker (Kamath, Thor et al. 2005; Norton, Pollock et al. 2008). However, the cause of PNC formation is not well understood. Dr. Sui Huang from Northwestern University has shown that inhibition of RNA polymerase III, and not RNA polymerase I or II, inhibits PNC prevalence, and that certain chemotherapeutic drugs can inhibit PNC prevalence. In collaboration with Dr. Huang's lab, I studied the effect of the chemotherapeutic compounds on RNA polymerase III transcription by transient transfection of Y1 and 7SK reporters in HeLa cells followed by analysis with the RNase T1 protection assay, as shown in Figure A-4A (Norton, Wang et al. 2009). The data demonstrates that some chemotherapeutic drugs, such as doxorubicin and bleomycin, strongly repress RNA polymerase III transcription, while others, such as actinomycin D and mercaptopurine, do not. The effect of the drugs on RNA polymerase III transcription and PNC prevalence was compared (Figure A-4B). PNC prevalence correlates with RNA polymerase III transcription when certain types of drugs are used, such as the nucleoside analogs. However, some drugs, such as actinomycin D and camptothecin strongly repress PNC development without effecting RNA polymerase III transcription, suggesting there is a different mechanism effecting PNC formation.

Figure A-1. CSB positively regulates U6-1 transcription

HeLa cells were transiently transfected with CSB siRNA (lane 2) or control siRNA (lane 3) and the U6-1 reporter or the Y1 reporter, or the reporter only (lane 1). 24 hours after transfection the total RNA was collected. The effectiveness of the knockdown was measured by reverse transcription PCR with primers specific for the CSB mRNA (top panel). The level of the U6-1 (middle panel) and Y1 (bottom panel) transcription was analyzed with the RNase T_1 protection assay. The P^{32} labeled probe was measured with a PhosphorImager.

Figure A-1. CSB positvely regulates U6-1 transcription

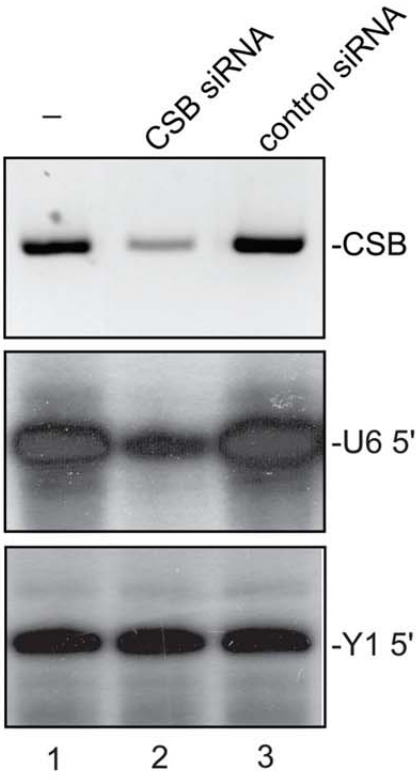


Figure A-2. Bacterial rRNAs co-purify with recombinant Rb

GST-Rb or GST was expressed in DH5a *E.coli* cells with IPTG induction and collected with glutathione sepharose agarose beads. The purifying protein was Proteinase K treated and then RNase-treated (lanes 6 vs. 9), or DNase-treated (lanes 7 vs. 10), or left untreated (lanes 5 vs. 8). The purifying nucleic acids were analyzed by agarose gel electrophoresis and ethidium bromide staining. Increasing mass of *E.coli* total RNA was analyzed in lanes 1-4 as a comparison.

Figure A-2. Bacterial rRNAs co-purify with recombinant Rb

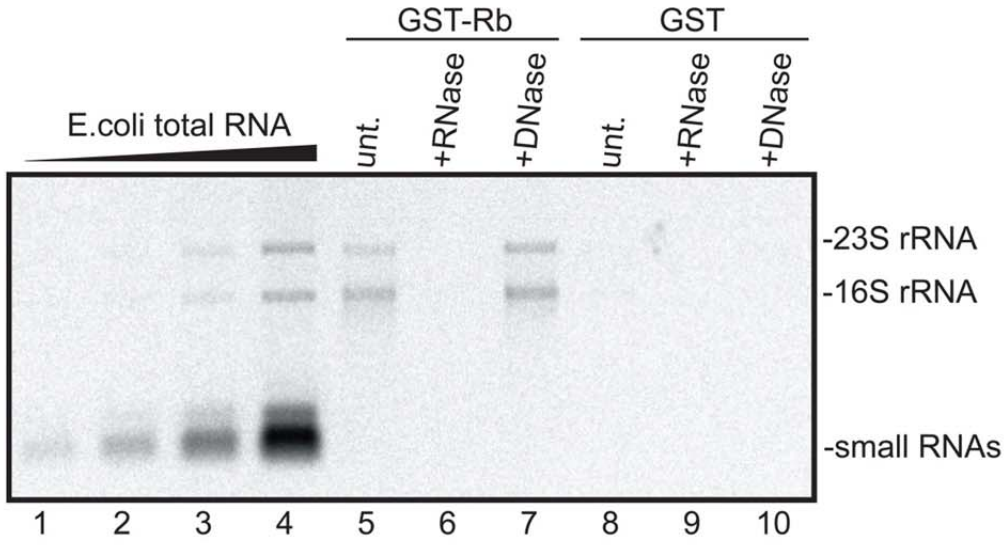


Figure A-3. Human rRNAs co-purify with endogenous human Rb

A. Human Rb was immunoprecipitated from MCF7 (left graph) or normal fibroblast (right graph) cell extract. The immunoprecipitated complexes were immobilized on Protein G beads. The nucleic acids were purified and analyzed for 18S and 5S rRNA. The rRNA was normalized to the rRNA purified by preimmune serum (PI).
B. The level of endogenous Rb in increasing amounts of MCF7 extract (lanes 1-3) and increasing amounts of normal fibroblast extract (lanes 4-6) was compared with an Rb Western Blot. Actin levels were measured as a loading control.
C. The co-purification of other rRNAs (5S, 5.8S and vault 1-1) from Rb in MCF7 cells was measured as in A. The recovery of Rb after immunoprecipitation was measured to be 4.8%.

Figure A-3. Human rRNAs co-purify with endogenous human Rb

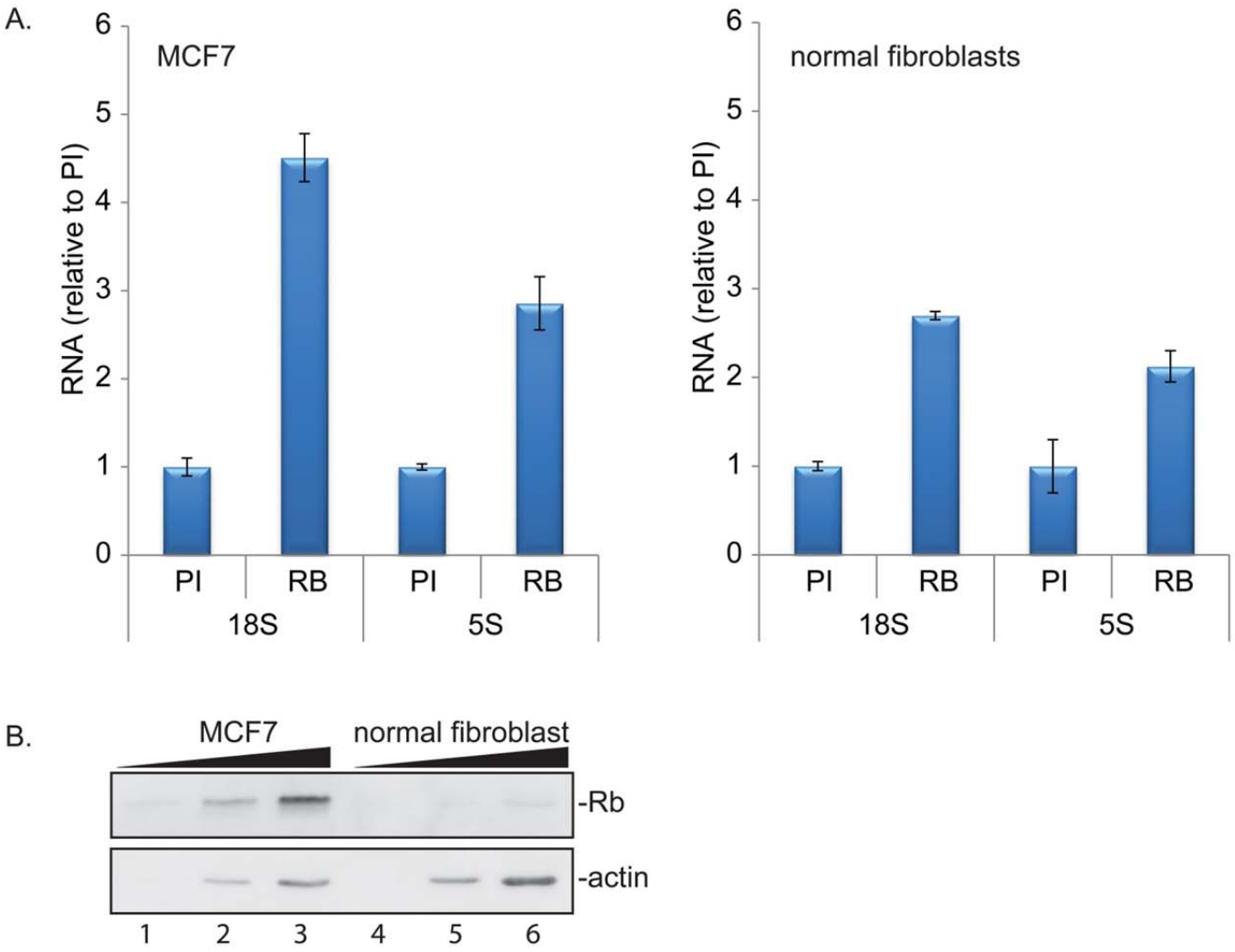


Figure A-3 (cont'd)

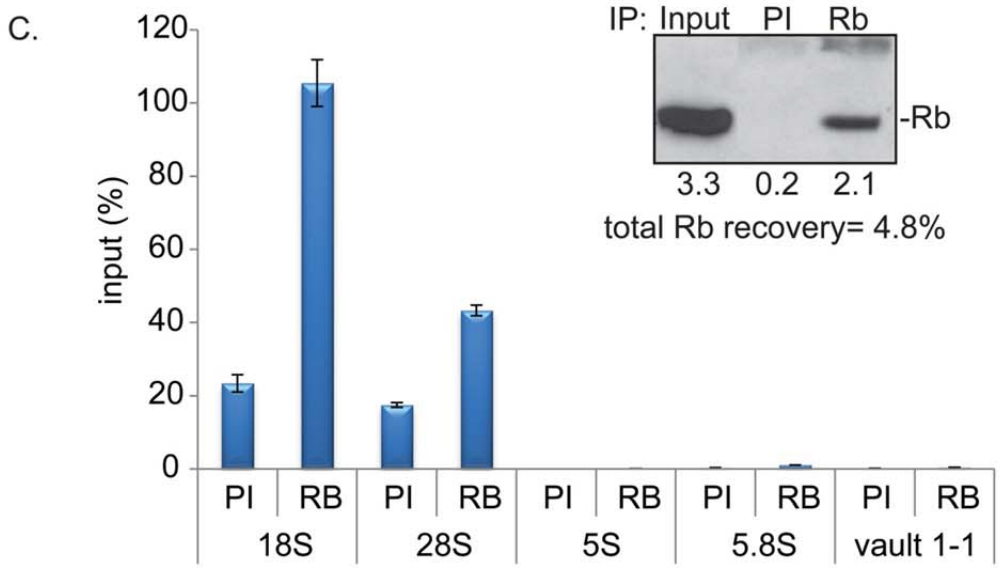
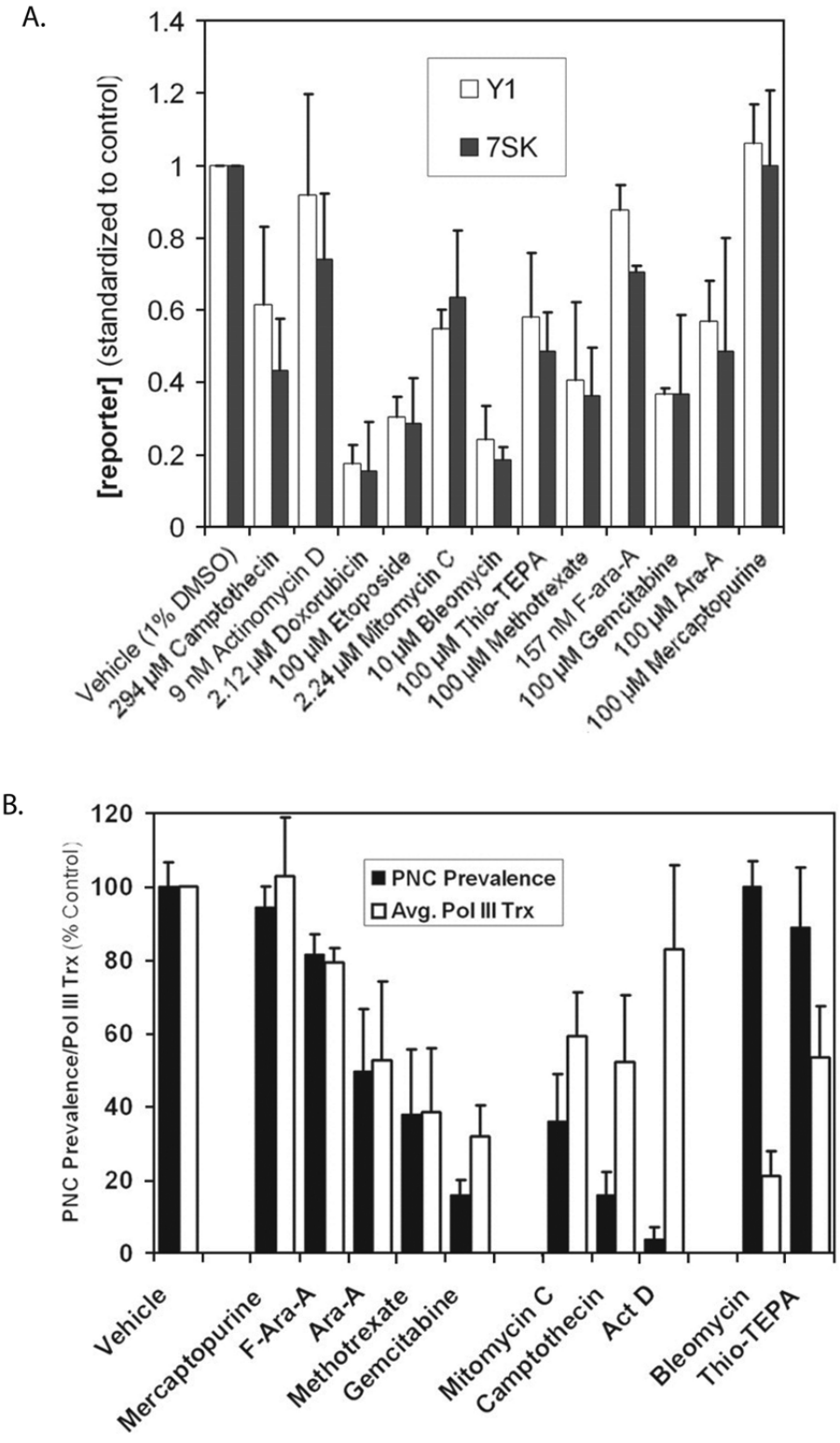


Figure A-4. RNA Polymerase III Transcription and PNC Formation

A. Y1 and 7SK reporters were transfected into Hela cells treated with various drugs known to effect PNC formation. The level of transcription was measured using the RNase T1 protection assay and quantified with Phosphoimager software.
B. The effect of the drugs on RNA polymerase III transcription is shown in comparison to PNC prevalence.

Figure A-4. RNA Polymerase III Transcription and PNC formation



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