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TRANSCRIPTIONAL REGULATION OF THE HUMAN SMALL NUCLEAR RNA GENE FAMILY

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TRANSCRIPTIONAL REGULATION OF THE HUMAN SMALL NUCLEAR RNA GENE FAMILY

By

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ABSTRACT

TRANSCRIPTIONAL REGULATION OF THE HUMAN SMALL NUCLEAR RNA GENE FAMILY

By

Gauri W. Jawdekar

Human RNA polymerase III synthesizes small, non-translated RNAs including 5S rRNA, tRNA, and U6 snRNA that control numerous critical steps during the flow of genetic information in biological systems. U6 snRNA genes and other related family members are unusual because they are transcribed by either RNA polymerase II or III depending on the arrangement of core promoter elements. Thus human snRNA genes in general provide a good model to study polymerase preference and activity during normal and deregulated growth.

One characteristic feature of all snRNA genes is the presence of an essential proximal sequence element (PSE) in the core promoter region that is recognized by the multi-subunit general transcription factor called $SNAP_C$. DNA binding by $SNAP_C$ is a crucial early event during preinitiation complex assembly, and is a target for regulatory intervention. The mechanism of DNA binding by $SNAP_C$ was investigated in this study. The SNAP50 subunit of $SNAP_C$ plays an important role in preinitiation complex assembly for RNA polymerase II and III transcription in a process involving an unorthodox but highly conserved zinc finger domain. This zinc finger domain functions directly in DNA binding and is essential for cooperative promoter recognition by $SNAP_C$.

The Retinoblastoma (RB) tumor suppressor protein represses U6 snRNA transcription by RNA polymerase III and interestingly can interact with the SNAP50 subunit of $SNAP_C$ suggesting that RB could impair DNA binding by $SNAP_C$ to repress transcription. However, studies from our lab suggest that RB does not affect DNA binding by $SNAP_C$ during repression. Therefore, to further understand the mechanism of RB repression of U6 snRNA transcription, I examined the role of RB co-factors such as histone deacetylases (HDACs) and components of the ATP-dependent SWI/SNF chromatin remodeling complex in RB repression. In this study I show that endogenous RB co-occupies the U6 snRNA promoter with HDACs and stimulates association of HDAC2 and Brg1 with the U6 promoter during repression. Interestingly, HDAC enzymatic activity is required for RB repression of chromatin template but not naked DNA template, suggesting that RB repression and HDAC activity are biochemically separable.

In a search for additional factors that associate with $SNAP_C$ and potentially regulate human snRNA transcription, a protein called HEXIM1 was identified. In this study, I show that HEXIM1 co-purifies with $SNAP_C$ and stimulates DNA binding by $SNAP_C$ and TBP. HEXIM1 positively regulates 7SK transcription by potentially enhancing preinitiation complex assembly by these factors. Interestingly, HEXIM1 and 7SK RNA are part of a complex that inhibits the activity of P-TEFb, which is required for transcription of HIV-1 and cellular genes by RNA polymerase II. Our observation that HEXIM1 stimulates transcription of its co-repressor partner suggests a mechanism by which P-TEFb activity and hence global RNA polymerase II transcription may be regulated.

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KEY TO ABBREVIATIONS

ATP	Adenosine triphosphate
Bdp1	B double prime
Brf1	TFIIIB related protein 1
Brf2	TFIIIB related protein2
CDK	Cyclin dependent kinase
CHH	Cartilage-hair hypoplasia
ChIP	Chromatin immunoprecipitation
CK2	Casein Kinase 2
Co-IP	Co-immunoprecipitation
CSB	Cokayne syndrome group B protein
CTD	Carboxy terminal domain
DNMT1	DNA methyl transferase 1
DSE	Distal sequence element
EDG1	Estrogen down-regulated gene 1
EMSA	Electrophoretic mobility shift assay
ERE	Estrogen receptor response element
FAA	Flame atomic absorption spectroscopy
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GST	Glutathione S transferase
HA	Heamagglutinin
HAT	Histone acetylase

HDAC	Histone deacetylase
HEXIM1	Hexamethylene bis-acetamide inducible 1
HIV	Human immunodeficiency virus
HP1	Heterochromatin protein 1
ICP-MS	Inductively coupled plasma resonance mass spectroscopy
ICR	Internal control region
LTR	Long terminal repeat
MBD	Methylated DNA binding protein
MRP	Mitochondrial RNA processing
mSNAPc	mini-SNAPc
mSNAPcγ4	mini-SNAPc gamma 4
Mu	Mutant
NLS	Nuclear localization signal
PCR	Polymerase chain reaction
PNC	Perinucleolar compartment
PSE	Proximal sequence element
P-TEFb	Positive transcription elongation factor b
RB	Retinoblastoma tumor suppressor
RNAi	RNA interference
RNAP	RNA polymerase
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase polymerase chain reaction
SANT	SWI-SNF, ADA, N-CoR and TFIIIB

siRNA	Short interfering RNA
$SNAP_C$	Small nuclear RNA activating protein complex
snRNA	Small nuclear RNA
snRNP	Small nuclear ribonucleoprotein
SRP	Signal recognition particle
STAF	Selenocysteine tRNA gene transcription activating factor
SV40	Simian virus 40
TBP	TATA- binding protein
TFIIA	Transcription factor II A
TFIIB	Transcription factor II B
TFIID	Transcription factor II D
TFIIF	Transcription factor II F
TFIIH	Transcription factor II H
TFIIIA	Transcription factor III A
TFIIIB	Transcription factor III B
TFIIIC	Transcription factor III C
tRNA	Transfer RNA
TSA	Trichostatin A
UV	Ultra violet
VSMC	Vascular smooth muscle cells
vRNA	Vault RNA
WCE	Whole cell extract
Wt	Wild type

CHAPTER 1

INTRODUCTION

RNA polymerase III transcription

In most eukaryotes three major related nuclear RNA polymerases are responsible for catalyzing the synthesis of RNA from DNA. RNA polymerase I is dedicated to transcribing only one set of genes, the tandemly repeated ribosomal RNA (rRNA) genes. RNA polymerase II transcribes protein coding messenger-RNA (mRNA) genes, micro RNA (miRNA), and some non-translated small nuclear RNA (snRNA) genes. In contrast, RNA polymerase III transcribes the well-studied 5S ribosomal RNA (rRNA) and transfer RNA (tRNA) genes, and a diverse collection of small nuclear RNA (snRNA) genes. One common feature shared by these RNA polymerase III transcribed genes is that they encode non-translated RNAs that are almost always shorter than ~400 base pairs (111).

1. Diverse cellular functions of genes transcribed by RNA polymerase III

The products of RNA polymerase III transcription such as U6 snRNA, 5S rRNA and tRNA have been best studied for their function in mRNA splicing and protein synthesis, respectively. However, recent evidence suggests that RNA polymerase III transcripts function for most, if not all, crucial steps during the flow of information in biological systems and for maintaining cellular homeostasis. Table 1 describes the various RNAs molecules transcribed by RNA polymerase III and their known functions.

Table 1-1. Diverse cellular functions of some non-translated RNAs transcribed by RNA polymerase III and II.

RNA polymerase III transcribes non-translated RNAs that are characteristically less than 400 base pairs long. These gene products play an important role in diverse cellular processes indicated in the table.

Gene product	Organism	RNAP specificity	RNA length	Cellular function	Reference
B2 RNA	Mouse	III	180	inhibition of RNAP II transcription upon heat shock	21
7SK snRNA	Human	III	330-332	inhibition of RNAP II elongation	94,137,138
U1 snRNA	Human	II	164	mRNA splicing	48
U2 snRNA	Human	II	186	mRNA splicing	48
U4 snRNA	Human	II	141-145	mRNA splicing	48
U5 snRNA	Human	II	116	mRNA splicing	48
U6 snRNA	Human	III	106-107	mRNA splicing	48
5S rRNA	Human	III	~120	protein synthesis	48
tRNA	Human	III	70-90	protein synthesis	48
U3	Human	III	217-241	rRNA processing	23,33
H1 RNA	Human	III	341-344	tRNA maturation	3
7SL RNA	Human	III	~140	protein translocation	19,131
MRP RNA	Human	III	267	RNA primer processing for mtDNA synthesis	8,14,45,110
MRP RNA	Yeast	III	340	exit from cell cycle progression at the end of mitosis	28
Y RNA	Human	III	84-112	degradation of misfolded non-coding RNA	11,74,81,115,116
Vault RNA	Human	III	86-142	nucleocytoplasmic trafficking, multi-drug resistance	31,65,66,92

1.1. RNA polymerase III and information flow in biological systems

The central dogma is described as the sequential transfer of genetic information from DNA to RNA by the process of transcription and from RNA to protein by the process of translation. Recent observations show that snRNA gene products transcribed by RNA polymerase III influence each step in these processes. For example, B2 RNA represses RNA polymerase II-driven transcription. When mouse cells are exposed to heat shock, RNA polymerase II transcription of genes including actin, histone H1 and hexokinase II is repressed, while transcription of heat shock genes including hsp70 increases. In response to heat shock, B2 RNA binds to core RNA polymerase II and forms transcriptionally inactive preinitiation complexes at specific gene promoters to inhibit mRNA synthesis by an unknown mechanism (21). Like B2 RNA, 7SK snRNA also inhibits RNA polymerase II transcription through its membership in the 7SK/HEXIM1 ribonucleoprotein complex that interacts with and inhibits the positive elongation factor b (P-TEFb) (94, 137, 138). P-TEFb, which is composed of cyclin T1 and cdk9, phosphorylates the carboxy terminal domain (CTD) of the largest subunit of RNA polymerase II to stimulate transcription elongation of cellular genes as well as HIV-1 transcription and replication from the HIV long terminal repeat (LTR) (83, 84, 147). A cellular function for 7SK snRNA that might be independent of P-TEFb has been recently described. si-RNA mediated depletion of endogenous 7SK caused a marked decrease in cellular growth that was accompanied with increasing apoptosis by 72 hrs post-transfection in HeLa cells (36).

Newly synthesized pre-mRNA products of RNA polymerase II transcription are almost always spliced before they can be translated into proteins. Splicing is carried out

by the uridine rich snRNAs that are members of the snRNA gene family. Interestingly, some snRNA genes like U6 are transcribed by RNA polymerase III, whereas other snRNA genes such as U1, U2, U4, and U5 are transcribed by RNA polymerase II. Both types of snRNA function in mRNA splicing as a part of the small nuclear ribonucleoprotein particles (snRNPs) (48). Once the mRNA is appropriately processed by the snRNPs, it is ready for protein translation in a process involving the RNA polymerase III-transcribed 5S rRNA and tRNA gene products. 5S rRNA and tRNA are themselves processed before becoming active. For example, U3 snRNA is involved in rRNA processing (23, 33), while H1 snRNA is part of the catalytic subunit of RNase P that functions in tRNA maturation (3). Finally 7SL RNA as a component of the signal recognition particle (SRP) directs integral membrane and secretory proteins to the endoplasmic reticulum during translation (19, 131). Thus numerous products of RNA polymerase III transcription perform critical functions during transmission of information from DNA to RNA to protein.

In addition, a role for the MRP RNA in DNA replication was described. MRP RNA, transcribed by RNA polymerase III, was originally identified as an essential component of a ribonucleoprotein particle with endoribonuclease activity (RNase MRP). RNase MRP in mouse cells was shown to be involved in cleavage of mitochondrial RNA, which functions as a primer for mitochondrial DNA replication (8). Subsequent studies have identified a role for MRP RNA in pre 5.8S rRNA processing in the nucleolus (14, 45, 110). Interestingly, mutations in the gene encoding MRP RNA in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* lead to a delay in exit from mitosis. This delay was caused by MRP RNA mediated destabilization of the CLB2

mRNA, which encodes the major mitotic B-cyclin (28). While the regulation of B-cyclin by RNA is a novel result in yeast, the mechanism for reduction in B-cyclin by the MRP RNA is yet to be elucidated. Nevertheless, this result is interesting given the fact that the RNA and protein components of the RNase MRP are highly conserved in all eukaryotes.

1.2. RNA polymerase III transcripts and cellular homeostasis

Products of RNA polymerase III transcription have been suggested to play a role in maintaining cellular homeostasis. Although the mechanisms for understanding DNA, mRNA, and protein quality control have been fairly well studied, not much is known about the cellular response to misfolded, abnormal non-coding RNAs. A role for Y RNAs that are transcribed by RNA polymerase III has been proposed in this process. Y RNA is associated with the Ro autoantigen (74), which was originally discovered in patients suffering from lupus erythematosis. Ro was subsequently shown to recognize misfolded 5S rRNA in *Xenopus* oocytes (115) and human U2 snRNA in response to UV irradiation (11). The Ro orthologue in the eubacteria *Deinococcus radiodurans* binds to several small RNAs, including one that resembles human Y RNA and contributes to resistance to UV irradiation thus allowing cell survival (10). Y RNA is speculated to sequester Ro in an inactive state by causing a steric hindrance in the misfolded RNA binding site. While the trigger for Y RNA disassociation from Ro remains to be identified, the Y RNA is replaced by misfolded RNA that is ultimately degraded by an unknown mechanism (81, 116).

Vault RNA (vRNA), a small non-translated RNA transcribed by RNA polymerase III, binds to certain chemotherapeutic compounds (31, 65, 66). vRNA is part of a large

cytoplasmic ribonucleoprotein complex called Vault. Vault particles appear to be localized in both the nucleus as well as the cytoplasm, and hence have been implicated in intracellular and nucleocytoplasmic transfer. Furthermore, Vault particles have been postulated to play an important role in intracellular detoxification, thus contributing to the multidrug resistance of cancer cells (92). For example, the interaction of vRNA with certain chemotherapeutic compounds has been suggested to play an important role in Vault function by participating in the detoxification process (31, 65, 66).

2. Regulation of RNA polymerase III transcription

For a long time the transcription of RNA polymerase III genes was believed to be constitutive and not subject to regulation. However, recent data have clearly shown that transcription of RNA polymerase III is controlled via several regulatory pathways in the cell during both normal as well as disease conditions.

2.1. Transcriptional regulation during cellular proliferation and differentiation

RNA polymerase III synthesizes structural and catalytic RNAs that are essential components of the RNA processing and protein synthesis machinery. The demand for these RNAs fluctuates depending on the metabolic state of the cell, and therefore RNA polymerase III transcription is regulated during cell growth and cell cycle progression (97). The oncogene c-Myc plays an important role in positively influencing cellular growth (109). Recent findings that Myc is a direct activator of 5S rRNA and tRNA (29) suggest that increasing products of RNA polymerase III transcription might be a way by which c-Myc can increase the biosynthetic capacity of a cell and therefore increase cell

growth. Once cells exit the cell cycle they either arrest in the G0 phase or differentiate terminally. Recent studies show that RNA polymerase III transcription is regulated in terminally differentiated mammalian cardiomyocytes. When serum starved cardiomyocytes were stimulated to differentiate in response to hypertrophic signals such as fetal calf serum (FCS), levels of RNA polymerase III-transcribed 5S rRNA, tRNA, and U6 snRNA were elevated. Additionally, whole cell extracts from FCS treated cardiomyocytes stimulated RNA polymerase III-transcribed templates more actively as compared to extracts from un-stimulated cells. Consistent with a positive role of c-Myc in terminally differentiated cells, the authors of this study show that the increase in RNA polymerase III transcripts is mediated by c-Myc (30). Interestingly, RNA polymerase III transcription is most active during the S and G2 phase of the cell cycle and is repressed during the M and G0/early G1 phases (22, 32, 73, 132). The oncoprotein protein kinase CK2 represses RNA polymerase III transcription in the M phase, but stimulates transcription in the S phase (58, 62). Thus, CK2 can both positively and negatively influence RNA polymerase III transcription. What then is the mechanism for repression of RNA polymerase III transcription during the G0/early G1 phase of the cell cycle? One obvious possibility is that the Retinoblastoma (RB) tumor suppressor protein contributes to the silencing of RNA polymerase III transcription at the G0/early G1 stage of the cell cycle. This link is made because hypo-phosphorylated RB is active in the early G1 phase, and is switched off by increased phosphorylation as cells progress through middle and late G1 into the S phase. That RB activity correlates with an increase in RNA polymerase III transcription from the early G1 to S phase suggests a role for RB in regulating these genes (114, 132). Indeed, RB represses global RNA polymerase III transcription (131).

2.2. RNA polymerase III transcription and disease

Products of RNA polymerase III transcription contribute to the cellular growth and are elevated in many cancers. The observation that RNA polymerase III transcription is regulated by two cardinal tumor suppressor proteins, p53 and RB, strongly suggests that deregulation of transcription due to inactivating mutations in p53 and RB, that are a common feature of almost all human cancers, might contribute to cancer initiation and/or progression. Indeed, elevated levels of RNA polymerase III transcripts were observed in primary fibroblasts cells from Li-Fraumeni patients. Li-Fraumeni syndrome is characterized by a predisposition to cancer due to inherited mutations in p53 (117). This result suggests that endogenous p53 regulates RNA polymerase III transcription. Moreover, RNA polymerase III activity is elevated in p53-/- and RB -/- tumor cells, further supporting the idea that deregulation of RNA polymerase III transcription may be an important component of the growth control function by these proteins (7, 12, 34, 52, 131, 133). While it has not yet been established that p53 and RB repress RNA polymerase III transcription to limit growth, it is widely accepted that the rate of RNA polymerase III is linked to cell growth. Thus it was proposed that this arm of the p53 and RB pathways functions to contribute to tumor suppression by restricting the biosynthetic capacity of the cell (130, 133).

Levels of the general transcription factors required for RNA polymerase III transcription are elevated in tumors. For example, RT-PCR analysis showed increased levels of mRNAs encoding all five subunits of TFIIIC2 in ovarian tumors analyzed compared to normal control mRNAs. The penta-protein TFIIIC2 complex is required for 5S rRNA and tRNA transcription. This was the first example showing overexpression of

RNA polymerase III transcription factors in cancer (134). Furthermore, increased RNA polymerase III transcription during simian virus 40 (SV40) induced transformation of mouse cells was ascribed to an increase in the level of the Bdp1 component of TFIIIB (24). Also, the SNAP43 and SNAP45 subunits of the snRNA specific $SNAP_C$ are overexpressed in certain tumors, indicating that snRNA genes with a requirement for $SNAP_C$ might also be deregulated in these cancers (102).

Elevated levels of RNA polymerase III transcripts have recently been associated with the perinucleolar compartment (PNC). The PNC is usually enriched in a subset of RNA polymerase III transcribed genes such as the 7SL RNA, H1, MRP, and Y RNA (27, 71, 85, 128) but not tRNA, 5S rRNA, or U6 snRNA (85). Although the exact function of the PNC is not clear, it is possible that the PNC functions in trafficking of a subset of newly synthesized RNA. Moreover, continuous transcription of these RNA polymerase III products is necessary for maintaining the structural integrity of the PNC. Interestingly, an increase in the number of PNCs was observed in transformed cells with metastatic advantage (64). As an increase in RNA polymerase III transcription is observed during transformation (131), it is possible to use these transcripts and PNCs as a prognostic marker to assess the stage of the cancer.

Several reports link the function of RNA polymerase III transcribed genes to many disease conditions. For example, mutations in the MRP RNA gene have been implicated as the cause of human cartilage-hair hypoplasia (CHH). CHH is a recessively inherited disorder that is characterized by disproportionate short stature, defective cellular immunity, and a predisposition to several cancers (104). The MRP RNA normally functions in 5.8S rRNA processing and in yeast also controls exit from mitosis. Real-time

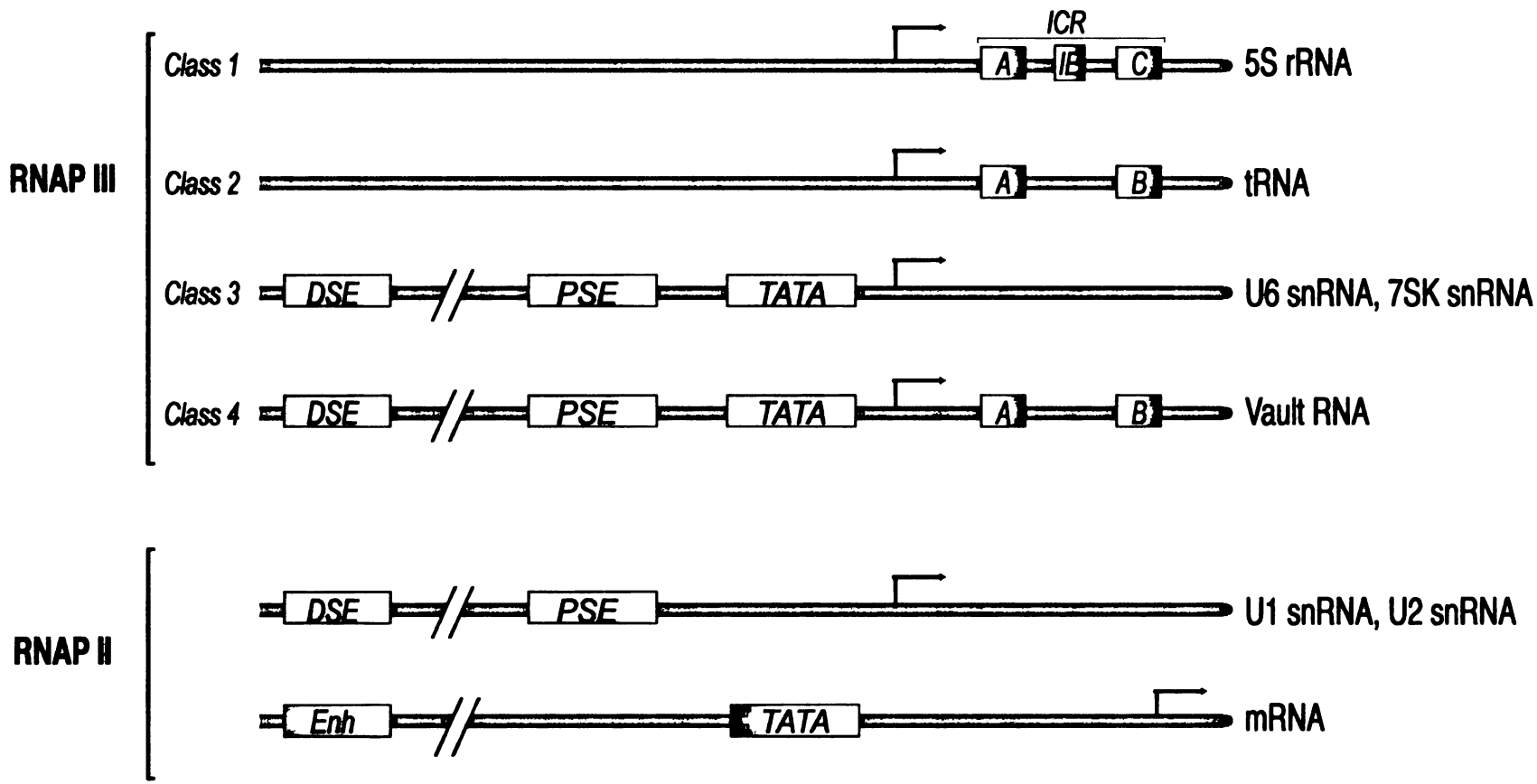
PCR analysis showed a reduction in the expression of MRP RNA in CHH patients, in some cases due to mutations located in the promoter region. Other mutations located in the transcribed region might cause decreased RNA stability. Nonetheless, the ratio of unprocessed 5.8S rRNA compared to processed 5.8S rRNA was altered when MRP mutations mimicking those found in CHH patients were introduced into the yeast orthologue of MRP. Moreover, micro-array analysis of leukocytes obtained from CHH patients showed an increase in the levels of several cytokines and cell cycle regulatory genes (46). Thus, these results suggest that CHH symptoms might be caused by a compromise in MRP RNA function.

3. Mechanisms of regulation of RNA polymerase III transcription- Promoter structure

Because the products of RNA polymerase III transcribed genes function in numerous cellular processes, RNA polymerase III regulation is critical to maintain cellular homeostasis. The first level of regulation is dependent upon the unique promoter structures of RNA polymerase III transcribed genes. As illustrated in Figure 1-1, these genes are grouped into four different classes. 5S rRNA genes (class 1) as well as tRNA and the Adenovirus (Ad) VAI genes (class 2) use intragenic promoter elements. 5S rRNA promoters contain an A box, an intermediate element (IE), and a C box that is conserved in the 5S promoters across species. Together, these sequence elements constitute the internal control region (ICR) that is required for transcription (48, 98, 111). tRNA promoters consists of an A and B box (26, 48, 111).

Figure 1-1. Schematic representation of the promoter elements of genes transcribed by RNA polymerase III and II.

RNA polymerase III transcribed genes can be grouped into four classes. Class 1 and class 2 genes such as 5S rRNA and tRNA, respectively contain intragenic promoter elements. Class 1 genes contain the A and C boxes that are separated by an internal element (IE). Together these three elements constitute the internal control element (ICR). Class 2 genes contain an A box and a B box. Class 3 genes exemplified by U6 and 7SK snRNA contain extragenic promoter elements including the distal sequence element (DSE), a proximal sequence element (PSE) and a TATA box. The promoter architecture of these genes is similar to other snRNA genes that are transcribed by RNA polymerase II such as U1, and U2 although these genes lack a TATA box, unlike the prototypical mRNA genes also transcribed by RNA polymerase II. The class 4 genes exemplified by Vault RNA contain both intragenic as well as extragenic promoter elements.


RNAP III
Class 1
ICR
A
IE
C
5S rRNA
Class 2
A
B
tRNA
Class 3
DSE
PSE
TATA
U6 snRNA, 7SK snRNA
Class 4
DSE
PSE
TATA
A
B
Vault RNA
RNAP II
DSE
PSE
U1 snRNA, U2 snRNA
Enh
TATA
mRNA

In contrast to the class 1 and 2 gene promoters, the class 3 promoters contain extragenic promoter elements. Examples of class 3 genes include U6 and 7SK snRNA genes. These genes are grouped into a family of genes called the snRNA gene family. Interestingly, some human snRNA genes such as U6 are transcribed by RNA polymerase III while other human snRNA genes such as U1 are transcribed by RNA polymerase II, although both function in the same spliceosomal complex. snRNA gene promoters contain a distal sequence element (DSE) and a proximal sequence element (PSE), regardless of RNA polymerase specificity. The DSE contains an octamer motif, which activates transcription from the core promoter, and is located in the regulatory region around -220 bp upstream of the transcription start site. An adjacent *SphI* postoctamer homology (SPH) motif also stimulates snRNA transcription (68). The essential PSE is located within the core promoter region at approximately -45 bp upstream of the transcription start site. Those snRNA genes transcribed by RNA polymerase III also contain a TATA box at position -25 bp (49, 69, 77). Interestingly, in vertebrate snRNA promoters, the TATA box is a crucial element for determining RNA polymerase III specificity (77). Thus, due to their similar promoter elements the snRNA gene family is a good model system to compare and contrast the mechanisms of RNA polymerase preference and activity during normal and deregulated growth.

RNA polymerase III transcribes yet another category of genes with hybrid promoter elements. These class 4 genes contain intragenic as well as extragenic promoter elements. In the case of vault RNA, selenocysteine tRNA, and the Epstein-Barr viral product EBER2 the promoter contains gene-external PSEs as well as gene-internal control elements (56, 57, 65, 96, 124, 126). Additionally, the U6 snRNA-like H1 RNA

gene promoter contains an internal A-box but not B-box homology region (1). Like H1 RNA, the promoters for the MRP, Y1 and Y3 genes also contain DSE, PSE, and a TATA-box elements, in addition to gene-internal A box. However, mutational studies indicate that the upstream sequences of the gene encoding MRP RNA are required for transcription in HeLa cell extracts, whereas internal sequences are not (123, 142).

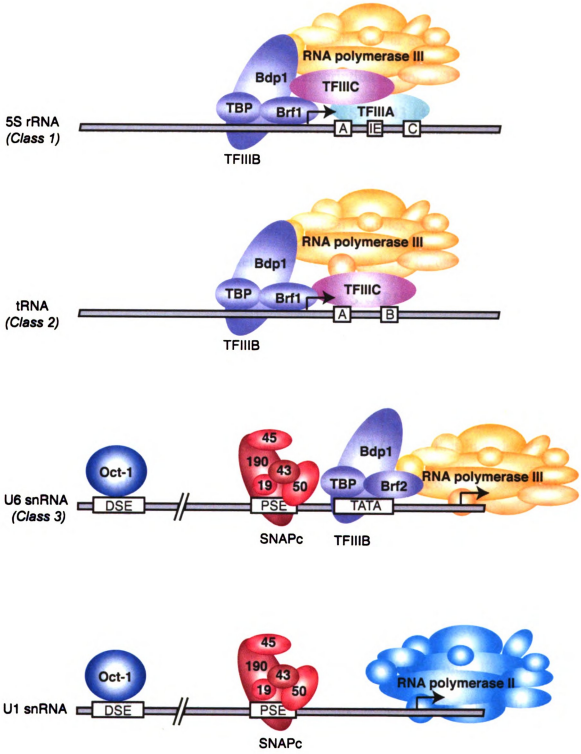
4. Mechanisms of regulation of RNA polymerase III transcription- General transcription machinery

The different classes of RNA polymerase III gene promoters recruit distinct sets of well characterized basal transcription factors as illustrated in Figure 1-2. These factors specialize in nucleating unique preinitiation complexes that ultimately converge on RNA polymerase III recruitment.

In class 1 promoters (5S rRNA), the ICR is recognized by TFIIIA, which is a founding member of the C2H2 zinc finger family of DNA binding proteins (88, 122). The binding of TFIIIA is followed by recruitment of TFIIIC (48, 111). In contrast to class 1 promoters, the A and B boxes of class 2 genes (tRNA) are directly recognized by TFIIIC without prior binding by TFIIIA (48, 111). Thus, TFIIIA acts as a specificity factor that influences promoter recognition function of TFIIIC, and directs TFIIIC to the 5S promoter. In both class 1 and class 2 promoters, TFIIIC binding is followed by recruitment of the TBP containing Brf1-TFIIIB complex, which in addition to TBP, consists of Brf1 and Bdp1 (111). Thus, TFIIIA and TFIIIC can be considered as recruitment factors that function to engage TFIIIB to promoters of different structures allowing the subsequent recruitment of RNA polymerase III.

Figure 1-2. The general transcription machinery required for RNA polymerase III transcription.

RNA polymerase III transcribed Class 1 and 2 genes both require TFIIIC and TFIIIB. The TFIIIB used by these genes is composed of Brf1, Bdp1, and the TATA-box binding protein (TBP). In addition, Class 1 genes additionally require TFIIIA. For Class 3 genes, the transcriptional activator protein, Oct-1, binds the DSE. The PSE is recognized by $SNAP_C$, which consists of at least SNAP19, SNAP43, SNAP45, SNAP50 and SNAP190. The TATA-box is recognized by TFIIIB, which is composed of Brf2, Bdp1, and TBP. The snRNA genes transcribed by RNA polymerase II, also require Oct-1 and $SNAP_C$.


RNA polymerase III
Bdp1
TFIIIC
TBP
Brf1
TFIIIA
5S rRNA
(Class 1)
A
IE
C
TFIIIB
RNA polymerase III
Bdp1
TBP
Brf1
TFIIIC
tRNA
(Class 2)
A
B
TFIIIB
45
190
43
19
50
Oct-1
Bdp1
TBP
Brf2
RNA polymerase III
U6 snRNA
(Class 3)
DSE
PSE
TATA
SNAPc
TFIIIB
45
190
43
19
50
Oct-1
RNA polymerase II
U1 snRNA
DSE
PSE
SNAPc

The PSE of class 3 RNA polymerase III transcribed as well as RNA polymerase II transcribed snRNA genes is recognized by the small nuclear activating protein complex ($SNAP_C$) (108), also known as the PSE transcription factor (PTF) (93). $SNAP_C$ is composed of at least five subunits, SNAP19 (43), SNAP43 (44, 140), SNAP45 (107, 140), SNAP50 (2, 42), and SNAP190 (135). SNAP190 interacts with all the other $SNAP_C$ subunits and acts as a central scaffold for the complex (79). Promoter recognition by $SNAP_C$ is a crucial early event during preinitiation complex assembly and therefore it might be a target for regulatory proteins. Several studies have focused on understanding the mechanism of DNA binding by $SNAP_C$. The SNAP190 subunit of $SNAP_C$ was initially shown to be in close contact with DNA in UV cross-linking experiments (139). Indeed, subsequent experiments showed that SNAP190 mediates specific DNA binding by $SNAP_C$ via a Myb- like DNA binding domain within its N-terminal region (135). A minimal $SNAP_C$ (mini-$SNAP_C$) consisting of SNAP190 (1-505) that includes its DNA binding domain, along with SNAP50, and SNAP43 has been shown to be sufficient for specific DNA binding (90) and transcription by RNA polymerase III and II *in vitro* (37). However, DNA binding by mini-$SNAP_C$ requires all three subunits (37, 51), suggesting that the SNAP190 DNA binding domain is required, but not sufficient. Consistent with the requirement of other subunits for DNA binding, SNAP50 was shown to be close to DNA in UV cross-linking experiments, indicating that SNAP50 most likely provides additional DNA contacts (42). Further evidence supporting a role for SNAP50 in DNA binding by SNAPC is discussed in Chapter 3. Interestingly, unlike the mini-$SNAP_C$ that binds DNA efficiently, the endogenous $SNAP_C$ binds poorly to DNA on its own. On a TATA-box containing U6 promoter, $SNAP_C$ is efficiently recruited to the DNA via at

least two cooperative binding interactions; one with Oct-1 and the other with TBP, such that each factor binds to its cognate promoter element [reviewed in (111)]. $SNAP_C$ aids recruitment of the TBP containing Brf2-TFIIIB complex, which in addition to TBP consists of Brf2 and Bdp1, thus nucleating RNA polymerase III- specific preinitiation complex assembly (108, 112). In contrast, on TATA-less snRNA promoters (U1 snRNA), $SNAP_C$ participates in RNA polymerase II-specific preinitiation complex assembly with TBP and other general transcription factors including TFIIA, TFIIB, TFIIE, and TFIIF (67). Thus, in contrast to most promoter recognition complexes that function in transcription by a single class of RNA polymerase, $SNAP_C$ is the most functionally versatile due to its role in both RNA polymerase III and II systems.

5. Mechanisms of regulation of RNA polymerase III transcription- Regulatory factors

While the general transcription factors required for RNA polymerase III transcription are well characterized much less is known about the factors that modulate RNA polymerase III transcription. This section describes some of the transcription factors, kinases, and tumor suppressor proteins that have been shown to regulate RNA polymerase III transcription.

5.1. Factors that positively regulate transcription

Oct-1 & STAF- As mentioned earlier, all human snRNA genes contain a DSE, which is recognized by the transcriptional activator protein Oct-1 (47, 48, 111). Invariably the DSE contains a SPH element, which is recognized by a C2H2 type zinc finger containing transcription factor called STAF (also known as the SPH binding factor

or SBF) (111). Chromatin immunoprecipitation (ChIP) experiments provide evidence that STAF binds to the DSE elements of both RNA polymerase II and III transcribed snRNA genes (80). Several experiments have established a role for Oct-1 as an activator of snRNA gene transcription. Oct-1 has been localized to snRNA promoter sequences *in vivo* by ChIP (145). The C-terminal region of SNAP190 inhibits $SNAP_C$ DNA binding whereas the Oct-1 POU domain relives this auto-inhibition through direct protein-protein interactions with SNAP190. This interaction allows the cooperative binding of Oct-1 and $SNAP_C$ to the DSE and PSE, respectively during activated transcription of human snRNA genes by both RNA polymerase III and II (25, 55, 89, 90). Interestingly, mapping of DNase I and micrococcal nuclease cleavage sites in chromatin suggested that the natural U6 and U1 promoters harbor a positioned nucleosome between the DSE and the PSE. Positioning of the nucleosome at this location was further confirmed using an in vitro chromatin assembly assay (4, 119, 145). The correct positioning of the nucleosome is suggested to place these two promoter elements into proximity such that Oct-1 and $SNAP_C$ can cooperatively bind to the DSE and PSE, respectively, to facilitate transcriptional activation (145). Thus, this is an example where a nucleosome is a functional component of the transcriptional activation process instead of causing repression.

Myc- The Myc oncoprotein is a transcription factor that is upregulated in many types of cancers. Among other functions, Myc regulates cell growth and division (17, 18). Endogenous Myc occupies 5S rRNA, $tRNA^{Tyr}$, and $tRNA^{Leu}$ genes, and stimulates RNA polymerase III transcription of these genes. These genes lack the E-box that is

characteristically recognized by Myc in its RNA polymerase I- and II-transcribed target genes, and thus it is thought that Myc is recruited instead via direct protein-protein interactions with Brf1-TFIIIB. It is postulated that the Myc-TFIIIB interaction recruits coactivators and chromatin-modifying activities, or abrogates the effects of negative regulators of RNA polymerase III transcription to stimulate transcription (29). In subsequent experiments the same group of researchers used an inducible Myc construct to show that a Myc/TFIIIB complex, that also contained the HAT GCN5 and the cofactor TRRAP, is recruited rapidly to the tRNA promoter. Concurrently, they observed hyperacetylation of promoter-proximal histone H3, but not H4, followed by RNA polymerase III recruitment. This observation is in contrast to the c-Myc activation of RNA polymerase II transcription, during which histone H4 acetylation is predominant (Robert J. White, personal communication). It should be noted that the U6 snRNA gene transcribed by RNA polymerase III does contain an E-box sequence upstream of the DSE; however, the role of the E-box sequence or Myc for U6 snRNA transcription is not yet known.

HEXIM1- Hexamethylene bis-acetamide inducible 1 protein (HEXIM1) was recently shown to interact with the RNA polymerase III transcribed 7SK snRNA in transient transfection experiments. 7SK RNA containing various deletions was expressed in human cells and the bound protein was identified by co-immunoprecipitation with a HEXIM1 specific antibody (20). Interestingly, HEXIM1 was also found to co-purify with endogenous $SNAP_C$, which is a transcription factor that recognizes the PSE in the 7SK snRNA gene promoter. Moreover, HEXIM1 was cross-linked to DNA in a PSE specific

manner (R. William Henry, unpublished results). These two observations suggested that HEXIM1 influences the DNA binding properties of $SNAP_C$ to potentially regulate transcription of its interacting partner 7SK snRNA. The role of HEXIM1 in the regulation of 7SK snRNA transcription was further investigated and the results are discussed in Chapter 4.

The HEXIM1/7SK snRNA ribonucloprotein complex can inhibit RNA polymerase II elongation by targeting the P-TEFb complex (87, 138). P-TEFb consists of cyclin T1 and cdk9 that phosphorylates the CTD of RNA polymerase II thus facilitating promoter clearance and subsequent transcription elongation of most mRNA genes and HIV-1 transcription (101). In the current model for P-TEFb inhibition, phosphorylation of the cdk9 component of P-TEFb at a key tyrosine residue at position 186 within the T-loop, that is required for P-TEFb kinase activity, is also required to associate with the HEXIM1/7SK RNP. Thus, the primed active form of P-TEFb is held inactive by the 7SK snRNP. The association between HEXIM1/7SK snRNP and P-TEFb is disrupted by various stress inducing agents including DNA damaging UV irradiation thereby allowing transcription of genes regulated by P-TEFb. In addition to transcription of cellular genes, P-TEFb is also required for HIV-1 transcription and replication. Interestingly, recent evidence suggests that HEXIM1 competes with the HIV-1 Tat protein for binding to the cyclin T1 component of P-TEFb thus decreasing levels of the active P-TEFb and interfering with HIV-1 transcription (113).

5.2. Factors that positively and negatively regulate transcription

Protein Kinase CK 2- Casein kinase 2 (CK2) is a highly conserved serine/threonine protein kinase which is implicated in the regulation of cellular growth (86, 99, 100), and deregulated expression of CK2 may lead to tumor progression (121). Several studies have indicated a role for CK2 in regulating RNA polymerase III transcription. For example, inhibition of CK2 in S phase extracts debilitated transcription, while inhibition of CK2 in M phase extracts restored transcription, suggesting that depending upon the phase of the cell cycle, CK2 is can activate transcription (S phase) or inhibit transcription (M phase). A positive role for CK2 was indicated due to the observation that both AdVAI, as well as U6 snRNA transcription were abolished in the presence of a CK2 substrate peptide, when extracts from S-phase cells were used to support transcription, indicating that CK2 is essential for transcription (58). The exact mechanism of CK2 regulation in the S phase is not yet known. However, CK2 phosphorylation of purified RNA polymerase III was essential for active transcription (59). Taken together these observations suggested a mechanism in which CK2 is proposed to target RNA polymerase III or a tightly associated factor to activate transcription. In contrast, AdVAI as well as U6 snRNA transcription was restored only in the presence of Bdp1 and a CK2 substrate peptide when mitotic cell extracts were used to support transcription. The restoration of transcription was not observed by other components of the RNA polymerase III preinitiation complex, such as TBP or by the polymerase itself, indicating that CK2 targets Bdp1 specifically to inhibit transcription during the M phase. Furthermore, CK2 phosphorylation of Bdp1 was shown to cause disassociation of Bdp1 from both tRNA as well as U6 snRNA promoters (22, 58). In yet

another report, CK2 also had a positive role in RNA polymerase III transcription by enhancing preinitiation complex assembly between Brf1-TFIIIB and TFIIIC at tRNA promoters (62). In addition to regulating TFIIIB, CK2 has recently been shown to regulate $SNAP_C$, a transcription factor that is required for snRNA gene transcription. Phosphorylation of SNAP190 by casein kinase 2 (CK2) abrogates DNA binding by $SNAP_C$; however, promoter recognition can be restored by cooperative binding of TBP to RNA polymerase III transcribed U6 snRNA promoter sequences containing both a PSE and a TATA-box, but not to RNA polymerase II transcribed U1 snRNA promoter sequences lacking a TATA-box (35). This observation suggests that post-translational modification of $SNAP_C$ by CK2 alters its DNA binding properties even though $SNAP_C$ is required for transcription by both RNA polymerase III and II. Thus, the snRNA gene family is a good model to compare and contrast the mechanism(s) of CK2- mediated regulation of gene transcription.

5.3. Factors that negatively influence transcription

p53 tumor suppressor protein- p53 is one of the most intensively studied tumor suppressor proteins due to its role in tumor suppression and the fact that the normal function of p53 is lost in almost all cancers in vertebrates (54). p53 plays a pivotal role in the DNA damage response pathway. RNA polymerase III transcription is regulated in response to DNA damage caused by UV irradiation. For example, transcription of U6 snRNA (and RNA polymerase II transcribed U1 snRNA) decreases in response to UV irradiation as measured by nuclear run-on assay. Interestingly, this effect was observed in MCF-7 cells that contain functional p53, but not in HeLa cells in which p53 is inactivated

by papillomavirus E6 protein. This observation suggested a role for p53 in regulation of these genes (7, 12, 34). Indeed, p53 can repress in vitro transcription of both U6 snRNA and 5S rRNA genes by RNA polymerase III (127). A direct role of p53 in repressing RNA polymerase III transcription is indicated by the observation that p53 can interact with the general transcription factors, TFIIIB and $SNAP_C$, required for RNA polymerase III transcription (15, 34, 118). p53 interaction with the TBP component of TFIIIB interferes with Brf1-TFIIIB promoter association at 5S rRNA and tRNA promoters resulting in a failure to assemble the preinitiation complex. However, the consequence of p53 interaction with $SNAP_C$ for preinitiation complex formation on U6 snRNA promoter is not yet known. Nonetheless, p53 can repress snRNA gene transcription by RNA polymerase II and III in response to DNA damage (34)

Chromosomal breakpoints referred to as fragile sites, often found in cancers, are formed at the RNA polymerase III transcribed 5S rRNA gene loci in addition to the RNA polymerase II transcribed U1 and U2 snRNA gene loci during Adenovirus serotype 12 (Ad12 infection). Interestingly, while p53 is required for Ad12-induced fragility at these sites (75), Cockayne syndrome group B (CSB) cells that fail to express functional CSB protein also contain these same fragile sites (141), thus suggesting an interplay between p53 and CSB. CSB is a DNA repair protein shown to play a role in the transcription coupled repair (TCR) pathway by recruiting the nucleotide excision repair apparatus in response to DNA damage (76). In addition, CSB is also thought to play a role in elongation by permitting resumption of elongation by polymerase that had been stalled due to highly structured RNA, such as RNA polymerase II-transcribed U1 and U2 snRNAs (141). A role for CSB in RNA polymerase III transcription is suggested by

recent ChIP experiments indicating that CSB occupies a variety of RNA polymerase III target genes, including 5S rRNA, U6 snRNA, 7SK snRNA, MRP, and Y RNA (Gridasova A.A. et. al. unpublished results). Interestingly, CSB dissociates from these gene promoters upon UV irradiation concomitant with an increased p53 promoter association. Moreover, in vitro recruitment assays show that p53 might displace CSB from promoters during repression of RNA polymerase III transcribed genes. These observations suggest an antagonistic relationship between p53 and CSB for regulating RNA polymerase III transcription (Gridasova A.A. et. al. unpublished results).

Retinoblastoma tumor suppressor protein- The Retinoblastoma (RB) susceptibility gene encodes a nuclear phosphoprotein that was originally isolated due to its frequent mutations observed in retinoblastoma, a rare pediatric tumor of the retina (72). Subsequently, mutations in the gene encoding the RB protein have been implicated in approximately 30% of all human tumors including osteosarcomas, small-cell lung carcinomas, cervical carcinomas, prostate carcinomas, breast carcinomas, and some forms of leukemia (91). Ectopic expression of RB in RB -/- cells obtained from various tumors suppressed growth and proliferation, soft agar colony formation, and tumorigenicity in nude mice (60, 103). Thus, RB has the credentials of a tumor suppressor protein. It is suggested that RB regulation of RNA polymerase III transcription may contribute to the growth suppressive function of RB.

The growth-suppressive function of RB is dependent on the pocket region, which extends from amino acids 379-792 and can be further subdivided into the A and B pocket. The A/B pocket is required but not sufficient for tumor suppression whereas the

C-terminal region of RB provides additional functions that are critical for tumor suppression. Indeed, a larger region of RB containing the A/B pocket and the C-terminal region is sufficient for regulation and tumor suppressor function because this same region rescued the lethal phenotype when reintroduced into RB-/- mice, thus allowing the mice to develop normally (103, 136). The activity of RB as a transcription factor is critical for its role in tumor suppression function. Interestingly, the same A/B pocket and the C-terminal region of RB that is required for its tumor suppressor function is also required to repress RNA polymerase III transcription *in vitro* (53, 133). In contrast, the A/B pocket alone is sufficient for RB repression of prototypical E2F-regulated RNA polymerase II transcribed target genes (13). These observations indicate that the tumor suppressor function of RB might be linked to its ability to regulate products of RNA polymerase III transcription. Indeed, RNA polymerase III transcription was elevated in RB -/- SAOS2 osteosarcoma cells compared to RB +/+ U2OS cells, while RNA polymerase II transcription remained largely unaffected. In a more rigorous test of endogenous RB function, a 5 fold increase in transcription of some RNA polymerase III genes was observed in nuclear run-on assays performed using primary embryonic fibroblast cells derived from RB-knockout mice compared to equivalent cells from wild type mice (70, 133). RB has been shown to inhibit the synthesis of 5S rRNA, tRNA, and U6 snRNA transcription by RNA polymerase III both in vivo and in vitro (52, 133). Thus, RB appears to be a global repressor of RNA polymerase III-transcribed genes.

Models of RB repression

Although RB is a global repressor of genes transcribed by RNA polymerase III, these genes contain diverse promoter elements and distinct general transcription machinery. How then does RB bring about repression of RNA polymerase III transcription? As discussed in this section, RB utilizes distinct mechanisms to repress RNA polymerase III transcription.

RNA polymerase III transcription of 5S rRNA (class1) and tRNA (class 2) genes utilize the Brf1-TFIIIB general transcription factor. TFIIIB is an initiation factor that is recruited to the promoter of these genes by the TFIIIC complex, which directly recognizes these promoters (112). RB targets the Brf1 component of TFIIIB complex, thereby disrupting the TFIIIB-TFIIIC interactions. Thus, RB prevents preinitiation complex assembly and subsequent recruitment of RNA polymerase III to the promoter (70, 120). This mechanism of RB repression is similar to that used by RB to inhibit transcription of RNA polymerase II transcribed, E2F-stimulated target genes such as DHFR, TK, cyclin A, cyclin E, cdk2, cdc2 that are important for cell cycle progression. In the E2F model, RB stably associates with the promoter via protein-protein interactions with E2F and represses transcription by disrupting the TFIID-TFIIA complex thereby blocking preinitiation complex assembly and subsequent RNA polymerase II recruitment (5, 39, 40, 50, 106).

In contrast to the mechanism used by RB to repress RNA polymerase III transcribed class 1and 2 genes, RB utilizes a novel mechanism to repress U6 snRNA (class 3) transcription by RNA polymerase III. RB specifically accesses the U6 snRNA promoter via protein-protein interactions with components of $SNAP_C$ and TFIIIB and

stably associates with the U6 snRNA promoter both in vitro and in vivo. This aspect of repression is similar to E2F where RB targets the RNA polymerase II transcription factor, E2F, for stable promoter association. RB, $SNAP_C$ and TFIIIB co-occupy the same U6 snRNA promoter in vivo as demonstrated by sequential ChIP experiments. Interestingly, RB and RNA polymerase III concurrently occupy a human U6 snRNA promoter during repression (53), in contrast to the E2F model wherein RB disrupts RNA polymerase II recruitment (9, 41, 61, 129). Our results suggest that RB inhibits steps subsequent to RNA polymerase III recruitment during U6 repression. For example, RB might target promoter escape, open complex formation, first dinucleotide bond formation, or elongation. *In vitro* $KMnO_4$ sensitivity assays performed in our laboratory indicate that RB might trap RNA polymerase III in an open complex and facilitate a DNA modification event, presumably methylation, to inhibit transcription (T. Selvakumar, unpublished results). Thus, it is possible that RB recruits corepressor(s) to the U6 snRNA promoter to repress transcription. It is intriguing to note that despite the same cohort of transcription factors, p53 disrupts RNA polymerase III recruitment during U6 repression (Gridasova, AA et. al. unpublished data). Thus, more insight into the tumor suppression function of RB and p53 can be obtained by comparing and contrasting the mechanisms used by these proteins to repress human U6 snRNA transcription.

While not much is known about the RB co-repressor proteins for regulating RNA polymerase III transcription, several corepressor protein partners have been associated with RB mediated repression of the E2F-stimulated RNA polymerase II target genes. RB interacts with co-repressors that can control the chromatin structure, and therefore, repress transcription by altering the state of chromatin surrounding a gene promoter. The

altered state of chromatin in turn might further limit access of transcription factors to gene promoters and ablate transcription. Emphasis herein is given only to a subset of these proteins that are pertinent to this document. Among other proteins, RB interacts with the ATP-dependent chromatin remodeling complex SWI/SNF that repositions promoter proximal nucleosomes to create repressive barriers within gene promoters, or to prevent DNA unwinding required for transcription factor binding (38, 144). RB has also been shown to associate with chromatin modifying proteins, including the class 1 histone deacetylases (HDACs) consisting of HDAC1, HDAC2, and HDAC3 (16). Deacetylation of histones was observed on E2F-regulated target gene promoters in correlation with RB repression. Furthermore, RB-mediated repression was inhibited when TSA, a HDAC-specific inhibitor, was used in the transcription assays (6, 78, 82, 143, 144). HDACs remove acetyl groups from lysine residues in histone tails resulting in condensation of chromatin. This altered state might obstruct transcription factors from accessing promoter elements or inhibit DNA unwinding required for transcription. Interestingly, RB has been associated with both HDAC and SWI/SNF activities as part of the same complex during repression. For example, studies indicate that RB recruits both HDACs and SWI/SNF as part of the same complex to regulate exit from G1. Subsequent disassembly of HDAC activity is then thought to allow the RB-SWI/SNF containing complex to regulate exit from S phase of the cell cycle (144). Interestingly, in addition to causing chromatin condensation to hinder transcription factor access to promoter DNA, RB recruited HDAC proteins may initiate a relay of histone modifications leading to transcriptional repression. For example, deacetylation of histone H3 at lysine residue 9 (H3K9) would facilitate the subsequent methylation of H3K9 by the histone methyl transferase SUV39H1. The

methylated H3K9 would in turn facilitate binding of the heterochromatin protein 1 (HP1) (146). Indeed, RB has been shown to associate with SUV39H1 and the methyl lysine binding protein HP1 in co-immunoprecipitation experiments, and use the concerted activities of SUV39H1 and HP1 to repress cyclin E expression (95, 125). Contrary to its role in promoting heterochromatic silencing in the context of RB-mediated repression, HP1 is thought to mask the methylated H3K9 such that it cannot be demethylated and subsequently acetylated. Additionally, RB was also associated with DNA methyl transferase 1 (DNMT1) in a complex that also included HDAC1 and E2F1, during the chromatographic purification of DNMT1. DNMT1 methylates cytosines in CpG islands in the promoter region of target genes to impede transcription factor access. Interestingly, the methyltransferase activity of DNMT1 was partially abolished in the presence of TrichostatinA (TSA), which is a HDAC inhibitor, suggesting that RB can engage the concerted activities of DNMT1 and HDACs for repression (105). Indeed it is suggested that the methylated CpGs are bound by methyl DNA binding protein MBD, which in turn can help coordinates HDAC activity to repress transcription (63). While RB can utilize a constellation of co-repressor proteins as part of a repressor complex, a common theme that emerges is that HDACs are almost always present in these complexes.

Although not much is known about corepressor proteins that RB might engage to repress RNA polymerase III transcription, human U6 snRNA gene transcription is a good model system to investigate the putative RB corepressor protein partners. As RB can stably occupy a U6 snRNA promoter (53), it allows us to study the mechanistic order of events that occur during RB repression. Chapter 2 describes the studies I performed to

investigate the role of RB co-factors in RB-mediated repression of U6 snRNA transcription.

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CHAPTER 2

RB RECRUITS HDAC ACTIVITY FOR REPRESSION OF HUMAN U6 snRNA TRANSCRIPTION BY RNA POLYMERASE III[1]

Abstract

The Retinoblastoma (RB) tumor suppressor protein is a global repressor of RNA polymerase III transcription. We have previously shown that RB, components of the preinitiation complex and RNA polymerase III co-occupy a U6 snRNA promoter during repression. To further elucidate the molecular mechanism underlying RB mediated repression of U6 snRNA transcription, the role of putative RB co-factors was examined. In this study I show that endogenous RB, HDACs and SWI/SNF chromatin remodeling proteins occupy a human U6 snRNA promoter. Interestingly, RB stimulates the binding to HDAC2 and Brg1 to a U6 promoter during repression. Importantly, HDAC enzymatic activity is required for RB repression of U6 transcription initiated from chromatin-assembled DNA but not from naked DNA templates. These results suggest that RB repression of U6 transcription is a multi-step process in which a HDAC2-dependent step(s) is required during repression of chromatin templates but not during the establishment of repression on naked DNA template. This biochemical separation of RB

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repression and HDAC activity will be a useful tool to understand the mechanistic order of events involved in this process.

Introduction

The gene encoding the Retinoblastoma (RB) tumor suppressor protein was identified due to its frequent mutation in the rare pediatric eye tumor, retinoblastoma. Subsequently, mutations in the retinoblastoma gene were also found in approximately 30% of all adult onset tumors including osteosarcomas, small-cell lung carcinomas, cervical carcinomas, prostate carcinomas, breast carcinomas, and some forms of leukemia (3, 7). Many studies over the years have established the credentials of RB as a tumor suppressor protein, and most support a central role for RB in constraining growth and proliferation (29). For example, overexpression of RB in RB -/- tumor cells suppressed growth and proliferation, soft agar colony formation, and tumorigenicity in nude mice, showing that RB restricts growth (12, 20). RB overexpression induces G1 arrest while the lack of RB in cells was correlated with accelerated G1 to S phase transition, suggesting that RB regulates cell cycle progression. RB is also implicated in other cellular functions such as DNA replication, DNA repair, apoptosis, and differentiation through its ability to repress E2F-stimulated transcription of target genes whose products influence these processes (2, 6, 7). Thus, RB function as a transcription factor is central for its tumor suppressor activity.

RNA polymerase III transcribed gene products are essential for cell growth and proliferation (8). Moreover, RB has been shown to be a global repressor of RNA polymerase III transcription. Indeed, we and others have shown that RB represses human

RNA polymerase III transcribed genes including 5S rRNA, tRNA, and U6 snRNA both in vivo and in vitro (10, 15, 30). For example, RNA polymerase III transcription of these genes was elevated in RB -/- SAOS2 human osteosarcoma cell line compared to RB +/+ U2OS human osteosarcoma cell line, while RNA polymerase II transcription remained largely unaffected (15, 30). As elevated RNA polymerase III activity and loss of RB function are both common features of transformed and tumor cells, it was proposed that RB regulates RNA polymerase III transcription to restrict growth. Recent studies have demonstrated that the A/B pocket domain and C-terminal region of RB (extending from amino acids 379-928), is required for efficient repression of RNA polymerase III transcription (11, 30). Interestingly, this same region of RB is also required for tumor suppressor function (20, 31). These observations indicate that repression of RNA polymerase III transcription by RB could contribute to its tumor suppressive potential.

While RB is a global repressor of RNA polymerase III transcription, RB utilizes distinct mechanisms of repression for different classes of genes. RNA polymerase III transcribed genes can be divided into four different classes. Class 1 and class 2 genes contain intragenic promoter elements exemplified by 5S rRNA and tRNA genes, respectively. In contrast, class 3 genes contain extragenic promoter elements exemplified by U6 snRNA gene. Class 4 genes contain both intragenic as well as extragenic promoter elements exemplified by the Vault RNA. The model for RB regulation of class 2 (tRNA) genes suggests that RB disrupts preinitiation complex assembly at the promoter. In this model, RB targets the Brf1-TFIIIB complex for repression. Brf1-TFIIIB complex contains the TATA-box binding protein (TBP) and Bdp1 in addition to Brf1, and is recruited to the promoter of these genes by the TFIIIC complex, which directly binds to

the promoter (23). RB-mediated disruption of the Brf1-TFIIB complex is proposed to abrogate recruitment of RNA polymerase III thereby repressing transcription (15, 26). RB utilizes a similar mechanism to repress RNA polymerase II transcribed genes, where RB interacts with the activator protein, E2F, and inhibits polymerase II recruitment (6, 7, 9, 22).

Unlike tRNA genes and E2F-regulated genes where RB prevents assembly of the preinitiation complex, RB utilizes a novel mechanism to repress U6 snRNA gene transcription by RNA polymerase III. At these genes, RB stably associates with the promoter, like E2F-mediated repression, but instead does not abrogate association of the general transcription machinery consisting of $SNAP_C$ and Brf2-TFIIIB, with the U6 promoter. Interestingly, RB and RNA polymerase III also simultaneously occupy a human U6 snRNA promoter in vivo and in vitro during repression (11). This last observation indicates that a third model of RB repression is required to explain RB repression at these genes wherein RB inhibits a step after RNA polymerase III recruitment, possibly affecting promoter escape, open complex formation, first di-nucleotide bond formation, or elongation. RB could inhibit these processes directly by interacting with RNA polymerase III; alternatively, RB could act indirectly by occluding binding of a critical transcription factor required for RNA polymerase III elongation or by recruiting a co-repressor(s) that inhibit RNA polymerase III function.

Several RB co-repressor proteins have been identified for RB repression of E2F-regulated genes transcribed by RNA polymerase II. For example, RB interacts with histone deacetylases (HDACs), and the SWI/SNF ATP-dependent chromatin remodeling complex that may reposition promoter proximal nucleosomes and affect access of

additional transcription factors (1, 5, 16, 17, 32). RB has been suggested to recruit multiple co-repressors as components of a single co-repressor complex. For example, a RB-HDAC-SWI/SNF complex regulates exit from the G1 phase of the cell cycle, while a RB-SWI/SNF complex regulates exit from the S phase (32). In addition to HDACs, RB also recruits methyltransferases, for example the histone methyltransferase SUV39H1, and DNA methyltransferase (DNMT) to E2F target genes (13, 18, 21, 28). RB is thought to utilize both histone deacetylase activity and SUV39H1 histone methylase activity sequentially to inhibit transcription of the cyclin E gene (34). These RB cofactors are also candidates as RB co-regulatory factors for RNA polymerase III transcription.

In this study, I have examined the role of RB co-factors in regulation of RNA polymerase III transcription. I found that the bona fide RB co-factors including HDAC2 and the Brg1 component of the SIW/SNF ATP-dependent chromatin remodeling complex occupy a human U6 promoter in cells that retain an intact RB pathway. Furthermore, RB stimulates association of HDAC2 and the Brg1 component of SWI/SNF with promoter DNA during repression of U6 transcription. Although RB can repress U6 transcription from both naked and chromatin DNA templates, the HDAC enzymatic activity is required for RB repression only when the U6 transcription template is assembled into chromatin, but not when the template is non-nucleosomal. These results suggest that RB repression of U6 transcription may be a multi-step process in which a HDAC2 dependent step(s) is not required during the establishment of repression on naked DNA template but is required during repression of chromatin templates.

Materials and Methods

Chromatin immunoprecipitation.

Human 184B5 cells and HeLa cells were grown to approximately 75% confluence and were then crosslinked with formaldehyde for 30 minutes. Chromatin was harvested as described before (11). 100 μL of soluble chromatin corresponding to approximately 1x x 10^7 cells was used per immunoprecipitation reaction (total volume 1 mL) along with the following antibodies: 2 μL of anti-$SNAP_C$ (CS143 Term Bleed), anti-TBP (SL2), or preimmune serum, or 1 μg of anti-RB (SC-7872), anti-HDAC1 (SC-7899), anti-HDAC2 (SC-7872), anti-Brg1 (SC-10768), anti-BRM (SC-6450), anti-CBP (SC-A22),α-p300 (SC-N15), anti-H3 (Abcam-ab1791), anti-AcH3 (Lys9/Lys14, Upstate) or 2 μg goat immunoglobin (IgG). After overnight incubation at 4°C, proteins G agarose beads were added for 3 hours at 4°C. After several washes, protein complexes were eluted in 300 μl of 0.1 M $NaHCO_3$ + 1% SDS for 30 minutes at room temperature. Cross-links were reversed overnight at 65°C and the recovered chromatin was suspended in 50 μL of TE buffer.

Sequential ChIPs were performed as described previously (11). Soluble chromatin was prepared from 184B5 cells as described above. 500 μL of chromatin, which is equivalent to 5 x 10^7 cells was used in a total volume of 5 mL for each of the primary immunoprecipitation reactions done with 5 μL of α-RB (MI170 Test Bleed 2), α-RNA polymerase III (MI172 Test Bleed 2) or preimmune sera. After 1 hour at room temperature, the immunoprecipitated material was collected using Protein G agarose beads for an additional hour at room temperature. The beads were then washed as described above. Next, immunoprecipitated material was released from the beads by

elution in a buffer containing 15 mM DTT. Subsequently, one-sixth of the eluted material was used in the second round of immunoprecipitation reaction. The immunoprecipitated material was then bound to protein G agarose beads and subsequently eluted in a buffer containing 0.1M $NaHCO_3$ + 1% SDS. After reversing the cross-links, the immunoprecipiated material was treated with Proteinase K, phenol extracted and ethanol precipitated. The recovered DNA was suspended in 50 μL of TE buffer. PCR analysis was performed using 5 μL of each immunoprecipitated sample or input chromatin. PCR products were separated by 2% TBE-agarose electrophoresis, stained with ethidium bromide, and visualized using Kodak imaging software.

The primers used to amplify each gene were:

U6 forward - 5'-GTACAAAATACGTGACGTAGAAAG-3'

U6 reverse – 5'-GGTGTTTCGTCCTTTCCAC-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' and

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

Protein purification and expression.

Recombinant GST-RB (379-928) was expressed and purified as described before (10).

Immunoprecipitation from an in vitro transcription reaction mixture.

In vitro transcription repression of a U6 promoter plasmid was performed as described before in the presence of GST-RB (379-928) or GST (10). One-third of the reaction was processed for RNase T1 protection to measure transcription. The remaining two-thirds was diluted to 1 mL with ChIP dilution buffer, cross-linked in 1% formaldehyde for 15 min at room temperature and subsequently quenched with 0.125 M glycine for 10 min at room temperature. Ten μL of the cross-linked material was used for immunoprecipitation reactions with preimmune sera or anti-RB (SC-7872), anti-HDAC1, anti-HDAC2, anti-BRM, and anti-Brg1 antibodies as above. Recovered plasmid DNA was analyzed by PCR using primers specific to the U6 promoter region. The sequence of the primers was described before (10).

In vitro repression assay of naked and chromatinized U6 template.

In vitro transcription repression of a naked U6 promoter plasmid was performed as described before in the presence of GST-RB (379-928) or GST (10), except 6 μL of HeLa cell nuclear extract was used to program transcription. U6 template DNA was chromatinized using the protocols described before (14, 33). For the experiment shown in Figure 2-4A, 1000 ng of GST-RB or GST was used in the absence or presence of 330 nM TSA or methanol control. For the experiment shown in Figure 2-5, 150 ng, 300 ng, 600 ng, or 1000ng of GST-RB was used in the absence or presence of 500 nM TSA.

Results

Chromatin remodeling proteins occupy the endogenous human U6 snRNA promoter

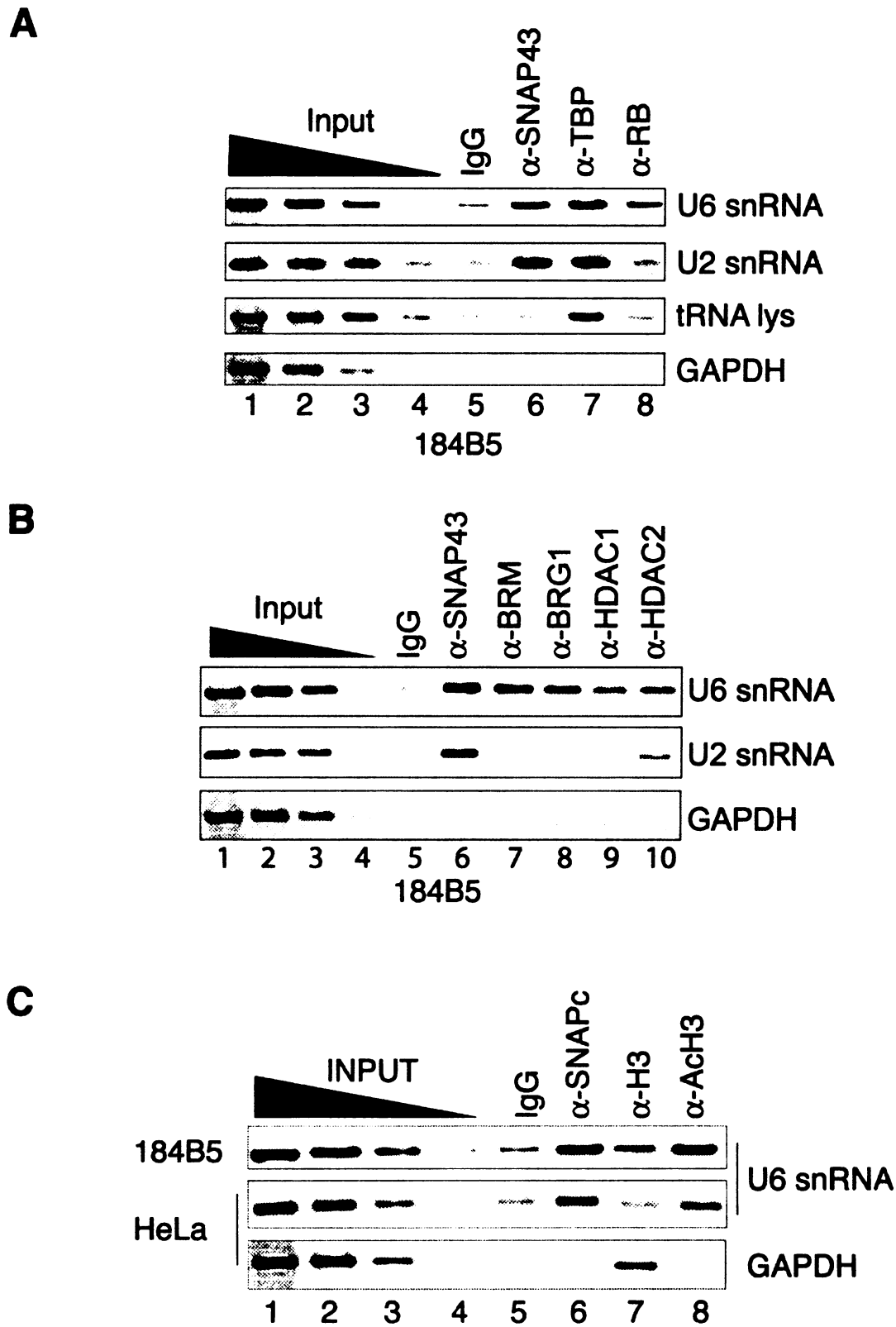
Human RNA polymerase III transcription of 5S rRNA, tRNA, and U6 snRNA genes is inhibited by RB (10). To further understand the mechanism of RB repression of endogenous class 2 (tRNA) and class 3 (U6 snRNA) genes, and whether RB can stably associate with these promoters, the in vivo occupancy of RB at a $tRNA^{Lys}$ and a U6 snRNA promoter was examined by ChIP assays. As shown in Figure 2-1A, the anti-RB immunoprecipitation was enriched in U6 snRNA promoter DNA but not U2 snRNA or $tRNA^{Lys}$ promoter DNA (lane 8). The immunoprecipitation performed with anti-SNAP43 antibody was significantly enriched for U6 snRNA and U2 snRNA promoter DNA but not for $tRNA^{Lys}$ or GAPDH exon 2 DNA (lane 6) as compared to the negative control IgG precipitation (lane 5). This result is expected, as $SNAP_C$ is required for snRNA transcription but not tRNA transcription. Furthermore, anti-TBP immunoprecipitation was also enriched for U6 snRNA, U2 snRNA, and $tRNA^{Lys}$ promoter DNA, consistent with a role for TBP in transcription of these genes (lane 7). That endogenous RB occupies the U6 but not the U2 snRNA gene promoter is consistent with our previous observation that RB preferentially regulates RNA polymerase III transcribed U6 snRNA but not RNA polymerase II transcribed U2 snRNA even though both genes contain similar promoter elements. Moreover, in the model for RB repression of tRNA genes wherein RB disrupts preinitiation complex formation, RB is not expected to be present at the promoter (11). These results show that endogenous RB can stably associate with U6 snRNA promoter DNA.

Figure 2-1. Endogenous chromatin modifying proteins associate with a human U6 snRNA promoter.

(A) RB occupies a U6 snRNA gene promoter in vivo. ChIP experiments from human 184B5 cells were performed using anti-SNAP43 (lane 6), anti-TBP (lane 7), and anti-RB (lane 8) antibodies and nonspecific IgG (lane 5). Precipitated DNA was analyzed by PCR amplification for enrichment of U6 snRNA, U2 snRNA, and tRNA promoters and negative control GAPDH exon 2.

(B) Endogenous HDAC1 and HDAC2 as well as the BRM and Brg1 subunits of the ATP-dependent chromatin remodeling complex occupy a U6 snRNA promoter. ChIP experiments were performed, using chromatin harvested from RB containing 184B5 cells with anti-SNAP43 (lane 6), anti-BRM (lane 7), anti-Brg1 (lane 8), anti-HDAC1 (lane 9), and anti-HDAC2 (lane 10) antibodies or nonspecific IgG (lane 4).

(C) Acetylation levels of endogenous histone H3 at U6 snRNA promoter are affected by RB status. Chromatin was harvested from RB containing normal human mammary epithelial cells (184B5) and HeLa cervical carcinoma cells containing RB lacking RB function, was used for ChIP with anti-SNAP43 (lane 6), anti-H3 (lane 7), and anti-AcH3 that recognizes acetylated histone H3 at lysine 9/14 (lane 8) and negative control IgG (lane 5). Enrichment of human U6 snRNA promoter DNA and GAPDH exon 2 DNA in the immunoprecipitated DNA was analyzed as above. In all the above experiments, lanes 1-4 show a 10-fold serial dilution of input chromatin from 10% to 0.01%.


A
Input
IgG
α-SNAP43
α-TBP
α-RB
U6 snRNA
U2 snRNA
tRNA lys
GAPDH
1 2 3 4 5 6 7 8
184B5
B
Input
IgG
α-SNAP43
α-BRM
α-BRG1
α-HDAC1
α-HDAC2
U6 snRNA
U2 snRNA
GAPDH
1 2 3 4 5 6 7 8 9 10
184B5
C
INPUT
IgG
α-SNAPc
α-H3
α-AcH3
184B5
HeLa
U6 snRNA
GAPDH
1 2 3 4 5 6 7 8

It was recently shown that a positioned nucleosome is located between the DSE and the PSE in the natural U6 snRNA gene promoter region and the correctly positioned nucleosome is suggested to bring these snRNA promoter elements into close proximity to facilitate cooperative binding by Oct-1 and $SNAP_C$ to the DSE and PSE, respectively, which is required for activated transcription (33). Thus, it is possible that RB recruits one or more chromatin modifying activities to influence nucleosome structure or position during U6 repression. As RB can recruit chromatin modifying proteins such as HDAC1 and HDAC2, as well as the Brg1 and BRM components of the ATP-dependent SWI/SNF chromatin remodeling complex, to repress the E2F-stimulated transcription of target genes (1, 16, 17, 32), the possibility that RB recruits these co-repressor proteins to the U6 snRNA promoter was examined. As a first step whether endogenous HDAC1 and HDAC2 as well as the Brg1 and BRM subunits of the SWI/SNF ATP-dependent chromatin remodeling complex occupy a human U6 snRNA promoter in vivo was examined using chromatin harvested from normal mammary 184B5 epithelial cells. As shown in Figure 2-1B, U6 promoter DNA, but not GAPDH exon 2 DNA, was significantly enriched in immunoprecipitations performed with anti-BRM (lane 7), anti-BRG1 (lane 8), anti-HDAC1 (lane 9), and anti-HDAC2 (lane 10) antibodies. Interestingly, no detectable or modest enrichment of U2 snRNA promoter DNA was observed in these immunoprecipitations, even though significant enrichment was seen in the anti-$SNAP_C$ immunoprecipitation (lane 6). This correlates with the lack of RB occupancy on the U2 snRNA promoter as shown in Figure 2-1A. These observations suggest that HDAC1, HDAC2, Brg1, and BRM proteins associate with the U6 snRNA promoter and may be involved in RB repression of U6 transcription.

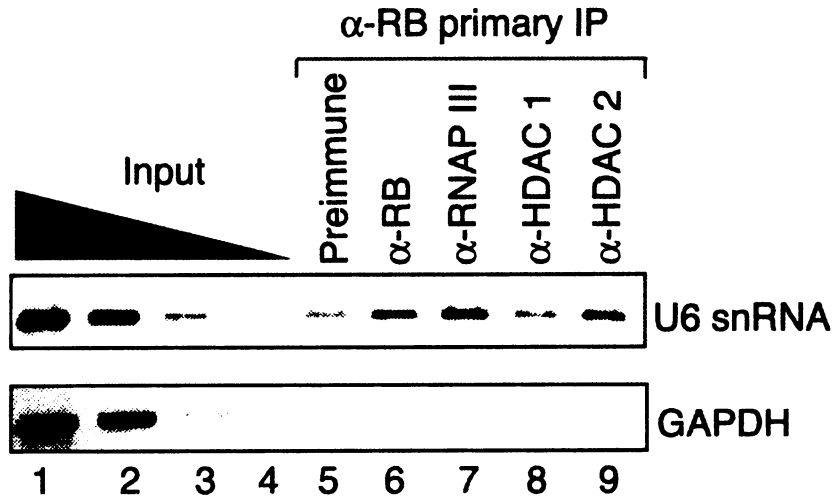
Next, ChIP experiments were performed to detect the presence of unmodified-histone H3 or acetylated (Ac) histone H3 at a human U6 snRNA promoter in vivo and whether acetylation status was affected by RB status. For this experiment chromatin was harvested from normal mammary 184B5 epithelial cells containing RB, and HeLa cervical carcinoma cells lacking functional RB. RB occupies the endogenous U6 promoter in 184B5 cell but not in HeLa cells, suggesting that U6 regulation may be lost in the HeLa cancer cells (11). As shown in Figure 2-1C, both total H3 and acetylated H3 occupy an endogenous U6 promoter in 184B5 cells. Very little detectable total H3 as compared to H3 that is acetylated occupied U6 promoter DNA in HeLa cells (lanes 7 and 8). Significant enrichment of the U6 promoter DNA, but not GAPDH exon2 DNA, is seen in the anti-SNAP43 immunoprecipitation from both 184B5 and HeLa cells (lane 6). That total H3 is seen at the U6 promoter in 184B5 cells, but not in HeLa cells, indicates that the presence of total H3 and post-translationally modified H3 may be contingent upon a functional RB pathway and associated RB-dependent co-factors that influence histone modification.

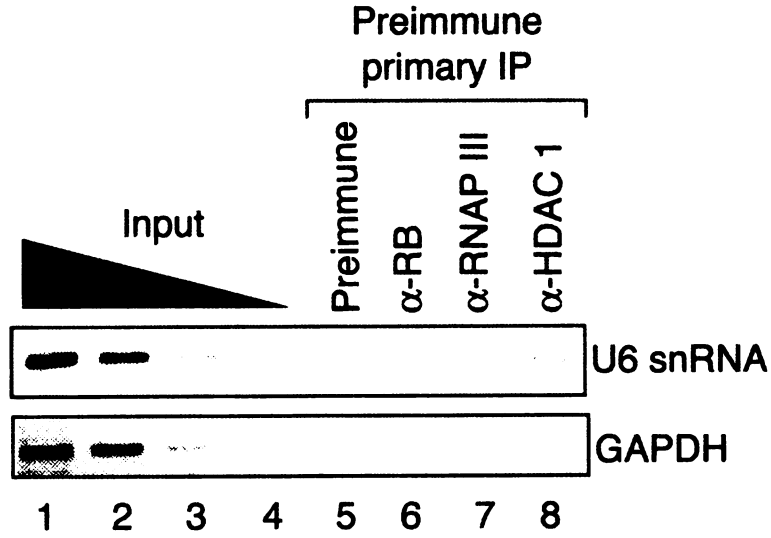
RB recruits HDAC proteins during repression

As endogenous HDACs occupy a human U6 snRNA gene promoter in cells containing functionally active RB, the hypothesis that RB and HDAC proteins associate simultaneously on the U6 promoter during repression was tested. Sequential ChIP was performed using chromatin harvested from 184B5 cells with either anti-RB or preimmune antibodies in the first round of immunoprecipitation. Associated factors were then eluted from protein G agarose beads and a second round of immunoprecipitation

Figure 2-2. RB concurrently occupies an endogenous U6 snRNA promoter with HDAC1, HDAC2 and RNA polymerase III.

Sequential ChIP was carried out from chromatin harvested from 184B5 cells using anti-RB antibodies (top panel) or nonspecific preimmune serum (bottom panel) in the primary immunoprecipitation reaction. Subsequently, secondary immunoprecipition reactions were carried using anti-RB (lane 6), anti-RNA polymerase III (lane 7), anti-HDAC1 (lane8) or anti-HDAC2 (lane 9). DNA precipitated after the secondary immunoprecipitation was analyzed for enrichment of U6 snRNA promoter DNA and GAPDH exon 2 DNA.


α-RB primary IP
Input
Preimmune
α-RB
α-RNAP III
α-HDAC 1
α-HDAC 2
U6 snRNA
GAPDH
1 2 3 4 5 6 7 8 9


Preimmune
primary IP
Input
Preimmune
α-RB
α-RNAP III
α-HDAC 1
U6 snRNA
GAPDH
1 2 3 4 5 6 7 8

was performed using the indicated antibodies. As shown in Figure 2-2 (top panel), significant enrichment of U6 promoter DNA was observed in the anti-RB/anti-RB (lane 6) and anti-RB/anti-RNA polymerase III (lane 7) immunoprecipitation reactions, but not in the negative control anti-RB/preimmune precipitation reaction (lane 5), consistent with previous data from our lab (11). Interestingly, U6 promoter DNA was enriched in the immunoprecipitated reactions performed using anti-RB antibodies followed by anti-HDAC2 antibodies (lane 8), but not by the anti-HDAC1 antibodies (lane 9). The lack of U6 promoter recovery with anti-HDAC1 antibodies in this experiment may be because the HDAC1 epitope is occluded; however, these anti-HDAC1 antibodies did recover HDAC1 in single round ChIP reactions (Figure 2-1B). Therefore, we favor the idea that RB and HDAC2, but not HDAC1, can simultaneously occupy an endogenous U6 promoter. As expected, no significant amount of U6 promoter DNA was obtained when the negative control preimmune sera was used in the first round of immunoprecipitation (Figure 2-2 bottom panel).

Next, whether HDACs and/or SWI/SNF proteins are recruited to the U6 promoter when RB is actively repressing transcription was examined. To test this idea, in vitro U6 transcription was performed in the presence or absence of GST-RB (379-928). A portion of the transcription reaction was tested for RB repression in a riboprobe protection assay. As shown in Figure 2-3A, GST-RB repressed U6 transcription relative to the untreated extract or extract treated with a comparable amount of GST (compare lane 2 to lanes 1 and 3). The remainder of each transcription reaction was cross-linked with formaldehyde for subsequent immunoprecipitation reactions using non-specific preimmune sera or

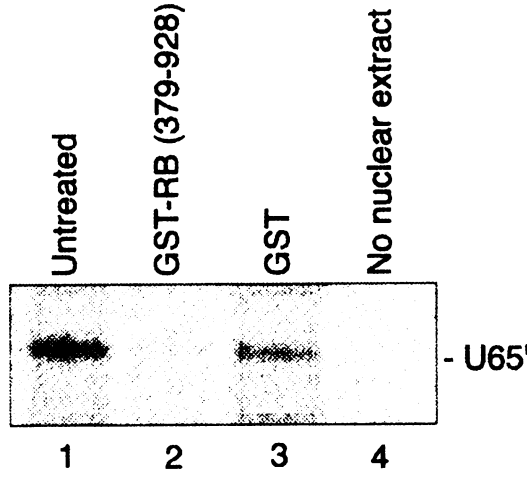
Figure 2-3. RB stimulates enrichment of HDACs and SWI/SNF at a U6 snRNA promoter during repression in vitro.

(A) U6 snRNA in vitro transcription was carried out in the in the absence of any added proteins (lane1), was effectively repressed by 1000 ng of recombinant GST-RB (379-928) (lane 2) but not by an equivalent amount of GST (lane 3). Transcription was also carried out in the absence of nuclear extract (lane 4). Portions of the U6 transcription reaction were cross-linked with formaldehyde as described before (11).

(B) Cross-linked in vitro transcription reactions were subjected to immunoprecipitation reactions using anti-RB (lane 3), anti-HDAC1 (lane 4), anti-HDAC2 (lane 5), anti-BRM (lane 6), or anti-BRG1 (lane 7) antibodies or nonspecific preimmune serum (lane 2). The recovery of immuoprecipitated material was analyzed by PCR for the presence of the U6 reporter construct (pU6/Hae/Ra.2) with primers specific to the promoter region in the plasmid.

Part A of this figure was performed by Heather A. Hirsch.

A


Untreated
GST-RB (379-928)
GST
No nuclear extract
- U65'
1
2
3
4

B

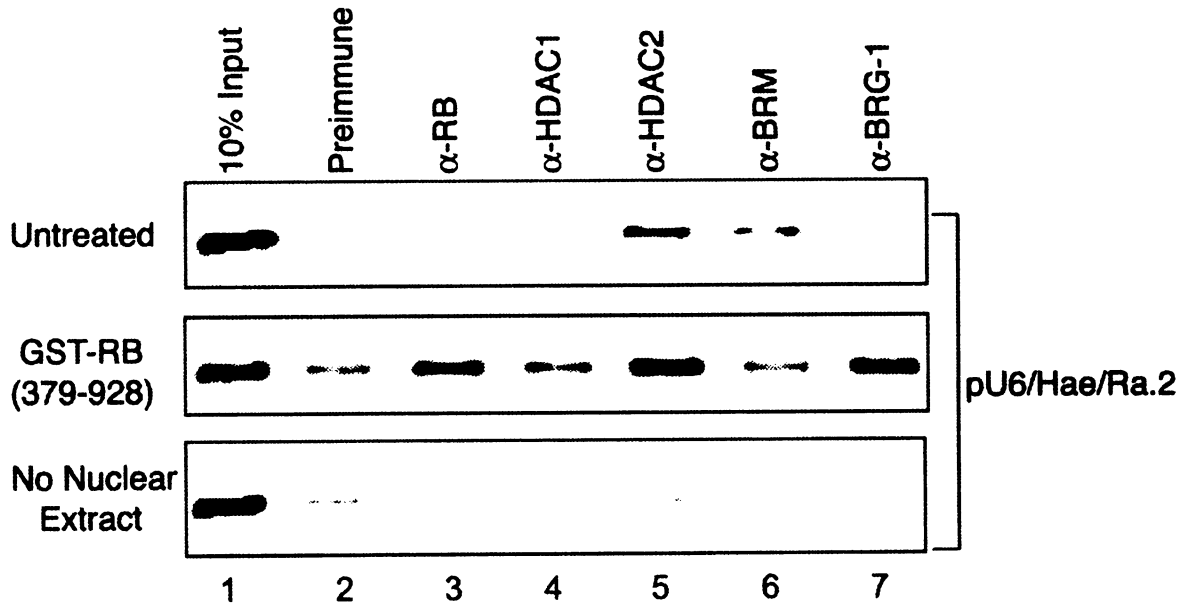

10% Input
Preimmune
α-RB
α-HDAC1
α-HDAC2
α-BRM
α-BRG-1
Untreated
GST-RB (379-928)
No Nuclear Extract
pU6/Hae/Ra.2
1
2
3
4
5
6
7

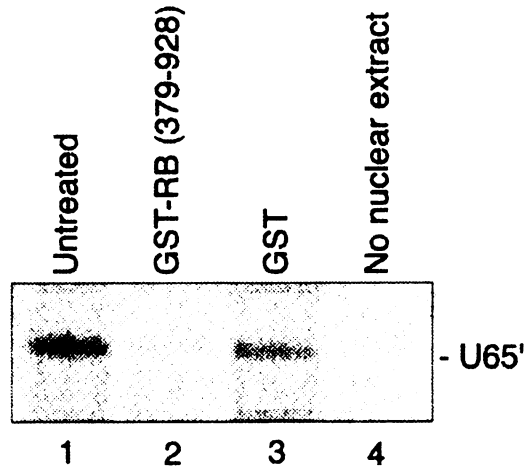
Figure 2-3. RB stimulates enrichment of HDACs and SWI/SNF at a U6 snRNA promoter during repression in vitro.

(A) U6 snRNA in vitro transcription was carried out in the in the absence of any added proteins (lane1), was effectively repressed by 1000 ng of recombinant GST-RB (379-928) (lane 2) but not by an equivalent amount of GST (lane 3). Transcription was also carried out in the absence of nuclear extract (lane 4). Portions of the U6 transcription reaction were cross-linked with formaldehyde as described before (11).

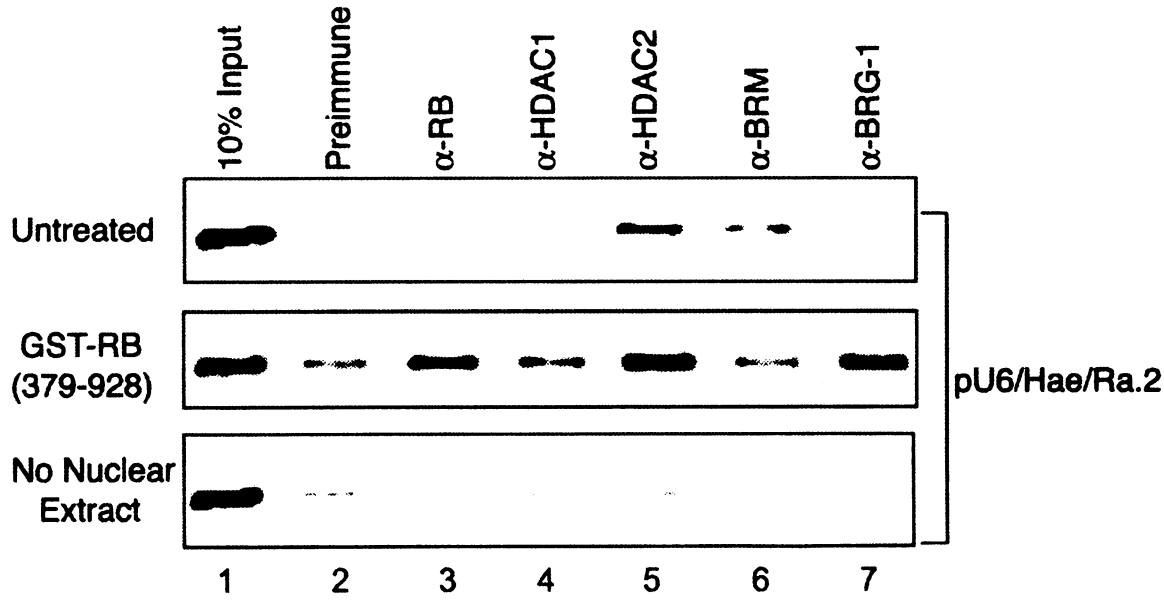
(B) Cross-linked in vitro transcription reactions were subjected to immunoprecipitation reactions using anti-RB (lane 3), anti-HDAC1 (lane 4), anti-HDAC2 (lane 5), anti-BRM (lane 6), or anti-BRG1 (lane 7) antibodies or nonspecific preimmune serum (lane 2). The recovery of immuoprecipitated material was analyzed by PCR for the presence of the U6 reporter construct (pU6/Hae/Ra.2) with primers specific to the promoter region in the plasmid.

Part A of this figure was performed by Heather A. Hirsch.

A


Untreated
GST-RB (379-928)
GST
No nuclear extract
- U65'
1
2
3
4

B


10% Input
Preimmune
α-RB
α-HDAC1
α-HDAC2
α-BRM
α-BRG-1
Untreated
GST-RB (379-928)
No Nuclear Extract
pU6/Hae/Ra.2
1
2
3
4
5
6
7

antibodies directed against RB, HDAC1, HDAC2, BRM, and BRG1. U6 promoter DNA was immunoprecipitated with anti-RB antibodies only when GST-RB was included in the transcription reaction (Figure 2-3B, lane 3). Similar experiments performed previously demonstrated that exogenously added GST-RB (379-928) does not associate with a promoter-less plasmid ((11), and data not shown). In the absence of added GST-RB, the anti-HDAC1 and anti-Brg1 immunoprecipitation resulted in no U6 promoter DNA recovery, whereas the anti-HDAC2 and anti-BRM immunoprecipitation led to weak U6 promoter recovery. Interestingly, U6 promoter DNA recovery in the anti-HDAC2 and anti-Brg1 immunoprecipitation was noticeably increased in the presence of GST-RB (lanes 5 and 7, compare the top and middle panels). Whereas GST-RB only modestly stimulated U6 promoter DNA recovery from the anti-HDAC1 and anti-BRM immunoprecipitations as compared to DNA recovery from the mock immunoprecipitation reaction carried out using preimmune sera (lanes 4 and 6, middle panel). Together, these results suggest that RB recruits HDAC2 and Brg1 to the U6 promoter either directly or indirectly.

HDAC activity is required for RB repression of U6 snRNA transcription from a chromatin template

The observation that RB stimulates promoter association of HDAC2 at the U6 promoter during repression suggested that HDAC activity is required for RB repression of RNA polymerase III transcription. To test this idea, RB repression assay was carried out in the absence or presence of Trichostatin A (TSA), which is a potent

inhibitor of HDAC activity. The deacetylase activity of HDACs is mostly associated with removing acetyl groups from histone tails that are part of a nucleosome. However, RB potently represses U6 transcription initiated from a non-nucleosomal naked DNA template, and the use of HDAC activity in this context would suggest that HDAC2 targets proteins other than nucleosomes, for example, U6 snRNA- specific transcription factors such as $SNAP_C$ or Brf2-TFIIIB, to exert repression. In contrast, if HDAC activity was required for RB repression of chromatin templates but not naked DNA templates, then it would indicate that HDAC activity targets nucleosomes to inhibit transcription. As shown in the Figure 2-4 top panel, U6 transcription carried out in the presence of methanol + TSA (lane 2) or methanol, which was used as a vehicle for TSA (lane 3), was not significantly affected as compared to the levels of transcription seen in lane 1. However, this same amount of TSA was sufficient to inhibit transcription of a tRNA-like (class 2) AdVAI gene (Figure 2-4, see lane 6 bottom panel). Thus, HDAC activity is not essential for U6 transcription, permitting testing of the hypothesis that HDAC activity is required for RB repression of naked DNA templates. Interestingly, GST-RB (379-928) repression of U6 transcription was comparable both in the absence or presence of TSA (compare lanes 4 and 5). Similar results were observed when sodium butyrate, a different HDAC inhibitor was used (data not shown). Thus, HDAC activity is not required for RB repression of U6 transcription initiated from a naked DNA template and altering the acetyation status of U6-specific transcription factors is not critical for repression.

As RB repression of naked U6 DNA template was refractory to HDAC inhibition, we next considered that RB directs HDAC activity towards nucleosomes during repression. To directly test the hypothesis that RB requires HDAC activity for inhibition

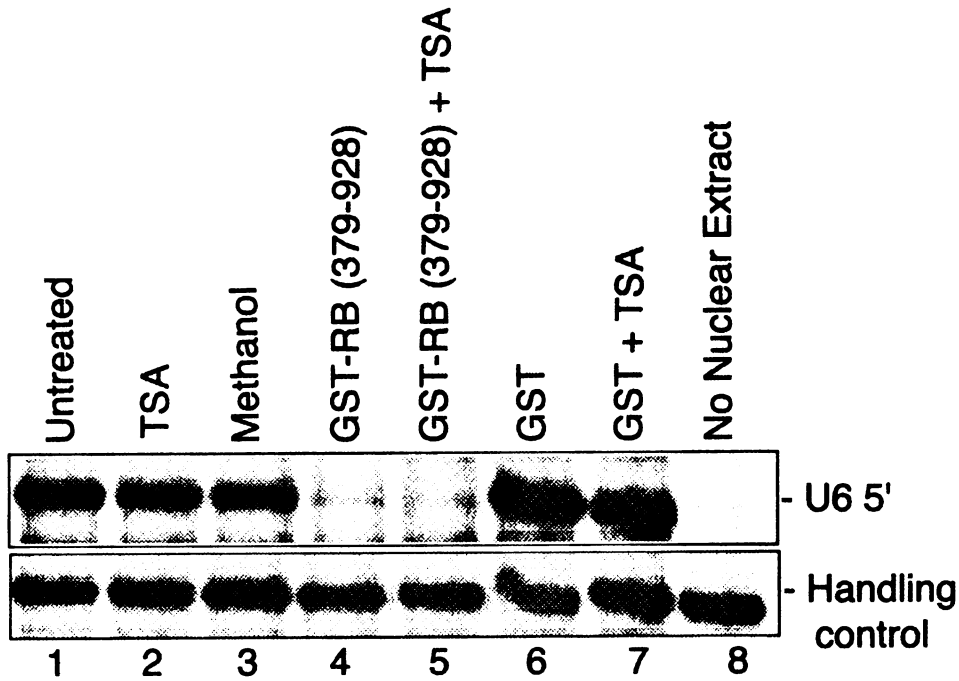
Figure 2-4. RB repression of U6 transcription and HDAC activity are biochemically separable activities.

RB repression of U6 transcription is not affected by concentrations of TSA that inhibit AdVAI transcriptioin.

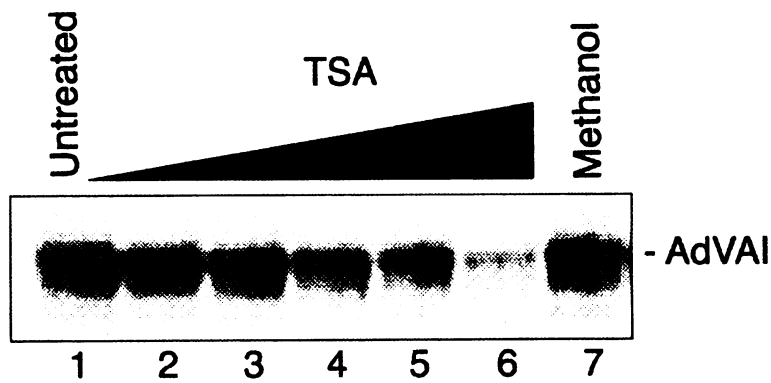
(A) U6 transcription was carried out in the presence of 200 nM Trichostatin A (TSA) (lane 2) or methanol, which was used as a carrier for TSA (lane 3). The ability of 1000 ng GST-RB (379-928) to repress U6 transcription in the absence (lane 4) or presence of TSA (lane 5) was tested. As a control 1000 ng GST was added to the transcription reactions in the absence (lane 6) or presence (lane 7) of TSA. Lane 1 represents U6 transcription supported with untreated extract, and lane 8 shows transcription reactions that were carried out in the absence of any nuclear extract.

(B) AdVAI transcription was carried out in the presence of increasing amounts of TSA (25 nM, 50 nM, 100 nM, 165 nM, and 330 nM) (lanes 2 to 6). Methanol (330 nM) was added to the transcription reaction in lane 7. Lane 1 shows AdVAI transcription supported by untreated extract.

A


Untreated
TSA
Methanol
GST-RB (379-928)
GST-RB (379-928) + TSA
GST
GST + TSA
No Nuclear Extract
- U6 5'
- Handling control
1 2 3 4 5 6 7 8

B


Untreated
TSA
Methanol
- AdVAI
1 2 3 4 5 6 7

of U6 transcription from nucleosomal templates, I established an in vitro transcription assay initiated from a chromatin template plasmid harboring the U6 snRNA promoter elements. The chromatin template was initially tested for its ability to support U6 transcription. As shown in Figure 2-5, comparable levels of U6 transcription were obtained from naked DNA and chromatin templates (lane 1, compare the first and third panel). When increasing amounts of GST-RB were added to the transcription reaction in the absence or presence of TSA, GST-RB repressed U6 transcription of naked DNA template to similar levels (compare lanes 2-4 in the first and second panels). Interestingly, in the absence of TSA lesser amounts of GST-RB were needed for efficient repression of chromatin templates as compared to naked DNA templates. However, in the presence of TSA, GST-RB did not repress U6 transcription to a similar extent from a chromatin template as it did from naked DNA templates. Thus, this result suggests that RB requires HDAC activity to repress U6 snRNA transcription in the context of chromatin.

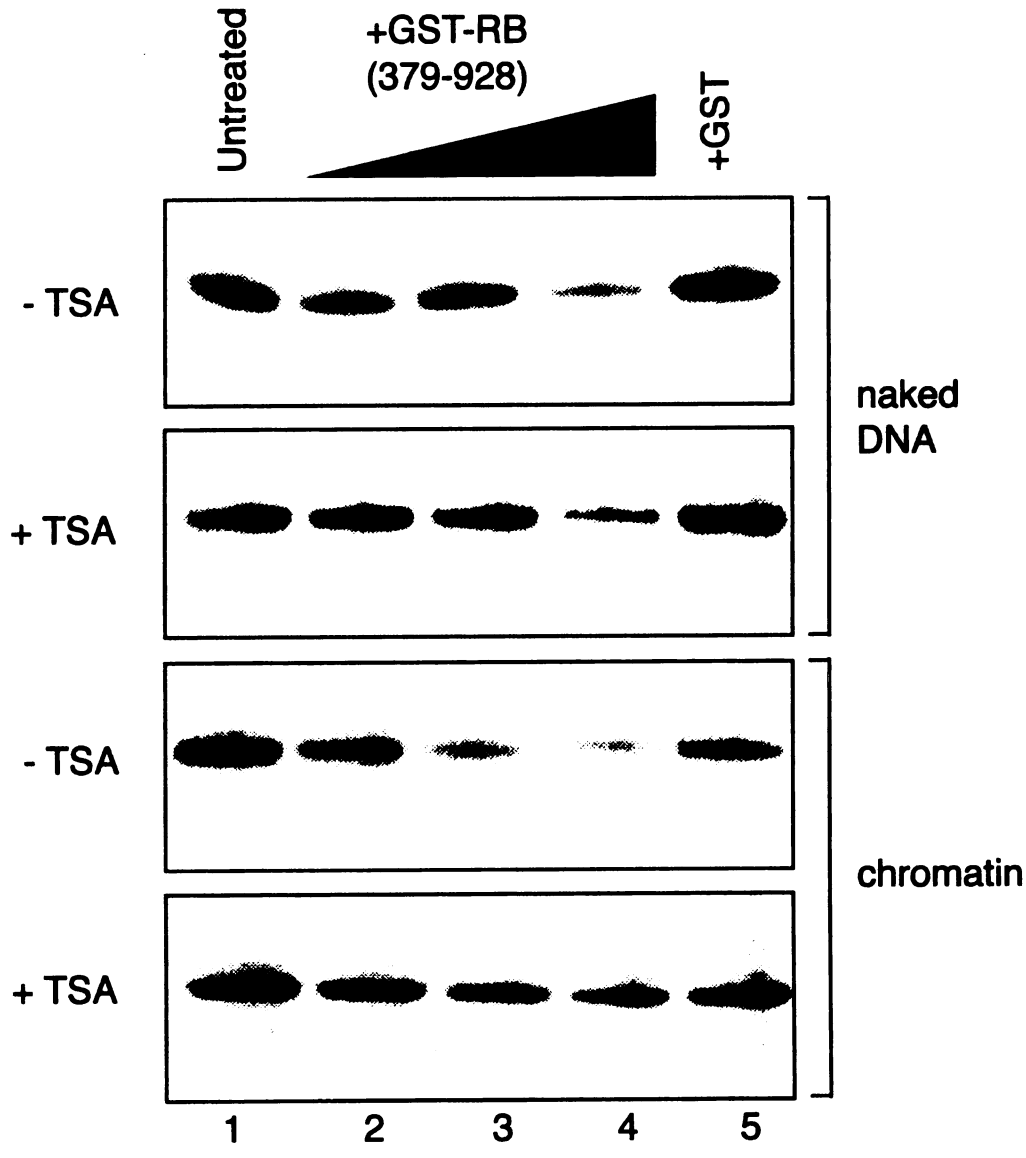
Discussion

RB is a global repressor of RNA polymerase III transcription. Further understanding the mechanism(s) of RB repression of these genes will provide insights into RB function. The data presented herein demonstrates a role for HDACs and components of the SWI/SNF ATP-dependent chromatin-remodeling complex in RB-mediated repression of U6 snRNA transcription.

On other target genes, RB recruits these co-factors to prevent transcription factor binding and polymerase recruitment. For example, RB recruits HDACs to deacetylate the

Figure 2-5. HDAC activity is required for RB repression of U6 transcription from chromatin DNA templates but not from naked DNA templates.

Approximately 6 μL of HeLa cell nuclear extract was incubated with a U6 promoter containing plasmid, or plasmid that had been assembled into chromatin. Increasing amounts; 300 ng (lane 2), 600 ng (lane 3), and 1000 ng (lane 4) of GST-RB (379-928) were tested for U6 repression in the presence of 500 μM Trichostatin A (TSA) or in reaction without any added TSA. 1000 ng GST (lane 5) was used as a negative control. Lane 1 shows the level of transcription supported by untreated extracts.


Untreated
+GST-RB
(379-928)
+GST
- TSA
+ TSA
naked
DNA
- TSA
+ TSA
chromatin
1
2
3
4
5

upstream binding factor (UBF), which is a RNA polymerase I-specific transcription factor. It is thought that deacetylation of UBF results in its inability to recruit the TBP-containing SL-1 complex (4, 19). Alternatively, RB utilizes HDACs and SWI/SNF to repress E2F-regulated genes by mainly altering the chromatin structure (1, 16, 17, 24, 27). Our observation that HDAC activity is required for RB repression of U6 transcription from a chromatin template, that is presumably reflective of its endogenous state but not from a naked DNA template, suggests that HDACs may influence the chromatin structure at a U6 promoter. Recent studies have indicated a role of chromatin structure in U6 snRNA transcription. For example, in vivo mapping studies have demonstrated the presence of a positioned nucleosome between the distal sequence element (DSE) and the proximal sequence (PSE). Correct positioning of the nucleosome is thought to bring these two promoter elements close to each other, thus facilitating cooperative binding of Oct1 and $SNAP_C$ to the DSE and PSE, respectively, to activate transcription (33). It is possible then to imagine that RB recruites HDAC and SWI/SNF to alter the nucleosome structure to repress transcription. For example, misaligning the nucleosome may disrupt the Oct1-SNAPc interaction and/or prevent the interaction of Oct1 with an unknown transcription factor required for promoter escape. Alternatively, RB could remodel nucleosomes that are near the start site of transcription; however, whether any nucleosome is located near the start site is not known. Moreover, RNA polymerase III is capable of transcribing through a nucleosome (25) and hence the positioned nucleosome in the U6 promoter region is the most likely RB target.

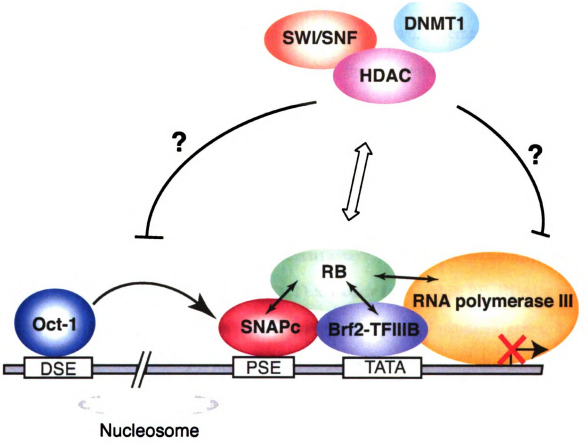
RB has also been shown to associate with multiple enzymatic activities in the same complex. For example, RB can form a repressor complex that contains either

SWI/SNF or HDACs and SWI/SNF. The RB-HDAC-SWI/SNF complex and the RB-SWI/SNF complex have been shown to repress cyclin E and cyclin A, respectively, whose genes products are required for cell cycle progression (32). The sequential disassembly of the RB-HDAC-SWI/SNF complex to one lacking HDAC activity is proposed as one mechanism governing RB repression of different genes at distinct phases of the cell cycle. Interestingly, we see that RB stimulates U6 promoter occupancy by HDAC2 and Brg1. Whether RB, HDAC2 and Brg1 are part of the same complex is not known.

Previous studies investigating the mechanisms of RB repression of U6 transcription have indicated that RB and the general transcription machinery, including $SNAP_C$ and TFIIIB, occupy a U6 snRNA promoter simultaneously (11). Furthermore, RB and RNA polymerase III concurrently occupy a U6 snRNA promoter during repression both in vitro and in vivo (11). These studies suggest that RB does not necessarily exclude RNA polymerase III from the promoter, but may prevent subsequent steps in transcription such as promoter escape by recruiting co-regulatory factors discussed in this current study. While RB repression naked U6 DNA templates is efficient, and RB stimulates association of HDAC2 and Brg1 with naked DNA templates, HDAC activity is invoked only when the U6 templates is nucleosomal. These observations suggest that RB repression may be a multi-step process, as illustrated in a speculative model depicted in Figure 2-6. In this model, RB establishes repression in the absence of nucleosomes and maintains repression by invoking chromatin-remodeling activity. RB establishes repression on naked DNA by interacting directly with RNA polymerase III, possibly to tether the polymerase to the general transcription machinery

Figure 2-6. Model for RB repression of human U6 snRNA gene transcription

RB co-factors such as HDACs and components of the SWI/SNF ATP-dependent chromatin remodeling complex are recruited to a U6 snRNA promoter during RB repression. These activities may inhibit transcription by altering the positioned nucleosome located between the distal sequence element (DSE) and the proximal sequence element (PSE). Oct-1 and SNAPC cooperatively bind to the DSE and PSE, respectively to activate transcription, which is facilitated in part by the positioned nucleosome. Alternatively, RB may recruit other co-repressor proteins such as DNMT1 as part of the HDAC-containing complex to modify the DNA at the start site of transcription, thus preventing polymerase escape. Direct interactions with RNA polymerase III allow RB to tether the polymerase to the general transcription machinery.


SWI/SNF
DNMT1
HDAC
?
?
RB
RNA polymerase III
Oct-1
SNAPc
Brf2-TFIIIB
DSE
PSE
TATA
Nucleosome

thus preventing promoter escape. Preliminary data from our lab indeed shows that RB interacts with RNA polymerase III both in vitro and in vivo (X Song, unpublished data). Subsequently, RB recruits a complex that contains HDACs and/or SWI/SNF that may alter the positioned nucleosome and prevent Oct-1 from binding to the PSE and/or causing disruption of the Oct1-$SNAP_C$ contacts. Whether Oct-1:DNA contacts are severed in a RB/HDAC-dependent manner is not yet known. Alternatively, RB may modify DNA around the start site of transcription, thus preventing polymerase escape. Data from our laboratory indicates that DNA near the start site of transcription is indeed modified during RB repression, and this modification is most likely methylation (T Selvakumar, unpublished data). Moreover, association of *de novo* DNA methyl transferase 1 (DNMT1) with the U6 snRNA gene promoter increases when RB is over-expressed in HeLa cells in transient transfections assays (G. Jawdekar unpublished data) raising the hypothesis that RB recruits DNMT1 to modify DNA. While RB, HDACs, and DNMT1 have been implicated in a repressor complex for E2F-regulated genes, it is possible that such a complex is also recruited to U6 snRNA gene promoters during RB-mediated repression transcription. By biochemically separating RB repression and HDAC activities we can now start understanding the mechanistic details of RB repression on both naked as well as chromatin DNA.

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CHAPTER 3

THE UNORTHODOX SNAP50 ZINC FINGER DOMAIN CONTRIBUTES TO CO-OPERATIVE PROMOTER RECOGNITION BY HUMAN $SNAP_C$[1]

Abstract

Human small nuclear (sn) RNA gene transcription by RNA polymerases II and III depends upon promoter recognition by the $SNAP_C$ general transcription factor. DNA binding by $SNAP_C$ involves direct DNA contacts by the SNAP190 subunit in cooperation with SNAP50 and SNAP43.The data presented herein shows that SNAP50 plays an important role in DNA binding by $SNAP_C$ through its zinc finger domain. The SNAP50 zinc finger domain contains fifteen cysteine and histidine residues configured in two potential zinc coordination arrangements. Individual alanine substitution of each cysteine and histidine residue demonstrated that eight sites are important for DNA binding by $SNAP_C$. However, metal-binding studies revealed that $SNAP_C$ contains a single zinc atom indicating that only one coordination site functions as a zinc finger. Of the eight residues critical for DNA binding, four cysteine residues were also essential for both U1 and U6 transcription by RNA polymerase II and III, respectively. Surprisingly, the remaining four residues, while critical for U1 transcription could support partial U6 transcription. DNA binding studies showed that defects in DNA binding by $SNAP_C$ alone could be suppressed through cooperative DNA binding with another member of the RNA

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polymerase III general transcription machinery, TFIIIB. These results suggest that these eight cysteine and histidine residues perform different functions during DNA binding with those residues involved in Zn coordination likely performing a dominant role in domain stabilization and the others involved in DNA binding. These data further define the unorthodox SNAP50 zinc finger region as an evolutionarily conserved DNA binding domain.

Introduction

The human snRNA gene family is unusual in that related member genes are transcribed by either RNA polymerase II or III depending upon their core promoter structures, and they thus serve as an interesting model to understand principles of polymerase selection and activity both during normal and deregulated growth (reviewed in (8, 12)). Human snRNA genes are defined by the presence of a diagnostic promoter element called the proximal sequence element (PSE). For both polymerase systems, the PSE recruits the general transcription factor called $SNAP_C$ (27), which is also known as PTF (25). $SNAP_C$ is composed of at least five subunits SNAP190, SNAP50, SNAP45, SNAP43, and SNAP19 (9-11, 26, 35). SNAP190 can interact with all the other subunits, and thus provides a central architectural backbone to the complex (22). SNAP190 directly interacts with the transcriptional activator protein Oct-1 during stimulated transcription of human snRNA genes (5, 24). In the RNA polymerase III pathway, SNAP190 also interacts with TBP to recruit Brf2-TFIIIB to the TATA box of human U6 snRNA genes (13, 23). Thus, the upstream signal from Oct-1 is conveyed through SNAP190, stimulating preinitiation complex assembly with other general transcription

factors as a prerequisite for RNA polymerase III recruitment. Interestingly, protein kinase CK2 can phosphorylate SNAP190 to impede DNA binding; however, promoter recognition by $SNAP_C$ can be restored by cooperative binding of TBP to those promoter sequences containing both a PSE and TATA box, but not sequences lacking the TATA element (6). This last observation suggests that CK2 can differentially influence RNA polymerase II and III snRNA transcription by covalent modification of $SNAP_C$ to alter its DNA binding properties even though $SNAP_C$ is shared between both polymerase systems.

The specific binding of $SNAP_C$ to the PSE is mediated by a Myb-like DNA binding domain within the N-terminal region of SNAP190. Other $SNAP_C$ subunits may additionally function in PSE recognition and provide stabilizing contacts with DNA. Indeed, UV cross-linking experiments suggest that both SNAP190 (36) and SNAP50 (9) are in close contact with DNA. In addition, SNAP50 contains an unusual arrangement of cysteine and histidine residues at the carboxy terminal region of the protein that is evolutionarily conserved. In other transcription factors, zinc finger motifs function for nucleic acid binding and/or protein-protein interactions (21). In the current study, we have focused on the function of the zinc finger region of SNAP50, and we show that this region is critical for DNA binding by $SNAP_C$. The arrangement of cysteine and histidine residues within SNAP50 further defines this region as an unorthodox zinc finger domain that functions in divergent preinitiation complex assembly pathways for RNA polymerase II and III transcription.

Materials and methods

Expression and purification of recombinant proteins

For the EMSA shown in Figure 2-1A, mini-$SNAP_C$ was assembled from subunits individually expressed in *E. coli* and purified as described before (13). The partial complex $SNAP_C\gamma4$ was obtained by co-expression in *E. coli* of SNAP190 (1-505), SNAP43, SNAP19, and either wild type or mutant SNAP50. The complex was purified as described (7). TBP was expressed as a GST fusion protein in *E. coli* and purified as described (13). The Brf2 and Bdp1 (1-470) proteins were expressed in *E. coli* and purified as described before (30). In all cases, GST tags were removed prior to use in functional assays by thrombin digestion during purification

Electrophoretic mobility shift assay

EMSA was performed in a 20 μL total volume using DNA probes containing a wild-type or mutant mouse U6 PSE with a mutant human U6 TATA box, or containing the wild-type human U6 sequence, as described previously (13). DNA binding reactions using only $SNAP_C$ were performed in a buffer containing 60 mM KCl, 20 mM HEPES pH 7.9, 5 mM MgCl2, 0.2 mM EDTA, 10% glycerol, 0.5 μg of poly(dI-dC), and 0.5 μg of pUC119 plasmid. Reactions were incubated for 20 min at room temperature after which 5,000 cpm of probe was added, and reactions were incubated an additional 20 min. Samples were fractionated on a 5% nondenaturing polyacrylamide gel (39:1) in TGE running buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA). Reactions containing both $SNAP_C\gamma4$ and TBP were performed in a buffer containing 100 mM KCl, 20 mM HEPES pH 7.9, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.07% Tween

20, 0.2 μg of poly(dG-dC), and 0.2 μg of pUC119 plasmid. The samples were fractionated on a 5% nondenaturing polyacrylamide gel (39:1) in TGEM running buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA, 5 mM $MgCl_2$). Approximately 1 ng of $SNAP_C\gamma4$, 50 ng human TBP, 240 ng Brf2, and 40 ng of Bdp1 (1-470) was used, as indicated.

GST-pulldown and immunoprecipitation assays

GST pulldown assays were done as described before (13). For the immunoprecipitation assays performed in Figure 3-2B, approximately 20 ng of each complex containing SNAP50 with the indicated point mutation was used with 1 μL of anti-SNAP43 antibody, preimmune sera, or buffer alone. Immunoprecipitation reactions were carried out in HEMGT-150 buffer (25 mM HEPES, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% Glycerol, 0.1% Tween-20, 150 mM KCl) containing protease inhibitors and 1 mM DTT at 4°C for 2 hr. The immunoprecipitated material was collected by incubation with Protein G agarose beads at 4°C for 2 hr. Beads were then collected, washed 5 times with HEMGT-150, and the bound protein was released by boiling in Laemmli buffer. Recovered proteins were resolved by 15% SDS-PAGE and were transferred to a nitrocellulose membrane for Western blotting using anti-HA antibodies.

In vitro transcription assays

Endogenous $SNAP_C$ was removed from HeLa extracts by anti-SNAP43 antibody immunodepletion as described before (10). In-vitro transcription of human U1 and U6 snRNA genes was performed for 1 hr at 30°C using the depleted extract. Approximately

5 ng of wild type and mutant $SNAP_C\gamma4$ complexes containing point mutations in SNAP50 were used for the reconstitution of both U1 and U6 transcription.

Zn binding studies

The amount of Zn associated with recombinant $SNAP_C$ was determined by two methods: 1) Inductively coupled plasma mass spectrometry (ICP-MS), and 2) Flame Atomic Absorption (FAA). For the ICP-MS studies, $SNAP_C$ protein concentration was determined by Bradford assay using BSA as a standard. The molecular weights of recombinant $SNAP_C\gamma4$ and $SNAP_C\gamma3$ were calculated as 159,735 and 148,432 g/mol, respectively. To measure Zn concentration, sample solutions were transferred to a Teflon vial and brought to dryness on a hot plate. Concentrated nitric acid was added and the sample was placed on the hot plate and hydrolyzed for 30 min. After cooling, the sample was diluted with water to bring the acid concentration to 2%, and 2% nitric acid was added to bring the solution to the desired volume. Ni and Zn standards (Spex Certiprep) in the concentration range from 0-1000 ppb were prepared in 2% nitric acid. Samples and standards were each mixed with 20 ppb indium and bismuth standard solution (Spex Certiprep) as internal standards. Samples were analyzed on an ICP-MS instrument (GV Instruments) with a flow rate of 0.5 mL/min. Zn66, Zn68 and Ni60 isotopes were measured and quantified. The responses for zinc and nickel were corrected according to indium and bismuth response. For the FAA studies, $SNAP_C$ protein concentration was determined by UV absorbance in 6 M urea at 280 nm. The molar extinction coefficient of 176,950 $cm^{-1}M^{-1}$ was used to calculate the molar concentration. $SNAP_C$ samples were transferred to a crucible, dried and ashed at 260 degrees. Concentrated nitric acid was

added to the ash and reheated until only white powder remained. The sample was reconstituted with 5% nitric acid. Zinc standards in the concentration range from 10-1000 ppb were prepared from zinc metal (Spectrum, 99.9 %) dissolved in concentrated nitric acid. The samples and standards were analyzed on the flame atomic absorption instrument (Varian SpectrAA-200) equipped with a zinc hollow cathode lamp operating at 213.9 nm. Aspiration rate was 1 mL/min.

For the Zinc analysis shown in Figure 2-6, wild type or mutant SNAP50 (315-411) was expressed as a N-terminal His-SUMO fusion protein from pET28a in *E. coli* RIL codonplus. Cells were grown in LB broth containing kanamycin (100 μg/ml) and chloramphenicol (50 μg/ml) supplemented with 50 μM $ZnCl_2$. Approximately 1 L of culture was used for the expression and purification of each protein. Cells were ruptured by sonication in buffer A (50 mM Tris pH 8.5, 350 mM NaCl, 10% glycerol, 10 mM imidazole, 1 mM β-mercaptoethanol) supplemented with 10 μM $ZnCl_2$. His-tagged recombinant proteins were purified by nickel column chromatography followed by elution with buffer A containing 400 mM imidazole without added zinc. Protein containing fractions were dialyzed against buffer A containing 1 mM DTT and no added zinc for 10 hrs at 4°C for subsequent FAA analysis, which was performed as above using approximately 2 mL of each protein solution (~1 mg/mL) with an aspiration rate of 5 mL/min. Protein concentration was determined in 6 M urea by UV absorbance at 280 nm. The molar extinction coefficient of 27305 $cm^{-1}M^{-1}$ was used to calculate the molar concentration of the wild type and mutant His-SUMO-SNAP50 (315-411) proteins. Zinc measurements and protein determination were done in triplicate.

Structural Modeling

Two protocols were followed to obtain *ab initio* predictions of SNAP50 (301-411). First, the amino acid sequence of SNAP50 was submitted to the Robetta server (19), and ten models were obtained. Second, lattice-based sampling from extended chains with MONSSTER (31) and the MMTSB Tool Set (4) were carried out. 2000 structures were generated in independent runs. The resulting structures were subjected to a short minimization with CHARMM (1) and evaluated with the scoring function DFIRE (37). Correlation-based scores were obtained from the original DFIRE scores according to a recently published method for enhancing scoring functions in protein structure prediction applications (32). The structures were then clustered and average correlation-based scores were compared between clusters. The structure with the highest correlation-based score from the cluster with the highest average correlation-based score and more than one member was then examined and subjected to further refinement through energy minimization and constrained short molecular dynamics simulations with CHARMM. The electrostatic potential on the surface of the final structure was calculated from solutions of the Poisson equation with the PBEQ module (18) in CHARMM. The program VMD was used for visualization of the final model and electrostatic surface maps (17).

Results

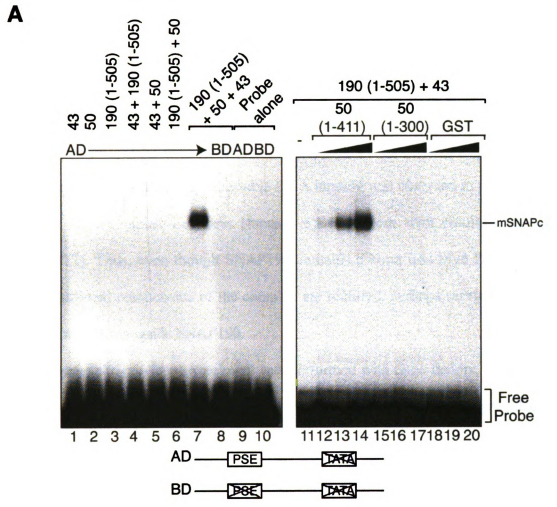
The C-terminal region of SNAP50 is required for DNA binding by $SNAP_C$.

Promoter recognition by $SNAP_C$ is a common early event in both RNA polymerase II- and III-specific pathways of pre-initiation complex formation at human

Figure 3-1. The C-terminal region of SNAP50 is required for DNA binding by $SNAP_C$.

(A) (Left panel) DNA binding by $SNAP_C$ requires cooperation among SNAP190, SNAP50, and SNAP43. Electrophoretic mobility shift assays (EMSA) were performed using a U6 probe containing a wild-type PSE and mutated TATA box (AD) or a U6 probe containing a mutated PSE and mutated TATA box (BD). DNA binding reactions were carried out with SNAP43, SNAP190 (1-505) or SNAP50 individually (100 ng each, lanes 1-3), or in pair wise combinations of SNAP43 and SNAP190 (1-505), SNAP43 and GST-SNAP50, and SNAP190 (1-505) and GST-SNAP50 (lanes 4-6). Formation of $SNAP_C$/DNA complex was observed in reactions containing all three subunits with the wild type PSE probe (lane 7) but not mutant PSE probe (lane 8). Reactions containing the wild type and mutant probes with no added proteins are contained in lanes 9 and 10, respectively. (Right panel) The SNAP50 C-terminal zinc finger domain is required for DNA binding by $SNAP_C$. EMSA were carried out with SNAP190 (1-505) and SNAP43 with 10, 30, or 100 ng of GST-SNAP50 (1-411), GST-SNAP50 (1-300), or GST, as indicated.

(B) The C-terminal SNAP50 zinc finger region is not required for interactions with SNAP43. Full-length and truncated SNAP50 proteins were labeled with [^{35}S]methionine and were used in GST pulldown assays with GST-SNAP43 (lane 3) and GST protein alone (lane 2). 10% of the total input for each protein is shown in lane 1. A schematic representation of the various SNAP50 proteins is shown at the left with the positions of the zinc finger domain and putative LxCxE RB interacting region (black box) indicated.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50 (1-411)
50 (1-300)
GST
AD
BD
ADBD
mSNAPc
Free Probe
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11 12 13 14 15 16 17 18 19 20
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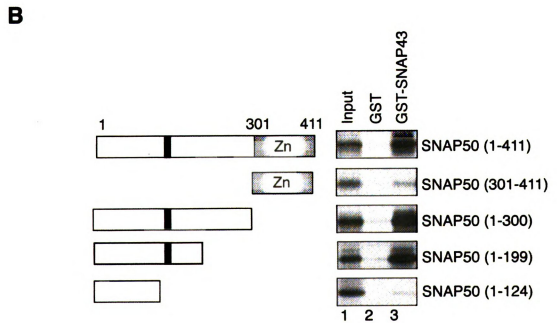
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GST
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SNAP50 (1-411)
SNAP50 (301-411)
SNAP50 (1-300)
SNAP50 (1-199)
SNAP50 (1-124)
1 2 3

snRNA genes. While the mechanism for PSE binding by $SNAP_C$ is not well understood, this initial event requires extensive cooperation among SNAP43, SNAP50, and SNAP190, as indicated by the data in Figure 3-1A. EMSA reactions containing all three subunits supported robust binding by mini-$SNAP_C$ to wild-type (lane 7) but not mutant PSE probes (lane 8), whereas no detectable DNA binding was observed in reactions that lacked all three complex members (lanes 1-6), consistent with results previously described (13). Thus, even though SNAP190 contains a bona fide Myb DNA binding domain, additional components of the complex are required, perhaps serving to directly recognize the PSE alongside SNAP190.

Whereas the previous experiment was performed with three factors, we postulate that the recognition of DNA by $SNAP_C$ likely involves direct DNA contacts provided by SNAP50 in addition to the contributions made by SNAP190. Firstly, SNAP50 was UV cross-linked to the PSE during DNA binding by endogenous $SNAP_C$ (9), indicating that SNAP50 is in close proximity to DNA during promoter recognition. Secondly, SNAP50 contains a putative zinc finger domain within the C-terminal region, and as zinc finger domains are typically involved in nucleic acid binding and/or protein-protein interactions, we hypothesized that this region of SNAP50 is involved in DNA binding by $SNAP_C$. To test this idea, EMSA were performed using mini-$SNAP_C$ containing SNAP50 that lacked the C-terminal cysteine/histidine-rich region. In support of the hypothesis, DNA binding by mini-$SNAP_C$ was completely ablated in reactions performed with SNAP50 (1-300) lacking the cysteine/histidine-rich region (lanes 15-17), as compared to comparable amounts of mini-$SNAP_C$ containing wild-type SNAP50 (lanes 12-14). In these reactions, the assembly of SNAP50 (1-300) into a complex along with SNAP43 and SNAP190 (1-

505) was as efficient as that observed for wild-type SNAP50 (data not shown), suggesting that those protein-protein interactions required for complex formation are not seriously jeopardized by removal of the cysteine/histidine-rich region. Indeed, strong pairwise interactions between SNAP50 and SNAP43, its major partner in $SNAP_C$, were maintained for truncated SNAP50 molecules lacking this region (Figure 3-1B). In GST-pulldown experiments, GST-SNAP43 interacted well with full-length SNAP50 (1-411), SNAP50 (1-300), and SNAP50 (1-199), but not with SNAP50 (1-124) nor with the cysteine/histidine- rich region alone (SNAP50 301-411). These data support the idea that the central region of SNAP50 participates in complex assembly with SNAP43 while the C-terminal cysteine/histidine-rich region constitutes a DNA binding domain within SNAP50.

The requirement for the SNAP50 cysteine/histidine rich region for DNA binding by $SNAP_C$ suggests that this region of SNAP50 constitutes a zinc finger domain. To determine whether zinc is indeed bound by $SNAP_C$, the ratio of zinc associated with a recombinant $SNAP_C$ containing HA epitope tagged SNAP50 along with SNAP190 (1-505), SNAP43, and SNAP19 (hereafter referred to as $SNAP_C\gamma4$) was determined by inductively coupled plasma mass spectrometry (ICP-MS). $SNAP_C\gamma4$ was chosen for this study because this recombinant complex, assembled by coexpression of each subunit in *E. coli*, is fully functional for PSE-specific DNA binding and for snRNA gene transcription by both RNA polymerases II and III (7), and zinc binding under functional conditions could be examined. As shown in Table 3-1, approximately 1.2 moles of Zn, as measured for both Zn66 and Zn68, were found associated with each mole of $SNAP_C\gamma4$, while little detectable Ni was associated with $SNAP_C\gamma4$. Similar data was obtained for

Table 3-1

This experiment was performed by Andrej Hanzlowsky (Dr. Jim Geiger's Lab).

Table 3-I

Zinc and Nickel analysis of recombinant SNAPc as determined by Inductively-Coupled Plasma Mass Spectrometry and Flame Atomic Absorption

Method 1: ICP- MS

Sample	[Zn] ppb	[Ni] ppb	[protein] mg/mL	n(Zn)/n(protein)	n(Ni)/n(protein)
SNAPcγ4 (1)	[a]1250.4	-	2.6	1.2 -	
	[b]1268.5	-	2.6	1.1 -	
	-	17.9	2.6	-	0.02
SNAPcγ4 (2)	[a]1415.5	-	2.9	1.2 -	
	[b]1443.6	-	2.9	1.2 -	
	-	35.5	2.9	-	0.03

a = total Zn calculated from Zn66
b = total Zn calculated from Zn68

$MW_{SNAPc\gamma4}$ ~ 159,735 g/mole
$MW_{SNAPc\gamma3}$ ~ 148,432 g/mole

Method 2: Atomic Absorption

Sample	[Zn] ppb	[protein] mg/mL	n(Zn)/n(protein)
SNAPcγ4 (1)	1956.8	5.3	0.90
SNAPcγ3	1642.2	4.1	0.91

two separate $SNAP_C\gamma4$ preparations. These results are comparable to those obtained by atomic absorption indicating a molar ratio (nZn/n $SNAP_C\gamma4$) of 0.9 for the four-subunit $SNAP_C\gamma4$ complex, and for the three-subunit $SNAP_C\gamma3$ complex lacking SNAP19. As no other $SNAP_C$ subunits besides SNAP50 contain suitable arrangements of cysteines and/or histidines for Zn coordination, the parsimonious explanation of these data is that $SNAP_C\gamma4$ contains a single SNAP50 subunit that binds a single Zn atom.

An evolutionarily conserved zinc-finger domain in human SNAP50 functions for DNA binding

The amino acid sequence for the human SNAP50 zinc finger domain is shown in Figure 3-2A, which reveals an unorthodox arrangement of cysteines and histidines within the C-terminal 110 amino acids. Typically, zinc finger domains that function for DNA binding are constituted by multiple repeats of related zinc finger motifs (21). However, the putative DNA binding domain of human SNAP50 contains nine cysteines and six histidines that can be grouped into region 1 loosely resembling a TFIIIA-like C2H2 zinc finger and region 2 resembling a glucocorticoid-like C2C2 zinc finger. While both types of motifs in other proteins are involved in direct DNA contacts, the discordant arrangement of zinc finger motifs suggests that DNA binding by SNAP50 is distinct from mechanisms employed by other zinc finger proteins. In addition, as $SNAP_C\gamma4$ contains only a single Zn atom, it is likely that only one of the potential Zn fingers actually coordinates Zn. Interestingly, the results of a BLAST homology search show that of the fifteen potential zinc coordination sites in this region, eight are highly conserved among putative SNAP50 homologues from mammals, insects (*Drosophila, Anopheles*), fish

(*Danio*), worms (*C. elegans*), plants (*Arabadopsis*), slime mold (*Dictyostelium*), and parasites from the *Trypanosoma, Entamoeba, Plasmodium*, and *Leishmania* genera. This comparison also shows that the critical zinc coordination sites within region 2 that constitute the glucocorticoid-like finger motif are well conserved whereas the TFIIIA-like C2H2 zinc finger motif within region 1 is not. Nonetheless, two histidines and one cysteine residue within region 1 are conserved raising the possibility that a function performed by this region is also maintained across divergent species. The consensus motif derived from this comparison ($L_{X4}G_{X6}H_{X3}C_XH_{X20-23}YP_{X11-12}C_{X2}C_{X18}P_{X3-4}$ $C_{X2}CF_{X3}H_{X1-4}G$) is distinct from any other family of zinc finger motifs (20), suggesting that this domain represents a novel zinc finger fold, and is hereafter referred to as the "SNAP finger" domain.

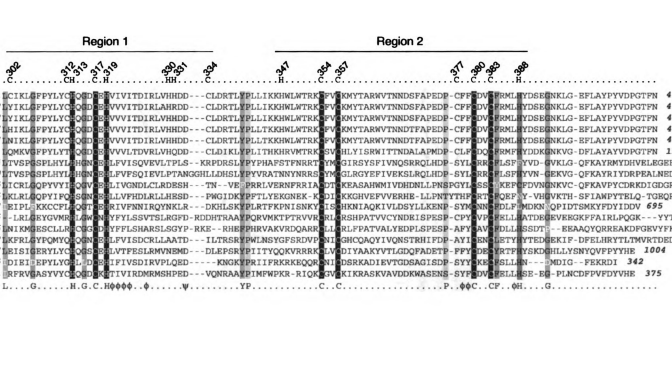
To examine the function of the cysteine and histidine residues within the C-terminal SNAP finger domain of SNAP50, each of these residues was changed to alanine for functional testing in the context of a partial $SNAP_C$ that contains SNAP190 (1-505), SNAP43, SNAP19, and the various derivatives of full-length HA-SNAP50. In addition, an arginine at position 385 was also substituted with alanine. First, to determine whether the targeted amino acids are critical for complex assembly a two-step affinity purification of wild type and mutant complexes were performed. Complexes were assembled by co-expressing four $SNAP_C$ subunits in *E. coli* followed by affinity purification of the complex via the GST tag contained on the amino terminus of SNAP190 (1-505). Complexes were liberated from the glutathione agarose beads by thrombin digestion, which recognizes its cognate site between the GST tag and the SNAP190 coding region. The soluble complexes were further purified by anti-SNAP43 immunoprecipitation, and

Figure 3-2. Single amino acid changes in the SNAP50 subunit abolish DNA binding by $SNAP_C$.

(A) Sequence alignment of human SNAP50 C-terminal amino acids (301-411) with corresponding regions from SNAP50 homologues of other species. Putative zinc fingers similar to TFIIIA ($Hx_5Hx_{10}Hx_3C$) and steroid receptors ($C_{X2}C_{X22}C_{X2}C$) are indicated as region 1 and region 2, respectively. The sequence of highly conserved amino acids derived from this alignment corresponds to $L_{X4}G_{X6}H_{X3}C_XH_{X20-23}YP_{X11-12}C_{X2}C_{X18}P_{X3-4}C_{X2}CF_{X3}H_{X1-4}G$. This alignment was performed using the Clustal W program. *Homo sapiens* (NP003075), *Cannis familiaris* (XP853813), *Bos taurus* (AAX08912), *Mus musculus* (NP084225), *Rattus norvegicus* (NP001013230), Drosophila melanogaster (NP724647), *Drosophila pseudoobscura* (EAL25490), *Trypanosoma brucei* (XP827295), *Arabidopsis thaliana* (AAO30067), *Caenorhabditis elegans*-1 (NP500819), *Caenorhabditis elegans*-2 (NP497807), *Plasmodium falciparum* (NP702469), *Danio rerio* (XP694501), *Leishmania major* (XP843572), *Anopheles gambiae* (XP310411), *Dictyostelium discoideum* (XP644064), *Entamoeba histolytica* (XP653151).

(B) Mutations in the SNAP50 zinc finger domain do not disrupt $SNAP_C$ assembly. Approximately 20 ng of each of the $SNAP_C\gamma4$ complex containing substitution mutations in HASNAP50 were affinity purified first using glutathione agarose to pull down GST-SNAP190 (1-505) followed by immunoprecipitation with a-SNAP43 antibodies. Associated wild type or mutant HA-SNAP50 was detected by anti-HA Western analysis (lanes 6-22). A titration of wild type $SNAP_C\gamma4$ using 8, 4 and 2% of the input material is shown in lanes 1-3, respectively. Lanes 4 and 5 contain wild type $SNAP_C\gamma4$ recovered with the protein-G agarose beads alone or with pre-immune serum, respectively. The bottom panel represents 4% of the input material directly analyzed by anti-HA Western analysis.

(C) Mutations in the SNAP50 zinc finger domain disrupt DNA binding by $SNAP_C$. Increasing amounts (3 and 10 ng) of $SNAP_C\gamma4$ with wild type (lanes 2 and 3) or mutant HA-SNAP50 (lanes 4-35) containing the indicated substitution mutations was tested in an EMSA for binding to a radiolabeled DNA probe containing a high affinity PSE and TATA box. Lane 1 shows the probe alone with no added proteins.


Region 1
Region 2
302
312
313
317
319
330
331
334
347
354
357
377
380
383
386
H sapiens 301 ECIKLSFPYLYCSQMDEEVIVITDIRLVHHDD---CLDRTLMPLLIKKHWLWTRKSFVSKMYTARWVTNNDSFAPEDP-CFPSDVSFRMLHYDSEGNKLG-SFLAYPYVDPGTFN 411
C familiaris 302 EYIKLGFPYLYCSQMDEEVVVITDIRLAHRDD---CLDRTLMPLLIKKHWLWTRKSFVSKMYTARWVTNNDSFAPEDP-CFPSDVSFRMLHYDSEGNKLG-SFLAYPYVDPGTFN 412
B taurus 302 EYIKLGFPYLYCSQMDEEVVVITDIRLVHHDD---CLDRTLMPLLIKKHWLWTRKSFVSKMYTARWVTNNDSFAPEDP-CFPSDVSFRMLHYDSEGNKLG-SFLAYPYVDPGTFN 412
M musculus 297 EHIKLGFPYLYCSQMDEEVVVITDIRLVHHDD---CLDRTLMPLLTKKHWLWTRKSFVSKMYTARWVTNNDTFAPEDP-CFPSDVSFRMLHYDSEGNKLG-SFLAYPYVDPGTFN 407
R norvegicus 297 EHIKLGFPYLYCSQMDEEVVVITDIRLVHHDD---CLDRTLMPLLTKKHWLWTRKSFVSKMYTARWVTNNDTFAPEDP-CFPSDVSFRMLHYDSEGNKLG-SFLAYPYVDPGTFN 407
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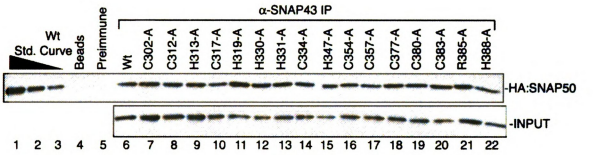
D melanogaster 254 LTVSPGSPLHYLFHQNDEELFVISQVEVLTPLS--KRFDRSLVPYPHAFSTFNRRTFVWGIRSYSPIVNQSRRQLHDP-SYLSRRSFLSFYVD-GVKLG-QFKAYRMYDHVELEGEEEDIKQQNLPS 377
D pseudoobscura 250 LTVSPGSPLHYLFHQNDEELFVPSQIEVLPTANGGHLLDHSLVPYVRATNNYNRRSFVWGLRGYEPIVEKSLRQLHDP-SYLSRRSFLSFTYVN-GEKVG-QFKAYRIYDRPEALNED 365
C elegans 1 305 ITCRLQQFPVVYICQMVPELLIVGNDLCLRDESH---TN--VE-PRRLVERNFRRIAVDTKEASAHWMIVDHDNLLPNSPGYLRSSE-KKFPFDVNINKVC-QFKAVPYCDRKDIGDGRQF-FTELDL 425
C elegans 2 296 KKLRLQQFVIFQQSMDEELLVPHDLRLLHRSD---PWGIDKTPFTLYEKGNEK-KKDIKKGHVPEFVVPERHE-LLPNTYTHPSRTYQRFY-VHQVKTH-SFIANPYTELQ-TGSQRGWPFGDFEQED 418
P falciparum 527 EIPL-KKCCFLQQTSEQIFVNNIRQYNKLR---DKHLSKYPLRTFKPNISNKVTISCHKNIAQKIVLDSYLLKENP-SYMGNNSMDLF-MDKN-QPIDTSMKYFDYIIDDV 695
T brucei 336 -LRLLEKYGVMRLLWNNYFYLSSVTSLRGFD-RDDHTRAAYPQRVMKTPTKVVRSRLQRSHPATVVCYNDKISPESP-CPYVVPYSKLLLATDEGEVEKGKFPAIRLPQGK---YYTA 448
L major 316 NIKMGESCLLRYCVGDEYFFLSHARSLSGYP-RKE--RHEYPHRVAKVRDQARRLLKRLFPATVALYEDPLSPESP-AFYSAVSYDLLLESSDYT--EEAAQYQRREAKDPGEVYFKAT 430
A gambiae 132 LKFRLQYPQMYQQNMEELFVISDCRLLAATD---ILTRSRYPWLNSYGFSRDVPNLSGHCQAQYIVQNSTRHIFDP-AYISRNSETYYTEDEKIF-DFELHRYTLTMVRTDEDADSEEASTS 244
D discoideum 897 EHISIGRKYLYCTQMDEELVTFESLRWVNEMD---DLRPSRYPHITTYQQKVRRRKTLVWDIYAAKYVTLGDQFADSTP-FFYLDRSKTTYYSKDGHLLYSNYQVFPYTHE 1004
E histolytica 245 TDIEIEFPYLYCSLYDEEIFIVSDIRVPLQED-----KNGKYPRIIFRKRKEQQRLNIDSRKADIEVTGDSAGISDP-SYYKRSWSLLDN---MDIG--FEKRDI 342
A thaliana 271 RFRVVASYVYCQMDKETIVIRDMRMSHPED---VQNRAAPIMFWPKR-RIQKLGVKIKRASKVAVDDKWAASENS-SYFDVSYSLLSE-EG-PLNCDFPVFDYVHF 375

B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Wt
Std. Curve
Beads
Preimmune
Wt
C302-A
C312-A
H313-A
C317-A
H319-A
H330-A
H331-A
C334-A
H347-A
C354-A
C357-A
C377-A
C380-A
C383-A
R385-A
H388-A
-HA:SNAP50
-INPUT
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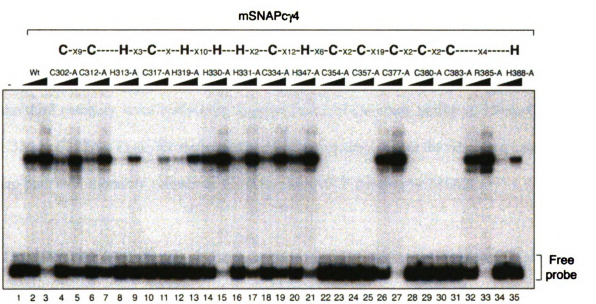
C


mSNAPcγ4
C-x9-C-----H-x3-C--x--H-x10-H---H-x2---C-x12-H-x6-C-x2-C-x19-C-x2-C-x2-C-----x4-----H
Wt
C302-A
C312-A
H313-A
C317-A
H319-A
H330-A
H331-A
C334-A
H347-A
C354-A
C357-A
C377-A
C380-A
C383-A
R385-A
H388-A
Free probe
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 34 35

the extent of SNAP50 association, as a measure of complex integrity, was determined by Western blot analysis against the HA-tag contained at the N-terminus of SNAP50. In this approach, any SNAP50 detected in this analysis had to be in a complex with both SNAP43 and SNAP190 (1-505). As shown in Figure 3-2B, the amounts of SNAP50 recovered after the two-step affinity purification were similar for the wild type and mutant complexes, suggesting that SNAP50 assembly into $SNAP_C$ is not markedly dependent upon any individual cysteine or histidine in this domain.

Next, partially purified wild type and mutant complexes were then tested for PSE binding function in EMSA to determine whether the cysteine and histidine residues within the SNAP finger domain are important for DNA binding by $SNAP_C$. The results revealed three categories of DNA binding effects, as shown in Figure 3-2C. The first category of mutants exhibited either wild-type DNA binding (H330A, H347A, C377A, R385) or modest defects (C302A, C312A, H331A, C334A) as compared to the complex containing wild-type SNAP50. The second category of mutant complexes exhibited severe defects in DNA binding (H313A, C317A, H319A, H388A), while complexes in the third category were completely impaired for DNA binding ability (C354A, C357A, C380A, C383A). Thus, the cysteine and histidine residues within the latter two categories are both evolutionarily conserved and critical for DNA binding by $SNAP_C$.

The SNAP50 zinc finger domain is required for RNA polymerase II and III transcription.

We next tested whether mutations that affected PSE-recognition by $SNAP_C$ were also critical for human U1 snRNA gene transcription by RNA polymerase II and U6

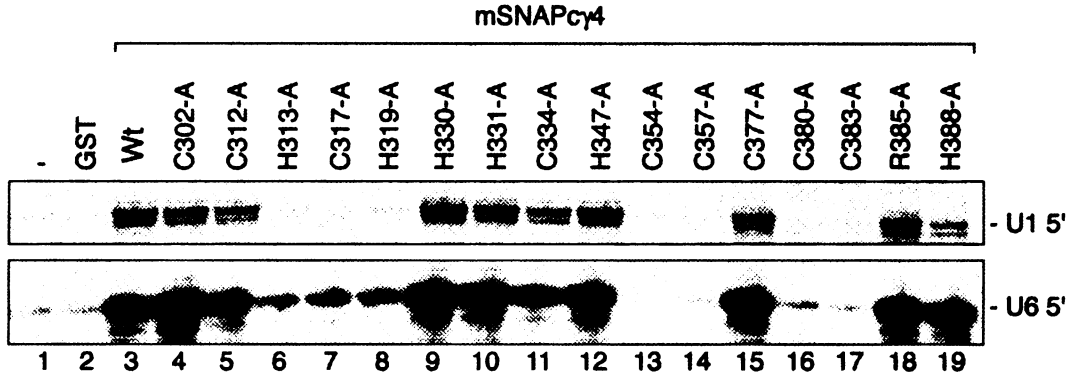
snRNA gene transcription by RNA polymerase III. For these experiments, we predicted that those complexes that exhibited no DNA binding ability would also be defective in transcription, confirming that DNA binding by $SNAP_C$ is an essential aspect of snRNA gene transcription. Secondly, any previously unappreciated function for SNAP50 in communication with other components of the general transcription machinery would be revealed as a defect in transcription for those complexes with wild type DNA binding activity. As the general transcription machinery for RNA polymerase II and III transcription are different, it was possible that certain mutations would result in transcriptional defects in only one polymerase system. However, as shown in Figure 3-3A, all complexes that were capable of DNA binding also functioned well for RNA polymerase II and III transcription with the extent of activity essentially parallel for both systems. Thus, those residues that are not evolutionarily conserved are also not critical for human snRNA gene transcription by either polymerase. In contrast, those mutant complexes that were crippled for DNA binding activity (C354A, C357A, C380A, C383A) did not support either RNA polymerase II or III transcription. We consider it likely that these critical cysteine residues within region 2 of the SNAP finger play a structural role for SNAP50 function and are likely important for Zn binding. Some unexpected differences between RNA polymerase II and III transcription were apparent for those mutant complexes that exhibited reduced, but not ablated, DNA binding. Some complexes (H313A, C317A, H319A) were moderately active for RNA polymerase III transcription but did not support RNA polymerase II transcription. Most noticeably, the complex containing SNAP50 H388A was fully functional for RNA polymerase III transcription. Thus, the DNA binding defects caused by altering these conserved residues,

Figure 3-3. The SNAP50 zinc finger domain is required for human snRNA gene transcription by RNA polymerases II and III.

(A) HeLa cell nuclear extract was depleted with anti-SNAP43 antibodies to immunodeplete endogenous $SNAP_C$. In vitro U1 and U6 transcription was then tested in the absence (lanes 1) or presence of purified $SNAP_C\gamma4$ (5 ng) containing wild type SNAP50 (lane 3) or mutant SNAP50 with the indicated alanine substitutions (lanes 4-19). Addition of GST alone did not reconstitute either U1 or U6 transcription as shown in lane 2.

(B) Summary of mutations in the SNAP50 zinc finger domain. Alanine substitution of cysteines and histidines within the SNAP50 zinc finger domain revealed three classes of phenotypes, as indicated.

A



B

Category	Phenotype	Mutation
I	Wild type DNA binding, U1 and U6 transcription	C302-A, C312-A, H330-A, H331-A, C334-A, H347-A, C377-A, R385-A
II	Diminished for DNA binding and transcription	C354-A, C357-A, C380-A, C383-A
III	Diminished for DNA binding and U1 transcription but active for U6 transcription	H313-A, C317-A, H319-A, H388-A

especially for the H388A substitution are suppressed during RNA polymerase III transcription.

Suppression of DNA binding defects by cooperation between TFIIIB and $SNAP_C$.

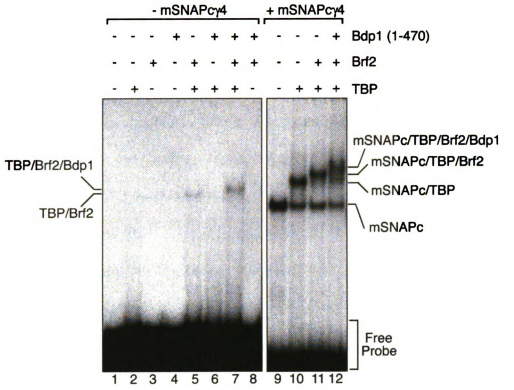
Based on the previously described observations, we hypothesized that the evolutionarily conserved residues in region 1 (H313, C317, and H319), as well as H388 in region 2 facilitate DNA contacts by $SNAP_C$, either directly or indirectly, and during RNA polymerase III transcription their activity was restored because TFIIIB stabilized mutant $SNAP_C$ binding to the PSE. To determine whether TFIIIB was capable of restoring DNA binding activity to $SNAP_C$, it was first necessary to establish an assay that measured DNA binding by TFIIIB and $SNAP_C$. Human U6 snRNA gene transcription relies on a variant of TFIIIB called Brf2-TFIIIB, which is composed of TBP, Brf2, and Bdp1 (30). In the following experiments, a truncated form of Bdp1 (1-470) was used because it supports wild-type U6 transcription (ref. (16), data not shown) and can be expressed and purified more easily than the full-length Bdp1 (1- 1338). As shown in Figure 3-4A (left panel), weak binding of Brf2-TFIIIB to U6 promoter probes is observed when all three components are included in the DNA binding reaction, whereas no binding was observed in any reactions that did not contain TBP, consistent with the observation that Brf2-TFIIIB is TATA-box dependent (data not shown). Weak, but nonetheless cooperative, DNA binding is also observed for TBP plus Brf2 at levels greater than that observed for either factor alone, as previously described (2, 13, 28). In contrast, $SNAP_C\gamma4$ bound well and effectively recruited TBP to DNA (Figure 3-4A right panel). Although Brf2 stimulated DNA binding by TBP, much shorter exposure times were used

Figure 3-4. $SNAP_C$ recruits Brf2-TFIIIB to U6 promoter DNA in vitro.

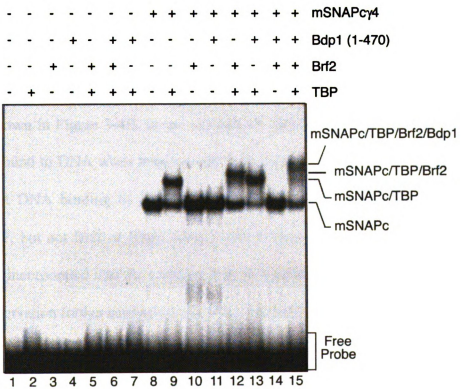
(A) $SNAP_C$ stimulates DNA binding by Brf2-TFIIIB. Electrophoretic mobility shift assays were performed using a U6 probe containing a wild-type mouse U6 PSE and a wild-type TATA-box. DNA binding was carried out in the absence (lanes 1-8) or presence (lanes 9-12) of wild type $SNAP_C\gamma4$. Reactions containing individual TBP, Brf2, and Bdp1 (1-470) subunits are shown in lanes 2-4. Reactions containing pair wise combinations of TBP with Brf2, TBP with Bdp1 (1-470), and Brf2 with Bdp1 (1-470) are shown in lanes 5, 6, and 8. DNA binding by the complete Brf2-TFIIIB complex in the absence of $SNAP_C$ is shown in lane 7. Additional reactions were performed with $SNAP_C$ alone (lane 9) or in combination with Brf2-TFIIIB subunits (lanes 10-12), as indicated. Lane 1 shows migration of the probe alone. The relative positions of the various $SNAP_C\gamma4$/Brf2-TFIIIB complexes are shown on the right.

(B) Coordinated DNA binding by $SNAP_C$ and TBP facilitates higher order complex assembly with Brf2 and Bdp1. DNA binding reactions were performed with the indicated combinations of $SNAP_C\gamma4$ and Brf2-TFIIIB subunits. These results suggest that preinitiation complex assembly follows the order $SNAP_C$>TBP>Brf2>Bdp1.

A


- mSNAPcγ4
+ mSNAPcγ4
Bdp1 (1-470)
Brf2
TBP
TBP/Brf2/Bdp1
TBP/Brf2
mSNAPc/TBP/Brf2/Bdp1
mSNAPc/TBP/Brf2
mSNAPc/TBP
mSNAPc
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12

B


mSNAPcγ4
Bdp1 (1-470)
Brf2
TBP
mSNAPc/TBP/Brf2/Bdp1
mSNAPc/TBP/Brf2
mSNAPc/TBP
mSNAPc
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

to visualize DNA binding for reactions containing $SNAP_C$ (right panel), and for exposures that exhibited prominent DNA complexes containing $SNAP_C$, the DNA binding by Brf2-TFIIIB alone was essentially undetectable (not shown, and Figure 3-4B). Thus, at least under these conditions, $SNAP_C$ plays a dominant role for TBP recruitment to U6 promoter DNA. Interestingly, serial addition of Brf2 then Bdp1 resulted in complexes that migrated incrementally more slowly, consistent with the idea that increasingly larger complexes are being assembled on the DNA. The amount of the $SNAP_C\gamma4$-DNA complex was not further affected by Brf2 and Bdp1. In contrast, the amount of the $SNAP_C\gamma4$/TBP-DNA complex was diminished by Brf2 addition, and the $SNAP_C\gamma4$/TBP/Brf2-DNA complex was diminished by Bdp1 addition. These last observations suggest a substrate-product relationship during complex assembly, and are consistent with the idea that Brf2 and Bdp1 both exhibit a binding preference for the higher order complexes containing TBP than that complex containing only $SNAP_C$.

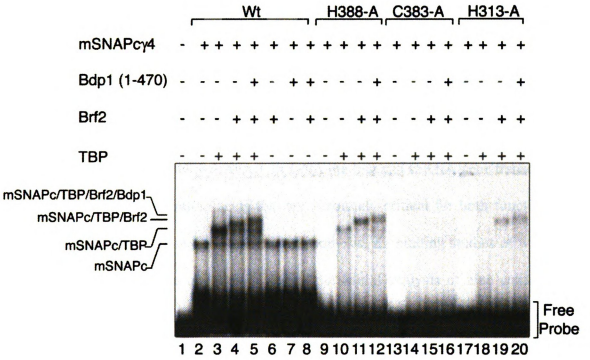
To further refine the pathway for assembly of $SNAP_C$ and Brf2-TFIIIB on U6 promoter DNA, combinations of each factor were tested for DNA binding ability in EMSA. As shown in Figure 3-4B, in the absence of $SNAP_C$, none of the Brf2-TFIIIB components bound to DNA when tested singly or in combination under conditions that support robust DNA binding by $SNAP_C\gamma4$. Interestingly, $SNAP_C\gamma4$ was capable of recruiting TBP, but not Brf2 or Bdp1, when tested in pair wise combination, whereas Brf2 could be incorporated into the complex only in reactions containing $SNAP_C\gamma4$ and TBP. This observation further supports the premise that Brf2 preferentially recognizes the $SNAP_C$/TBP promoter-bound complex. Similarly, Bdp1 was not recruited to the $SNAP_C$/TBP complex, but again it required the presence of Brf2 for DNA association.

Based on these results, we propose a sequential assembly pathway with the initial promoter recognition performed by $SNAP_C$ and TBP followed by Brf2 and Bdp1. The eventual recruitment of Bdp1 is predicted to enable RNA polymerase III recruitment under transcription conditions.

To test the hypothesis that Brf2-TFIIIB can suppress DNA binding defects in $SNAP_C$, recombinant $SNAP_C$ containing wild type or mutant SNAP50 were tested for DNA binding in the absence or presence of each component of Brf2-TFIIIB (Figure 3-5). For this assay, examples from each category of mutant SNAP50 were tested including H388A and H313A that were fully or partially active in U6 transcription, respectively, as well as C383A that was devoid of measurable activity for either DNA binding or transcription. In these assays, the same U6 promoter sequence was used for DNA binding as that contained on the reporter plasmids that were previously used for in vitro U6 transcription. As expected, wild-type recombinant $SNAP_C$ bound DNA well and supported robust recruitment of Brf2-TFIIIB. In contrast, mutant $SNAP_C$ containing SNAP50 (H388A), SNAP50 (C383A), or SNAP50 (H313A) were each inactive when tested alone for DNA binding. Thus, both wild-type and mutant $SNAP_C$ bound with reduced affinity to the U6 promoter sequences tested in this assay relative to those experiments done with the artificial high affinity PSE promoter probes as shown in Figures 3-2 and 3-4. Importantly, DNA binding by the SNAP50 (H388A)-containing complex was restored by TBP, although not to levels seen with the wild type SNAP50 complex, and higher order complex formation with Brf2 and Bdp1 occurred at nearly wild type levels. Thus, Brf2-TFIIIB was capable of restoring DNA binding activity to mutant $SNAP_C$. This result stands in contrast to the SNAP50 (C383A)-containing

Figure 3-5. TFIIIB suppresses DNA binding defects in $SNAP_C$.

DNA binding reactions were carried out as previously described for the artificial AC probe instead using the natural U6 promoter containing DSE, PSE, and TATA-box elements. Reactions were performed with 8 ng of $SNAP_C\gamma4$ containing either wild type SNAP50 (lanes 2-8) or mutant SNAP50 with the substitutions H388A (lanes 9-12), C383A (lanes 13-16), and H313A (lanes 17-20). Reactions additionally containing TBP, TBP and Brf2, or TBP, Brf2 and Bdp1 (1-470) were performed as indicated. DNA binding by mutant $SNAP_C\gamma4$ harboring the H388A mutation was restored by TBP alone whereas TBP plus Brf2 were required to restore DNA binding by $SNAP_C\gamma4$ harboring the SNAP50 (H313A) mutation. No DNA binding under any condition was observed for $SNAP_C\gamma4$ containing the SNAP50 (C383A) substitution.


Wt
H388-A
C383-A
H313-A
mSNAPcγ4 - + + + + + + + + + + + + + + + + + +
Bdp1 (1-470) - - - - + - + + - - - + - - - + - - - +
Brf2 - - - + + + - + - - + + - - + + - - + +
TBP - - + + + - - - - + + + - + + + - + + +
mSNAPc/TBP/Brf2/Bdp1
mSNAPc/TBP/Brf2
mSNAPc/TBP
mSNAPc
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

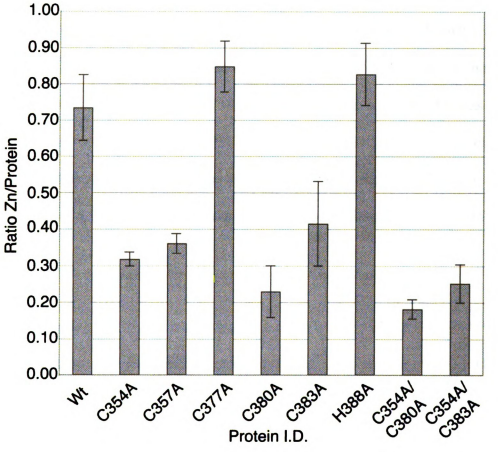
complex that was unable to bind DNA under any conditions. Interestingly, TBP did not restore DNA binding by the SNAP50 (H313A)-containing complex but Brf2 plus TBP did, suggesting that the H313A substitution presents a more dramatic defect to DNA binding by $SNAP_C$. Nonetheless, Bdp1 could be recruited at reduced levels by this $SNAP_C^{(H313A)}$/TBP/Brf2 complex, consistent with the markedly reduced transcription supported by this complex for in vitro U6 transcription.

The C-terminal SNAP50 zinc finger binds zinc.

As the previous analyses revealed that certain amino acids within the SNAP finger domain are differentially required for DNA binding and snRNA gene transcription, we postulated that those amino acids that are absolutely critical for both functions are also important for zinc binding. To test this hypothesis, Zn binding studies of wild type and mutant SNAP50 were undertaken. While our initial analysis of zinc content was performed for the four-member complex ($SNAP_C\gamma4$), suitable amounts of mutant complexes for zinc analysis were not obtained in this context. Nor were we able to obtain sufficient amounts of full-length SNAP50 (1- 411) or truncated SNAP50 (301-411) for these studies when expressed individually in *E. coli*. However, suitable amounts of truncated SNAP50 (315-411) were obtained when expressed as fusion protein with a His-SUMO N-terminal tag, and zinc binding studies of this protein were therefore pursued. As shown in Figure 3-6, analysis of SNAP50 (315-411) by flame atomic absorption showed that this region of SNAP50 bound substantial amounts of zinc (~0.7 mole zinc/mole protein). This level of zinc binding by the isolated SNAP50 zinc finger domain is comparable to that seen for full-length SNAP50 (1-411) in the context of the four-

Figure 3-6. The SNAP50 zinc finger domain binds zinc.

Wild type and mutant SNAP50 (315-411) containing the indicated mutations were tested for Zn content by flame atomic absorption, and the calculated molar ratios of zinc to protein are shown. Error bars indicate the relative standard deviation.


1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Ratio Zn/Protein
Wt
C354A
C357A
C377A
C380A
C383A
H388A
C354A/ C380A
C354A/ C383A
Protein I.D.

protein complex $SNAP_C\gamma4$ (~0.9 mole zinc/mole protein), indicating that the SNAP finger domain is likely responsible for zinc binding by $SNAP_C$. Zinc binding by SNAP50 (315- 411) was markedly reduced by individual alanine substitution at positions C354, C357, C380, and C383, whereas alanine substitutions of C377 and H388 did not substantially affect zinc binding. Overall, these data indicate that those cysteines within the $C354_{X2}C357_{X22}C380_{X2}C383$ motif are important for zinc binding by $SNAP_C$, whereas the adjacent C377 and H388 residues are not. Moreover, SNAP50 (315-411) harboring either the C354A:C380A or C354A:C383A double alanine substitutions was further incapacitated as compared to SNAP50 C354A or C383A, but zinc levels were comparable to the reduced levels observed for SNAP50 harboring the single C380A substitution. Thus, C380 plays a more critical role in zinc binding than C354 and C383.

Discussion

DNA binding by $SNAP_C$ is a cooperative event wherein SNAP190, SNAP50, and SNAP43 are all required for promoter recognition (ref. (13), and Figure 3-1). Our data demonstrate that an unorthodox zinc finger domain in SNAP50 plays a critical role in this process. A comparison of this region with SNAP50 homologues from other species revealed that the arrangement of many cysteine and histidine residues within the SNAP50 C-terminal region is remarkably well conserved (Figure 3-2A), and a mutational analysis of all histidine and cysteine amino acids throughout this region showed that these highly conserved residues are critical for DNA binding by $SNAP_C$.

The high degree of sequence conservation within the SNAP finger domain throughout evolution suggests that SNAP50 function is conserved in other species.

Indeed, *Drosophila* PBP50 (Proximal element binding protein 50 kDa), a homologue of human SNAP50, makes direct DNA contacts within the U6 and U1 promoters although the promoter sequences recognized by human $SNAP_C$ and *Drosophila* PBP are different (34). Besides SNAP50, *Drosophila* also maintains homologues of SNAP43 and SNAP190, but not of SNAP45 or SNAP19. The identification of SNAP50- and SNAP43-related proteins in *Trypanosoma* and *Leishmania* suggests an ancient function for $SNAP_C$ in non-translated RNA production, in this case RNA polymerase II transcription of spliced leader (SL) RNA. *Trypanosoma* $SNAP_C$ also contains a Myb domain-containing protein (3, 29) reminiscent of human SNAP190, which contains an unusual Myb DNA binding domain (35). The conservation of these three subunits throughout evolution remarkably parallels the experimental definition of a minimal human $SNAP_C$ composed of SNAP190 (1-505), SNAP50, and SNAP43 that retains full activity in RNA polymerase II and III transcription (7, 24).

Interestingly, the overall spacing of potential zinc coordination sites within SNAP50 does not resemble other known zinc finger motifs, although the arrangement for a subset of these cysteine residues resembles that of hormone receptor DNA binding domains (20), consistent with a role in DNA binding for this region in SNAP50. Nonetheless, the unusual arrangement of zinc coordination sites combined with secondary structure predictions suggest that human SNAP50 is the founding member for a novel class of zinc finger domains that we refer to as the SNAP finger domain.

To date, an experimental structure of SNAP50 has not been determined. Therefore, computational methods were employed to predict model structures to assist our understanding of the mechanism for DNA binding by SNAP50. Comparative

modeling, which is often successful in other cases, was not possible because no sequence homologs of SNAP50 with known structures are available. However, as the SNAP finger domain is sufficiently short (~100 residues) *ab initio* modeling based only on the amino acid sequence and the predicted secondary structure was performed. Although *ab initio* structure prediction methods in general cannot accurately predict protein structures at the level of experimental structures, it is often possible to obtain approximate models of relatively small domains (<100 residues) with an overall root mean square deviation of 5-10 Å from the correct, native structure.

The first round of *ab initio* prediction with the Robetta server resulted in ten models for the SNAP finger domain. The resulting models were substantially different; however, the conserved cysteines 354, 357, 380, and 383 were in sufficiently close proximity to serve as zinc coordination sites in 5 out of the 10 models. Such a result is non-trivial given that the pair C354/C357 is separated by 22 residues from C380/C383, which lends support to the hypothesis that these four cysteines coordinate zinc in a novel zinc finger fold topology. In order to examine a wider range of possible structures and arrive at a model for the entire SNAP finger domain, additional *ab initio* sampling was carried out under the constraint that the two pairs of residues, C354/C380 and C357/C383, are each in close proximity. The best-scoring model consists mainly of β-sheets and a small α-helical segment according to the predicted secondary structure (Figure 3-7A), and the predicted structure is shown in Figure 3-7B. Submission of the model shown to the DALI server (14, 15) resulted in two known structures with remotely similar topology:domain II from calpain, a cysteine protease (PDB ID: 1KXR) and a beta-propeller domain of sialidase (PDB ID: 1EUT). However, the structural similarity is

Figure 3-7. Model for the SNAP50 zinc finger domain.

(A) Predicted secondary structure of SNAP50 (301-411) from SABLE (39).

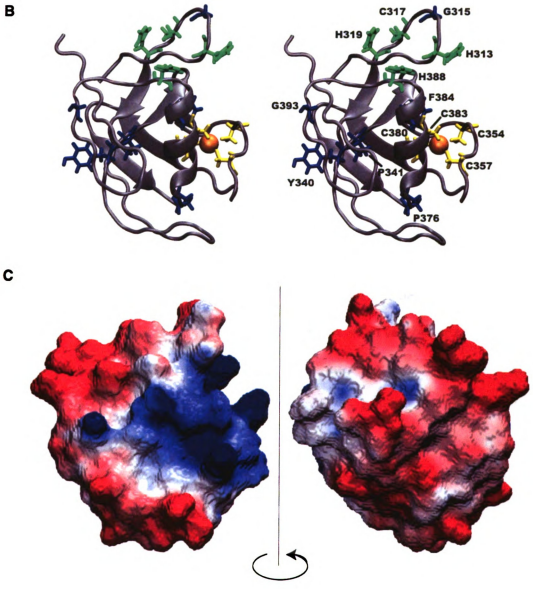
(B) Stereo image of the predicted model structure for the C-terminal domain of SNAP50 (residues 305-411). Conserved cysteines C354, C357, C380, C383 (yellow) are shown in a zinc coordination geometry (zinc is shown in orange). The conserved residues H313, C317, H319, and H388 are shown in green, and other conserved residues are shown in blue.

(C) Electrostatic potential projected onto the molecular surface of the SNAP50 model in the same orientation as in figure 7B (left) and rotated to show the back (right).

Modeling of SNAP50 zinc finger domain was done by Dr. Michael Feig.

A


300 310 320 330 340 350 360 370 380 390 400
DLCIKLGFPYLYCQGDEEVIVITDIRLVHHDDCLDRTLYPLLIKKHWLWTRKCFVCKMYTARWVTNNDSFAPEDPCFFCDVCFRMLYDSEGNKLGEFLAYPYVDPGTFN
CCEEECCCCCEEEEECCCCCEEEEEEECEEECCCCCCCCCCCCEEEEEEEECCCCCEEEEEEECCCCCEEEECCCCCCCCCCCCCHHHHHHHHHHCCCCCCCCCCCCEEEEEECCCCCCC


B
C317
G315
H319
H313
H388
F384
G393
C383
C380
C354
C357
Y340
P376
C
C

sufficiently low to suggest that the predicted SNAP finger fold has a novel architecture. The current model for the SNAP finger domain highlights the zinc coordination by C354, C357, C380, and C383, and is independently supported by experimental data indicating that these four cysteine residues are involved in zinc binding. The proposed model is vaguely reminiscent of the GATA-1 zinc finger motif wherein an α-helix plays a critical role in DNA recognition (33), but is substantially different because of a much longer inserted sequence between the two pairs of cysteines. In the SNAP50 model, other highly conserved residues, G315, P341, and P376, are located at critical turn regions for stabilization of the proposed structure.

Interestingly, this SNAP50 model also brings the conserved residues H313, C317, C319 in proximity with H388, which could provide an alternate metal binding site. We note, though, that the binding of an additional metal atom to $SNAP_C$ is not supported by the experimental data, and the exact function of these residues remains unclear. These residues may stabilize structures involved in DNA binding by SNAP50, or instead participate directly in DNA contacts. Of note, these residues are located adjacently to the α-helix, which in other zinc finger proteins is frequently used to make specific base contacts within the major groove during DNA binding (20). However, only the C-terminus of the corresponding α-helix within the SNAP finger domain model is fully exposed, which would likely limit major groove contacts. Thus, stable DNA binding through this region may require additional contacts by flanking residues. Interestingly, the electrostatic surface potential for the SNAP finger domain (Figure 3-7C) shows that the presented model clearly distinguishes between positively and negatively charged faces. A large well-defined positively charged surface patch surrounding the α-helix

suggests the potential for DNA binding, although a specific mode of interaction between SNAP50 and DNA cannot yet be predicted because of the uncertainty associated with this working model. Nonetheless, the current study provides insight into the DNA binding properties of $SNAP_C$, and identifies the evolutionarily conserved zinc finger domain of SNAP50 as critical for promoter recognition and human snRNA gene transcription.

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CHAPTER 4

THE HEXIM1 REPRESSOR OF P-TEFB STIMULATES TRANSCRIPTION OF ITS 7SK snRNA COREPRESSOR PARTNER BY RNA POLYMERASE III

Abstract

The HEXIM1/7SK snRNA ribonucleoprotein complex inhibits RNA polymerase II transcription of HIV-1 and cellular genes through interactions with the elongation factor P-TEFb. The 7SK promoter is a type 3 RNA polymerase III promoter, with a core consisting of a TATA box recognized by TBP and a proximal sequence element (PSE) recognized by $SNAP_C$. Here we show that HEXIM1 co-purifies and is associated with $SNAP_C$. HEXIM1 occupies an endogenous 7SK snRNA gene promoter and stimulates 7SK snRNA gene transcription in vivo. Consistent with a role as an activator of RNA polymerase III transcription, HEXIM1 stimulates the binding of $SNAP_C$ and TBP to DNA containing their cognate promoter elements. The cyclinT1 and cdk9 components of P-TEFb are dispensable for 7SK transcription by RNA polymerase III, but are required for U1 snRNA gene transcription by RNA polymerase II. These findings reveal a novel role for HEXIM1 as an activator of expression of its 7SK snRNA co-repressor partner and implicate this regulatory circuit as a potential attenuator of global RNA polymerase II transcription.

Introduction

Small nuclear (sn) RNA genes are among the most actively transcribed genes in human cells with initiation events occurring approximately every 2-4 seconds during periods of active growth (6). While initially believed to be constitutively transcribed and relatively immune to regulatory influence, the transcription of human snRNA genes is regulated both during the cell cycle (19) and in response to DNA damage (10). Many factors that have been extensively investigated because of their regulatory roles during human neoplasm initiation and progression also regulate human snRNA gene transcription. These include the p53 and Retinoblastoma tumor suppressor proteins, as well as the protein kinase CK2 and c-Myc oncoproteins (3, 4, 9, 10, 17, 20). That these critical tumor-related factors control human snRNA gene transcription portends a global role for snRNAs in controlling cellular growth.

The human snRNA gene products play central roles in global gene expression, most commonly through their function in RNA processing (26). Examples include the U-rich snRNAs U1, U2, U4, U5, and U6 that are required for messenger RNA splicing. In addition to their classical roles in RNA metabolism, snRNA complexes can also stimulate RNA polymerase II transcription possibly by coupling transcription initiation or elongation to downstream splicing events (8). More recently, U1 snRNA was shown to associate with TFIIH and to stimulate RNA polymerase II transcription (24), perhaps by stimulating TFIIH activity during transcriptional initiation. The cyclin H/cdk7 components of TFIIH phosphorylate the carboxy terminal domain (CTD) of the RNA polymerase II largest subunit to stimulate early initiation events (1, 7), but whether U1 snRNA modulates that process is not known. Additional CTD phosphorylation by the

cyclin T1/cdk9 kinase component of P-TEFb stimulates RNA polymerase II elongation (29). The P-TEFb transcriptional elongation factor, originally characterized as a RNA polymerase II elongation factor in Drosophila, was subsequently linked to HIV-1 transcription and replication initiated from the HIV long terminal repeat (LTR) (28, 46). Interestingly, P-TEFb kinase activity and consequently RNA polymerase II elongation from the HIV-1 LTR are inhibited by a ribonucleoprotein complex composed of HEXIM1 and 7SK snRNA (34, 42). Thus, U1 and 7SK snRNAs associate with the cyclin/cdk complexes, TFIIH and P-TEFb, respectively, but with opposite effects on gene transcription. These observations suggest that the relative levels of U1 and 7SK snRNA in the cell may control cellular transcription potential and furthermore, the regulated transcription of snRNA genes may be important for maintaining this balance.

Human snRNA genes can be grouped according to their promoter structures that dictate polymerase specificity for transcription. Some genes such as U1 and U2 are transcribed by RNA polymerase II, and others such as U6 and 7SK are transcribed by RNA polymerase III (11, 15). The use of different polymerases and transcription machinery may allow the cell to discretely control the transcription of distinct classes of snRNA genes. Regardless of polymerase choice, human snRNA genes contain a distal sequence element (DSE) that functions as an enhancer of transcription and contains binding sites for the Oct-1 (32) and STAF (33, 36) activator proteins. Each gene also contains a proximal sequence element (PSE) that is located within the core promoter region that is recognized by the general transcription factor called the snRNA activating protein complex ($SNAP_C$) (35), or the PSE-binding transcription factor (PTF) (32). A TATA box element located at a fixed distance from the PSE determines the initial

pathway of preinitiation complex assembly for transcription by RNA polymerase III (25). In this context, the TATA-box is recognized by the TATA-box binding protein (TBP) as part of the snRNA-specific version of the TFIIIB complex (38, 41). The assembly of $SNAP_C$ along with TFIIIB permits RNA polymerase III recruitment, whereas the absence of the TATA box by default directs PSE- and $SNAP_C$-dependent preinitiation complex assembly with TFIIA, TFIIB, TFIIE, and TFIIF for transcription by RNA polymerase II (23).

To identify factors that associate with $SNAP_C$ and are potentially involved in human snRNA gene transcription, a biochemical fractionation of $SNAP_C$ from HeLa cell extracts was performed. We report that endogenous HEXIM1 associates with $SNAP_C$ during chromatographic purification and furthermore, HEXIM1 associates with human snRNA genes in vivo as detected by chromatin immunoprecipitation (ChIP). In vitro, recombinant HEXIM1 stimulates promoter recognition by $SNAP_C$ and by TBP to their cognate cis elements within a 7SK-like probe, and in vivo HEXIM1 activates 7SK snRNA, thus revealing a novel positive function for HEXIM1 in regulating RNA polymerase III transcription. Other components of P-TEFb including cyclin T1 and cdk9 are dispensable for U6 and 7SK snRNA gene transcription by RNA polymerase III in vitro, but are required for U1 transcription by RNA polymerase II, suggesting that the function of HEXIM1 for RNA polymerase III transcription is separate from its role in regulating P-TEFb kinase activity.

Methods and Materials

UV cross-linking.

The conditions for UV cross linking are essentially as described (35), except that reactions were performed with highly purified $SNAP_C$ fractions. The purification of $SNAP_C$ was described previously (14).

Electrophoretic mobility shift assays.

EMSA were performed essentially as described (16) using recombinant HEXIM1 expressed in *E. coli* as a N-terminal GST-fusion protein (GST-HEXIM1). After protein expression, GST-HEXIM1 was affinity-purified by binding to glutathione agarose beads followed by thrombin digestion to release the untagged full-length HEXIM1 protein. As indicated, reactions also included recombinant full-length human TBP or mini-$SNAP_C$ containing SNAP190 (1-505), SNAP43, and SNAP50. The amounts of each protein are indicated in the figure legends. The radiolabeled DNA probes used contained a high affinity wild-type mouse U6 PSE and human U6 TATA box. Additional reactions were performed with probes that contain debilitating mutations in each element. Protein-DNA complexes were separated on a 5% polyacrylamide gel in 0.5X Tris-borate-EDTA (TBE) running buffer at 150 V. The HEXIM1-TBP complexes were resolved in running buffer containing Tris-glycine-EDTA-Mg^{2+} (TGEM).

Chromatin immunoprecipitation assay.

Normal human mammary epithelial cells (184B5) were maintained in Dulbecco's minimum essential media (Gibco) containing 5% fetal bovine serum (Gibco), and

penicillin-streptomycin at 37°C with 5% CO_2. ChIP assays were performed as described previously (18) using 184B5 cells that were grown to ~75% density. Cells were treated with 1% formaldehyde for 30 min at room temperature. After cell lysis, the DNA was fragmented into 500-800 bp fragments by sonication. Immunoprecipitation reactions were performed overnight at 4°C using chromatin from approximately 10^7 cells per reaction and approximately 1μg of each antibody. The anti-HEXIM1 antibody (CS162) was generated in rabbits using a peptide (CSH419) corresponding to the C-terminal 18 amino acids of HEXIM1. The anti-SNAP43 (CS48, (14)) and anti-TBP (SL2, (27)) antibodies were described previously. The anti-RNA polymerase III antibody (MI170) was raised in rabbits against the C-terminal 18 amino acids of hRPC155 as previously described (40). The RNA polymerase II antibody (8WG16) was purchased from Covance Research Products. The anti-cdk9 (SC-484) and anti-cycT1 (SC-8127) antibodies were purchased from Santa Cruz Biotechnology. Immunoprecipitated DNA was analyzed by PCR using primers specific to the genes indicated. The primers used for amplification of each gene are as follows:

U6 forward: 5'-AAGACGCGCAGGCAAAACG-3'

U6 reverse: 5'-CGGTGTTTCGTCCTTTC-3'

7SK forward: 5'-TTTTGGGAATAAATGATATTTG-3'

7SK reverse: 5'- GAGGTACCCAGGCGGCGCACAAG-3'

U1 forward: 5'- CACGAAGGAGTTCCCGTG-3'

U1 reverse: 5'-CCCTGCCAGGTAAGTATG-3'

U1 upstream forward: 5'-GAACTTACTGGGATCTGG-3'

U1 upstream reverse: 5'-GAGACAACTGAGCCACTTG-3'

GAPDH forward: 5'-AGGTCATCCCTGAGCTGAAC-3'

GAPDH reverse: 5'- GCAATGCCAGCCCCAGCGTC-3'

PCR products were separated by 1.5 % agarose gel electrophoresis in 0.5X TBE buffer, stained with ethidium bromide, and visualized with Kodak imaging software.

In vitro transcription.

Antibody immunodepletion of HeLa cell nuclear extracts were performed as described (13) and the depleted extracts were used for in vitro transcription assays for 1hr at 30°C. In vitro transcription of human U6 snRNA, U1 snRNA, AdML, and AdVAI genes were performed as described (25, 35). 7SK transcription was analyzed by a riboprobe protection assay using the plasmid pBS-7SK, containing the 7SK promoter from –245 to +1 fused to an inverted β-globin sequence, and conditions identical to those used for U6 in vitro transcription. Transcripts were separated by denaturing PAGE and visualized by autoradiography.

Transient transfection assay.

For the HEXIM1 knockdown experiment, control siRNA and the HEXIM1-specific annealed siRNA (si-RNA ID# 17952) were purchased from Ambion Biotechnology (5'-GGAUCCGAGCCGAGAUGUU-3'). HeLa cells were grown to 50-60% confluence in a six-well plate and transiently transfected with 0.2 nmol of the siRNA using Lipofectamine 2000 (Invitrogen). After 8 h, the medium containing the transfection reagent was supplemented with Dulbecco's modified Eagle's medium with 5% fetal

bovine serum and antibiotics. Cells were also co-transfected with 300 ng of the pBS-7SK reporter plasmid containing an inverted β-globin sequence driven by the 7SK snRNA core promoter (-245 to +1). The pBS-U1 reporter plasmid was constructed by replacing the 7SK promoter region with that of U1. The pU6/Hae/Ra.2 has already been described (25). Cells were harvested 30 hr post transfection. For HEXIM1 overexpression, HeLa cells were transiently transfected with 10 ng pCGN-HEXIM1 plasmid or pCGN empty vector and 300 ng of the pBS-7SK reporter plasmid. Total RNA was extracted using TRIZOL reagent (Invitrogen). Equal amounts of total RNA were further used in an RNase protection assay with a radiolabeled probe specific to the inverted β-globin sequence.

Results

Endogenous HEXIM1 co-purifies with $SNAP_C$.

Previous UV cross linking experiments performed with partially-purified $SNAP_C$ /PTF, hereafter referred to as $SNAP_C$, revealed that two subunits, SNAP190/PTFα and SNAP50/PTFβ, were in close proximity to the PSE during DNA binding by $SNAP_C$ (12, 45). However, a third unidentified factor of 70 kDa apparent molecular weight was also covalently attached to the DNA in a PSE-specific manner (35). As no subunits within $SNAP_C$ correspond to this size, this observation suggested that an additional unknown factor could function in human snRNA gene transcription through interactions with the general transcription factor $SNAP_C$ and with DNA. Therefore, an extensive biochemical fractionation of $SNAP_C$ was employed to identity this associated factor. The purification scheme for endogenous $SNAP_C$ (Figure 4-1A) is the same as that previously described

(14). Typically, the $SNAP_C$ present in the last Mono-S step of fractionation has been purified approximately 104-fold to the point that individual proteins can be isolated and identified (data not shown).

To determine whether a similar pattern of protein cross-linking to PSE probes was possible with highly purified $SNAP_C$ as was previously described for crude fractions, UV cross linking experiments were performed with fractions obtained after the Mono-S step of fractionation. DNA binding reactions were performed with radioactive wild type (wt) or mutant (mu) PSE probes containing bromodeoxyuridine. Reactions were also performed in the presence of excess unlabeled wt or mu PSE competitor DNA. After DNA binding, reactions were cross linked with UV light, digested with micrococcal nuclease, and the molecular weight of proteins cross linked to DNA were estimated by SDS-PAGE and autoradiography. As shown in Figure 4-1B, three proteins of approximately 70, 54, and 50 kDa were detected in reactions performed with the wt PSE probe either alone (lane 2) or with mu PSE competitor DNA (lane 5). No cross linking was observed in reactions using the mu PSE probe (lane 3) or with wt PSE probe plus wt PSE competitor DNA (lane 4). Thus, all three proteins are specifically bound to DNA in a PSE-dependent fashion. Cross linking of the 70 and 50 kDa proteins in this experiment is similar to that previously described using less purified $SNAP_C$-enriched fractions (35).

To determine the identity of the cross-linked proteins, the Mono-S fractions were separated by SDS-PAGE and proteins were detected by Coomassie blue staining. Subsequently, those proteins that were of similar molecular weight as the radiolabeled species observed in the UV cross-linking experiment were excised for lysyl endopeptidase digestion and N-terminal peptide sequencing. All peptides that were

Figure 4-1. Endogenous HEXIM1 associates with $SNAP_C$.

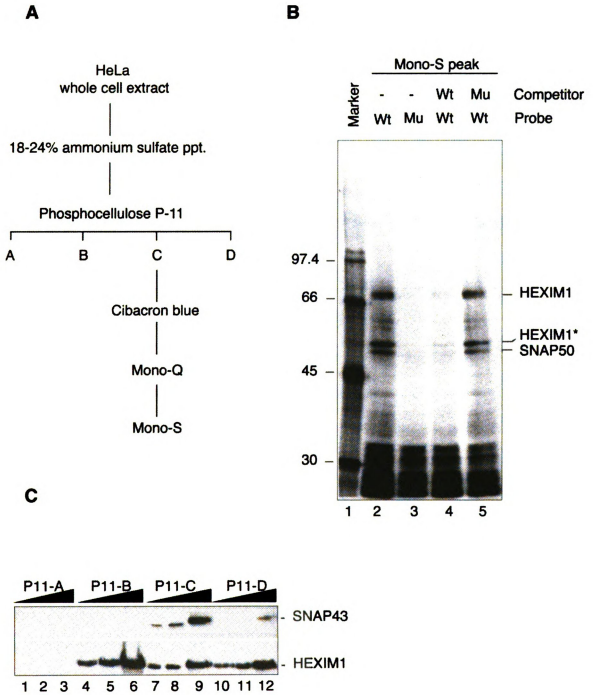
(A) Schematic representation of the chromatographic steps used to purify SNAPc.

(B) A polypeptide of ~ 70 kDa is cross-linked specifically to the PSE during DNA binding by SNAPC. DNA binding reactions were performed using Mono-S fractions enriched in $SNAP_C$ and homogeneously labeled probes that were substituted with bromodeoxyuridine and contained either a wild-type (wt) or mutated (mu) mouse U6 PSE (12). Reactions were performed with the wt or mu probes alone (lanes 2 and 3), or wt probe in the presence of excess wt (lane 4) or mu (lane 5) unlabeled competitor DNA. Proteins were cross-linked to DNA with UV light, digested with DNase I and micrococcal nuclease, and size fractionated by 12.5% SDS-PAGE. Proteins covalently linked to the remnants of the radiolabeled probe DNA were visualized by autoradiography. Lane 1 contains protein size markers. N-terminal sequencing of the 50, 54, and 70 kDa proteins from untreated Mono-S fractions identified the proteins to be SNAP50 and HEXIM1, as indicated.

(C) A minor population of cellular HEXIM1 co-purifies with $SNAP_C$. The phosphocellulose P-11 fractions were characterized for the presence of HEXIM1 and SNAP43 by Western blot analysis. Increasing amounts of P11-A (lanes 1 to 3), P11-B (lanes 4 to 6), P11- C (lanes 7 to 9), and P11-D (lanes 10 to 12) were separated by SDSPAGE electrophoresis, and the presence of SNAPC and HEXIM1 were detected by Western analysis.

(D) HEXIM1 co-fractionates extensively with SNAPC. Twenty mL of highly purified SNAPC fractions from the Mono-S stage of purification were analyzed by
Western analysis using antibodies against HEXIM1 (top panel) or SNAP43 (middle panel). Five mL of the same fractions were also analyzed by EMSA for PSE-binding activity (bottom panel) using radioactive probes containing a high-affinity mouse U6 PSE and TATA box. The positions of unbound probe (free probe) and SNAPC bound to DNA are indicated. The peak of SNAPC DNA binding activity is contained in fractions 61 to 66, whereas the peak of HEXIM1 is found from fractions 58 to 69.

This experiment was performed by R. William Henry.


A
HeLa
whole cell extract
18-24% ammonium sulfate ppt.
Phosphocellulose P-11
A
B
C
D
Cibacron blue
Mono-Q
Mono-S
B
Mono-S peak
Marker
- - Wt Mu Competitor
Wt Mu Wt Wt Probe
97.4
66
45
30
HEXIM1
HEXIM1*
SNAP50
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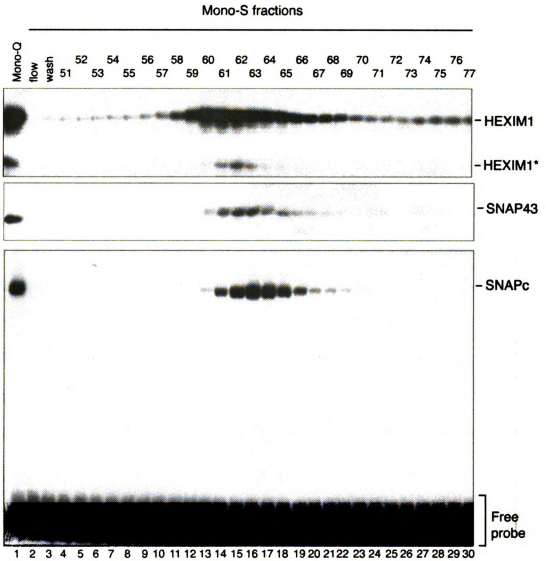
P11-B
P11-C
P11-D
SNAP43
HEXIM1
1 2 3 4 5 6 7 8 9 10 11 12

D


Mono-S fractions
Mono-Q flow wash
51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77
HEXIM1
HEXIM1*
SNAP43
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 23 24 25 26 27 28 29 30

obtained from the 70 and 54 kDa proteins correspond to HEXIM1 and peptides from the 50 kDa protein were found to belong to SNAP50, previously identified as contacting the PSE during DNA binding by $SNAP_C$ (12). For all three cases, no peptides from other proteins were obtained, including the recently identified HEXIM2 protein that shares extensive similarity with HEXIM1 (2, 44). We speculate that the smaller HEXIM1 protein (labeled HEXIM1*) is a proteolytic product of larger full length HEXIM1. Thus, HEXIM1 present in the $SNAP_C$-enriched fractions is in close proximity to the PSE during DNA binding by $SNAP_C$.

Next, antibodies were generated against HEXIM1 to follow the fractionation of HEXIM1 during $SNAP_C$ purification. As shown in Figure 4-1C, most $SNAP_C$ is detected in the P11-C fraction (lanes 7-9), as expected, whereas most HEXIM1 is present in the P11- B (lanes 4-6) and P11-D (lanes 10-12) fractions, with a similar but lesser amount detected in the P11-C fraction containing $SNAP_C$. Neither $SNAP_C$ nor HEXIM1 were detected in the P11-A fraction (lanes 1-3). Therefore, a substantial but minor proportion of HEXIM1 co-fractionates with $SNAP_C$ during phosphocellulose chromatography. The population of HEXIM1 that does co-purify with $SNAP_C$ was more extensively analyzed as shown in Figure 1D. Fractions obtained from the Mono-S step of fractionation revealed an extensive co-purification of HEXIM1 (top panel) and SNAP43 (middle panel). The fractionation pattern for SNAP43 closely resembled the pattern of $SNAP_C$ DNA binding activity in electrophoretic mobility shift assay (EMSA; bottom panel). However, significant amounts of HEXIM1 were also detected in fractions that were devoid of SNAP43 and $SNAP_C$ DNA binding activity, indicating that even at this late stage of purification, HEXIM1 can be chromatographically separated from $SNAP_C$. Only

modest amounts of HEXIM1 associated with $SNAP_C$ during co-immunoprecipitation from HeLa nuclear extracts or $SNAP_C$-enriched fractions, and the $SNAP_C$/DNA complex was not obviously super shifted using anti-HEXIM1 antibodies (data not shown), suggesting that HEXIM1 is not a stable component of endogenous $SNAP_C$. Together, these data indicate that HEXIM1 and $SNAP_C$ may only loosely associate.

HEXIM1 cooperates with $SNAP_C$ and TBP for snRNA gene promoter recognition

The observation that HEXIM1 was cross linked to DNA in a PSE-specific fashion indicated that HEXIM1 could target these promoters through cooperative DNA binding with $SNAP_C$ or TBP, which also function for both RNA polymerase II and III transcription of snRNA genes. Therefore, EMSAs were performed to investigate whether HEXIM1 could cooperate with $SNAP_C$ and TBP for promoter recognition. In the following experiments, low levels of each factor were purposefully chosen to minimize DNA binding by each factor alone. As shown in Figure 4-2A, neither HEXIM1 nor recombinant mini-$SNAP_C$ alone bound strongly to wtPSE/wtTATA probe DNA (lanes 2 and 3, respectively), but inclusion of both factors resulted in cooperative complex formation (lane 4) that was not observed in reactions containing $SNAP_C$ plus comparable amounts of GST (lane 5). A similar pattern of cooperation between HEXIM1 and $SNAP_C$ continued with the wtPSE/muTATA box probe (lanes 11-15), but complex formation was not observed in reactions performed with probes containing mutations in the PSE, regardless of the TATA box status (lanes 6-10 and 16-20). The HEXIM1-$SNAP_C$ complex was super shifted by anti-HEXIM1 and anti-SNAP190 antibodies, but not by IgG (data not shown), indicating that both factors are components of this complex.

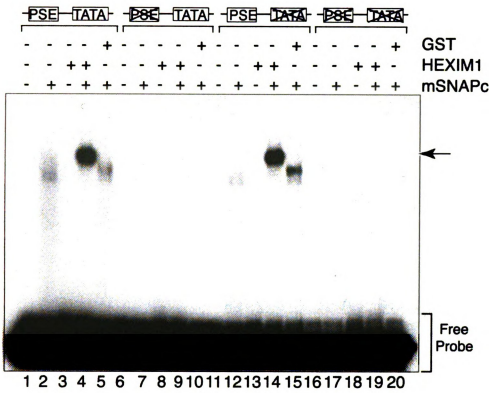
Figure 4-2. HEXIM1 cooperates with $SNAP_C$ and TBP for DNA binding.

(A) HEXIM1 cooperates with $SNAP_C$ for DNA binding in a PSE-specific manner. Approximately 30 ng of mini-$SNAP_C$ ($mSNAP_C$) containing SNAP190 (1-505), SNAP43, and SNAP50, was used for DNA binding either alone or with 100 ng of HEXIM1 or GST. Reactions containing only HEXIM1 were also performed as indicated. Reactions were performed with dsDNA probes containing combinations of wt and mu PSE with wt and mu TATA as shown schematically. Reactions containing only the dsDNA probes are shown in lanes 1, 6, 11, and 16.

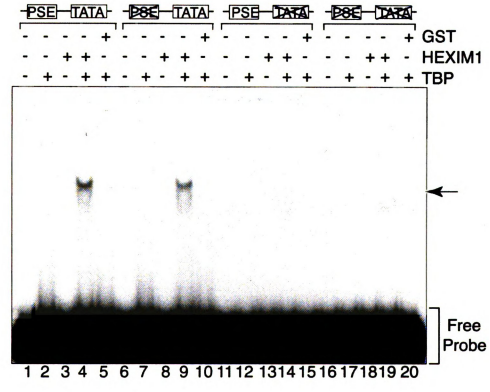
(B) HEXIM1 cooperates with TBP for DNA binding in a TATA box specific manner. Approximately 100 ng of TBP was tested for DNA binding either alone, or with 100ng of HEXIM1 or GST, as indicated. Reactions containing HEXIM1 alone were also performed, as indicated. The identity of each probe is shown schematically above the figure.

(C) HEXIM1 stimulates preinitiation complex assembly on snRNA promoters. HEXIM1 stimulates $SNAP_C$ and TBP binding. Similar amounts of $SNAP_C$, HEXIM1 and TBP as above were used and tested for DNA binding on probes containing combnations of wt or mu PSE with wt or mu TATA box. Reactions containing $SNAP_C$ and TBP are shown in lane 2. The presumptive identities of the various protein-DNA complexes are shown on the left.

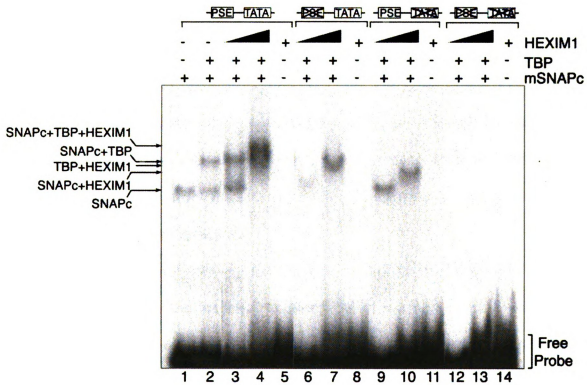
A


PSE
TATA
PSE
TATA
PSE
TATA
PSE
TATA
GST
HEXIM1
mSNAPc
Free
Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

B


PSE
TATA
PSE
TATA
PSE
TATA
PSE
TATA
GST
HEXIM1
TBP
Free
Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

C


PSE
TATA
HEXIM1
TBP
mSNAPc
SNAPc+TBP+HEXIM1
SNAPc+TBP
TBP+HEXIM1
SNAPc+HEXIM1
SNAPc
Free
Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Therefore, HEXIM1 and mini-$SNAP_C$ can cooperate for DNA binding in a PSE-dependent fashion. This result can also explain the PSE dependent cross linking of HEXIM1 to DNA previously observed in the UV cross linking experiments with highly purified $SNAP_C$ fractions (Figure 4-1).

We next tested whether HEXIM1 can influence DNA binding by TBP (Figure 4-2B). Neither HEXIM1 nor TBP alone bound to the wtPSE/wtTATA box (lanes 2 and 3, respectively), but cooperative DNA binding was observed in reactions containing both factors (lane 4), and in this experiment, GST did not influence TBP binding (lane 5). In contrast to the PSE-dependent cooperation between HEXIM1 and $SNAP_C$, the cooperative DNA binding by HEXIM1 and TBP was TATA box dependent, as PSE mutation did not affect complex formation (lanes 6-10), whereas no cooperative complex formation was observed in reactions where the probes contained mutations in the TATA box (lanes 11-20). Thus, HEXIM1 can cooperate with either $SNAP_C$ or TBP, and the ability of HEXIM1 to form complexes on DNA is dictated by the DNA binding specificity of its cooperating partner.

TBP recruitment by $SNAP_C$ is thought to be part of the preinitiation complex assembly pathway. We therefore tested whether HEXIM1 can influence TBP recruitment by $SNAP_C$. As shown in Figure 4-2C, inclusion of HEXIM1 in reactions that contain $SNAP_C$ and TBP caused a noticeable retardation in mobility of the $SNAP_C$-TBP-DNA complex, consistent with the idea that higher order complexes are being formed (compare lanes 3 and 4 with lane 2). Interestingly, this effect was not observed on probes that contained muPSE/wtTATA, wtPSE/muTATA or muPSE/muTATA sequences. Only the HEXIM1-TBP complex was seen with the probe containing a muPSE/wtTATA box,

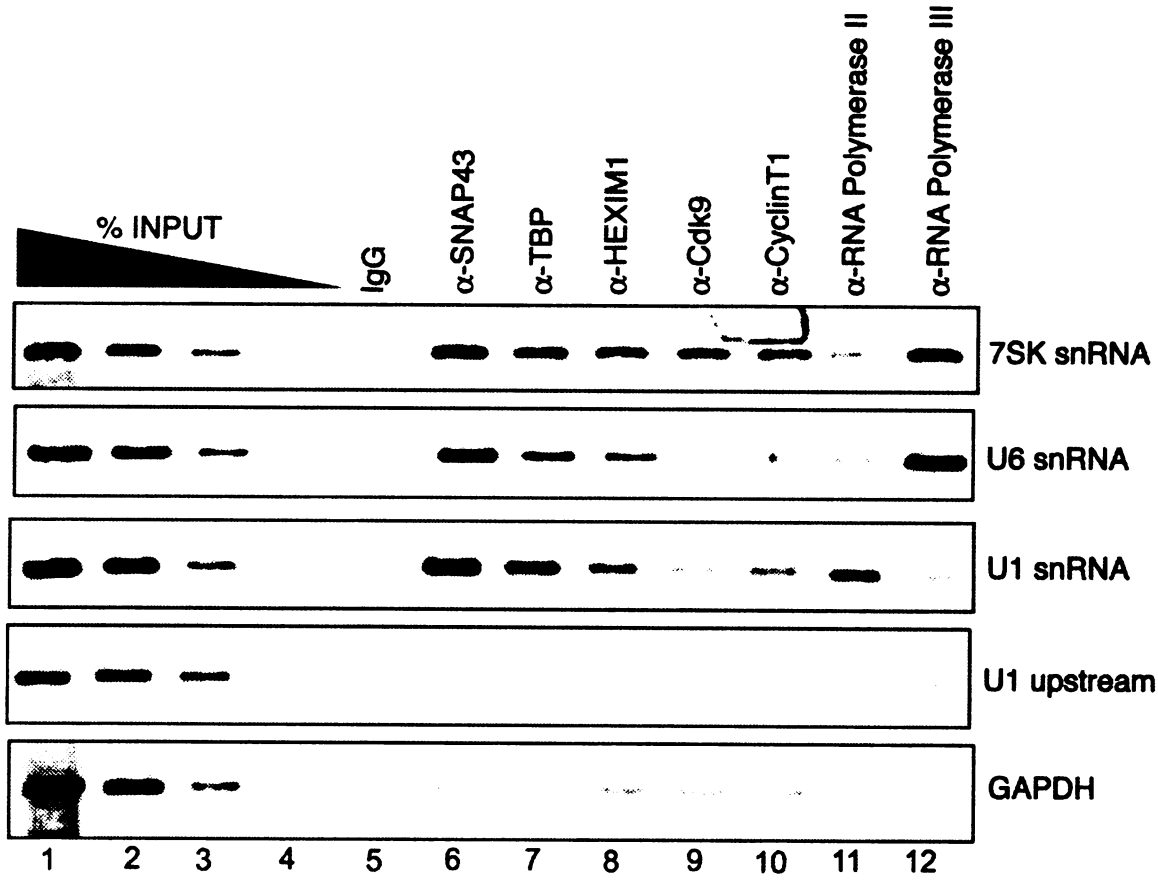
(lanes 6 and 7) while the HEXIM1-$SNAP_C$ complex was seen with the probe containing wtPSE/muTATA box (lane 9 and 10). It should be noted that $SNAP_C$ and TBP were both detected in the HEXIM1-$SNAP_C$-TBP-DNA complex, but not HEXIM1, using antibody supershift assays (data not shown). It is possible that HEXIM1 only transiently associates with the DNA and therefore was not detected in the HEXIM1-$SNAP_C$-TBP-DNA complex. Nonetheless, together these observations suggest that HEXIM1 stimulates DNA binding by $SNAP_C$ and TBP.

Differential snRNA promoter occupancy in vivo by P-TEFb subunits.

The association between HEXIM1 and $SNAP_C$ observed during biochemical purification of $SNAP_C$ suggests that HEXIM1 may play a role in human snRNA gene transcription. Therefore, chromatin immunoprecipitation experiments were performed to determine whether endogenous HEXIM1 associates with various snRNA gene promoters, including the 7SK and U6 snRNA genes that are transcribed by RNA polymerase III, and the U1 snRNA gene that is transcribed by RNA polymerase II. In addition to HEXIM1, immunoprecipitation reactions were performed using antibodies directed against $SNAP_C$, TBP, and RNA polymerases II and III. As HEXIM1 is a component of P-TEFb, snRNA gene promoter association by the cyclinT1 and cdk9 subunits of P-TEFb subunits were examined (Figure 4-3). As expected, the $SNAP_C$ and TBP immunoprecipitated samples were enriched for all snRNA gene promoters examined (lanes 6 and 7), relative to the IgG control (lane 5), whereas RNA polymerase III was present only at the U6 and 7SK genes (lane12), and RNA polymerase II only at the U1 snRNA gene promoter (lane 11). Interestingly, 7SK promoter DNA was enriched in all P-TEFb specific

Figure 4-3. HEXIM1 occupies endogenous snRNA promoters.

Chromatin was harvested from human mammary epithelial cells (184B5) as described previously (18) and immunoprecipitation reactions were performed using IgG (lane 5) or anti-SNAP43 (lane 6), anti-TBP (lane 7), anti-HEXIM1 (lane 8), anti-Cdk9 (lane 9), anti-Cyclin T1 (lane 10), anti -RNAP II (lane 11), and anti-RNAP III (lane 12) antibodies. Immunoprecipitated DNA was analyzed by PCR for enrichment of 7SK snRNA, U6 snRNA, and U1 snRNA core promoters using primers specific for each gene. Enrichment of GAPDH exon 2 and U1 upstream DNA were examined as negative controls. Amplification for a 10-fold serial dilution (10% to 0.01%) of input chromatin is shown in lanes 1-4.


% INPUT
IgG
α-SNAP43
α-TBP
α-HEXIM1
α-Cdk9
α-CyclinT1
α-RNA Polymerase II
α-RNA Polymerase III
7SK snRNA
U6 snRNA
U1 snRNA
U1 upstream
GAPDH
1
2
3
4
5
6
7
8
9
10
11
12

immunoprecipitations using HEXIM1 (lane 8), cdk9 (lane 9), and cyclin T1 (lane 10) antibodies, whereas U6 promoter DNA was only enriched in the HEXIM1-specific reactions. U1 promoter recovery by P-TEFb antibodies resembled the U6 pattern, with strong recovery by HEXIM1 antibodies. The modest U1 promoter enrichment by cyclin T1 immunoprecipitation suggests that the cyclin/cdk component of P-TEFb could regulate U1 transcription by RNA polymerase II. In all immunoprecipitations, enrichment of the U1 upstream region and GAPDH exon 1 was not observed. These results indicate that multiple components of endogenous P-TEFb associate with the 7SK gene promoter in vivo and thus P-TEFb might regulate 7SK transcription by RNA polymerase III. Furthermore, 7SK, U6, and U1 transcription may be differentially regulated by P-TEFb even though the 7SK and U6 promoters are highly similar and all genes share a requirement for the PSE, and thus $SNAP_C$, for efficient transcription.

The cyclin/cdk component of P-TEFb is not required for RNA polymerase III transcription.

Whereas RNA polymerase II elongation is regulated by P-TEFb, which phosphorylates the CTD of the RNA polymerase II largest subunit, a direct role for the cyclin/cdk sub-complex of PTEFb in snRNA gene transcription by RNA polymerase II or III has not yet been established. RNA polymerase III does not contain a similar proven target for P-TEFb phosphorylation and thus it might be immune to P-TEFb action. Nonetheless, P-TEFb could participate in regulation of snRNA gene transcription by RNA polymerase III through an unanticipated mechanism.

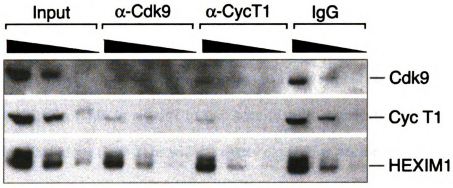
To test whether P-TEFb is required for snRNA gene expression, P-TEFb was removed from HeLa nuclear extracts by immunodepletion using antibodies directed against individual P-TEFb subunits, and the effect on in vitro transcription was determined. As shown in Figure 4-4A, Western blot analysis revealed that treatment of extracts with antibodies against cdk9 (lanes 4-6) and cyclin T1 (lanes 7-9) resulted in an approximate 90% reduction of both cdk9 (top panel) and cyclin T1 (middle panel) relative to the IgG treated extracts (lanes 10-12) and untreated nuclear extracts (lanes 1-3). Endogenous HEXIM1 levels were reduced by approximately 50% in the P-TEFb-depleted samples. Thus, multiple components of P-TEFb are effectively removed by either treatment. This result is also consistent with the idea that not all HEXIM1 is associated with the catalytic P-TEFb subcomplex. As shown in Figure 4-4B, U1 transcription by RNA polymerase II was markedly inhibited for both the cdk9 (lane 2) and cyclin T1 (lane 3) depleted extracts relative to transcription levels supported by the IgG depleted (lane 4) or mock treated extracts (lane 5). Under these conditions adenovirus major late (AdML) transcription was modestly affected only in the cdk9 immunodepleted extracts (data not shown). Thus, P-TEFb is required for U1 in vitro transcription by RNA polymerase II, possibly for CTD phosphorylation, and is consistent with a previously postulated role for P-TEFb in 3' end formation of U2 snRNA (21, 30). In contrast with RNA polymerase II snRNA gene transcription, neither 7SK nor U6 snRNA gene transcription was substantially affected by P-TEFb depletion, indicating that P-TEFb is not essential for in vitro snRNA gene transcription by RNA polymerase III. Even though cdk9 and cyclin T1 associate with the 7SK snRNA gene promoter in vivo, the apparent bystander status for cyclin T1 and cdk9 during RNA polymerase III

Figure 4-4. P-TEFb is differentially used for snRNA gene transcription by RNA polymerases II and III in vitro.

(A) Multiple P-TEFb subunits are removed from extracts by antibody immunodepletion. HeLa cell nuclear extracts were subjected to immunodepletion using anti-Cdk9 (lanes 4-6), anti-Cyclin T1 (lanes 7-9), and IgG (lanes 10-12) antibodies that were covalently cross-linked to protein G agarose beads. Lanes 1-3 contain a titration of the untreated extract. A portion of the extracts was separated by 12.5% SDS-PAGE, and analyzed by Western blot analysis using antibodies directed against cdk9, cyclin T1, and HEXIM1, as indicated.

(B) P-TEFb is required for U1 transcription by RNA polymerase II, but not for 7SK and U6 snRNA transcription by RNA polymerase III. In-vitro transcription assays for U1, U6, and 7SK snRNA genes were performed with HeLa nuclear extract (lane 1) or extract that had been immunodepleted for P-TEFb components (lanes 2 and 3). Lanes 4 and 5 show transcription from extract that was mock depleted with IgG or beads, respectively.

A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α-CycT1
IgG
Cdk9
Cyc T1
HEXIM1
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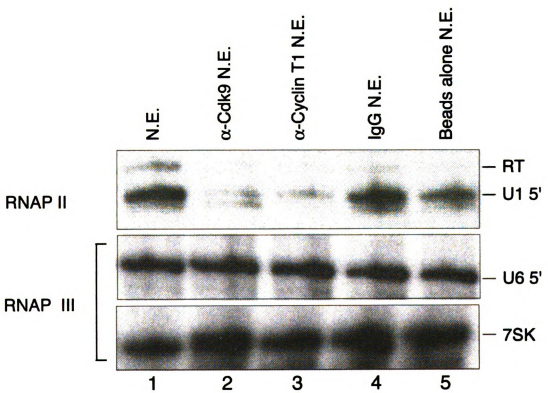
B


N.E.
α-Cdk9 N.E.
α-Cyclin T1 N.E.
IgG N.E.
Beads alone N.E.
RNAP II
RNAP III
RT
U1 5'
U6 5'
7SK
1 2 3 4 5

transcription suggests that the 7SK gene might be a site for assembling the inactive P-TEFb ribonucleoprotein complex, perhaps as the 7SK snRNA is being transcribed.

HEXIM1 activates 7SK transcription in vivo

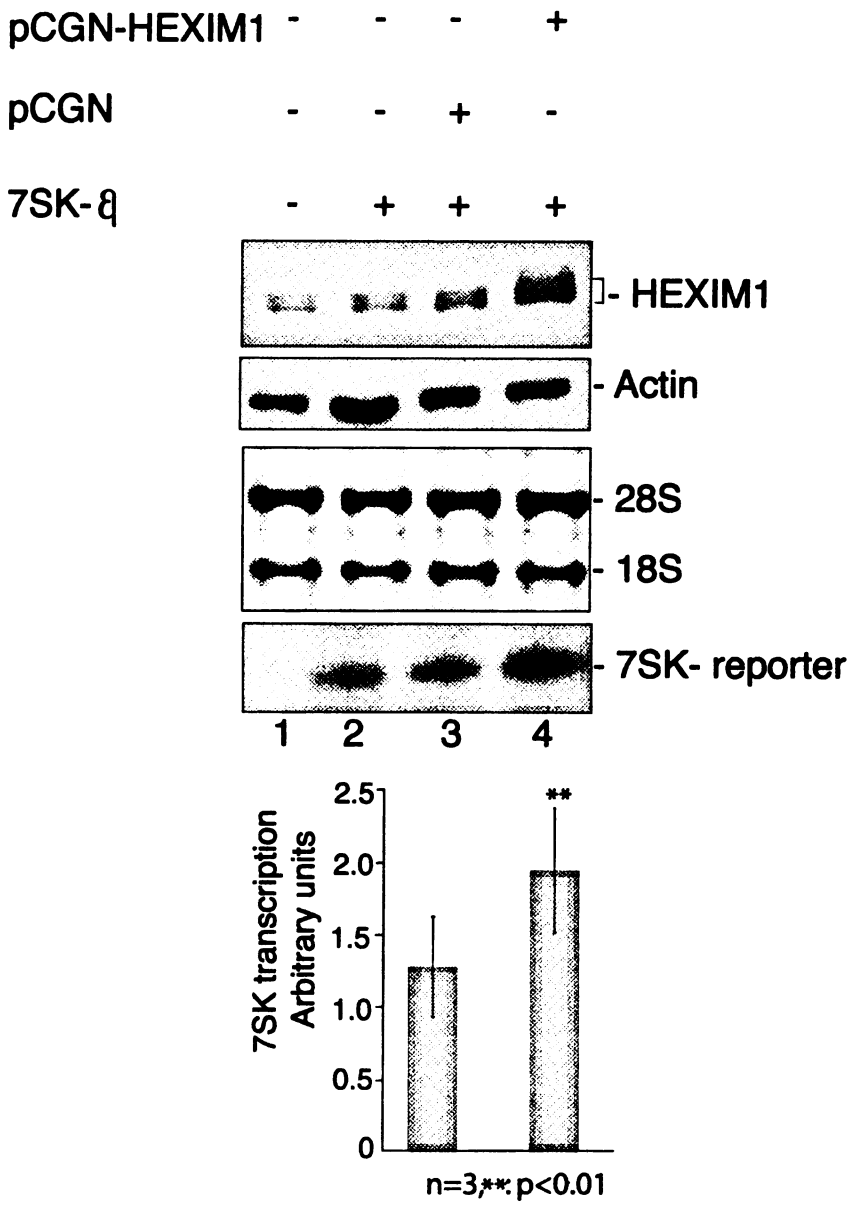
Next the effect of HEXIM1 over-expression on 7SK reporter gene expression was tested (Figure 4-5A). Over-expression of HEXIM1 resulted in a modest, but reproducible, increase in 7SK transcription (lane 4) relative to that seen in cells transfected with the 7SK reporter plasmid either alone (lane 2) or with an empty expression vector (lane 3). In this experiment, over-expression of HEXIM1 did not affect endogenous actin or rRNA levels. We note that HEXIM1 over-expression did not significantly affect 7SK transcription during nuclear run-on assays in HeLa cells (data not shown), suggesting that in vivo HEXIM1 levels are not limiting for 7SK transcription unless the 7SK gene copy number is increased such as during transient transfection. We were unable to achieve a substantial reduction in endogenous HEXIM1 levels by anti-HEXIM1 immunodepletion (data not shown), and thus could not determine whether HEXIM1 contributes to snRNA gene transcription in vitro. Nonetheless, the observation that HEXIM1 is present at snRNA promoters in vivo suggests that this protein could directly regulate transcription. To examine the possibility that HEXIM1 regulates RNA polymerase III transcription of snRNA genes, the steady state levels of HEXIM1 were reduced by using siRNA specific for HEXIM1 and the effect on 7SK reporter gene expression was tested. As 7SK snRNA binds directly to HEXIM1, any change in 7SK snRNA levels may confound the assessment of HEXIM1 function, and therefore the 7SK snRNA encoding sequence in this reporter gene was replaced with an inverted β-globin sequence. As shown in Figure

Figure 4-5. HEIM1 positively regulates 7SK snRNA transcription in vivo.

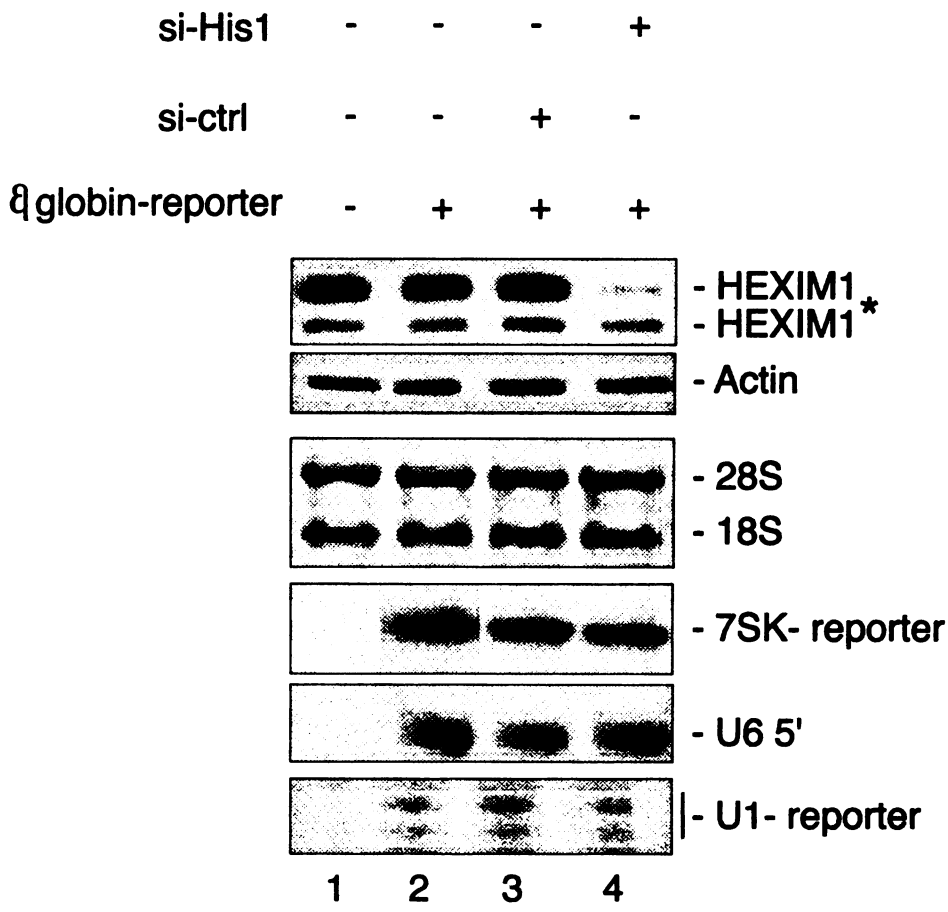
(A) Over-expression of HEXIM1 increases 7SK snRNA transcription. HeLa cells were cotransfected with pBS-7SK reporter gene alone (lane 2) or with either pCGN (lane 3) or pCGN-HA-HEXIM1 (lane 4). Cells were harvested 24 hr after transfection and processed for whole cells extract preparation and total RNA collection. Endogenous HEXIM1 and actin levels were measured by Western blot analysis. Endogenous 18S and 28S rRNA levels were monitored by agarose gel electrophoresis and ethidium bromide staining. 7SK snRNA reporter gene transcription was measured by RNase T1 protection assay and phosphoimager analysis. The experiment was performed five times and the average 7SK reporter gene activity is shown in the graph at the bottom. Statistical significance was estimated using a Student's T-test and error bars are the standard deviation.

(B) siRNA mediated reduction in HEXIM1 does not affect 7SK transcription. Hela cells were
cotransfected with an inverted β-globin reporter construct driven by a human 7SK snRNA promoter (pBS-7SK) alone (lane 1) or along with either the control siRNA (lane 2) or siHis1 (lane 3). 30 hr after transfection cells were harvested and processed for whole cell extract preparation and total RNA collection as in (A). This experiment was performed three times.

A


pCGN-HEXIM1 - - - +
pCGN - - + -
7SK-ß - + + +
- HEXIM1
- Actin
- 28S
- 18S
- 7SK- reporter
1 2 3 4
7SK transcription
Arbitrary units
2.5
2.0
1.5
1.0
0.5
0
**
n=3;**: p<0.01

B


si-His1 - - - +
si-ctrl - - + -
ßglobin-reporter - + + +
- HEXIM1
- HEXIM1*
- Actin
- 28S
- 18S
- 7SK- reporter
- U6 5'
- U1- reporter
1 2 3 4

4-5B, transient transfection of the si-HEXIM1caused 90% reduction of endogenous HEXIM1 (lane 4) relative to levels observed during transfection of the control siRNA (si-ctrl lane 3). Actin levels were similar for each treatment as were the steady state 18S and 28S rRNA levels. Interestingly, 7SK reporter gene transcription by RNA polymerase III was not affected with HEXIM1 knock down, suggesting that endogenous HEXIM1 is not essential for transcription. Interestingly, a lower migrating form of HEXIM1 (labeled as HEXIM1*) was not affected in this knockdown. The sequence of this lower migrating form of HEXIM1 is not known and whether it plays a role in snRNA transcription is yet to be determined. Nonetheless, the over-expression and knockdown data suggests that HEXIM1 stimulates 7SK snRNA transcription although HEXIM1 is not essential for this process.

Discussion

As the products of human snRNA genes play critical roles in numerous steps of productive global gene expression, the rate of cellular growth and the ability to proliferate may be sensitive to steady state snRNA levels. In particular, global RNA polymerase II transcription may be sensitive to the cellular levels of U1 and 7SK snRNA that associate with the cyclin/cdk complexes, TFIIH and P-TEFb, respectively (24, 34, 42). Both of these complexes directly regulate RNA polymerase II transcription. Interestingly, HEXIM1 forms a cyclin/cdk inhibitor complex with 7SK snRNA to down regulate P-TEFb activity (31, 43). In this context, HEXIM1 is thought to play a negative role in RNA polymerase II transcription. The data presented herein reveal an unexpected

positive role for HEXIM1 in transcription of its 7SK snRNA co-repressor partner by RNA polymerase III.

The role of HEXIM1 in snRNA gene transcription was first suggested because HEXIM1 associated with the general transcription factor $SNAP_C$ during chromatographic fractionation of HeLa cell extracts. As $SNAP_C$ is required for transcription of human snRNA genes including U1, U6, and 7SK, a role for HEXIM1 in transcription of all these genes was postulated. Whereas our results suggest a positive role for HEXIM1 in 7SK snRNA gene regulation, however, HEXIM1 function does not seem to be essential for snRNA gene transcription, even though endogenous HEXIM1 was resident at these gene promoters in vivo. While efficient HEXIM1 knockdown was observed, a shorter form of HEXIM1 (HEXIM1*) was not affected. Interestingly, HEXIM1* and not HEXIM1 associates with the DNA-binding domain within SNAP50 (22), when this region of GST-SNAP50 was used as a bait to pull out interacting proteins from HeLa cell nuclear extract (data not shown). The sequence of HEXIM1* is not known, but it seems likely that it may have internally deleted sequences, as both N- and C-terminal specific antibodies were able to recognize HEXIM1* in a Western analysis (data not show). Thus it is likely that an unidentified splice variant of HEXIM1 exists in the cell and may play a role in the regulation of snRNA gene transcription.

U6 transcription has been reconstituted in vitro with recombinant factors and highly purified RNAP III, and in this system no requirement for HEXIM1 was observed (5). Perhaps traces of HEXIM1 co-purify with RNAP III or with $SNAP_C$ purified from insect cells. It seems more likely; however, that the role of HEXIM1 can be bypassed in the in vitro transcription system, For example, if HEXIM1 serves to facilitate

preinitiation complex assembly, this function might be dispensable in vitro where the template is naked DNA and the transcription initiation factors are in excess (20), and thus HEXIM1 activity for U6 regulation may be restricted to a context that is not yet appreciated.

Our data further indicate that only a minor proportion of HEXIM1 co-fractionates with $SNAP_C$, suggesting that HEXIM1 partitions into multiple complexes consistent with the idea that HEXIM1 plays multiple independent roles in the cell (43). Indeed, the function of HEXIM1 for RNA polymerase III transcription is independent of P-TEFb kinase activity, as 7SK in vitro transcription is not sensitive to cyclinT1 and cdk9 levels. Thus, it is interesting that cyclinT1 and cdk9 were both found associated with the endogenous 7SK snRNA gene promoter. One possibility is that P-TEFb kinase activity is required for RNA polymerase III transcription in the cell, but this requirement is not revealed by in vitro assays. However, the catalytic subunits of P-TEFb were not detected at the highly related U6 snRNA gene promoter, and although the role of HEXIM1 for U6 transcription by RNA polymerase III is not known, this observation suggests that RNA polymerase III phosphorylation by P-TEFb is not essential for RNA polymerase III transcription. An alternative explanation is that the 7SK snRNA gene serves as a site for P-TEFb assembly of the cyclinT/cdk9 kinase complex with the regulatory HEXIM1/7SK snRNA complex, as the 7SK snRNA is being transcribed.

As endogenous HEXIM1 associates with PSE-containing promoter sequences within the cell an important question remains as to how HEXIM1 is targeted to these genes. We observed that at protein concentrations where little DNA binding is detected by either $SNAP_C$ or HEXIM1 alone, HEXIM1 cooperates with $SNAP_C$ for PSE-

dependent promoter recognition. Furthermore, HEXIM1 also cooperated with TBP for TATA-box dependent binding, suggesting that HEXIM1 plays a more general role to assert its influence on 7SK snRNA gene transcription by facilitating cooperative preinitiation complex assembly at snRNA gene promoters.

Human 7SK snRNA, along with other snRNAs, are expressed at high levels in the cell. In part, these levels are maintained by exceptional transcription efficiencies dictated by the typical promoter structure of snRNA genes (37). In fact, the relatively compact and powerful promoters of some snRNA genes have lent themselves to widespread use in biotechnology and medical applications to drive high-level expression of effector RNA molecules. However, snRNA gene promoters are sensitive to complex regulatory control that may have downstream effects on global RNA production and cellular proliferation. It is especially intriguing that 7SK snRNA gene transcription is regulated by its functional partner HEXIM1. We speculate that P-TEFb stimulates HEXIM1 production by RNA polymerase II, and in turn, HEXIM1 stimulates 7SK snRNA gene transcription in a process that is expected to down regulate P-TEFb activity as increased levels of the HEXIM1/7SK snRNA complex are formed. Thus, one possibility is that HEXIM1 levels may function as a barometer for cellular gene expression levels via feedback regulation of its co-repressor partner. It is further possible that HEXIM1 contributes to cell growth control in specialized contexts. For example, an intriguing idea is that HEXIM1 antagonizes HIV-1 transcription by directly maintaining P-TEFb in an inactive state and ensuring that the levels of its 7SK snRNA co-repressor partner are adequate for this function. Indeed, a recent model was proposed wherein the HIV-1 Tat protein competes with HEXIM1 for binding to the cyclin T1 component of P-TEFb to increase levels of

active P-TEFb and HIV-1 gene transcription (39). It will be important to determine the context, timing, and cell-type specificity for HEXIM1 regulation of 7SK transcription.

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CHAPTER 5

SUMMARY

Non-translated RNAs synthesized by RNA polymerase III contribute to the biosynthetic growth capacity of a cell (6). Deregulated transcription of these RNAs may cause unrestricted growth and therefore it is important to regulate RNA polymerase III transcription. Interestingly, the RB and p53 tumor suppressor proteins (2, 9) as well as the CK2 and Myc oncoproteins (1, 3) that are important for regulating cell proliferation, also regulate transcription of these genes. This observation indicates that transcriptional regulation of these RNA polymerase III transcribed genes may play an important role in controlling cellular proliferation.

Genes transcribed by RNA polymerases III include those encoding 5S rRNA, tRNA and U6 snRNA that are molecular components of the cellular machinery governing multiple steps in the flow of genetic information in cells. Of these genes, the human U6 snRNA gene is interesting because subtle changes in the core promoter architecture can switch transcription from RNA polymerase III to II (5). Thus the U6 snRNA gene and related family members provide a good model system to study the molecular mechanism of polymerase preference and activity during both normal and deregulated growth.

All human snRNA genes contain a proximal sequence element (PSE) located in the core promoter region that is recognized by the general transcription factor called $SNAP_C$. $SNAP_C$ plays a pivotal role in snRNA gene transcription by providing core promoter recognition and coordinating TBP activity as part of nucleating the preinitiation

complex assembly for both RNA polymerase II and III. In contrast to other promoter recognition complexes such as SL1 and TFIIIC that specialize in transcription by a single RNA polymerase, $SNAP_C$ is more functionally versatile due to its role in both RNA polymerase II and III transcription. $SNAP_C$ is composed of at least five subunits namely, SNAP19, SNAP43, SNAP45, SNAP50, and SNAP190. Although SNAP190 has been shown to contain a Myb domain in its sequence that contributes to DNA binding by $SNAP_C$, it is not sufficient for this process (10). Indeed DNA binding by $SNAP_C$ is a cooperative event wherein SNAP190, SNAP50, and SNAP43 are all required ((7) and Figure 3-1A). These observations suggested that additional contacts are necessary for DNA binding. A role for SNAP50 in this process was suggested because cross-linking experiments showed that SNAP50 is in close proximity to the DNA (4). My mutagenesis studies revealed that SNAP50 does indeed play a role in $SNAP_C$ DNA binding through a highly conserved zinc finger domain in its C-terminus, that functions for preinitiation complex assembly for both RNA polymerase II and III transcription.

As $SNAP_C$ binding to the PSE is a crucial early event in the preinitiation complex assembly at snRNA promoters, it is a target for regulatory factors. Indeed the Retinoblastoma (RB) tumor suppressor protein, which was shown to repress of U6 snRNA gene transcription, does interact with the SNAP50 subunit of $SNAP_C$ (8), This observation suggested that the RB-SNAP50 interaction could disable DNA binding by $SNAP_C$ thus explaining the mechanism for RB repression. However, subsequent studies have shown that RB does not affect DNA binding by $SNAP_C$, indicating that RB may use some other mechanism. For example, it may recruit co-factors to repress U6 transcription. For other RB target genes such as the RNA polymerase II transcribed E2F-

regulated genes required for progression through the cell cycle, RB has been shown to recruit histone deacetylases (HDACs) and components of the SWI/SNF ATP-remodeling complexes (12). Therefore, I examined the role of these RB co-factors during repression of U6 transcription. My studies revealed a role for HDACs and SWI/SNF in RB repression of U6 snRNA transcription. My data further indicates that endogenous HDACs and SWI/SNF proteins associate with the U6 promoters in 184B5 cells that retain RB function but not in HeLa cells in which the RB function is compromised. These observations suggest that recruitment of co-repressor to the U6 snRNA promoter may be dependent upon RB function. As a first step to address whether RB recruits co-repressor proteins to the U6 promoter in vivo I have successfully established RB overexpression in HeLa cells using transient transfection assays. Interestingly, my preliminary data suggests that one other RB co-factor called DNA methyl transferase 1 (DNMT1) associates with a U6 promoter in vivo only in the RB-transfected cells but not in a control empty vector-transfected or mock-treated cells as assayed by transient transfection assay followed by ChIP experiments. These results suggest that HDAC, SWI/SNF, DNMT1 could be involved in RB-mediated repression of U6 transcription.

My results further indicate that RB repression and HDAC activity are biochemically separable. Using this system, it will be possible to determine whether RB co-factors are recruited sequentially to the U6 promoter and whether chromatin affects this process. For example, RB can form different repressor complexes that contain HDACs or HDACs plus SWI/SNF. Indeed a RB-HDAC-SWI/SNF complex and the RB-SWI/SNF complex have been shown to repress cyclin E and cyclin A, respectively, whose genes products are required for cell cycle progression The sequential disassembly

of the RB-SWI/SNF/HDAC complex to one lacking HDAC activity is proposed as one mechanism governing RB repression of different genes at distinct phases of the cell cycle (11). It is possible that RB may utilize a similar mechanism wherein it associates with multiple corepressor complexes. My studies indicate that RB stimulates U6 promoter association by HDAC2 and the Brg1 component of SWI/SNF complex (Chapter 2), Thus, an important future avenue of research will be to determine if and how RB coordinates the activity of multiple co-repressor proteins for repression of U6 transcription.

RB repression of U6 snRNA transcription is a good model system to understand the mechanistic details of RB repression, as we have established in vitro repression assays using naked- as well as chromatin-DNA templates, and in vivo assays to study promoter association by RB and its cofactors. A link between the tumor suppressor function of RB and U6 repression has been suggested due to the observation that the region encompassing the A/B pocket and the C-terminus of RB are the same regions required for tumor suppression and U6 repression by RB (9). These studies will help clarify the mechanism of RB repression as an important tool to understand RB activity during tumor suppression.

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APPENDIX A

MULTIPLE SUBUNITS OF $SNAP_C$ CO-EXPRESSED IN *E. COLI* ARE ACTIVE FOR TRANSCRIPTION BY HUMAN RNA POLYMERASE II AND III[1]

DNA binding by $SNAP_C$ is a crucial early event during preinitiation complex assembly for both RNA polymerase II and III transcribed human snRNA genes. Although mini-$SNAP_C$ ($mSNAP_C$) that assembled by mixing the individual subunits namely SNAP190 (1-505), SNAP50, and SNAP43 is able to function in DNA binding, it is crippled for *in vitro* transcription (1). One possible explanation is that a post-translational modification of $SNAP_C$ is crucial for its activity and that this event is not successfully recapitulated in *E. coli* cells. This might also explain the observation that $SNAP_C$ expressed in a *Baculaovirus* expression system functions for transcription (2). Another possibility is that $SNAP_C$ does not efficiently obtain its fully active, native conformation when its subunits are expressed separately and reassembled. To test this hypothesis we devised a co-expression system wherein the individual subunits of $mSNAP_C$ were simultaneously expressed in the same *E. coli* cell. The co-expressed $SNAP_C$ is referred to as $mSNAP_C\gamma4$ hereafter.

[1]Figures A-1B and A-1C used in this document were published in the following manuscript: Andrej Hanzlowsky, Blanka Jelencic, Gauri W. Jawdekar, Craig S. Hinkley, James H. Geiger, and R. William Henry (2006) Co-expression of multiple subunits enables recombinant $SNAP_C$ assembly and function for transcription by human RNA polymerase II and III. *Protein Expression and Purification Expression* Vol.48; pp.215-223

mSNAP$_C$γ4 is functional for DNA binding and TBP recruitment to a U6 snRNA promoter

We wanted to determine whether mSNAP$_C$γ4 was functional. The composition of mSNAP$_C$γ4 obtained by using a co-expression system is shown in Figure A-1A. The ability of mSNAP$_C$γ4 to bind DNA in an electrophoretic mobility shift assay was tested as shown in Figure A-1B. Increasing amounts of mSNAP$_C$γ4 was able to bind wtPSE/wtTATA probe DNA (lanes 2 and 3) and wtPSE/muTATA probe DNA (lanes 10 and 11), however failed to bind to a muPSE/wtTATA DNA probe (lanes 6 and 7) and muPSE/muTATA DNA probe (lanes 14 and 15). The mSNAP$_C$γ4 was functional for TBP recruitment to a probe containing wtPSE/wtTATA DNA (lane 4) but not to a wtPSE/muTATA DNA (lane 12). These results show that the mSNAP$_C$γ4 obtained using the co-expression system does indeed behave similarly to mSNAP$_C$ for DNA binding and TBP recruitment.

mSNAP$_C$γ4 is able to reconstitute human U1 and U6 snRNA transcription *in vitro*

We next wanted to test whether mSNAP$_C$γ4 was able to reconstitute *in vitro* transcription initiated from a plasmid containing the human U1 snRNA promoter as shown in Figure A-2A. HeLa cell nuclear extract was either mock depleted with pre-immune rabbit sera or depleted with anti-SNAP43 to remove endogenous SNAP$_C$. In the absence of endogenous SNAP$_C$, U1 transcription is reduced considerably but not in the mock depleted extract (compare lanes 1 and 2 with lane 3). When increasing amounts of mSNAP$_C$γ4 were added to the transcription reaction U1 transcription was restored (lanes 4 to 10). Similarly, as shown in Figure A-2B, U6 snRNA transcription was also restored

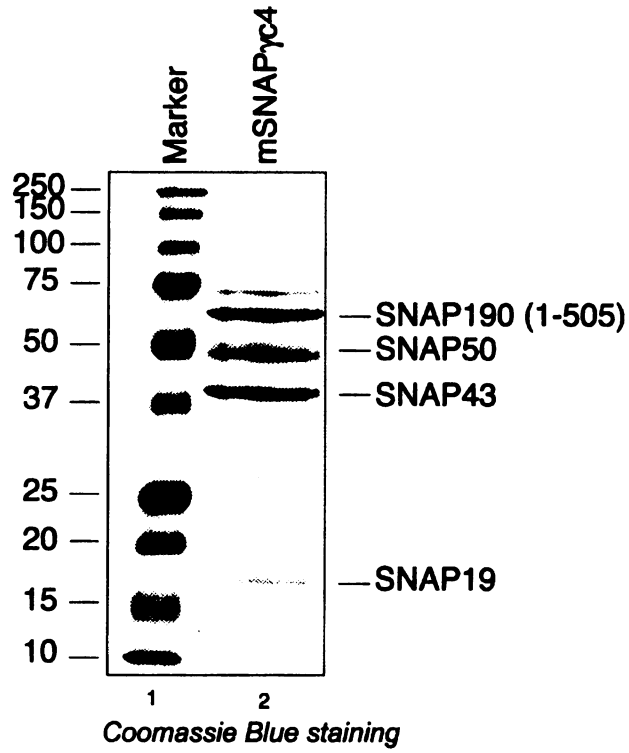
by increasing amounts of mSNAP$_C\gamma$4 (lanes 3 to 9), but not by a non-specific protein like GST (lane 10). These results show that mSNAP$_C\gamma$4 is indeed functional in an *in vitro* transcription assay. Thus we have been able to establish a system for co-purification of functional mSNAP$_C\gamma$4 that contains SNAP190 (1-505), SNAP10, SNAP43, and SNAP19. This system is overall far superior as it yields nearly a pure and homogenous complex, and the quantity of protein obtained is suitable for further structure-function characterization of this multi-protein transcription factor.

Figure A-1. mSNAP$_C\gamma$4 is competent for DNA binding and TBP promoter recruitment

(A) Purified recombinant mSNAP$_C\gamma$4 was separated by 15% SDS-PAGE and visualized by staining with Coomassie blue (lane 2). Lane 1 contains a protein size markers.

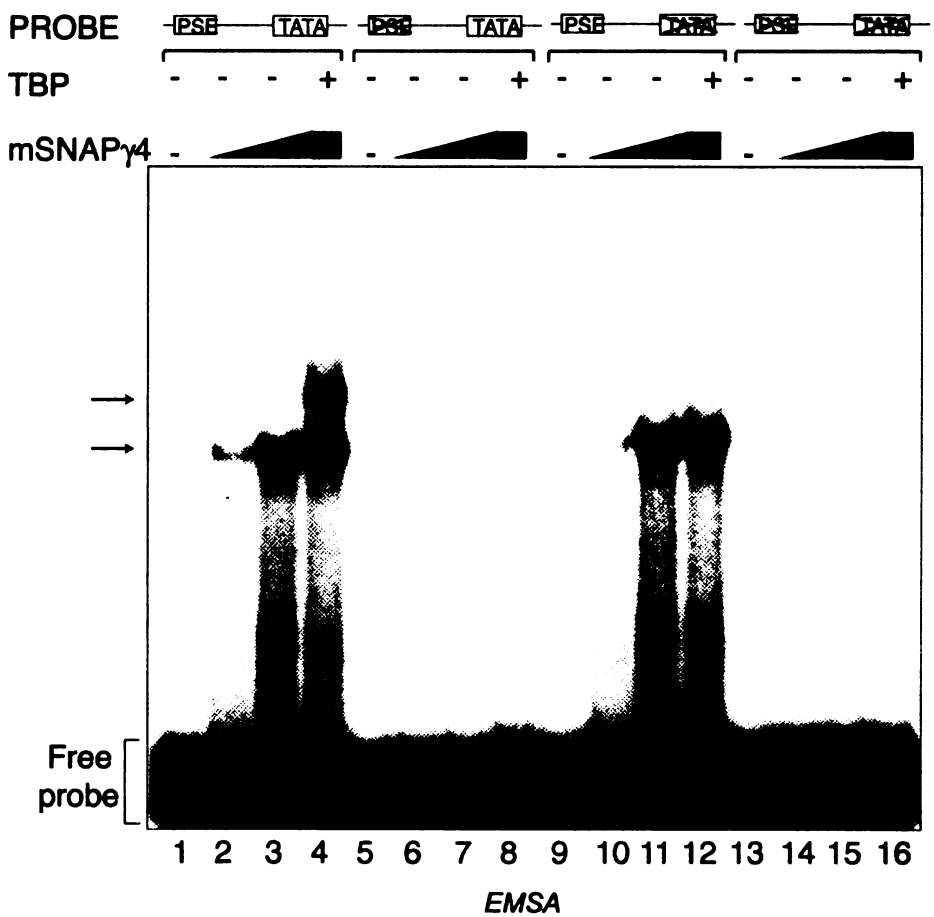
(B) Increasing amounts of mSNAP$_C\gamma$4 (3 ng and 10 ng) were added to EMSA reactions containing dsDNA probes harboring a wt PSE and wt TATA, mu PSE and wt TATA, wt PSE and mu TATA, or mu PSE and mu TATA box, as indicated. Lanes 4, 8, 12, and 16 contain approximately 50 ng of recombinant human TBP in addition to 10ng of mSNAP$_C\gamma$4. Reactions containing only the DNA probe are shown in lanes 1, 5, 9, and 13.

A



Coomassie Blue staining

B



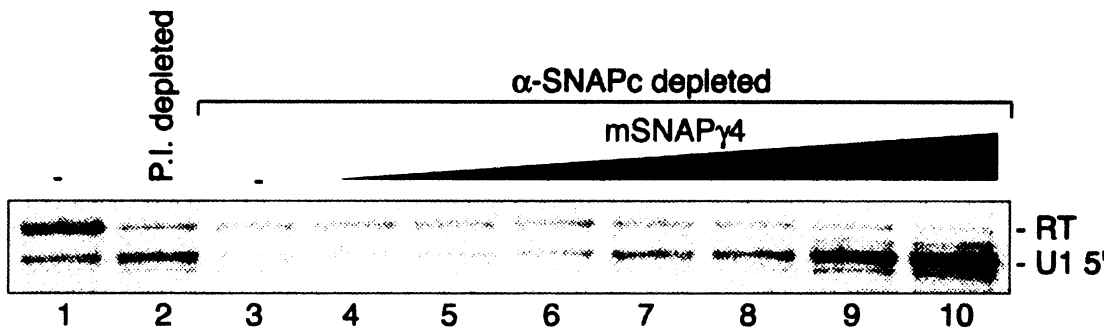
EMSA

Figure A-2. mSNAP$_C$γ4 supports human snRNA gene transcription *in vitro* by both RNA polymerase II and III

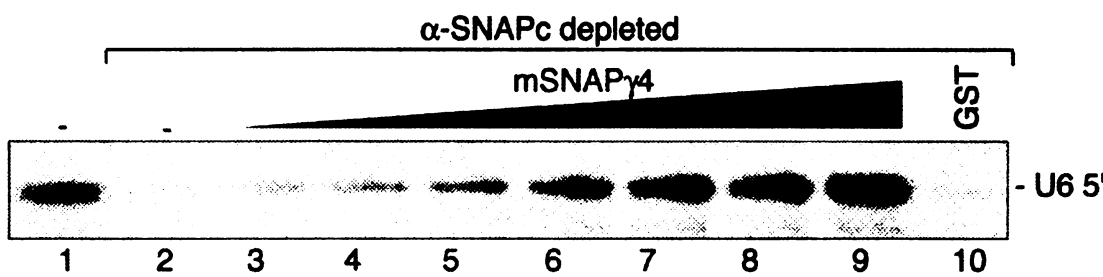
(A) The HeLa cell nuclear extract used for human U1 *in vitro* transcription assay was either mock depleted with a preimmune rabbit sera or anti-SNAP43 antisera to immunodeplete endogenous SNAP$_C$. Reduction of the U1 signal upon removal of endogenous SNAP$_C$ is shown in lane 3. Increasing amounts of mSNAP$_C$γ4 (0.3 ng, 1 ng, 3 ng, 10 ng, 30 ng, 100 ng, or 300 ng) was able to reconstitute correctly initiated transcription from a human U1 promoter as shown in lanes 4 to 10. Lanes 1 and 2 show the U1 signal obtained from either untreated or mock-depleted reactions. RT represents a read-through transcript.

(B) *In vitro* transcription of human U6 snRNA was carried out using HeLa cell nuclear extract that was treated as before in. Increasing amounts of mSNAP$_C$γ4 (0.3 ng, 1 ng, 3 ng, 10 ng, 30 ng, 100 ng, or 300 ng) was able to reconstitute correctly initiated transcription from a human U6 promoter as shown in lanes 3 to 9. Lane 2 shows the reduced U6 signal upon removal of endogenous SNAP$_C$. Approximately 300ng of GST was added to the transcription reaction instead of mSNAP$_C$γ4 as shown in lane 10.

A


P.I. depleted
α-SNAPc depleted
mSNAPγ4
-
-
- RT
- U1 5'
1
2
3
4
5
6
7
8
9
10

B


α-SNAPc depleted
mSNAPγ4
GST
-
-
- U6 5'
1
2
3
4
5
6
7
8
9
10

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APPENDIX B

DIFFERENTIAL U6 snRNA PROMOTER ASSOCIATION IN VIVO BY RB FAMILY MEMBERS

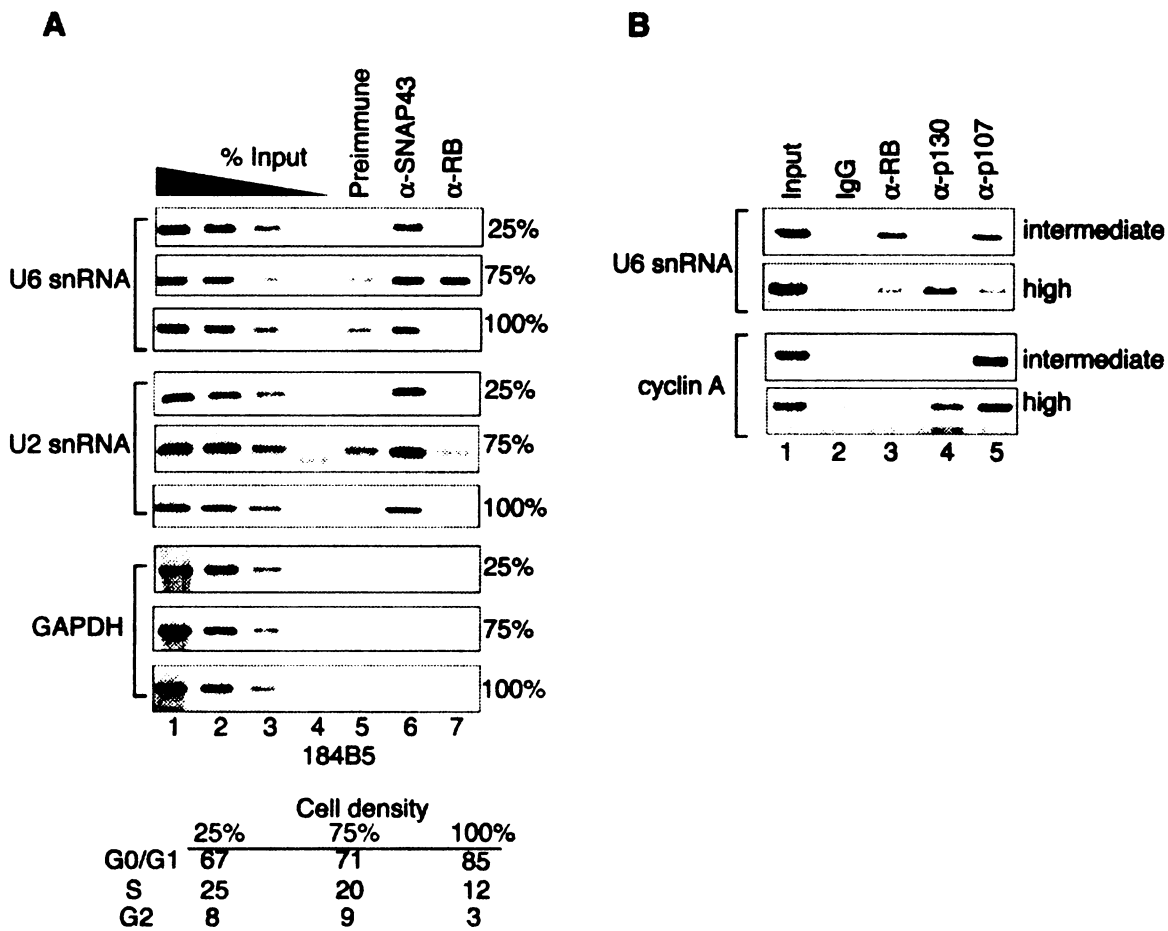
Pocket proteins associate with a U6 sNRNA promoter in vivo.

The Retinoblastoma (RB) tumor suppressor protein is thought to contribute to growth control through its regulation of RNA polymerase III transcription Therefore to test whether RB function at U6 snRNA promoters is linked to growth, chromatin immunoprecipitations assay was performed from normal mammary epithelial cells (184B5) harvested at increasing cell densities. Populations of cells were harvested at low (~25%), intermediate (~75%), and high (~100%) cell densities. As shown in Figure B-1A, both U6 (top panel) and U2 (middle panel) promoter DNA were significantly enriched in chromatin immunoprecipitations performed with anti-SNAP43 antibodies (lane 6) at all cell densities as compared the GAPDH exon2 negative control (bottom panel). Interestingly, measurable levels of RB were detected at the U6 snRNA promoter only in those cells harvested at intermediate cell density, but not at the low or high densities (lane 7). The pattern for RB enrichment in these experiments suggests that RB may regulate these genes when cells are actively growing but not when the cells have exited the cell cycle. Therefore, to characterize the relative percentage of cells in each phase of the cell cycle, cells grown at the different densities were analyzed for DNA content by flow cytometry. Indeed, the relative number if cells in G0/G1 increases as the cell density increases, indicating that when grown to 100% confluence most cells have

Figure B-1. U6 snRNA promoter occupancy by RB and RB family members is cell density dependent.

(A.) U6 promoter occupancy by RB is sensitive to the cell density. Chromatin was harvested form normal human mammary epithelial cells (184B5) grown to approximately 25%, 75%, and 100% cell density. Immunoprecipitation reactions from each chromatin sample were performed using rabbit pre-immune sera (lane 5), anti-SNAP43 (lane 6), or anti-RB antibodies (lane 7). Immunoprecipitated material was analyzed for U6 snRNA and U2 snRNA promoter DNA (upper and middle panels, respectively) or GAPDH exon 2 DNA (bottom panel) by PCR using primers specific to each gene. Lanes 1-4 represent 10-fold serial dilution (10% to 0.001%) of input chromatin. Cells grown to similar density were processed for propidium iodide staining and the relative percentages of cells in G0/G1, S, or G2 phase of the cell cycle were determined using FACS analysis.

(B) Cell density influences distinct RB family member association with the human U6 promoter. Chromatin was collected from human 184B5 cells that were grown to either intermediate or high densities. Immunoprecipitation reactions were performed using IgG (lane 2), anti-RB (lane 3), anti-p130 (lane 4), or anti-p107 (lane 5) antibodies. Immunoprecipitated DNA was analyzed for U6 snRNA (upper panel) and Cyclin A (lower panel) promoter DNA by PCR. Lane 1 indicates the input DNA.



Cell density

	25%	75%	100%
G0/G1	67	71	85
S	25	20	12
G2	8	9	3

likely exited the cell cycle. However, the difference in cell cycle profile for cells grown at low and intermediate densities is subtle, and thus other contributing factors may govern whether RB regulates U6 transcription. One possible explanation could be that at lower cell-density most of the RB protein is in the hyperphosphorylated form and as the cell density increases there is a shift to the hyphosphorylated form of RB, which is believed to be transcriptionally active. Indeed there has been a report showing that as cell density increased from 13% to 43% there was an increase in hyperphosphorylated-RB as observed by Western blot analysis. However, as the cell density reached 75% and 100% there was more hypophosphorylated-RB (1).

Prior studies have suggested that all three members of the RB family can regulate RNA polymerase III transcription (3, 5). Therefore, I wanted to determine whether p107 and p130 also associate with the U6 snRNA promoter. Chromatin immunoprecipitation assays were performed from cells grown to intermediate or high cell density and using antibodies for each RB family member. Enrichment of the cyclin A promoter was examined as a positive control for the p107 and p130 immunoprecipitations (6). As shown in Figure B-1B, the U6 snRNA promoter was enriched in the α-RB and α-p107 immunoprecipitations at the intermediate cell density conditions, but not when cells were grown at high densities. RB was not present at the cyclin A promoter during either growth condition whereas the cyclin A promoter was enriched in the p107 immunoprecipitation from cells grown at both intermediate and high density. In contrast, p130 associates with both promoters only at high cell densities. None of the RB pocket proteins was detected at the U6 promoter at low cell density nor was enrichment of the GAPDH exon 2 DNA observed (data not shown). Together these results suggest that all

three RB family members may participate in regulation of U6 snRNA expression but under different growth conditions. Data from our lab indeed shows that p107 and p130 also repress U6 snRNA transcription in vitro by RNA polymerase III (Xianzhou Song, unpublished data). Thus my data has revealed that both RB and p107 associate with U6 snRNA promoter DNA when cells are actively dividing. When cells have exited the cell cycle due to contact inhibition, neither RB nor p107 occupy a U6 snRNA promoter, but instead p130 now associates with the U6 promoter. Previously, p130 was shown to be mostly active in the G0/G1 phase while p107 is active in the S-phase (reviewed in (4). These observations are consistent with previous reports that both p107 and p130 can regulate RNA polymerase III transcription (5) My data indicates that RB and p107 may regulate RNA polymerase III activity during active growth and transition through the cell cycle whereas p130 may be active during growth arrest, and suggest that pocket protein family members regulate human U6 snRNA gene transcription at distinct phases of cell growth.

Methods and Materials

Human mammary epithelial 184B5 cells were used for the cell density experiments. The cells were seeded as follows: $0.15x10^7$ cells into 40 plates (25%), $0.6x10^7$ cell into 10 plates (75%), and $1.2x10^7$ cells into 5 plates (100%). After 48 hrs cells were harvested form each density pool and processed for collecting chromatin as described before (2). A portion of the chromatin corresponding to $1x10^7$ cells was then used for each immunoprecipitation reaction using anti-SNAP43 (CS48), anti-RB (SC-1538) antibodies, or an irrelevant preimmune serum. For the ChIP experiment shown in Figure B-1B, anti-

p107 (SC-318), anti-p130 (SC-317) antibodies were additionally used. Immunoprecipitated DNA was examined for enrichment of U6 snRNA, U2 snRNA, and Cyclin A promoter DNA or GAPDH exon2 DNA using PCR amplification. For the FACS analysis done Figure B-1A, a portion of the harvested cells were washed and fixed with ice cold 70% ethanol. Subsequently, cells were stained with Propidium Iodide and the DNA content was analyzed using a FACS Vantage flow cytometer.

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APPENDIX C

RB INTERACTS WITH MULTIPLE COMPONENTS OF THE U6 snRNA-SPECIFIC GENERAL TRANSCRIPTION MACHINERY FOR PROMOTER RECRUITMENT[1]

RB interacts with the U6 snRNA-specific general transcription machinery.

For RB to enact repression of U6 snRNA transcription RB might be recruited to the promoter either directly by binding to specific DNA control elements or through interactions with the general transcription factors. As RB does not have a DNA-binding domain, RB most likely is recruited to the DNA via interactions with $SNAP_C$ and TFIIIB. Therefore, GST pulldown experiments were performed to determine the region(s) of SNAP50 that is required for interaction with RB. The truncated SNAP50 mutant proteins used are represented in Figure C-1A. GST-RB (379-928) and the ^{35}S-labeled SNAP50 proteins were expressed as before (4). GST-RB (379-928) interacted with the full-length SNAP50 (1-411) protein and this interaction was specific, as no interaction was seen with GST (lanes 2 and 3). SNAP50 (1-300) and SNAP50 (301-411) proteins interacted with GST-RB (379-928) suggesting that there might be two regions in SNAP50 involved in RB interaction. A bigger C-terminal deletion of SNAP50 containing amino acids 1-199 also interacted with RB, however the region in SNAP50 containing amino acids 1-124 and 123-199 did not interact with RB suggesting that the region in SNAP50 around amino acids 123/124 could be important for RB interaction. Interestingly, a LxCxE motif

[1] Figure C-2 used in this document was published in the following manuscript: Heather A. Hirsch, Gauri W. Jawdekar, Kang-Ae Lee, LiPing Gu, and R. William Henry (2004) Distinct mechanisms for repression of RNA polymerase III transcription by the Retinoblastoma tumor suppressor protein. *Molecular and Cellular Biology* Vol.24; pp.5989-5999

is present within this region, amino acid residues 109-113. The LxCxE sequence, which is a known motif for RB binding (3), may be important for SNAP50 interaction with RB. However, a truncation of SNAP50 containing amino acids 123-300, which lacks the LxCxE motif, also interacts strongly with RB, suggesting that a region outside the LxCxE motif may provide a region for interaction with RB. Whether the LxCxE motif can contribute to interactions with RB is not known. Other amino-terminal truncations tested, including SNAP50 (301-411), SNAP50 (123-411), SNAP50 (200-411) lacking the LxCxE motif interacted weakly with RB. Together these data provide evidence that the region of SNAP50 between amino acids 1-199, 123-300, and 301-411 may be important for RB interaction and provide a basis for further mutational analysis.

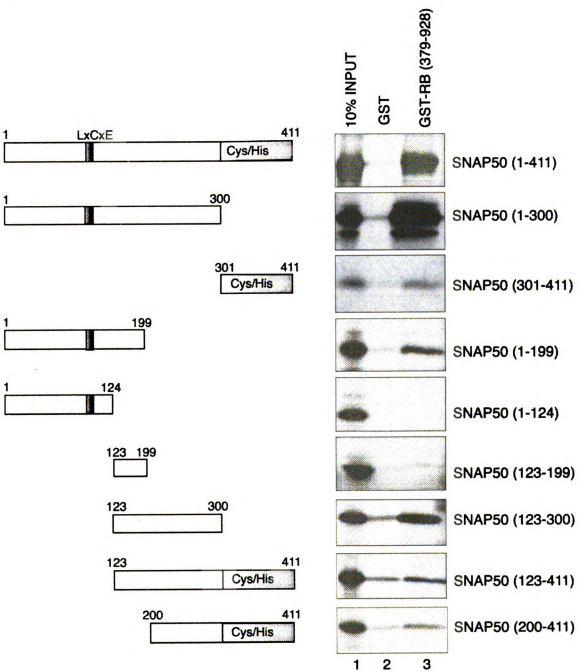
The region(s) in Bdp1 that are important for RB interaction were mapped in a GST pulldown assay. C- and N- terminal deletion mutants of Bdp1 were cloned and expressed as 35S-labeled proteins. The Bdp1 protein contains a SANT domain in the N-terminal amino acids. Though the exact function of the SANT domain is not known, in other proteins it has been implicated in DNA binding (1). Bdp1 contains a series of repeats in the C-terminal two-thirds of the protein with potential phosphorylation sites. As shown in Figure C-1B, full length Bdp1 (1-1338) protein, Bdp1 (471-1338) protein, and Bdp1 (823-1338) protein interacted strongly with GST-RB (379-928) (lane 2) but not with GST or beads alone (lanes 3 and 4). However, the N-terminal region containing amino acids 1-470 did not interact with GST-RB (379-928), suggesting that the C-terminal two-thirds of Bdp1 is important for RB interaction and the amino acids between 823-1338 are sufficient for this interaction. RB could potentially be recruited to the U6 promoter via these interactions.

Figure C-1. Characterization of the regions in SNAP50 and Bdp1 that are required for RB interaction.

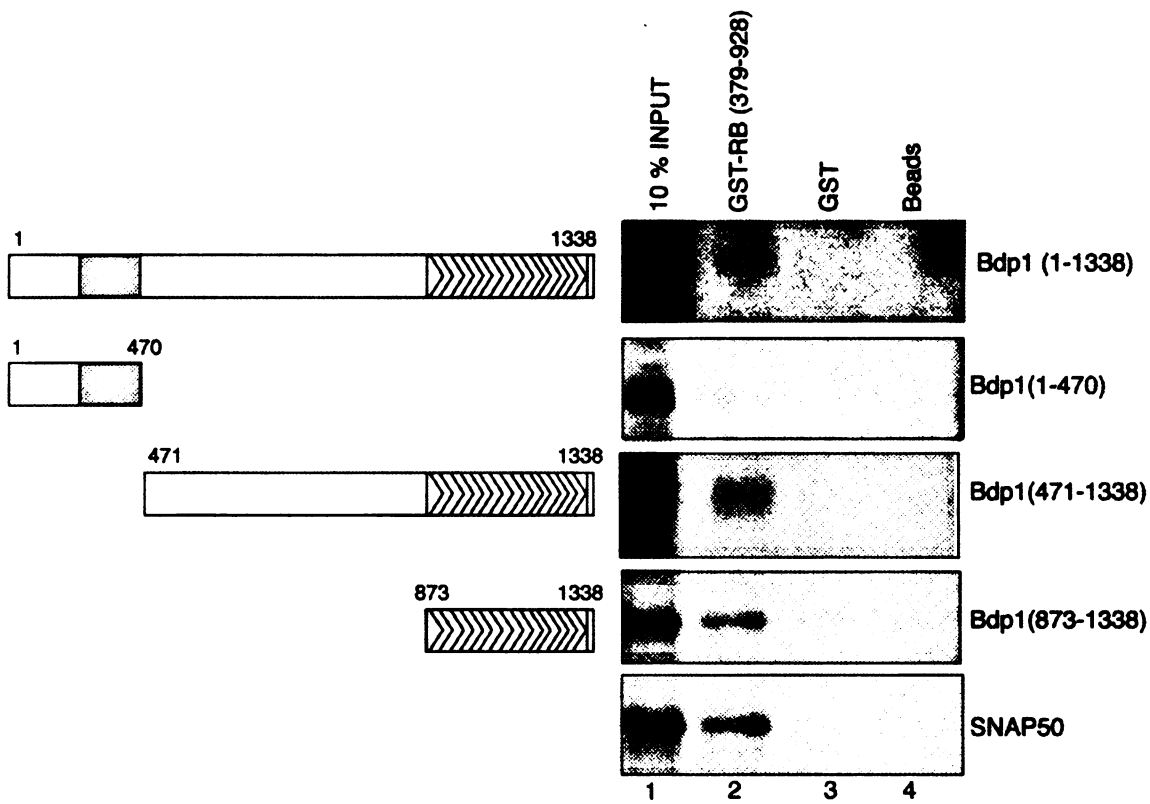
(A) The RB pocket domain interacts with at least three regions in SNAP50. Schematic representation of SNAP50 proteins containing the indicated deletions is shown in the left panel. GST pulldown analysis was performed to map the regions in SNAP50 that interact with RB. SNAP50 proteins containing deletions were expressed in vitro using rabbit reticulocyte lysate and labeled with ^{35}S-methionine. Equal amounts of the SNAP50 proteins were incubated with approximately 1 μg GST-RB (379-928) (lane 3) and 1 μg GST (lane 2). Complexes were collected with glutathione agarose beads for 2 hrs at 4°C. Bound proteins were washed and eluted in Laemmlie buffer. Proteins were separated by SDS-PAGE and visualized by autoradiography. Lane 1 shows 10% of the input.

(B) The C-terminal region of Bdp1 is important for RB interaction. Schematic representation of the Bdp1 proteins harboring the indicated deletions are shown in the left panel. GST pulldown assays were carried out with ^{35}S-methionine-labeled Bdp1 proteins and GST-RB (379-928) as in (A).

A


10% INPUT
GST
GST-RB (379-928)
1
LxCxE
411
Cys/His
SNAP50 (1-411)
300
SNAP50 (1-300)
301
SNAP50 (301-411)
199
SNAP50 (1-199)
124
SNAP50 (1-124)
123
SNAP50 (123-199)
SNAP50 (123-300)
SNAP50 (123-411)
200
SNAP50 (200-411)
1 2 3

B


10 % INPUT
GST-RB (379-928)
GST
Beads
1
1338
Bdp1 (1-1338)
1
470
Bdp1(1-470)
471
1338
Bdp1(471-1338)
873
1338
Bdp1(873-1338)
SNAP50
1
2
3
4

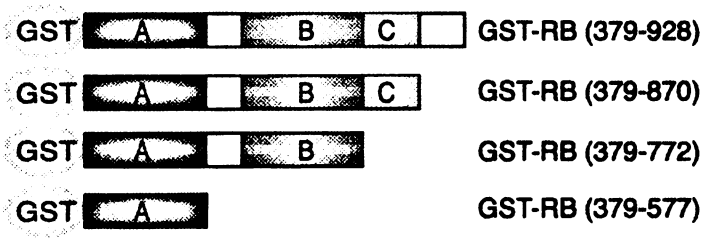
To further understand the mechanism of RB repression of U6 transcription, the RB domains that are required for repression of U6 transcription were characterized. Therefore, RB proteins containing the A domain but lacking additional regions of the carboxy terminus were analyzed. Previous observations show that RB interacts with $SNAP_C$ (4) and TFIIIB (2, 6). That RB does not occupy a human U1 snRNA gene promoter even though $SNAP_C$ is required for transcription of U1 by RNA polymerase II, suggests that RB interaction with $SNAP_C$ and TFIIIB represents a mechanism by which RB is specifically recruited to the U6 snRNA promoter to repress transcription. GST pull down experiments were performed to identify the regions of RB necessary for interaction with components of $SNAP_C$ and TFIIIB. Each component of the basal transcription machinery was individually expressed and labeled with ^{35}S-methionine in rabbit reticulocyte lysate. Expression of these labeled proteins is shown in Figure C-2B (lane 1). Equivalent amounts of GST-RB (379-928) or GST-RB proteins containing the indicated truncations were incubated with the labeled proteins. Strong interactions between GST-RB (379-928) and two components of $SNAP_C$, SNAP43 and SNAP50 were observed. Specific interactions of GST-RB (379-928) with the TBP, Brf1, and Bdp1 components of TFIIIB were also observed, although the interactions with TFIIIB appear to be weaker as compared to those with $SNAP_C$ components. These interactions are specific because neither GST nor beads alone bound to any of the $SNAP_C$ or TFIIIB proteins (lanes 6 and 7). In contrast GST-RB (379-928) did not interact with Brf2 or Oct-1 in these assays (lane 2). More interestingly, GST-RB (379-870), which repressed U6 transcription, maintained the ability to interact with components of $SNAP_C$ and TFIIIB (lane 3), but GST-RB (379-772), which failed to repress U6 transcription, showed

Figure C-2. The A/B pocket domain and the C-terminal region of RB are required for interactions with RNA polymerase III-general transcription machinery.

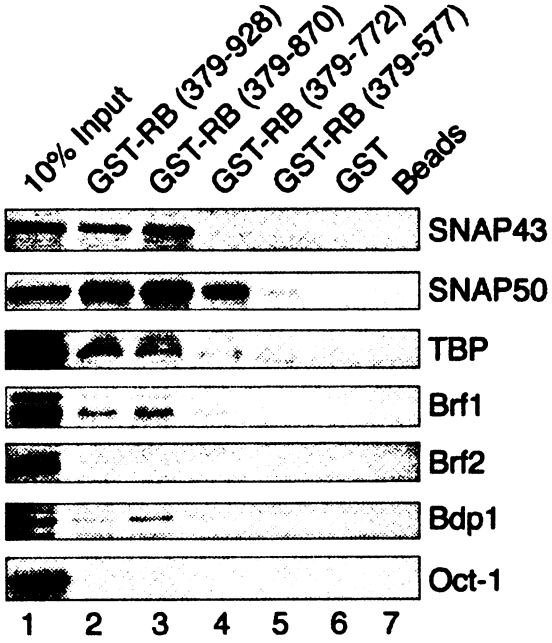
(A) Schematic representation of the GST-RB proteins containing the indicated deletions.

(B) Characterization of the RB regions required for interactions with RNA polymerase III basal transcription machinery. GST-pull down analysis was performed to determine the region of RB that can interact with each component of RNA polymerase III basal machinery. SNAP43, SNAP50, TBP, Brf1, Brf2, Bdp1, and Oct-1 were expressed in vitro and labeled with ^{35}S-methionine. Lane 1 shows 10% of each protein that was added to the reaction. The various GST-RB proteins containing deletions were incubated with each ^{35}S-methionine labeled protein (lanes 2-5). GST or beads alone were used as controls (lanes 6 and 7). The 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.

A



B



GST-RB Protein ID	U6 snRNA repression	SNAP43	SNAP50	TBP	Bdp1	Brf2
GST-RB (379-928)	+	+	+	+	+	-
GST-RB (379-870)	+	+	+	+	+	-
GST-RB (379-772)	-	-	+	-	-	-
GST-RB (379-577)	-	-	-	-	-	-

reduced interactions with SNAP43, TBP, Bdp1, and Brf1 (lane 4). Thus these interactions might be critical for RB repression of U6 snRNA transcription (summarized in the table). Although GST-RB (379-772) interacted strongly with SNAP50, this interaction alone is not sufficient to maintain RB repression. Finally, GST-RB (379-577), which contains only the A domain, was not able to interact with any of the $SNAP_C$ or TFIIIB proteins (lane 5) consistent with its inability to repress transcription. These observations show that the A/B pocket domain and the C-terminal region of RB are important for interactions with the U6 snRNA-specific general transcription factors. It is possible that RB is recruited to the promoter DNA via multiple protein-protein interactions with $SNAP_C$ and TFIIIB. Sequential ChIP experiments indeed show that RB co-occupies the same U6 snRNA gene promoter with $SNAP_C$ and TFIIIB in vivo (5). Interestingly, proteins harboring progressively increasing C-terminal deletions lose their ability to repress transcription, indicating that the A/B pocket domain and the C region are necessary for repression of RNA polymerase III transcription (5).

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