# REGULATION OF U6 SMALL NUCLEAR RNA TRANSCRIPTION BY THE RETINOBLASTOMA TUMOR SUPPRESSOR PROTEIN

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**ABSTRACT**

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The Retinoblastoma tumor suppressor protein (RB) participates in many cellular functions including cell cycle progression, apoptosis, differentiation, and growth control. Key to the ability to participate in each of these processes is RB's ability to regulate gene expression. Postulated repression mechanisms suggest that RB directly blocks pre-initiation complex assembly or recruits additional co-factors such as histone deacetylases or ATP-dependent chromatin remodeling machines whose activities impair RNA polymerase II access to promoters. RB also represses transcription of non-translated genes that are transcribed by RNA polymerases I and III, potentially to control cell growth. To further understand how RB regulates gene expression, we examined RB repression of two classes of RNA polymerase III transcribed genes, the Adenovirus 2 VAI gene (similar to tRNA genes in promoter architecture and factor requirements) and the U6 snRNA gene.

Herein we demonstrate that RB associates with the endogenous U6 snRNA promoter *in vivo*, an important step in the repression of a target gene. RB did not however, associate with RNA polymerase II transcribed snRNA genes (U1 and U2) or repress transcription of the U1 snRNA gene. We also demonstrate that the general transcription factors snRNA activating protein complex (SNAPc) and TFIIIB are important for RB repression of human U6 snRNA gene transcription by RNA polymerase III. RB interacts with these

basal factors that are required for RNA polymerase III transcription providing a potential mechanism for RB recruitment to target genes. Together, SNAPc and TFIIIB act cooperatively to recruit RB to a U6 snRNA promoter *in vitro* and TFIIIB acts as a selectivity factor specifically recruiting RB to RNA polymerase III transcribed snRNA genes. Additionally, we show that RB co-occupies the U6 promoter with RNA polymerase III *in vivo* and RB repression *in vitro* does not preclude RNA polymerase III recruitment. These results suggest a novel mechanism wherein RB represses transcription at steps subsequent to RNA polymerase recruitment to gene promoters.

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# KEY TO ABBREVIATIONS

| | |
|---|---|
| ATP | Adenosine triphosphate |
| Bdp1 | B double prime |
| Brf-1 | TFIIB related protein 1 |
| Brf-1_v2 | TFIIIB related protein 1 splice variant 2 |
| Brf-2 | TFIIB related protein 2 |
| Cdc2 | Cell division cycle 2 protein |
| CDK | Cyclin dependent kinase |
| ChIP | Chromatin immunoprecipitation |
| Co-IP | Co-immunoprecipitation |
| DHFR | Dihydrofolate reductase |
| DNA | Deoxyribonucleic acid |
| EMSA | Electrophoretic mobility shift assay |
| GAPDH | Glyceraldehyde – 3 – phosphate dehydrogenase |
| HAT | Histone acetyl transferase |
| HDAC | Histone deacetylase |
| HMT | Histone methyltransferase |
| HP1 | Heterochromatin protein 1 |
| ICR | Internal control region |
| MCM | Mini-chromosome maintenance protein |
| MEF | Mouse embryonic fibroblast |
| mSNAPc | Mini-SNAPc |

| | |
|---|---|
| NTP | Nucleotide triphosphate |
| PcG | Polycomb group complex |
| PCNA | Proliferating cell nuclear antigen |
| PCR | Polymerase chain reaction |
| RB | Retinoblastoma tumor suppressor protein |
| RNA | Ribonucleic acid |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| RRM2 | ribonucleotide reductase M2 |
| SNAPc | Small nuclear RNA activating protein complex |
| SnRNA | Small nuclear RNA |
| SuVAR39H1 | Suppressor of varigation |
| TBP | TATA binding protein |
| TFIIA | Transcription factor II A |
| TFIIB | Transcription factor II B |
| TFIID | Transcription factor II D |
| TFIIF | Transcription factor II F |
| TFIIIA | Transcription factor III A |
| TFIIIB | Transcription factor III B |
| TFIIIC | Transcription factor III C |
| TK | Thymidine kinase |
| TS | Thymidylate synthase |

# Chapter 1

## Introduction

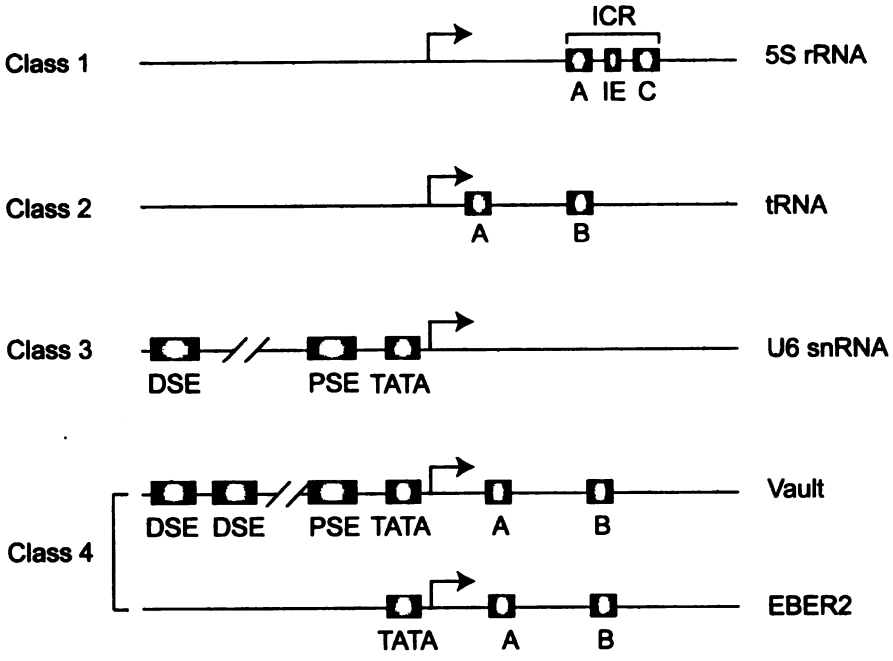
## RNA Polymerase III

In eukaryotic organisms, nuclear genes are transcribed by three highly related RNA polymerases: RNA polymerase I, II, and III. Each of these polymerases is responsible for transcription of a distinct subset of genes. RNA polymerase I synthesizes a single tandemly arrayed set of non-translated structural RNAs that assemble into ribosomes. RNA polymerase II transcribes a diverse set of genes including protein-encoding genes and some non-translated uridine rich snRNA genes. RNA polymerase III produces an assorted collection of non-translated structural and catalytic RNAs including small RNAs that are usually shorter in length than 400 nucleotides (36, 113).

### *Structure of genes transcribed by RNA polymerase III*

The genes transcribed by RNA polymerase III fall into four classes characterized by promoter architecture (Figure I-1). Class 1 and 2 genes, represented by the 5S ribosomal RNA (5S rRNA) and transfer RNA (tRNA) genes respectively, contain required gene internal promoter elements. Class 3 genes are characterized by external promoter elements and are represented by the U6 snRNA gene and the 7SK gene. Class 4 genes exhibit a combination of the elements found in the first three classes.

**Figure I-1: Representation of the three classes of genes transcribed by RNA polymerase III.**
Class 1 genes such as 5S rRNA genes contain gene internal promoter elements including A and C boxes that are separed by an intermediate element (IE). Together, these elements comprise the internal control region (ICR) that is required for gene expression. Class 2 genes such as the mammalian tRNA gene also contain gene internal cis-acting elements referred to as the A and B boxes. Class 3 genes (U6 snRNA) are characterized by gene external promoter elements including the distal sequence element (DSE), the proximal sequence element (PSE) and a TATA element. Class 4 genes such as the vault RNA genes or EBER gene from the Epstein Barr virus, contain a mixture of all three classes of promoter elements.


ICR
Class 1
5S rRNA
A IE C
Class 2
tRNA
A
B
Class 3
U6 snRNA
DSE
PSE TATA
Vault
DSE DSE
PSE TATA
A
B
Class 4
EBER2
TATA
A
B

5S rRNA genes (class 1) contain an A box, an intermediate element (IE), and a C box that is highly conserved through many species. These sequence elements combined constitute the internal control region (ICR) that is required for transcription (5, 95-97). For example, in *Xenopus laevis*, the A box is located at +50 to +64, the IE between +67 and + 72, and the C box is located from +80 to +97 (96). The spacing of these elements is important, as effective transcription of 5S rRNA genes is intolerant of changes in spacing (96). In *S. cerevisiae*, only the C box is required for efficient expression of 5S genes (10).

Class 2 gene promoters, Ad2 VAI (adenovirus gene product similar to tRNA) and tRNA genes, consist of an A box (e.g. *X. laevis* leucine tRNA +8 to +19) and B box (+52 to +62) region downstream of the start site of transcription (35, 119). These intragenic sequences are extremely well conserved from species to species perhaps in part because these regions encode the T and D loop regions that are critical for tRNA function. In contrast, the spacing between the A and B boxes is variable. However, recently it has been shown that some extragenic sequences may be necessary for class 2 gene transcription. For example, yeast U6 snRNA genes appear to have an extragenic TATA like element (7). Similarly, some tRNA genes in *D. melanogastor* also appear to contain TATA elements upstream of the start site of transcription that may be important for efficient gene expression (127). Additionally, the –30 to –25 bp region of C elegans tRNA genes may also contain sequences required for the transcription of these genes (68).

Class 3 genes as represented by the mammalian U6 snRNA and 7SK genes, are characterized by gene external core promoter elements. The enhancer regions of these genes contain a distal sequence element (DSE) that includes octamer sites, as well as sites for other transcriptional activators such as Spl and Staf (69, 112). The DSE activates transcription from the core promoter of Class 3 genes and can be located at variable positions upstream of the core promoter from species to species. In mammalian systems the DSE is located approximately 239 bp upstream of the start site of transcription (121, 150). The core promoter regions of class 3 genes consist of two essential elements: the proximal sequence element (PSE) and a TATA element. These cis-acting elements are located at approximately -46 and -20 bp upstream of the start site of transcription, respectively. The spacing of these two elements is invariant in human genes.

Class 4 genes represent a mix of characteristic sequence elements found in the other three classes of RNA polymerase III genes. These class 4 genes include vault genes (130, 132), selenocysteine tRNA, EBER2 (Epstein-Barr viral product) (47, 48), MRP RNase (involved in maturation of mitochondrial DNA replication), Y1 and Y3 (unknown function). In the case of vault RNAs and selenocysteine tRNA, the promoters of these genes contain gene external PSEs as well as gene internal control elements. Additionally, many human vault RNA genes contain distal sequence elements similar to those found in snRNA genes. The Epstein-Barr gene, EBER contains a TATA element as well as gene internal A and B box.

***RNA polymerase III Basal Transcription Machinery***

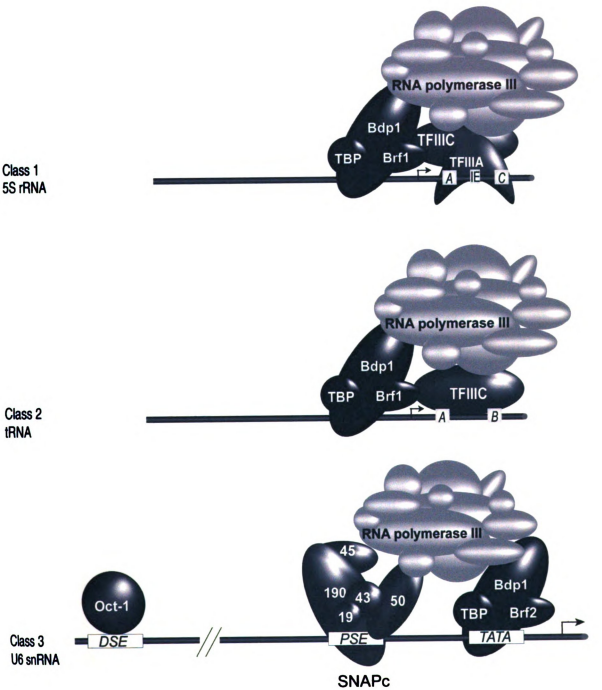
Just as each class of RNA polymerase III transcribed gene contains characteristic promoter sequences, each class of RNA polymerase III transcribed genes has distinct, specific basal machinery requirements, as discussed in more detail in the following sections (figure I-2). 5S rRNA (class 1) genes require the binding of TFIIIA to the ICR of the promoter followed by recruitment of TFIIIC. Interaction of TFIIIB with TFIIIC allows TFIIIB to associate with the promoter, subsequently recruiting RNA polymerase III. For preinitiation complex formation to occur at tRNA (class 2) genes, TFIIIC must bind to both the A and B box of the promoters followed by interaction with TFIIIB. Positioning of TFIIIB near the start site of transcription allows for RNA polymerase III recruitment. U6 snRNA genes (class 3) require different factors. This set of genes requires cooperative interactions between Oct-1, SNAPc (snRNA activating protein complex), and a variant TFIIIB. At this time, the factor requirements for class 4 genes are unclear. One possibility is that a blend of class 2 and class 3 transcription factors are required, depending on specific promoter structure (47, 48, 130, 132).

*TFIIIA*:

TFIIIA is a basal factor required to recruit TFIIIC specifically to 5S rRNA genes. A founding member of C2H2 family of zinc finger proteins, *Xenopus laevis* TFIIIA was the first eukaryotic transcription factor to be purified to homogeneity and to have its cDNA

**Figure I-2: Factor requirements for RNA polymerase III transcribed genes.**

Both class 1 and class 2 RNA polymerase III transcribed genes require the TFIIIC complex and a TFIIIB complex consisting of Bdp1, TBP, and Brf1. Class 1 genes have an additional requirement for TFIIIA which provides DNA binding specificity during pre-initiation complex formation. Class 3 genes have very different factor requirements. Transcriptional activators such as Oct-1 bind to the DSE to activate transcription. A multi-protein complex called SNAPc binds to the PSE to nucleate pre-initiation complex assembly and is important for subsequent gene expression. An alternative TFIIIB complex consisting of Bdp1, Brf2, and Bdp1 associates with the TATA element and may also play an important role in RNA polymerase III recruitment.


Class 1
5S rRNA
RNA polymerase III
Bdp1
TFIIIC
TBP
Brf1
TFIIIA
A
B
C
Class 2
tRNA
RNA polymerase III
Bdp1
TBP
Brf1
TFIIIC
A
B
Class 3
U6 snRNA
RNA polymerase III
45
190
43
50
19
Oct-1
Bdp1
TBP
Brf2
DSE
PSE
TATA
SNAPc

cloned (37). When TFIIIA binds to the promoter of the 5S RNA gene, its nine zinc fingers are aligned over the ICR with the C terminus of the protein near the 5' end of the promoter and its N-terminus at the 3' end of the promoter. The C block of the promoter is recognized by the three end most zinc fingers at the N terminus and the A block is recognized by zinc fingers 7-9 (18, 33, 89). The middle three fingers adopt a completely different conformation in order to span the intervening DNA. After binding to 5S rRNA genes, TFIIIA provides a platform for the recruitment of TFIIIC, which has little affinity for the 5S rRNA gene alone.

*TFIIIC*

TFIIIC is a required basal transcription factor that enables pre-initiation complex formation to occur at all RNA polymerase III transcribed promoters in yeast and the 5S and tRNA genes in mammalian systems. The mammalian U6 snRNA gene does not require TFIIIC but rather another basal factor, SNAPc, which is discussed in detail below. Productive recruitment of TFIIIC to 5S rRNA promoters requires interaction with TFIIIA, whereas recruitment to tRNA promoters and yeast U6 snRNA promoters is mediated through sequence specific DNA contacts by TFIIIC. Yeast TFIIIC consists of six subunits (36, 113) whereas human TFIIIC includes 5 subunits. The TFIIIC complex can be separated into two domains separated by a flexible linker: $\tau_A$ which binds weakly to the A box and $\tau_B$ which binds with high affinity to the B box (78). The linker between the domains confers a flexibility to the TFIIIC protein complex that allows for binding to tRNA promoters that have varied distances between the A and B boxes. Stable association of TFIIIC with promoters allows for recruitment and correct positioning of

TFIIIB. TFIIIC has also been shown to contact RNA polymerase III itself (50) suggesting that although TFIIIB alone is sufficient to recruit polymerase, contacts with TFIIIC may also be important.

*SNAPc*

Mammalian class 3 RNA polymerase III transcribed genes have slightly different factor requirements, perhaps in part due to the presence of unique gene external promoter elements. The snRNA activating protein complex (SNAPc) also called the proximal element transcription factor (PTF) (2, 146, 147) is required for snRNA expression including the U6 snRNA gene which is representative of Class 3 genes. SNAPc is a multi-protein complex consisting of at least five subunits as designated by apparent molecular mass: SNAP190 (PTFα) (32, 145), SNAP50 (PTFβ) (2, 41), SNAP45 (PTFδ) (108, 147), SNAP43 (PTFγ) (43, 147), and SNAP19 (42). SNAPc is specifically recruited to the PSE of snRNA genes and is important for nucleation of pre-initiation complex formation (41-43, 72, 109, 145). Further characterization of the SNAP complex demonstrated that the minimal complex (mSNAPc) necessary for DNA binding and transcriptional activation consists of SNAP190 (1-505), SNAP43, and SNAP50 (44, 74, 83). SNAPc interacts with TBP and cooperatively binds to DNA with TBP (82) suggesting that SNAPc plays a role in recruitment of TFIIIB to U6 promoters.

*TFIIIB*

TFIIIB plays a central role in the establishment of a pre-initiation complex at the promoters of RNA polymerase III transcribed genes. TFIIIB is recruited to promoters

through interactions with TFIIIC (58-60) or SNAPc (13, 44). Subsequent to recruitment, TFIIIB is responsible for bringing RNA polymerase to promoters to initiate transcription (57, 133, 144).

Three proteins compose TFIIIB complexes: the TATA binding protein (TBP), TFIIB related factor (Brf), and B" (Bdp) (58-60, 64, 100, 117, 129). Most of the information currently available for how TFIIIB complexes are assembled and how they function for RNA polymerase III transcription comes from studies in *S. cerevisiae*. In yeast, TBP and Brf1 form a very stable complex (designated B') from which the less tightly associated B" is separable by chromatography (58). The Brf1 subunit plays a central role in maintaining TFIIIB complex integrity through extensive contacts with TBP and Bdp1. Less is known about the TFIIIB complexes that function in human systems. It appears that multiple variant complexes may exist that function at different classes of RNA polymerase III transcribed genes (81, 114, 125). Human Brf1 and yeast Brf are highly homologous, especially in the TFIIB related N-terminal half (133). Human Bdp1 is a very large protein that has some similarity (~40%) to yeast B" along a 400 amino acid stretch that encompasses the indispensable SANT domain (114).

At the human U6 promoter, the TATA box directly recruits TBP, which is likely to be important for recruiting additional BRF-related factor(s), and Bdp1 (114). In contrast to most RNA polymerase III-transcribed genes, human U6 snRNA gene transcription does not require Brf1 (79, 81). While the TFIIIB complex containing Brf1 and TBP clearly does not function for human U6 transcription (81), the exact nature of the TBP complex

that does function at human U6 promoters is currently unclear. Two proteins analogous to Brf1 have been shown to be important for U6 snRNA gene expression. Brf2 associates with U6 promoters *in vivo* (114) and is necessary for gene expression as determined by immunodepletion-reconstitution experiments (13, 114). Additionally, *in vitro* transcription experiments performed using highly purified recombinant SNAPc, TBP, Bdp1, Brf2 and chromatographic fractions enriched for RNA polymerase III support U6 snRNA transcription, suggesting that these proteins define the minimal set of factors required for U6 transcription (13). However, a splice variant of Brf1 called Brf1_v2 has also been shown to participate in U6 gene expression in immunodepletion experiments (79). Compared to Brf1, Brf2 has a conserved zinc finger and core domains and a divergent C-terminus. Brf1_v2 lacks the zinc finger and 1st repeat domain conserved in Brf1 and TFIIB. The role that each of these proteins plays in U6 snRNA gene expression is unclear. However, both proteins may be in a complex that is required for U6 snRNA transcription.

***RNA polymerase III enzyme***

RNA polymerase III has been well defined in *S. cerevisiae* and has been shown to have 17 subunits. RNA polymerase III contains ten subunits that are unique to RNA polymerase III and are designated the C subunits. The two common subunits shared by RNA polymerase I and III are designated the AC subunits. RNA polymerase III also contains five subunits common to all three polymerases (ABC subunits) (46). Human RNA polymerase III has been characterized both by chromatography (133, 134) and from cell lines expressing epitope tagged subunits (51, 133, 134). All yeast subunits except

RPC37 have been disrupted and shown to be essential for viability (11). All of the human RNA polymerase III subunits have now been characterized by mass spectrometry after purification from human cell lines contained double epitope tagged HsRPC4 (51). This analysis found human orthologues to all of the yeast RNA polymerase III subunits except for RPC10, which may not have been detected due to small size.

## The Retinoblastoma Tumor Suppressor Protein

The Retinoblastoma tumor suppressor protein (RB) is a critical regulator of important cellular processes including cell cycle progression (24, 25, 39, 40, 56, 80, 86, 101, 111, 135, 149), DNA replication (105), growth regulation (118, 140), differentiation (12, 38, 90, 99, 118, 126, 148), and apoptosis (49, 148). RB is frequently mutated in a variety of human malignancies and tumors. Approximately 30% of all human cancers are deficient for RB activity, and the RB signal transduction pathway is disrupted upstream in 50% of human cancers. RB was originally identified as a result of frequent mutation in the rare pediatric eye tumor (34, 67). Cancers that have been shown to be deficient in RB activity include osteosarcomas, small cell lung cancers, bladder, prostate, and breast cancer (17).

RB belongs to a family of pocket proteins that also includes p107 and p130 (135). These proteins were originally characterized by the presence of a large A/B pocket domain that was identified by the ability to bind to viral oncoproteins, including SV40 Large T antigen, Adenovirus E1a, and Papilloma virus E7 (27, 28). The Large A/B pocket extends from amino acids 379-869 and is required for tumor suppression. This region can be

further subdivided into small A/B pocket (393-768) and the C pocket (768-869) (138). The small A/B pocket is sufficient for repression of transcription (14).

Classically, RB has been studied in the context of cell cycle progression. RB mediates the G1/S transition of the cell cycle by regulating the expression of genes required to enter into S phase. Additionally, RB is a phosphoprotein that is phosphorylated in a cell cycle dependent manner. The hypophosphorylated form of the protein, considered the active form of the protein, binds to and regulates target cellular factors. Phosphorylation by cylin D/cdk4 (30, 61) during late G1 or by Cyclin E/cdk2 (22, 23, 26, 87) during early G1/S phase inactivates RB such that it can no longer bind to target factors.

***RB plays an essential role in development***

A powerful tool for dissecting the function of genes of interest is the technology that allows for targeted gene disruption (knock out) or ectopic gene expression (knock in) within a model organism. "Knock-out" or "knock-in" mice provide a wealth of valuable information about the functions of RB family members in an *in vivo* and physiologically relevant setting. Transgenic mice over-expressing RB show dwarfism by day 15 embryonic development (E15), indicating that RB regulation is required for proper development (4). However the lack of RB also adversely affects development. Gene targeted *Rb-/-* embryos die between E13.5 and E15.5 (16, 53, 65). Additionally, increased apoptosis is observed in the nervous system as early as E11.5 and is particularly evident in the hindbrain, spinal cord, and trigeminal and dorsal root ganglia. Ectopic mitoses are also observed especially in the hindbrain. *Rb-/-* embryos exhibit defective hematopoiesis,

manifested as an increased number of immature nucleated erythrocytes. Introduction of a RB transgene into knockout mice fully rescues developmental defects indicating the importance of this gene in normal development (4).

Unlike human retinoblastoma patients, mouse *RB* chimeras develop pituitary gland tumors rather than retinoblastomas (75, 143). A similar phenotype is observed in *Rb+/-* mice, in that tumors develop in the brain and pituitary gland. These tumors exhibit loss of heterozygosity of the remaining wild type allele, demonstrating that RB is a tumor suppressor in mice as well as in humans. Although *Rb+/-* mice are cancer prone, they do not accurately reiterate the tumor spectrum observed in human retinoblastoma patients. This phenomenon may be explained by the fact that other RB family members could compensate for the loss of RB in the eye. Mice homozygous for p107 and p130 knock outs are viable, fertile and healthy (19, 66). *p107-p130* double knock out mice experience neonatal lethality and most *Rb+/- p107-/-* mice are growth retarded, and increased mortality of these mice is observed in the first three weeks after birth. Although *Rb+/-p107-/-* pups that survive to adulthood do not show increased cancer predisposition compared to *Rb+/-* mice, they develop multiple dysplasic lesions of the retina. Unlike RB, p107 and p130 are not required for embryonic development, and *p107+/-, p130+/-, p130-/-, p107-/-* mice do not exhibit increased incidence of tumor development. Triple knockout embryonic fibroblasts have normal growth characteristics but impaired differentiation capacity (21, 110). Triple knockout mouse embryonic fibroblasts have a shorter cell cycle and can spontaneously immortalize. These cells are also resistant to G1 arrest following DNA damage, contact inhibition or serum starvation.

***Targets for RB regulation***

Originally, genes proposed to be repressed by RB were known E2F responsive genes. It had been shown that RB could convert a positive acting E2F site to a negatively acting element in transient transfection experiments (137). Based on the data presented in this study, genes that had E2F binding sites or were shown genetically or biochemically to be dependent on E2F were postulated RB targets. Proposed target genes include S phase cyclins and cdks, E2F family members, other pocket protein family members, genes that function for DNA replication and nucleotide biosynthesis pathways (1, 56, 84, 86, 118, 136).

Many studies have been performed to identify the true physiological targets of RB. RT-PCR and Northern blot analysis using mouse embryonic fibroblasts in which each individual RB family member is knocked out were used to study relative amounts of target gene expression in the absence of RB family members (52). This study showed a de-repression of cyclin E and p107 at the G0/G1 check point in the absence of RB. Additionally, b-myb, cdc2, E2F-1, thymidylate sythase (TS), ribonucleotide reductase M2 (RRM2), cyclin A2 and dihydroxyfolate reductase (DHFR) were shown to be de-repressed at the G0/G1 interface in the absence of both p107 and p130. Another group of researchers employed DNA micro-array chips to study global changes in gene expression under conditions in which a constitutively active RB was over-expressed in tissue culture cells (77). This study identified 341 targets that were reproducibly repressed at least 1.7 fold in multiple cell lines. These targets fell into four categories including: (1) DNA

replication, (2) DNA repair, (3) chromatin structure and transcription, and (4) G2/M progression. While many of the genes identified in this study have previously been shown to be E2F dependent, several novel targets identified have not been previously linked to E2F. The micro-array analysis pinpointed several genes also identified previously by RT-PCR analysis (52) including cyclin A, RRM2, thymidine kinase, TS, cyclin E, cdc2 and DHFR. Additionally, this screen also indicated that MCM6, MCM7, DNA polymerase δ, topoisomerase IIα, PCNA, Cdk2, cyclin B1, and HMG2 were repressed by the ectopically expressed RB. Together these two studies provide a pool of candidate genes regulated by RB family members. However, these studies do not distinguish between direct and indirect regulation of gene expression.

In order to determine which genes RB family members directly target, it is important to study RB on the level of target gene promoter occupancy. Several groups have undertaken this question by using chromatin immunoprecipitation (ChIP) to analyze RB family member promoter occupancy. ChIP analysis provides a powerful tool for understanding direct gene targets for proteins of interest because it relies on *in vivo* endogenous proteins bound to genes in a physiologically relevant chromatin structure rather than on over-expression and reporter gene assays. The results of these studies however, are contradictory. Wells and co-workers (139) report that RB and RB family members occupy the TK promoter during G0; DHFR, cdc2, and cyclin E during G0 and G1/S; and b-myb in asynchronous cell populations (139). A second group, Takahashi and co-workers (124), were unable to detect RB at any promoter studied. They instead observed p130 at E2F1, cdc6, p107, b-myb, and cyclin A promoters during G0 and

G0/G1 suggesting that p130 is the major player in cell cycle progression. One explanation for this discrepancy may be that these two groups worked in two different systems: mouse and human, respectively. RB family members may regulate cell cycle progression and growth control differently in these two species. Additionally, the experiments were performed from different cell types indicating that there may be tissue specific regulation by RB family members.

***Mechanisms for RB repression of RNA polymerase II transcribed genes***

The function of RB as a tumor suppressor is linked to its ability to regulate gene expression. Therefore, to fully understand the contribution of RB to cellular proliferation observed during carcinogenesis, it is important to determine the mechanisms that RB uses to regulate gene activity. Direct repression of transcription by RB implicates protein-protein interactions between RB and transcriptional activators belonging to the E2F family. E2F binds to enhancer regions of E2F responsive gene promoters to stimulate transcription by RNA polymerase II. By binding to E2F at gene promoters, RB can potentially directly block activation function of E2F by obscuring the trans-activation domain that facilitates pre-initiation complex assembly, or RB can recruit co-regulatory factors that alter chromatin structure surrounding these genes to repress transcription.

*Disruption of pre-initiation complex formation*

One critical step in the activation of transcription for most RNA polymerase II

transcribed genes is the establishment of a stable preinitiation complex (PIC) (93). Preinitiation complexes are thought to assemble in a step-wise fashion (93, 103, 104). First the TBP containing factor, TFIID, binds to TATA elements, nucleating preinitiation complex formation and recruiting additional basal factors. Next, TFIIA and TFIIB are recruited to the start site of transcription, followed by association of TFIIF in complex with RNA polymerase. Potentially, RB may repress transcription by interfering with one of these steps.

Elegant studies using purified factors in an *in vitro* transcription assay show that RB does in fact repress transcription through disruption of pre-initiation complex formation (107). Specifically, RB inhibits the formation of the TFIID-TFIIA (DA) complex. However, if the DA complex is allowed to bind to DNA prior to the addition of RB, it becomes resistant to RB repression. Together, these results suggest that RB represses transcription by blocking pre-initiation complex formation thereby not allowing for RNA polymerase II association and subsequent gene expression.

*RB and histone modification: HDACs*

Covalent modification of histones and change chromatin structure is critical for efficient gene regulation (3, 55). Histone tails in nucleosomes at the promoters of active genes are generally acetylated on lysine residues. This acetylation neutralizes the positive charge of the lysine that binds tightly to the surrounding negatively charged DNA. The resulting unwrapping of the histone tails loosens the chromatin structure, allowing access to transcription factors and RNA polymerase.

One potential mechanism that RB may use to repress transcription of target RNA polymerase II transcribed genes is the recruitment of histone deacetylase (HDAC) activities. HDACs are co-repressor complexes that enzymatically remove the acetyl groups from the lysines of histone tails. Several groups have shown that RB interacts with class I HDACs and that a deacetylase activity co-purifies with RB (6, 31, 73, 76, 99, 101, 149). Potentially, this HDAC activity could deacetylate histone tails at RB target genes, causing a net compaction of surrounding chromatin. This alteration of chromatin structure, resulting in a loss of accessibility to transcription factors and RNA polymerase, would then effectively repress transcription of the gene. Nonetheless, HDAC activity does not account for all of RB's repression capability. The addition of HDAC inhibitors only partially reverses RB repression, suggesting that RB has HDAC independent as well as HDAC dependent repression mechanisms (6, 76). The HDAC independent repression may include mechanisms such as masking of the E2F trans-activation domain or preinitiation complex disruption. Additionally, there appears to be some promoter selectivity for RB repression through histone deacetylases (73).

The true target of RB associated deacetylation activity is unclear. There is no direct *in vivo* evidence that the HDACs recruited by RB repress transcription by deacetylation of the histone tails in the promoters of target genes. While a corresponding lack of acetylation has been observed, it may be an indirect effect perhaps caused by failure to recruit the proper HAT complex. Another possibility is that the true target of RB-associated deacetylase activity is not histone tails but rather other acetylated factors

important for gene expression. In fact, one group studying RB repression of RNA polymerase I transcription demonstrated that acetylation of the required factor, UBF, was important for active transcription of rRNA genes (9). Moreover, this group also showed that deacetylation of UBF by RB associated HDACs results in loss of gene expression, suggesting that HDACs may have substrates in addition to histone tails. Altogether, studies to date indicate that HDAC activity is important for the regulation of select promoters even if the mechanism of repression is still as yet unclear.

*RB and chromatin remodeling: SWI/SNF*

Chromatin remodeling is another fundamental process in the regulation of gene expression. Many chromatin-remodeling complexes reposition nucleosomes that create repressive barriers within gene promoters. Repositioning these nucleosomes alters the local chromatin structure and increases promoter access to factors necessary for active transcription of the gene (85). Chromatin remodeling is also another key process that RB may influence in order to repress transcription. Several groups have demonstrated that RB can interact with mammalian orthologues of the Drosophila Brahma protein: hBRM and Brg-1 (120, 122, 128, 149). These paralogous human proteins are the large ATPase subunits of the human SWI/SNF complexes. SWI/SNF is an ATP-dependent chromatin remodeling complex that is required for the activation of many RNA polymerase II transcribed genes (94). SWI/SNF appears to be required for RB regulation of cell cycle progression (149). In contrast to the activation activity normally associated with chromatin remodeling, when SWI/SNF is recruited to target genes by RB, the overall effect is negative, perhaps by repositioning nucleosomes in a fashion that would obscure

the promoter. Interestingly, RB may recruit both SWI/SNF and HDACs to the same promoter to repress transcription through a combination of histone modification and chromatin remodeling. RB appears to regulate exit from $G_1$ phase of the cell cycle through the recruitment of both HDACs and SWI/SNF, whereas exit from S phase is regulated through RB recruitment of SWI/SNF alone. Again these results suggest multiple mechanisms of RB repression that may be dependent on promoter selectivity.

*RB and co-repressor complexes: Polycomb*

Additionally, RB may regulate gene expression and consequently cell cycle through recruitment of Polycomb (PcG) complexes. Members of the Polycomb family have roles in the negative regulation of *Hox* genes during development (98, 115) and also serve as critical regulators of cell cycle progression (54, 70). Polycomb complexes bind to Polycomb response elements (PRE) in target genes to establish a silenced chromatin state (98) and also contribute to transcriptional memory such that once silenced in a cell, a gene will also be silenced in progeny cells (8).

RB has been shown to associate with a PcG complex containing HPC2, Ring1, and Bmi-1 in an interaction mediated by CtBP (20). Specifically, this HDAC-independent mechanism appears to arrest cells in the G2 phase of the cell cycle by repressing the expression of cyclin A and cdc2. This PcG complex also associates with the cyclin A promoter under conditions of cell cycle arrest. The authors of this study suggest that this mechanism of repression may occur under conditions of irreversible growth arrest

whereas HDAC-dependent RB repression may occur in the case of reversible cell cycle arrest.

*RB and histone methylation: HP1 – SuVar39*

RB may also regulate gene expression by recruitment of HP1 and SUV39H1 (88, 131). SUV39H1 specifically methylates lysine 9 on histone H3 tails. (91, 102). The resulting methylation on K9 of H3 tails induces the formation of a high affinity-binding site on the chromatin for proteins of the heterochromatin protein 1 (HP1) family, which are involved in heterochromatin silencing (29).

RB associates with SUV39H1 in co-immunoprecipitation experiments and directly interacts with HP1 (88). A histone methyltransferase activity also co-purifies with RB. Furthermore, HP1 and RB are both recruited to a methylated H3 peptide, suggesting that HP1 can recognize RB and methylated histone tails simultaneously. *In vivo* chromatin IP data demonstrates that HP1 is present at the cyclin E promoter in RB+/+ cells and that the H3 tails at these promoters are methylated, whereas HP1 does not associate with this promoter in the absence of RB and the H3 tails exhibit lower methylation. Collectively the results suggest that RB recruits the SUV39H1 enzyme to target promoters where it methylates K9H3, providing a binding site for HP1. Since methylation cannot occur at a lysine residue that is acetylated (102), the deacetylase activity associated with RB may be required as a preceding step to SUV39H1 mediated methylation. Potentially the role for HP1 in this repression is not to promote the formation of heterochromatin but rather to mask the methylated lysine 9 in histone H3 tails such that it cannot be de-methylated and

subsequently acetylated. These results indicate that the SUV39H1-HP1 complex is not only involved in heterochromatic silencing but also has a role in repression of euchromatic genes by RB and perhaps other co-repressor proteins.

*RB and DNA methylation: DMNT1*

DNA methylation at CpG dinucleotides is essential for embryonic development and is catalyzed by three DNA methyltransferases, DMNT1, DMNT3a and DMNT3b, which have differential capacities for maintenance and *de novo* methylation (71, 92). During chromatographic purification of the DMNT1 methyltransferase activity, RB was shown to co-purify in a complex with DMNT1 (106) that also includes E2F and HDAC1. The methyltransferase activity that is isolated by immunoprecipitation for RB and DNMT1 appears to work co-operatively with RB and HDAC1 to repress transcription. Potentially, the DNA binding domains of the associated E2F proteins provide the necessary specificity to recruit the repression activities to target genes. The results of this study establish a link between DNA methylation, sequence specific DNA binding activity, histone deacetylation as well as a growth regulatory pathway this is disrupted in the majority of cancer cells.

## *RB regulation of RNA polymerase III*

In addition to regulating genes expressed by RNA polymerase II, RB also regulates genes transcribed by RNA polymerase III. RB globally represses RNA polymerase III transcripts both *in vivo* and *in vitro,* including RNA polymerase III transcribed snRNA genes (15, 45, 62, 116, 123, 142). Interestingly, nuclear run-on assays in RB-/- mouse

embryonic fibroblasts suggest that while global RNAP III activity is elevated in these cells, RNAP II transcription is largely unaffected (142). Regulation of RNA polymerase III transcribed genes may be important for controlling the growth potential of the cell. Genes transcribed by RNA polymerase III encode small RNAs that act as metabolic machinery and thus contribute to the biosynthetic capacity of the cell. Thus, cells lacking RB would be expected to have increased proliferation potential as a result of increased available biosynthetic machinery, and this potential may contribute to unregulated growth during tumor formation.

How RB regulates RNA polymerase III activity in the cell is not clear. RNA polymerase III transcriptional activity is under cell cycle control, with higher levels observed in the late G1, S and G2 phases of the cell cycle than in G0 and early G1. The increase in RNA polymerase III activity correlates with an increase in phosphorylated RB (inactive form) during the G1 phase of the cell cycle, suggesting a link between RB regulation and RNA polymerase III activity (116, 141). Over-expression of RB during transient transfection assays in RB-/- SaOS-2 osteosarcoma cells results in the repression of tRNA genes as well as Ad2 VAI genes.

One proposed mechanism for RB repression of 5S rRNA and tRNA transcription by RNA polymerase III is the disruption of interactions between basal transcription factors required for pre-initiation complex formation. RB has been shown to associate with both TFIIIB (63) and TFIIIC2 (15). Specifically, RB associates with the Brfl and TBP subunits of TFIIIB in co-immunoprecipitation experiments and co-purifies with TFIIIB

biochemically. RB can also interact with TFIIIC2 in GST pull-down experiments performed in HeLa cell nuclear extracts (15). RB however does not appear to interact with TFIIIA (63) or RNA polymerase III. Sutcliffe and collaborators (123) demonstrated that RB interaction with TFIIIB precludes TFIIIB interaction with TFIIIC. Since TFIIIB can no longer associate with promoters through interactions with TFIIIC, RNA polymerase III is not recruited to the start site of transcription, effectively abolishing gene expression.

Less is known about how RB regulates the expression of class 3 RNA polymerase III transcribed genes. RB represses transcription of the human U6 gene by RNA polymerase III both *in vivo* and *in vitro*. However, U6 snRNA genes differ significantly from other genes transcribed by RNA polymerase III. Importantly, these genes contain snRNA-specific promoter elements and do not employ the same TFIIIB and TFIIIC complexes implicated in repression of tRNA genes. One suggestion is that the factors recognizing these sequence elements are important for regulating U6 transcription specifically with respect to RB.

Because the promoter elements are simple and well defined and the factor requirements are known, RNA polymerase III genes provide an excellent model system for understanding regulation of eukaryotic gene expression. We therefore wished to exploit this model system to better understand how the Retinoblastoma tumor suppressor protein functions to repress transcription.

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# Chapter 2

## The retinoblastoma tumor suppressor protein targets distinct general transcription factors to regulate RNA polymerase III gene expression[1].


### Abstract

The retinoblastoma protein (RB) represses RNA polymerase III transcription effectively both *in vivo* and *in vitro*. Here we demonstrate that the general transcription factors SNAPc and TBP are important for RB repression of human U6 snRNA gene transcription by RNA polymerase III. RB is associated with SNAPc as detected by both co-immunoprecipitation of endogenous RB with SNAPc and co-fractionation of RB and SNAPc during chromatographic purification. RB also interacts with two SNAPc subunits, SNAP43 and SNAP50. TBP or a combination of TBP plus SNAPc restores efficient U6 transcription from RB-treated, extracts indicating that TBP is also involved in RB regulation. In contrast, the TBP-containing complex TFIIIB restores adenovirus VAI but not human U6 transcription in RB-treated extracts suggesting that TFIIIB is important for RB regulation of tRNA-like genes. These results suggest that different classes of RNA polymerase III-transcribed genes have distinct general transcription factor requirements for repression by RB.


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## Introduction

RB is a tumor suppressor that controls cell growth by influencing cell-cycle progression (8, 12, 44), differentiation (5, 15, 44), and apoptosis (23, 68). Mutations in the gene encoding RB are associated with diverse human cancers (21, 22, 27, 45). RB function is also compromised in other human malignancies through disruption of upstream control pathways or downstream targets of RB (reviewed in 58). The function of RB as a tumor suppressor is linked to its ability to regulate gene expression. Therefore, to fully understand the contribution of RB to cellular proliferation observed during carcinogenesis, it is important to determine the mechanisms that RB uses to regulate gene activity.

An understanding of RB function in gene regulation was revealed through its role as a modulator of E2F transcription factor activity (16, 24, 25, 59). However, RB controls additional cellular functions beyond regulating E2F activity. The intracellular concentration of RB exceeds the concentration of E2F (58) and interactions between RB and other transcription factors have been described (10, 34, 51). Thus, further activities performed by RB involve regulation of other genes besides E2F-responsive genes. Interestingly, RB is not limited to regulating mRNA production by RNA polymerase II but also inhibits the synthesis of ribosomal RNAs by RNA polymerase I (4) and of 5S rRNA, tRNA, and U6 snRNA by RNA polymerase III (63). It was proposed that loss of control of these genes is an important step in tumor progression because the products of

genes transcribed by RNA polymerases I and III are important determinants of biosynthetic capacity (reviewed in 61). Repressed synthesis of non-translated RNAs is expected to inhibit cell proliferation presenting a significant hurdle to unregulated cell growth. Therefore, control of RNA polymerase I and III transcriptional activity may represent an essential component of growth regulation by RB.

How RB regulates RNA polymerase III activity in the cell is not clear. RNA polymerase III transcriptional activity is under cell cycle control with higher levels observed in the late G1, S and G2 phases of the cell cycle than G0 and early G1 (62). The increase in RNA polymerase III activity correlates with an increase in phosphorylated RB during the G1 phase of the cell cycle. This is important because the function of RB is controlled by phosphorylation (6, 38). Hypo-phosphorylated RB can interact with potential target proteins to regulate their activity whereas hyper-phosphorylated RB cannot interact and therefore is inactive (58). RNA polymerase III activity is maximal during the cell cycle when RB is inactive. This implies that hypo-phosphorylated RB may target factors that function in RNA polymerase III transcription. The correlation between RB levels and RNA polymerase III activity has been further demonstrated *in vivo* by transient transfection assays of adenovirus (Ad) VAI gene transcription. Transcription of this gene by RNA polymerase III is elevated in a human osteosarcoma cell line (SAOS2) that is RB-deficient compared to an osteosarcoma cell line (U2OS) that contains functional RB. Over-expressing RB in SAOS2 cells represses RNA polymerase III transcription whereas RNA polymerase II transcription from the HIV LTR is unaffected. Furthermore, in nuclear run-off assays, RNA polymerase III-specific transcription is diminished in nuclei

isolated from wildtype mouse embryonic fibroblasts compared to nuclei isolated from mouse RB-/- embryonic fibroblasts whereas wholesale RNA polymerase II activity is unchanged in RB+/+ and RB-/- embryonic fibroblasts (63). These experiments suggest that RNA polymerase III activity *in vivo* is regulated by RB.

We have focused on understanding the contribution of RB to repression of RNA polymerase III activity. Genes transcribed by RNA polymerase III can be subdivided into four classes. Class 1 and class 2 genes contain gene-internal promoter elements exemplified by the 5S rRNA and tRNA genes, respectively. Class 3 genes contain gene external promoter elements exemplified by the human U6 snRNA genes. A fourth class exemplified by the Vault RNA genes contain both external and internal promoter elements (54). RNA polymerase III-transcribed genes also have distinct general transcription factor requirements consistent with their different promoter architectures. 5S rRNA genes require TFIIIA, TFIIIB, and TFIIIC, whereas tRNA gene transcription only requires a subset of these factors, TFIIIB and TFIIIC, for full activity (50, 52, 64). In contrast, human U6 gene transcription requires the snRNA activating protein complex or SNAPc (48), which is also known as PTF (42). While TFIIIC is not required, the requirement for TFIIIB is controversial (39, 57). In addition to these general transcription factors, other transcription activator proteins including Oct-1 (3), Sp1 (30), and STAF (43, 49) positively regulate U6 snRNA gene transcription.

The mechanism that RB utilizes to repress RNA polymerase III transcription is not known. However, potential targets for regulating RNA polymerase III activity include

TFIIIB and the TFIIIC2 form of TFIIIC (7, 29). Human TFIIIB consists of the TATA box binding protein (TBP) and a tightly associated factor called TFIIB-related factor or BRF (39, 57). By analogy with yeast TFIIIB (26), a loosely associated factor referred to as B" may also be a component of human TFIIIB. BRF and TBP associate with RB during chromatographic fractionation of cellular extracts and during co-immunoprecipitation experiments (29). TFIIIC2 is a multi-protein complex containing five proteins (67). RB can also interact with TFIIIC2 in GST-pulldown experiments from HeLa nuclear extracts (7). Together, these data indicate that RB can interact with the RNA polymerase III general transcription machinery, and this may be important for RB repression of RNA polymerase III-specific gene transcription.

It is not known whether RB targets similar factors to regulate all classes of RNA polymerase III transcribed genes. In contrast to the clear requirement of TFIIIB and TFIIIC2 for RNA polymerase III transcription of genes containing gene-internal promoter elements, neither TFIIIC (55) nor a TFIIIB complex of BRF and TBP is required for human U6 snRNA gene transcription *in vitro* (17, 39). Potentially, a different form of TFIIIB may function both for U6 gene transcription and regulation by RB. Other alternative spliced forms of BRF have been identified and one form referred to as hBRF2 functions for human U6 transcription *in vitro* (37). It is also possible that RB targets other factors to regulate human U6 snRNA genes. One potential target is SNAPc. SNAPc is a multi-protein complex composed of at least five proteins SNAP19 (19), SNAP43/PTFγ (20, 66), SNAP45/PTFδ (47, 66), SNAP50/PTFβ (1, 18) and SNAP190/PTFα (65). In

addition, SNAPc associates with TBP (20). SNAPc binds to the proximal sequence element (PSE) contained in the core promoter regions of human U6 snRNA genes and interacts with TBP bound to the TATA box. Together SNAPc and TBP act cooperatively to facilitate transcription by RNA polymerase III (40). The binding of these factors to the promoter is a crucial early step in pre-initiation complex assembly at these genes and therefore SNAPc and TBP are attractive targets for regulating human snRNA gene transcription.

Our results suggest that RB regulates different RNA polymerase III-transcribed genes by targeting different components of the general transcription machinery. The general transcription factor TFIIIB, composed of BRF and TBP, functionally restores Ad VAI gene transcription but not human U6 snRNA gene transcription in RB-treated extracts. In contrast, a combination of the general transcription factors SNAPc and TBP act cooperatively to reconstitute U6 snRNA gene transcription after RB treatment indicating that these factors are also important for RB regulation of RNA polymerase III activity. Depleting extracts with GST-RB (379-928) resulted in a reduction in SNAP43 levels consistent with the idea that RB is targeting SNAPc. In HeLa cell nuclear extracts, a sub-population of RB is associated with SNAPc and this association may be direct because RB interacts effectively with two components of SNAPc. These data indicate that the general transcription factors SNAPc and TFIIIB provide an important targeting mechanism governing RB function for different classes of RNA polymerase III-transcribed genes.

## Materials and Methods

**Expression and purification of recombinant proteins:** The region of RB corresponding to amino acids 379 to 928 was amplified by polymerase chain reaction and cloned into a pET11c-based expression vector to generate pGST-RB (379-928). This contains an N-terminal GST tag fused in frame with RB (379-928). Both SNAP43 (1-368) and SNAP50 (1-411) were constructed in a similar manner to generate pGST-SNAP43 (1-368) and pGST-SNAP50 (1-411). GST-fusion proteins were expressed in *E. coli* BL21 DE3 and extracts were prepared by sonication. Proteins were purified by binding to glutathione agarose beads (Sigma) followed by extensive washing in HEMGT-150 buffer (20 mM Hepes pH 7.9; 0.5 mM EDTA; 10 mM $MgCl_2$; 10% glycerol; 0.1% Tween-20) containing protease inhibitors (0.1 mM PMSF, 1 mM sodium bis-sulfate, 1 mM benzamidine, 1 μM pepstatin A) and 1 mM DTT. Bound proteins were then used directly for GST pulldown assays. For *in vitro* transcription experiments, GST-RB (379-928) and GST were eluted from beads in HEMGT-150 buffer containing 50 mM glutathione for 1 hr at 4°C. Eluted proteins were dialyzed against Dignam buffer D (9) and concentrated by centrifugation through YM10 centricon columns (Millipore) to give a final concentration of at least 200 ng/μl. Protein expression levels, efficiency of binding to glutathione agarose beads, and protein concentrations were monitored by SDS-PAGE and staining with Coomassie blue.

**In vitro transcription assays:** *In vitro* transcription of the adenovirus VAI and human U6 snRNA genes were performed essentially as described (31, 32, 48). For repression assays depicted in Figure 1, HeLa cell nuclear extracts were pre-incubated with purified recombinant proteins at 30°C for 30 minutes. The amounts of each protein used are indicated in the figure legend. Twenty microliter transcription reactions were initiated by addition of 0.25 μg DNA templates (pBSM13+VAI; pU6/Hae/RA.2), rNTPs, and transcription buffer. Transcription reactions were performed for 1 hour at 30° C. Transcripts were separated by denaturing PAGE and visualized by autoradiography or by phosphoimager analysis (Molecular Dynamics).

**RB affinity depletion assays:** For human U6 snRNA gene repression assays shown in Figures 2A, 8 μL HeLa cell nuclear extract (7.5 μg/ μL) was pre-incubated with 1, 2, or 3 μL of purified GST-RB (379-928) or GST (each at 1 μg/ μL) for 30 min at 30°C. GST-RB (379-928) and GST were removed by affinity purification with glutathione sepharose beads (Pharmacia) added at a 1:1 ratio of beads to extract. Samples were then incubated at 4°C for 4 hours and centrifuged to remove associated proteins. One half of each supernatant (4.5, 5.0, and 5.5 μL of the 1, 2, and 3 μg treated samples, respectively) was used for *in vitro* transcription assays as described above. For the experiment shown in Figure 2B, the affinity depletion reactions were scaled up to include 32 μL nuclear extract (7.5 μg/ μL) plus 10 μg GST-RB (379-928) (200 ng/ μL). For Ad VAI gene repression assays shown in Figure 2C, 24 μL HeLa cell nuclear extract (7.5 μg/ μL) was

pre-incubated with approximately 14 μg purified GST-RB (379-928) (200 ng/ μL). Similar pre-incubation reactions were performed with either Dignam buffer D (mock depleted) or GST protein at equivalent amounts as used for GST-RB (379-928). After affinity depletion, supernatants were used immediately in adenovirus VAI and human U6 transcription reactions as described previously. Transcription reactions were also supplemented with chromatographic fractions containing SNAPc (Mono-Q peak fraction; approx. 0.3 mg/ml protein; ref. 20) or TFIIIB (PII-B; approx. 0.6 mg/ml; ref. 32). For the experiment presented in Figure 2B, 10 ng of recombinant human TBP (Promega) was also added as indicated. For the experiment presented in Figure 2D, 20 μL HeLa nuclear extract was treated with 6 μg of purified GST-RB (379-928) or GST as described above. 15 μL of each supernatant and proteins bound to the beads after extensive washing were separated by 12.5 % SDS-PAGE and tested by western blot analysis using rabbit anti-SNAP43 antiserum (CS48; ref. 20) or mouse monoclonal antibodies (SL2; ref. 33).

**RB/SNAPc co-immunoprecipitation:** Approximately 300 μL HeLa cell nuclear extract was incubated with 2 μg mouse anti-RB (clone G3-245; Pharmingen) or anti-Haemaglutinin (12CA5) antibodies overnight at 4°C. Samples were diluted with 1 mL of HEMGT-150 containing protease inhibitors and 20 μL Protein-G agarose beads (Gibco-BRL) were added to each reaction. Samples were further incubated at 4°C for 4 hours. Antibody beads were washed in HEMGT-150 containing protease inhibitors (3 x 1 mL), bound proteins were eluted by boiling in 1x Laemmli buffer prior to size fractionation by 12.5% SDS-PAGE. SNAP43 was detected by western blot analysis using antibodies

specific to SNAP43 (CS48; ref. 20). To perform the reciprocal immunoprecipitations, approximately 300 µL of HeLa cell nuclear extract was incubated with 50 µL protein-A agarose beads (Boehringer Mannheim) pre-coupled with either rabbit anti-SNAP43 or pre-immune antibodies. Reactions were incubated for 2 hr at 4°C with mixing. Beads were washed extensively in Dignam buffer D (100 mM KCl) containing protease inhibitors and bound proteins were then competitively eluted in 200 µL Dignam buffer D containing either a specific peptide (CSH375: ref. 20) or non-specific peptide each at 1 mg/mL. Eluted samples were precipitated with trichloroacetic acid. Precipitates were re-dissolved in 1x Laemmli buffer and size fractionated by 12.5% SDS-PAGE. Full length RB was detected by western blot analysis using mouse monoclonal antibodies directed against an epitope contained within amino acids 300-380 (G3-245; Pharmingen).

**Protein Chromatography and EMSA analysis:** Nuclear extracts were prepared from HeLa cells by the method of Dignam et al. (1983). SNAPc- and TFIIIB-containing fractions were generated essentially as described previously (20, 32, 48). The SNAPc fractions used are from the Mono-Q step of purification. The TFIIIB fractions used are from the P11-B step of purification. PSE-specific DNA binding by SNAPc was assayed by EMSA as described (48).

**GST-pulldown assays**: Individual SNAPc subunits and full-length RB were individually expressed *in vitro* using rabbit reticulocyte lysates (TNT-Promega) and proteins were labeled with $^{35}$S-methionine. GST-pulldown reactions were performed using 20 µL

glutathione agarose beads containing approximately 1 μg of GST-RB (379-928), GST-SNAP50 (1-411), GST-SNAP43 (1-368) and GST or beads alone. These were individually incubated with 10 μL of $^{35}$S-labelled proteins for 2 hours at 4°C in 1 mL HEMGT-150 containing protease inhibitors and 1 mM DTT. The specific combinations of proteins used are indicated in the figure legend. Beads were washed extensively in HEMGT-150 and bound proteins were separated by 17% SDS-PAGE. Proteins were stained with Coomassie blue to ensure equivalent loading of GST tagged proteins in each sample. Associated radioactive proteins were detected by autoradiography.

**EMSA experiments:** See appendix A

## Results

### RB represses RNA polymerase III transcription

RB is an important regulator of cellular growth and its ability to perform this function can partially be attributed to regulation of RNA polymerase III activity. RB contains 928 amino acids and can be divided into at least three regions: the N-terminal region from amino acids 1-378, the A/B region from 393-772, and the C region from 768-869. Most functions ascribed to RB including tumor suppressor activity and interactions with regulatory target proteins require either the A/B and/or C regions (56, 60).

To determine the function of RB in regulating RNA polymerase III activity, recombinant RB containing the A/B and C regions was tested for its ability to repress *in vitro*

transcription by RNA polymerase III. Specifically, *in vitro* transcription assays of the Ad VAI gene and a human U6 snRNA gene were performed to compare RB regulation of RNA polymerase III transcription for genes containing gene-internal (class 2) and gene-external (class 3) promoter elements. A schematic representation of the core-promoters of the genes used for this study is shown in Figure 2-1A. The Ad VAI gene contains gene-internal A and B box control elements that are also characteristic of human tRNA genes. The core-promoter regions of human U6 snRNA genes contain a PSE and a TATA box. In addition, the U6 gene contains a DSE that recruits Oct-1 to activate U6 transcription. The GST-RB (379-928) and GST proteins typically used for these experiments are shown in Figure 2-1B. GST-RB (379-928) and GST were each expressed in *E. coli* and were purified to homogeneity by affinity purification using glutathione agarose beads. In each case, the full-length protein is the most prevalent species observed. To determine the effect of these proteins on RNA polymerase III transcription, increasing amounts of purified GST-RB (379-928) and GST were added to HeLa cell nuclear extracts and these were tested for ability to support Ad VAI transcription. As shown in Figure 2-1C, GST-RB (379-928) inhibited transcription of the Ad VAI gene (top panel: lanes 2-5) compared to levels observed for the untreated extract (lane 1). This repression appears to be specific because addition of equivalent amounts of the GST control protein had no significant effect on Ad VAI transcription (lanes 6-9). The repression observed for RB is not limited to "classical" RNA polymerase III transcribed genes containing gene internal promoter

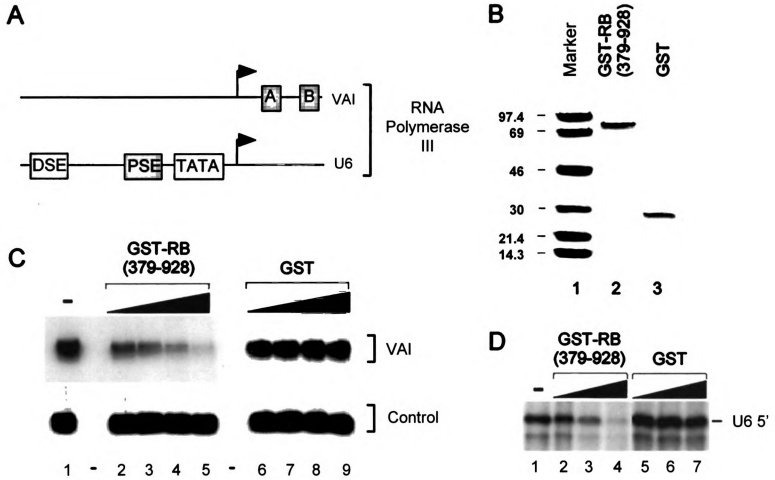
**Figure 2-1: RB represses *in vitro* transcription by RNA polymerase III.**

(A) Schematic representation of the adenovirus VAI and human U6 snRNA promoters.

(B) Analysis of GST-RB (379-928) and GST proteins used in transcription reactions. GST-RB (379-928) (lane 2) and GST (lane 3) were expressed in *E. coli* and purified by affinity chromatography using glutathione agarose beads and competitive elution with glutathione. After dialysis against Dignam buffer D proteins were separated by SDS-PAGE and visualized by staining with Coomassie blue. Lane 1 contains a protein size standard.

(C) GST-RB (379-928) represses adenovirus VAI transcription by RNA polymerase III. Approximately 2 μL of HeLa cell nuclear extract (approx. 7.5 μg/μL) was incubated with 200, 400, 800, and 1200 ng of GST-RB (379-928) (lanes 2-5) or GST protein (lanes 6-9) at 30°C for 30 minutes. *In vitro* transcription of the Ad VAI gene (top panel) was then initiated by addition of template, cold rNTPs, [$\alpha^{32}$P]-CTP, and transcription buffer. Lane 1 shows the level of transcription with the untreated extract. Sample handling was monitored by a non-specific RNA handling control transcript (bottom).

(D) GST-RB (379-928) represses human U6 snRNA gene transcription by RNA polymerase III. Approximately 2 μL of HeLa cell nuclear extract was incubated with 100, 250, and 500 ng of GST-RB (379-928) (lanes 2-4) or GST protein (lanes 5-7). Lane 1 shows the level of transcription with the untreated extract. Correctly initiated transcripts from the U6 promoter (labeled U6 5') were detected by RNase T1 protection essentially as described (31).


A
A
B
VAI
RNA
Polymerase
III
DSE
PSE
TATA
U6
B
Marker
GST-RB
(379-928)
GST
97.4
69
46
30
21.4
14.3
1 2 3
C
GST-RB
(379-928)
GST
-
VAI
Control
1 - 2 3 4 5 - 6 7 8 9
D
GST-RB
(379-928)
GST
-
U6 5'
1 2 3 4 5 6 7

elements. As shown in Figure 2-1D, increasing amounts of GST-RB (379-928) significantly reduced RNA polymerase III transcription that is correctly initiated from the human U6 promoter (lanes 2-4). Again, comparable levels of the GST control protein had no significant effect in these assays (lanes 5-7). Therefore, RB effectively represses *in vitro* transcription by RNA polymerase III.

**The human U6 snRNA and adenovirus VAI promoters have distinct factor requirements for transcriptional repression by RB.**

In order to repress gene transcription by RNA polymerase III, RB must target specific factors required for transcription of these genes. To identify these factors, chromatographic fractions previously demonstrated to reconstitute human U6 and Ad VAI transcription were tested for their ability to restore transcription in extracts that were treated with GST-RB (379-928). Potentially, these chromatographic fractions also contain the factor(s) that are targeted by RB and these may act as a dominant inhibitor of RB function. Both Ad VAI and human U6 transcription can be reconstituted by using a combination of fractions obtained from the purification of HeLa cell extracts over a phosphocellulose P-11 column. These fractions include the P11-B fraction (containing RNA polymerase III as well as 0.38M TFIIIB; ref. 32) and the P11-C fraction (containing RNA polymerase III, TFIIIC and SNAPc). For human U6 transcription, the P11-C fraction can be replaced with a chromatographic fraction further enriched for SNAPc (Mono-Q) and additional recombinant TBP (48). When recombinant TBP and chromatographic fractions containing SNAPc were initially tested for their ability to directly restore human U6 transcription in RB treated extracts, none were able to counter

RB repression (data not shown), perhaps because these reactions contain an excess of RB (379-928). Therefore, whether chromatographic fractions could restore transcription in RB-repressed extracts that have had the GST-RB (379-928) removed by affinity purification was tested. First, the amounts of GST-RB (379-928) required for specific repression under these conditions were established. To perform these experiments, GST-RB (379-928) or GST was pre-incubated with HeLa cell nuclear extracts. Subsequently, GST-RB (379-928) was removed by affinity purification with glutathione agarose beads and extracts were then tested for ability to support U6 transcription. As shown in Figure 2-2A, diminished U6 transcription was observed with extracts affinity depleted with increasing amounts of GST-RB (379-928) (lanes 3-5) compared to extracts treated with similar amounts of GST protein (lanes 6-8). Therefore, GST-RB (379-928) can specifically repress human U6 transcription under these conditions and transcription levels remain low after removal of GST-RB (379-928) by affinity depletion.

To determine the identity of factors targeted by GST-RB (379-928), the ability of chromatographic fractions to reconstitute human U6 gene transcription in GST-RB (379-928) affinity depleted extracts was then assessed. In the experiment shown in Figure 2-2B, the GST-RB (379-928) depletion conditions are similar to that shown in lane 5 of Figure 2-2A; however, less extract was tested for transcription thus the starting levels of

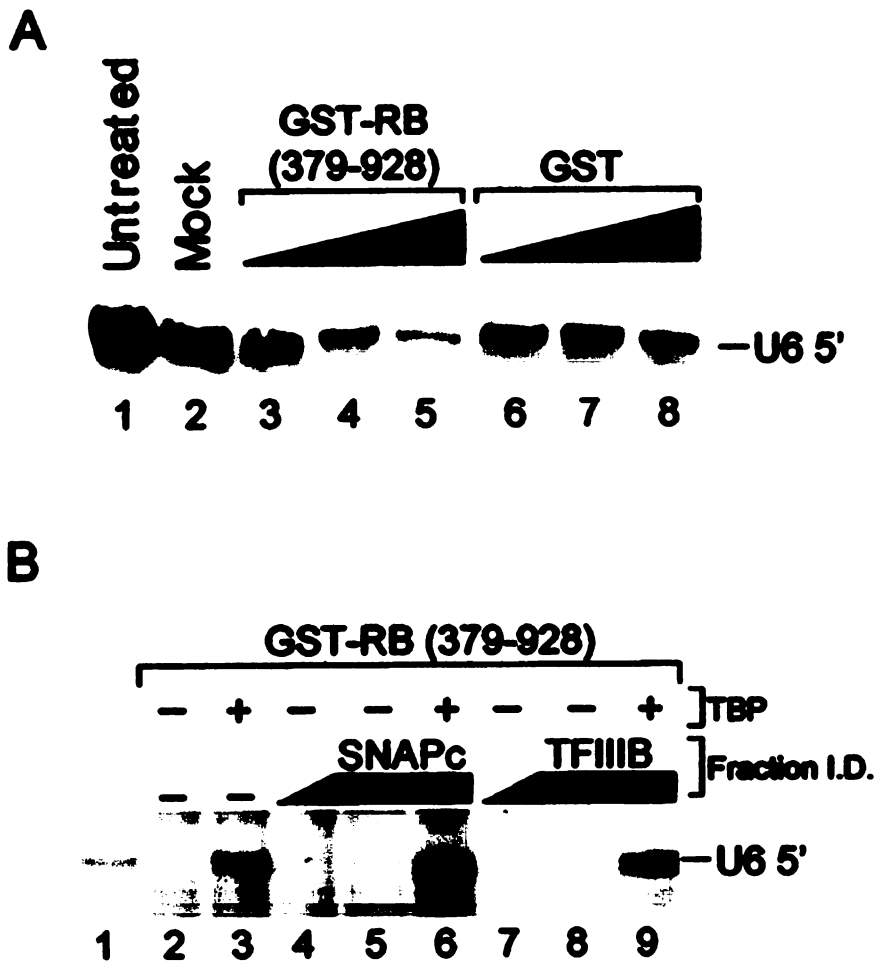
**Figure 2-2. Different factors reconstitute adenovirus VAI and human U6 snRNA gene transcription in GST-RB (379-928) treated extracts.**

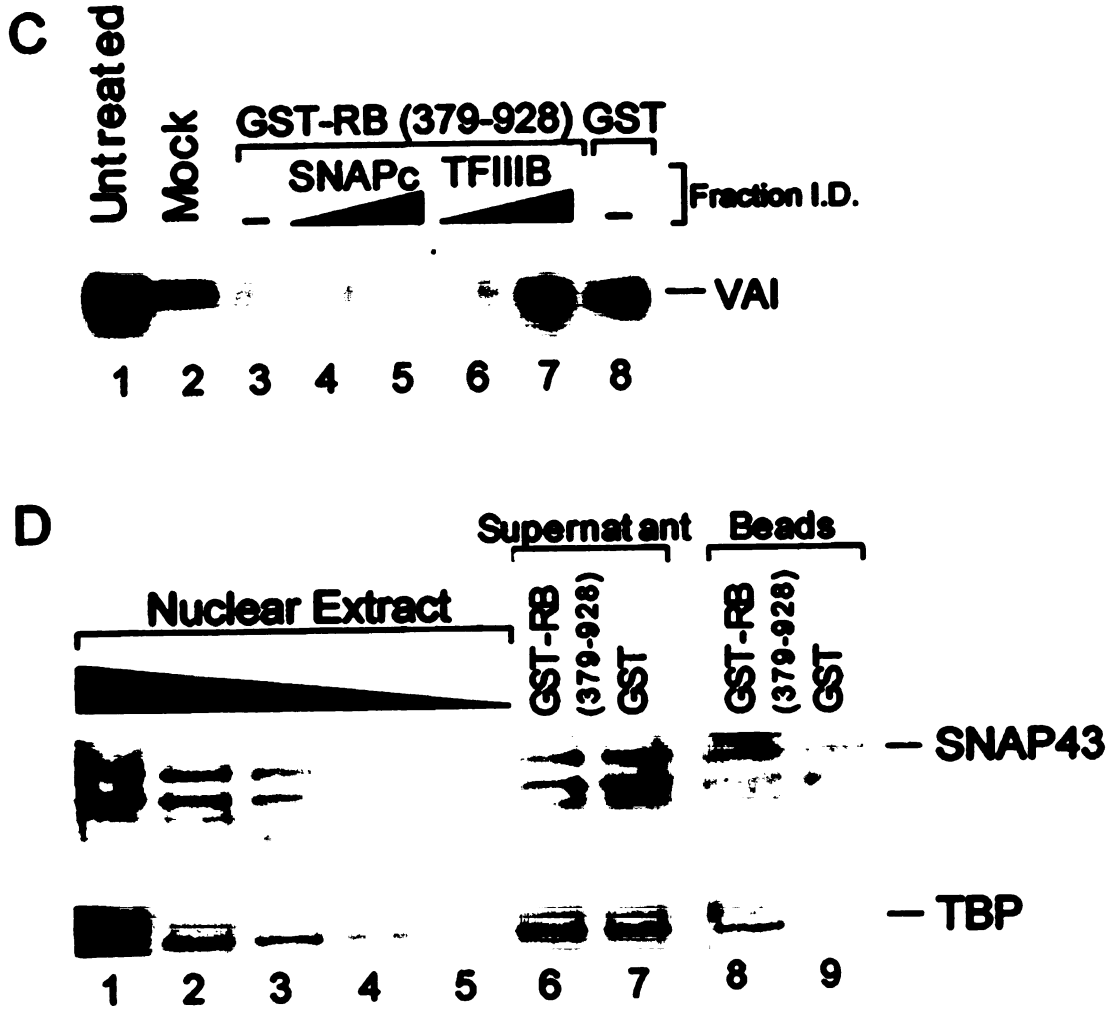
(A) GST-RB (379-928) affinity depletion specifically inhibits U6 transcription. HeLa cell nuclear extracts (8 μL) were incubated with Dignam buffer D (mock lane 2) or 1, 2, or 3 μg purified GST-RB (379-928) (lanes 3-5) or GST (lanes 6-8) for 30 minutes at 30°C. The recombinant proteins and associated factors were then removed by affinity purification with glutathione agarose. One half of each treated extract was then tested for the ability to support human U6 snRNA transcription. Lane 1 shows the transcription supported by 4 μL of the untreated extract.

(B) SNAPc but not TFIIIB acts cooperatively with TBP to reconstitute human U6 snRNA gene transcription. HeLa cell extracts were incubated with GST-RB (379-928) (lanes 2-9). After treating with glutathione agarose beads, 5 μL of each treated extract was tested for the ability to support U6 transcription in the absence (lane 2) or presence of chromatographic fractions containing SNAPc (2.7, 8, and 8 μL, lanes 4-6) or TFIIIB (2.7, 8, and 8 μL; lanes 7-9). Reactions shown in lanes 3, 6, and 9 were also complemented with 10 ng recombinant TBP (Promega). Lane 1 shows transcription supported by 2 μL of the untreated extract.

(C) TFIIIB but not SNAPc reconstitutes adenovirus VAI gene transcription. HeLa cell nuclear extracts were incubated with Dignam buffer D (lane 2), GST-RB (379-928) (lanes 3-7), or GST (lane 8) for 30 min at 30°C. GST-RB (379-928) and GST were removed by incubating treated extracts with glutathione agarose beads for 4 hr at 4°C. 8 μL of depleted extracts were then tested for ability to support VAI gene transcription in the absence (lane 3 and 8) or presence of chromatographic fractions containing SNAPc (2.7 and 8 μl; lanes 4 and 5, respectively) or TFIIIB (2.7 and 8 μl; lanes 6 and 7, respectively). Lane 1 shows transcription supported by 2 μL of the untreated extract.

(D) SNAPc levels are reduced in RB-treated extracts. HeLa cell nuclear extract was treated with 6 μg of GST-RB (379-928) or GST as described above. 15 μL of depleted extracts and proteins associated with the beads after extensive washing were separated by 12.5% SDS-PAGE and tested by western blot analysis using rabbit anti-SNAP43 antisera (top panel). The membrane was then stripped and reprobed using mouse anti-TBP antibodies (bottom panel). Lanes 1-5 contain 12, 6, 3, 1.5, and 0.75 μL of HeLa cell nuclear extract, respectively. The GST-RB (379-928)- and GST-treated extracts are shown in lanes 6 and 7, respectively. Lanes 8 and 9 contain proteins associated with the GST-RB (379-928) and GST agarose beads, respectively.


A
Untreated
Mock
GST-RB
(379-928)
GST
U6 5'
1 2 3 4 5 6 7 8
B
GST-RB (379-928)
TBP
SNAPc
TFIIIB
Fraction I.D.
U6 5'
1 2 3 4 5 6 7 8 9


C
Untreated
Mock
GST-RB (379-928)
GST
SNAPc
TFIIIB
Fraction I.D.
VAI
1 2 3 4 5 6 7 8
D
Supernatant
Beads
Nuclear Extract
GST-RB (379-928)
GST
GST-RB (379-928)
GST
SNAP43
TBP
1 2 3 4 5 6 7 8 9

transcription is reduced. Under these conditions, U6 transcription is abolished by GST-RB (379-928) affinity depletion (lane 2) compared to the untreated extract (lane 1). Addition of recombinant TBP alone restored significant activity to depleted extracts (lane 3). Therefore, TBP or a TBP-containing complex required for U6 snRNA gene transcription is functionally limiting in these RB-treated extracts. Neither fractions containing SNAPc (lanes 4, 5) nor TFIIIB (lanes 7, 8) restored transcription when added alone even though these fractions contain significant levels of TBP. However, when chromatographic fractions containing SNAPc are complemented with recombinant TBP, enhanced activity is now observed (lane 6). This level is approximately 3-fold greater than that observed for TBP alone (lane 3) and significantly greater than SNAPc alone (lane 5). The cooperative effect observed for TBP with SNAPc is specific because this is not observed with TBP complemented with chromatographic fractions containing TFIIIB (lane 9). Therefore, SNAPc alone can not restore transcription, but together SNAPc and recombinant TBP act cooperatively to restore U6 snRNA transcriptional activity to fractions treated with GST-RB (379-928).

The above results suggest that RB may target TBP or alternatively target SNAPc to control TBP activity whereas TFIIIB may not be involved in RB repression of these genes. However, TFIIIB was previously described as a target for RB repression of RNA polymerase III activity (7, 29). To determine whether RNA Polymerase III-transcribed genes with intragenic promoter elements have similar factor requirements for relief from RB repression, the ability of chromatographic fractions to restore Ad VAI transcription was also tested. As shown in Figure 2-2C, the GST-RB (379-928) treated extract is

compromised for Ad VAI transcriptional activity (lane 3) as compared to either mock treated (lane 2) or untreated HeLa cell nuclear extracts (lane 1). Transcriptional activity was not restored by addition of chromatographic fractions containing SNAPc (lanes 4, 5). This result was expected because SNAPc is not required for transcription of these genes. However, Ad VAI transcriptional activity is effectively reconstituted by addition of increasing amounts of chromatographic fractions containing TFIIIB (lanes 6, 7). Transcription in these samples is comparable to levels obtained with either the mock treated (lane 2) or GST-treated (lane 8) extracts. Therefore, fractions containing TFIIIB effectively reconstitute Ad VAI transcription and this is specific because restoration is not observed with SNAPc-containing fractions. This data is consistent with that previously described (7, 29) and supports the hypothesis that TFIIIB is one target for gene regulation by RB.

One explanation for the reduced U6 transcription following affinity depletion with GST-RB (379-928) is that TBP or higher order complexes containing TBP and SNAPc are removed. Thus, affinity depletion of nuclear extracts using GST-RB (379-928) should also result in a measurable reduction in the levels of these factors. Therefore, a western blot analysis was performed using anti-SNAP43 antibodies (CS48; Henry et al. 1995) and anti-TBP antibodies (SL2; ref. 33) to determine whether treatment of nuclear extracts with GST-RB (379-928) alters the levels of SNAPc or TBP. As shown in Figure 2D (top panel), depleting extracts with GST-RB (379-928) results in a significant decrease in SNAP43 levels (lane 6) compared to amounts present in extracts depleted with the GST control protein (lane 7). Comparison of SNAP43 in the GST-RB (379-928) treated

sample and in decreasing amounts of HeLa cell nuclear extract (lanes 1-5) indicate that at least 50% of endogenous SNAP43 was removed by affinity depletion with GST-RB (379-928). This same membrane was then re-probed using antibodies directed against TBP and the results are shown in Figure 2-2D (lower panel). In this case, no significant difference in TBP levels was observed for the GST-RB (379-928) and GST treated samples suggesting that TBP is not effectively depleted in these assays. This may mean that RB does not target TBP. Alternatively, since TBP is present in numerous TBP-containing complexes and RB may target only some of these complexes, the removal of a minor proportion of the total TBP may not be detectable in these assays. Indeed, TBP was associated with the GST-RB (379-928) agarose beads (lane 8) but not with the GST-agarose beads (lane 9) suggesting that a minor proportion of the total TBP can associate specifically with GST-RB (379-928). Our results are also consistent with the notion that GST-RB (379-928) is targeting SNAPc in a stable fashion.

**Endogenous RB associates with SNAPc**

The above experiments suggest that high levels of GST-RB (379-928) can target SNAPc to potentially control human U6 gene transcription. However, it is important to determine whether an association between endogenous RB and SNAPc is possible. To determine whether endogenous RB and SNAPc can interact, co-immunoprecipitation experiments were performed by incubating HeLa cell nuclear extracts with either anti-HA or anti-RB antibodies. Proteins bound by these antibodies were eluted by boiling in Laemmli buffer and separated by SDS-PAGE for analysis by anti-SNAP43 western blotting. As shown in

**Figure 2-3. Endogenous RB is associated with SNAPc.**

(A) Endogenous SNAP43 is co-immunoprecipitated with RB. Approximately 300 μL HeLa cell nuclear extract was incubated with mouse anti-RB (G3-245; Pharmingen) (lane 5) or anti-HA (12CA5) (lane 4) antibodies overnight. Protein-antibody complexes were removed by affinity purification using Protein-G agarose beads (Gibco-BRL). The beads were washed extensively and bound proteins were resolved by 12.5% SDS-PAGE. SNAP43 association was detected by western blot analysis using antibodies specific to SNAP43 (CS48; ref. 20). Lanes 1-3 show the amount of SNAP43 present in 10, 3, and 1 μL nuclear extract.

(B) Endogenous RB is co-immunoprecipitated with SNAPc. HeLa cell nuclear extracts were incubated with antibodies directed against the SNAP43 subunit of SNAPc (lanes 6 and 7) or rabbit pre-immune antibodies (lanes 4 and 5) covalently coupled to protein A agarose beads (Boehringer Mannheim). After protein binding and extensive washing, each reaction was divided in half. Bound proteins were competitively eluted in buffer containing either a specific peptide (CSH375: ref. 20) (lanes 4 and 6) or an irrelevant peptide (CSH374) (lanes 5 and 7). Eluted proteins were precipitated with TCA, size fractionated by 12.5% SDS-PAGE and the levels of RB were detected by western blot analysis. Lanes 1-3 show the levels of RB detected in 10, 3, and 1 μL of HeLa cell nuclear extract after precipitation with TCA. The experiment in part B was performed by R. William Henry.

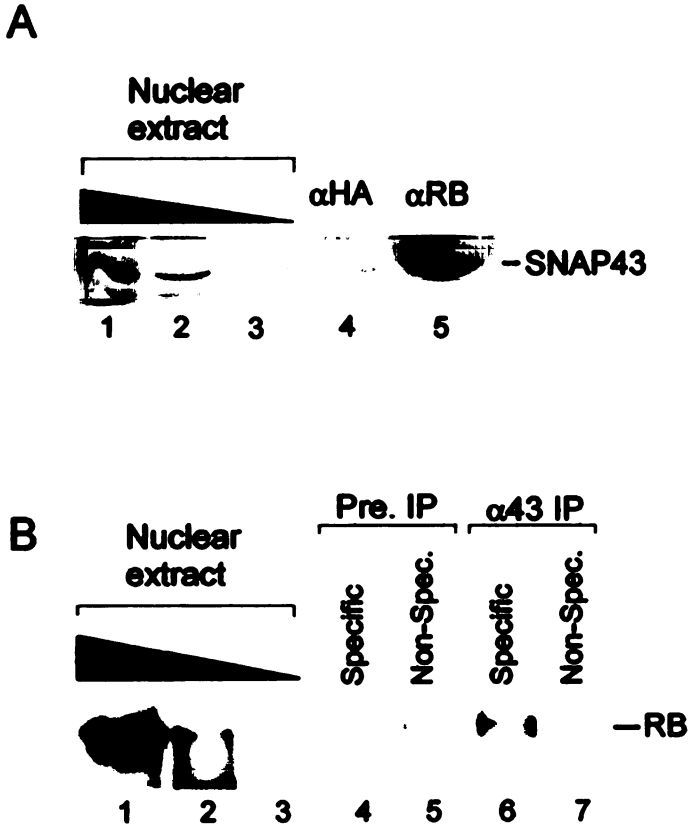

A
Nuclear extract
αHA
αRB
-SNAP43
1 2 3 4 5
B
Nuclear extract
Pre. IP
α43 IP
Specific
Non-Spec.
Specific
Non-Spec.
—RB
1 2 3 4 5 6 7

Figure 2-3A, significant levels of SNAP43 were detected in the samples immunoprecipitated with the anti-RB (lane 5) but not with the anti-HA (lane 4) antibodies. To confirm these results, the reciprocal experiment was performed testing the co-immunoprecipitation of RB through immunoprecipitation of the SNAP43 subunit of SNAPc (Figure 2-3B). In these experiments the immunoprecipitation methodology was modified to reduce the high non-specific background that was observed due to cross-reaction of the secondary antibody with the heavy chain from the anti-SNAP43 antibodies. HeLa cell nuclear extracts were incubated with agarose beads covalently crosslinked with either rabbit anti-SNAP43 (CS48: ref. 20) or preimmune antibodies. Each sample was then divided in half. Bound proteins were competitively eluted either with the specific peptide used to generate the anti-SNAP43 antibodies (1mg/ml, peptide CSH375: ref. 20) or a non-specific peptide. These elution conditions were chosen to minimize the disruption of protein-protein interactions and reduce contamination of the eluted proteins with the heavy chain from the SNAP43 antibodies. Eluted proteins were then concentrated by precipitation with trichloroacetic acid, size fractionated by 12.5 % SDS-PAGE, and analyzed by western blot analysis using antibodies directed against RB. As shown in Figure 3, a significant amount of endogenous RB is co-immunoprecipitated using anti-SNAP43 antibodies and eluted with the specific peptide (lane 6). The co-immunoprecipitation of RB with SNAPc is specific because it is not non-specifically eluted from the anti-SNAP43 antibodies (lane 7) and it also is not observed under any immunoprecipitation conditions using pre-immune antibodies (lanes 4, 5). Therefore, a sub-population of endogenous RB associates with endogenous SNAPc.

If the association between RB and SNAPc is stable, then it is possible that a significant amount of endogenous RB will co-fractionate with SNAPc during extensive chromatographic purification of SNAPc. Therefore, to further test the association between endogenous RB and SNAPc, SNAPc was purified from HeLa cell extracts and the co-purification of RB was monitored by western blot analysis. The purification scheme typically used to fractionate SNAPc is shown in Figure 2-4A. This scheme applies to the purification of SNAPc both from HeLa cell nuclear and S-100 extracts. Briefly, HeLa cell extracts were selectively precipitated by ammonium sulfate prior to fractionation using a phosphocellulose P-11 column. Proteins bound to this column were step eluted in buffers containing increasing concentrations of KCl to generate the P11-A, B, C, and D fractions. The majority of SNAPc is present in the P11-C fraction. This was then directly passed over a Cibacron blue affigel column (CB) and bound proteins were eluted with a linear gradient from 500 mM KCl (0% ethylene glycol) to 2.5 M KCl (25 % ethylene glycol). Fractions generated from this column were dialyzed against Q100 buffer and then tested for DNA binding activity as previously described (48). Electromobility shift analysis (EMSA) of the CB fractions revealed that the majority of PSE binding activity is present in CB fractions 29-59 (Figure 2-4B). This peak corresponds to fractions eluted at KCl concentrations between 1.8 to 2.5 M KCl and is indicative of SNAPc activity. These fractions were then tested for the presence of RB by western blot analysis and the results are shown in Figure 2-4C. A significant level of RB is present in the CB fractions with a peak observed in fractions 29-39 indicating that RB

**Figure 2-4. Endogenous RB co-fractionates with SNAPc during chromatographic purification.**

(A) Schematic representation of the chromatographic purification used to purify SNAPc.

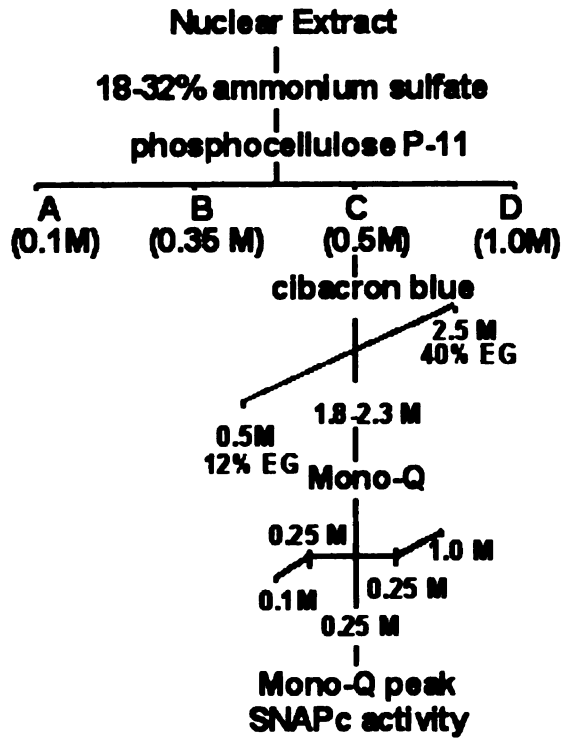
(B) Characterization of chromatographic fractions for PSE binding activity. Approximately 10 μL aliquots of the P11-C (load; ca. 0.5 mg/ml protein) and fractions obtained from the cibacron blue (CB) step of purification were tested for DNA binding activity by electromobility shift assay using radioactive probes containing a high affinity mouse U6 PSE and TATA box. The positions of the unbound probe (free probe) and SNAPc bound to DNA (SNAPc) are labeled. The peak of SNAPc is contained in fractions 27-59 and corresponds to fractions eluted in buffer containing between 1.8-2.5 M KCl.

(C) Anti-RB western blot analysis of SNAPc -containing fractions shown in B. Approximately 10 μL aliquots of each fraction were tested for the presence of RB using mouse anti-RB monoclonal antibodies that recognize an epitope between amino acids 300-380 of human RB (Pharmingen, antibody G3-245). Significant levels of RB are detected in CB fractions 29-39.

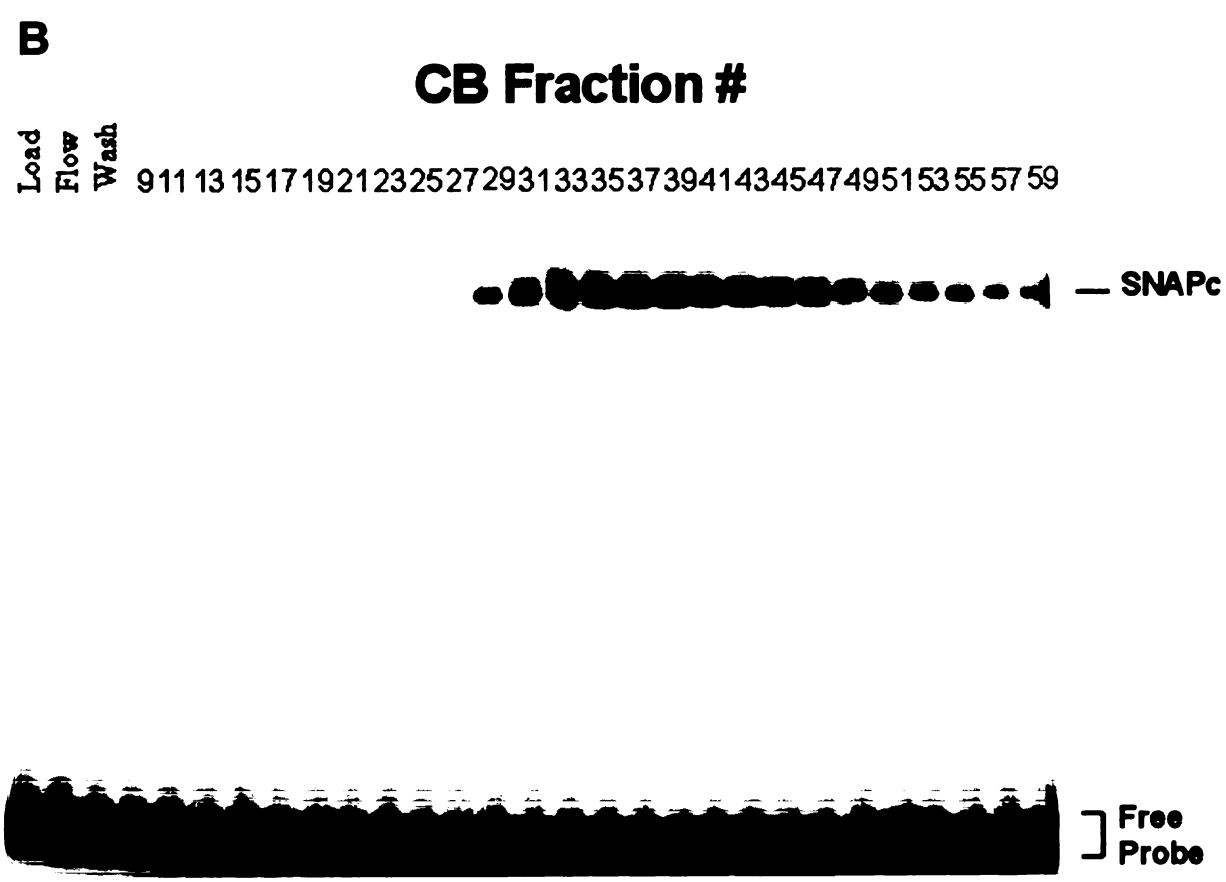
(D) Anti-RB western blot analysis of highly purified SNAPc fractions. The peak of SNAPc activity from the cibacron blue column (CB 29-59) was pooled and further purified by anion exchange column chromatography using a mono-Q HR 5/5 column (Pharmacia). The peak of SNAPc elutes from this column as a single peak in buffer containing 250 mM KCl. Increasing amounts of the Mono-Q peak fractions (lanes 1-3: approximately 0.8, 2.4, and 7.5 μg total protein, respectively) and nuclear extract starting material (lanes 4-6: approximately 10, 30, and 100 μg total protein, respectively) were analyzed by 12.5% SDS-PAGE and western blotting using antibodies directed against RB.

These experiments were performed by R. William Henry.

A


Nuclear Extract
18-32% ammonium sulfate
phosphocellulose P-11
A
(0.1M)
B
(0.35 M)
C
(0.5M)
D
(1.0M)
cibacron blue
2.5 M
40% EG
1.8-2.3 M
0.5M
12% EG
Mono-Q
0.25 M
1.0 M
0.1M
0.25 M
0.25 M
Mono-Q peak
SNAPc activity

B

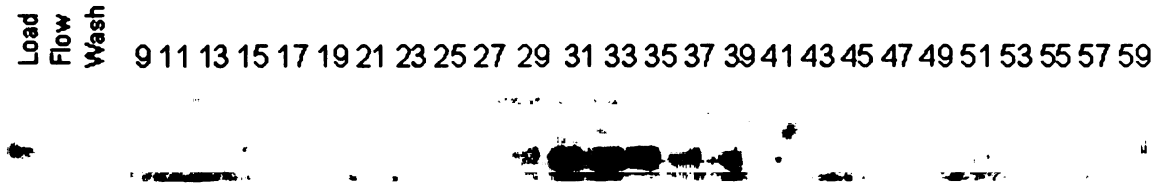

CB Fraction #
Load
Flow
Wash
9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
SNAPc
Free
Probe

C

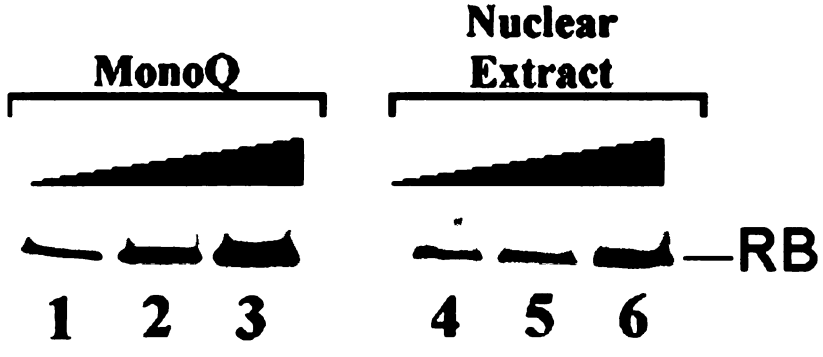
Load

D

C


CB Fraction #
Load
Flow
Wash
9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59

D


MonoQ
Nuclear
Extract
RB
1 2 3
4 5 6

co-fractionates with SNAPc. However, RB is present only in a subset of the fractions containing significant levels of SNAPc activity. For example, CB fractions 39, 41, and 43 contain similar levels of DNA binding activity and yet contain distinctly different levels of RB. This suggests that RB is associated with a sub-population of SNAPc.

To further characterize the association of RB with SNAPc, the peak of DNA binding activity from the Cibacron blue column was purified by anion exchange chromatography using a Mono-Q column (Pharmacia). The vast majority of SNAPc is eluted from this column isocratically in buffer containing 250 mM KCl. By this stage of purification SNAPc has been purified approximately 2000-fold (data not shown). To test for the presence of RB in these fractions, increasing amounts of the Mono-Q peak (3, 10, and 30 μL: ca. 0.25 mg/mL protein) were analyzed for the presence of RB and the results are shown in Figure 2-4D. Again, significant levels of RB are present in these fractions containing highly purified SNAPc (lanes 1-3). More RB is detected in these Mono-Q fractions than that in HeLa cell nuclear extracts (1, 3, and 10 μL; ca. 10 mg/mL protein) shown in lanes 4-6. These data indicate that RB is approximately 1-3 fold more concentrated in the Mono-Q fractions than in HeLa cell nuclear extracts. Correspondingly, this represents an estimated 30-100 fold purification of RB during the purification of SNAPc. Therefore, a sub-population of endogenous RB is associated with a sub-population of endogenous SNAPc.

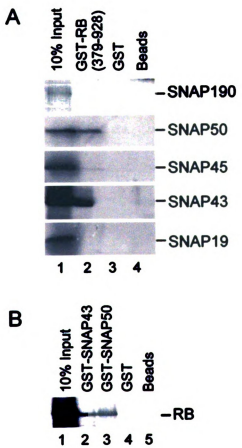
**RB can interact with SNAPc**

RB associates with SNAPc as detected both by co-immunoprecipitation from nuclear extracts and co-fractionation during SNAPc purification. However, it is not known whether this association is mediated by additional factors or RB directly targets SNAPc. Therefore, to determine whether direct interactions between RB and SNAPc are possible, GST-pulldown assays were performed with GST-RB (379-928) and individual subunits of SNAPc. Each SNAPc subunit was expressed separately in rabbit reticulocyte lysates and proteins were labeled with $^{35}$S-methionine. These were then mixed with GST-RB (379-928) that was pre-bound to glutathione agarose beads. Proteins were also tested for interactions with GST protein or beads alone as negative controls. The results of these GST pulldown assays are shown in Figure 5A. Significant interactions were observed between GST-RB (379-928) and two SNAPc subunits: SNAP43 and SNAP50. These interactions are specific as no interaction was detected between these proteins and either the GST or beads-alone control samples. In contrast, no significant interactions were observed between GST-RB (379-928) and any other SNAPc subunits. As an additional control, the reciprocal experiment was performed as shown in Figure 5B. Full-length RB (1-928) was expressed *in vitro* and labeled with $^{35}$S-methionine. This was tested for interactions with GST-SNAP43 and GST-SNAP50. Again, full-length RB interacted with both SNAP43 and SNAP50, and this is specific because little cross-reaction with the GST-alone or beads-alone samples was observed. Therefore, the association of RB with

**Figure 2-5. RB interacts with two components of SNAPc.**

(A) GST-pulldown experiment performed to test interactions between individual SNAPc subunits and GST-RB (379-928). Each SNAPc subunit was expressed *in vitro* using rabbit reticulocyte lysates and proteins were labeled with $^{35}$S-methionine. Lane 1 contains 10% of the $^{35}$S-labeled proteins used as inputs. These were tested for interactions with GST-RB (379-928) (lane 2), GST (lane 3), or glutathione agarose beads alone (lane 4). Proteins were size fractionated by 12.5% SDS-PAGE and visualized by autoradiography. The identity of each SNAPc subunit is indicated.

(B) Full length RB was expressed *in vitro* and labeled with $^{35}$S-methionine. Lane 1 contains 10% of $^{35}$S-labeled RB used as input. This was tested for interaction with GST-SNAP43 (lane 2), GST-SNAP50 (lane 3), GST (lane 4), and beads alone (lane 5) as above.


A
10% Input
GST-RB (379-928)
GST
Beads
SNAP190
SNAP50
SNAP45
SNAP43
SNAP19
1 2 3 4
B
10% Input
GST-SNAP43
GST-SNAP50
GST
Beads
RB
1 2 3 4 5

SNAPc previously observed may involve direct protein-protein interactions between RB and SNAPc.

**Discussion**

RB is an important tumor suppressor protein that acts to regulate cell growth in part by controlling progression through the cell cycle. Typically, RB is thought to act by regulating expression of genes that are important for executing specific cell cycle functions. The discovery that RB represses transcription by RNA polymerases I (4) and III (63) reveals that RB also acts to regulate expression of highly transcribed genes encoding non-translated RNAs.

The ability of RB to regulate gene expression is determined by targeting RB to specific gene promoters. Control of RNA polymerase II transcription typically involves recruiting RB to gene promoters by direct protein-protein interactions with the transcriptional regulatory protein E2F (11). Interactions between RB and other regulatory proteins are also important for RB function. Nonetheless, RB does not directly regulate transcription of most genes by RNA polymerase II. In contrast, RB appears to generally repress RNA polymerase III activity (63). This suggests that RB interacts with the general transcriptional machinery required for RNA polymerase III transcription to effect repression. Indeed, the general transcription factor TFIIIB, composed of TBP and BRF, is important for expression of 5S rRNA and tRNA genes and also appears important for regulation of these genes by RB. In our experiments, addition of chromatographic fractions containing TFIIIB restored adenovirus VAI transcription to extracts that were

affinity depleted using GST-RB (379-928). This result suggests that TFIIIB is limiting for VAI transcription in extracts affinity depleted using GST-RB (379-928) and is consistent with the previously suggested hypothesis that RB targets TFIIIB for repression of these genes (7, 29).

The human U6 gene family has distinctly different promoter elements contained entirely in the 5' region flanking these genes. *In vitro*, RB also effectively represses transcription of these genes and in our assays repression of U6 gene transcription is observed at moderately lower concentrations of RB than that required for repression of adenovirus VAI gene transcription (Figure 2-1 and data not shown). Many possible explanations might explain the differential regulation of these two genes by RB, but one explanation that we favor is that RB is specifically interacting with different factors specialized for transcription of these genes and these interactions are important for repression. Transcription of human U6 snRNA genes by RNA polymerase III requires the general transcription factors SNAPc, BRF2, and TBP (37, 48), whereas TFIIIB, consisting of BRF and TBP, appears not to be required (39). Fractions containing TFIIIB were unable to restore U6 snRNA gene transcription in extracts that were affinity depleted using GST-RB (379-928). Therefore, TFIIIB appears to be important for RB repression of adenovirus VAI but not human U6 snRNA gene transcription.

In order to test whether the general transcription factor SNAPc has a role in mediating RB regulation, chromatographic fractions containing SNAPc were added to GST-RB (379-928) treated extracts. In these experiments, SNAPc fractions did not restore U6

snRNA gene transcription, which would suggest that SNAPc is not involved in RB repression. One possibility is that RB also present in these fractions is repressing SNAPc activity. We consider this possibility unlikely because SNAPc fractions obtained by biochemical fractionation efficiently restore U6 transcription in extracts immuno-depleted of endogenous SNAPc (data not shown) suggesting that the levels of RB present are not inhibitory for SNAPc function. A second possibility is that other factors not contained in the SNAPc fractions are targeted and removed from extracts by affinity depletion using GST-RB (379-928). Indeed, recombinant TBP alone restored transcription from the U6 promoter when added to GST-RB (379-928) affinity depleted extracts. This result suggests that TBP or higher order complexes containing TBP are important for RB regulation of human U6 snRNA genes.

Although recombinant TBP alone restored U6 transcription, the levels of transcription observed were increased by addition of fractions containing SNAPc, but not TFIIIB, to reactions containing recombinant TBP. This result suggests that SNAPc is also involved in the pathway for RB regulation of human U6 genes. Consistent with these observations, the levels of endogenous SNAPc were reduced in nuclear extracts affinity depleted with an excess of GST-RB (379-928). One function of SNAPc is to recruit TBP to the TATA box contained in human U6 snRNA gene promoters (40). However, high levels of recombinant TBP can overcome the requirement for SNAPc for U6 transcription (data not shown). The ability of TBP to function alone in these assays, therefore, may be because high levels of TBP can bypass the requirement for SNAPc. Thus, RB may control SNAPc to affect TBP activity at these promoters. If RB targets SNAPc to repress

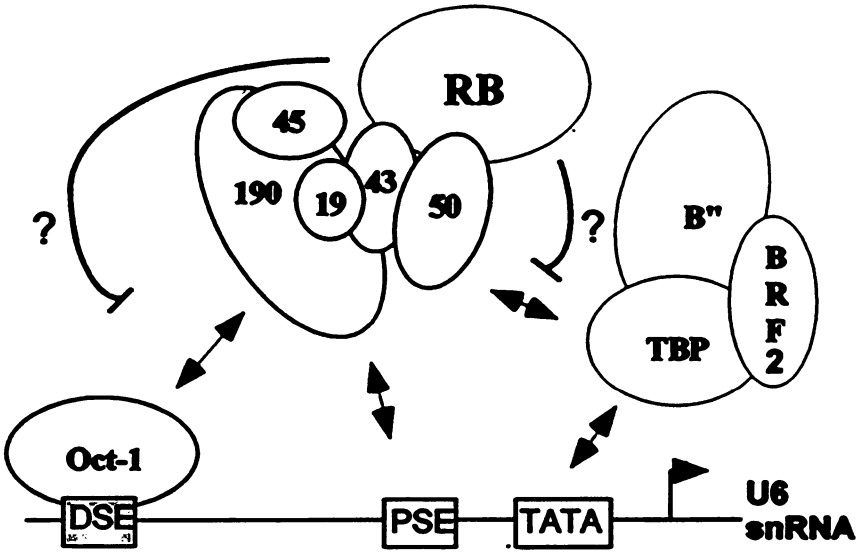
human U6 transcription then it was expected that there should be a measurable association between endogenous RB and SNAPc. Indeed, a fraction of the total endogenous RB associates with SNAPc. First, a modest level of RB was observed to co-immunoprecipitate with SNAPc. Furthermore, co-purification of RB with SNAPc was observed during the biochemical fractionation of SNAPc. In support of these results, RB can interact with the SNAP43 and SNAP50 subunits of SNAPc, which suggests that direct interactions between RB and SNAPc may be important for regulating U6 gene expression. These results, however, do not rule out the possibility that additional factors may also contribute to the function of RB at these promoters. It remains to be determined whether the recently described alternatively spliced forms of human BRF (37) also participate in RB repression of human U6 gene transcription.

For RNA polymerase II transcription, two models have been proposed to explain the mechanism for gene repression by RB. In one model, RB binds E2F at the promoter to abrogate the potential of E2F to activate transcription. E2F recruits the general transcription factor TFIID to the promoter in a TFIIA-dependent manner and this is sensitive to the presence of RB. After TFIID-TFIIA complex formation, gene expression becomes resistant to repression by RB (46). In a second model, RB represses transcription of some cell cycle responsive genes that contain E2F binding sites via recruitment of a histone deactylase (HDAC) (2, 35, 36). Thus, transcriptional repression by RB may involve modification of chromatin structure. HDACs remove acetyl groups from histones and consequently re-configure the chromatin structure to a non-permissive state for transcription. The recruitment of HDACs by RB may be direct (13, 36) or

require a tethering protein (28). However, not all promoters are sensitive to recruitment of HDACs and thus it appears that these two models for RB repression are promoter selective (35).

How does RB regulate U6 snRNA gene transcription by RNA polymerase III? Potentially, histone deacetylation is an attractive model to explain regulation of U6 snRNA gene transcription *in vivo*. In chromatin reconstitution experiments, the human U6 snRNA gene contains a positioned nucleosome located between the distal sequence element (DSE) and the PSE (53). Transcription of the U6 wild-type gene is enhanced after chromatin assembly, and thus, modification of histones potentially could repress gene activity. However, in our *in vitro* system we observe repression of RNA polymerase III transcription using naked DNA templates. Thus, it appears that chromatin modification is not essential for repression of RNA polymerase III *in vitro*. In an alternative model, RB acts to inhibit pre-initiation complex assembly at human U6 promoters. For example, RB could disrupt interaction between the transcriptional activator Oct-1 and SNAPc (Figure 4-6). Direct contacts between the Oct-1 POU domain and the SNAP190 subunit of SNAPc facilitate SNAPc binding to the PSE (14, 41). Interactions between RB and SNAPc may prevent interactions between Oct-1 and SNAPc and therefore prevent recruitment of SNAPc to human U6 gene promoters. This model is reminiscent of the role of RB for repressing RNA polymerase II transcription by modulating E2F-mediated pre-initiation complex assembly with TFIID and TFIIA (46).

**Figure 2-6. Model for RB repression of human U6 snRNA gene transcription.** There are several mechanisms by which RB may repress U6 snRNA gene expression by RNA polymerase III. RB could interact with SNAPc and prevent DNA binding by SNAPc. Alternatively, RB could disrupt protein-protein communications that are important for U6 transcription. Targeting interactions between SNAPc and either the transcriptional activator protein Oct-1 or TBP would be predicted to repress human U6 snRNA gene transcription. Finally, RB may also interact with both SNAPc and TBP/BRF2/B" simultaneously to block further pre-initiation complex assembly.


RB
45
190
19
43
50
?
?
B"
BRF2
TBP
Oct-1
DSE
PSE
TATA
U6
snRNA

RB may also interfere with pre-initiation complex assembly by preventing DNA binding by SNAPc or TBP. By binding to SNAPc, RB may directly prevent this core promoter complex from binding to the PSE in the promoter of human U6 snRNA genes. Another interesting possibility is that RB disrupts communication between SNAPc and TBP. The binding of SNAPc to the PSE and recombinant TBP to the TATA box is cooperative and this is important for transcription of the U6 snRNA gene (40). Therefore, RB could disrupt TBP recruitment by interfering with the function of SNAPc for TBP recruitment. Interestingly, SNAPc interacts well with TBP and this may involve the SNAP43 subunit (20), which also binds RB in our assays. Thus, potential interactions between SNAP43 and RB may modulate simultaneous interaction between SNAP43 and TBP.

RB plays an important role in coordinating RNA polymerase III activity and our data indicates that RB does this by targeting TFIIIB and SNAPc /TBP. Clearly, regulating TBP is important and RB appears to target core-promoter complexes that are important for TBP function. TFIIIB and SNAPc play crucial early roles in pre-initiation complex assembly at RNA polymerase III-gene promoters and therefore, these are attractive targets for regulating RNA polymerase III activity. By understanding the mechanisms by which expression of non-translated RNAs is controlled, we can define the contribution of these RNAs to the regulation of normal cell growth. Importantly, the availability of essential non-translated RNAs could act to limit cell growth and RB repression of genes encoding these RNAs would likely have to be overcome prior to tumor progression.

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# Chapter 3

## Retinoblastoma protein and RNA polymerase III concurrently occupy a repressed human U6 promoter


## Abstract

The Retinoblastoma protein (RB) regulates the cell cycle and differentiation by repressing RNA polymerase II transcription of key target genes. Postulated repression mechanisms suggest that RB directly blocks pre-initiation complex assembly or recruits additional co-factors such as histone deacetylases or ATP-dependent chromatin remodeling machines whose activities impair RNA polymerase II access to promoters. RB also represses transcription of non-translated RNAs that are transcribed by RNA polymerases I and III, potentially to control cell growth. To analyze the mechanism by which RB represses RNA polymerase III transcription we examined human U6 snRNA gene transcription by RNA polymerase III. Herein, we show that RB co-occupies the U6 promoter with RNA polymerase III *in vivo* and RB repression *in vitro* does not preclude RNA polymerase III recruitment. These results suggest a novel mechanism wherein RB represses transcription at steps subsequent to RNA polymerase recruitment to gene promoters.

**Introduction**

The Retinoblastoma protein (RB) provides important functions that contribute to cell cycle control, cellular differentiation, apoptosis, and general growth control. Mutations in the gene encoding RB are found in a wide variety of human cancers (14), highlighting the central importance of this protein as a tumor suppressor. Typically, RB functions to repress RNA polymerase II transcription of genes that act at control points in these key cellular processes (6, 7, 8). The classical understanding of RB function is exemplified by cell cycle control by RB (reviewed in 30). For example, RB can target cell cycle control genes that are required to enter into S phase and whose promoters contain binding sites for the E2F family of transcriptional activator proteins (4, 11, 20, 31). By binding to E2F at gene promoters, RB can directly block the transactivation function of E2F (21). Alternatively, RB can recruit co-regulatory proteins, such as histone deacetylases (1, 16, 17) or ATP-dependent chromatin remodeling machines (25, 28, 37) that alter chromatin structure surrounding these genes to repress transcription.

In addition to regulating transcription of key protein-encoding genes by RNA polymerase II, RB can also repress transcription of non-translated RNAs that are transcribed by RNA polymerases I (3) and III (35). Interestingly, transcription of diverse RNA polymerase III-transcribed genes is elevated in cell lines whose RB function is disrupted, suggesting that RB can play an important role in regulating RNA polymerase III transcription generally (35). In contrast, global RNA polymerase II transcription in these cells is unaffected. It was proposed that RB may regulate transcription of key biosynthetic genes

by RNA polymerase I and III to control growth (32). This suggestion is consistent with observations that RNA polymerase I and III transcription in eukaryotic cells is tightly regulated in response to cell growth rate and differentiation status, as well as during the cell cycle (23, 33, 34). Cells lacking RB would be expected to have increased proliferation potential as a result of increased available biosynthetic machinery and this increased potential may contribute to unregulated growth during tumor formation.

Even though RB can globally regulate RNA polymerase III transcription, genes transcribed by this polymerase exhibit diverse promoter architectures and factor requirements for transcription. This factor diversity presents a puzzle as to how RB can recognize and regulate general RNA polymerase III transcription. The RNA polymerase III-specific factor TFIIIB, which is an essential factor required for the transcription of most RNA polymerase III-transcribed genes that contain intragenic promoter elements, is one proposed target for RB (15, 26, 35). Human TFIIIB is composed of the TATA box binding protein (TBP) and at least two additional TBP associated factors called Brf1 (18, 29) and hBdp1 (22). Together these factors form a platform at the promoter that is recognized by RNA polymerase III. Within TFIIIB, the Brf1 component is one target for RB (15, 26). Indeed, RB can disrupt interactions between TFIIIB and TFIIIC (26) and may prevent pre-initiation complex assembly at some RNA polymerase III-transcribed genes.

In contrast to most RNA polymerase III-transcribed genes, human U6 snRNA gene transcription does not require Brf1 (18) and yet is regulated by RB (13). Thus, additional

factors must be important for RB regulation of these genes. Clues as to the identity of the potential targets for RB regulation of these genes are derived from the architecture of the U6 promoter. The U6 core promoter region contains a proximal sequence element (PSE) and a TATA box, which are centered around -57 and -27 bp upstream from the start site, respectively. The PSE recruits a multi-protein complex called the small nuclear RNA activating protein complex (SNAPc) (10) that is also known as the proximal sequence element transcription factor (PTF) (19, 36). Endogenous RB associates with SNAPc both during co-immunoprecipitation and chromatographic purification of SNAPc, and RB can interact with the SNAP43 and SNAP50 subunits of SNAPc (13). Thus, SNAPc may contribute to the ability of RB to specifically target human U6 snRNA genes. Although SNAPc 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 (13).

It is interesting that human U6 snRNA genes are transcribed by RNA polymerase III, but contain extragenic promoter sequences like typical protein-encoding genes transcribed by RNA polymerase II. Thus, these genes appear to be a hybrid between genes transcribed by RNA polymerase II and III. Consequently, an investigation of human U6 snRNA gene transcription was initiated to examine the mechanism for RB repression of these unusual genes. In contrast with previous hypotheses that suggest RB disrupts pre-initiation complex assembly and RNA polymerase recruitment, the data presented herein suggests that RNA polymerase III can occupy a human U6 promoter simultaneously with RB and under conditions where *in vitro* U6 transcription is repressed. Together, these

observations suggest a novel mechanism for RB repression where RB likely interferes with critical steps in transcription that occur subsequently to RNA polymerase recruitment.

## Materials and Methods:

**Tissue culture:** Human mammary epithelieal cells (184B5) were a gift from Susan Conrad. Cells were maintained in Dulbecco's Minimum Essential Media (DMEM - Gibco) plus 10% fetal bovine serum (Gibco), 200 mM glutamine, and penicillin-streptomycin in 37°C incubator with 5% $CO_2$.

**Antibodies:** Anti-SNAP43 (CS48) and anti-TBP (SL2) antibodies have been described previously (10). Anti-hBdp1 (CS913) and α-BRF1/BRF2 (CS1043) antibodies were gifts from Nouria Hernandez (22). The anti-Galectin-3 antibody (Mac-2) was a gift from Patty Voss and John Wang (Michigan State University). Mouse anti-RB antibodies (G3-245) used for western analysis were purchased from Pharmingen. The goat anti-RB antibodies (C-15) and pre-immune control IgGs were purchased from Santa Cruz Biotechnologies. The RNA polymerase II antibodies (Covance Research Products – 8WG16) were gifts from Fransisco Herrera and Steve Triezenberg (Michigan State University)

**Chromatin immunoprecipitation:** Chromatin immunoprecipitations were performed similarly to that described previously (Braunstein et al., 1993). Human 184B5 cells were grown to 75% confluency and then crosslinked with formaldehyde for 30 minutes. After cell lysis and sonication, chromatin immunoprecipitations were performed

(approximately 1 x $10^7$ cells per immunoprecipitation) in Dilution buffer containing 400 mM NaCl using 1 ug of each antibody overnight at 4°C. Cross-links were reversed at 65°C overnight and recovered chromatin was suspended in 50 µl TE buffer. PCR analysis was performed using 5 µL of immunoprecipitated chromatin or input chromatin. The primers used for each gene are:

U6 forward – 5'-GTACAAAATACGTGACGTAGAAAG- 3',

U6 reverse – 5'-GGTGTTTCGTCCTTTCCAC- 3',

U1 forward – 5'-CACGAAGGAGTTCCCGTG-3',

U1 reverse – 5'-CCCTGCCAGGTAAGTATG-3',

U2 forward – 5'-AGGGCGTCAATAGCGCTGTGG-3',

U2 reverse – 5'-TGCGCTCGCCTTCGCGCCCGCCG-3',

GAPDH forward – 5'-AGGTCATCCCTGAGCTGAAC-3', and

GAPDH reverse – 5'-GCAATGCCAGCCCCAGCGTC-3'.

PCR products were separated by 2% agarose electrophoresis in TBE buffer and visualized using Kodak imaging software.

**Recombinant protein expression and purification:**

*Mini-SNAPc:* Recombinant SNAP43, SNAP50, and SNAP190 (1-505) were expressed individually in *E. coli* BL21 DE3 codon+ cells (Stratagene) using the vectors pGST-SNAP43, pGST-SNAP50, and pGST-SNAP190 (1-505), respectively (12). The GST-tagged proteins were purified by binding to glutathione agarose beads and cut with thrombin in HEMGT-150 buffer (20 mM HEPES pH 7.9, 5 mM EDTA, 10 mM MgCl2, 10% glycerol (v/v), 0.1 % Tween-20 (v/v), and 150 mM KCl). Mini-SNAPc was

assembled from approximately 100 μg of each protein at room temperature for 2 hours. Assembled mini-SNAPc was purified by chromatography using a Mono-Q (HR 5/5) column (Pharmacia) and eluted proteins were assayed for SNAPc activity by EMSA. The purified mini-SNAPc was estimated to contain approximately 5 ng/uL of each SNAPc protein.

*TBP:* TBP was expressed as a GST-fusion protein in *E. coli* BL21 DE3 at 37 °C in ZB-M9 media as previously described (12). GST-TBP was bound to glutathione agarose beads prior to cleavage with thrombin as described above. TBP was concentrated using a centricon YM-10 spin column (Millipore) and dialyzed against Dignam buffer D (Dignam et al., 1983).

*Brf2 and hBdp1:* Recombinant BRF2 and hBdp1 were expressed as previously described (22) using the pSBET-hBRFU and pSBET-hB' expression plasmids, respectively. Recombinant hBdp1 was affinity purified using Ni-NTA beads (Qiagen) as described (22). Subsequently, hBdp1 was dialyzed against Dignam buffer D containing 80 mM KCl prior to concentration by centrifugation using a centricon YM-30 spin column (Millipore).

*GST-RB (379-928) and GST:* Recombinant GST-RB (379-928) and GST were expressed and purified as described previously (13).

***In vitro* transcription:** In vitro transcription assays were performed essentially as described previously (9). 2 μl of HeLa cell nuclear extract was used for U6 and Ad VAI reactions, 4 μl HeLa nuclear extract was used for each AdML reaction, and 9 μl whole cell extract was used for each U1 transcription reaction. The templates used for each

reaction are pU6/Hae/RA.2, M13-VAI, M13-AdML, and pU1 for the U6, VAI, AdML, and U1 transcription reactions, respectively. Transcription reactions were supplemented with 200 and 800 ng of GST-RB (379-928) or GST as designated in the figure legend.

**Co-immunoprecipitation and GST-Pull down experiments:** Approximately 300 µL of HeLa cell nuclear extract (ca. 8 mg/mL) was incubated with 2 µg of antibodies directed against Bdp1 (CS913; (22), BRF1/BRF2 (CS1043; (22)), or 1 µg rabbit IgG overnight at 4°C as indicated in figure legend. Reactions were diluted to 1 ml in HEMGT-150 buffer and stable complexes were affinity purified by incubation with Protein-G Fast Flow sepharose beads (Upstate Biotechnology) for 4 hours at 4°C. Beads were washed 3 times in HEMGT-150 and boiled for 3 minutes in Laemmli Buffer. Bound proteins were separated by 12.5% SDS-PAGE, transferred to nitrocellulose and associated proteins were determined by western blot analysis using antibodies directed against RB (G3-245; Pharmingen). The same membrane was stripped and probed sequentially with antibodies directed against TBP (SL-2) and then Galectin 3 (Mac-2). GST pull down experiments were performed as described previously (13).

**Electrophoretic Mobility Shift Assays:**

The EMSA experiments shown in figure 2 were performed in 10 mM HEPES, pH 7.9, 30 mM Tris-HCl, pH 8.4, 60 mM KCl, 7.5 mM $MgCl_2$, 20% glycerol, 6 mM β-mercaptoethanol, 20 mM DTT and 50 mM NaF. Each binding reaction was supplemented with 0.2 µg pUC119 DNA and 0.6 µg poly dGdC-dGdC (Amersham-Pharmacia). Approximately 15 ng SNAPc, 200 ng TBP, 100 ng Brf2, and 20 ng Bdp1 were used as

indicated. Reactions were incubated on ice for 20 minutes prior to addition of radiolabeled probe. DNA binding reactions were carried out at 30°C for 30 minutes. Approximately 20 ng Bdp1 was then added to appropriate DNA binding reactions for 40 minutes at room temperature. Next, approximately 3 μg GST-RB (379-928) or GST was added to appropriate reactions for 20 minutes at room temperature. Resulting DNA-proteins complexes were separated on a 4% polyacrylamide gel in 0.5x TBE running buffer at 150V. Complexes were visualized by autoradiography and phosphoimager analysis.

**Sequential Chromatin Immunoprecipitations:**

Soluble chromatin fraction was prepared from 184B5 cells as above. Primary IPs were performed scaled up 5 fold (~ 5 x $10^7$ cells per IP) using 5 μg α-RB, α-SNAP43, α-RNAPII, and IgG. The immunoprecipitations were performed at room temperature for 1 hour and then incubated with protein G agarose for an additional hour at room temperature. After extensive washing, precipitated protein-DNA complexes were eluted in dilution buffer containing 15 mM DTT at room temperature for 30 minutes. One sixth of the recovered material was used for a second ChIP and material was processed exactly the same as chromatin immunoprecipitations described in figure 3-1.

**Cross-linked Immunoprecipitation of Repressed Promoters:**

*In vitro* transcription reactions were performed as described above with the following exceptions: transcription was carried out for 15 minutes rather than 1 hour and 0.25 μg pUC119 was added to each reaction to serve as a negative control DNA. Each of the 20

μl transcription reactions was set up in triplicate due to volume constraints and all three reactions were pooled at the end of the 15 minute transcription time. One third of this reaction was processed as described above by T1 RNase protection (Figure 4A). The remaining two-thirds was diluted to 1 ml with ChIP Dilution buffer and was cross-linked in 1% formaldehyde for 15 minutes at room temperature and quenched in 0.125 M glycine. Ten μl of each cross-linked reaction was used as starting material to perform sequential immunoprecipitations as described in the sequential chromatin immunoprecipitation section. RSP22 and USP21 primers were used in PCR reactions for analysis of the negative control pUC119 plasmid.

## Results

### Endogenous RB occupies a human U6 snRNA promoter

A key step in the direct regulation of gene expression by any transcription factor is the ability of that factor to associate with promoter regions of target genes. In order for RB to efficiently regulate U6 expression, RB may be directed to U6 promoters either by direct binding to specific DNA control elements or through recruitment by other trans-acting factors. Additional data suggest that RB can directly interact with SNAPc on promoter DNA *in vitro* rather than direct binding to DNA sequences in the U6 promoters region as determined by EMSA (Chapter 4). However, it is also important to determine whether RB association at human U6 promoters can be observed *in vivo*. To determine whether RB occupies human snRNA promoters in the cell, chromatin immunoprecipitation experiments (ChIP) were performed. Normal human mammary epithelial cells (184B5) were treated with formaldehyde to covalently cross-link proteins

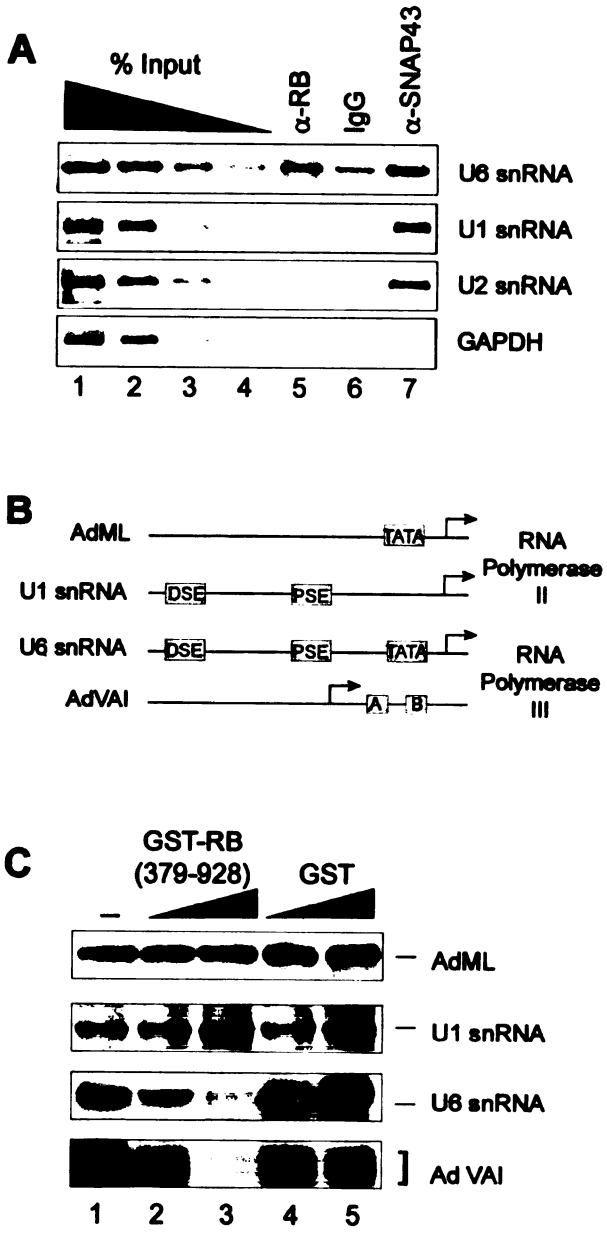
to DNA and other neighboring proteins. Subsequently, lysates were prepared and sonicated to fragment genomic DNA into segments approximately 500-800 bp in size such that there should not be more than one promoter per segment. Lysates were then subjected to immunoprecipitation using antibodies directed against SNAP43 or RB, and the resultant protein-DNA complexes were affinity purified using protein-G agarose beads. Ideally, proteins precipitated in this manner will carry along DNA segments to which they are covalently cross-linked. The recovered DNA segments were analyzed by PCR using primers specific to human U1, U2, and U6 snRNA promoters as well as primers specific to GAPDH exon 2 as a negative control. A ten-fold serial dilution of input chromatin was also analyzed for each gene of interest to serve as a standard curve, allowing estimation of relative levels of promoter DNA precipitated and to illustrate that PCR conditions for each set of primers was in the dynamic range. As shown in Figure 3-1A, U6 promoter DNA was immunoprecipitated with anti-RB (lane 5) and anti-SNAP43 antibodies (lane 7) but not with pre-immune serum (lane 6). The level of U6 promoter enrichment is significant as compared to the levels of enrichment of the negative control GAPDH exon 2 DNA. This observation indicates that RB is present at this human U6 gene promoter in these cells. In contrast, the level of RB associated with the U1 and U2 snRNA gene promoters is not significantly higher than that observed for the GAPDH negative control, indicating that RB does not associate with the promoters of the RNA polymerase II-transcribed U1 and U2 snRNA genes. Thus, RB can associate *in vivo* with the promoters of RNA polymerase III-transcribed snRNA genes but not with those transcribed by RNA polymerase II.

**Figure 3-1: RB selectively occupies U6 snRNA promoters *in vivo* and specifically represses U6 snRNA transcription *in vitro*.**

(A) Chromatin immunoprecipitation experiments were performed from human 184B5 cells using antibodies specific to RB (lane 5), SNAP43 (lane7) and IgG control (lane 6). Precipitated DNAs were analyzed by PCR for enrichment of U6 snRNA, U1 snRNA, U2 snRNA promoters and GAPDH exon 2 as a negative control. Lanes 1-4 show a 10 fold serial dilution from 10% to 0.01% of input chromatin.

(B) Schematic representation of selected RNA polymerase II and RNA polymerase III promoters used for in vitro transcription assays.

(C) In vitro transcription assays for the RNA polymerase II and RNA polymerase III-transcribed genes were performed using HeLa cell nuclear extract. 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.


A
% Input
α-RB
IgG
α-SNAP43
U6 snRNA
U1 snRNA
U2 snRNA
GAPDH
1 2 3 4 5 6 7
B
AdML
TATA
RNA Polymerase II
U1 snRNA
DSE
PSE
U6 snRNA
DSE
PSE
TATA
RNA Polymerase III
AdVAI
A
B
C
GST-RB (379-928)
GST
–
AdML
U1 snRNA
U6 snRNA
Ad VAI
1 2 3 4 5

The specific association of RB with a human U6 promoter but not with the U1 or U2 snRNA promoters raised the possibility that RB occupancy of snRNA promoters correlates with the ability of RB to repress gene expression. To determine whether RB represses expression of RNA polymerase II-transcribed snRNA genes, *in vitro* repression assays were performed. A schematic representation of the promoters tested is shown in Figure 3-1B. Two classes of RNA polymerase II-specific promoters were assayed for susceptibility to RB repression: the adenovirus major late gene (AdML) promoter and a human U1 snRNA promoter. Two classes of RNA polymerase III-specific promoters were also examined: the adenovirus VAI (Ad VAI) gene and a human U6 snRNA promoter. For these *in vitro* repression experiments, HeLa cell extracts were pre-incubated with increasing amounts of GST-RB containing amino acids 379-928, hereafter referred to as GST-RB, or GST proteins. As shown in Figure 3-1C, addition of increasing amounts of GST-RB decreased RNA polymerase III transcription from the AD VAI and U6 snRNA promoters, consistent with our previous observations (13). Repression of both human U6 and Ad VAI by GST-RB is specific to RB since the addition of equivalent amounts of GST protein did not reduce the relative amount of gene expression observed (lanes 4 and 5). In contrast, addition of GST-RB did not repress expression from either the AdML or U1 snRNA promoters. Together, these results suggest that RB effectively represses expression of these two RNA polymerase III-transcribed genes but not the RNA polymerase II-transcribed genes. These results are consistent with the observation that RB is present at a human U6 promoter but not at the U1 and U2 promoters *in vivo*.

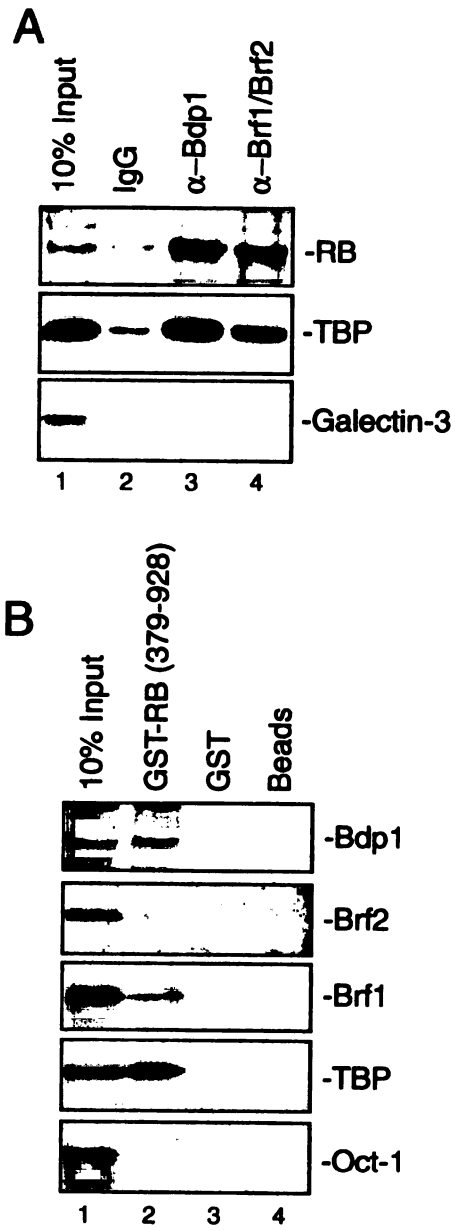
**RB promoter recruitment depends upon SNAPc and an snRNA-specific TFIIIB complex**

Our previous data suggests that RB may target SNAPc to regulate human U6 snRNA gene transcription (13). However, SNAPc is present at both RNA polymerase II- and III-transcribed snRNA genes (U1 and U6, respectively), whereas RB is present at only the U6 promoter, and RB does not repress human U1 transcription *in vitro*. Therefore, we tested whether other factors present at U6 snRNA promoters may lend specificity to RB recruitment. Unlike U1 snRNA promoters, the U6 core promoter contains a TATA box that recruits TBP and additional components of a TFIIIB complex including Bdp1 and Brf2 (2, 22, 27, 12). To determine whether RB can associate with TFIIIB, co-immunoprecipitation experiments were performed. HeLa cell nuclear extracts were incubated with antibodies specific to Bdp1 or with antibodies that recognize both Brf1 and Brf2 (22). As a negative control, co-immunoprecipitations were also performed using IgG. Immunoprecipitated protein complexes were analyzed by Western blot using antibodies specific to RB. As shown in Figure 3-2A (top panel), a significant amount of RB precipitated with Bdp1 (lane 3) as well as with Brf1/Brf2 (lane 4). The association between RB and both Bdp1 and the Brf proteins appears specific because RB was not immunoprecipitated using IgG (lane 2). This membrane was then re-probed with anti-TBP antibodies (middle panel) to determine whether TBP associates with these proteins. Indeed, significant amounts of TBP co-immunoprecipitated with Bdp1 and with Brf-1/Brf2. Finally, the membrane was stripped and re-probed with an antibody specific to Galectin-3 as a negative control (bottom panel). Galectin-3 functions in mRNA splicing (5) and is not expected to associate with TFIIIB. In contrast to the observed association of

**Figure 3-2: RB interacts with components of TFIIIB complexes**

(A) Co-immunoprecipitation assays were performed from HeLa cell extract using IgG (lane 2), αBdp1 (lane 3), or αBrf1/2 (lane 4) antibodies. Western blot analysis were performed to detect RB, TBP and Galectin-3 association.

(B) GST pull-down assays were performed using recombinant GST-RB (379-928) (lane 2), GST (lane 3), or glutathione agarose beads (lane 4). Interactions were tested with the indicated in vitro translated, $^{35}$S-methionine labeled proteins. Associated proteins were size fractionated by SDS-PAGE and visualized by autoradiography.


A
10% Input
IgG
α–Bdp1
α–Brf1/Brf2
-RB
-TBP
-Galectin-3
1 2 3 4
B
10% Input
GST-RB (379-928)
GST
Beads
-Bdp1
-Brf2
-Brf1
-TBP
-Oct-1
1 2 3 4

the TFIIIB proteins with both RB and TBP, Galectin-3 was not precipitated under any conditions. Therefore, these results suggest that endogenous RB can associate with components of TFIIIB complexes.

To determine whether RB can interact directly with individual components of TFIIIB complexes, GST-pulldown experiments were performed. As shown in Figure 3-2B, significant amounts of Bdp1, Brf-1, and TBP interacted with GST-RB. These interactions appear specific because no interaction with GST or the beads alone were observed. In contrast, neither Brf-2 nor Oct-1 interact with GST-RB. Therefore, RB can interact with all three proteins (Bdp1, Brf-1, and TBP) comprising the TFIIIB complex involved in tRNA gene expression but only two out of three proteins (Bdp1, Brf2, and TBP) that are recruited to the TATA element of human U6 snRNA genes.

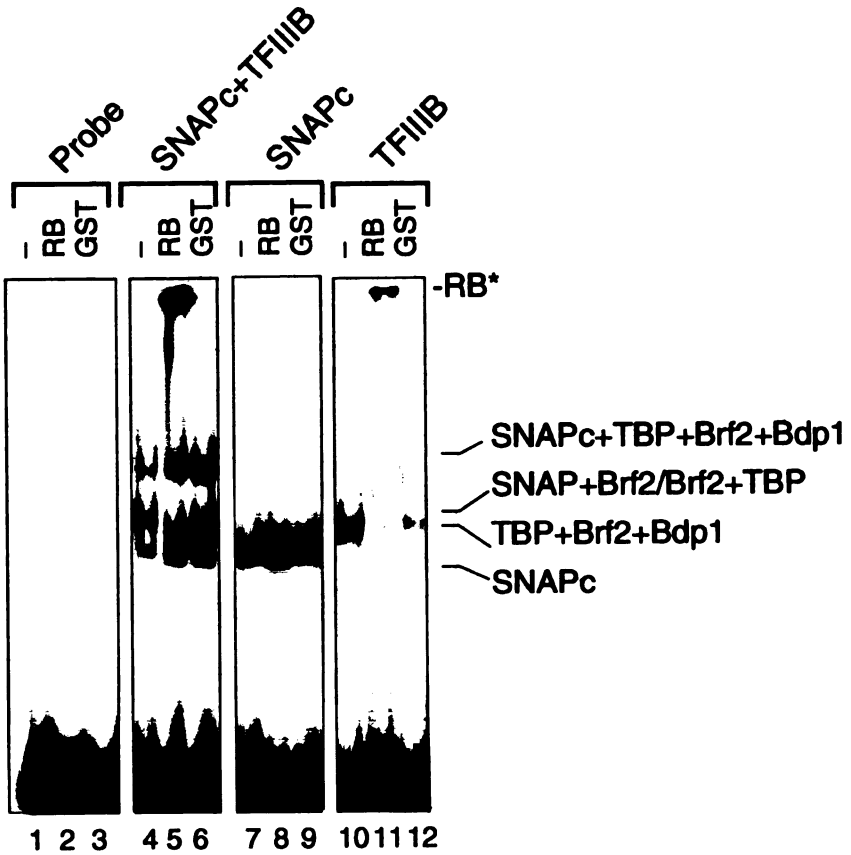
One possibility for how RB represses RNA polymerase III transcription is that RB interferes with the DNA binding of the RNA polymerase III transcription machinery (26). We and others (15, 26) have shown that RB can interact with components of TFIIIB, and therefore, we used electrophoretic mobility shift assays (EMSA) to determine whether RB affects the recruitment of TFIIIB to the U6 promoter (Figure 3-3A). Neither GST-RB nor GST were capable of binding alone to the DNA probe containing a high affinity PSE and wild type TATA box (compare lanes 2 and 3 to lane 1). However, addition of GST-RB to reactions containing recombinant mini- SNAPc plus TFIIIB (TBP, Brf2, and Bdp1) resulted in the formation of a slower migrating complex (labeled RB*, lane 5) that was not observed in the presence of comparable amounts of GST (lane 6) or in reactions

**Figure 3-3: TFIIIB acts as a selectivity factor to recruit RB specifically to RNA polymerase III transcribed snRNA promoters.**

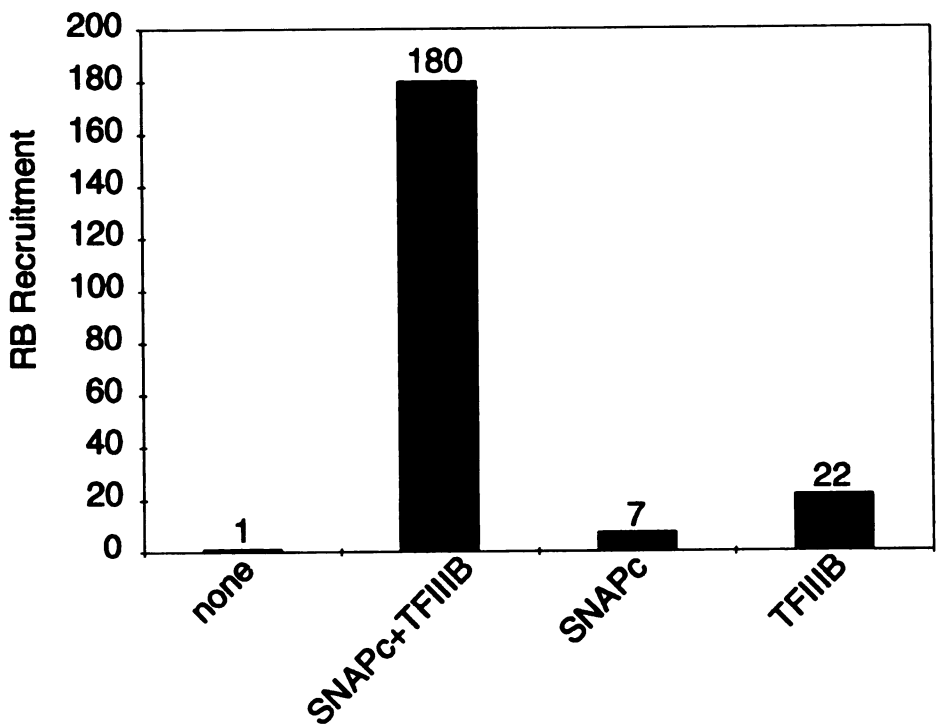
(A) Electrophoretic mobility shift assays were performed using a $^{32}P$ labeled DNA probe containing a high affinity PSE and wild type TATA. Recombinant mini-SNAPc, recombinant TFIIIB, and GST-RB (379-928) were added to DNA binding reactions as indicated. DNA-protein complexes were resolved by polyacrylamide electrophoresis and visualized by autoradiography.

(B) Phospho-imager analysis quantitation for RB recruitment. The lower mobility complex formed by the addition of RB was quantitated for net intensity value. Each value was normalized to the probe alone shift such that the figure reflects fold increase in RB recruitment.

A


Probe
SNAPc+TFIIIB
SNAPc
TFIIIB
- RB GST
- RB GST
- RB GST
- RB GST
-RB*
SNAPc+TBP+Brf2+Bdp1
SNAP+Brf2/Brf2+TBP
TBP+Brf2+Bdp1
SNAPc
1 2 3 4 5 6 7 8 9 10 11 12

B


RB Recruitment
200
180
160
140
120
100
80
60
40
20
0
180
1
7
22
none
SNAPc+TFIIIB
SNAPc
TFIIIB

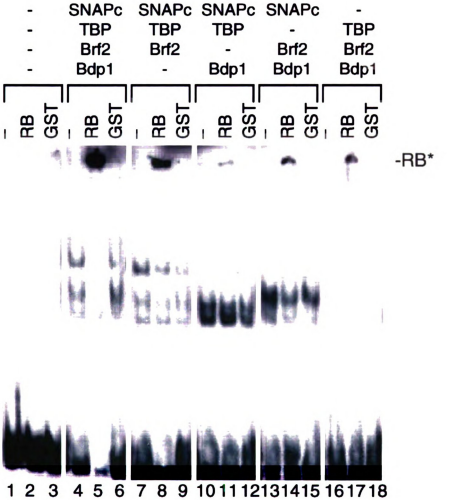
containing the general factors alone (lane 4). This suggests that RB recruitment to the promoter is dependent on the presence of TFIIIB and SNAPc. To determine the relative contribution of TFIIIB and SNAPc for RB recruitment in these assays, each of these factors was tested individually with GST-RB and quantitation of this recruitment is shown in figure 3-3B. Weak recruitment of GST-RB was observed in the presence of SNAPc alone (lane 8) or TFIIIB alone (lane 11). More specifically, GST-RB recruitment by SNAPc plus TFIIIB was 26-fold better than SNAPc alone and 8-fold better than TFIIIB alone. In these experiments, both GST-RB and GST diminished TFIIIB binding (lane 12), suggesting that TFIIIB association with this promoter is unstable. It is possible that SNAPc stabilizes TFIIIB on DNA while TFIIIB provides stronger RB contacts. Altogether, these results indicate that efficient RB recruitment depends upon multiple interactions between RB and the general transcription machinery. More importantly, the presence of RB does not prevent binding of the general transcription machinery to the promoter.

To determine the contribution of each of the individual factors found at U6 snRNA promoters to RB recruitment, EMSA experiments were performed in which a single component was left out of the DNA binding reaction. These single-elimination reactions were then tested for the ability to recruit RB to a DNA probe containing a wild type PSE and TATA elements similar to those found at U6 promoters. A representative result from these EMSA experiments is shown in Figure 3-4A. The amount of RB recruitment was measured using phospho-imager analysis and is illustrated in the graph shown in Figure

**Figure 3-4: Contribution of individual components of TFIIIB to RB recruitment to a U6 snRNA promoter:**

(A) EMSA experiments were performed to determine which components of TFIIIB and SNAPc are important for RB recruitment to a U6 snRNA promoter. DNA binding reactions similar to those performed in the previous figure were assembled minus one individual component as shown above. The amount of recruitment for each combination was then determined by phospho-imager analysis.

(B) Graphical representation of the amount of RB recruitment observed for each combination of SNAPc and TFIIIB components. The lower mobility complex formed by the addition of RB was quantitated for net intensity value. Each value was normalized to the amount of RB recruited when all proteins are present. This value was set to 100% such that the recruitment of RB is expressed as a percentage.


A
-
-
-
-
SNAPc
TBP
Brf2
Bdp1
SNAPc
TBP
Brf2
-
SNAPc
TBP
-
Bdp1
SNAPc
-
Brf2
Bdp1
-
TBP
Brf2
Bdp1
-
RB
GST
-RB*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
RB Recruitment
100
50
0
Probe
All proteins
- Bdp1
- Brf2
- TBP
- SNAPc

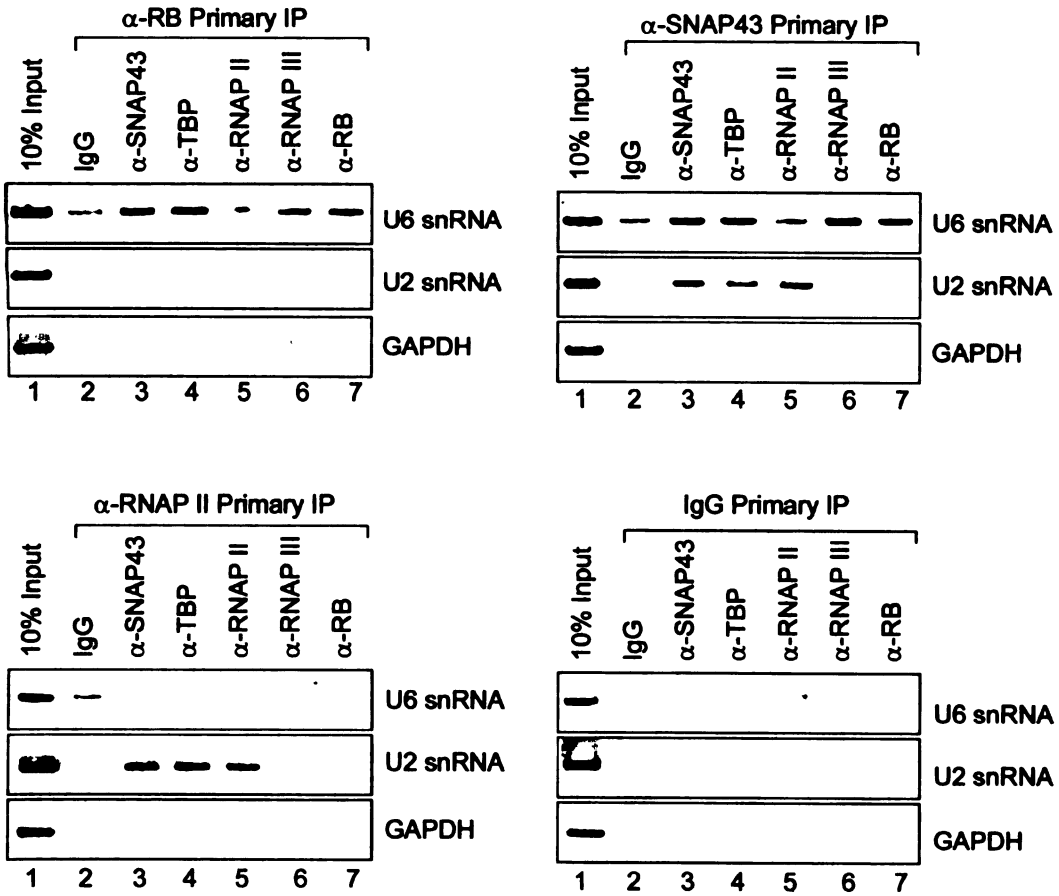
3-4B. The amount of RB recruitment when all components of SNAPc and TFIIIB are present (Figure 3-4A lanes 4-6) was considered 100%. In DNA binding reactions missing Bdp1 (Figure 3-4A lanes 7-9), the amount of RB recruitment was decreased to 40% (2.5 fold decrease) of the amount of RB recruitment observed when all of the factors (SNAPc, TBP, Bdp1, and Brf2) are present (Figure 3-4A lanes 4-6). Interestingly, the absence of Brf2 (Figure 3-4A lanes 10-12) results in a drastic drop in RB recruitment. Without Brf2, the amount of RB recruitment observed is 9% (11 fold decrease) of that seen when all factors are present. This result is surprising since there was no detectible association between RB and Brf2 in the GST-pull down experiment shown above. Leaving TBP out of the DNA binding reactions (Figure 3-4A lanes 13-15) results in 15% (7 fold decrease) RB recruitment as compared to the amount of RB recruitment seen with all SNAPc and TFIIIB proteins. Lanes 16-18 show the effects of leaving SNAPc out of the DNA binding reactions. Longer exposure of these experiments show that TFIIIB can bind to the DNA probe by itself although much less efficiently than when SNAPc is also present. The amount of RB recruited to the U6 promoter *in vitro* in the absence of SNAPc is approximately 15% (7 fold decrease) of the amount of RB recruitment observed when all components of SNAPc and TFIIIB are present. Overall these experiments indicate that while TFIIIB is important for RB recruitment to the U6 promoter, it is not sufficient to do so alone. Therefore, both SNAPc and TFIIIB play important cooperative roles in the recruitment of RB to human U6 snRNA promoters.

**RB and RNA polymerase III co-occupy a human U6 promoter in vivo and in vitro**

The data above suggest that rather than preventing the binding of TFIIIB to the snRNA gene promoter, RB is capable of co-occupying the same U6 promoter with TFIIIB and SNAPc. To establish whether these factors co-occupy the same promoter and whether RB precludes binding of RNA polymerase III to the U6 snRNA gene promoter *in vivo*, sequential chromatin immunoprecipitations were performed (Figure 3-5). Soluble chromatin fractions isolated from cells were subjected to a primary α-RB immunoprecipitation (top left panel) 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. As expected, secondary immunoprecipitations performed using α-RB antibodies (lane 7) significantly enriched U6 promoter DNA compared to reactions performed with IgG (lane 1) or RNA polymerase II (lane 5). Secondary immunoprecipitations performed using α-SNAP43 (lane 3) and α-TBP (lane 4) antibodies also enriched U6 promoter DNA, consistent with results in the above EMSA (see Figure 2C). Interestingly, U6 promoter DNA was also enriched in the α-RNA polymerase III immunoprecipitation. None of the secondary precipitations significantly enriched for U2 snRNA promoter DNA or GADPH exon 2 DNA, suggesting that the observed association between RB and RNA polymerase III with this human U6 promoter is specific.

To confirm these observations, similar sequential ChIP assays were performed using α-SNAP43 antibodies in the first round of immunoprecipitation (top right panel) to enrich for both a U6 snRNA promoter, which directs RNA polymerase III transcription, and U2

**Figure 3-5: RB co-occupies the same endogenous U6 snRNA promoter as SNAPc, TFIIIB and RNA polymerase III.**
Sequential chromatin immunoprecipitations were performed from human 184B5 cells to determine RB co-occupancy with other factors. Precipitated material was recovered after the second immunoprecipitation and analyzed by PCR for enrichment of U6 and U2 promoter DNA or GAPDH exon 2 as a negative control. Lane 1 shows 10% input chromatin.


α-RB Primary IP
10% Input
IgG
α-SNAP43
α-TBP
α-RNAP II
α-RNAP III
α-RB
U6 snRNA
U2 snRNA
GAPDH
1 2 3 4 5 6 7
α-SNAP43 Primary IP
10% Input
IgG
α-SNAP43
α-TBP
α-RNAP II
α-RNAP III
α-RB
U6 snRNA
U2 snRNA
GAPDH
1 2 3 4 5 6 7
α-RNAP II Primary IP
10% Input
IgG
α-SNAP43
α-TBP
α-RNAP II
α-RNAP III
α-RB
U6 snRNA
U2 snRNA
GAPDH
1 2 3 4 5 6 7
IgG Primary IP
10% Input
IgG
α-SNAP43
α-TBP
α-RNAP II
α-RNAP III
α-RB
U6 snRNA
U2 snRNA
GAPDH
1 2 3 4 5 6 7

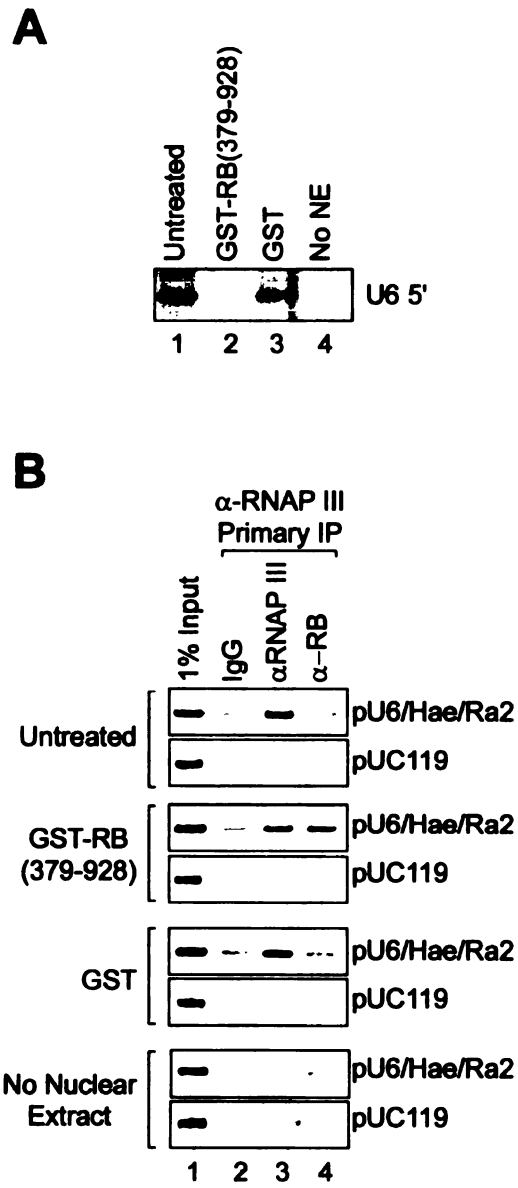
snRNA promoters, which direct RNA polymerase II transcription. As expected, α-RNA polymerase III immunoprecipitation enriched for U6 but not U2 snRNA promoters whereas α-RNA polymerase II immunoprecipitation enriched for U2 but not U6 snRNA promoters. Furthermore, secondary immunoprecipitations with either α-SNAP43 or α-TBP antibodies enriched 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 (Henry et al., 1998; Henry et al., 1995). Importantly, secondary immunoprecipitations with α-RB antibodies enriched for U6 but not U2 snRNA promoter sequences. This result confirms the original observation that RB and SNAPc can reside simultaneously at the same U6 gene. In contrast, none of the secondary immunoprecipitations enriched U6 DNA when primary immunoprecipitations were performed using either α-RNA polymerase II or IgG antibodies (bottom left and bottom right panel, respectively). RNA polymerase II was found simultaneously with SNAP43 and TBP, but not with RB, on U2 promoters. Taken together, these results suggest that RB does not prevent binding of SNAPc, TFIIIB, or RNA polymerase III to the U6 snRNA gene promoter. Rather, RB can co-occupy the same U6 snRNA promoter as SNAPc (α-SNAP43 IP), TFIIIB (α-TBP IP), and RNA polymerase III (α-RNAPIII IP). This result is unexpected because previous models suggested that RB functions to prevent RNA polymerase access to the promoter.

To directly test whether RB repression of U6 transcription occurs simultaneously with the association of the RNA polymerase III transcription machinery, *in vitro* U6 transcription reactions were performed with HeLa cell nuclear extracts that were either left untreated

**Figure 3-6: RB and RNA polymerase III co-occupy the same repressed U6 snRNA promoter *in vitro*.**

**(A)** In vitro transcription reactions were performed using HeLa cell extracts. Lane 1 shows untreated extract and lane 4 shows no addition of nuclear extract. 500 ng GST-RB(379-928) or GST was added to lanes 2 and 3 respectively.

**(B)** Sequential crosslinked immunoprecipitations were performed from the transcription reactions from part A using α-RNA polymerase III antibodies for the primary immunoprecipitation and IgG (lane 2), α-RNA polymerase III (lane 3), and α-RB (lane 4) antibodies for the secondary immunoprecipitation. PCR analysis was performed on the recovered DNA using primers specific to the U6/Hae/RA.2 reporter or pUC119 as a negative control.


A
Untreated
GST-RB(379-928)
GST
No NE
U6 5'
1 2 3 4
B
α-RNAP III
Primary IP
1% Input
IgG
αRNAP III
α-RB
Untreated
GST-RB
(379-928)
GST
No Nuclear
Extract
pU6/Hae/Ra2
pUC119
1 2 3 4

or treated with GST-RB or GST. Portions of these reactions 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 α-RNA polymerase III immunoprecipitation. Secondary immunoprecipitations were then performed using IgG, α-RNA polymerase III, or α-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 reactions. Figure 3-6A shows the level of U6 gene transcription from samples containing HeLa cell nuclear extract (lane1), extract plus GST-RB (lane 2), or extract plus GST (lane 3). Transcription from samples lacking any nuclear extract is shown in lane 4. As expected, GST-RB effectively repressed U6 transcription as compared to the effect of GST. The result of the sequential immunoprecipitations from these transcription reactions is shown in Figure 3-6B. RNA polymerase III was detected at the U6 promoter-containing plasmid in transcription reactions performed with untreated nuclear extract (top panel) but not in reactions to which no extract was added (bottom panel). Significantly, RNA polymerase III was also detected at this promoter DNA in transcription reactions that were completely repressed by GST-RB (second panel). In these GST-RB repressed transcription assays, RB also associated with the U6 promoter but not with the irrelevant plasmid. The pattern of factor association with the U6 promoter plasmid in the GST-treated samples (third panel) was similar to that observed with samples containing the untreated nuclear extract. Together, these observations indicate that under repressed conditions, RB does not displace RNA polymerase III, but rather RB and RNA polymerase III can co-occupy the same repressed promoter.

**Discussion**

To better understand how RB acts to prevent tumor progression it is important to understand how RB regulates gene expression. Previous studies illustrate that RB can repress transcription by RNA polymerase I (3), II (31), and III (35) suggesting a role for RB in the regulation of a diverse set of genes. This versatility allows RB to participate in many processes critical to normal cell function including cell cycle progression, apoptosis, DNA replication, and differentiation. RB's contribution to each of these processes may play a role in the suppression of tumor formation.

Additionally, RB may regulate cell growth by the regulation of RNA polymerase III gene expression. The non-translated genes transcribed by RNA polymerase III provide metabolic building blocks that increase the biosynthetic capacity of the cell. An increase in a cell's biosynthetic capacity elevates the cell's growth potential, which unchecked, could lead to tumorigenesis. Bypass of RB regulation of non-translated RNAs could represent a significant obstacle to overcome during the progression of tumor formation.

RB can repress transcription by RNA polymerase III both *in vivo* (35) and *in vitro* (15, 35) including transcription of U6 snRNA genes (13). The data herein illustrate that RB can associate with the U6 snRNA promoter, a step that may be important for subsequent repression of transcription. The chromatin immunoprecipitation assays clearly show the RB protein occupying U6 snRNA promoter *in vivo* suggesting that RB does in fact target this gene for transcriptional regulation. In contrast, RB is not present at the U1 and U2

RNA polymerase II transcribed snRNA genes. Interestingly, RB occupancy of snRNA promoters *in vivo* correlates with the ability to repress transcription as demonstrated by *in vitro* transcription experiments. Whereas RB can repress U6 snRNA transcription by RNA polymerase III, it is unable to repress transcription by RNA polymerase II initiated from a U1 snRNA promoter. Indeed, the data presented herein, illustrate that RB preferentially targets and represses RNA polymerase III transcribed snRNA genes.

One potential mechanism for the recruitment of RB to target promoters is through interaction with general factors required for transcription. In the case of the U6 snRNA gene both SNAPc and TFIIIB could be potential recruitment factors for RB. Previous studies demonstrated that RB can associate with SNAPc (13). However, if SNAPc alone were capable of mediating RB repression of U6 snRNA genes, then it should also direct RB repression activities to other genes at which SNAPc is present such as the U1 or U2 snRNA genes. Because RB was not detected at these genes, it is possible that SNAPc is not the sole determining factor for selective RB targeting RNA polymerase III transcribed snRNA genes. One obvious difference between snRNA gene transcribed by RNA polymerase II and III is the presence of a TATA element in core promoter region of RNAP III type snRNA genes. One possibility is that proteins or protein complexes recruited to this distinguishing promoter element are candidate selectivity factors directing RB solely to RNAP III transcribed snRNA genes rather than all snRNA genes.

Indeed, TFIIIB is one candidate selectivity factor that binds to the TATA elements of U6 snRNA promoters. The results in Figure 3-2 demonstrate that TFIIIB associates with endogenous RB by co-immunoprecipitation and that individual components of TFIIIB (Bdp1 and TBP) interact with RB in GST-pulldown experiments. Thus, an alternative TFIIIB complex consisting of TBP, Brf2, and Bdp1 that associates with U6 snRNA promoters via the TATA element may be important for ensuring efficient RB recruitment to human U6 snRNA genes. In EMSA experiments, TFIIIB, like SNAPc, can recruit RB to U6 promoter DNA. RB recruitment is significantly enhanced in the presence of SNAPc, suggesting cooperative interactions between TFIIIB and SNAPc direct RB to the U6 snRNA promoter. Additionally, all components of SNAPc and TFIIIB are necessary for efficient recruitment of RB.

The results of the EMSA experiments indicate that RB can interact with SNAPc and TFIIIB on a U6 snRNA DNA probe rather than simply displacing these factors to repress transcription. This result is also supported by the promoter occupancy studies of the U6 snRNA gene using sequential chromatin immunoprecipitation analysis. Sequential chromatin immunoprecipitation analysis revealed that RB, SNAPc and TFIIIB are present at the same U6 snRNA promoter *in vivo* again indicating that association of these basal factors is not interupted by RB. The unique surprising result from these sequential ChIP experiments is that RB and RNA polymerase III occupy the same U6 snRNA promoter. Further *in vitro* analysis also indicates the RB and RNA polymerase III can occupy the same repressed U6 snRNA promoter.

The results presented herein are inconsistent with the previous model in which RB represses transcription by blocking the binding of RNA polymerase III to cognate promoters. Sutcliffe and co-workers (26) proposed a mechanism of RNA polymerase III transcriptional repression in which RB sequesters required factors. RB was proposed to bind to TFIIIB interrupting important interactions with TFIIIC. Since interaction with TFIIIC is critical for recruitment to tRNA genes, RB effectively sequesters TFIIIB away from the promoter thereby not allowing pre-initiation complex formation to occur. Similarly, RB was proposed to repress some RNA polymerase II transcribed genes by targeting the TFIID-TFIIA complex formation step to prevent PIC formation for RNA polymerase II (21). Since RB and RNA polymerase III occupy the same promoter under repressed conditions, RB is clearly not using a commonly accepted mechanism, disrupting pre-initiation complex formation, to repress transcription at the U6 snRNA promoter.

Again, the results described herein disagree with accepted mechanisms of RB repression. Instead, we propose that, at least for human U6 transcription, RB impedes critical steps in RNA polymerase III transcription that occur subsequently to polymerase recruitment to the promoter. For example, RB may create a physical barrier in the chromatin at the start site of transcription that would block the progression of the polymerase. Potentially RB could recruit histone modification activities that would modify local chromatin structure preventing transcription by RNA polymerase III. However, several studies have indicated that HDAC mediated RB repression is promoter specific and may not occur at all RB regulated promoters (16). HDAC mediated repression of U6 genes may be

unlikely as data from other groups suggest that RB can repress expression of U6 gene in an HDAC independent fashion (26).

Another method by with RB could use to create a physical barrier in chromatin to prevent U6 gene expression is through the recruitment of ATP-dependent chromatin remodeling complexes such as SWI-SNF. Previous studies have demonstrated interaction between RB and SWI-SNF complexes and that RB required SWI-SNF to repress transcription initiated from some promoters (28, 37). *In vivo*, the U6 promoter contains a positioned nucleosome between the DSE and PSE that is vital to proper gene expression (38). Recruitement of chromatin remodeling factors by RB to a U6 promoter could potentially mis-align this nucleosome and thus effectively abolish U6 gene expression.

Additionally, RB may hinder steps that allow for promoter escape and subsequent transcription of the U6 snRNA gene. Furthermore, RB could inhibit steps that convert an initiating polymerase to an elongating polymerase such as phosphorylation of the enzyme. Perhaps RB interferes with elongation resulting in a stalled complex in the middle of the U6 snRNA gene. Whichever case may apply, it represents a novel mechanism for RB regulation of gene expression.

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# Chapter 4

## The intact Large A/B pocket domain and C-terminus of RB is required for efficient repression of RNA polymerase III transcription.


## Abstract

The Retinoblastoma tumor suppressor protein plays a central role in many cellular processes including cell cycle regulation, apoptosis, differentiation, and growth control. This protein contributes to growth control through its regulation of RNA polymerase III transcription. Here we dissect the region of RB required for repression of transcription and interaction with basal RNA polymerase III transcription factors. The region of RB between amino acids 379-870 is sufficient to repress both U6 snRNA and Ad VAI transcription since the ability to regulate transcription is lost in a RB mutant protein containing only amino acids 379-772. Interestingly this phenomenon correlates with the ability to interact with SNAP43, a component of the SNAP complex required for snRNA expression.


## Introduction

RB is an important regulator of eukaryotic cellular growth. Its ability to perform this function can partially be attributed to regulation of RNA polymerase III activity (8, 18, 19). RB contains 928 amino acids and can be divided into at least three regions: the N-terminal region from amino acids 1-378, the A/B region from 393-772, and the C region from 768-869 (16, 17). Most functions ascribed to RB including tumor suppressor

activity and interactions with regulatory target proteins require either the A/B and/or C regions. When re-introduced into RB -/- mice, the region of RB between amino acids 379-928 was sufficient to rescue the phenotype and allow mice to develop normally (21). This same region of RB also appears to be the minimal region necessary for effective tumor suppression (21). Interestingly, the regions required for tumor suppression and transcription repression are different. Previous data has shown that the region from 379-792 was sufficient to repress transcription from RNA polymerase II transcribed reporter constructs (3). Additionally, the region of RB from amino acids 379-772 was shown to be sufficient to recruit histone deacetylase activity to repress RNA polymerase II transcription (11).

RB interacts with several components of RNA polymerase III basal machinery. Both SNAP43 and SNAP50 of the snRNA activating protein complex (SNAPc) interact strongly with RB (7). RB also interacts with TATA binding protein, TBP, (9 and HAH unpublished data) and B double prime, Bdp1, which are components of all TFIIIB complexes. The TFIIB related factor 1, Brf1, a component of TFIIIB complexes involved in tRNA transcription, also weakly interacts with RB (9 and HAH unpublished data). RB does not appear to interact with RNA polymerase III itself (15 and HAH unpublished results). Additionally, RB has been shown to interact with TFIIIC (4, 15), a basal factor required for tRNA and 5s rRNA expression, although this complex will not be studied further in this paper. Further characterization of RB interaction with basal factors and further insight into the mechanisms RB uses to regulate gene expression is essential in understanding the function of this critical tumor suppressor protein function.

## Materials and Methods

**Tissue culture:** Human mammary epithelieal cells (184B5) were a gift from Susan Conrad. Cells were maintained in Dulbecco's Minimum Essential Media (DMEM - Gibco) plus 10% Fetal Bovine serum (Gibco), 200 mM Glutamine, and penicillin-streptomycin in 37°C incubator with 5% $CO_2$.

**Recombinant protein expression and purification:**

*Mini-SNAPc:* Plasmids encoding GST-SNAP43 Ex-XB, pET-GST-SNAP50, and pET-GST-SNAP190(1-505) were transformed into *E. coli* BL21 DE3 codon + (Stratagene) and grown at 37°C in ZBM9 media until OD at 600 nm is 0.5. Temperature was lowered to 16°C and protein expression was induced with 1 mM IPTG for 20 hours. Pelleted bacteria were resuspended in HEMGT-150 (20 mM Hepes pH 7.9; 0.5 mM EDTA; 10 mM $MgCl_2$; 10% glycerol; 0.1% Tween-20) containing protease inhibitors (0.1 mM PMSF, 1 mM sodium bis-sulfate, 1 mM benzamidine, 1 μM pepstatin A) and 1 mM DTT and sonicated using a Branson sonifier 4 times for 30 pulses output=6 duty 60%. Debris was pelleted at 13,000 RPM at 4°C for 30 minutes. Soluble protein was bound to glutathione sepharose (Pharmacia) overnight at 4°C and washed 4 times in HEMGT-150 plus protease inhibitors and twice in HEMGT-150 without protease inhibitors. Proteins were cleaved from the beads using 20 units thrombin (Sigma) on ice for 1.5 hours vortexing every 15 minutes. SNAP50 and SNAP190(1-505) were collected as fractions in HEMGT-150. Appropriated fractions were pooled and dialyzed against Dignam buffer D80 (5). SNAP43 was collected in step wise salt gradient (150 to 1000 mM KCL in HEMGT buffer) and appropriate fractions pooled. Mini-SNAPc was assembled from

approximately 100 μg of each protein at room temperature for 2 hours. The assembly reaction was diluted such that the final salt concentration was less than 80 mM KCl and the final glycerol concentration was less than 5%. The diluted assembly reaction was allowed to equilibrilate on ice for 1 hour. Assembled mini-SNAPc was then loaded onto a 1 ml Mono-Q column. Bound proteins were eluted from the column in 0.5 ml fractions in a 0 to 500 mM salt gradient in Dignam buffer D80 (5% glycerol). Fractions were adjusted to 20% glycerol and assayed for SNAPc activity by SDS-PAGE and EMSA. The peak of SNAPc activity corresponded to fractions 20-24 (~380-420 mM KCl). Recombinant RB proteins were expressed and purified as before (7) with the exception that RB mutants were grown at a temperature of 30°C to an O.D. of 0.6 and induced at 16°C for 20 hours.

**Chromatin immunoprecipitation:** Chromatin immunoprecipitations were performed similarly to that described previously (2). Human 184B5 cells were grown to 75% confluency and then crosslinked with formaldehyde for 30 minutes. After cell lysis and sonication, chromatin immunoprecipitations were performed (approximately 1 x $10^7$ cells per immunoprecipitation) in Dilution buffer containing 400 mM NaCl using 1 ug of each antibody overnight at 4°C. Cross-links were reversed at 65°C overnight and recovered chromatin was suspended in 50 μl TE buffer. PCR analysis was performed using 5 μL of immunoprecipitated chromatin or input chromatin. The primers used for each gene are:

U6 forward – 5'-GTACAAAATACGTGACGTAGAAAG-3',

U6 reverse – 5'-GGTGTTTCGTCCTTTCCAC-3',

U1 forward – 5'-CACGAAGGAGTTCCCGTG-3',

U1 reverse – 5'-CCCTGCCAGGTAAGTATG-3',

U2 forward – 5'-AGGGCGTCAATAGCGCTGTGG-3',

U2 reverse – 5'-TGCGCTCGCCTTCGCGCCCGCCG-3',

GAPDH forward – 5'-AGGTCATCCCTGAGCTGAAC-3',

GAPDH reverse – 5'-GCAATGCCAGCCCCAGCGTC-3',

tRNA-lys forward – 5'-GGTTTCCCTCAAGGAGGGGG-3',

tRNA-lys reverse – 5'-GCCCGGATAGCTCAGTCGGTAG-3'

PCR products were separated by 2% TBE-agarose electrophoresis and visualized using Kodak imaging software.

***In vitro* transcription:** In vitro transcription assays were performed essentially as described previously (6). 2 µl of HeLa cell nuclear extract (~15 mg/ml) was used for the transcription reactions using 0.25 µg pU6/Hae/RA.2 or M13-VAI reporter plasmids. Transcription reactions were supplemented with 250 and 1000 ng of GST-RB (379-928), GST-RB (379-870), GST-RB (379-772), GST-RB (379-577) or GST as designated in the figure legend.

**Cross-linked immunoprecipitation of repressed promoters:** *In vitro* transcription reactions were performed as described above with the following exceptions. Three-quarters of this reaction was processed as described above by T1 RNase protection (Figure 4A). The remaining fourth was diluted to 150 µl with ChIP Dilution buffer (as described in chapter 3) and was cross-linked in 1% formaldehyde for 15 minutes at room temperature and quenched in 0.125 M glycine. Ten µl of each cross-linked reaction was

used as starting material to perform immunoprecipitations as described in the immunoprecipitation section in chapter 3 using 10 ml of glutathione sepharose beads (Pharmacia). Recovered DNA was analyzed using the following primers:

U6 forward – 5'-GTACAAAATACGTGACGTAGAAAG-3',

U6 reverse – 5'-GGTGTTTCGTCCTTTCCAC-3',

VAI forward – 5'-TCCGTGGTCTGGTGG-3',

VAI reverse – 5'-CGGGGTTCGAACCCGG-3'.

**Electrophoretic Mobility Shift Assays:**

DNA binding reactions were performed in 60 mM KCl, 20 mM HEPES pH 7.9, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 0.5 μg poly(dI-dC), and 0.5μg pUC119 plasmid. Approximately 25 ng of SNAPc were incubated with increasing amount of GST-RB (379-928) or GST at room temperature for 20 minutes. 5000 CPM DNA probe (prepared as described previously (6)) containing with a high affinity PSE or mutant PSE was added to each reaction and incubated for 30 minutes at room temperature. The resulting complexes were separated by 5% Tris-Glycine polyacrylamide containing 5% glycerol and visualized by autoradiography. For the EMSA performed in figure 2, 1 μg goat α-RB (Santa Cruz) antibodies or goat IgG was added to the binding reactions at the times indicated.

**GST-Pulldown:** GST-pulldowns were performed as described previously (7).

## Results

### Characterization of the RNA polymerase III repression domain in RB

In order to determine the minimal region of RB required to regulate RNA polymerase III transcription, C-terminal truncation mutagenesis was performed on RB separating the protein into its pocket regions (Figure 4-1A) based on the structural domains observed in the RB crystal structure (10). These mutantions were created by Ms. Liping Gu in the Henry lab. Truncated RB proteins were expressed as GST fusion proteins in *E. coli* and affinity purified using gluthione-sepharose resin. Eluted proteins were desalted and concentrated using spin columns. Each RB protein was purified to near homogeneity (>90%) as shown by SDS-PAGE analysis and subsequent coomassie blue staining (Figure 4-1B).

In vitro repression assays were used to test the ability of the RB truncation protein mutants to repress RNA polymerase III transcription. The two representative RNA polymerase III promoters examined, U6 snRNA and Adenovirus VAI, are diagramed in Figure 4-1C. For these experiments, HeLa nuclear extracts were pre-incubated with GST-RB, the RB truncation mutants, or GST as a negative control. Transcription was initiated by the addition of template and nucleotides. Correctly initiated transcripts were visualized by autoradiography. As shown in Figure 4-1D, addition of increasing amounts of GST-RB (379-928) (lanes 2-3) significantly decreases the amount of transcription correctly initiated from the U6 snRNA or VAI promoters as compared to untreated extract (lane 1)

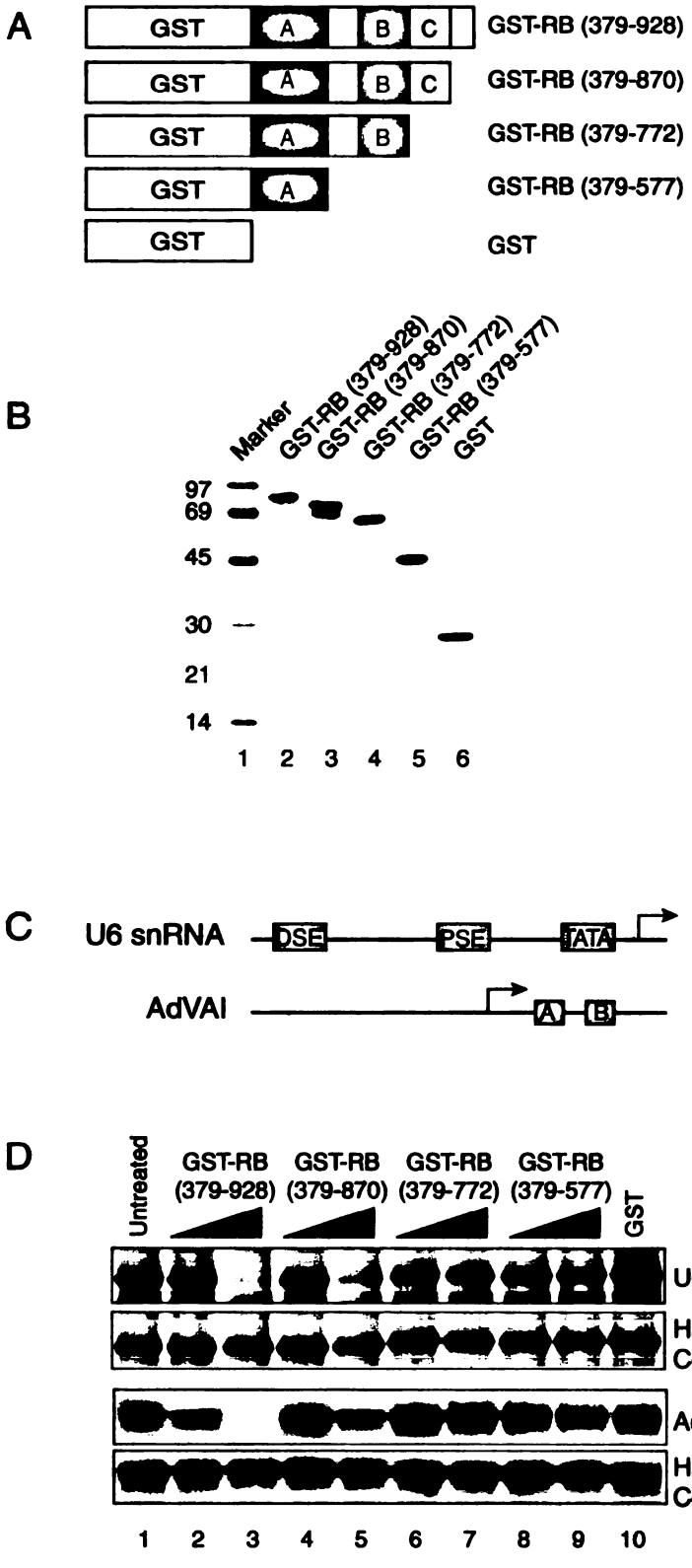
**Figure 4-1: Amino acids 379-870 of RB contain the minimal region necessary to repress RNA polymerase III transcription.**

(A) Schematic representation of the RB truncation mutants used in these experiments.

(B) Analysis of GST-RB truncation mutants and GST proteins used in transcription reactions. 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 using glutathione agarose beads and competitive elution with glutathione. After dialysis against Dignam buffer D proteins were separated by SDS-PAGE and visualized by staining with Coomassie blue. Lane 1 contains a protein size standard.

(C) Schematic representation of the adenovirus VAI and human U6 snRNA promoters.

(D) GST-RB (379-928) and GST-RB (379-870) repress adenovirus U6 snRNA and VAI transcription by RNA polymerase III. Approximately 2 μL of HeLa cell nuclear extract (approx. 7.5 μg/μL) was incubated with 250 and 1000 ng of GST-RB (379-928) (lanes 2-3), GST-RB (379-870) (lanes 4-5), GST-RB (379-772) (lanes 6-7), GST-RB (379-577) (lanes 8-9) or GST protein (lanes 10-11) at 30°C for 30 minutes. Lane 1 shows the level of transcription with the untreated extract. Correctly initiated transcripts from the U6 promoter (labeled U6 5') were detected by RNase T1 protection essentially as described in Chapters 2 and 3 (20). *In vitro* transcription of the Ad VAI gene (bottom) was then initiated by addition of template, cold rNTPs, [$\alpha^{32}$P]CTP, and transcription buffer. Sample handling was monitored by a non-specific RNA handling control transcript (bottom panels).


A
GST
A
B
C
GST-RB (379-928)
GST-RB (379-870)
GST-RB (379-772)
GST-RB (379-577)
GST
B
Marker
GST-RB (379-928)
GST-RB (379-870)
GST-RB (379-772)
GST-RB (379-577)
GST
97
69
45
30
21
14
1 2 3 4 5 6
C
U6 snRNA
DSE
PSE
TATA
AdVAI
A
B
D
Untreated
GST-RB (379-928)
GST-RB (379-870)
GST-RB (379-772)
GST-RB (379-577)
GST
U6 5'
Handling Control
Ad VAI
Handling Control
1 2 3 4 5 6 7 8 9 10

and extract treated with a molar equivalent of GST (lane 10). Removal of the extreme C-terminus (GST-RB (379-870)) results in lowered, but still significant repression (lanes 4-5) of U6 snRNA and VAI gene expression. However, deletion of the C-pocket (GST-RB (379-772)) results in abolition of RB's repressor ability (lanes 6-7) even though A/B pocket integrity should still be maintained in this mutant. In fact, genes transcribed by RNA polymerase II have been shown to be regulated by RB truncation mutants containing only amino acids 379-772 (3, 11). Similarly, the A pocket alone (GST-RB (379-577)) was unable to repress U6 or VAI gene expression. Together, these results suggest that the A/B pocket as well as the C region of RB is necessary for repression of RNA polymerase III repression. Interestingly, this corresponds to the region of RB that is required for growth suppression.

**RB occupancy of RNA polymerase III promoters *in vitro* and *in vivo***

To ascertain whether the ability to repress transcription correlates with the ability to occupy a target RNA polymerase III transcribed promoter, cross-linked immuno-precipitations were performed from the transcription reactions used in the previous figure. *In vitro* U6 transcription reactions were performed with HeLa cell nuclear extracts that were either left untreated or treated with GST-RB mutants or GST. Portions of these reactions were analyzed by RNase protection assays for full-length transcripts driven from the U6 promoter (see above) while the remainder was subjected to formaldehyde cross-linking followed by affinity purification, using glutathione sepharose to isolate the RB mutant proteins. The precipitates were then analyzed for the presence of the U6 reporter construct (pU6/Hae/RA.2) or the Ad VAI reporter construct (M13-Ad VAI)

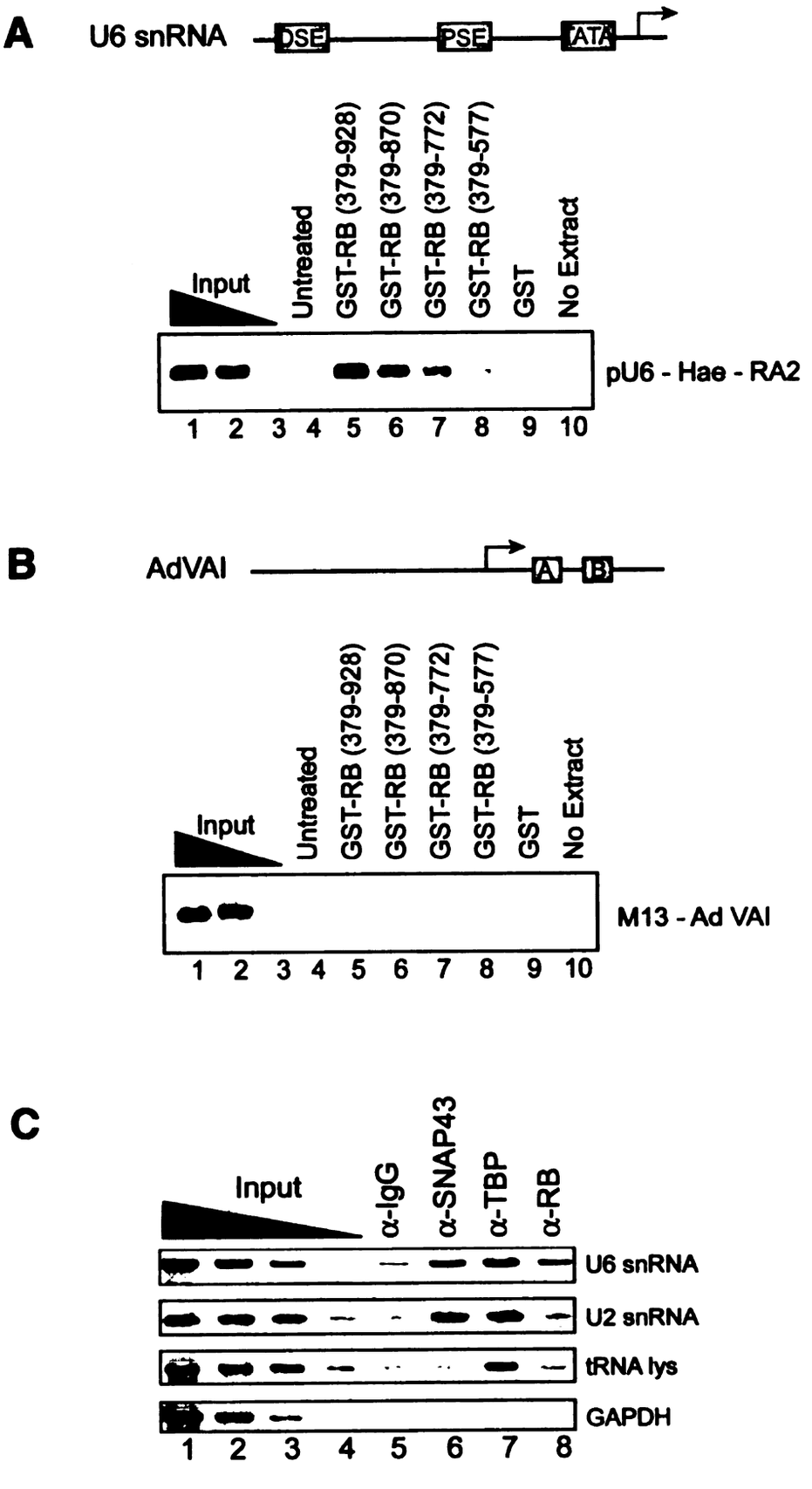
**Figure 4-2: RB occupancy of RNA polymerase III transcribed promoters *in vitro* and *in vivo***

(A) GST-pull down experiments were performed from the crosslinked U6 5' transcription reactions in figure 4-1D. Associated plasmid DNA was detected by PCR using primers specific to U6 promoter.

(B) GST-pull down experiments were performed from the crosslinked Ad VAI transcription reactions in figure 4-1D. Associated plasmid DNA was detected by PCR using primers specific to M13-Ad VAI promoter.

(C) Chromatin immunoprecipitation experiment to determine RB occupancy of class 2 and class 3 promoters *in vivo*. Chromatin immunoprecipitation experiments were performed similar to those described in Chapter 3. A ten fold serial dilution of input chromatin from 10% to 0.01% is shown in lanes 1-4. Immunoprecipitation reactions were set up using pre-immune sera (lane 5), α-SNAP43 (lane 6), α-RB (lane 7), or α-TBP (lane 8). Recovered DNA was analyzed using primers specific to U2 and U6 snRNA promoters, tRNA (lys), and GAPDH.

Part C of this figure was performed by Gauri Jawdekar.


A
U6 snRNA
DSE
PSE
TATA
Input
Untreated
GST-RB (379-928)
GST-RB (379-870)
GST-RB (379-772)
GST-RB (379-577)
GST
No Extract
pU6 - Hae - RA2
1 2 3 4 5 6 7 8 9 10
B
AdVAI
A
B
Input
Untreated
GST-RB (379-928)
GST-RB (379-870)
GST-RB (379-772)
GST-RB (379-577)
GST
No Extract
M13 - Ad VAI
1 2 3 4 5 6 7 8 9 10
C
Input
α-IgG
α-SNAP43
α-TBP
α-RB
U6 snRNA
U2 snRNA
tRNA lys
GAPDH
1 2 3 4 5 6 7 8

using primers specific to each of these plasmids. As shown in Figure 4-2A, GST-RB (379-928) exhibits significant amounts of U6 promoter occupancy compared to untreated, GST treated or reactions containing no nuclear extract. This result correlates to its ability to completely repress transcription. Additionally, GST-RB (379-870) also associates with the U6 reporter construct, although to a lesser extent, suggesting that the lowered ability to repress transcription may be due to a lowered affinity for the U6 promoter. Again this result suggests an important role for the extreme C-terminus of RB in the regulation of U6 snRNA gene expression. Interestingly, GST-RB (379-772) can also occupy the U6 reporter construct promoter to some extent, even though it does not repress transcription, indicating that recruitment to the promoter may not be enough for repression and that there may be additional co-factors required to repress transcription. These potential co-repressors may require the intact C pocket region of RB to be recruited to the U6 promoter. The RB mutant containing amino acids 379-577 did not appear to support significant promoter occupancy similar to its inability to repress transcription. Surprisingly, none of the RB mutants occupied the Ad VAI reporter constructs even though they were capable of repressing transcription to varying extents (Figure 4-2B). This result implies that RB may have fundamentally different mechanisms for regulating the expression of class 2 versus class 3 RNA polymerase III transcribed genes.

To further define the mechanisms used for repression of class 2 and class 3, the *in vivo* RB occupancy of a tRNA(lys) and a U6 snRNA promoter were analyzed by chromatin imunoprecipitation. Normal human mammary epithelial cells (184B5) were treated with formaldehyde to covalently cross-link proteins to DNA and other neighboring proteins.

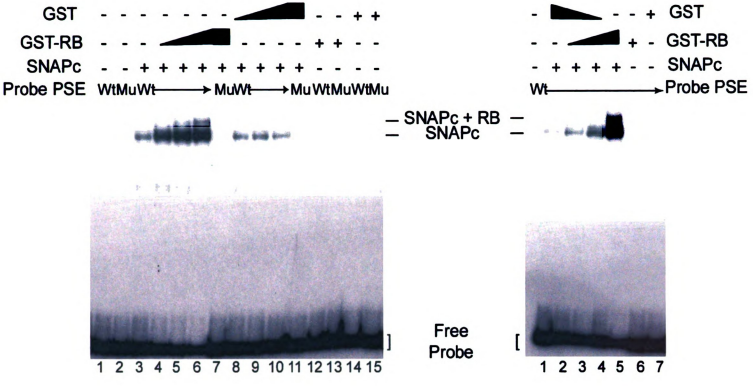
Subsequently, lysates were prepared and sonicated to fragment genomic DNA into segments approximately 500-800 bp in size such that there should not be more than one promoter per segment. Lysates were then subjected to immunoprecipitation using antibodies directed against SNAP43, RB or TBP, and the resultant protein-DNA complexes were affinity purified using protein-G agarose beads. The recovered DNA segments were analyzed by PCR using primers specific to human tRNA, U2 and U6 snRNA promoters as well as primers specific to GAPDH exon 2 as a negative control. A ten fold serial dilution of input chromatin was also analyzed for each gene of interest to serve as a standard curve, allowing estimation of relative levels of promoter DNA precipitated and to illustrate that PCR conditions for each set of primers was in the dynamic range. As shown in Figure 4-2C, immunoprecipitations performed with α-SNAP43 antibodies were significantly enriched for U6 snRNA and U2 snRNA promoter DNA but not tRNA(lys) or GAPDH as compared to IgG precipitations as expected (23). Similarly, precipitations performed with α-TBP antibodies were enriched for U6 snRNA, U2 snRNA, and tRNA(lys) promoter DNA as has been shown previously (23). As observed previously, immunoprecipitations performed using α-RB antibodies were enriched for U6 snRNA promoter DNA but not U2 or GAPDH promoter DNA. Interestingly, the α-RB immunprecipitations were not enriched for tRNA promoter DNA, correlating to the results seen in the *in vitro* assays shown above. Together, these results suggest that RB may regulate RNA polymerase III gene expression by two different mechanisms.

**Characterization of the region of RB interacts with SNAPc bound to the PSE**

In order for RB to efficiently regulate U6 expression, RB may be directed to U6 promoters either by direct binding to specific DNA control elements or through recruitment by other trans-acting factors. Interaction between RB and SNAPc has been shown previously, and therefore, it is possible that RB could be recruited to human U6 snRNA promoters through interaction with SNAPc. To determine whether RB can interact with SNAPc bound to DNA, electrophoretic mobility shift assays (EMSA) were performed. It should be noted here that these assays were performed using a much different gel system as compared to chapter 3. This tris-glycine gel system is optimized for the best possible SNAPc-RB interaction, all other gel conditions did not support SNAPc and RB interaction. The reason that this type of gel system was not used in chapter 3 is that the TFIIIB complexes will not form. For these experiments, a mini-SNAPc (12) was assembled from recombinant proteins (SNAP43, SNAP50, and SNAP190 (1-505)) and was incubated with a radio-labeled probe containing either a high affinity or mutant PSE in the presence and absence of GST-RB (379-928). Since RB interacts efficiently with both SNAP43 and SNAP50 (7), a complex containing these factors could suffice to recruit RB to the U6 promoter. As shown in Figure 4-3, DNA binding reactions performed with increasing amounts of GST-RB (379-928) result in a protein-DNA complex with slower mobility than that observed for reactions containing only mini-SNAPc (compare lanes 4-6 to lane 3), suggesting that GST-RB (379-928) is forming a complex with mini-SNAPc on DNA. In contrast, addition of a GST control protein had no effect on DNA binding by mini-SNAPc (lanes 8 -10). In all cases, DNA binding is dependent upon the PSE because no binding of any type is observed for

**Figure 4-3: SNAPc can recruit RB to promoter DNA.**

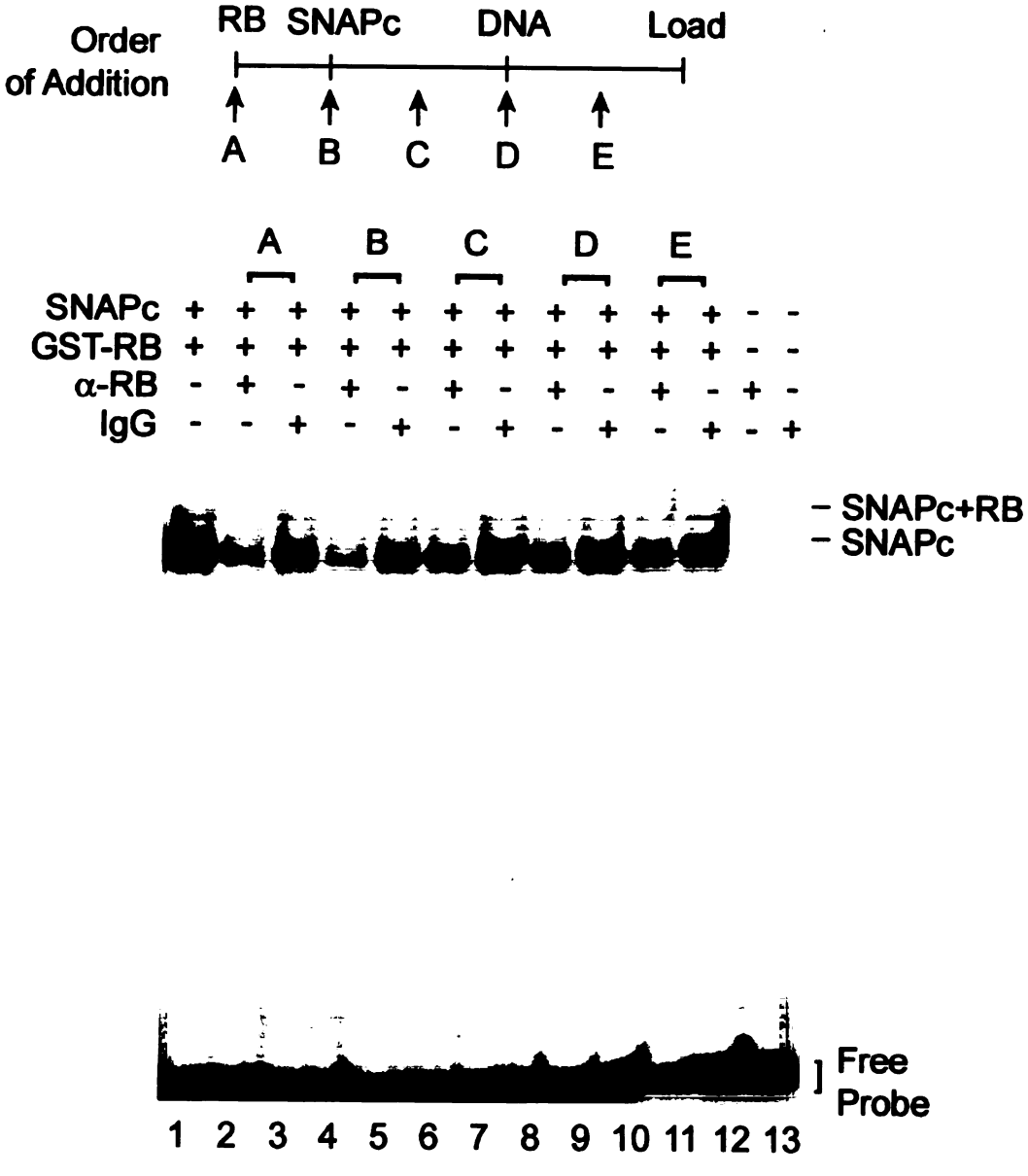
Left panel: Electrophoretic mobility shift assays were performed with purified recombinant mini-SNAPc alone (lane 3) or with mini-SNAPc plus 300 ng, 1000 ng and 3000 ng GST-RB (379-928) (lanes 4 -7) or GST (lanes 8-11). Radio-labeled DNA probe containing either wild type or mutant PSE was then incubated with each reaction. The resulting protein-DNA complexes were resolved by 5% acrylamide gel electrophoresis and were visualized by autoradiography. Addition of RB to reactions containing SNAPc results in formation of a lower mobility complex (lanes 4-6) that is PSE specific (lane 7) whereas GST does not affect SNAPc/DNA binding. Addition of GST-RB also results in increased SNAPc binding activity whereas GST alone does not. As shown in lanes 12 and 13, RB alone does not bind to DNA. GST also does not bind DNA (lanes 14 and 15). Right Panel: A cross titration of GST-RB (379-928) and GST was performed to illustrate that the increased DNA binding activity observed in the left panel is a specific effect of GST-RB and not a non-specific protein effect.


GST
GST-RB
SNAPc
Probe PSE
Wt Mu Wt
Mu Wt
Mu Wt Mu Wt Mu
SNAPc + RB
SNAPc
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1 2 3 4 5 6 7

reactions containing a mutant PSE probe (lanes 7 and 11). Neither GST-RB (379-928) nor GST bind to DNA containing either a wild type or mutant PSE (lanes 12-15). Therefore, RB can associate with SNAPc bound to the promoter of a U6 gene *in vitro*. Interestingly, RB also appears to enhance mini-SNAPc binding to the PSE. In order to determine whether the observed effects of GST-RB (379-928) are perhaps due non-specific protein effects, EMSA analysis was performed maintaining the total protein level constant while varying the proportion of GST-RB (379-928) and GST in each reaction. When the total amount of protein is kept constant, an increasing proportion of GST-RB (379-928) correlates with an increased amount of mini-SNAPc bound to the PSE (Figure 4-3; lanes 2-5), suggesting that RB can enhance DNA binding by SNAPc or that RB has associated with the complex and that the supershifted complex is not resolvable in this system.

To further demonstrate that the effects of RB on DNA binding by mini-SNAPc are indeed due to RB, EMSA analysis was performed with mini-SNAPc plus GST-RB (379-928) in the presence of an antibody specific to the extreme C-terminus of RB. As shown in Figure 4-4, addition of the anti-RB antibody disrupted the slower mobility complex, consisting of SNAPc and RB, and also abolished the enhanced DNA binding by SNAPc (compare lane 2 to lane 1). An order of addition experiment was also performed to determine whether the timing of antibody addition relative to DNA binding by SNAPc and RB was important. Pre-incubating GST-RB (379-928) with the anti-RB antibodies (lane 4) or adding the anti-RB antibodies, SNAPc, and RB at the same time (lane 6) results in the loss of the lower mobility complex and also in reduced DNA binding by

**Figure 4-4: Addition of α-RB antibodies precludes interaction with SNAPc.**
The effects of anti-RB antibodies on DNA binding by GST-RB (379-928) and SNAPc when added at various incubation time points were studied in electrophoretic mobility shift assays. DNA binding reactions were set up by the addition of RB followed by SNAPc and lastly, DNA. Equivalent amounts of anti-RB antibodies or non-specific IgG antibodies were added with RB (A), with SNAPc (B), after SNAPc (C), with DNA (D), or after DNA (E).

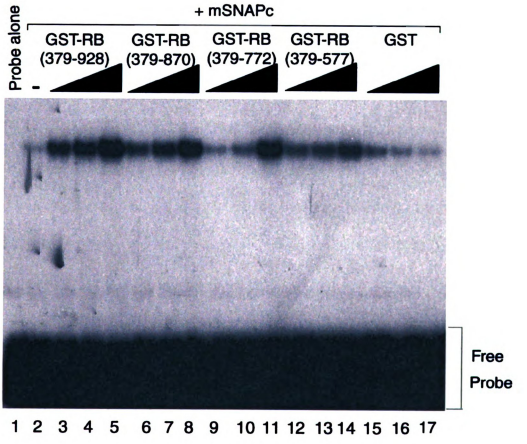

Order of Addition
RB
SNAPc
DNA
Load
A
B
C
D
E
SNAPc + + + + + + + + + + - -
GST-RB + + + + + + + + + + - -
α-RB - + - + - + - + - + - + -
IgG - - + - + - + - + - + - +
– SNAPc+RB
– SNAPc
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13

mini-SNAPc. However, the formation of the lower mobility complex was unaffected when the anti-RB antibodies were added after GST-RB (379-928) and mini-SNAPc interacted with each other. For example, addition of the anti-RB antibodies prior to DNA binding by GST-RB (379-928) plus mini-SNAPc (lane 8) or after DNA binding (lane 10) had no effect, which suggests that the epitope on RB recognized by this antibody is masked by pre-incubating RB with mini-SNAPc. Non-specific rabbit IgG antibodies had no effect on DNA binding by mini-SNAPc and GST-RB (379-928) (lanes 3, 5, 7, 9, and 11) indicating that the effect of the anti-RB antibodies is specific. These observations suggest RB is in fact part of the lower mobility complex and RB does not displace SNAPc from DNA.

Since RB can interact with SNAPc bound to the PSE in EMSA experiments, the region of RB required for this interaction was determined. Using DNA binding assays similar to those described above, RB mutant proteins were tested for the ability to associate with SNAPc bound to DNA. Increasing amounts of GST-RB truncation proteins were incubated with SNAPc prior to DNA binding and were resolved using polyacrylamide gel electrophoresis. Resulting protein-DNA complexes were visualized by autoradiography. For this experiment, lowered amounts of RB proteins were used that would result in enhanced binding of SNAPc but not the formation of the upper complex. As shown in Figure 4-5, the amount of binding observed from SNAPc alone is illustrated in lane 2. Addition of increasing amounts of GST-RB (379-928) (lanes 3-5) results in a significant increase in SNAPc binding as compared to GST (lanes 15-17), recapitulating the results seen above and indicating RB association with SNAPc bound to DNA. Similarly, GST-

**Figure 4-5: Characterization of the region of RB necessary for interaction with SNAPc bound to U6 promoter DNA.**
EMSA analysis was done to determine which region of RB can associate with SNAPc on DNA. Purified recombinant mini-SNAPc alone (lane 2) or mini-SNAPc plus 30 ng, 100ng, or 300 ng GST-RB (379-928) (lanes 3-5), GST-RB (379-870) (lanes 6-8), GST-RB (379-772) (lanes 9-11), GST-RB (379-577) (lanes 12-14) or GST (lanes 15-17) were incubated for 20 minutes at room temperature. Radio-labeled DNA probe containing a wild type PSE was then incubated with each reaction. The resulting protein-DNA complexes were resolved by 5% acrylamide gel electrophoresis and were visualized by autoradiography.

This experiment was performed by Gauri Jawdekar.


+ mSNAPc
Probe alone
GST-RB (379-928)
GST-RB (379-870)
GST-RB (379-772)
GST-RB (379-577)
GST
-
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

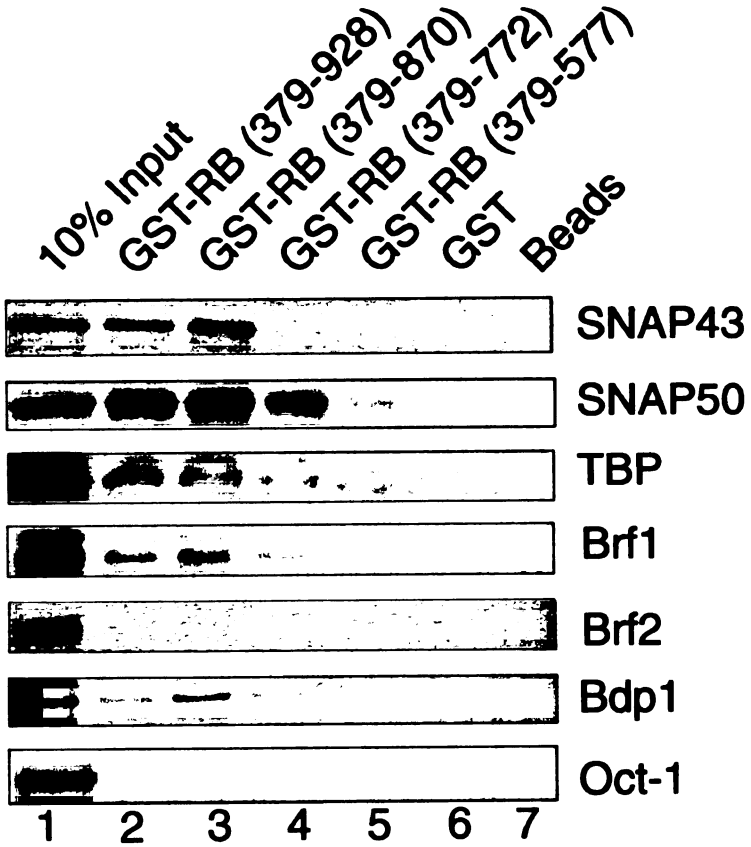
RB (379-870) (lanes 6-8) also increases binding indicating that this region of RB can still associate with the U6 snRNA promoter *in vitro*, which is consistent with repression and promoter occupancy data. However, GST-RB (379-772) (lanes 9-11) seems to associate with SNAPc bound to DNA with a slightly lower affinity correlating to its lack of ability to repress transcription. GST-RB (379-577) (lanes12-14) does not seem to have as significant effect on SNAPc binding as the other truncation mutants, again consistent with transcription data showing a lack of repression.

**RB interaction with components of RNA polymerase III basal machinery**

To better understand how RB is acting to regulate expression of genes transcribed by RNA polymerase III, it is important to determine which regions of RB are necessary for interaction with components of core promoter complexes expected to be present at RNA polymerase III promoters. Interaction with basal machinery (i.e. SNAPc and TFIIIB) represents a mechanism by which RB is recruited to target promoters to repress transcription. To determine which regions of RB are requisite for interaction with these basal factors, GST pull down experiments were performed. RB mutant proteins or GST were mixed with components of RNA polymerase III basal machinery that were individually expressed in rabbit reticulocyte lysate and labeled with $^{35}$S-methionine. Expression of these $^{35}$S-labeled proteins is shown in Figure 4-6 (lane 1). Stably interacting proteins were isolated using glutathione sepharose beads, washed extensively and stable protein complexes were eluted into Laemmli buffer. Recovered proteins were

**Figure 4-6: Characterization of the region in RB that can interact with RNA polymerase III basal machinery.**
GST-pull down analysis to determine the region of RB that can interact with each component of RNA polymerase III basal machinery. SNAP43, SNAP50, TBP, Brf1, Brf2, and Oct-1 were expressed *in vitro* and labeled with $^{35}$S-methionine. 10% input for each *in vitro* expressed protein is shown in lane 1. Each protein was incubated with GST-RB truncation proteins or GST and stable protein complexes were purified using glutathione sepharose. The beads were extensively washed and bound proteins were separated by SDS-PAGE. Associated proteins were visualized by autoradiography.

This experiment was performed by Gauri Jawdekar.


10% Input
GST-RB (379-928)
GST-RB (379-870)
GST-RB (379-772)
GST-RB (379-577)
GST
Beads
SNAP43
SNAP50
TBP
Brf1
Brf2
Bdp1
Oct-1
1 2 3 4 5 6 7

separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. As shown in figure 4-6, SNAP43 interacts strongly with the full length GST-RB (379-928) (lane 2) but not with GST (lane 6) or beads alone (lane 7) demonstrating the specificity of interaction of SNAP43 with GST-RB (379-928). SNAP43 also interacts with GST-RB (379-870) (lane 3) suggesting that the extreme C-terminus of RB is not critical for SNAP43 interaction. However, when the region of RB containing the C pocket of RB is removed, interaction is abolished, suggesting that the C pocket of RB is required for SNAP43 interaction. Further truncation of the RB protein resulting in the A pocket alone (lane 5) also lacks the ability to interact with SNAP43. A similar pattern of RB interaction is also observed for TBP in that the removal of the C pocket results in lowered capacity for interaction. Together these results indicate that the A/B pocket domain as well as the C pocket is crucial for RB's interaction with both SNAP43 and TBP.

Unlike SNAP43, the C pocket of RB does not appear to be required for SNAP50 interaction. SNAP50 interacts strongly with GST-RB (379-928) and GST-RB (379-870) but not GST or beads alone as was the case with SNAP43. Interestingly, SNAP50 can still interact with GST-RB (379-772) in which the C-pocket has been removed. GST-RB (379-577) did not interact with SNAP50 indicating that the intact A/B pocket is required for interaction. These data imply that the A/B pocket of RB is required and sufficient for interaction with SNAP50. Similarly, Brf1 and Bdp1 also interact with GST-RB (379-928), GST-RB (379-870), and GST-RB (379-772) although to a lesser extent than

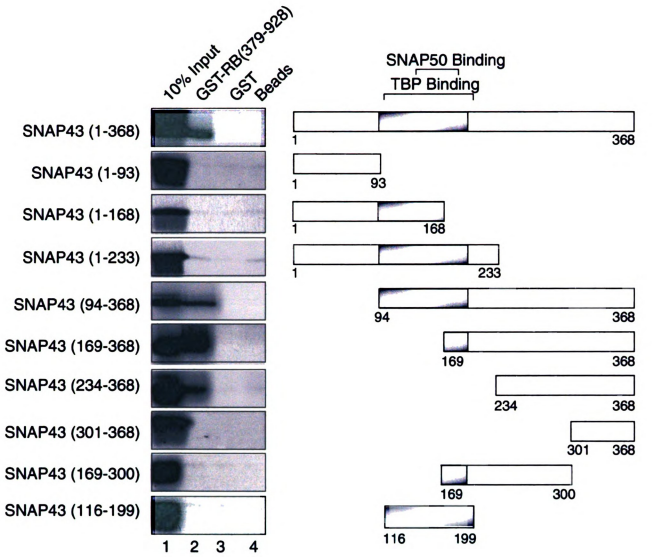
SNAP50. Oddly, GST-RB (379-870) interacts more strongly than GST-RB (379-928). As expected, none of the RB mutants tested interact with the transcriptional activator Oct-1 or with Brf2, a component of variant TFIIIB complex both of which are important for expression of snRNA genes transcribed by RNA polymerase III. Overall, these assays. indicate that interaction with SNAP43 and TBP require the A/B pocket and C pocket of RB whereas SNAP50, Brf1, and Bdp1 require only the A/B pocket

**The region of SNAP43 contained within amino acids 234-368 is sufficient for RB interaction**

Since there is a strong correlation between the region of RB that interacts with SNAP43 and the region that is able to repress U6 snRNA transcription, the RB interaction domain within SNAP43 was further characterized using SNAP43 truncation mutant proteins. Each truncation mutant as shown in Figure 4-7 was tested for RB interaction in GST-pull down assays similar to those described above. As shown in Figure 4-7, SNAP43 (1-368) interacts strongly with RB whereas SNAP43 (1-93), SNAP43 (1-168), and SNAP43 (1-233) do not. This suggests that the region of SNAP43 between 1-233 is not sufficient for interaction with RB. Therefore, N-terminal truncation mutants of SNAP43 were tested for interaction with RB. Truncation mutant proteins containing amino acids SNAP43 (94-368), SNAP43 (169-368), and SNAP43 (234-368) interact strongly with RB. However, a truncation proteins containing amino acids 169-300 no longer interacts with RB, suggesting that amino acids 301 to 368 are important for interaction. Accordingly, a SNAP43 truncation containing amino acids 301 to 368 was tested and was shown not to interact with RB. This result suggests that amino acids 301 to 368 are required for

**Figure 4-7: SNAP43 (234-368) is the minimal region required for interaction with RB**

GST-pull down analysis to determine the minimal region of SNAP43 required to interact with RB. Individual SNAP43 mutants (cloned by Liping Gu) were expressed in vitro and labeled with $^{35}$S-methionine. These proteins were incubated with GST-RB (379-928) (lane 2), GST (lane 3), or beads alone (lane 4). Bound proteins were washed extensively and separated by 17% SDS-PAGE. Proteins were visualized by autoradiography. 10% input is shown in lane 1.


10% Input
GST-RB(379-928)
GST Beads
SNAP50 Binding
TBP Binding
SNAP43 (1-368)
SNAP43 (1-93)
SNAP43 (1-168)
SNAP43 (1-233)
SNAP43 (94-368)
SNAP43 (169-368)
SNAP43 (234-368)
SNAP43 (301-368)
SNAP43 (169-300)
SNAP43 (116-199)
1 2 3 4
1 368
1 93
1 168
1 233
94 368
169 368
234 368
301 368
169 300
116 199

interaction with RB but are not alone sufficient to support RB interaction. As shown in Figure 4-7, the smallest region of SNAP43 that interacts with RB in this assay is SNAP43 (234-268). Interestingly, RB does not interact with SNAP43 (116-199), which has been shown to interact with both SNAP50 and TBP. Altogether, the results of this assay suggest that the region of SNAP43 containing amino acids 234-368 is sufficient for interaction with RB.

## Discussion

As discussed earlier, RB plays a central role in many cellular processes. Studies from other labs have determined that distinct regions of RB are necessary for participation in different cellular processes. The A and B pockets of RB are sufficient to regulate cell cycle progression (13, 14), interact with E2F or HDACs (3, 11, 22), and repress transcription by RNA polymerase II (1, 3, 14). Conversely, growth control and tumor suppression have been assigned to the A, B, and C pockets (17). In fact, tumor suppression can be rescued by re-introduction of RB 379-928 into Rb-/- mouse embryonic fibroblasts and can reverse embryonic lethality and other development defects in Rb-/- mice (21).

RB's role in growth regulation may significantly depend on it's ability to regulate transcription by RNA polymerase III. It was therefore interesting to determine whether the region of RB that repressed RNA polymerase III activity correlates to the region sufficient to regulate growth control. As shown by the mutational analysis of RB, the minimal region of RB necessary for repression of RNA polymerase III transcription is

amino acids 379-870. This region of RB was sufficient for repression of both U6 snRNA and Ad VAI transcription if not quite as potent as the truncation protein 379-928. Potentially, the extreme C-terminus of RB has important contributions to RNA polymerase IIII repression but is not absolutely required. The RB truncation mutant containing amino acids 379-772 is not sufficient to support repression of RNA polymerase III even though it can repress RNA polymerase II transcription (3, 11) suggesting an required role for the C-pocket. The results of this study suggest a link between regulation of RNA polymerase III transcription, growth control, and tumor suppression based on the regions of RB required for each of these processes.

In order to repress U6 snRNA transcription, RB is recruited to the promoter via a complex network of interactions. RB can interact with multiple components of complexes needed for pre-initiation complex formation at the U6 snRNA gene. Previously, SNAPc has been shown to be an important determinant in the regulation of U6 snRNA genes by RB. RB association with SNAPc has been described by a variety of methods including co-immunoprecipitation, biochemical co-purification, and GST-pull down analysis in which RB was shown to preferentially interact with the SNAP43 and SNAP50 subunits (7). Treatment of nuclear extracts with RB to deplete for RB interacting factors exhibit a marked depletion of SNAPc as well as comprised ability to continue to support correctly initiated U6 gene expression. When supplemented with a combination of SNAPc and TBP, transcription of U6 snRNA genes is restored, suggesting that while SNAPc is important to the ability of RB to regulate U6 genes, it is not sufficient to do so alone. Data presented in this report demonstrates that RB can in fact be recruited to a U6

promoter *in vitro* by interaction with SNAPc albeit not very efficiently. An alternative form of TFIIIB that assembles at the TATA box of U6 snRNA promoters also plays a role in the recruitment of RB (Chapter 3). Endogenous RB co-immunoprecipitates with TFIIIB complexes. Additionally, TBP and Bdp1 in the TFIIIB complex specific for U6 snRNA transcription also interact significantly. Together, interactions with SNAPc and TFIIIB act cooperatively to recruit RB to the U6 snRNA promoter.

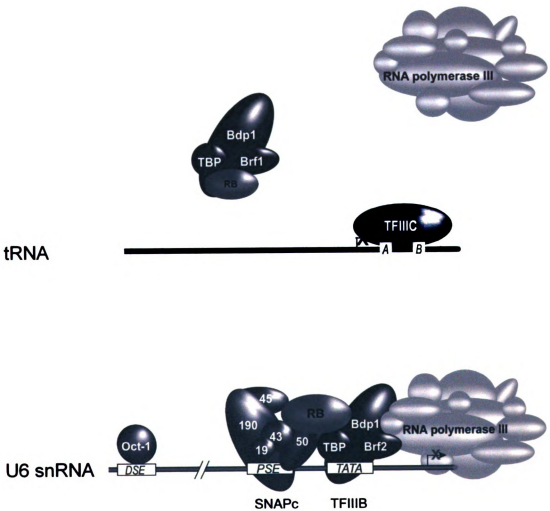
Structure function analysis of RB interaction with these basal factors RB illustrates that all three pocket domain are important for protein-protein interactions. The A/B pocket + C pocket regions are sufficient for interaction with SNAP43 and TFIIIB components and the C pocket is critical for this interaction. Conversely, the A and B pockets were sufficient to interact with SNAP50 and removal of the B region abolishes the interaction. Interestingly, the loss of ability to repress U6 snRNA transcription (removal of the C pocket) correlates with the loss of ability to interact with SNAP43 and a lowered affinity for the U6 promoter. Similarly, the region of RB containing the A, B, and C pockets was sufficient to significantly associate with SNAPc bound to a U6 snRNA promoter in EMSA experiments.

The mechanisms RB uses to repress RNA polymerase III are still unclear. One proposed model for RB repression of tRNA genes suggests that RB target the TFIIIB complex involved in tRNA expression (20). Through direct interactions with the Brf-1 subunit, RB inhibits important interactions with TFIIIC. It has been proposed that RB disrupts pre-initiation complex formation including polymerase recruitment at tRNA gene

promoters (Figure 4-8) (9). In this model, RB is not expected to be present at a tRNA promoter as it has sequestered TFIIIB away from the target gene (15). The data presented in this study are consistent with this mechanism, as RB does not appear to associate with tRNA promoters *in vivo* or *in vitro* under conditions of repression.

However, RB appears to employ a different mechanism for the repression of class 3 genes (U6 snRNA). As shown by both *in vivo* and *in vitro* promoter occupancy experiments, RB occupies the U6 snRNA promoter (a class 3 gene) in contrast to a lack of promoter occupancy at a tRNA promoter (class 2) (Figure 4-8). One explanation for RB association at the U6 snRNA promoter and not Ad VAI or tRNA promoter lies in the strength of interaction with the basal factors. Overall, RB interacts more strongly with SNAPc components than with TFIIIB component and this strength of interaction could contribute to a more stable complex formation at the U6 snRNA promoter. Additionally, SNAPc and TFIIIB bind very tightly to the promoter elements in the U6 snRNA gene perhaps making them more resistant to RB displacement and encouraging a stable complex including RB to form on the DNA.

**Figure 4-8: RB repression of class 2 and class 3 RNA polymerase III transcribed genes.**
RB potentially uses to different mechanisms to repress RNA polymerase III transcription of different classes of promoters. For repression of tRNA genes, RB may specifically target the TFIIIB complex, sequestering it away from the tRNA promoter interrupting pre-initiation complex formation. In this model RB would not be expected to associate with the target promoter. Conversely, RB does associate with the U6 snRNA promoter through recruitment by SNAPc and TFIIIB. The molecular step inhibited by RB is still not yet known but may be subsequent to pre-initiation complex assembly.


RNA polymerase III
Bdp1
TBP
Brf1
RB
tRNA
TFIIIC
A
B
U6 snRNA
Oct-1
DSE
PSE
TATA
45
190
43
19
50
RB
Bdp1
TBP
Brf2
RNA polymerase III
SNAPc
TFIIIB

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# Chapter 5

## Summary

The Retinoblastoma tumor suppressor protein plays a central role in many regulatory processes in the cell including cell cycle progression, apoptosis, DNA replication and repair, differentiation, and growth control. The key to RB's ability to function in all of these cellular processes is the ability to act as a transcription factor to regulate the transcription of genes involved each process. Previous studies illustrate that RB can repress transcription by RNA polymerase I (3), II (31), and III (35) suggesting a role for RB in the regulation of a diverse set of genes. This versatility allows RB to participate in many processes critical to normal cell function. RB's contribution to each of these processes may play a role in the suppression of tumor formation.

RB acts to regulate cell growth in part by controlling the production of biosynthetic building blocks. The discovery that RB represses transcription by RNA polymerases I (3) and III (18) reveals that RB also acts to regulate expression of highly transcribed genes encoding non-translated RNAs. By understanding the mechanisms by which expression of non-translated RNAs is controlled, we can define the contribution of these RNAs to the regulation of normal cell growth. Importantly, the availability of essential non-translated RNAs could act to limit cell growth and RB repression of genes encoding these RNAs would likely have to be overcome prior to tumor progression.

Unlike regulation of RNA polymerase II, in which only small subset of genes is regulated, RB appears to regulate global RNA polymerase III activity. Nuclear run off assays performed from cells that were deficient for RB activity either through mutation (osteosarcoma cells) or from targeted gene disruption (*Rb-/-* mouse embryonic fibroblasts) show a net elevation in RNA polymerase III activity as compared to matched sister cell lines with active RB (9, 18). While the RNA polymerase III activity increases in RB deficient cells, wholesale RNA polymerase II activity is largely unaffected. Similarly, RNA polymerase III activity is repressed when RB is re-introduced into these cells via transient overexpression (9, 18).

Transcription by RNA polymerase III is also regulated in a cell cycle dependent manner (15, 17). Transcription of these genes is low in $G_0$ and $G_1$, increases and peaks in S phase, decreases by $G_2$ and is severely diminished in M phase. The lack of transcription by RNA polymerase III corresponds to phases of the cell cycle during which RB is expected to be active, whereas expression RNA polymerase III transcribed genes is high during cell cycle phases when RB is phosphorylated and inactive. Furthermore, over-expression of cyclin D/cdk4 and cyclin E/cdk2 activates transcription by RNA polymerase III in transient transfection assays (15). Together these two results suggest that inactivation of RB is important for the expression of RNA polymerase III transcribed genes.

The mechanisms RB uses to repress RNA polymerase III are still unclear. One proposed model for RB repression of tRNA genes suggests that RB target the TFIIIB complex

involved in tRNA expression (18). Through direct interactions with the Brf-1 subunit, RB inhibits important interactions with TFIIIC. It has been proposed that RB disrupts pre-initiation complex formation including polymerase recruitment at tRNA gene promoters (Figure 4-8) (9). Similarly, RB was proposed to repress some RNA polymerase II transcribed genes by targeting the TFIID-TFIIA complex formation step to prevent pre-initiation complex formation for RNA polymerase II (11). In this model, RB is not expected to be present at a tRNA promoter as it has sequestered TFIIIB away from the target gene (16). However, not all RNA polymerase III transcribed gene have a Brf-1 requirement (10, 14). For example, the class 3 genes such as U6 snRNA do not require Brf-1 or the TFIIIB complex containing Brf-1 for efficient expression. Nonetheless, these genes are still repressed by RB suggesting that RB has multiple mechanisms for repressing RNA polymerase III transcribed genes. I therefore wanted to explore the different mechanisms used by RB to repress RNA polymerase III transcription paying special attention to the class 3 genes.

At the beginning of my thesis research, I needed to establish a functional assay to study the effects of RB on transcription by RNA polymerase III. I therefore developed *in vitro* repression assays using two representative RNA polymerase III transcribed genes. I chose the Ad VAI gene as a representative of class 2 genes and the U6 snRNA gene as a representative of class 3 genes. Both of the genes tested were repressed in the presence of recombinant RB but not by the control GST protein. Overall, these experiments recapitulated the original observation that RB repressed transcription by RNA polymerase III.

In order for a repressor or activator transcription factor to directly affect the transcription of a target gene, it may associate with the promoter of that target gene. Therefore, it was important to determine whether RB could associate with U6 promoter *in vivo*. To this end, chromatin immunoprecipitation assays, a powerful tool to study *in vivo* promoter occupancy, were developed using normal human mammary epithelial cells. The chromatin immunoprecipitation assays clearly show the RB protein occupying U6 snRNA promoter *in vivo* suggesting that RB does in fact target this gene for transcriptional regulation. In contrast, RB is not present at the U1 and U2 RNA polymerase II transcribed snRNA genes. Interestingly, RB occupancy of snRNA promoters *in vivo* correlates with the ability to repress transcription as demonstrated by *in vitro* transcription experiments. Whereas RB can repress U6 snRNA transcription by RNA polymerase III, it is unable to repress transcription by RNA polymerase II initiated from a U1 snRNA promoter. Indeed, the data presented herein, illustrate that RB preferentially targets and represses RNA polymerase III transcribed snRNA genes.

To efficiently regulate U6 expression, RB may be directed to U6 promoters either by direct binding to specific DNA control elements or through recruitment by other trans-acting factors. Since RB does not seem to have innate DNA binding capability, it is most likely recruited to target promoters through protein-protein interactsions. Potentially, RB could be recruited to a U6 snRNA promoter by the protein complexes that bind to U6 snRNA promoter elements such as the proximal sequence element and TATA box.

The snRNA activating protein complex (SNAPc) is one potential candidate protein complex for recruiting RB to a U6 snRNA promoter. This protein complex consisting of at least five subunits binds to the PSE to nucleate pre-initiation complex formation and is important for subsequent gene expression (4-8, 12, 13, 19). SNAPc is an important determinant in the regulation of U6 snRNA genes by RB. Treatment of nuclear extracts with RB to deplete for RB interacting factors exhibit a marked depletion of SNAPc as well as comprised ability to continue to support correctly initiated U6 gene expression. When supplemented with a combination of SNAPc and TBP, transcription of U6 snRNA genes is restored, suggesting that while SNAPc is important to the ability of RB to regulate U6 genes, it is not sufficient to do so alone. Potentially, TBP or TBP containing complexes are also important for RB regulation of U6 snRNA genes. Importantly, the TBP containing TFIIIB complex utilized in tRNA transcription was not able to restore U6 snRNA transcription in these GST-RB treated extracts. RB association with SNAPc has been described by a variety of methods including co-immunoprecipitation, biochemical co-purification, and GST-pull down analysis in which RB was shown to preferentially interact with the SNAP43 and SNAP50 subunits. Data presented in this report demonstrates that RB can in fact be recruited to a U6 promoter *in vitro* by interaction with SNAPc albeit not very efficiently. Together, these data suggest that SNAPc is important for RB repression of U6 snRNA transcription but it is not the sole factor targeted during RB repression.

When searching for clues as to what other factor(s) play a role recruiting RB to U6 snRNA to subsequently repress transcription, the results of chromatin

immunoprecipitation and *in vitro* transcription experiments presented strong evidence for an additional candidate target for RB repression. Interestingly, RB was only present at RNA polymerase III transcribed snRNA genes *in vivo* and capable of transcriptional repression of only RNA polymerase III transcribed snRNA genes *in vitro*. It would stand to reason that if SNAPc alone were capable of mediating RB repression of U6 snRNA genes, then it should also direct RB repression activities to other genes at which SNAPc is present. This is clearly not the case, suggesting that SNAPc is not the sole determining factor for RB selectively targeting the RNA polymerase III transcribed snRNA genes. The obvious difference between snRNA genes transcribed by RNA polymerase II and III is the presence of a TATA element in core promoter region of RNA polymerase III transcribed snRNA genes. It follows that proteins or proteins complexes that are recruited to this distinguishing promoter element are candidate selectivity factors directing RB solely to RNAP III transcribed snRNA genes rather than all snRNA genes.

An alternative TFIIIB complex consisting of TBP, Brf2, and Bdp1 associates with U6 snRNA promotes (but not U1) *in vivo* through association with the TATA element and is thought to recruit RNA polymerase III for ensuing gene expression (1, 2, 14). As a candidate target for mediating RB repression of U6 genes, I demonstrated by coimmunoprecipitation, that TFIIIB associates with endogenous RB and that individual components of TFIIIB (Bdp1 and TBP) interact with RB in GST-pulldown experiments. TFIIIB, like SNAPc, can recruit RB to a U6 promoter but most likely does not act unaided to mediate RB association. *In vitro* RB promoter recruitment is significantly enhanced in the presence of SNAPc, suggesting cooperative interactions between TFIIIB

and SNAPc to direct RB to the U6 snRNA promoter. Surprisingly, Brf2, which does not interact directly with RB, appears to play a vital role in RB interaction at U6 promoters *in vitro*. Potentially, Brf2 could function within the TFIIIB complex to present the proper façade for RB association. Alternatively, Brf2 may help to stabilize Bdp1 binding at the TATA element increasing the chances of a stable Bdp1-RB interaction. RB does however interact with the two subunits (Bdp1 and TBP) that are common to both types of TFIIIB complexes suggesting a potential mechanism for targeting RB to promoters of RNA polymerase III transcribed genes.

Importantly, EMSA experiments show that RB does not repress transcription of U6 snRNA genes simply by displacing required general factors. In fact, RB appears to be able to bind to both SNAPc and TFIIIB bound to a U6 snRNA promoter. This idea of RB co-occupying a U6 snRNA promoter with required general factors is further recapitulated in the results of sequential chromatin immunoprecipitation experiment. These experiments clearly show RB, SNAPc, and TFIIIB occupying the same U6 snRNA promoter *in vivo*.

One potential mechanism RB could use to repress transcription is to block RNA polymerase III access to the U6 snRNA promoter. For example RB could be recruited to the promoter through interaction with both SNAPc and TFIIIB and further block the interactions necessary for RNA polymerase III association. Surprisingly, sequential chromatin immunoprecipitation experiments indicate that this may not be the case. These assays show that RB and RNA polymerase III can co-occupy the same U6 snRNA

promoter *in vivo*. This experiment, however, does not tell us whether these co-occupied promoter are repressed. Therefore, I returned to an *in vitro* repression system to determine whether RB and RNA polymerase III co-occupy a repressed U6 snRNA promoter. These experiments plainly indicate that RB and RNA polymerase III can co-occupy the same repressed U6 snRNA promoter.

The results presented herein are inconsistent with the previous model in which RB represses transcription by blocking the binding of RNA polymerase III to cognate promoters. Sutcliffe and co-workers (26) proposed a mechanism of RNA polymerase III transcriptional repression in which RB sequesters required factors. RB was proposed to bind to TFIIIB interrupting important interactions with TFIIIC. Since interaction with TFIIIC is critical for recruitment to tRNA genes, RB effectively sequesters TFIIIB away from the promoter thereby not allowing pre-initiation complex formation to occur. Since RB and RNA polymerase III occupy the same promoter under repressed conditions, RB is clearly not using a commonly accepted mechanism, disrupting pre-initiation complex formation, to repress transcription at the U6 snRNA promoter. Instead, we propose that, at least for human U6 transcription, RB impedes critical steps in RNA polymerase III transcription that occur subsequently to polymerase recruitment to the promoter.

How then does RB regulate transcription of U6 snRNA genes? We suggest that RB is recruited to U6 core promoter regions cooperatively by both SNAPc and TFIIIB where it

can subsequently repress transcription through many potential mechanisms. Potentially RB could recruit histone modification activities that would modify local chromatin structure preventing transcription by RNA polymerase III. However, in an *in vitro* system we observe repression of RNA polymerase III transcription using naked DNA templates. Thus, it appears that chromatin modification is not essential for repression of RNA polymerase III *in vitro*. Additionally, HDAC mediated repression of U6 genes may be unlikely as data from other groups suggest that RB can still repress expression of U6 gene even in the presence of HDAC inhibitors (16).

Another method by with RB could prevent U6 gene expression is through the recruitment of ATP-dependent chromatin remodeling complexes such as SWI-SNF. *In vivo*, the U6 promoter contains a positioned nucleosome between the DSE and PSE that is vital to proper gene expression. Association of RB in combination with chromatin remodeling factors a U6 promoter could potentially mis-align this nucleosome and thus effectively abolish U6 gene expression.

Additionally, RB may hinder steps that allow for promoter escape and subsequent transcription of the U6 snRNA gene. Furthermore, RB could inhibit steps that convert an initiating polymerase to an elongating polymerase such as phosphorylation of the enzyme. Perhaps RB interferes with elongation resulting in a stalled complex in the middle of the U6 snRNA gene. Whichever case may apply, it represents a novel mechanism for RB regulation of gene expression.

Why regulate snRNA gene expression at all? RB regulation of snRNA gene expression serves as a brake system to help shut down growth capacity of the cell. Small non-translated RNAs such as snRNAs or tRNA are building blocks that contribute to the biosynthetic capacity of the cell and thereby increase growth potential. The cell's ability to overcome the "brake system" imposed by RB would directly influence the cell's growth potential possibly encouraging progression into a tumor state.

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# Appendix A

## Recruitment of Basal Factors to the U6 snRNA promoter

Figure A-2 was included in the following manuscript:

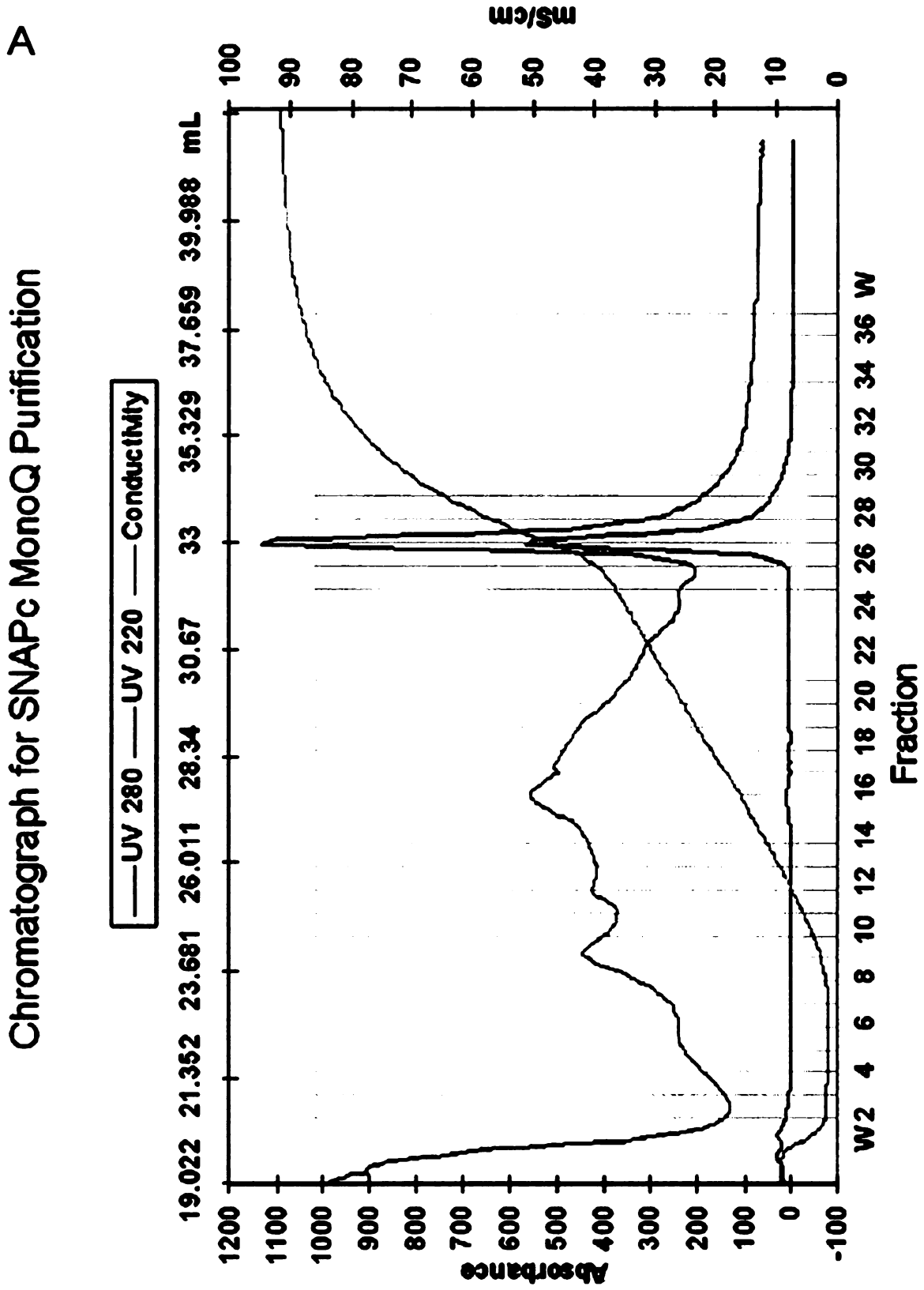
**Hinkley, C. S., Hirsch, H. A., Gu, L., Lamere, B., and Henry, R. W.**, (2003). The Small Nuclear RNA-activating Protein 190 Myb DNA Binding Domain Stimulates TATA Box-binding Protein-TATA Box Recognition. J. Biol. Chem. 278: 18649-18657.

**SNAPc assembly at human U6 snRNA promoter *in vitro***

To study the effects of RB on SNAPc binding to the U6 snRNA promoter, an assay for studying SNAPc binding to the proximal sequence element was required. To this end, the components of mini-SNAPc: SNAP190 (1-505), SNAP50, and SNAP43 were expressed as GST-fusion proteins in *E. coli* (3, 5) and purified by affinity chromatography using glutathione sepharose. The proteins were cleaved from the affinity matrix using thrombin and assayed for purity using SDS-PAGE analysis. Both SNAP43 and SNAP190 (1-505) purified to >90% homogeneity whereas SNAP50 contained at least three major contaminating proteins. These purified proteins were assembled into a mini-SNAPc complex and further purified by anion exchange chromatography to remove contaminating proteins and unincorporated subunits. The resulting fractions were analyzed for SNAPc activity by EMSA and analyzed for purity by SDS-PAGE analysis. Figure A-1A illustrates the chromatogram associated with SNAPc purification over a mono-Q column. Protein concentration was monitored by UV absorbance at both 280 and 220 nm wavelengths. Figure A-1B shows the SDS-PAGE analysis of the fractions illustrating the removal of contaminating bands in fractions 20 to 22 as compared to the starting material. As shown in Figure A-1C, SNAPc activity, as monitored by DNA binding, peaks in fractions 20 to 22.

**Figure A-1: SNAPc assembly and binding to U6 snRNA promoter DNA *in vitro*:** (A) Chromatograph of protein fractions obtained during column chromatographic purification of SNAPc. (B) SDS-PAGE of SNAPc starting material and eluted column fractions. (C) EMSA analysis for SNAPc activity.

A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mS/cm
Absorbance
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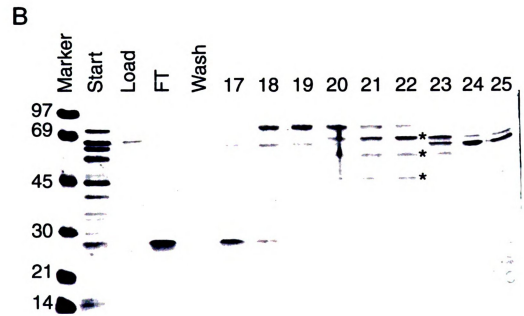
B
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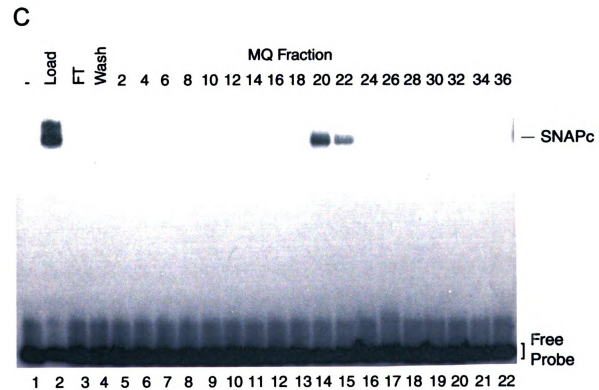
C
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- 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
— SNAPc
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

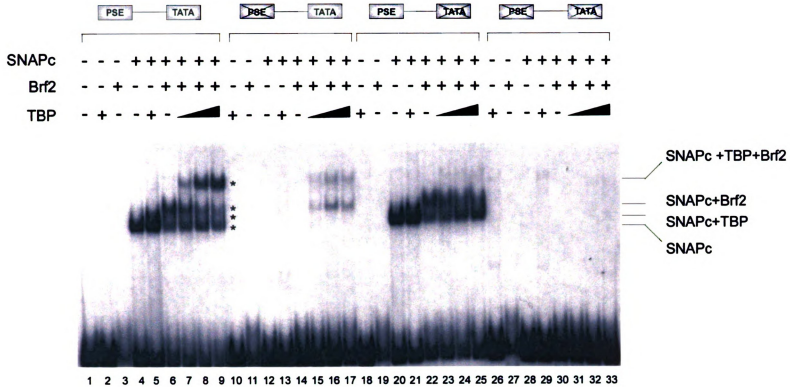
**SNAPc stimulates TFIIIB assembly at human U6 promoters**

To study the effects of RB on both SNAPc and TFIIIB complexes at the U6 promoter, an assay for measuring the assembly of both of the complexes needed to be established. It has already been shown that SNAPc and TBP act cooperatively to bind to U6 promoter DNA (3-5). Additionally, other studies have shown that TBP+Brf2 and TBP+Brf2+Bdp1 can form complexes on 7SK DNA probes that are similar to U6 promoters (1, 2). However, these papers do not take SNAPc into consideration when looking at TFIIIB complex association. Therefore both Brf2 and Bdp1 association with the U6 snRNA promoters in the presence of SNAPc and TBP were analyzed.

*Brf2:*

SNAPc binding to the PSE is a crucial early event during the assembly of a functional pre-initiation complex at human U6 promoters. As described, one function of SNAPc is to stimulate TBP recruitment to the TATA box. However, additional events are required including the assembly of additional TFIIIB components at these promoters. As a marker for TFIIIB assembly, we followed the binding of Brf2 to human U6 promoter probes in electrophoretic mobility shifts assays. As shown in Figure A-2, neither TBP alone (lanes 2, 10, 18, and 26) nor Brf2 alone (lanes 3, 11, 19, and 27) bound efficiently to any of the U6 promoter probes. In contrast, mini-SNAPc alone bound efficiently to wild-type PSE probes (lanes 4 and 20) but not to mutant PSE probes (lanes 12 and 28) as expected. When recombinant TBP was added to reactions containing mini-SNAPc, modest formation of a slower migrating complex was observed (lane 5: labeled mini-SNAPc +

**Figure A-2: Mini-SNAP$_c$ can recruit Brf2 to a U6 snRNA promoter.**
Electrophoretic mobility shift assays were performed with 1μg rTBP (lanes 2, 10, 18, and 26), 120 ng Brf2 (lanes 3, 11, 19, and 27), 10 ng mini-SNAP$_c$ (lanes 4, 12, 20, and 28), rTBP and mini-SNAP$_c$ (lanes 5, 13, 21, and 29), Brf2 and mini-SNAP$_c$ (lanes 6, 14, 22, and 30), or Brf2, mini-SNAP$_c$, and increasing amounts of rTBP (0.1, 0.3 and 1.0 μg: lanes 7-9, 15-17, 23-25, and 31-33, respectively). The DNA probes used contain a high-affinity mouse U6 snRNA PSE and a human U6 snRNA TATA box (lanes 1-9), a mutant PSE and wild-type TATA box (lanes 10-17), a wild-type PSE and mutant TATA box (lanes 18-25), or a mutant PSE and mutant TATA box (lanes 26-33). Lane 1 contains probe alone. The presence (+) or absence (-) of rTBP, Brf2, and mini-SNAP$_c$ is indicated above each lane. The positions of protein/DNA complexes are indicated.


PSE
TATA
PSE
TATA
PSE
TATA
PSE
TATA
SNAPc
Brf2
TBP
SNAPc +TBP+Brf2
SNAPc+Brf2
SNAPc+TBP
SNAPc
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33

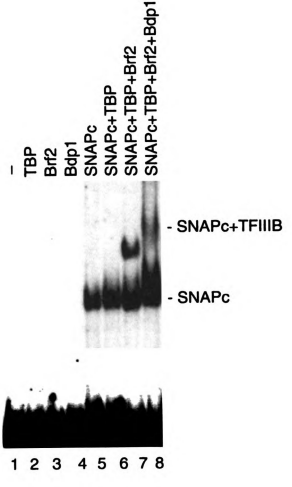
rTBP) and surprisingly, complex formation was not affected by TATA box mutation (lane 21). This complex was inferred to contain both mini-SNAPc and TBP because it was only observed in the presence of both mini-SNAPc and TBP. SNAPc stimulation of TBP binding was also less than that observed in experiments presented in Figures 1 and 3 presumably because these experiments were performed using different electrophoresis conditions. Interestingly, a slower migrating complex was now observed when Brf2 was added to reactions containing mini-SNAPc (lane 6: labeled mini-SNAPc + Brf2). This complex contained Brf2 because antibodies directed against the histidine tag on Brf2 were able to supershift this complex (data not shown). Mutation of the PSE severely debilitated SNAPc/Brf2 complex formation (lane 14) whereas TATA box mutations did not affect this complex (lane 22). Thus, mini-SNAPc can recruit Brf2 to a U6 promoter in a PSE-dependent manner. Next, the ability of SNAPc/Brf2 to recruit TBP was tested. As increasing amounts of TBP were added to reactions containing mini-SNAPc and Brf2 (lanes 7-9), formation of a new complex was observed (labeled mini-SNAPc + Brf2 + rTBP). The presence of Brf2 in this new complex was confirmed using antibody supershift assays (data not shown). In these reactions, the levels of the SNAPc/Brf2 complex was reduced concomitantly with added TBP whereas SNAPc binding was unchanged, suggesting that TBP preferentially recognized the SNAPc/Brf2 complex to form the SNAPc/Brf2/TBP complex. Mutation of the PSE severely impaired, but did not completely abrogate, the formation of this new complex (lanes 15-17). This observation suggests that TBP and Brf2 together can help stabilize SNAPc binding to a weak PSE. In these reactions, a faster migrating complex is also observed, which co-migrates with the SNAPc/Brf2 complex. However, this complex likely corresponds to a complex

containing Brf2 and TBP because the SNAPc/Brf2 complex does not form on probes containing a mutated PSE (lane 14). Indeed, in subsequent experiments the SNAPc/Brf2 and TBP/Brf2 complexes were observed to co-migrate (data not shown). As expected, when mutations were introduced into both the PSE and TATA box, DNA binding by all factors was abolished (lanes 26-33). Therefore, SNAPc can recruit Brf2 in a PSE-specific manner and together, these factors further stimulate TBP recruitment to a human U6 promoter.

*Bdp1:*

Although TFIIIB complex formation at U6 snRNA promoters can be partially monitored by incorporation of Brf2, Bdp1 must also associate with the U6 promoter to complete the complex. Therefore, recombinant Bdp1 was expressed in *E. coli*, purified, and introduced into the DNA binding reactions described above to create a complex containing SNAPc and TFIIIB bound to the U6 snRNA promoter *in vitro*. As shown in figure A-3, TBP, Brf2, and Bdp1 do not bind to DNA alone (lanes 2-4). The complex formed by the addition of SNAPc to U6 probe is shown in lane 5. Addition of TBP to SNAPc is shown in lane 6 resulting in increased binding of SNAPc. Addition of Brf2 to these proteins and DNA (lane 7) results in a lower mobility complex consistent to those seen in the figure above. Addition of Bdp1 (lane 8) to these reactions results in the formation of a lower mobility complex indicated in the figure as SNAPc + TFIIIB. This complex is inferred to contain SNAP and TFIIIB.

**Figure A-3: SNAPc and TFIIIB assembly at a U6 snRNA promoter *in vitro*.**
The assembly of SNAPc and TFIIIB complexes was analyzed using EMSA assays as described in figure A-2. Lane 1 contains probe alone illustrating the position of free probe. TBP (300 ng), Brf2 (200 ng), and Bdp1 (40 ng) alone do not bind to the probe (lanes 2-4 respectively). The amount of binding supported by SNAPc (25 ng) is shown in lane 5 and is labeled as SNAPc. Addition of TBP to SNAPc containing reactions is shown in lane 6. SNAPc, TBP, and Brf2 (lane 7) create a lower mobility complex similar to that observed in the previous figure. The complex formed by both SNAPc and TFIIIB is labeled as such in lane 8.


-
TBP
Brf2
Bdp1
SNAPc
SNAPc+TBP
SNAPc+TBP+Brf2
SNAPc+TBP+Brf2+Bdp1
- SNAPc+TFIIIB
- SNAPc
1 2 3 4 5 6 7 8

## Materials and Methods:

### Recombinant protein expression and purification:

***Mini-SNAPc:*** GST-SNAP43 Ex-XB, pET-GST-SNAP50, and pET-GST-SNAP190(1-505) were transformed into E. Coli BL21 DE3 codon + (Stratagene) and grown at 37°C in ZBM9 media until $OD_{600}$= 0.5. Temperature was lowered to 16°C and protein expression was induced with 1 mM IPTG for 20 hours. Pelleted bacteria were resuspended in HEMGT-150 and sonicated using a Branson sonifier 4 times for 30 pulses output=6 duty 60%. Debris was pelleted at 13,000 RPM at 4°C for 30 minutes. Soluble protein was bound to glutahione sepharose (Pharmacia) overnight at 4°C and washed 4 times in HEMGT-150 plus protease inhibitors and twice in HEMGT-150 without protease inhibitors. Proteins were cleaved from the beads using 20 units Thrombin (Sigma) on ice for 1.5 hours vortexing every 15 minutes. SNAP50 and SNAP190(1-505) were collected as fractions in HEMGT-150. Appropriated fractions were pooled and dialyzed against Dignam Buffer D80. SNAP43 was collected in step-wise salt gradient (150 to 1000 mM KCL in HEMGT buffer) and appropriate fractions pooled. Mini-SNAPc was assembled from approximately 100 µg of each protein at room temperature for 2 hours. The assembly reaction was diluted such that the final salt concentration was less than 80 mM KCl and the final glycerol concentration was less than 5%. The diluted assembly reaction was allowed to equilibrilate on ice for 1 hour. Assembled mini-SNAPc was then loaded onto a 1 ml Mono-Q column. Bound proteins were eluted from the column in 0.5 ml fractions in a 0 to 500 mM salt gradient in Dignam Buffer D80 (5% glycerol). Fractions were adjusted to 20% glycerol and assayed for SNAPc activity by

SDS-PAGE and EMSA. The peak of SNAPc activity corresponded to fractions 20-24 (~380-420 mM KCl).

***TBP:*** TBP was expressed as a GST-fusion protein in *E. Coli* BL21 DE3 at 37 °C in ZB-M9 media and induced with 1 mM IPTG for 3 hours. Pelleted bacteria were resuspended in HEMGT-150 and sonicated using a Branson sonifier 4 times for 30 pulses output=6 duty 60%. Debris was pelleted at 13,000 RPM at 4°C for 30 minutes. Soluble protein was bound to glutahione sepharose (Pharmacia) overnight at 4°C and washed 4 times in HEMGT-150 plus protease inhibitors and twice in HEMGT-150 without protease inhibitors. TBP was cleaved from the beads using 20 units Thrombin (Sigma) on ice for 1.5 hours vortexing every 15 minutes. Appropriate fractions were pooled, concentrated to a working concentration of 400 ng/µl using centricon YM-10 spin columns (Millipore), and dialyzed against Dignam Buffer-D80.

***Brf2:*** pSBET-hBRFU plasmid was a gift from N. Hernandez. BRFU was expressed and purified as described previously (7).

***Bdp1:*** pSBET-hB" plasmid was a gift from N. Hernadez. hB" was transformed into E. Coli BL21 DE3 codon + (Stratagene) and grown at 37°C until $OD_{600}$= 0.5. Temperature was lowered to 16°C and protein expression was induced with 1 mM IPTG for 20 hours. hB" was affinity purified using Ni-NTA beads (Qiagen) as described previously (7). hB" was dialyzed against Dignam Buffer D-80 and concentrated using centricon YM-30 spin columns (Millipore).

**Electrophoretic mobility shift assays:**

***SNAPc*:** Approximately 25 ng of SNAPc was used in EMSA using DNA probes containing a wild-type or mutant mouse U6 PSE with a wild-type or mutant human U6 TATA box as described previously (4, 6). All DNA binding reactions were performed in 20 ul total volume in a buffer containing 60mM KCl, 20 mM HEPES pH 7.9, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 0.5 μg poly(dI-dC), and 0.5μg pUC119 plasmid. Reactions were incubated 20 minutes at room temperature after which 5000 cpm of probe was added and reactions were incubated an additional 20 minutes. Samples were fractionated on a 5% nondenaturing polyacrylamide gel (39 : 1) in TGE running buffer (50 mM Tris base, 380 mM glycine, 2 mM EDTA).

***SNAPc and TFIIIB*:** Reactions also containing Brf2 were performed in a buffer containing 50 mM NaF, 10 mM HEPES pH 7.9, 20 mM Tris pH 8.4, 60 mM KCl, 7.5 mM $MgCl_2$, 10% glycerol, 6 mM B-mercaptoethanol, 12 mM DTT, 0.2 μg poly(dG-dC), and 0.2 μg pUC119 plasmid. Reactions were incubated 20 minutes at on ice after which 5000 cpm of probe was added and reactions were incubated an additional 30 minutes at 30°C. The samples were fractionated on a 4% nondenaturing polyacrylamide gel (39:1) containing 1 X TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA) and 2.5% glycerol in 0.5 X TBE running buffer.

## References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2. **Cabart, P., and S. Murphy.** 2001. BRFU, a TFIIB-like factor, is directly recruited to the TATA-box of polymerase III small nuclear RNA gene promoters through its interaction with TATA-binding protein. J Biol Chem **276:**43056-64.
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4. **Mittal, V., and N. Hernandez.** 1997. Role for the amino-terminal region of human TBP in U6 snRNA transcription. Science **275:**1136-40.
5. **Mittal, V., B. Ma, and N. Hernandez.** 1999. SNAP(c): a core promoter factor with a built-in DNA-binding damper that is deactivated by the Oct-1 POU domain. Genes Dev **13:**1807-21.
6. **Sadowski, C. L., R. W. Henry, S. M. Lobo, and N. Hernandez.** 1993. Targeting TBP to a non-TATA box cis-regulatory element: a TBP-containing complex activates transcription from snRNA promoters through the PSE. Genes Dev **7:**1535-48.
7. **Schramm, L., P. S. Pendergrast, Y. Sun, and N. Hernandez.** 2000. Different human TFIIIB activities direct RNA polymerase III transcription from TATA-containing and TATA-less promoters. Genes Dev **14:**2650-63.

# Appendix B:

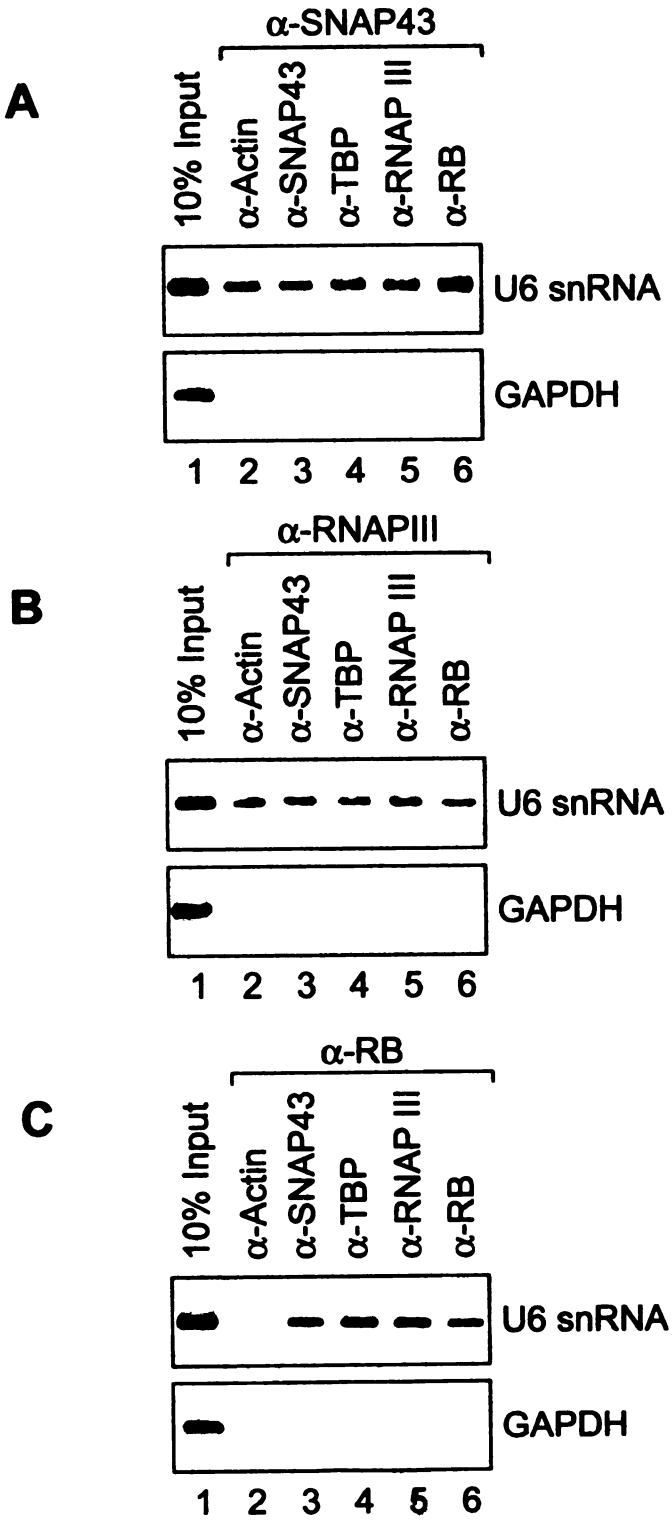
# Potential co-factors that regulate snRNA gene expression

**Actin association with potentially active U6 snRNA promoters**

Previous studies have shown that actin proteins may be in complexes with chromatin remodeling machines. Potentially actin and actin related proteins may be important for regulation of gene expression (1, 6). Data produced in our laboratory by Ms. Liping Gu suggests that actin may be in a complex with SNAPc and may therefore contribute to the regulation of snRNA genes.

In order to determine whether actin is present at the U6 snRNA promoter, perhaps through recruitment by SNAPc, sequential chromatin immunoprecipitations were performed (Figure B-1) similar to those described in chapter 3. Sequential ChIP assays were performed using α-SNAP43 (Figure B-1A), α-RNAPIII (Figure B-1B), or α-RB (Figure B-1C) antibodies in the first round of immunoprecipitation. As expected, secondary immunoprecipitations using either α-SNAP43 (lane 3), α-TBP (lane 4), α-RNA polymerase III (lane 5) or α-RB (lane 6) antibodies enriched the U6 snRNA promoter DNA but not GAPDH DNA, consistent with the data shown in chapter 3. Interestingly, when secondary precipitations were performed using α-actin antibodies (lane 2) were enriched for U6 snRNA promoter DNA only when primary precipitations

**Figure B-1: Actin and SNAPc or RNA polymerase III co-occupy the same U6 snRNA promoter *in vivo*.**
Sequential chromatin immunoprecipitations were performed from human 184B5 cells to determine actin co-occupancy with other factors. Precipitated material was recovered after the second immunoprecipitation and analyzed by PCR for enrichment of U6 DNA or GAPDH exon 2 as a negative control. Lane 1 shows 10% input chromatin.

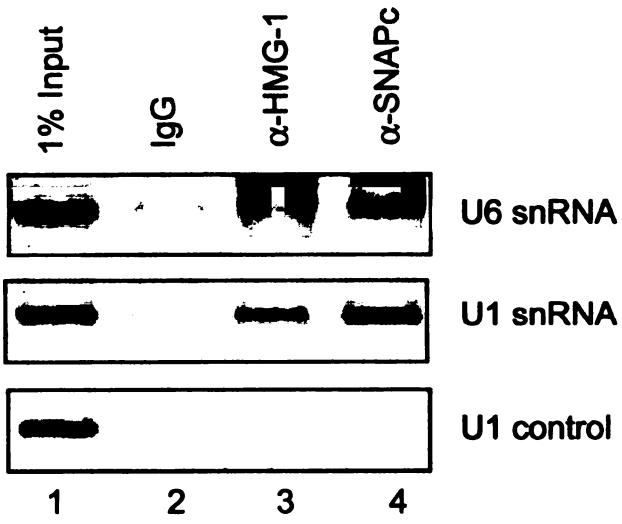

A
α-SNAP43
10% Input
α-Actin
α-SNAP43
α-TBP
α-RNAP III
α-RB
U6 snRNA
GAPDH
1 2 3 4 5 6
B
α-RNAPIII
10% Input
α-Actin
α-SNAP43
α-TBP
α-RNAP III
α-RB
U6 snRNA
GAPDH
1 2 3 4 5 6
C
α-RB
10% Input
α-Actin
α-SNAP43
α-TBP
α-RNAP III
α-RB
U6 snRNA
GAPDH
1 2 3 4 5 6

were performed with α-SNAP43 or α-RNAPIII. Secondary immunoprecipitations using the α-actin antibodies from samples immunoprecipitated first with α-RB antibodies were not enriched for U6 snRNA promoter DNA. If it is assumed that the U6 genes precipitated with α-RB antibodies in the first round are in the repressed state, it may be the case that actin is associating with only the actively transcribed U6 snRNA gene perhaps suggesting that actin plays a role in the activation of U6 snRNA genes.

**HMG-1 association with snRNA promoters:**

High mobility group protein (HMG 1&2) bind to DNA without sequence specificity similar to histone proteins and appear to have a role in the assembly of nucleoprotein complexes *in vivo* (8, 10). It is thought that HMG proteins may act like H1 linker histones during higher order chromatin assembly. These proteins are sometimes referred to as architectural transcription factors due to their ability to help stabilize higher order structures containing chromatin and other transcription factors at the promotes of specific genes. For example, HMG proteins have been shown to interact with the transcriptional activator Oct-1 and help is bind stably to DNA (11). Interestingly, the region of snRNA genes between the Oct-1 binding sites in the DSE and region containing the PSE have been shown to be resistant to micro-coccal nuclease digestion indicating the presence of a higher order chromatin structure (9). Two possible explanations for this protection of promoter DNA is the presence of a nucleosomes or a higher order complex composed of HMG proteins and DNA.

**Figure B-2: HMG-1 occupies snRNA promoters *in vivo*.**
Chromatin immunoprecipations were performed from HeLa cells using antibodies specific to HMG-1 (lane 3) and SNAP43 (lane 4). The precipitated DNA was then analyzed by PCR for the presence of U1 and U6 snRNA promoter DNA or the U1 upstream negative control. 1% input is shown in lane 1.


1% Input
IgG
α-HMG-1
α-SNAPc
U6 snRNA
U1 snRNA
U1 control
1
2
3
4

In order to determine whether HMG proteins are present at snRNA promoters, chromatin immunoprecipitations were performed from HeLa similar to the experiments described previously. As shown in Figure B-2, chromatin immunoprecipitations performed with α-SNAP43 antibodies were enriched for U6 and U1 snRNA promoter DNA but not the U1 upstream control DNA as compared to precipitations performed using control IgG. Additionally, chromatin immunoprecipitations performed using α-HMG antibodies were significantly enriched for both U6 and U1 snRNA promoter DNA indicating that HMG-1 proteins are present at snRNA promoters *in vivo*.

**p70: a potential co-repressor for RB?**

During the biochemical purification of SNAPc, a protein of 70 kDa was identified that co-purified with SNAPc. This protein, designated p70, bears some sequence similarity to yeast Bpd1. p70 has been shown to interact with SNAP43 and TBP by GST pull down analysis (Brandon Lamere, unpublished data) and can be directly crosslinked to U6 promoter DNA during UV-photocrosslinking experiments using biochemically purified SNAPc (R. William Henry, unpublished data). Thus far, the function of p70 remains unknown. It is not required for snRNA transcription (3) and immuno-depeletion of this factor does not affect U1 or U6 transcription (R. William Henry unpublished data).

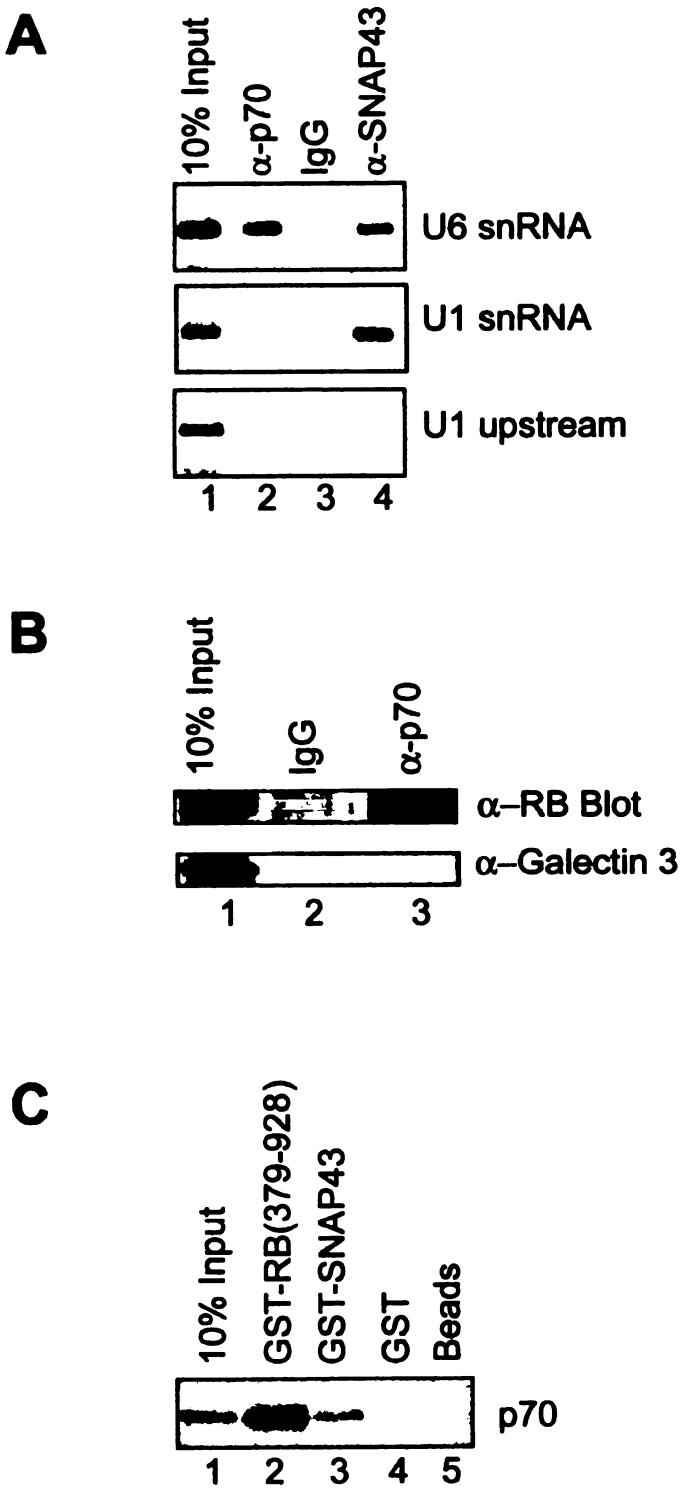
To better define a role for p70 in snRNA transcription, chromatin immunoprecipitation experiments were performed from HeLa cells to see if p70 associates with snRNA promoters (Figure B-3A). As expected, chromatin immunoprecipitations using α-SNAP43 antibodies (lane 4) were enriched for both U6

**Figure B-3: p70 associates with the U6 snRNA promoter *in vivo* and interacts with RB.**

(A) Chromatin immunoprecipitation to determine the *in vivo* occupancy of p70 at snRNA promoters. Chromatin immunoprecipations were performed from HeLa cells using antibodies specific to p70 (lane 2) and SNAP43 (lane 4). The precipitated DNA was then analyzed by PCR for the presence of U1 and U6 snRNA promoter DNA or the U1 upstream negative control. 10% input is shown in lane 1.

(B) p70 associates with endogenous RB. Co-immunoprecipitation was performed from HeLa cell nuclear extract using antibodies specific to p70. RB association was determined by Western Blot analysis using α-Galectin 3 antibodies as a negative control. 10% input is shown in lane 1.

(C) p70 directly interacts with GST-RB (379-928). ) GST pull-down assays were performed using recombinant GST-RB (379-928) (lane 2), GST (lane 3), or glutathione agarose beads (lane 4). Interactions were tested with the indicated in vitro translated, 35S-methionine labeled p70 protein. Associated proteins were size fractionated by SDS-PAGE and visualized by autoradiography.


A
10% Input
α-p70
IgG
α-SNAP43
U6 snRNA
U1 snRNA
U1 upstream
1 2 3 4
B
10% Input
IgG
α-p70
α–RB Blot
α–Galectin 3
1 2 3
C
10% Input
GST-RB(379-928)
GST-SNAP43
GST
Beads
p70
1 2 3 4 5

and U1 snRNA promoter DNA as compared to control IgG precipitations (lane 3). Additionally these IPs were not enriched for the U1 upstream control DNA supporting the specificity of the precipitations. When the chromatin immunoprecipitations were performed using antibodies specific for p70 (lane 2), only the U6 snRNA promoter was precipitated in the reaction. This result suggests that p70 is specifically recruited to the RNA polymerase III transcribed snRNA genes and that p70 could potentially play a role in U6 snRNA gene expression.

Interestingly, p70 was present at the same snRNA promoter to which RB is specifically recruited, suggesting that p70 may have a connection to RB regulation of U6 snRNA genes. Accordingly, association between p70 and RB was investigated. As shown in Figure B-3B, immunoprecipition of HeLa cell extract using p70 (lane 3) demonstrates RB association as analyzed by Western Blot. This association appears to be specific, as Galectin-3 is not precipitated with the p70 antibody. Together these results provide evidence for association of endogenous p70 with endogenous RB.

To determine whether p70 directly interacts with RB, GST-pull down experiments were performed similar to those described in previous chapters. For these experiments, p70 was expressed *in vitro* and labeled with 35S-methionine. The expression of this protein is shown in lane 1 of Figure B-3C. These *in vitro* expressed proteins were then mixed with GST-RB (379-928) or GST. Stable protein complexes were isolated with glutathione sepharose and size fractionated by 12.5% SDS-PAGE. Proteins were then visualized by autoradiography. As shown in lane 2, p70 interacts very strongly with GST-RB (379-

928). This interaction is specific as p70 did not interact with GST (lane 3) or beads alone (lane 4). The results of this experiment indicate that p70 can directly interact with RB. Overall the experiments suggest that p70 may have a role in U6 snRNA transcription potentially in connection with RB.

## Materials and Methods:

**Antibodies:** Anti-SNAP43 (CS48) and anti-TBP (SL2) antibodies have been described previously (5, 7). Anti-p70 (CS160) antibodies were gifts from Nouria Hernandez. The anti-Galectin-3 antibody (Mac-2) was a gift from Patty Voss and John Wang (Michigan State University). Mouse anti-RB antibodies (G3-245) used for western analysis were purchased from Pharmingen. The goat anti-RB antibodies (C-15), HMG-1, and pre-immune control IgGs were purchased from Santa Cruz Biotechnologies. Actin antibodies were purchased from Sigma.

**Chromatin immunoprecipitation:** Chromatin immunoprecipitations were performed similarly to that described previously (2). Human 184B5 or HeLa cells were grown to 75% confluency and then crosslinked with formaldehyde for 30 minutes. After cell lysis and sonication, chromatin immunoprecipitations were performed (approximately 1 x $10^7$ cells per immunoprecipitation) in dilution buffer containing 400 mM NaCl using 1 ug of each antibody overnight at 4°C. Protein complexes were isolated using Protein-G Fast Flow sepharose beads (Upstate Biotechnology) and were washed extensively as described previously (2). Cross-links were reversed at 65°C overnight and recovered chromatin was

suspended in 50 µl TE buffer. PCR analysis was performed using 5 µL of immunoprecipitated chromatin or input chromatin. The primers used for each gene are:

U6 forward – 5'-GTACAAAATACGTGACGTAGAAAG-3',

U6 reverse – 5'-GGTGTTTCGTCCTTTCCAC-3',

U1 forward – 5'-CACGAAGGAGTTCCCGTG-3',

U1 reverse – 5'-CCCTGCCAGGTAAGTATG-3',

U1 upstream control forward – 5'-GAACTTACTGGGATCTGG-3',

U1 upstream control reverse – 5'-GAGACAACTGAGCCACTTG-3',

GAPDH forward – 5'-GGTCATCCCTGAGCTGAAC-3', and

GAPDH reverse – 5'-GCAATGCCAGCCCCAGCGTC-3'.

PCR products were separated by 2% TBE-agarose electrophoresis and visualized using Kodak imaging software.

**Sequential Chromatin Immunoprecipitations:**

Soluble chromatin fraction was prepared from 184B5 cells as above. Primary IPs were performed scaled up 5x (~ 5 x $10^7$ cells per IP) using 5 µg α-RB, α-SNAP43, α-RNAPIII. The immunoprecipitations were performed at room temperature for 1 hour and then incubated with protein G agarose for an additional hour at room temperature. After extensive washing, precipitated protein-DNA complexes were eluted in elution buffer containing 15 mM DTT + 1% SDS at room temperature for 30 minutes. One sixth of the recovered material was used for second ChIP and material was processed exactly the same as chromatin immunoprecipitations described in above.

**Co-immunoprecipitation:** Approximately 300 µL of HeLa cell nuclear extract (ca. 8 mg/mL) was incubated with 2 µl of antibodies directed against p70 (CS160) or 1 µg rabbit IgG overnight at 4°C as indicated in figure legend. Reactions were diluted to 1ml in HEMGT-150 buffer and stable complexes were affinity purified by incubation with Protein-G Fast Flow sepharose beads (Upstate Biotechnology) for 4 hours at 4°C. Beads were washed 3 times in HEMGT-150 and boiled for 3 minutes in Laemmli Buffer. Bound proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose and associated proteins were determined by western blot analysis using antibodies directed against RB (G3-245; Pharmingen). The same membrane was stripped and probed with antibodies directed against Galectin 3 (Mac-2) (4).

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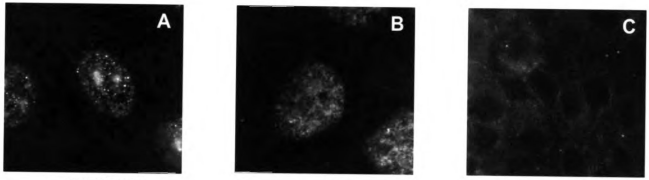
# Appendix C

## Subcellular localization of SNAPc and TFIIIB components

To determine whether components of SNAPc and TFIIIB localize to specific parts of the cell, indirect immunofluorescence was performed using antibodies to SNAP43, TBP and Bdp1. As shown in figure C-1A, SNAP43 does in fact localize to discrete subnuclear locations. Two distinct types of subnuclear particles that appear contain SNAP43. SNAP43 appears to be present in are smaller speckles that number between 15 and 20 per nucleus. SNAP43 also appears to be present in larger dots in the nucleus that number from 1 to 3 copies in the nucleus. These dots do not appear to nucleoli which are visible under phase contrast light microscopy.

The subcellular localization of Bdp1 was also investigated. As shown in Figure C-1C, Bdp1 seemed to localize diffusely in the cytoplasm and not at all in the nucleus. This was an unexpected result since transcription factors are expected to be in the nucleus and this protein has been shown to occupy the U6 snRNA promote *in vivo* (3). As a positive control for nuclear staining, α-TBP antibodies (Figure C-1B) were also used indicating that the permeabilization was sufficient to detect nuclear proteins.

**Figure C-1: Subcellular Localization of components of SNAPc and TFIIIB.**


A
B
C
α-SNAP43
α-TBP
α-Bdp1
D
E
Pre-immune
Secondary only

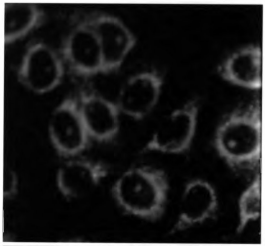
One possibility to account for the lack of Bdp1 nuclear staining is that the cells used in this experiment were very nearly 100% confluent and most likely growth arrested by contact inhibition. Potentially, if RNA polymerase III genes were not being actively transcribed due to growth arrest, there would be no need for an RNA polymerase III specific transcription factor in the nucleus. To directly test this hypothesis, coverslips were prepared such that the cell densities were approximately 50%, 75, or 100% and then analyzed for Bdp1 localization. As shown in Figure C-2, cells at 50% exhibit Bdp1 localization around the periphery of the nucleus although from the staining in this experiment it is not possible to determine whether this staining is perinuclear or associated with the inside of the nuclear membrane. Cells at 100% confluency show a similar pattern to the previous figure and seem to perhaps have less Bdp1 than the other two cell densities. Interestingly, at 75% confluency, when the cells are growing efficiently, there is more staining of the nuclear periphery perhaps indicating an increase in protein expression and also the emergence of one to two large dots in the nucleus itself. These dots may indicate a sub-compartment of nuclease dedicated to RNA polymerase III transcription.

## Materials and Methods:

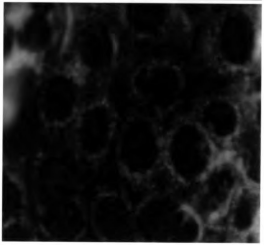
**Tissue culture:** Human mammary epithelieal cells (184B5) were a gift from Susan Conrad. Cells were maintained in Dulbecco's minimum essential media (DMEM - Gibco) plus 10% fetal bovine serum (Gibco), 200 mM Glutamine, and penicillin-streptomycin in 37°C incubator with 5% $CO_2$. For the purpose of immunofluorescent

**Figure C-2: Cell density affects subcellular localization of Bdp1.**

50% Confluent



75% Confluent



100% Confluent

studies, these cells were grown directly on poly-lysine treated coverslips (Thermonox – Nunc).

**Antibodies:** Anti-SNAP43 (CS48) and polyclonal anti-TBP antibodies have been described previously (1, 2). Anti-hBdp1 (CS913) antibodies were a gift from Nouria Hernandez (3). Secondary goat α-rabbit antibodies conjugated to Alexa 488 chromaphore were purchased from Molecular Probes.

**Indirect Immunofluorescence:** Cells on coverslips were fixed for 15 minutes at –20°C in 50:50 acetone-methanol and then washed three times in PBS. The coverslips were then pre-blocked in 10% BSA for 15 minutes at room temperature and washed two times in PBS. Coverslips were incubated in primary antibodies inverted on parafilm for 30 minutes at 37°C. Primary antibodies were used at a 1:100 dilution in 1% BSA in PBS. The coverslips were washed three times with PBS for 10 minutes at room temperature. Coverslips were incubated in secondary antibodies inverted on parafilm for 30 minutes at 37°C in the dark. Secondary antibodies were used at a 1:200 dilution in 1% BSA in PBS. The coverslips were washed three times with PBS for 10 minutes at room temperature. The coverslips were then washed three times with water for 10 minutes at room temperature. Coverslips were drained and mounted on microscope slides in Permafluor (Fisher). Immunofluorescent images were taken using computer assisted fluorescent laser scanning confocal microscopy on a Meridian-Insight microscope.

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