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

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Date

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

By

Tharakeswari Selvakumar

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ABSTRACT

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

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The Retinoblastoma tumor suppressor (RB) protein restricts unregulated cell division by repressing transcription of genes whose products are essential for cell growth and division. RB represses a subset of RNA Polymerase (Pol) II-transcribed genes that contain E2F binding sites and are important for progression into S-phase. RB also acts as a general repressor of Pol I and Pol III transcription. Considering that Pol I and Pol III transcribed products are essential for cell growth and division, repression of transcription by Pol I and Pol III can be an important aspect of the growth-suppressive function of RB. The growth inhibitory function of RB is linked to its anti-tumorigenic potential, and it is likely that understanding RB repression of Pol I and Pol III transcription can elucidate some important aspects of the RB tumor suppression mechanism. My study examines the mechanism for RB repression of U6 transcription by Pol III.

Sequence comparison among the nine U6 copies in humans revealed that all the functional U6 copies are enriched in CpG dinucleotides at the promoter regions compared to the non-functional copies, and as the CpG sequence is the primary target for methylation in humans, this indicated a potential involvement

of DNA methylation in regulation of U6 transcription. Existing evidence indicating a repressive role for DNA methylation on transcription, led to the hypothesis that DNA methylation contributes to U6 repression. In support, in vitro transcription from pre-methylated U6 templates demonstrated that DNA methylation has an inhibitory effect on U6 transcription.

Earlier studies indicated that RB interacts with DNA methyltransferase (DNMT) 1 and DNMT1 activity contributes to RB repression of E2F-transactivated Pol II transcription. Consistently, RB was found to direct recruitment of DNA methylation and DNA methyltransferases 1 and 3A to the U6 promoter during repression of Pol III transcription. Also, siRNA-mediated depletion of DNMT1, 3A and 3B in RB positive cells resulted in enhanced U6 transcription, suggesting a repressive role for DNA methyltransferases in U6 transcription. The results presented here implicate RB-directed promoter DNA methylation as an important aspect of the mechanism for RB-mediated repression of human U6 transcription.

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KEY TO SYMBOLS AND ABBREVIATIONS

ATP	Adenosine triphosphate
Bdp1	B double prime 1 protein
Brf-1	TFIIB related factor 1
Brf-2	TFIIB related factor 2
Cdk	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
C-Terminal	Carboxy-terminal
Da	Dalton
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DSE	Distal sequence element
GAPDH	Glyceraldehyde 3- phosphate dehydrogenase
HDAC	Histone deacetylase
ICR	Internal control region
MeCP	Methyl CpG binding protein
MBD	Methyl binding domain
NTP	Nucleotide triphosphate
PcG	Polycomb group complex
PCR	Polymerase chain reaction

Pol	Polymerase
PSE	Proximal sequence element
RB	Retinoblastoma tumor suppressor protein
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RT-PCR	Reverse transcriptase polymerase chain reaction
SAM	S-adenosyl methionine
SAH	S-adenosyl homocysteine
SNAPc	Small nuclear RNA activating protein complex
snRNA	Small nuclear RNA
TBP	TATA binding protein
TFIIB	Transcription factor IIB
TFIIIA	Transcription factor IIIA
TFIIIB	Transcription factor IIIB
TFIIIC	Transcription factor IIIC
Topo	Topoisomerase
tRNA	transfer RNA

CHAPTER I

INTRODUCTION

1. The Retinoblastoma Protein

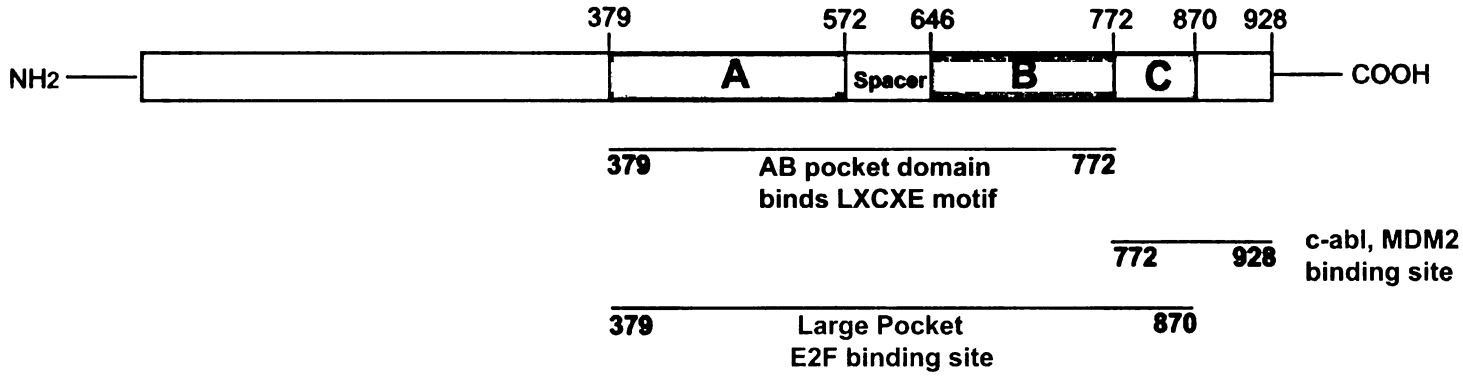
The human Retinoblastoma susceptibility gene (RB1) was identified at genetic locus 13q14 on account of homozygous deletions and tumor-specific expression abnormalities in cases of retinoblastomas and sarcomas (53, 56, 116). The product of the RB1 gene, the Retinoblastoma protein (RB), controls the cell-cycle and facilitates G1 growth arrest (92, 155, 167, 221). As a result, RB exerts an anti-proliferative effect against unregulated cell growth and division and functions as a checkpoint against tumorigenesis (66). RB binds and inactivates E2F transactivator proteins (E2Fs) 1, 2 and 3 (117). Some of the E2F-activated genes that are important for cell cycle progression include c-myc, B-myb, cdc2, dihydrofolate reductase and thymidine kinase (18, 25, 41, 146, 210). These genes contain variant forms of the consensus nucleotide sequence TTTCGCGC for E2F binding in their promoters (112, 146). RB is thought to repress transcription of E2F target genes by binding and inactivating E2F (51, 68, 179). Evidence suggesting active repression of transcription by the RB-E2F complex at targeted gene promoters containing E2F binding sites has been reported (44, 113, 167, 191, 222). The hypophosphorylated form of RB is thought to be the active form whereas the hyperphosphorylated form is inactive with respect to transcriptional repression at E2F target genes (25, 27). Cyclins D1, D2 and D3 as complexes

with CDK4/CDK6 (47, 102, 221) and the Cyclin E-CDK2 complex (78) phosphorylate RB and the resultant inactivation during mid to late G1 permits cell cycle progression into S phase (47, 78, 102, 221).

2. RB functional domains

RB contains the A and B pocket domains that are common to the related proteins p107 and p130, and which are involved in regulating cell cycle progression (Figure 1-1) (30, 36, 46). The A (amino acids 379-572) and B pocket (amino acids 646-772) domains (85, 88, 97, 107) are conserved across species (31, 115) and are crucial for the tumor suppressor function of RB (166). The spacer region from amino acids 573-645 serves a structural role to facilitate folding of the A/B pocket. The amino acid sequence of the spacer is not crucial for A/B pocket activity (85, 88, 107).Viral oncoproteins such as the adenoviral E1A, SV40 large T antigen and human papillomavirus E7 interact with RB at the A/B pocket domain to inhibit RB function in infected cells (30, 85, 86, 88, 97). The region encompassing amino acids 379-928 comprising the A/B pocket and the C terminal regions was found to be the minimal region required for tumor suppression (241). Many naturally occurring mutations observed in the Rb locus in tumors disrupt the pocket domain (63, 83) indicating that the pocket domain plays a crucial role in tumor suppression by RB. Factors that interact with RB at the pocket domain such as viral oncoproteins (115, 129, 232) and co-repressor proteins such as HDACs 1and 2 and BRG1 (20, 43, 130, 132), contain an LXCXE motif that mediates their binding to RB. Structural analysis of the pocket domain

Figure 1-1: Schematic representation of the RB functional domains. The A (amino acids 379-572) and B pocket (amino acids 646-772) domains (85, 88, 97) and the C pocket (amino acids 772-870) (225) are represented. The A/B pocket domain is involved in binding to proteins containing an LXCXE motif, such as viral oncoproteins (115, 129, 232) and co-repressor proteins such as HDACs 1 and 2 and BRG1 (20, 43, 130, 132). The spacer region is shown (amino acids 572-646) (85, 88). The Large Pocket (amino acids 379-870) comprising the A/B pocket and the C pocket which is involved in E2F binding is shown (77, 166). C terminal binding site for c-abl and MDM2 (at amino acids 772-870) (225, 238) are indicated.



The Retinoblastoma Protein

showed that the B domain contains the LXCXE binding site (115). However, the A domain was found to be important for maintenance of the active conformation of the B domain (105, 115). E2Fs do not contain an LXCXE motif, and they bind to RB via its Large Pocket domain comprising the A/B and C pocket (amino acids 379-870) regions (77, 87, 107, 115, 166, 225). The C Pocket domain (amino acids 772-870) binds c-abl tyrosine kinase and MDM2 (107, 225, 238). The tyrosine kinase function of c-abl is inhibited when it is bound to RB (225) and this interaction is observed to be important for growth suppression by RB (226). The significance of the interaction between RB and MDM2 is not well characterized (66). The amino terminal region of RB contains cdk phosphorylation sites (31) and is thought to contribute to the tumor suppressive effect of RB (66, 173). However, contradictory evidence indicating that the removal of the amino-terminal region of RB led to enhanced tumor suppressive potential of RB has also been presented (166, 240).

3. RB-E2F pathway

The role of the RB-E2F regulatory mechanism in cell cycle control has been studied extensively (92, 167, 255). Overexpression of dominant-negative DP1, which is an E2F binding partner, inhibits progression in S phase and highlights the importance of E2F function and interaction with DP1 in S phase progression (237). In another study, E2F3 knockout mice were delayed in entering S phase (89). In tumors triggered by T antigen expression, which inactivates RB (and p53) leading to the release of E2F, backcrossing the mice in

an E2F1-/- background impaired tumor growth. This suggested that the release of E2F1 is important for tumor growth when RB is inactivated (202). Also, crossing RB -/- mice into an E2F1-/- background caused a significant reduction in ectopic cell cycle entry in the CNS and lens compared to RB-/- only mice (212). These lines of evidence suggest that the RB-E2F genetic interaction is involved in regulating cell cycle progression and tumor growth.

4. RB repression of Pol I and Pol II transcription

RB represses transcription by RNA Polymerases I, II and III (227, 230). This section will focus on RB repression of Pol I and E2F-mediated Pol II transcription. Studies done by Cavanaugh et al., (23) have found that RB can inhibit Pol I transcriptional activation by UBF by binding to UBF through the RB pocket domain. RB can repress E2F-mediated Pol II transcription by binding to the E2F activation domain directly and blocking the transactivation function of E2F (51, 68). A second mechanism where RB recruits co-repressor complexes to E2F-target promoters has also been proposed (66). HDAC activity is required for RB repression of two E2F target genes, thymidine kinase and DHFR (130). hBRM (part of the hSWI/SNF chromatin remodeling complex) can cooperate in RB-mediated repression of E2F transcriptional activation (211). RB-mediated growth arrest can also occur in an E2F-independent manner. Overexpression of RB continues to cause growth arrest in the presence of a dominant negative E2F, suggesting that there are other additional RB targets for growth arrest (66, 248).

Possibly, RB repression of general Pol I and Pol III transcription may be another important aspect of its growth arrest and tumor suppression mechanism.

5. RB repression of RNA polymerase III transcription

RB represses general RNA polymerase III transcription (230). On comparing two osteosarcoma cell lines and the RB negative SAOS2 cells had elevated levels of Pol III transcription relative to the RB positive U2OS cells (230). Moreover, primary fibroblasts from RB knockout mice had higher Pol III activity when compared to equivalent cells from wild type mice, suggesting that RB causes repression of Pol III transcription (230).

RB repression of Pol III transcription can contribute to its anti-growth effect. Pol III-transcribed products such as the U6 snRNA, tRNA, and 5SrRNA are required for key cellular processes such as protein synthesis and splicing which are important for normal cellular growth rate and division. During rapid growth and cell division in tumors, elevated levels of biosynthesis of cellular products are needed, thereby raising the demand for higher cellular levels of the protein synthesis (227) and possibly splicing machinery. When quiescent cells were subject to mitogenic stimulation to grow and divide, production of rRNA and tRNA increased along with a corresponding increase in protein synthetic functions (178). The rate of growth is directly proportional to the rate of protein accumulation which in turn is dependent on the rate of protein synthesis (6). These results suggest a clear correlation between the cellular levels of the protein synthesis to the cellular growth rate (227). Therefore it is possible that by

repressing transcription of Pol III products, RB exerts its anti-growth effect. As RB executes its tumor suppressor function by acting as a checkpoint against unwarranted cellular biosynthesis, growth and division (66, 221, 227), RB mediated repression of Pol III transcription could be an integral aspect of its tumor suppression function.

One study has demonstrated that the activities of Pol III (and also Pol I) are elevated in murine tumors, whereas Pol II activity remained unaffected (189). The RB domains required for tumor suppression and those required for repressing Pol III transcription largely coincide (23, 230), indicating a potential link between the Pol III repression and tumor suppression functions of RB. The region encompassing amino acids 379-928 in the RB protein was the minimal region required for tumor suppression. This region was also the minimal region required for efficient repression of Pol III transcription. On the contrary, the region between amino acids 379-792 was the minimal region for repression of Pol II transcription. This suggests that tumor suppression by RB requires other additional functions apart from Pol II transcriptional repression, possibly, repression activity targeting Pol III transcription. Also many transforming agents such as oncoproteins from the simian virus 40, papovavirus, hepatitis B virus and chemical carcinogens cause elevated Pol III transcription (49, 133, 190, 194, 219, 229). Naturally-occurring mutations that inactivate RB as a tumor suppressor were found to render RB inactive for repression of Pol III transcription also (103, 221, 231). Also, adenoviral E1A oncoprotein that binds and inactivates RB for tumor suppression inactivates RB as a repressor of Pol III transcription (230).

These evidence lend support to the idea that the tumor suppressor function and Pol III repressor functions of RB are linked. Detailed studies focused on understanding the mechanism for RB repression of Pol III transcription can help us gain insights into its tumor suppression mechanisms.

6. Mechanism for RB repression of Pol III transcription

Pol III promoters have been classified as types 1 (5S rRNA), 2 (AdVAI and tRNA) and 3 (U6 snRNA) (Figure 1-2) (186). The type I Pol III promoters contain an A box, C Box and the Intermediate element (IE) together comprising the Internal Control Region (ICR) (19, 158-160). The type 2 promoters in AdVAI and tRNA genes consist of an A box and a B box (1, 57, 81, 192). The type III promoters found in U6 snRNA, 7SK and H1 RNA genes are extragenic and consist a TATA Box, Proximal Sequence Element (PSE) and Distal Sequence Element (DSE) (76, 111, 126, 135). The TFIIIA, TFIIIB and TFIIIC factors are required for transcription of type I Pol III promoters, whereas only TFIIIB and TFIIIC are required in the case of type 2 promoters (Figure 1-3). TFIIIA is a Zinc finger containing DNA binding protein that binds to the ICR (Internal Control Region) of type I promoters (139). TFIIIB used in both type 1 and type 2 promoters consists of TBP, Bdp1 and Brf1 whereas the type 3 promoter uses TFIIIB that comprises TBP, Bdp1 and Brf2 (127, 140, 186, 187, 203, 208, 209, 220, 228, 233).

Figure 1-2: Types of RNA polymerase III promoters. Type 1 promoter in 5S rRNA gene has an internal control region (ICR) consisting an A box, intermediate element and C box. Type 2 promoter found in tRNA genes consists of an A box and a B box. Type 3 promoter in the U6 snRNA gene consists of a DSE (Distal Sequence Element), Proximal Sequence Element (PSE) and TATA Box. Tn represents the termination signal. Diagram adapted from (186).

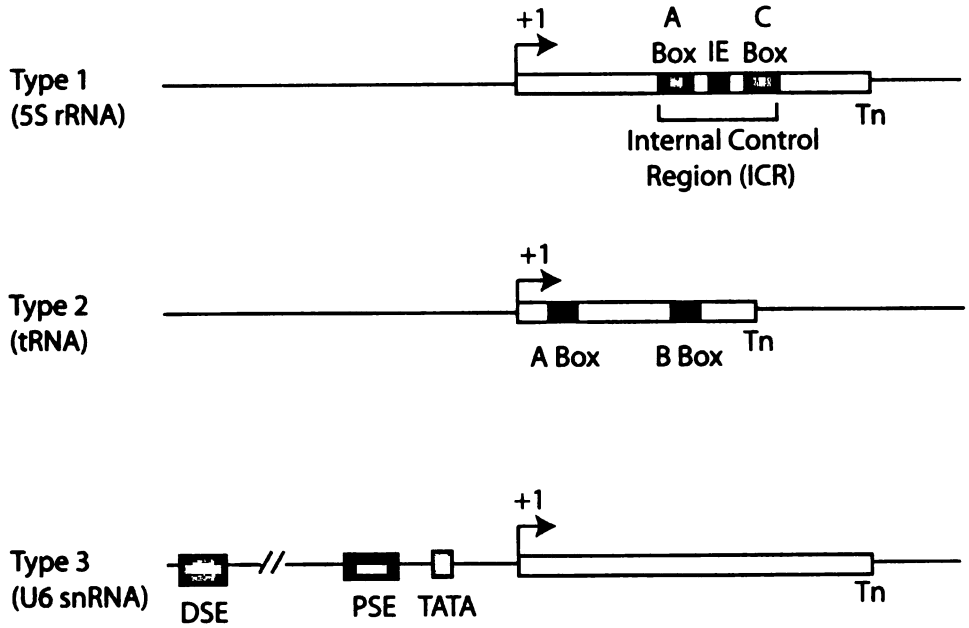
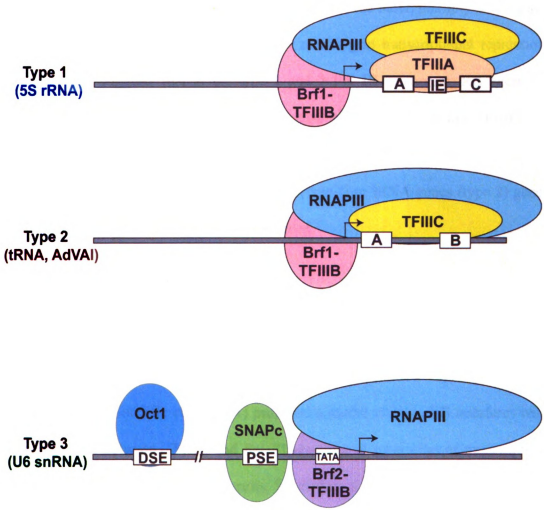

Type 1
(5S rRNA)
+1
A
Box
IE
C
Box
Tn
Internal Control
Region (ICR)
Type 2
(tRNA)
+1
A Box
B Box
Tn
Type 3
(U6 snRNA)
+1
DSE
PSE
TATA
Tn

Figure 1-3: General transcription machinery for RNA polymerase III transcription. The type I promoter requires TFIIIA, TFIIIC and Brf1-TFIIIB. TFIIIC and Brf1-TFIIIB are required for transcription from type 2 promoters. Type III promoter requires Brf2-TFIIIB, and SNAPc. Oct1 enhances type 3 transcription but is not essential (186).


Type 1
(5S rRNA)
RNAPIII
TFIIIC
TFIIIA
A
IE
C
Brf1-
TFIIIB
Type 2
(tRNA, AdVAI)
RNAPIII
TFIIIC
A
B
Brf1-
TFIIIB
Type 3
(U6 snRNA)
Oct1
SNAPc
RNAPIII
DSE
PSE
TATA
Brf2-
TFIIIB

Based on studies carried out on Alu and AdVAI transcription (that use the Brf1-TFIIIB), Chu et al., (32) proposed that RB can inhibit transcription of Pol III-transcribed genes that use Brf1-TFIIIB which are the type 1 and type 2 Pol III promoters, by binding to the Brf1 component of TFIIIB via the RB A domain, and to TFIIIC via the RB B domain. The authors proposed a model wherein the binding of either TFIIIB or TFIIIC to RB displaces the other, possibly leading to a failure to recruit TFIIIB to the promoter resulting in transcriptional repression. This study suggests that at the type 1 and 2 Pol III promoters, RB exerts its repressive effect by sequestering transcription factors TFIIIB and/or TFIIIC, but not by directly getting recruited at the target promoter. The observations that RB does not occupy the endogenous 5SrRNA (type I) or tRNA genes (type 2) genes are consistent with the above mentioned idea.

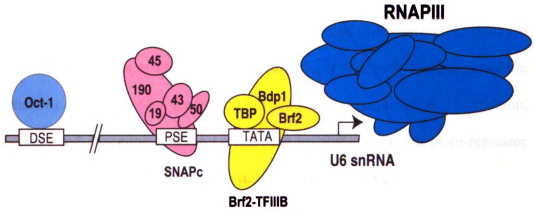
However, this model does not explain the mechanism for RB repression of U6 transcription, which is independent of TFIIIC and the Brf1 component of TFIIIB. In trying to understand the mechanism for RB repression of U6 transcription, Sutcliffe et al., (201) proposed a model wherein RB interferes with interactions between TFIIIB and Pol III resulting in repression of transcription. Using co-immunoprecipitation results, Sutcliffe et al., (201) demonstrated that RB disrupts interactions between TFIIIB and Pol III in the absence of DNA. Based on evidence that once TFIIIB gets to the promoter, it recruits Pol III via interactions between TFIIIB and Pol III (seen in the tRNA and 5SrRNA gene systems) (101) it was reasoned that disruption of interaction between TFIIIB and Pol III by RB can

lead to repression of transcription on account of failure to recruit Pol III to the U6 promoter. In contrast, studies done by Hirsch et al., (80) have shown that RB and Pol III co-occupy the U6 promoter, suggesting that RB does not interfere with recruitment of Pol III to the promoter. Furthermore, in contrast to the type 1 and 2 Pol III promoters, RB association with the promoter DNA seems to be important for repression from the U6 promoter, as seen from the observation that truncations in the RB protein that debilitated RB association with the U6 promoter also inactivated RB repression (80). It is possible that RB association with the U6 promoter is important for targeting recruitment of co-repressor proteins which can cause transcriptional repression. Thus, RB may not cause repression by interfering with Pol III recruitment, instead, by recruiting co-repressor proteins that cause chromatin modifications resulting in a chromatin configuration that is non-conducive for polymerase escape into the transcribed region, leading to repression.

7. RB represses U6 snRNA gene transcription

Earlier studies have demonstrated RB repression of the human U6 snRNA transcription (79, 80, 114). Factors involved in transcription of the U6 snRNA gene include the SNAP complex (SNAPc) bound to the PSE; a variant form of TFIIIB consisting of TBP, Brf2 and Bdp1 proteins bound to the TATA box; and the Oct-1 protein bound to the DSE (Figure 1-4) (186). SNAPc contains five subunits; designated SNAP190, SNAP50, SNAP45, SNAP43 and SNAP19 (3, 71-73, 181, 235, 245). SNAP190 binds DNA via its Myb domain. Crosslinking experiments have shown that SNAP190 and SNAP50 are in close contact with

Figure 1-4: The U6 snRNA transcription machinery. The U6 snRNA promoter is a type 3 RNA Polymerase III promoter. The key promoter elements comprise the TATA Box, PSE (Proximal Sequence Element) and DSE (Distal Sequence Element). The Brf2 containing TFIIIB complex containing also TBP and Bdp1 binds the TATA box. The multi-protein snRNA activator protein complex (SNAPc) consisting of SNAP190, SNAP50, SNAP45, SNAP43 and SNAP19 subunits binds the PSE. The Oct1 activator protein binds the DSE (186).


RNAPIII
45
190
43
19
50
Oct-1
Bdp1
TBP
Brf2
DSE
PSE
TATA
U6 snRNA
SNAPc
Brf2-TFIIIB

DNA (71, 141, 244) suggesting that SNAP190 and SNAP50 are the DNA binding components of the SNAP complex. The DSE is bound by the POU domain containing Oct1 activator protein. The endogenous human U6 promoter contains a positioned nucleosome between the DSE and the PSE, thereby promoting interactions between the Oct1 POU domain and SNAPc leading to transcriptional activation of U6 (200, 251).

RB co-occupies the U6 promoter with SNAP43, TBP and Pol III (80). RB also interacts with U6 transcription machinery components SNAP43, SNAP50, TBP and Bdp1 (79, 80). These results suggest that RB interactions with U6 transcription factors at the U6 promoter might interfere with the transactivation functions of these factors, contributing to repression of transcription. In addition, as discussed in section 6, RB repression can involve recruitment of co-repressor proteins to the U6 promoter to cause transcriptional repression

8. RB co-repressor proteins

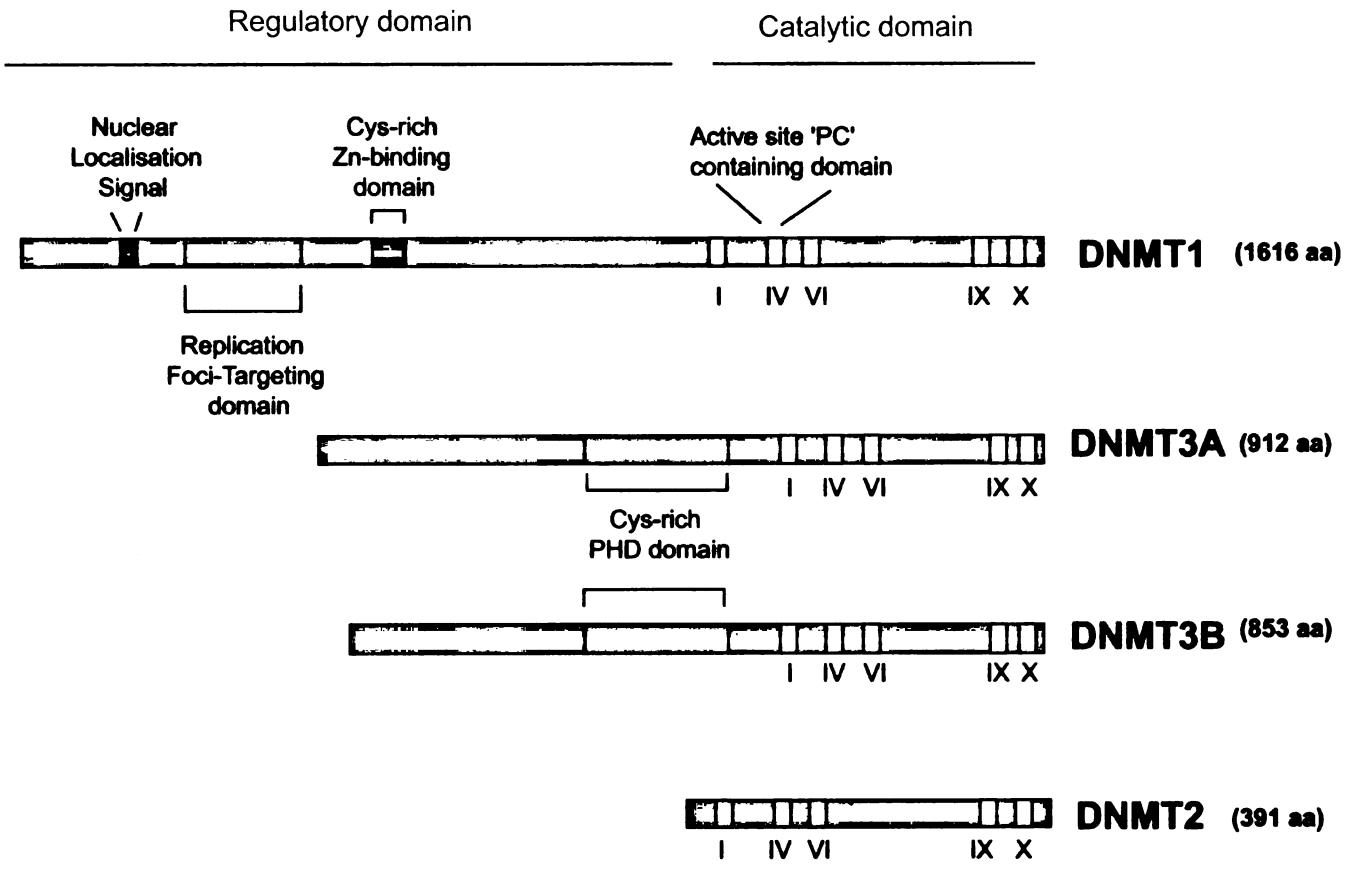
Additional mechanisms proposed for RB-mediated repression of target gene transcription involve the role of co-repressor proteins in causing chromatin modifications that impede transcription (50). Studies have revealed functional interactions between RB and other co-repressor proteins during repression of target genes (50). DNA methyltransferases, methyl CpG binding proteins, histone deacetylases, components of the SWI/SNF chromatin remodeling complex, histone methyltransferases, Polycomb group proteins (40, 90, 91) and DNA topoisomerases (14) are among those factors known to regulate RB function.

Sequence analysis of the nine human U6 copies that have identical coding region sequence revealed an enrichment of CpG dinucleotides in the functional U6 copies compared to the non-functional copies. CpG dinucleotides are frequents targets of methylation in mammalian cells. This suggested a role for DNA methylation and DNA methyltransferases in U6 regulation. As will be discussed in Chapter Two, I have investigated the role of DNA methylation in U6 transcriptional regulation and also analysed the correlation between RB function and DNA methylation in regulating U6 transcription. Evidence for RB directed recruitment of DNA methyltransferases and DNA methylation to the U6 promoter is presented, suggesting involvement of DNMT activity as an important aspect of the mechanism for RB repression of U6 transcription. In mammalian cells, there are three functional DNA methyltransferase enzymes that have been identified; DNMT1, DNMT3A and DNMT3B. The following subsection 8.1 focuses on DNA methyltransferases and DNA methylation.

8.1. DNA methyltransferases

DNA methylation in mammalian cells is regulated by three DNA methyltransferases (DNMTs); designated DNMT1, DNMT3A and DNMT3B (174) (Figure 1-5). Another methyltransferase, DNMT2, has been cloned and characterized but lacks catalytic activity (154, 242). DNMTs target cytosines in CpG dinucleotide sequences and methylate the cytosine at the 5 position. This reaction is catalysed by an active site cysteine (11). DNMT1 was the first

Figure 1-5: Schematic representation of the DNMT family proteins. DNMTs 1, 3A and 3B contain a regulatory domain and catalytic domain. Conserved motifs among the DNMT proteins are indicated as I, IV, VI, IX, X. DNMT1 contains domains for nuclear localization (NLS), replication foci targeting and a Zinc-binding cysteine rich region. DNMTs 3A and B contain a cysteine- rich PHD domain in their regulatory domain. Diagram adapted from (174).


Regulatory domain
Catalytic domain
Nuclear Localisation Signal
Cys-rich Zn-binding domain
Active site 'PC' containing domain
DNMT1 (1616 aa)
I IV VI IX X
Replication Foci-Targeting domain
DNMT3A (912 aa)
I IV VI IX X
Cys-rich PHD domain
DNMT3B (853 aa)
I IV VI IX X
DNMT2 (391 aa)
I IV VI IX X

methyltransferase to be discovered (10) followed by the discovery of the DNMT3 family of methyltransferases (153).

DNMT1 is considered to be the maintenance methyltransferase that recognizes and binds hemi-methylated CpG to methylate the unmethylated cytosine in the complementary DNA strand (162, 163). DNMT1 is the most abundant methyltransferase in somatic cells (176). DNMT1 is thought to copy methylation patterns after replication as seen from its ability to localize to replication foci and to interact with PCNA (33, 119, 125). DNMT1 is required for proper embryonic development, imprinting and X-inactivation as observed from effects in DNMT1 knockout mice. DNMT1 depletion arrests embryonic development and causes a 70% reduction in genomic 5meCpG content (8, 123, 124). However, DNMT1 knockdown in adenocarcinoma cells showed about 80% normal methylation and did not suffer remarkable growth defects. This suggested that DNMT3 family methyltransferases may also be involved in maintenance methylation in certain situations (172). Alternatively, other DNMTs that are yet to be discovered might perform redundant methyltransferase function.

Studies done by Robertson et al., (175) have found that DNMT1 can interact with RB both in vivo and in vitro and that the C706F mutation in RB abolished interaction with DNMT1 and also RB repression activity. DNMT1 co-purifies with RB, E2F1 and HDAC1 and cooperates with RB to repress E2F-dependent transcription. Expression of DNMT1 enhanced repression of transcription by RB when RB was tethered to an E2F responsive promoter. Similarly, expression of RB enhanced the repressive effect of DNMT1 when

DNMT1 was tethered to the same promoter, suggesting a cooperative functional interaction between RB and DNMT1 in repressing transcription from an E2F target gene. Repression by either tethered RB or tethered DNMT1 was relieved by the addition of the HDAC inhibitor TSA. Expression of RB, DNMT1 and E2F1 in combination led to the most efficient transcriptional repression from a natural E2F responsive promoter, in comparison to transcriptional repression seen with expression of E2F1 and RB or with E2F1, RB and DNMT1 expressed individually. Consistently, transcription from an E2F unresponsive promoter remained unaffected even upon combined expression of RB, DNMT1 and E2F1 (175).

Genes encoding the DNMT3 family of methyltransferases were first cloned and characterized by Okano et al., in 1998 (153). Mice knockouts for the DNMT3 family methyltransferases showed loss of de novo methylation following embryo implantation (152). In vitro studies as well as studies done in DNMT3 knockout mice revealed that DNMT3 enzymes have an equal preference for hemi-methylated and unmethylated DNA substrates, leading to their classification as de novo methyltransferases (153). DNMT3A knockout mice die at 4 weeks after birth and DNMT3B knockout mice are not viable (152). In vitro assays performed with recombinant proteins expressed in baculovirus showed that DNMT3A was more active than DNMT3B. DNMT3B was found to be involved in maintenance of DNA methylation of minor satellite repeats adjacent to centromeres. Mutations in the catalytic domain of the human DNMT3B are associated with the ICF syndrome (Immunodeficiency, Centromere instability and Facial anomalies

syndrome), a rare autosomal recessive disorder. Patients with ICF syndrome suffer from immunodeficiency, chromosomal abnormalities and facial abnormalities (62, 152, 239).

DNMT1 methyltransferase is considered to be the maintenance methyltransferase while the DNMT3 family of methyltransferases are thought to perform the de novo methylation. Studies done by Lei et al., (118) showed that DNMT1 knockout embryonic cells retained de novo methylation. Expression of DNMT1 and DNMT3A in *Drosophila melanogaster* showed that DNMT1 had no denovo methylation property whereas expression of DNMT3A led to low levels of methylation (131). Also, in ES cells, homozygous deletions of Dnmt3a and Dnmt3b led to no alterations in pre-existing methylation patterns but homozygous deletion of Dnmt1 led to about 70% reduction in cytosine methylation (124, 152).

However, recent evidence support a reinterpretation of these observations. Earlier studies by Vertino et al., (214) showed that overexpression of DNMT1 in cancer cell lines resulted in de novo methylation. Also, Rhee et al., (172) reported that 80% normal methylation patterns are retained in somatic cells that lack DNMT1 but contain normal expression levels of DNMT3A and DNMT3B. It is possible that other methyltransferases can compensate for the loss of DNMT1 function. It is also possible that all three DNMTs have both de novo and methyltransferase activities, but the different methlytransferases are involved in methylating DNA in certain genomic regions via their interactions with other DNA binding proteins (174).

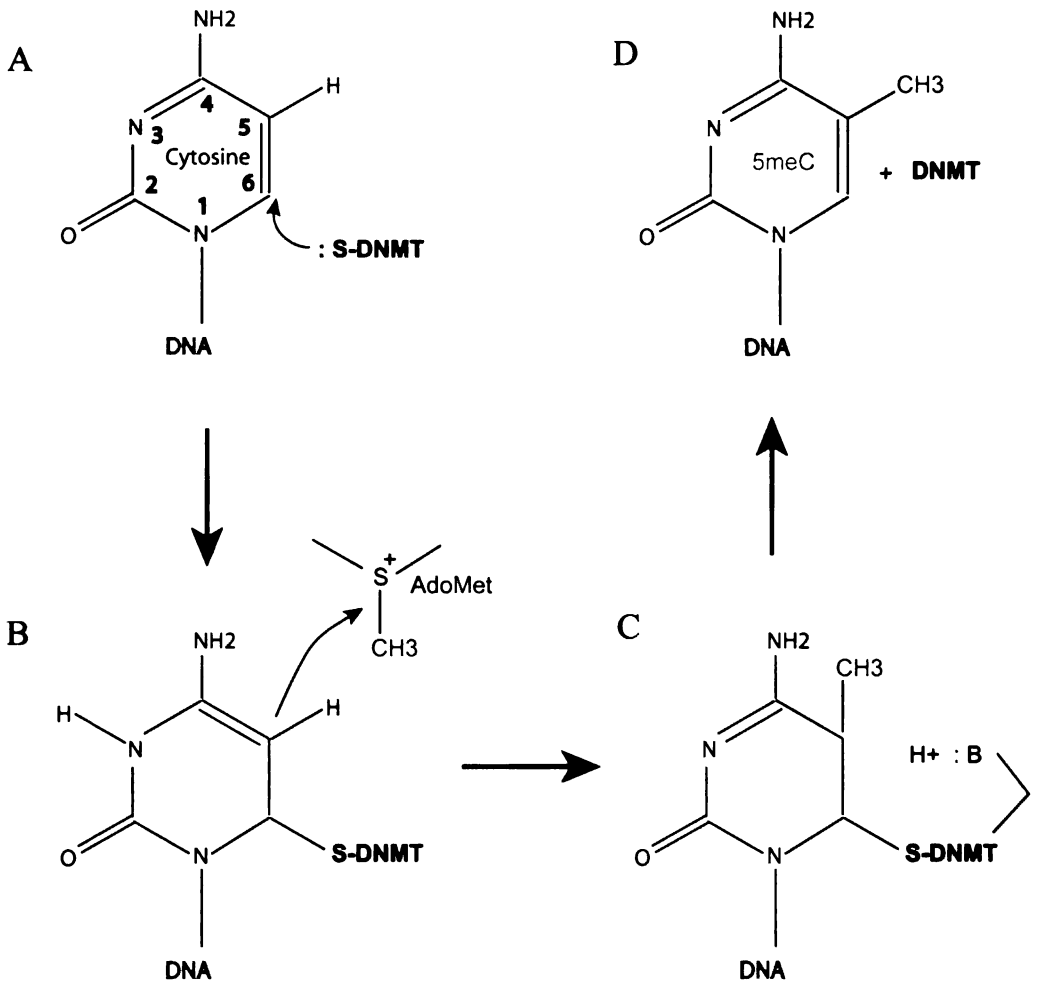
8.1.1. DNA methylation mechanism

The following reaction mechanism for cytosine methylation by DNA methyltransferases has been proposed (Figure 1-6) (11, 26, 183). A cysteine thiolate group in the enzyme active site adds covalently to the C6 position of the target cytosine residue, pushing electrons to the C5 position to make the carbanion, which attacks the methyl group of S-adenosyl methionine (AdoMet, SAM). After methyl transfer, abstraction of a proton from the C5 position allows reformation of the C5-C6 double bond and release of the enzyme. DNMTs have a conserved prolycysteinyl active site dipeptide that provides the cysteine thiolate. Motifs I and X form the SAM binding site. Motif IV contains the active site prolyl-cysteinyl active site. Motif VI contains an important glutamyl residue for proton abstraction process in the enzyme catalysis mechanism. There is a motif VIII downstream of motif VI (not indicated in Figure 1-5).The target recognition domain is usually located between motifs VIII and IX and is involved in making base-specific contacts in the major groove of DNA. Motif IX is involved in maintaining the structure of the target recognition domain (11).

8.1.2. DNA methylation and cancer

Aberrations in methylation patterns are frequently observed in cancers (48, 58, 75, 196). Global hypomethylation along with region-specific hypermethylation is often seen in tumor cells (7, 93). Considering that maintenance of normal DNA methylation patterns is important for tumor suppression (detailed in the following paragraphs in this section), and that RB can

Figure 1-6: Proposed mechanism for DNA methylation. (A). Cysteine thiolate of DNMT attacks carbon 6 of the cytosine residue, pushing electrons to the C5 position. (B). Enamine attack on the methyl group of Adomet (SAM) occurs. (C). Methyl group transfer to carbon 5 and abstraction of a proton from carbon 5 follows (26, 183). (D). Reformation of the C5-C6 double bond followed by release of the methyltransferase enzyme occurs. Diagram adapted from Ref (11).


A
NH2
H
4
5
N
3
Cytosine
2
6
1
O
N
: S-DNMT
DNA
D
NH2
CH3
N
5meC
+ DNMT
O
N
DNA
B
S+
AdoMet
CH3
NH2
H
H
N
O
N
S-DNMT
DNA
C
NH2
CH3
N
H+ : B
O
N
S-DNMT
DNA

direct DNA methylation to target gene during repression (discussed in Chapter Two), RB functional association with DNMT activity could be an important aspect of the tumor suppression mechanism of RB.

A majority of the hypomethylation events associated with cancer are seen in repetitive and parasitic elements which are normally heavily methylated, possibly leading to an increase in transcription from these elements causing genomic instability and malignant growth. One idea is that the ancestral function of DNA methylation is to prevent the spread of these parasitic elements to protect the genome against unrestricted transpositions (13, 243). This genome defense system could later have evolved as a gene regulatory system (174). CpG methylation plays a role in X-chromosome inactivation in females and in genomic imprinting. Silencing associated with promoter hypermethylation is seen in the case of several genes such as those involved in tumor suppression for e.g., RB, DNA mismatch repair, cell adhesion and DNA damage protection mechanisms (174).

Promoter hypermethylation at the RB gene (196) is observed in familial cases of retinoblastoma and similarly, the VHL (von Hippel Lindau) gene promoter is hypermethylated in renal cancer (74). In sporadic cases of colorectal carcinomas exhibiting microsatellite instability, elevated levels of promoter hypermethylation of the mismatch repair gene hMLH1 was observed (75). Aberrant hypermethylation can occur early in tumorigenesis, leading to misregulated gene regulation predisposing cells to malignant transformation. Aberrant methylation patterns were observed in pre-neoplastic lesions and the

frequency of aberrant DNA methylation events increased with disease progression. (9, 150, 234). This supports the idea that DNA hypermethylation can directly contribute to tumorigenesis.

Tumors exhibit misregulated DNA methylation with both hypomethylation as well as region-specific hypermethylation occurring in different genomic regions in the same cell (174). The global loss of DNA methylation also plays an important role in the cellular transformation process. Primary tumor samples from humans and rodents show demethylation and re-expression of transposable elements (52, 60). DNA methylation can contribute to genome stability by inhibiting homologous recombination between repeats (37). Deleterious consequences of recombination between repeats in humans have been reported (165, 180, 195). In the fungal species *Ascobolus immerses*, induced methylation of a recombination hotspot reduced the frequency of crossing-over in this region by several hundred fold (134). In mammalian cells, V(D)J recombination is reduced more than 100-fold when the recombining genomic region is methylated (84). Repetitive elements are found to be demethylated in tumors and the degree of hypomethylation correlated with disease progression (168). Some potential mechanisms by which DNA methylation might downregulate homologous recombination include masking the recombination initiation site, maintenance of a highly condensed chromatin structure, destabilization of the recombination intermediate or interfering with the assembly of the recombination machinery (174).

8.1.3. DNA methylation and transcriptional repression

DNA methylation has been associated with transcriptional silencing (12, 35, 93, 100, 164, 249). Promoters enriched for cytosine methylation are usually transcriptionally silent and condensed into nuclease-resistant chromatin structures that contain hypoacetylated histones (45, 93). In some promoters, CpG methylation can interfere with transcription factor binding, resulting in transcriptional repression (207). An alternative model for transcriptional repression can involve methyl CpG binding proteins (MeCPs) (detailed in section 8.1.6). MeCP2 contains a methyl-CpG binding domain (MBD) that recognizes and binds to symmetrically methylated CpG dinucleotides, and a transcriptional repression domain (TRD) (122, 142, 218). Other methyl CpG binding proteins, MBDs 1-4, have also been identified (174). Evidence for MeCP2-mediated recruitment of HDAC machinery to repress transcription has been reported (94, 144). Methyl CpG binding proteins recognize and bind methylated DNA, directing recruitment of HDAC activity which can result in tighter chromatin packaging and possible prevention of access to transcription factors and/or polymerase escape (94, 144, 177). In *Xenopus*, the Mi2 chromatin remodeling complex contains MBD3 and Rpd3 (HDAC) (216). Evidence suggesting that MBD2, HDAC1 and HDAC2 are components of the MeCP1 repressor complex in HeLa cells has been reported (148). The combined effect of DNA methylation and HDAC function has been demonstrated by robust activation of target gene expression in the presence of inhibitors of both DNA methylation and HDAC activity, whereas using either one of these inhibitors alone had little to no effect

on gene activation (22). Also MeCP2 directed recruitment of histone methyltransferase activity catalyzing H3K9 methylation during transcriptional repression by MeCP2 has been demonstrated (55). Another mechanism proposed for the mechanism of transcriptional repression by DNA methylation is that the steric obstruction caused by binding of methyl CpG binding proteins prevents transcription factor recruitment (174).

8.1.4. DNA demethylation

DNA methylation is involved in transcriptional silencing by establishing a chromatin state that does not support transcription. DNA methylation is thought to play an important role in X chromosome inactivation, genomic imprinting and tissue-specific gene-silencing, where the methyl marks are made permanently (16, 136). DNA methylation is also involved in transcriptional repression of genomic regions that need to be permanently silenced, such as the parasitic elements and transposons, to prevent their amplification in the genome (13, 174, 243). However, DNA methylation is also involved in silencing genes that need to be switched between ON and OFF states. This necessitates a reversal mechanism for DNA methylation. DNA methylation is subject to reprogramming especially during development (16, 171, 193). Cyclical DNA methylation and demethylation events have been observed in a transcriptionally active promoter (138). Genome-wide demethylation and remethylation are seen during gametogenesis and post-fertilization. Also, localized DNA demethylation occurs at specific genes during differentiation (16, 17, 29, 110). DNA demethylation is important for epigenetic

reprogramming in Xenopus oocytes (193). Although there is abundant information about DNA methylation, there is much less known about DNA demethylation.

DNA demethylation can be either a passive or an active process, or a combination of both (99). Passive demethylation involves a lack of DNMT activity through cycles of replication, whereas active demethylation involves enzymatic functions that demethylate DNA (16, 110). There are at least three proposed mechanisms for active DNA demethylation (99). The first mechanism involves the direct replacement of the methyl group with a hydrogen atom. The human MBD2 protein was thought to demethylate DNA by this mechanism (15, 169). However, this reaction is thermodynamically unfavorable as it involves the breakage of a carbon-carbon covalent bond. This result has not been reproduced by others (16, 110, 148, 205). Moreover studies done by Kress et al., (109) have demonstrated that active cytosine demethylation involved DNA strand breaks. This observation does not agree with the proposed mechanism that involves the direct replacement of the methyl group with a hydrogen atom in the C5 position of the methylated cytosine, because this mechanism does not involve the formation of DNA strand breakage.

The remaining two proposed mechanisms involve DNA strand breaks and repair processes as part of the demethylation process (95, 104). The second mechanism involves DNA glycosylases, which cleave the methyl cytosine base from the deoxyribose moiety (base-excision model) (95, 96). For example, a G/T mismatch repair DNA glycosylase initiates DNA demethylation by breaking the

glycosidic bond of 5-methyl cytosine, leaving an abasic site that is processed by an AP endonuclease and other DNA repair enzymes (254). Human MBD4 has 5-methylcytosine DNA-glycosylase activity (5-MCDG) that leads to DNA demethylation in vitro. Overexpression of a human 5-MCDG in human embryonic kidney cells led to promoter demethylation of a hormone-regulated reporter gene (252-254).

The third mechanism proposed for DNA demethylation involves the removal of the methylated nucleotide by nucleotide excision and then replacement with unmethylated cytosine by DNA repair mechanisms (223, 224). Recent studies by Barreto et al., (5) demonstrated that Gadd 45 alpha (growth arrest and DNA-damage inducible protein alpha), which is involved in nucleotide excision repair, leads to gene activation by active demethylation of target promoter. Evidence for functional co-operation between Gadd 45 alpha and XPG, which is a DNA repair endonuclease, in DNA demethylation was also presented in this study (5).

8.1.5. Non-CpG methylation in mammalian DNA

As will be discussed in Chapter 2, evidence for RB-directed methylation of cytosine residues in a non CpG but in a CNG sequence (at the U6 start site) (i.e., the outside cytosine in the CCGG sequence) was observed. Also, data suggesting that cytosine methylation in a non CpG sequence context can also contribute to transcriptional repression is presented in Chapter 2. This observation revealed a novel link for RB-directed methylation of cytosines in non CpG

sequences to transcriptional repression. This section will focus on elaborating non CpG methylation in mammalian cells.

DNA methylation of cytosines at non CpG sequences were first identified by Salomon and Kaye in 1970 (182). Analysis of DNA isolated from newborn mice and mouse embryo cultures showed that 5meCpG dinucleotides were not the only methylated species, because 5meCpT, 5meCpC and 5meCp5meC species were also found (182). Studies by Jones and colleagues (151) have shown that upon induction of hypermethylation by treatment with DNA synthesis inhibitors, apart from CpG methylation, methylation of cytosines in CpA, CpT and CpC sequences was also observed in hamster fibrosarcoma cells. Woodcock et al., (236) found that human spleen DNA contains 5meCpA, 5meCpT and 5meCpC dinucleotides.

Methylation of cytosines in CpNpG sequences have been reported by Clark et al., (34). In plant genomes, the existence of methylated cytosines in CpG dinucleotides and CpNpG sequences is well known (61). But in mammalian DNA, much focus was given to cytosine methylation in CpG dinucleotides only. Only few studies have emphasized the presence of cytosine methylation in CpNpG sequences and also in other asymmetric sequences (156). Evidence for methylation of cytosines in CpApG, CpCpG, CpTpG and CpGpG sequences is available (34). Furthermore, bisulfite sequencing analysis of stably transfected pre-methylated DNA revealed efficient maintenance of CpNpG methylation, and also de-novo methylation of previously unmethylated CpNpG sites have been found (34).

Studies by Pradhan et al., (162) found that the human DNMT1 enzyme can methylate CpCpG, CpTpG and CpApG sequences in vitro, in addition to CpG, as seen from DNMT1 crosslinking to these duplex oligonucleotides in the presence of SAM. The efficiency of complex formation was in the order CpG > CpCpG > CpApG = CpTpG. Ramsahoye et al., (170) reported that DNMT3A enzyme can methylate cytosines in CpA and CpT dinucleotides. These studies indicate that it is possible that cytosine methylation can occur outside the context of the cognate CpG sequence. My results indicate a novel link to methylation of cytosines in a CNG sequence context to transcriptional repression (in Chapter 2).

8.1.6. Methyl CpG binding proteins

Methyl CpG binding proteins are involved in transcriptional repression, by binding to methylated CpG possibly by targeting the recruitment of co-repressor proteins such as sin 3 and HDACs (94, 144) and components of the SWI/SNF chromatin remodeling machinery (67). The first Methyl CpG binding proteins to be identified were MeCP1 and MeCP2. MeCP1 was identified as a nuclear factor that can discriminate between methylated and unmethylated DNA (137). MeCP2 was another factor that bound specifically to methylated CpG (143). MeCP2 is a chromatin-associated nuclear protein that binds to chromosomes that contain methylated DNA (142). A short region in the N-terminus of MeCP2 (of about 70 amino acid residues) contains the methyl CpG binding domain (MBD) (143).

A sequence homology search using the MBD of MeCP2 led to the identification of MBD1 (38). MBD1 binds methylated DNA and can repress transcription in vitro. Later database searches led to the discovery of MBD2, MBD3 and MBD4 (69). The sequence similarity between MeCP2 and MBDs 1-4 is largely limited to their MBD region (4). MBD1 is a 70 kDa protein that has cysteine-rich regions (CXXC motifs) related to those in DNMT1 (38). MBD2 is thought to have DNA demethylase function, but the findings remain controversial (15, 148, 216). MBD3 shares high homology (80% similar, 72% identical) to MBD2b which is the shorter splice variant form of MBD2 (216, 250). MBD4 has sequence homology to bacterial DNA repair enzymes at the C-terminus including *E. coli* Mut Y (an 8-oxoGA mispair-specific adenine-glycosylase), Mig from *Methanobacterium thermoautotrophicum* (a GT-mismatch specific glycosylase), Endo III from *E. coli* (a thymine glycol glycosylase) and UV-endonuclease of *Micrococcus luteus* (4). Splice variant forms of MBDs 1-4 have also been reported (70).

Studies done by Hendrich and Bird (69) have found that MBD1, MBD2 and MBD4 can bind to methylated DNA in vitro. MBD3 did not bind methylated DNA. GFP-tagging and cellular localization studies have identified that MBDs 1, 2 and 4 preferentially localized to genomic regions that were rich in methylated DNA such as major satellite DNA. On the other hand, MBD3 did not localize to methylated regions despite its high homology to MBD2b (69). Studies to understand the sequence specificities of these methyl-CpG binding proteins support the proposal that differences in CpG density of various genomic regions

and differences in dissociation constants of these methyl CpG binding proteins with methylated DNA serve to distinguish binding of these various methyl binding proteins to specific regions of the genome (4). In mouse, MeCP2 localizes to pericentromeric chromatin whereas MBD1 is localized to the hypermethylated region of chromosome 1q12 (54). MeCP2 does not require prior disruption of nucleosomal chromatin to bind to the genome. MeCP2 can bind nucleosomal DNA (24, 145). These findings lead to a model wherein MeCP2 and possibly the other methyl CpG binding MBDs can access chromatin first to target co-repressor complexes to cause chromatin modifications, leading to repression (4).

Methyl CpG binding proteins have been associated with transcriptional repression in several studies (94, 142, 147, 148, 216, 250). MeCP2 represses transcription in vitro and in vivo from methylated promoters but not from non-methylated promoters (98, 142). The transcription repression domain (TRD) of MeCP2 was found to associate with co-repressor complexes containing Sin3 and HDAC activity (94, 144). MeCP2-mediated silencing was relieved by TSA, suggesting a relationship between DNA methylation and HDAC function in transcriptional repression (94, 144). Similar studies have found that MBD1, MBD2 and MBD3 also associate with histone deacetylases (147, 148, 216, 250). MBD1 can affect transcription from both methylated and unmethylated promoters. One of the three CXXC motifs can bind DNA regardless of the methylation status. MBD1 represses transcription though the co-operation between its MBD, CXXC motifs and TRD (54). MBD2 is part of an MeCP2 repressor complex that contains HDAC1 and 2 and RbAp48/46 the latter of which are histone binding proteins (4,

38, 142, 148). MBD2b was also shown to repress transcription in a deacetylase-dependent manner (142). MBD3 has been identified as part of the Mi2/NuRD complex which has both histone deacetylase function and nucleosome remodeling activity indicating a potential link between DNA methylation, histone deacetylation and nucleosome remodeling activities (216, 217, 250).

The presence of different cellular MBDs in the cell suggests the binding of different MBDs to different genomic regions governed by CpG density and the binding affinities for these Methyl CpG containing proteins to methylated DNA. Despite a conserved MBD, significant differences in the functions, cellular localization and binding specificities to cofactors, and to DNA have been observed. It is possible that the regions outside the MBD also regulate important functional aspects of these proteins. MeCP2 is found to associate with DNA more stably than the MBD2/MeCP1 complex. MeCP2 binds single methylated CpG but MeCP1 can bind only to heavily methylated DNA. MBD1 can affect transcription form both methylated and unmethylated promoters. The molecular mechanism underlying these unique aspects of the seemingly similar Methyl-CpG binding proteins remains to be understood (4).

8.2. Histone deacetylases and RB-mediated transcriptional repression

Considering the presence of a positioned nucleosome which contributes to U6 transcriptional activation (200, 251), the activity of chromatin modifiers such as HDACs and the SWI/SNF chromatin remodeling machinery which can modulate the location of this nucleosome can play a role in U6 transcriptional

regulation. Also the presence of HDACs 1 and 2 and the BRG1 component of the SWI/SNF complex on the endogenous U6 promoter in RB positive cells suggests a role for these factors in U6 transcriptional regulation (discussed in the Appendix). Additional links between RB function and the activity of HDACs (discussed in this section) and the SWI/SNF complex (discussed in section 8.3) reported in studies by other groups suggests an important role for HDACs and the SWI/SNF complex in RB repression of U6 transcription. Also the presence of HDACs 1 and 2 and the BRG1 component of the SWI/SNF complex on the endogenous U6 promoter in RB positive cells suggests a role for these factors in U6 transcriptional regulation.

Histone deacetylases comprise seven families of which HDACs 1-3 are known to interact with RB (20, 28, 65, 130, 132). In a recent study to analyze the acetylation status of histones H3 and H4 at several cell-cycle regulated genes containing E2F sites, hypoacetylation of H3 and H4 was observed in quiescent cells but hyperacetylation of H3 and H4 was observed in late G1 and into S phase, indicating the importance of histone acetylation status of E2F target genes in cell cycle regulation (204). RB-directed recruitment of HDACs to target genes led to histone deacetylation and correlated with transcriptional repression (130). Inhibition of HDAC activity with Trichostatin A (TSA) relieved RB repression of certain genes. HDAC activity was found be important for RB repression of cyclin E and DHFR genes (required for G1 to S phase progression) during early to mid G1 phase, suggesting that HDAC activity is crucial for RB to induce G1 arrest (247). HDACs 1 and 2 contain the LXCXE motif that is found in many RB-

binding proteins including viral oncoproteins such as E1A and E7 (115). However, HDAC 3, which also binds to RB, does not contain this motif (64). E2Fs do not contain an LXCXE motif, and interact with RB via a distinct site than that which interacts with HDACs. This can allow the formation of an RB-E2F-HDAC complex that gets recruited to E2F site containing promoters (20, 28, 39, 130, 132). In some studies, LXCXE binding mutants of RB (defective for E1A and E7 binding) were unable to bind HDAC1 and HDAC2, and were defective in active transcriptional repression and in induction of growth arrest of long term assays (39). However, upon transient overexpression of the RB mutants, there was an observed increase in percentage of cells that were in G1 (39). There is also evidence suggesting that similar RB mutants that were defective for E7 binding could still bind HDAC1 to mediate transcriptional silencing and induce G1 arrest (42). In another study, an RB mutant for HDAC1 binding was as effective as wild type RB in arresting growth of RB negative cells, however the mutant RB was defective in the ability to cause irreversible growth arrest in differentiated myocytes (28). Nevertheless, in each of these studies, the ability of RB to interact with HDACs correlated with its ability to induce growth arrest or maintain differentiation, highlighting the importance of RB-HDAC interaction in RB function (246).

8.3. SWI/SNF and RB-mediated repression

ATP-dependent chromatin remodeling complexes constitute an important component of the chromatin modulatory machinery in the cells (106). The yeast SWI/SNF chromatin remodeling complex was discovered first among a group of many chromatin remodeling complexes which contain an ATPase catalytic subunit. The human homologs of SWI2/SNF2 are the BRG1 and hBRM proteins which along with BAFs (BRG1 associating factors) constitute the human SWI/SNF (hSWI/SNF) (157). In vitro, SWI/SNF and other chromatin remodeling complexes are able to catalyze the interconversion between 'closed' and 'remodeled' chromatin states in an ATP-dependent manner (128, 185). SWI/SNF-like complexes are known to be involved in both transcriptional activation and repression, as observed in a genetic study where disruption of the SWI/SNF complex led to transcriptional activation of one set of genes and to repression of another set of genes (82).

RB can interact with both BRG1 and hBRM (43, 199). There is evidence linking HDAC function and BRG1-containing nucleosome remodeling complexes in co-operative involvement with RB-mediated transcriptional repression (43). Overexpression of BRG1 in cells deficient in BRG1 and hBRM led to growth arrest, and this was dependent on the ability to interact with RB (43). Overexpression of a dominant negative form of BRG1 or hBRM that is mutant for the ATPase catalytic domain inhibited RB-mediated growth arrest (43, 198) suggesting that the chromatin remodeling function of SWI/SNF (which is ATPase dependent) can play an important role in RB-mediated repression. In C33A cells

that are deficient for BRG1, hBRM and RB, expression of BRG1 was essential for ectopically expressed RB to arrest cell growth (197, 247). While RB was sufficient for repression of transcription from a transiently transfected reporter, repression of a stably integrated identical reporter required both RB and BRG1 activities, suggesting a requirement for chromatin remodeling by BRG1 for RB repression (247). Although BRG1 has an LXCXE motif, it does not seem to require it for binding to RB, thereby possibly allowing the association of RB, HDAC and SWI/SNF in a single complex which can lead to transcriptional repression (247).

8.4. Other co-repressor proteins

8.4.1. Histone Methyltransferases

RB transcriptional repression also involves the histone methyltransferase SUV39H1, which is responsible for histone H3K9 methylation. Studies done by Nielsen et al., (149) have found that RB targets H3 methylation and HP1 (heterochromatic protein that binds methyl lysine) to the cyclin E gene during repression.

Interesting links between histone methylation and DNA methylation have also been reported (108, 205). Studies done in *Neurospora* have indicated that chromatin modifications facilitated by histone methylation were necessary for DNA methylation to occur, although the mechanism by which this occurs is unclear. Also, the observed co-localization between DNMT3 and HP1 suggests a functional link between histone methylation and DNA methylation (2, 108, 205).

8.4.2. Topoisomerases

Topoisomerases (Topo) are ATP-dependent enzymes that remove positive supercoils in DNA by causing DNA strand breakage and re-ligation (14). Topoisomerases are important for several cellular functions such as transcription, transcription, chromatin segregation, cell cycle progression and DNA repair (21). Topo I makes a single-stranded break whereas Topo II causes double-stranded breaks (21). In mammalian cells, Topo II is present as two isoforms Topo IIα and Topo IIβ encoded by different genes (206). Studies by Bhat et al., (14) have found a functional interaction between RB and Topo IIα, including physical association between RB pocket domain and Topo IIα and inhibition of TopoIIα by RB, both in vitro and in vivo (14) although the mechanism for inhibition of Topo IIα activity by RB remains uncharacterized. My studies have shown the presence of Topo IIα and Topo IIβ on the endogenous U6 promoter in RB positive cells (discussed in Appendix). These findings suggest a possible RB repression mechanism involving also the inhibition of topoisomerase activity.

8.4.3. Polycomb (PcG) group proteins

PcG proteins are classically known for their repressive effect on Hox genes. This repression coupled with transcriptional activation by trithorax proteins is important for establishing the pattern of Hox expression required for proper embryonic development (40, 59, 104, 121, 161, 188). Apart from Hox gene expression, PcG proteins are also involved in cell cycle regulation (90, 120, 215).

Mice that lack Bmi-1 show severe defects in lymphoid proliferation (213). Bmi-1 represses expression of p16 which is a cdk4/6 inhibitor. The absence of Bmi-1 leads to accumulation of p16 which blocks Cyclin D/cdk4 activity and consequent accumulation of hypophosphorylated RB and growth arrest (90, 91). Another member of the PcG proteins M33/CBX2/HPC1 is required for normal cellular proliferation. In contrast to Bmi-1 and M33, other PcG proteins such as EED and HPC2 act as negative regulators of the cell cycle (184).

Studies done by Dahiya et al., (40) have found that the polycomb group protein HPC1 in association with Ring1 protein acts as an HDAC-independent co-repressor for RB, causing repression of cdc2 and cyclin A genes leading to G2 arrest. This suggests co-operativity between PcG proteins and RB in transcriptional repression (40).

8.5. Summary

Deducing from studies discussed in this chapter, a model for RB repression of Pol III transcription can be proposed. Considering the observation that the presence of RB on the U6 promoter is important for transcriptional repression, it is possible that RB recruitment to the U6 promoter directs recruitment of other co-repressor proteins that can lead to inhibition of transcription. The observed enrichment of CpG dinucleotides in the functional U6 copies compared to the non-functional U6 copies, indicates possible involvement of DNMTs in RB repression of Pol III transcription. Evidence for RB-directed DNMT recruitment and DNA methylation, along with evidence for methylation leading to inhibition of transcription are presented in chapter 2. It is possible that

RB-directed DNMT recruitment and DNA methylation at the U6 promoter can lead to recruitment of HDACs and the SWI/SNF complex which can function to alter the location of the positioned nucleosome leading to inactivation of transcription. The presence of HDACs 1 and 2 and BRG1 on the U6 promoter in RB positive cells are supportive of this model.

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CHAPTER TWO

THE RETINOBLASTOMA TUMOR SUPPRESSOR PROTEIN REPRESSES U6 snRNA TRANSCRIPTION BY A MECHANISM INVOLVING PROMOTER DNA METHYLATION

ABSTRACT

The Retinoblastoma tumor suppressor (RB) protein functions as a cellular checkpoint at the G1 to S phase transition, thereby guarding against unregulated cell growth and division characteristic of tumors. RB represses transcription of gene products that facilitate the biosynthesis of cellular products whose levels determine the rate of cellular growth and concomitant cell division. RB function as a transcriptional repressor can be an important aspect of its tumor suppressor function. RB represses global Pol I and Pol III transcription as well as specific Pol II transcribed genes that are E2F regulated. The study presented herein analyzes the mechanism for RB repression of transcription from a type 3 RNA Polymerase III promoter, using the human U6 snRNA gene as a model system. My results demonstrate a role for DNA methyltransferase function in repression of U6 transcription by RB. I present in vitro and in vivo evidence that RB directs increased methylation of a conserved start site CpG at the U6 promoter. Moreover, RB directed recruitment of the DNA methyltransferases DNMT1 and DNMT3A to the U6 promoter. Depletion of DNMT1, 3A and 3B by si-RNA led to enhanced U6 transcription, demonstrating a repressive role for DNA methyltransferases in

U6 transcription. Consistently, transcription from a methylated U6 reporter was diminished compared to unmethylated template. This study provides previously unreported evidence for RB directed DNA methylation at a target gene during transcriptional repression. Considering that the role of RB as a transcriptional repressor is linked to its role as a tumor suppressor, the results presented in this study can be useful towards our understanding about RB function in tumor suppression.

Introduction

RB functions as a cellular safeguard against unregulated cellular growth by regulating the G1 to S phase transition phase of the cell cycle. RB is a transcriptional repressor of genes whose products are important for progression into S phase (14, 40). Some of the Pol II-transcribed genes that are targets of E2F-mediated transactivation, such as c-myc, B-myb, cdc2, dihydrofolate reductase and thymidine kinase, are repressed by RB (5, 26). RB also acts as a general repressor of Pol III and Pol I (4, 44). Products of Pol III and Pol I transcription include ribosomal RNAs and tRNAs, the structural components of the protein synthesis machinery, and uridine rich small nuclear RNAs that are structural components of the splicing machinery. Unregulated cellular growth observed in tumors requires elevated levels of these Pol III and Pol I products, necessitating the cell to have a mechanism to repress Pol III and Pol I transcription to suppress tumor development. This raises the possibility that RB might exert its tumor suppressive effects by acting as a repressor of Pol III and Pol I transcription (43).

Several lines of evidence suggest a potential link between the tumor suppressive capacity and the Pol III and Pol I repressor functions of RB. Naturally occurring RB mutations in tumors that rendered RB inactive as a tumor suppressor also debilitate its ability to repress Pol III and Pol I transcription (19, 40, 45), whereas the adenoviral E1A oncoprotein that binds and inactivates RB also interferes with its ability to repress Pol III transcription (44). Moreover, the functional domains within RB required for transcriptional repression and for tumor suppression largely coincide (4, 44). The minimal region of the RB protein required for tumor suppression comprises amino acids 378-928, which includes the A and B pocket domains and the C-terminal end of the protein (46). Studies done by Hirsch et al., have revealed that this region was also the minimal region for efficient repression of Pol III transcription (17).

RB is known to be a master regulator of the cell cycle and a key tumor suppressor that represses diverse types of genes that require distinct transcription machinery. Consequently, RB uses distinct mechanisms at different types of target genes in order to cause gene-silencing. RB repression of Pol II transcribed E2F target genes, by binding to and inactivating E2F proteins thereby inhibiting the E2F transactivation function, is a mechanism for regulating G1 to S phase transition and cell cycle progression by RB (1, 6, 9, 19, 41, 42). Studies done by Cavanaugh et al., (4) have found that RB represses Pol I transcription by binding to and inactivating the UBF transcription factor that is involved in rDNA transcriptional activation. Studies directed towards understanding RB repression

of Pol III transcription have indicated that RB interferes with critical transcription factors. Studies carried out on Alu and AdVAI transcription repression mechanisms have proposed that RB represses Alu and AdVAI genes which are type 2 Pol III promoters via interactions with TFIIIB and TFIIIC (7).

It is possible that at type I and type II Pol III promoters, RB exerts its repressive effect by binding to and sequestering the transcription factors TFIIIB and TFIIIC but not by getting directly recruited to the promoter. In support, RB does not occupy the endogenous 5SrRNA (type I) or tRNA genes (type 2) genes although it represses these genes (17). In contrast, RB occupied the U6 promoter (17). Truncations in the RB protein that debilitated RB association with the U6 promoter also inactivated RB repression (17) indicating that RB association with the promoter DNA is important for transcriptional repression from the U6 promoter. These results suggest that RB might use distinct mechanisms at different types of Pol III promoters to repress transcription.

With respect to type 3 Pol III promoter system exemplified by the U6 snRNA gene, White et al., (38) proposed that RB might cause repression of transcription by interfering with interaction between TFIIIB and Pol III, resulting in an inability to recruit Pol III to the promoter. On the contrary, studies done by Hirsch et al., (17) have found that RB and Pol III co-occupy the U6 promoter, suggesting that RB does not exclude the polymerase from recruitment to the promoter. This raises the possibility that RB might be using a different

mechanism to repress U6 transcription that does not exclude Pol III from getting recruited to the promoter. Alternatively, RB could direct recruitment of co-repressor protein(s) to the U6 promoter to lead to transcriptional repression.

In this study, I present evidence that DNA methylation contributes to RB repression of the U6 snRNA gene. Promoter CpG methylation and the function of DNA methylation have previously been associated with gene silencing (18, 31). RB interacts with the DNA methyltransferase DNMT1, and the co-operative role of DNMT1 is thought to be important for RB repression of specific E2F-regulated RB target genes that are transcribed by Pol II (30). Methylation of rDNA repeat sequences leading to inhibition of UBF transcription factor binding to the rRNA core promoter DNA has been proposed as a mechanism for repression of rDNA transcription by Pol I (34). The study presented here shows that DNA methylation plays a role in silencing a Pol III promoter. Specifically, my results indicate that RB directly employs the cellular DNA methylation machinery to cause promoter-proximal methylation at the U6 gene to cause gene-silencing. This study illuminates a previously undescribed role for DNA methylation as a repressive mechanism targeting Pol III-dependent transcription and lends further support to previous findings that have hinted at a close cooperative function for DNMTs with RB function in Pol II and Pol I transcriptional repression (30, 34).

Materials and Methods

Images in this thesis are presented in color.

Chromatin Immunoprecipitation (ChIP): Chromatin immunoprecipitation reactions were done as described previously (17). Chromatin was collected from HeLa, MCF7 and 184B5 cells that were grown to approximately 75% confluency. After harvesting by trypsinization, cells were fixed with 1% formaldehyde for 30 mins followed by washing with PBS, buffer I (10 mM HEPES pH 6.5, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100) and buffer II (10 mM HEPES pH 6.5, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl). Cells were then suspended in 1 ml lysis buffer per 10^8 cells (Lysis Buffer comprises 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, 0.5 μM phenylmethylsulfonyl fluoride (PMSF), 1 μM pepstatin A, 1 mM sodium bisulfite, 1 mM benzamidine, 1 mM DTT) followed by sonication to obtain soluble chromatin. Immunoprecipitation reactions were set up in dilution buffer (20 mM Tris pH 8.0, 2 mM EDTA, 1% Triton X-100, 0.5 μM PMSF, 1 mM DTT) with chromatin equivalent of 10^7 cells using 1μg antibody in a total volume of 1 ml. Immunocomplexes were recovered using Protein-G agarose beads. The beads were washed once with TSE (20 mM Tris pH 8.0, 0.1% SDS, 2 mM EDTA, 1% Triton X-100), TSE with 250 mM NaCl, TSE with 500 mM NaCl, Buffer III (10 mM Tris pH 8.0, 1 mM EDTA, 0.25 M LiCl, 1% NP-40, 1% deoxycholate) and TE (20 mM Tris pH 8.0, 2 mM EDTA). Complexes were eluted from beads with elution buffer (0.1 M NaHCO3 + 1% SDS) and then reverse crosslinked by incubating at 65° C overnight. DNA was then recovered

after phenol-chloroform extraction followed by ethanol precipitation. Primers for PCR amplification of the U6, U1, U2 and GAPDH loci were described previously (17). For amplification of 5S rRNA loci the primers used were 5' GGCCATACCACCCTGAACGC 3' and 5' CAGCACCCGGTATTCCCAGG 3'. The 7SK promoter region was amplified using the following primers.

5' TTTTGGGAATAAATGATATTTG 3' and

5' GAGGTACCCAGGCGGCGCACAAG 3'

PCR products were then separated on a 2% 0.5X TBE agarose gel and images recorded with Kodak imaging software.

In vitro transcription: In vitro transcription reactions were performed as described previously (17). Transcript levels were analyzed using the body-labelled riboprobe protection method (17). 250 ng of pU6/Hae/RA.2 plasmid DNA was incubated with HeLa nuclear extract with appropriate recombinant proteins. Transcription was done at 30 °C for 30 minutes and was stopped by adding stop mix (0.3 M sodium acetate pH 7.0, 0.5% SDS, 2.5 mM EDTA). Proteinase K digestion (20 μg/ml) was then done at 37° C for 1 hr. Nucleic acids were recovered by phenol extraction and ethanol precipitation. The nucleic acid pellet was then resuspended in 30μl FAHB (80% formamide, 400 mM NaCl, 40 mM PIPES pH 6.4 and 1 mM EDTA pH 8.0) containing 300,000 cpm riboprobe (body-labelled with α-P^{32} CTP) complementary to the transcripts whose synthesis was driven from the U6 promoter. Hybridization was done overnight at 61° C, followed by digestion with T1 RNAase (at 10U/ml) for 30 mins at 30° C to select

for U6 promoter-driven transcripts protected from hybridization to the riboprobe. The T1 RNAse was then inactivated by treatment with SDS (0.5% final concentration) and Proteinase K (20 μg/ml). Protected RNA transcripts were recovered by phenol extraction and ethanol precipitation and the RNA pellet was dissolved in FALB (90% formamide, 0.1 % Bromophenol blue and 0.1 % xylene cyalanol) and were analysed by fractionation on a 6% urea – polyacrylamide denaturing gel followed by exposure to film.

Transient transfections: HeLa cells were plated at $3x10^6$ cells per plate onto 15 cm diameter tissue culture plates in DMEM containing 5% FBS and antibiotics (penicillin and streptomycin). Transfection was done 24 hrs after plating. 10 μl Lipofectamine 2000 (Invitrogen) was mixed with 250 μl DMEM lacking serum and antibiotics and incubated at room temperature for 15 minutes. This solution was then mixed with 250 μl DMEM lacking serum and antibiotics, containing appropriate amount of plasmid DNA (pCMV-RB or pCMV-empty vector) and incubated for 15 minutes at room temperature. This DNA mix (total volume 500 μl) containing lipofectamine and plasmid DNA in DMEM was then added to cells in fresh 10 ml DMEM free of serum and antibiotics. Cells were incubated at 37° C, 5% CO_2 for 5 to 7 hours and then 10 ml media containing serum and antibiotics was added. After 24 hours, cells were harvested by scrapping and analyzed by Westen blotting for RB expression.

Methylation analysis (in vivo): Genomic DNA was harvested from HeLa or MCF7 cells that were resuspended in 10 mM Tris–HCl (pH 8.0), 10 mM EDTA at 10^7 cells/ml. Cells were treated with SDS (final concentration 0.5%) and Proteinase K (200 μg/ml) followed by incubation at 55° C for 2 hrs. Sodium chloride was added to a final concentration of 0.2 M and the resultant mixture extracted with phenol twice, followed by one extraction with chloroform. RNAase A (final concentration 25 μg/ml) was added to a final concentration of 25 μg/ml for 1 hr at 37° C. The DNA samples were extracted with phenol : chloroform (1:1) once followed by extraction with chloroform only. DNA was precipitated with 1.5 volumes of ethanol and resuspended in TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA).

100 ng of genomic DNA was incubated with either Taa I (Fermentas), HpyCH4 III, Ava II (New England Biolabs (NEB)) or no restriction enzyme overnight at either 37° C (HpyCH4 III and Ava II) or 65° C (Taa I). Digested DNA was recovered by phenol extraction and ethanol precipitation, followed by PCR analysis. Primer sequences used for amplification of the region spanning the U6 start site that contained 1 Taa I/HpyCH4 III site were 5' AAGTATTTCGATTTCTTGGC 3' and 5' AATATGGAACGCTTCACG 3'. Primers for amplification of the GAPDH region exon 2 that had no Taa I/HpyCH4 III site are 5' AGGTCATCCCTGAGCTGAAC 3' and 5' GCAATGCCAGCCCCAGCGTC 3'.

For methylation analysis after transient RB transfections, genomic DNA was harvested from HeLa cells transiently transfected with RB expression plasmid (as described earlier in this section) and restriction digestion was done as explained above. PCR analysis was carried out with the following primers for the U6, GAPDH region 1 and GAPDH region 2.

U6: 5'AAG TAT TTC GAT TTC TTG GC 3' and

5' AAT ATG GAA CGC TTC ACG 3'

GAPDH region 1: 5' CAT CAA GAA GGT GGT GAA GCA GGC 3' and

5' GCA ATG CCA GCC CCA GCG TC 3'

GAPDH region 2: 5' CAT TGA CCT CAA CTA CAT GG 3' and

5' CCT GGA AGA TGG TGA TGG G 3'

Methylation analysis (in vitro): In vitro transcription was performed as explained previously in this section. DNA was recovered and resuspended in 51 µl water and 6 µl restriction digestion buffer (NEB buffer 1). Each sample was split into three aliquots of 19 µl each and 1 µl of restriction enzyme (Hpa II/ Msp I, purchased from NEB) or water (for no restriction enzyme control) was added and incubated overnight at 37° C. DNA was recovered by phenol extraction and ethanol precipitation and resuspended in 20 µl water. Each sample was diluted 1:250 in water and 3 µl from this was used for PCR analysis. Primer sequences used for PCR analysis of regions R1, R2 and R3 in Figure 2-4D are as follows.

R1: 5' TTT CTT GGG TAG TTT GCA G 3' and

5' GTC CTC TGC TGC CTT CAG TG 3'

R2: 5' AAG CAA CCA TAG TAC GCG CCC 3' and

5' GGT CGA GGT GCC GTA AAG CAC 3'

R3: 5' CCC ATG ATT CCT TCA TAT TTG C 3' and

5' CAA GTT ACG GTA AGC ATA TG 3'

RNAi: MCF7 cells were plated at a density of $2x10^5$ cells per well in 6 well plates. Transient transfection was carried out with serum and antibiotic-free DMEM. siRNA targeting DNMT1, DNMT3A and DNMT3B (at a final concentration of 200 nM) or equivalent amount of control siRNA and reporter plasmid DNA was mixed with 250 µl DMEM. 2.5 µl Lipofectamine 2000 (Invitrogen) was mixed with 250 µl DMEM and incubated at room temperature for 15 mins. Solutions containing lipofectamine and the siRNA and plasmid reporter were then mixed and incubated at room temperature for 15 mins. This mix was then added to cells containing 500 µl DMEM. Cells were incubated at 37 °C, 5% CO2 for 5 to 7 hours and then 1.5 ml serum and antibiotic containing media was added. After 24 hrs following transfection, RNA was isolated using Trizol reagent.

siRNA information:

DNMT1-siRNA 1: Oligo ID- HSS102859 (Invitrogen)

Oligo 1: 5' AAA GAU GGA CAG CUU CUC AUU UGU C 3'

Oligo 2: 5' GAC AAA UGA GAA GCU GUC CAU CUU U 3'

DNMT1-siRNA 2: Oligo ID- HSS102861 (Invitrogen)

Oligo 1: 5' UUC CUU GAU GGA CUC AUC CGA UUU G 3'

Oligo 2: 5' CAA AUC GGA UGA GUC CAU CAA GGA A 3'

DNMT3A-siRNA 1: Oligo ID- HSS141868 (Invitrogen)

Oligo 1: 5' UAC ACC AGC CGC UCU CUU GUG CGC U 3'

Oligo 2: 5' AGC GCA CAA GAG AGC GGC UGG UGU A 3'

DNMT3A-siRNA 2: Oligo ID- HSS141870 (Invitrogen)

Oligo 1: 5' UUC UUU GGC AUC AAU CAU CAC AGG G 3'

Oligo 2: 5' CCC UGU GAU GAU UGA UGC CAA AGA A 3'

DNMT3B-siRNA 1: Oligo ID- HSS102865 (Invitrogen)

Oligo 1: 5' UAG GAG ACG AGC UUA UUG AAG GUG G 3'

Oligo 2: 5' CCA CCU UCA AUA AGC UCG UCU CCU A 3'

DNMT3B-siRNA 2: Oligo ID- HSS102867 (Invitrogen)

Oligo 1: 5' UUG AGA UGC CUG GUG UCU CCC UUC A 3'

Oligo 2: 5' UGA AGG GAG ACA CCA GGC AUC UCA A 3'

Negative Control siRNA: Cat. No. 12935-300 (Invitrogen)

RNA isolation: Cells were washed in 2 ml PBS following removal of medium. Cells were lyzed in 1 ml Trizol and were resuspended well by repeated pippetting. The contents were transferred to a centrifuge tube followed by addition of 200 µl chloroform. The tubes were vortexed well and spun at 4° C for phase separation.

The upper aqueous phase was transferred to a new tube followed by isopropanol precipitation. The pellets were washed with 75% ethanol and then air dried before suspension in 25 µl TE. RNA quantifications were performed using Nanodrop spectrophotometer (Thermo Scientific) and by agarose gel electrophoresis. 800 ng RNA was hybridized to riboprobe for RNAase protection analysis.

Reverse Transcriptase PCR (RT-PCR): 0.2 µg RNA was mixed with 1 µl RNase free DNase (10U/µl) and the final volume was adjusted to 10 µl with water and incubated at 37° C for 10 minutes to digest DNA. Following this, DNase inactivation was done at 75° C for 10 minutes. First strand DNA synthesis was performed by adding 0.5 µg oligo (dT) 12-18 (from Invitrogen) and 1µl 10mM dNTP mix. Samples were then incubated at 65° C for 5 minutes and then tubes transferred to ice. 4 µl 5X first strand buffer (Invitrogen) and 2 µl 0.1 M DTT (Invitrogen) were added and incubated at 42° C for 2 minutes. Tubes were placed on ice and 1 µl Superscript II (Reverse Transcriptase, 200 U/µl, Invitrogen) was added, the contents mixed thoroughly (total volume now was 20 µl) and then incubated at 42° C for 1 hour after reverse transcription. The tubes were incubated at 70° C for 15 minutes to inactivate the reverse transcriptase. From this cDNA stock, 1 µl was used as template for PCR amplification with 10 pmoles appropriate primers in a 50 µl PCR reaction. The primers used for amplification of DNMT3A cDNA was

5' CGT TGG CAT CCA CTG TGA ATG A 3' and

5' TTA CAC ACA CGC AAA ATA CTC CTT 3'

and those for beta-actin amplification were

5' CCA TCG AGC ACG GCA TCG TCA CCA 3' and

5' CTC GGT GAG GAT CTT CAT GAG GTA GT 3' (27).

Tissue culture: HeLa and MCF7 cells were grown in DMEM containing 5% fetal bovine serum (Gibco) and penicillin-streptomycin. SAOS2 and U2OS cells were grown in DMEM containing 10% fetal bovine serum (Gibco) and penicillin-streptomycin. The SOAS2 derived cell line inducible for RB induction was a gift from Dr. Liang Zhu (Albert Einstein College of Medicine of Yeshiva University). These cells were normally cultured in 15 cm diameter cell culture plates in DMEM containing 10% FBS and penicillin-streptomycin in 1μg/ml tetracycline. RB expression was induced by removing media containing tetracycline, washing the plates twice with 10 ml PBS followed by addition of media that is free of tetracycline.

In vitro methylation: pU6/Hae/RA.2 was methylated by incubating with 4 units M.Sss I methylase (M0226 NEB) per μg plasmid along with methylase buffer (NEB) and SAM (final concentration 160 μM) in a total volume of 20 μl. Tubes were incubated at 37° C overnight. DNA was then recovered by phenol extraction and ethanol precipitation and the DNA pellet was resuspended in water. The final DNA concentration was quantified by spectrophotometry and also by agarose gel

electrophoresis. The indicated amounts of this methylated DNA were used in vitro transcription reactions. Methylation with Msp I methylase was performed similarly. In the case of methylation with both methylases, the methylation reactions were performed sequentially. Plasmid DNA was first methylated with M.SssI, DNA recovered by phenol extraction and ethanol precipitation. DNA was then methylated with Msp I methylase (M0215 NEB) followed by phenol extraction and ethanol precipitation and used in transcription reactions.

Protein expression and Purification: GST-RB (379-928) was expressed and purified as described previously (16).

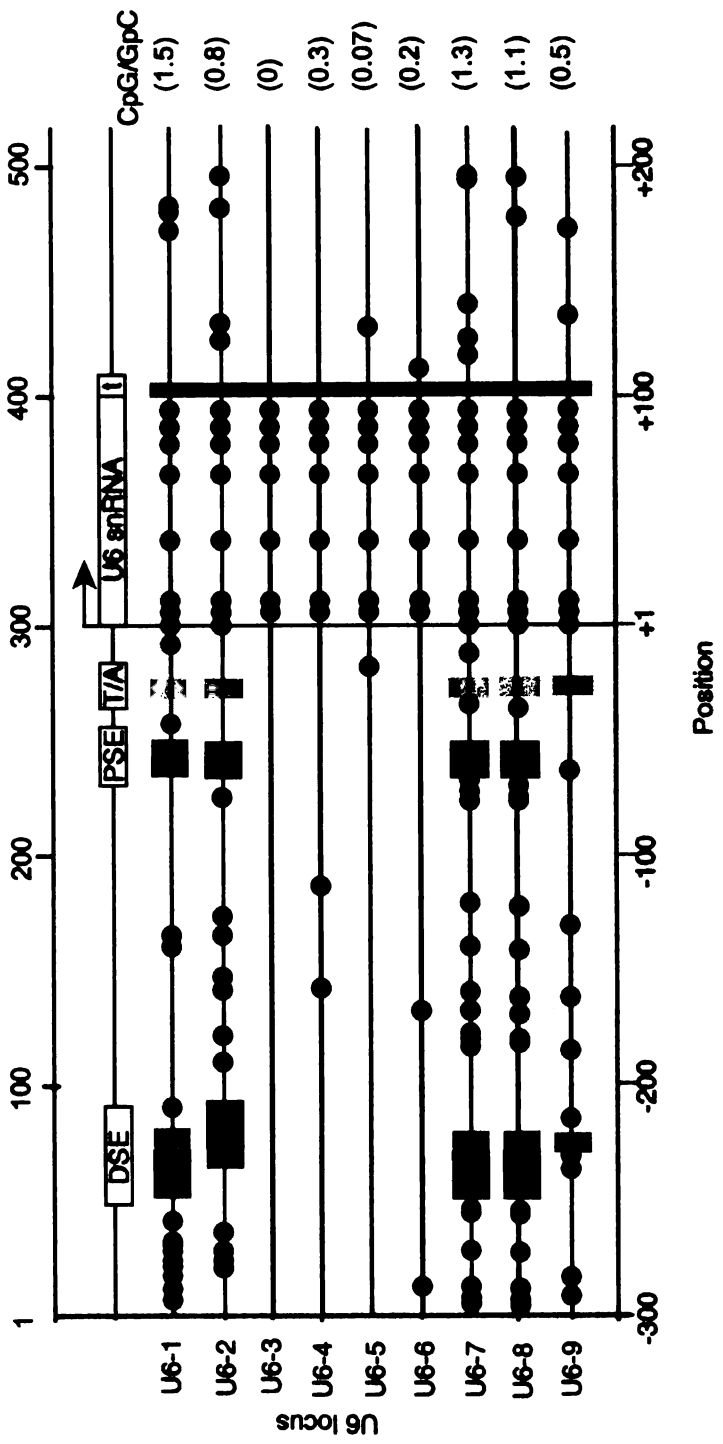
Plasmid constructs: pU6/Hae/RA.2 was used as the U6 reporter (23) and pBS-Y1-997 containing the Y1 promoter and the reverse beta-globin reporter were used in this study.

Antibodies: anti-SNAP43 (CS48), anti-TBP (SL2) are previously described (15, 33). IgG (Gibco), anti- DNTM1 (Imgenex IMG-261), anti-DNMT3A (Imgenex IMG-268A), anti-DNMT3B (Abcam ab2851) were used in this study.

Results

In mammalian genomes, DNA methylation at the 5 position of cytosine residues is often used as a mechanism to silence transcription. CpG dinucleotide enrichment seen in many functional promoters in the genome suggests an important role for DNA methylation in gene regulation. In *H. sapiens*, the U6 snRNA gene is present as nine copies, five of which are functional (U6- 1, 2, 7, 8 and 9) for U6 transcription and four are non-functional (U6- 3, 4, 5, 6). All the nine U6 copies have identical coding regions but vary only at the promoter region (10). The non-functional U6 copies – U6-3, 4, 5 and 6 lack the TATA Box, PSE and DSE (Figure 2-1). Sequence analysis (from -300 to +200) of all the nine copies of the human U6 gene revealed that the promoter proximal regions of all the functional copies were comparatively more enriched in CpG dinucleotides than the non-functional copies (Figure 2-1). The ratio of the number of CpG dinucleotides to the number of GpC dinucleotides was also higher for U6 copies 1, 2, 7, 8 and 9 than U6- 3, 4, 5 and 6 (Figure 2-1). CpG dinucleotides are frequent targets of methylation at the 5 position of cytosine, catalysed by the DNMT family of proteins DNMT1, DNMT3A and DNMT3B. Considering the pre-existing body of knowledge that suggests a link between RB and DNA methyltransferases (however, demonstrated only in Pol II and Pol I systems), and the role of DNA methylation in gene silencing, I hypothesized that CpG dinucleotides could be playing an important role in regulating U6 transcription. Therefore, I examined the potential role for DNA methylation and DNA methyltransferases in RB repression of U6 transcription. Firstly, I was

Figure 2-1: CpG plot of all the nine U6 copies. The positions of CpG dinucleotides (represented by a red dot) were plotted for all the nine U6 genes copies. The general promoter structure of the U6 genes is depicted. The non-functional copies U6-3, 4, 5 and 6 lack the U6 promoter elements DSE (green box), PSE (blue box) and TATA Box (pink box) (10). The U6-9 gene lacks a PSE consensus but recruits SNAPc (10). The transcriptional start site is indicated by the green line. The position of the termination sequence is indicated as 't'. The ratio of the number of CpG dinucleotide to GpC dinucleotide contained between -300 to +1 for each of these copies is indicated.


CpG/GpC
(1.5)
(0.8)
(0)
(0.3)
(0.07)
(0.2)
(1.3)
(1.1)
(0.5)
U6 snRNA
PSE
T/A
DSE
500
400
300
200
100
1
+200
+100
+1
-100
-200
-300
Position
U6 locus
U6-1
U6-2
U6-3
U6-4
U6-5
U6-6
U6-7
U6-8
U6-9

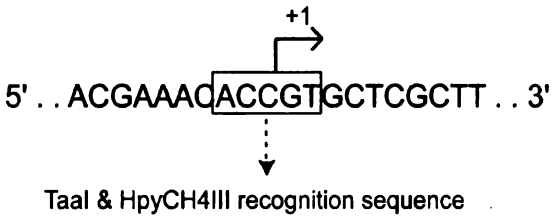
interested in knowing whether there was any link between the presence of CpG methylation and RB activity status. For this, I analyzed the methylation status of a highly conserved start site CpG dinucleotide in cells that either have active or inactive RB. I have analysed the U6-1 gene in all the experiments presented in this study.

The conserved U6 start site CpG is methylated in cells containing active RB but not in cells lacking RB activity

To examine whether promoter CpG methylation is linked to RB function, a comparative analysis of the methylation status of the start site CpG was done using genomic DNA harvested from cells containing active RB and those that lack functional RB. Methylation status of the start site CpG was analysed in HeLa (lacking functional RB) and MCF7 cells (that contain functional RB) using a restriction digestion-based approach. The U6-1 start site contains the sequence ACCGT, which is cleaved by isoschizomers Taa I and HpyCH4 III depending on the methylation status of the second cytosine in the recognition sequence (Figure 2-2A). Methylation at the 5 position of the second cytosine impairs digestion by Taa I but not HpyCH4 III (Figure 2-2B). Restriction digestion by either enzyme was measured by PCR amplification across this restriction site. Effective cutting would lead to the absence of PCR product but protection from cutting by methylation would result in a PCR product. Restriction digestion of the ACCGT sequence at the U6 start site by Taa I was impaired in MCF7 genomic DNA (Figure 2-2C, lane 8) but not in HeLa genomic DNA, whereas digestion by

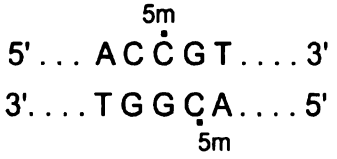
Figure 2-2: The U6 start site CpG is methylated in vivo in MCF7 but not HeLa cells. (A) Nucleotide sequence (from -10 to +10) of the U6 snRNA gene. The U6 start site region contains a recognition sequence for isoschizomers Taa I and HpyCH4 III. (B) Methylation of the indicated cytosines at the 5 position impairs Taa I digestion but not HpyCH4 III digestion. (C) Restriction digestion of genomic DNA from MCF7 or HeLa cells using Taa I or HpyCH4 III enzymes followed by PCR amplification of the U6 snRNA gene (lanes 5 to 8) or the GAPDH exon 2 region (negative control)(lanes 1 to 4). Lanes 1 to 3 and lanes 5 to 7 (Input titrations: 100%, 10%, 1%) are negative control reactions in which the genomic DNA was incubated with no restriction enzyme at either 65 °C (optimum temperature at which Taa I digestion was done) or at 37°C (optimum temperature at which HpyCH4 III digestion was done), respectively. (D) Ratio of normalized U6 PCR product intensity of Taa I to HpyCH4 III treated DNA was calculated for HeLa and MCF7 cells. Normalization was done to corresponding 10% input. Quantitation from three experimental measurements was performed and the 'p value' for MCF7 in comparison to HeLa is indicated. 'p value' calculations were done using two-tailed student T-test.

A



U6 template (in vivo)

B



TaaI digestion impaired
HpyCH4III digestion occurs

C

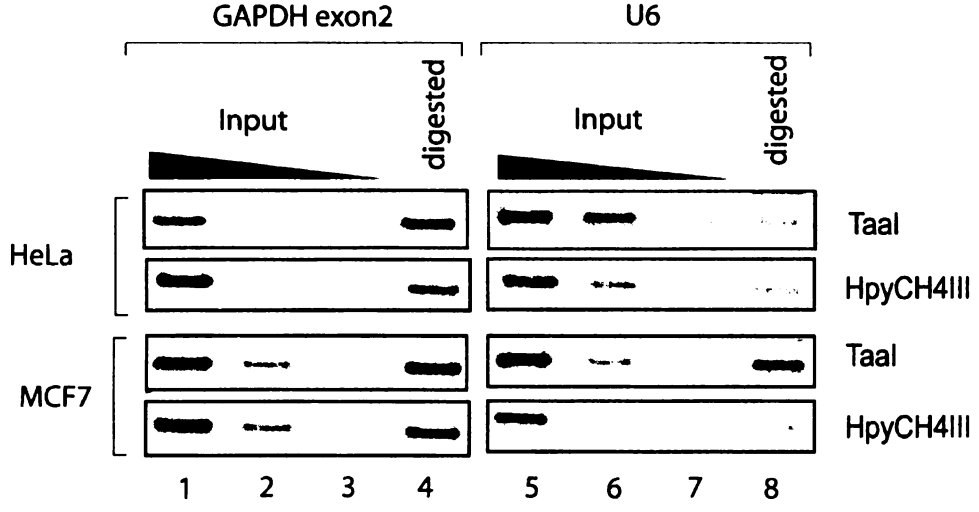
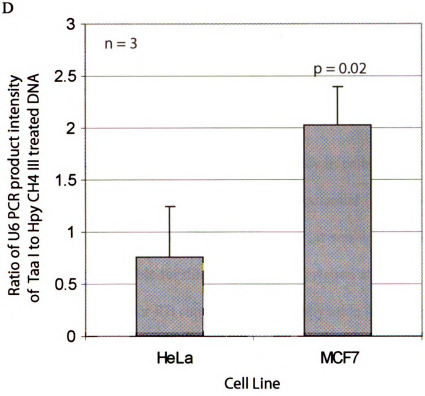


Figure 2-2 contd.

D



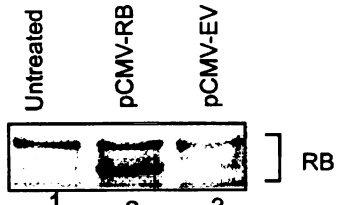
HpyCH4 III was not impaired in either of the two cell lines, indicating that the start site CpG is methylated only in cells containing active RB. This suggests a positive correlation between U6 promoter methylation and RB functional status. PCR amplification across GAPDH exon 2 that has no Taa I/HpyCH4 III restriction site serves as a control for presence of equal amounts of total DNA (Figure 2-2C, lanes 1-4). Quantitation results (Figure 2-2 D) from three experimental measurements showed approximately 2.7 fold enhanced methylation in MCF7 cells when compared to HeLa cells.

Transient RB expression in cells lacking RB function results in start site CpG methylation

The presence of promoter CpG methylation only in cells containing active RB but not in cells lacking functional RB suggests a potential link between DNA methylation and RB function at the U6 gene. However, it was unclear whether RB is the factor that is responsible for directing CpG methylation at the U6 promoter. Therefore, to address whether RB can direct DNA methylation at the U6 start site, RB was transiently expressed in HeLa cells (which lack functional RB), followed by analysis of the U6 start site methylation status. Panel A of Figure 2-3 is a Western blot showing RB expression in HeLa cells transfected with the RB expression vector pCMV-RB. Genomic DNA from HeLa cells that were either untreated or transfected with pCMV-RB or empty vector (pCMV-EV) was analysed for start site methylation status using restriction digestion with Taa I and HpyCH4 III enzymes. PCR amplification across the U6 start site or GAPDH

Figure 2-3: The U6 start site CpG gets methylated in vivo in HeLa cells in response to transient RB expression. (A) Western Blot showing RB expression in HeLa cells transiently transfected with the RB expression vector (pCMV-RB) (lane 2). (B) Schematic representation of the number of restriction enzyme sites in the genomic regions analyzed by PCR amplification. (C) Restriction digestion of genomic DNA from HeLa cells that were either untreated (lanes 1 to 4), or transfected with pCMV-RB (lanes 5 to 8) or pCMV-EV (lanes 9 to 12) using Taa I or HpyCH4 III or Ava II enzymes, followed by PCR amplification of the U6 snRNA gene or GAPDH region 1 or region 2. (D) Ratio of normalized PCR product intensity for Taa I to Hpy CH4 III treated DNA was calculated for the U6 locus, GAPDH region 1 and GAPDH region 2 for untreated HeLa cells and HeLa cells treated with either pCMV-RB or pCMV-EV. Quantitation from two experimental repeats was performed. Normalization was done to the corresponding Ava II digested sample. The respective 'p values' for the U6 locus, GAPDH region 1 and GAPDH region 2 for HeLa cells treated with pCMV-RB or pCMV-EV were calculated in comparison to HeLa cells that were untreated. 'p value' < 0.05 is indicated with an asterisk (*). 'p values' were calculated using two-tailed student T-test.

A



B

	No. Restriction Sites		
	U6	GAPDH region1	GAPDH region2
TaaI/HpyCH4III	1 (at start site)	0	1
AvaII	0	0	0

C

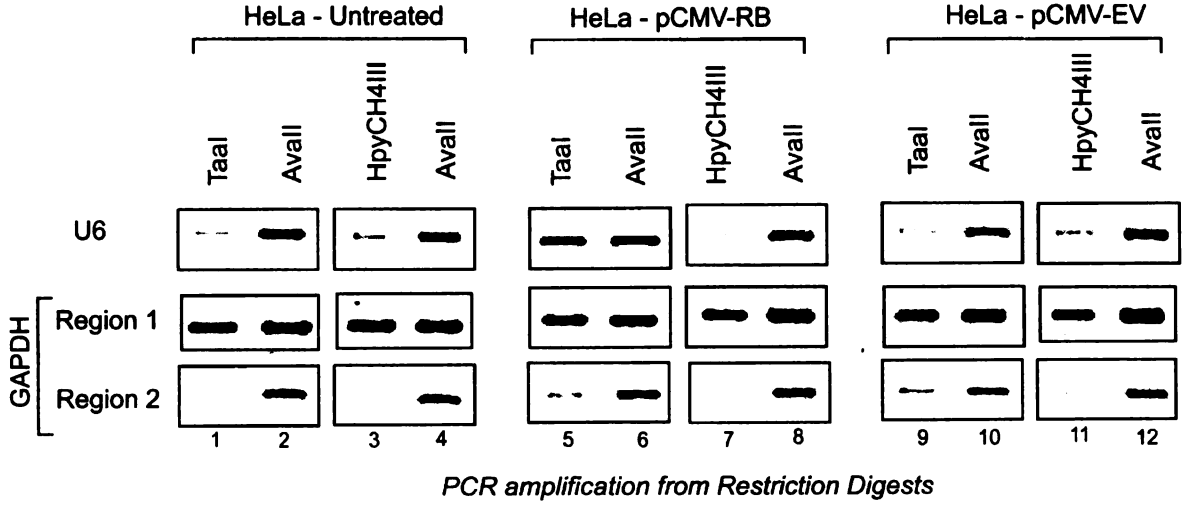
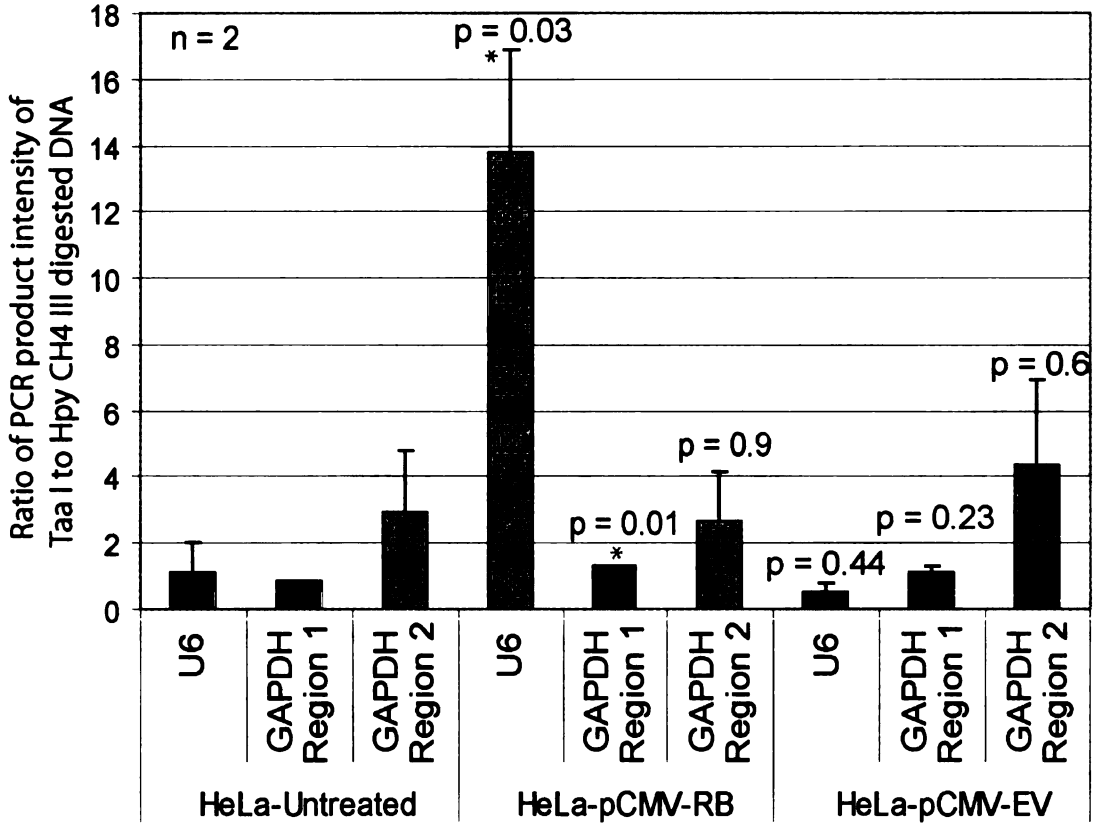


Figure 2-3 cond.

D



region 2 each having one Taa I/HpyCH4 III recognition site or GAPDH region 1 that has no recognition site for Taa I/HpyCH4 III was done and results shown in panel C of Figure 2-3. There are no Ava II sites in either of the three regions analysed by PCR. PCR amplification across the U6 start site revealed that expression of RB in HeLa cells resulted in start site CpG methylation as seen from impaired digestion by Taa I (but not HpyCH4 III digestion) in HeLa cells transfected with pCMV-RB (lanes 5 and 7, Figure 2-3C). Taa I digestion at the U6 start site was efficient in HeLa cells transfected with pCMV-EV (empty vector) (lane 9, Figure 2-3C) or untreated cells (lane 1, Figure 2-3C). Following restriction with Taa I or HpyCH4 III, PCR amplification across GAPDH region 1 which has no Taa I/HpyCH4 III recognition site showed comparable levels of PCR products in HeLa-untreated, HeLa-pCMV-RB treated or HeLa-PCMV-EV treated cells (GAPDH region 1, lanes 1, 3, 5, 7, 9 and 11, Figure 2-3C), suggesting the presence of equal amounts of genomic DNA. Amplification across GAPDH region 2 that has one Taa I/HpyCH4 III recognition site showed efficient cutting by both Taa I and HpyCH4 III in HeLa-untreated, HeLa-pCMV-RB treated or HeLa-pCMV-EV treated cells (GAPDH region 2, lane 1, 3, 5, 7, 9 and 11, Figure 2-3C), indicating that the impairment of Taa I digestion was specific to the U6 start site. Restriction by HpyCH4 III was efficient at U6 and GAPDH region 2 in all the conditions. As expected, following Ava II restriction, PCR amplification of the three genomic regions was comparable in all the conditions, because of the absence of an Ava II recognition site in all the three regions tested by PCR. This serves as a control for the presence of equal amounts of total DNA.

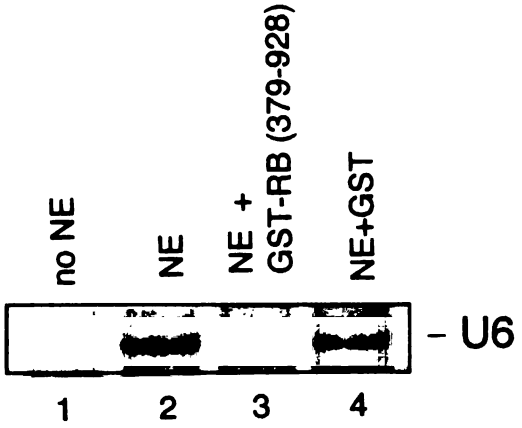
This study revealed that RB can direct promoter CpG methylation at the U6 gene in vivo. Quantitation results (Figure 2-3D) from two experimental repeats shows 14-fold increased methylation at the U6 locus in HeLa cells transfected with pCMV-RB compared to HeLa cells that were either untreated or transfected with pCMV-EV.

Start site CpG gets methylated in vitro during RB mediated repression of U6 transcription

Next I examined whether RB directs promoter methylation during repression of U6 transcription. RB has been shown to repress U6 transcription in vitro (16, 17). In this in vitro transcriptional assay system for RB repression, we tested the methylation status of the start site CpG of the U6 template to see whether RB directs promoter methylation during repression of U6 transcription. The U6 template for in vitro transcription has the CCGG sequence at the start site (Figure 2-4B). This base change from CCGT sequence observed at the U6 start site in vivo, to CCGG in vitro allows examination of possible CNG methylation that can occur in an RB-dependent manner. A restriction enzyme digestion analysis using Hpa II and Msp I (which are isoschizomers that recognize and cleave the sequence CCGG in a methylation status dependent manner) was done. Methylation at the 5 position of the second cytosine in the CCGG recognition sequence impairs Hpa II but not Msp I digestion, whereas the methylation patterns meCCGG and meCmeCGG were found to impair digestion by both Hpa II and Msp I (Figure 2-4B). Panel A of Figure 2-4 shows that RB represses U6

Figure 2-4: The start site CpG of the U6 snRNA gene becomes methylated in response to nuclear extract and GST-RB addition in vitro. A. Products of in vitro transcription reaction, analysed by riboprobe protection assay. B. Schematic representation of the methylation sensitivity of Hpa II and Msp I restriction enzymes. C. Schematic representation of the pU6HaeRA2 plasmid containing the U6 promoter elements – DSE, PSE and TATA Box and also the start site CCGG sequence and the termination sequence (a stretch of four thymines). R1 is the plasmid region containing the U6 start site CCGG. R2 contains a CCGG sequence outside the U6 promoter region. R3 contains a CCGG sequence within the U6 promoter. D. PCR amplification of regions R1, R2 and R3 of pU6HaeRA2 plasmid from in vitro transcription reactions set up with no NE, NE only or NE along with GST-RB or GST(negative control) digested with Hpa II or Msp I. Samples incubated with no restriction enzyme (no RE) are negative controls (E). Ratio of normalized U6 PCR product intensity was calculated for samples digested with Hpa II or Msp I after treatment with no nuclear extract (no NE), nuclear extract only (NE), nuclear extract with RB (NE + RB) or nuclear extract with GST (NE + GST). Quantitation from two experimental measurements was performed. Normalization was done to the corresponding no restriction enzyme (no RE) samples. The respective 'p values' were calculated (by two-tailed student T-test) in comparison to the corresponding 'no NE' sample and are indicated. 'p value' < 0.05 marked with an asterisk (*). 'p value' calculations were done comparing 'NE+RB' sample to 'NE' sample for both Hpa II and Msp I digested samples (marked as **).

A



In vitro transcription

B

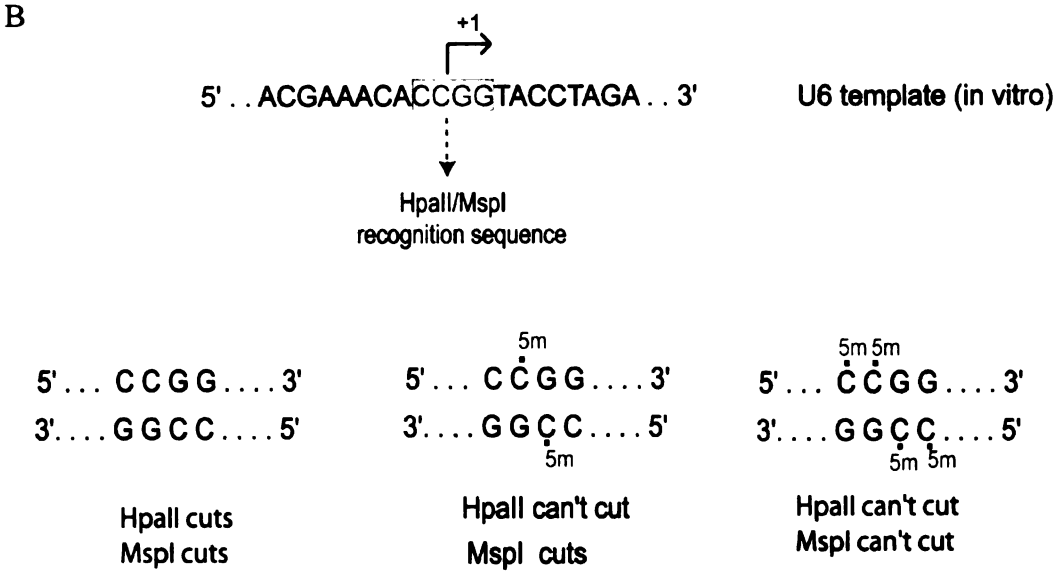
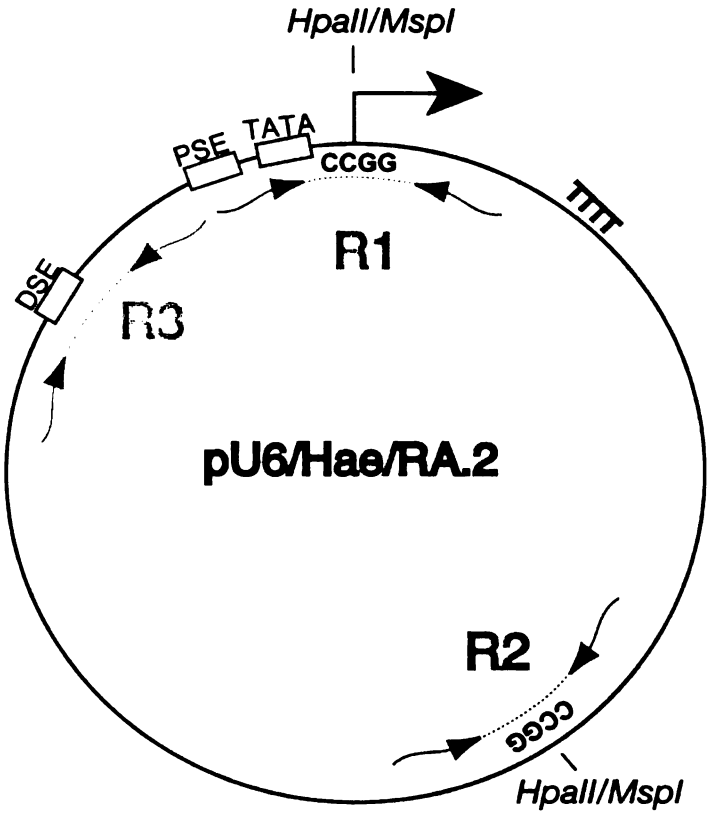
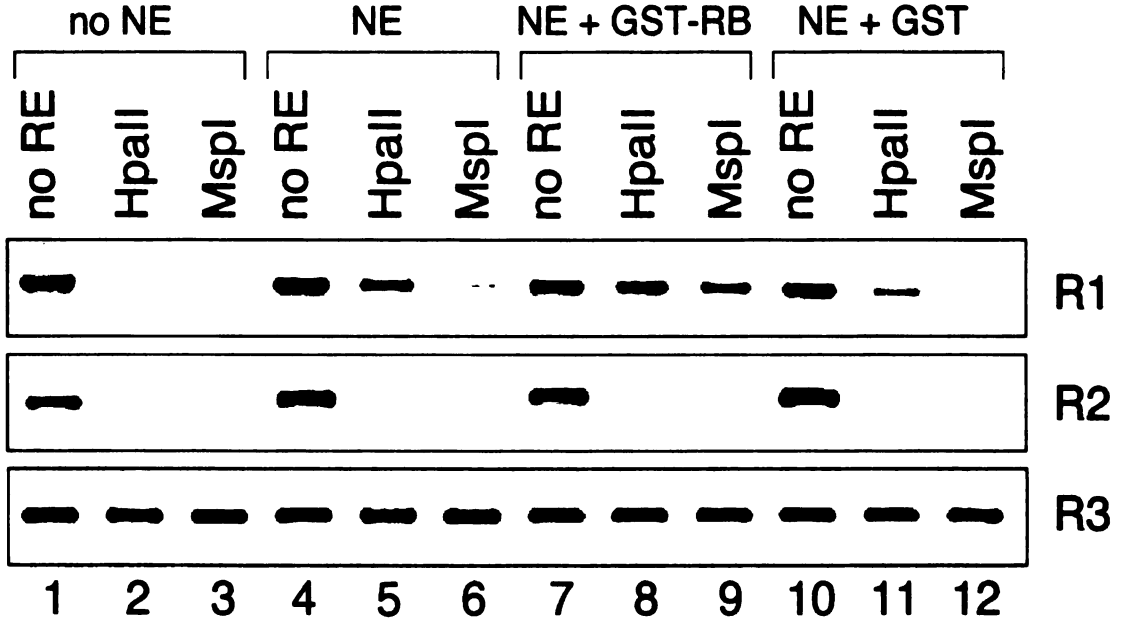


Figure 2-4 contd.

C



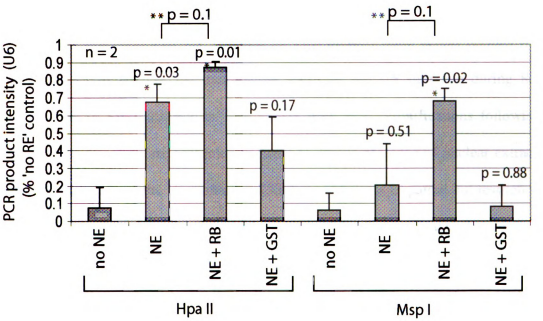
D



PCR amplification from Restriction Digests

Figure 2-4 contd.

E



transcription in vitro as detected by riboprobe protection. Panel C of Figure 2-4 is a schematic representation of the U6 reporter plasmid used for U6 transcription. The U6 promoter elements DSE, PSE, TATA Box, termination sequence and the start site CCGG sequence are indicated. Region R1 represents a region of the plasmid encompassing the start site CCGG recognition sequence for Hpa II/Msp I restriction enzymes. R2 is a region outside the U6 promoter that has a CCGG sequence. Region R3 has no CCGG sequence.

An aliquot of the transcription reactions shown in panel A was used for restriction digestion analysis followed by PCR amplification across regions R1, R2 and R3 to analyze the methylation status of the U6 start site during RB repression of U6 transcription. The results from PCR amplifications following restriction digestion are presented in panel D. In the presence of nuclear extract, Hpa II digestion of the U6 start site CCGG sequence was impaired but restriction by Msp I was efficient (lanes 5 and 6, Figure 2-4D). This indicated that the start site CpG methylation was induced by factor(s) in the nuclear extract. In the presence of RB along with nuclear extract, this methylation event (at the start site CpG dinucleotide) was found to be enhanced as seen from the enhanced insensitivity to Hpa II digestion when compared to samples treated with nuclear extract and GST (by 2.2 fold) or with nuclear extract only (by 1.3 fold) (lane 8 compared to lane 11 and 5, Figure 2-4D and quantitation in Figure 2-4E). In addition, in RB treated samples, along with impaired Hpa II digestion, Msp I enzyme was also unable to cut the start site CCGG sequence. This suggests that

the outside cytosine of the CCGG sequence was also methylated in the presence of RB. This effect was specific to the U6 start site as restriction digestion of the CCGG sequence in region R2 which is outside the U6 promoter is unaffected by nuclear extract or recombinant protein addition. Comparable levels of PCR amplification product from region R3 confirms the presence of equal amounts of total DNA. Reactions done with no nuclear extract (lanes 1-3, Figure 2-4D) or nuclear extract and GST (lanes 10-12, Figure 24D) serve as controls. Figure 2-4E shows quantitation results from two experimental repeats. Insensitivity to Hpa II digestion was increased by 1.3 fold in the presence of nuclear extract and RB compared to reaction containing nuclear extract only. Insensitivity to Msp I digestion was increased by 3.5 fold in the presence of nuclear extract and RB in comparison to reaction containing nuclear extract only. 'p values' were calculated in comparison to the corresponding 'no NE' sample. 'p values' < 0.01 are marked with an asterisk.

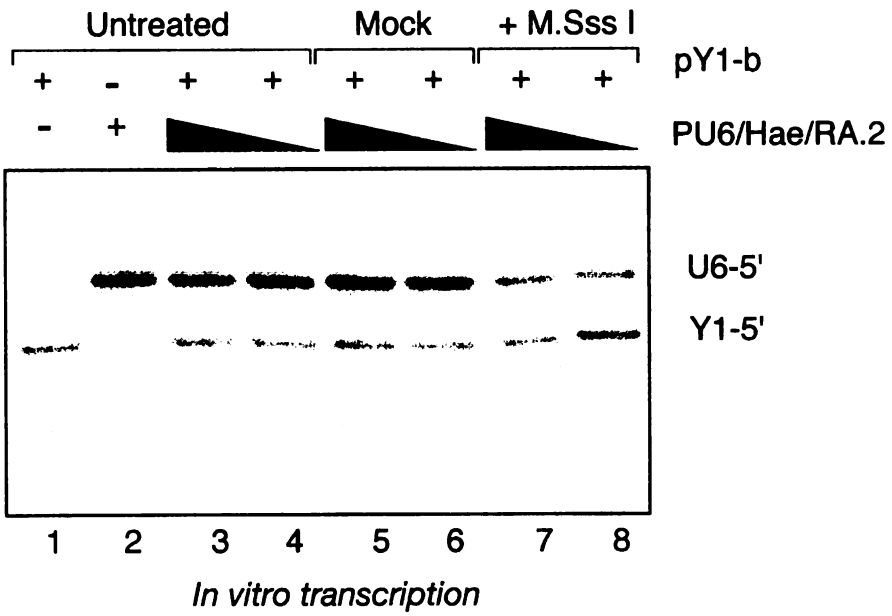
This analysis revealed that during RB repression of U6 transcription, the start site CCGG sequence undergoes methylation at both the cytosines. Enhanced start site CpG methylation (in comparison to samples treated with nuclear extract and GST, or with nuclear extract only) was seen in RB-treated samples. Additional methylation of the outside cytosine of the CCGG sequence was induced specifically only in the presence of RB. Evidence for methylation of the cytosines in CpNpG sequences (8) in mammalian DNA led us to speculate that RB can direct methyltransferase activity that can cause methylation of both the

cytosines in the CCGG sequence. Studies suggesting that DNMT1 can also methylate CCG, CTG and CAG sequences in addition to CpG (28) lend support to the idea that multiple cytosines can get methylated possibly by RB directed recruitment of one or more methyltransferase activities that can methylate cytosines in the CpG as well as in non-CpG sequence contexts. The above mentioned result provides evidence for RB directed cytosine methylation at the U6 promoter during repression of U6 transcription, suggesting that RB employs DNA methylation as part of its transcriptional repression mechanism.

CpG methylation of the U6 template results in reduced U6 transcription

To examine whether DNA methylation is sufficient to repress U6 transcription, the U6 template was methylated in vitro with M.Sss I methylase which methylates CpG dinucleotides. U6 transcription was done in vitro with CpG methylated, mock treated or untreated U6 reporter plasmid (Figure 2-5). U6 transcription from methylated template was reduced (lane 7 and 8, Figure 2-5) by 2 to 3-fold when compared to transcription from unmethylated template (lanes 3 and 4, Figure 2-5) or mock treated (lanes 5 and 6, Figure 2-5) U6 reporter plasmid. Untreated Y1 reporter plasmid was included in reactions in lanes 3 to 8 of Figure 2-5 as an internal control for transcription. Transcription with the U6 reporter only (lane 2, Figure 2-5) or the Y1 reporter only (lane 1, Figure 2-5) are also shown. This result demonstrated that CpG methylation by itself has a repressive effect on U6 transcription.

Figure 2-5: CpG methylation leads to reduced U6 transcription. U6 reporter plasmid was methylated with M.Sss I CpG methylase and used in transcription reaction in vitro alongside U6 reporter plasmids that were either untreated or mock treated for methylation. Lanes 7 and 8 contain transcription reactions done with 250 ng and 125 ng respectively of methylase treated plasmid. Lanes 3 and 4 contain transcription products from titrations of untreated plasmid. Lanes 5 and 6 contain transcription products from titrations of mock treated plasmid (incubated with methylase buffer only, in the absence of CpG methylase enzyme). In reactions from lane 3 to 8, 250 ng of untreated Y1 reporter plasmid was also included in the transcription reactions to serve as an internal control for transcription. Lane 2 contains transcription reaction carried out with U6 reporter only (no Y1). Lane 1 contains reactions carried out with Y1 reporter only.


Untreated
Mock
+ M.Sss I
pY1-b
+ - + + + + + +
- + PU6/Hae/RA.2
U6-5'
Y1-5'
1 2 3 4 5 6 7 8
In vitro transcription

Considering that during repression of U6 transcription in vitro, RB induced methylation of the outside cytosine in the start site CCGG sequence, I questioned whether this additional cytosine methylation at a non CpG sequence can also contribute to the repressive effect of DNA methylation on U6 transcription. Therefore, to examine the combined effect of promoter CpG methylation along with start site methylation of both the cytosines in the CCGG sequence, the U6 template was methylated with both SssI methylase, which methylated all CpG dinucleotides, and Msp I methylase, which methylates the first cytosine of the start-site CCGG sequence (Figure 2-6A).

Results of in vitro transcription from U6 template methylated with either M.Sss I or Msp I only or with both M.Sss I and Msp I methylases in comparison to untreated or mock treated plasmids are shown in Figure 2-6. Transcription from plasmid methylated with M.Sss I (lanes 4 and 5, Figure 2-6B) was reduced by 2.3 fold, when compared to untreated plasmid (lanes 2 and 3, Figure 2-6B) which is in agreement with fold reduction in transcription shown in Figure 2-5. Transcription from U6 plasmid methylated with both M.Sss I and Msp I (lanes 8 and 9, Figure 2-6B) was reduced by 3.3 fold when compared to the untreated and therefore had the most repressive effect on U6 transcription. Transcription from Msp I treated (lanes 6 and 7, Figure 2-6B) plasmid showed a 1.7 fold reduction compared to untreated and therefore had the least effect on U6 transcription. Y1 transcription was used as an internal control for transcription. Transcription reactions carried out with no reporter DNA (lane 1, Figure 2-6B) or with U6

Figure 2-6: Start site proximal cytosine methylation adds to the repressive effect caused by promoter CpG methylation on U6 repression. (A) Schematic representation of the methylase activity of M.Sss I and Msp I methylases. M.Sss I methylates all the CpG dinucleotides in the U6 reporter plasmid (only a section of the U6 reporter is shown). Msp I methylates the outside cytosine in the CCGG sequence. There is only a single CCGG sequence (which occurs at the start site) in the U6 promoter containing region of the reporter plasmid. (B) U6 transcription was performed in vitro from reporter plasmid (125ng or 250ng) that was untreated (lanes 2 and 3), methylated with M.Sss I CpG methylase (lanes 4 and 5), Msp I methylase (lanes 6 and 7), both M.Sss I and Msp I methylases (lanes 8 and 9) or mock treated (lanes 10 and 11) and products analysed by riboprobe protection. Transcription performed with no DNA (lane 1), with U6 reporter only (lanes 12 and 13) and Y1 reporter only (lane 14) are also shown. Bands corresponding to U6 and Y1 transcription are marked as U6-5' and Y1-5' respectively. (C) U6 template that was either untreated, methylated with M.SssI and Msp I, or mock treated plasmid was titrated from 7.5 ng to 125 ng in two fold steps and U6 transcription measured by riboprobe protection. 250 ng untreated Y1 reporter plasmid was added in all the reactions to serve as an internal control for transcription. (D) Graphical representation of data from quantitation of the results shown in C.

A

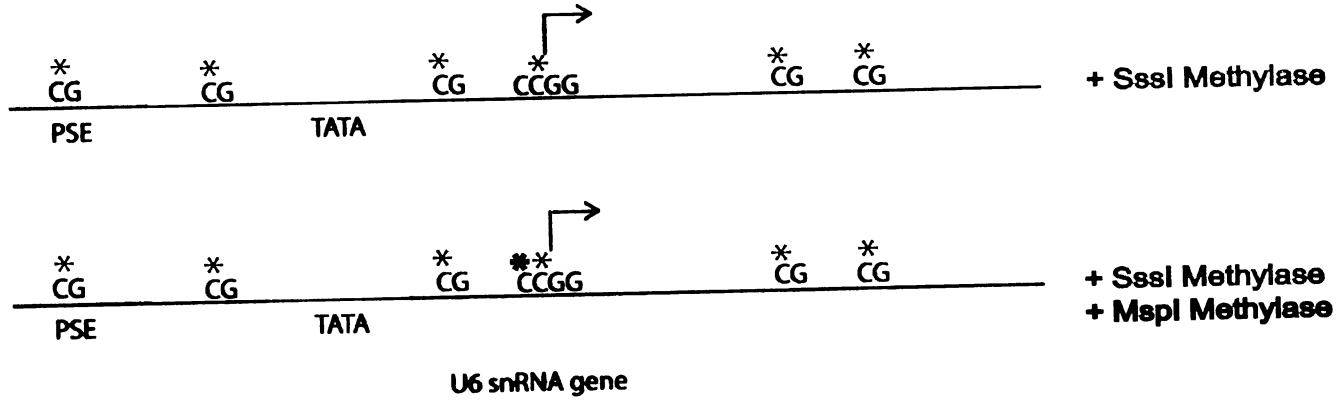

CG
CG
CG
CCGG
CG
CG
PSE
TATA
+ SssI Methylase
CG
CG
CG
CCGG
CG
CG
PSE
TATA
+ SssI Methylase
+ MspI Methylase
U6 snRNA gene

Figure 2-6 contd.

B

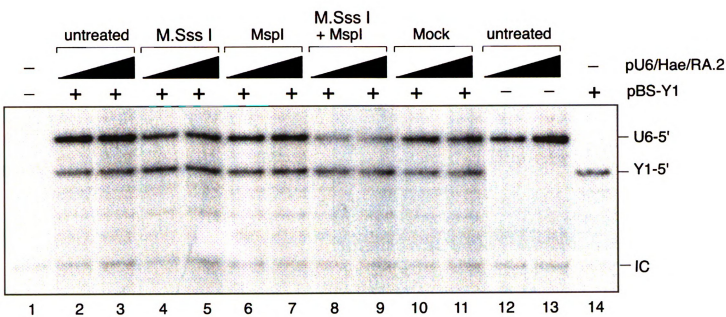
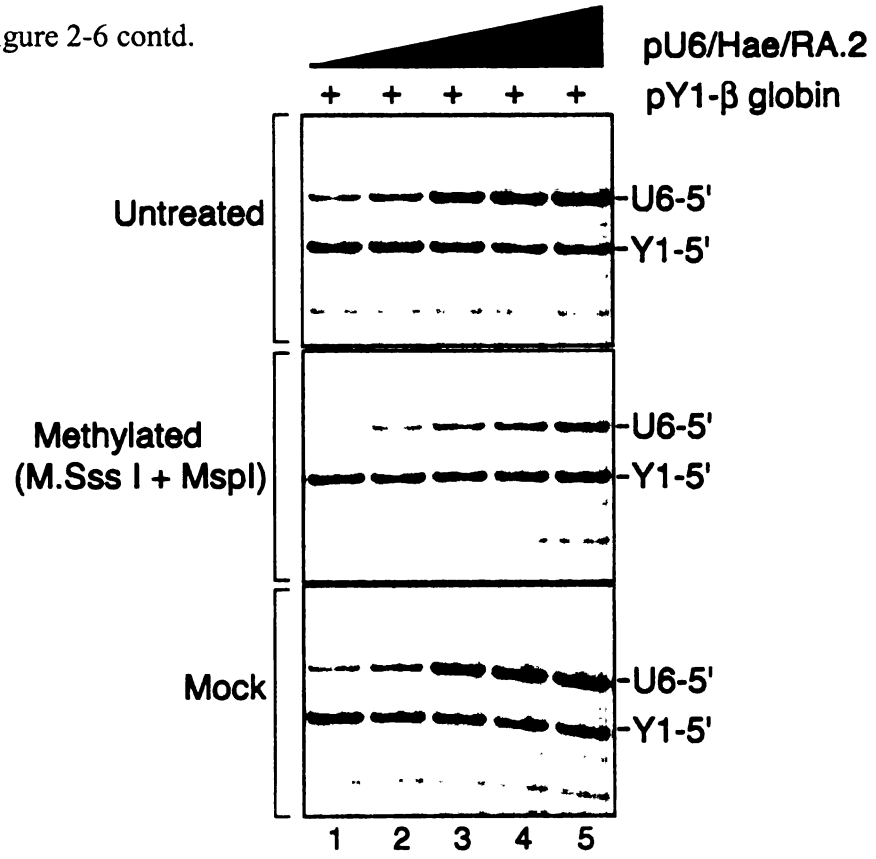
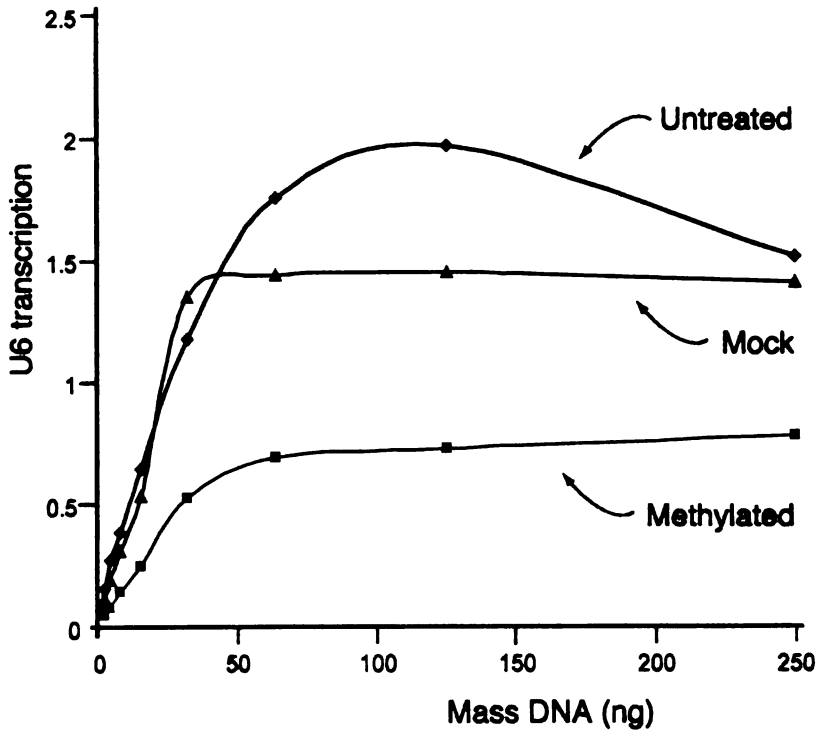


Figure 2-6 contd.

C



D



reporter only (lanes 12 and 13, Figure 2-6B) or Y1 reporter only (lane 14, Figure 2-6B) are also shown. 'IC' represents the loading control band.

To make sure that we were carrying out these transcription reactions with template amounts that were not limiting for transcription (which can affect the fold differences in transcription seen), I titrated the U6 template (from 0.95ng to 250 ng in two-fold increments). The indicated amounts of U6 template that was either treated with both methylases or mock treated or untreated plasmid were used in transcription reactions (Figure 2-6C, shown only 7.5 ng to 125 ng titration). Quantitation of this data presented in panel D of Figure 2-6 again confirmed the result presented in panel B of Figure 2-6. At template amounts (from 50 ng and higher) that were not limiting for transcription, I saw at least 3-fold reduction in U6 transcription from template that was treated with both methylases in comparison to the mock treated and untreated templates.

These results indicate that methylation of the outside cytosine at the start site CCGG sequence along with methylation of all the CpG dinucleotides in the U6 reporter plasmid further enhanced the repressive effect that CpG methylation had on U6 transcription. This suggests that RB could be employing CpNpG methylation along with CpG methylation at the U6 promoter to cause efficient repression of transcription. The observations that CpNpG and CpG methylation can repress U6 transcription and that CpNpG methylation adds to the repressive effect of CpG methylation lead us to propose that RB repression can be mediated by the recruitment of methyltransferase activity that leads to methylation of

cytosine residues in the CpG as well as non-CpG sequence contexts. Reports suggesting that cytosine methylation in CpCpG contexts can be catalysed by DNMT1 (28) and that DNMT3A can methylate cytosines in CpA and CpT dinucleotides (29) lend support to the idea that RB can direct DNMT activity to catalyze cytosine methylation at CpG and at non CpG sequences to cause transcriptional repression. However, methylation of only start-site cytosines (using Hpa II and Msp I methylases) without methylating the other CpG dinucleotides seemed to have no effect on U6 transcription (data not shown). These results suggest that the combined methylation of the start site cytosines and the other promoter CpGs is important for exerting the repressive effect on U6 transcription.

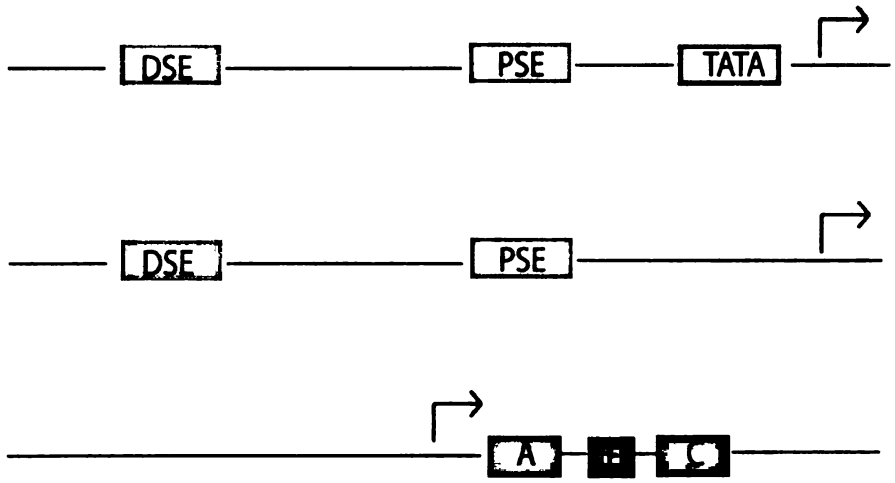
DNMT1 and DNMT3A occupy the endogenous U6 promoter in RB positive cells but not cells lacking RB activity

Many lines of evidence presented here suggest a correlation between RB repression of U6 transcription and DNA methylation at the U6 promoter. In mammalian cells DNA methylation is carried out primarily by the DNMTs 1, 3A and 3B. So I questioned whether these DNA methyltransferases occupied the U6 promoter and analysed for possible correlation between RB functional status and DNMT occupancy at the U6 promoter. Robertson et al., (30, 32) have suggested that DNMT1, which is a maintenance methyltransferase and the most abundant DNMT in somatic cells, cooperates with RB to repress target gene transcription by RNA polymerase II. Therefore, I investigated the presence of DNMT1 at

endogenous U6 promoters. To examine potential correlation between RB functional status and DNMT occupancy status at the U6 promoter, we carried out comparative ChIP analyses with MCF7 (that has active RB) and HeLa cells (which lack functional RB). Results presented in Figure 2-7B show that DNMT1 occupies the U6 promoter only in MCF7 cells but not in HeLa cells. DNMT1 did not occupy the U1 promoter (which is not an RB target gene) in either cell line. Interestingly, DNMT1 did not occupy the 5S rRNA gene although RB represses 5S rRNA transcription. Although the 5S rRNA gene is subject to RB regulation, RB does not occupy the 5S rRNA promoter. On the other hand, the U6 gene is sensitive to RB regulation and also is targeted for occupancy by RB (Figure 2-7A). Therefore, DNMT1 occupancy on the U6 promoter but not 5S rRNA promoter (lane 6, Figure 2-7B) indicated that DNMT1 occupancy on target genes correlates with RB sensitivity and RB occupancy on the target gene. GAPDH exon 2 serves as a negative control for PCR amplification. Immunoprecipitations done with antibodies against SNAP 43 (lane 5, Figure 2-7B) and TBP (lane 7, Figure 2-7B) serve as positive controls. Negative control immunoprecipitation was done with IgG. Figure 2-7C shows quantitation of the data shown in Figure 2-7B. In MCF7 cells, approximately 5-fold enrichment of the signal corresponding to DNMT1 occupancy on U6 was observed compared to the IgG control. The signal corresponding to DNMT1 occupancy on the U6 gene was negligible in HeLa cells. No significant signal intensity was observed for DNMT1 occupancy on the U1 and 5S rRNA genes in both cell lines. Chromatin immunoprecipitation from 184B5 cells (derived from normal mammary tissue) also showed the occupancy

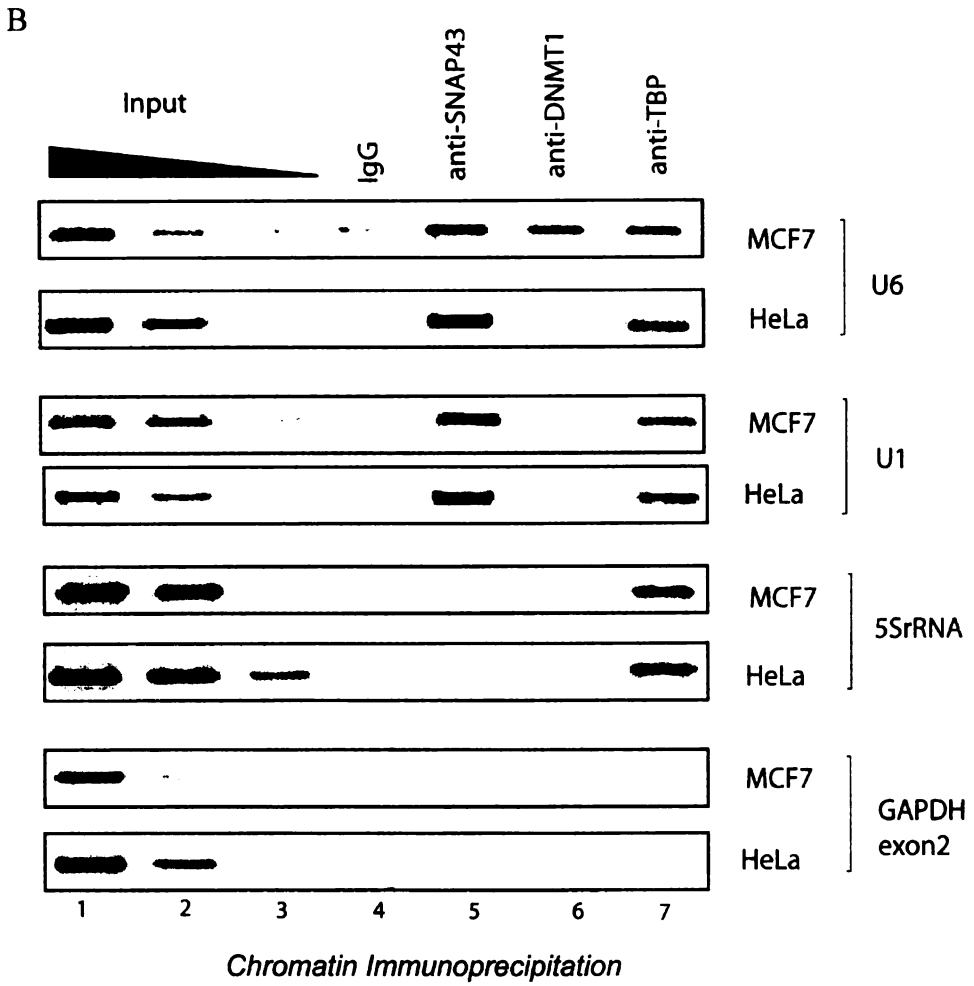
Figure 2-7: DNMT1 association with the endogenous U6 promoter coincides with RB activity status. (A) Promoter structure of the U6, U1 snRNA and 5S rRNA genes, their RNA Polymerase specificity, sensitivity to RB repression and the occupancy status of RB on these genes. (B) Chromatin from MCF7 or HeLa cells and was used in immunoprecipitation reactions with α-SNAP43 (lane 5) or α- DNMT1 (lane 6) or α-TBP (lane 7) or non-specific IgG (negative control) (lane 4). PCR analysis was done to test the enrichment of DNA fragments containing U6, U1 or 5SrRNA promoter regions in the immunoprecipitated DNA. GAPDH exon 2 served as the negative control. Lanes 1-3, input titration (1%, 0.1% and 0.01). (C) Quantitation of the ChIP result in B. PCR product intensity was normalized to 1% Input. Results for the U6, U1 and 5S rRNA loci for the MCF7 and HeLa cells are shown. (D) Chromatin harvested from 184B5 cells was used in immunoprecipitation with antibodies against SNAP43 (lane 6), DNMT1 (lane 7), TBP (lane 8) or IgG (lane 5). PCR amplification was done for U6, U1, U2, 7SK, 5S rRNA and GAPDH loci. Input titrations 10%, 1%, 0.1%, 0.01% are in lanes 1, 2, 3 and 4 respectively. (E) Quantitation of ChIP results from two experimental repeats from 184B5 cells. Results for immunoprecipitations of the U6, U1, U2, 5S rRNA and GAPDH loci with IgG, anti-SNAP43 and anti-DNMT1 antibody from 184B5 cells are shown. PCR product intensity was normalized to the corresponding 1% input. The 'p value' for the U6 immunoprecipitation with anti-DNMT1 antibody was calculated in comparison to the 'IgG control' using two-tailed student T-test.

A



	RNAP specificity	RB sensitivity	RB occupancy
U6 snRNA	RNAP III	+	+
U1 snRNA	RNAP II	–	–
5S rRNA	RNAP III	+	–

Figure 2-7 contd.



C Figure 2-7 contd.

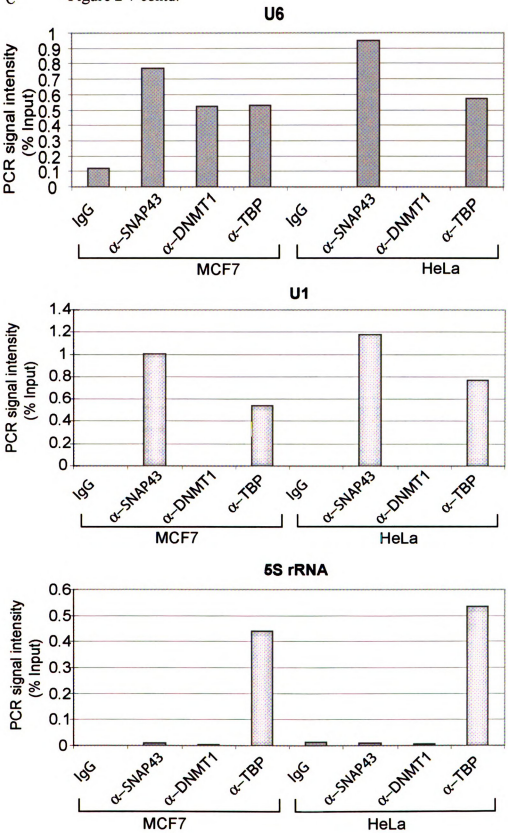


Figure 2-7 contd.

D

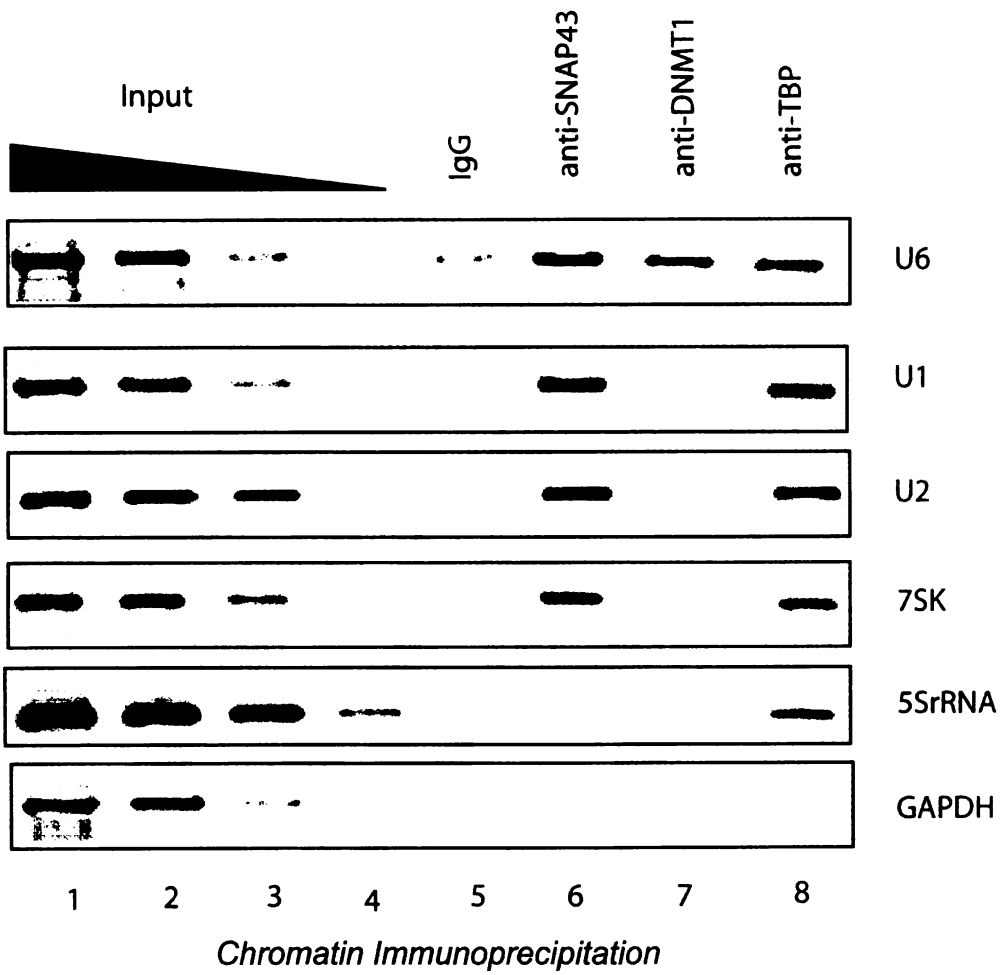
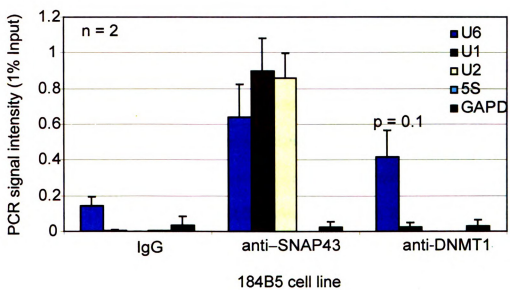


Figure 2-7 contd.

E



of DNMT1 on the U6 promoter and not U1, U2, 7SK or 5S rRNA promoters (Figure 2-7D). Quantitation of two experimental repeats of chromatin immunoprecipitation with IgG, anti-SNAP43 or anti-DNMT1 antibodies using 184B5 cells are shown in Figure 2-7E. Approximately 2.7 fold enrichment of the signal corresponding to DNMT1 occupancy on the U6 gene was observed when compared to the 'IgG control'. However, the 'p value' measured was 0.1, indicating a 90% confidence level for this result. No significant signal intensity corresponding to DNMT1 occupancy on the U1, U2, 5S rRNA and 7SK genes was observed.

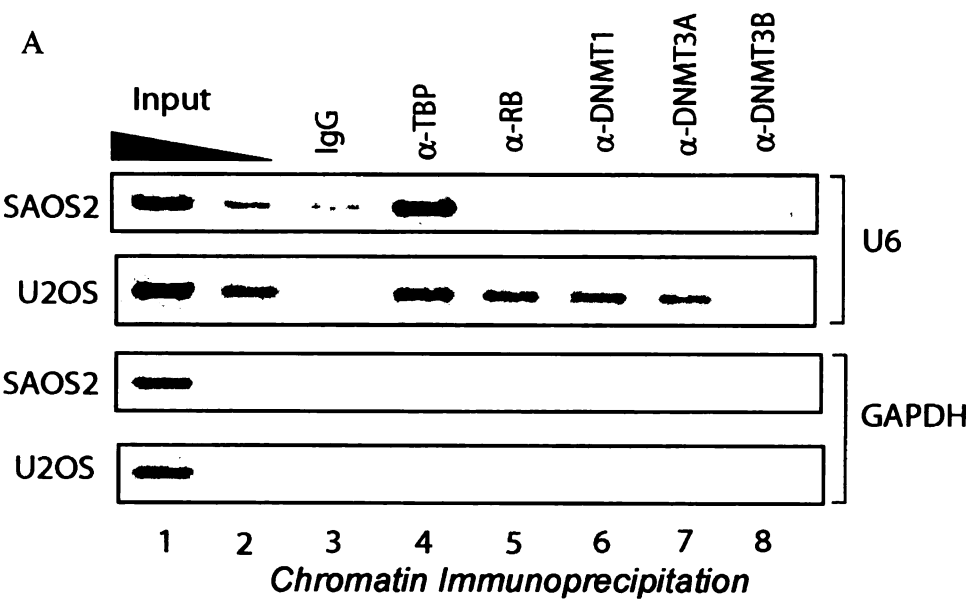
Following this, I extended the analysis to look for the occupancy of the other major methyltransferases DNMT3A and DNMT3B on the endogenous U6 promoter and whether there was any correlation with RB functional status. For this, chromatin immunoprecipitation was done from two osteosarcoma cell lines SAOS2 and U2OS cells of which SAOS2 does not contain RB activity whereas U2OS is positive for RB activity (Figure 2-8A). I observed that along with RB, DNMT1 and DNMT3A occupied the endogenous U6 promoter only in U2OS cells but not in SAOS2 cells, indicating a positive correlation between RB functional status and occupancy of DNMT1 and DNMT3A at the endogenous U6 promoter. Quantitation of chromatin immunoprecipitation results from two experimental repeats is shown in the lower panel of Figure 2-8A. At least 3-fold enrichment of the DNMT1 signal and a 4-fold enrichment of the DNMT3A signal in comparison to IgG was observed. The 'p values' corresponding to DNMT1 and

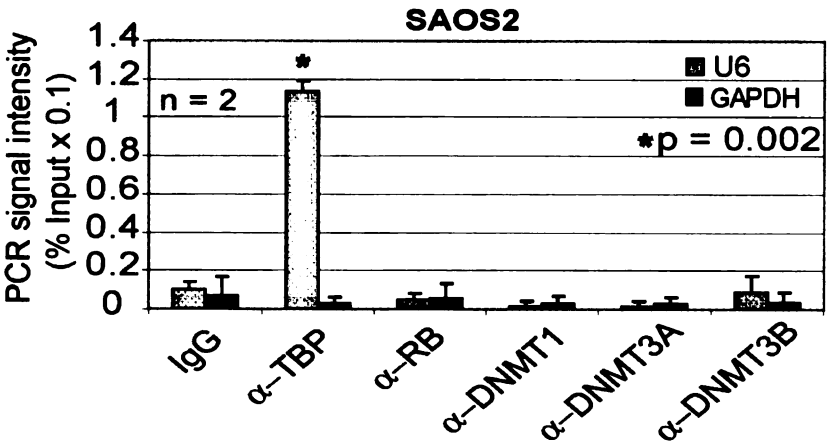
DNMT3A were 0.1 and 0.06 respectively. At least 90% confidence level can be attributed to this result.

Transient expression of RB results in recruitment of DNMT1 and 3A on the endogenous U6 promoter

The results mentioned above led us to speculate that RB directs DNMT recruitment to the U6 promoter. To examine whether RB directs recruitment of DNMTs to the U6 promoter, RB expression was induced in cells lacking RB and DNMT association with the U6 promoter was analysed by chromatin immunoprecipitation. A SAOS2 derived inducible cell line was induced to express RB upon tetracycline removal. Figure 2-8B is a Western blot showing RB expression upon tet removal. Following induction of RB expression, chromatin immunoprecipitation was done to look for RB recruitment to the U6 promoter as a first step. Panel C of Figure 2-8 shows recruitment of RB to the endogenous U6 promoter upon induction of RB expression by Tet removal. GAPDH exon 2 serves as the negative control. Then occupancy status of the DNMTs 1, 3A and 3B were examined after induction of RB expression. Figure 2-8D shows that upon induction of RB expression by tet removal, RB and also DNMTs 1 and 3A get recruited to the U6 promoter, suggesting that RB can direct the recruitment of DNMT1 and DNMT3A to the U6 promoter in vivo. The DNMT occupancy profile from ChIP analysis after induction of RB expression is in agreement with comparative analysis among U2OS and SAOS2 cells (Figure 2-8A). Quantitation of the result in Figure 2-8D is shown in its lower panel. Approximately 3-fold

Figure 2-8: RB recruits DNMT1 and DNMT3A to the endogenous U6 promoter: (A) Chromatin immunoprecipitation from two osteosarcoma cell lines SAOS2 (RB negative) and U2OS (RB positive) was performed with antibodies raised against RB (lane 5), DNMT1 (lane 6), DNMT3A (lane 7) and DNMT3B (lane 8). Immunoprecipitation performed with IgG (lane 3) and anti-TBP (lane 4) antibody serve as the negative and positive controls respectively for immunoprecipitation of crosslinked DNA. Lanes 1 and 2 contain input titration from 0.1% to 0.01% respectively. PCR amplification of the U6 promoter or GAPDH exon 2 (negative control for immunoprecipitation) was done. *Lower Panel.* Quantitation from two experimental repeats of the representative result shown in the top panel. PCR product intensity was normalized to the corresponding 0.1% input and the respective 'p values' were calculated in comparison to 'IgG' control and are indicated. (B) The SAOS2 derived inducible cell line was cultured in media containing Tetracycline (1 μg/ml). Western blot analysis shows expression of RB (by tet removal) after 48 hours after induction. (C) SAOS2 inducible cells were induced for RB expression, chromatin harvested at 48 hrs after RB induction, and immunoprecipitation was done with antibodies against TBP (lane 4), RB (lane 5) or non-specific IgG (lane 3). PCR amplification of U6 promoter region or GAPDH exon 2 region (negative control for immunoprecipitation) was done. Lanes 1 and 2 contain 0.1% and 0.01% input respectively. Chromatin immunoprecipitation was also done from cells that were not induced for RB expression (panels labelled as +TET). (D) RB expression was induced as explained above and chromatin immunoprecipitation was done with antibodies raised against TBP (lane 4), RB (lane 5), DNMT1 (lane 6), DNMT3A (lane 7), DNMT3B (lane 8) or non-specific IgG (lane 3). PCR amplification of U6 promoter DNA was done. Lanes 1 and 2 contain 0.1% and 0.01% input respectively. *Lower Panel.* Quantitation of results in the top panel. PCR product intensity was normalized to corresponding 0.1% input.


A
Input
IgG
α-TBP
α-RB
α-DNMT1
α-DNMT3A
α-DNMT3B
SAOS2
U2OS
U6
SAOS2
U2OS
GAPDH
1 2 3 4 5 6 7 8
Chromatin Immunoprecipitation


SAOS2
PCR signal intensity (% Input x 0.1)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
n = 2
U6
GAPDH
*p = 0.002
IgG
α-TBP
α-RB
α-DNMT1
α-DNMT3A
α-DNMT3B

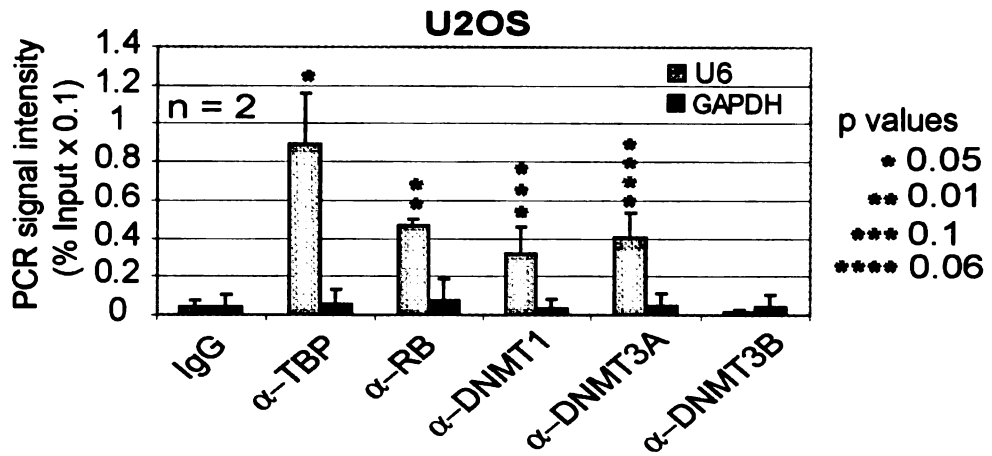
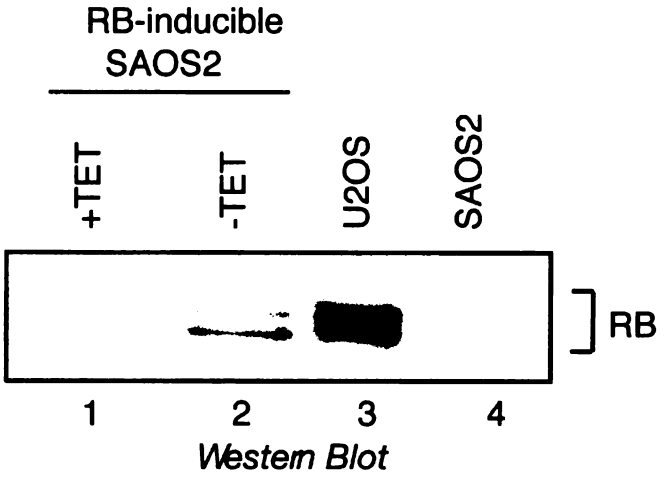

U2OS
PCR signal intensity (% Input x 0.1)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
n = 2
U6
GAPDH
p values
* 0.05
** 0.01
*** 0.1
**** 0.06
IgG
α-TBP
α-RB
α-DNMT1
α-DNMT3A
α-DNMT3B

Figure 2-8 contd.

B



C

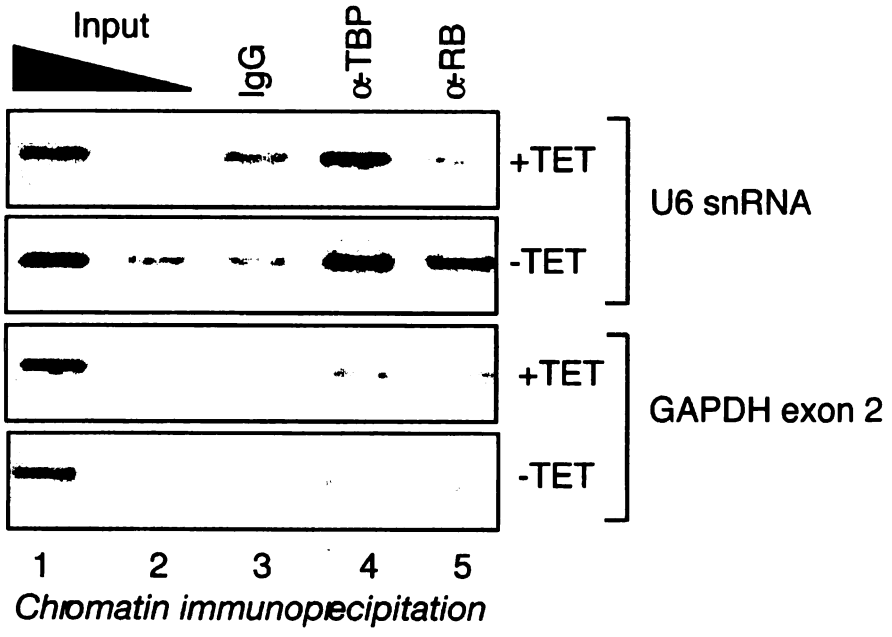
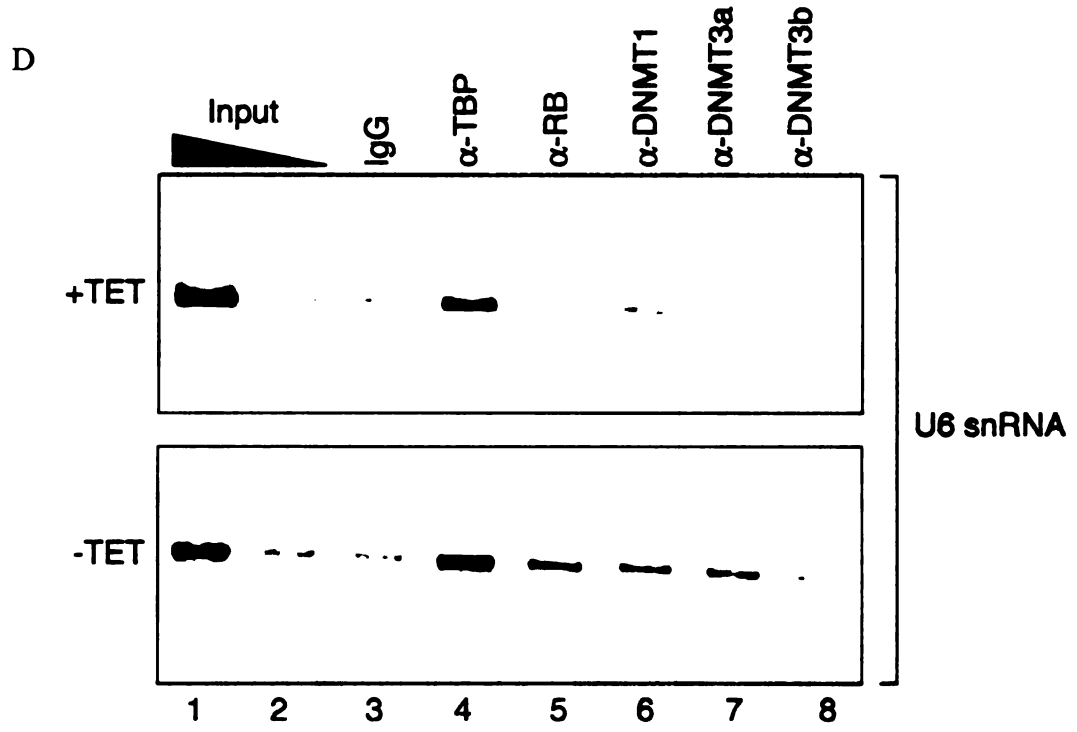
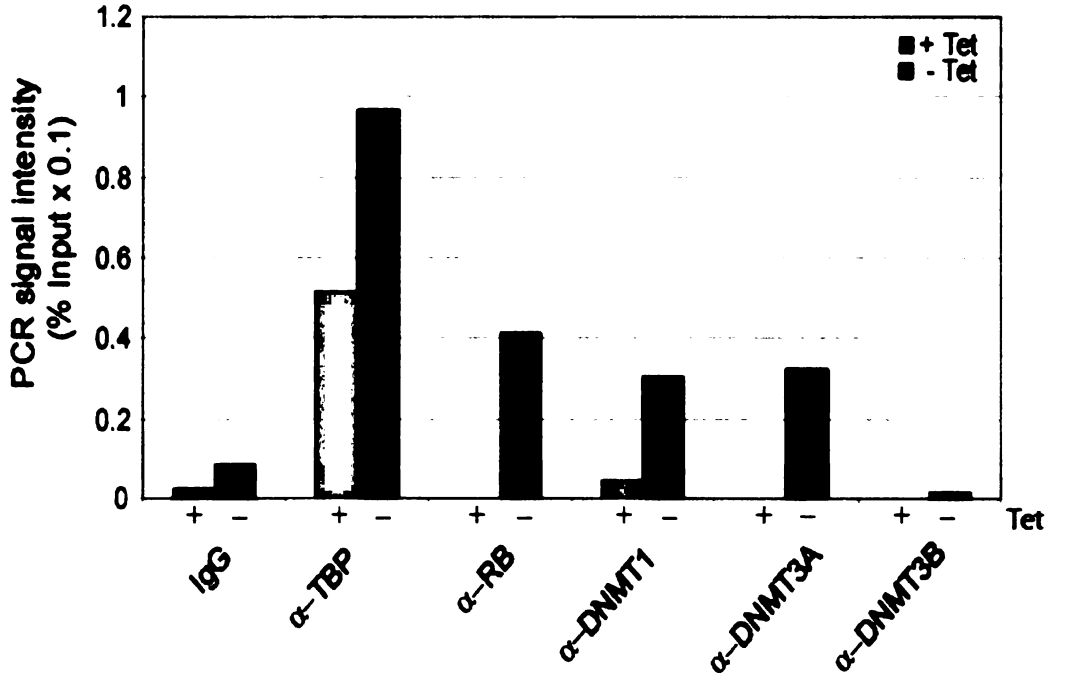


Figure 2-8 contd.





enrichment of DNMT1 and DNMT3A occupancy and a 4-fold enrichment of RB occupancy on the U6 gene were observed upon induction of RB expression and tetracycline removal.

Depletion of DNMTs in RB positive cells results in up regulation of U6 transcription

Following up on the evidence that RB can direct DNA methyltransferases to the U6 promoter, I was interested in knowing the function of DNMT activity on U6 transcription. For this, I examined the effect of si-RNA mediated depletion of the DNMT1, 3A and 3B methyltransferases on U6 transcription from a transiently transfected U6 reporter in cells containing functional RB. U6 transcription from the reporter plasmid was measured by riboprobe protection (Figure 2-9A). Depletion of DNMTs 1, 3A and 3B results in increased U6 transcription by 1.5 to 2 fold (Figure 2-9B) when compared to U6 transcription in cells treated with negative control siRNA (lane 9, Figure 2-9A) or cells treated with no siRNA (lane 2, Figure 2-9A). Panel B shows quantitation data from three measurements of U6 transcription. Figure 2-9A lower panel shows RT-PCR analysis showing corresponding reduction in levels of DNMT1, 3A or 3B in response to si-RNA treatment. At least 75% reduction in steady state mRNA levels of DNMT1 and DNMT3A and approximately 50% reduction was observed in the case of DNMT3B. Although RB was not observed to direct recruitment of DNMT3B to the U6 promoter, DNMT3B depletion led to enhanced U6 transcription. This suggests an indirect role for DNMT3B in repressing U6

Figure 2-9: Depletion of DNMT1, 3A and 3B in RB positive cells causes upregulation of U6 transcription. (A) U6 reporter plasmid was transiently transfected into MCF7 cells that were treated with two different sets of siRNA oligos each against DNMT1 (lanes 3-4), DNMT3A (lanes 5-6), DNMT3B (lanes 7-8) or with control siRNA (lane 9) or no siRNA (lane 2). Untreated (lane 2) HeLa cells also serve as negative control for U6 transcription. U6 transcription levels were analysed using riboprobe protection assay. 'U6' refers to the U6 promoter-driven transcript and 'IC' refers to internal control. Middle panel shows equal amount of total RNA in all lanes. 28S rRNA, 18S rRNA and small RNA are shown. Lower panel shows RT-PCR analysis for estimating steady state mRNA levels of DNMT1, DNMT3A, DNMT3B and actin after the indicated treatments. (B) Quantitation from 3 measurements of U6 transcription levels in response to the indicated siRNA treatments. The respective 'p values' were calculated (using two-tailed student T-test) in comparison to the 'negative control siRNA' control and was found to be <0.01 for all the DNMT siRNA treatments.

A

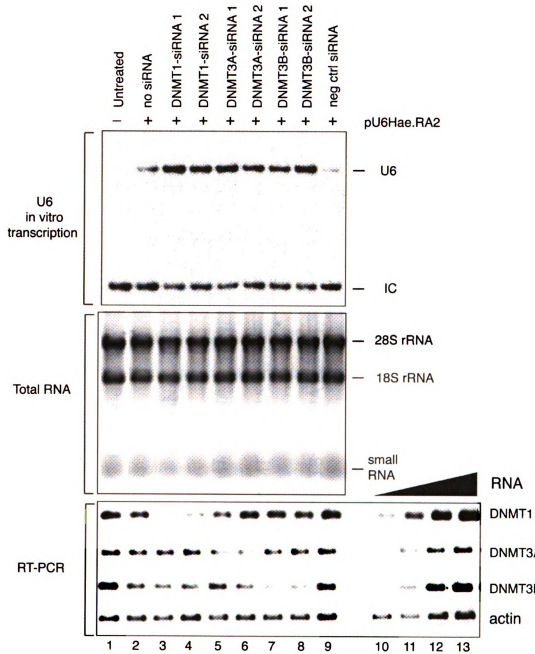
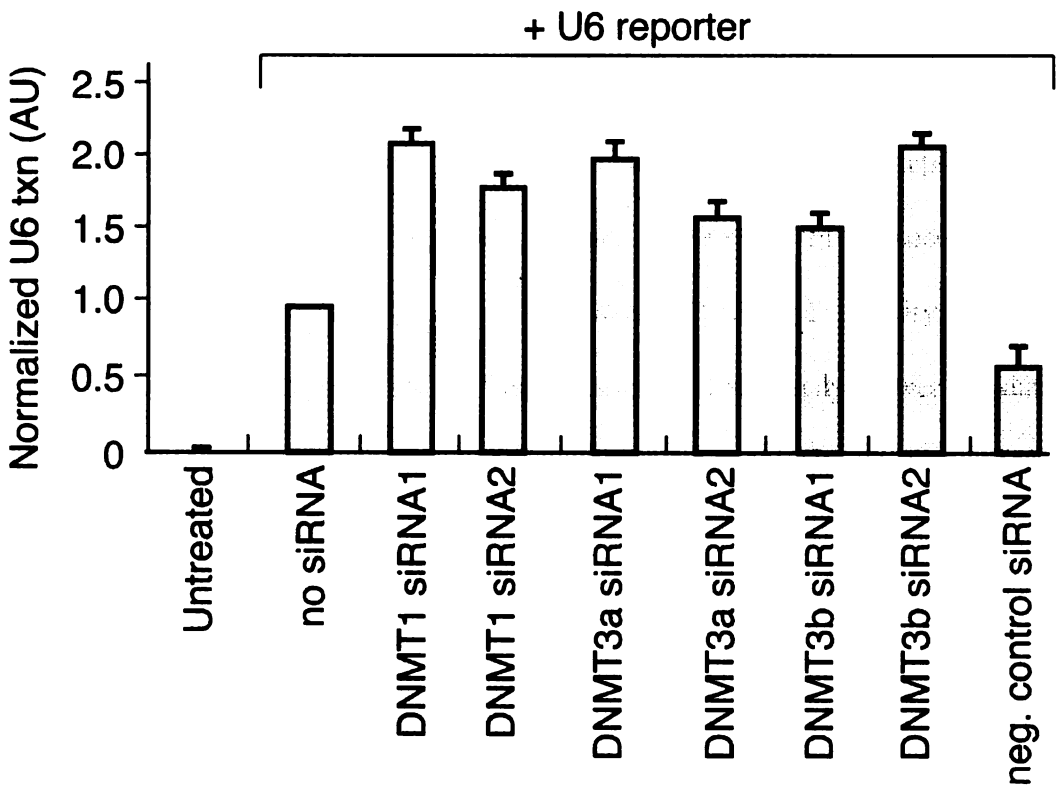

Untreated
no siRNA
DNMT1-siRNA 1
DNMT1-siRNA 2
DNMT3A-siRNA 1
DNMT3A-siRNA 2
DNMT3B-siRNA 1
DNMT3B-siRNA 2
neg ctrl siRNA
pU6Hae.RA2
U6
in vitro
transcription
U6
IC
Total RNA
28S rRNA
18S rRNA
small
RNA
RNA
RT-PCR
DNMT1
DNMT3A
DNMT3B
actin
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 2-9 contd.

B



transcription, for example, DNMT3B could repress transcription of some other gene whose product is important for U6 transcription. Another possibility could be that DNMT3B association with the U6 promoter is transient compared to DNMT1 and 3A association with the U6 promoter. On the other hand, it is possible that DNMT3B was not detected on the U6 promoter due to low efficiency of the antibody in recognizing DNMT3B. Quantitation results from three experimental measurements are shown in Figure 2-9B. Approximately 1.5 to 2 fold enhanced U6 transcription was observed in response to depletion of DNMT1, DNMT3A or DNMT3B. The 'p values' were <0.01 in the case of each of the DNMT depletions. This result indicated that DNMT activity plays a repressive role in U6 transcription. Considering the previously presented data suggesting RB directed recruitment of DNMT1 and 3A to the U6 promoter, along with this observation that DNMT activity has an inhibitory effect on U6 transcription, I propose that RB recruits DNMT activity to the U6 promoter, leading to promoter CpG methylation as part of the U6 repression mechanism.

Discussion

RB performs diverse functions in the cell, where it is involved in regulating key processes such as cell proliferation, differentiation and apoptosis. RB is a master regulator of the cell cycle and functions as a checkpoint against unwarranted cell growth and proliferation. RB exerts its anti-growth effect by acting as a repressor of transcription of a variety of genes whose products are

important for cell cycle proliferation (14, 40). RB functions as a transcriptional repressor by targeting genes transcribed by Polymerases I, II and III. RB represses general Pol I and Pol III transcription and represses Pol II transcription from certain E2F-regulated genes. Considering the varied promoter architecture and the diverse transcription machinery involved in transcribing the diverse population of genes that are targeted by RB for repression, it is evident that RB can employ diverse mechanisms to cause repression at target genes.

RB repression of Pol III transcription can play a crucial role in its tumor suppression function, as many of the Pol III transcribed products are inevitable building blocks of the cell and unregulated cell growth which is a characteristic feature in tumors necessitates high levels of Pol III products. This is supported by the observation that cancer cells had elevated levels of Pol III activity (36, 43). Keeping the levels of Pol III transcription under control can possibly be a crucial aspect of the tumor suppression mechanism. RB which is a key tumor suppressor, functions as a repressor of general RNA polymerase III transcription (43). Several lines of evidence indicate a potential link between the tumor suppression mechanism and Pol III repression function of RB (4, 16, 17, 19, 40, 43, 45, 46). An understanding of the mechanism by which RB restricts Pol III transcription can lead to important clues relating to the tumor suppression function of RB.

RB exerts its repressive effect on all the three types of Pol III promoters, although RB is found to physically associate with only the type III Pol III promoter, U6 snRNA (17). This suggests that RB repression of Pol III

transcription works by diverse mechanisms depending on the type of Pol III promoter targeted. On type 1 and type II promoters, where RB recruitment is not observed, it is likely that RB represses transcription by sequestering transcription factors Brf1-TFIIIB and TFIIIC (7, 38). On the contrary, on a type III promoter, which uses a variant TFIIIB (Brf2-TFIIIB) and is independent of TFIIIC for transcription, association of RB to promoter proximal DNA seems to be important for repression (17). My results indicate that RB association with the U6 promoter directs recruitment of DNMT1 and DNMT3A resulting in promoter DNA methylation. I have also presented evidence in vitro that a methylated U6 template is less supportive of transcription than the unmethylated template demonstrating that DNA methylation has an inhibitory effect on U6 transcription. Coherent with a repressive role for DNA methylation, siRNA-mediated knockdown of the DNMTs 1, 3A and 3B resulted in stimulation of U6 transcription in vivo in cells containing functional RB. Furthermore, RB-directed methylation of the conserved start site CpG has been shown both in vivo and in vitro. Our results indicate that DNA methylation can play an important role in RB repression of U6 transcription.

How does DNA methylation lead to repression of transcription? One possible mechanism by which DNA methylation can inhibit U6 transcription is by preventing the binding of transcription factors to their respective promoter elements resulting in failure to transcribe , as seen in the case of rDNA where UBF binding is inhibited (34). Another model for DNA methylation leading to U6

transcriptional repression could be DNMT1 and/or DNMT3A mediated recruitment of histone deacetylases to the U6 promoter resulting in a repressive chromatin state. Evidence that DNMT1 and DNMT3A associate with HDAC activity and repress target gene transcription in a TSA sensitive manner (11, 25) support this model. It is also likely that DNA methylation can lead to recruitment of Methyl CpG binding protein 2 (MeCP2) (22) which can then recruit HDACs to the U6 promoter to cause transcriptional repression (3, 24, 25). A third model for the U6 repression mechanism can involve DNA methylation induced recruitment of MeCP2 followed by histone methyltransferase activity to the U6 promoter to lead to repression. Fuks et al., (12) have reported that in the case of the H19 gene, MeCP2 facilitates H3K9 methylation to reinforce a repressive chromatin state. Histone methylation in turn can recruit HP1 and associated heterochromatin related proteins resulting in gene-silencing by heterochromatin formation (2, 20, 21). A fourth model for U6 repression is that once the DNMTs get recruited to the U6 promoter they in turn lead to recruitment of multi-protein complexes such as the NoRC complex via interaction with its TIP5 component. NoRC consists of the TIP5 and the SNF2 proteins and is involved in repression of rDNA transcription (13). TIP5 can interact with DNA methyltransferases and histone deacetylases (35, 47). SNF2 induces nucleosomal movement in an ATP and histone H4 dependent manner (37). It is possible that DNMTs that get recruited to the U6 promoter lead to recruitment of the NoRC complex which can cause nucleosomal remodeling resulting in transcriptional repression. In addition to the NoRC there can be other

complexes such as the Mi-2 complex that contains chromatin remodeling, histone deacetylation and methyl CpG binding proteins which can get recruited as result of DNA methylation at the U6 promoter resulting in transcriptional repression (39).

Further work in understanding the mechanism by which RB represses U6 transcription can be directed towards exploring whether DNA methylation can lead to recruitment of Methyl-CpG binding proteins such as MeCP2, NoRC complex and the Mi-2 complex to the U6 promoter. Any changes in the nucleosomal organization at the U6 gene that are induced in an RB-dependent manner can be examined using micrococcal nuclease digestion analysis. Knowing whether U6 promoter methylation can inhibit transcription factor binding will also be important to understanding the U6 transcriptional repression mechanism. Understanding RB repression of the highly transcribed Pol III genes will elucidate the molecular mechanism by which RB acts as a potent transcriptional repressor and its functional interactions with other co-repressor proteins, and can add to the existing knowledge about general transcriptional repression mechanisms.

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CHAPTER THREE

SUMMARY

RB represses general RNA Polymerase III transcription (17). Pol III-transcribed genes have varied promoter structures and distinct transcription factor requirements (9). Based on this, Pol III promoters have been categorized as type 1, 2, or 3 promoters (9). RB repression can function by distinct mechanisms at the different types of Pol III promoters (discussed in Chapter One) (3, 4, 13). My study is focused on understanding the mechanism for RB repression of the human U6 snRNA gene which is a type 3 Pol III promoter. On comparing the promoter proximal sequences of the nine human U6 copies, I observed the presence of CpG enrichment only in the active copies (U6-1, 2, 7, 8 and 9) but not in the inactive copies (U6-3, 4, 5 and 6) (Figure 2-1). This suggested a possible role for promoter DNA methylation in regulating U6 gene transcription. Therefore, I carried out further analyses to investigate whether DNA methylation is involved in U6 transcriptional regulation.

DNA methylation has been associated with gene silencing. A link between RB–mediated transcriptional repression of Pol II transcribed E2F target genes and DNMT function has been demonstrated by studies done by Robertson et al (8). Considering this, I questioned whether DNA methylation can be involved in RB repression of Pol III transcription. Firstly, I analysed whether there was any link between RB functional status and DNA methylation at the U6 promoter. Indeed, I

observed that in cells containing functional RB, a highly conserved start site CpG was methylated but not in cells that lack functional RB (Figure 2-2). This result indicated a positive correlation between RB functional status and promoter DNA methylation at the U6 gene.

Furthermore, to know whether the promoter DNA methylation observed in RB active cells, was directed in an RB-specific manner, I analysed the methylation status of the start site CpG after transient overexpression of RB in cells that lack functional RB (Figure 2-3). Results indicated that the start site CpG was indeed methylated upon overexpression of RB suggesting that the promoter DNA methylation was RB directed. Following this, I questioned whether the observed promoter DNA methylation directed by RB is relevant in the context of repression of U6 transcription by RB. For this, I analysed the methylation status of the start site CpG of the U6 template during repression of U6 transcription by RB in vitro (Figure 2-4). The results indicated during repression of U6 transcription, RB directs methylation of the U6 template at the cytosines in CpG as well as CpNpG sequences. This suggested that the RB repression mechanism can involve DNA methylation. To examine whether this is the case, firstly I needed to know whether DNA methylation can silence U6 transcription.

To analyze whether DNA methylation can repress U6 transcription, I performed in vitro U6 transcription with U6 template pre-methylated at all CpGs with M.Sss I methylase. The level of transcription from the methylated U6 template was compared to that from untreated or mock treated U6 template (Figure 2-5). Results from this study indicated that U6 transcription from a

methylated U6 template was reduced by 2 fold when compared to that of untreated or mock treated plasmid suggesting that methylation has an inhibitory effect on U6 transcription.

Considering that during repression of U6 transcription in vitro, RB induced methylation at the outside cytosine in the CCGG sequence at start site (inferred from MspI insensitivity), I examined the combined effect of promoter CpG methylation and methylation of the outside cytosine in the CCGG sequence. U6 template that was sequentially methylated with M.Sss I and the Msp I was used in transcription reaction in vitro. On comparing to template that was only methylated with either M.Sss I or Msp I, methylation at both cytosines in the start site CCGG sequence added to the repressive effect of CpG methylation (Figure 2-6A). The reduction in U6 transcription due to CpG methylation was about 2 fold, consistent with previous data (Figure 2-5). Methylating the U6 template with both M.Sss I and Msp I led to a reduction in U6 transcription by 3 fold, indicating that the RB directed methylation event at the U6 promoter can impede transcription. In vitro transcription with further detailed titration of the U6 template methylated with both M. SssI and Msp I was done, to ensure that the effect in transcription that I saw was in a range where the template amount was not limiting, which if it was the case can skew the number of fold reduction in U6 transcription that was observed. Results indicated that the inhibitory effect on U6 transcription due to promoter CpG methylation along with start site CCGG methylation (at both cytosines) occurred at a range where template amount was not limiting and the

results also further affirmed that the fold reduction in U6 transcription caused by methylation with M.Sss I and Msp I was 3 fold (Figure 2-6B and C).

The results so far have indicated a role for RB-directed DNA methylation in repression of U6 transcription. The next step of the analysis was on examining the role of factors that can lead to promoter methylation at the U6 gene. In mammalian cells, there are three DNA methyltransferase enzymes that are implicated in cytosine methylation, DNMT1, DNMT3A and DNMT3B. Of these enzymes, DNMT1 interacts with RB, and was shown to play a cooperative role in RB repression of an E2F target gene (8). Therefore, as a first step, I examined whether DNMT1 occupied the U6 promoter and whether there was any correlation between functional status and RB occupancy status to DNMT1 occupancy status. Comparative analysis among Hela and MCF7 cells revealed that DNMT1 occupied the U6 promoter in cells containing functional RB (MCF7) and not in cells lacking functional RB (Hela) (Figure 2-7). Furthermore, the occupancy status of DNMT1 also correlated positively with RB occupancy, for DNMT1 occupied only the U6 promoter and not the 5S rRNA promoter. Although both U6 and 5S rRNA genes are sensitive to RB repression, RB gets recruited only to the U6 promoter. This suggested that RB can possibly direct DNMT1 recruitment to the U6 promoter once it gets to the promoter. Experiment directed towards analyzing whether RB can direct recruitment of DNMT recruitment to the U6 promoter is presented in Figure 2-8.

To know the occupancy status of the other DNMTs, DNMT3A and DNMT3B also on the U6 promoter and their relationship to RB functional status,

chromatin immunoprecipitation was done from two osteosarcoma cell lines, SAOS2 (RB negative) and U2OS (RB positive). Results demonstrated that RB, DNMT1 and DNMT3A but not DNMT3B occupied the U6 promoter in RB positive cells but not RB negative cells (Figure 2-8A). This indicated that DNMT1 and DNMT3A can be involved in regulation of U6 transcription. To know whether these factors are involved in RB regulation of U6 transcription, it is important to know whether RB directs recruitment of these factors to the promoter. Induction of RB expression in a SAOS2 derived cell line resulted in recruitment of RB, DNMT1 and DNMT3A to the U6 promoter, suggesting that RB can direct recruitment on the DNMTs 1 and 3A to the U6 promoter (Figure 2-8D).

Considering that RB can recruit DNMTs to the U6 promoter, the next step was to know the effect of DNMT function on U6 transcription. For this, an siRNA-mediated depletion of DNMT1, 3A and 3B was done in RB containing cells, and the effect on U6 transcription was measured. Results show that upon depletion of either DNMT1, 3A or 3B, U6 transcription was enhanced by 1.5 to 2-fold in 3 measurements of transcript levels (Figure 2-9). This indicated that DNMT function can have a repressive effect on U6 transcription.

Results from this study have suggested the involvement of DNA methylation in RB repression of U6 transcription. RB-directed recruitment of DNMTs and associated DNA methylation as part of the RB repression mechanism have been demonstrated. The involvement of DNMT function in the RB repression mechanism on a Pol III promoter is shown here for the first time.

The role of other enzymatic functions such HDACs (2, 6, 18, 19) and SWI/SNF (10-12) in RB repression of target genes (E2F dependent) has been studied by other groups. Preliminary evidence suggesting involvement of HDACs and SWI/SNF in regulation of U6 transcription is presented in the Appendix. HDACs and BRG1 are known to play a cooperative role in RB repression of E2F target genes that were tested (19). The observation that HDACs 1 and 2 occupy the U6 promoter in RB positive cells but not in RB negative cells suggest a possible link between HDAC activity and RB function at the U6 gene (Figure AP-2). The observation that MeCP2 mediated transcriptional repression was dependent on HDAC activity indicates a functional interplay between DNA methylation and HDAC function (5, 7). Examining whether MeCP2 can recruit HDAC activity to the U6 promoter upon binding to promoter methyl CpG can discover any existing link between DNMT and HDAC activity in regulating U6 transcription. The identification of the Mi2/NuRD complex which has both HDAC and nucleosome remodeling activities and the Methyl CpG binding protein MBD3 suggests a functional link between DNA methylation, nucleosome remodeling and histone deacetylation (14-16). Also, the presence of BRG1 on the U6 promoter, the ATPase subunit of the SWI/SNF chromatin remodeling complex, suggests a potential involvement of SWI/SNF activity in regulating U6 transcription (Figure AP-3). However, further studies need to be carried out to test the role of these factors in RB repression of U6 transcription.

U6 promoter occupancy by Topo II isoforms is suggestive of potential involvement of Topo II function in U6 transcriptional regulation. Furthermore,

evidence suggesting a possible role for RB acting as an inhibitor of Topo II function has been presented in the Appendix section (Figure AP-1). Interaction between RB and Topo IIα has been reported and evidence for RB acting as an inhibitor of Topo II α function has been presented by Bhat et al., (1). Considering these details, exploring the role of Topo II function in RB repression of U6 transcription will be an exciting area for future research.

Whether DNA methylation is a hallmark of RB directed transcriptional repression or is there any specificity to the recruitment of DNA methyltransferase function to the various RB target genes can be explored in the future. Identification of factors contributing to the specificity in recruitment of DNMT function, if any, can be done. It will be useful to know the identity of other genes that are RB targets for repression that get methylated in an RB-dependent manner. The importance of RB directed DNA methylation of target genes in its tumor suppression function can be investigated. For example, it will be useful to examine whether RB directed DNA methylation of target genes is impaired in cancers caused by a loss of RB or the presence of non-functional RB. Also, taking into account the above evidence suggesting the involvement of various enzymatic functions such as DNMTs, HDACs, SWI/SNF and Topoisomerases, a compelling area for future research will be to unravel the functional interplay between these various enzymatic activities in regulating RB repression of U6 transcription.

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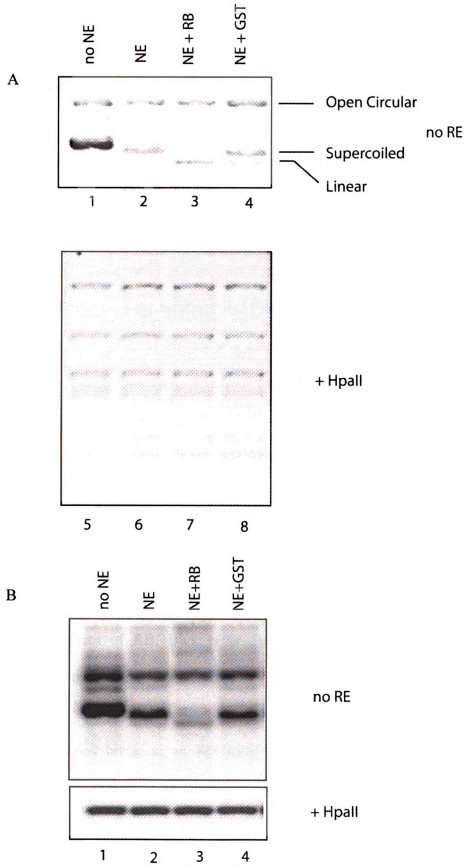
APPENDIX

AP-1. The Retinoblastoma tumor suppressor protein induces a double stranded break in template DNA

Ethidium bromide staining of template DNA recovered from in vitro transcription reactions showed that in the presence of RB, there was a loss of the supercoiled species of the plasmid template with concomitant appearance of a linear species (Figure AP-1.1 A, open circular, supercoiled and linear forms of DNA are indicated). Southern blot hybridization was done with a U6 promoter specific oligonucleotide, to affirm that the new linear species is indeed the U6 template plasmid (Figure AP-1.1 B). The results from this experiment indicate that RB induced the formation of a double stranded break in the U6 template DNA. This occurred in response to the presence of RB specifically, as this was not seen in GST treated samples or samples treated with nuclear extract only (Figure AP-1.1 A and B). There was an observed loss in ethidium bromide staining of the DNA recovered from the transcription reaction containing RB, raising the question as to whether there is loss of DNA. However, restriction digestion (with Hpa II) , followed by ethidium bromide staining and southern blot analysis of the recovered template DNA from in vitro transcription assay indicated the presence of equal amounts of total DNA in all the reactions. One explanation could be that RB-mediated inhibition of the re-ligation function of the DNA topoisomerases present across the DNA template, led to a non-uniform population of DNA fragments of a wide distribution of sizes such that the mass of DNA for any given size is below the threshold for detection for ethidium bromide.

Figure AP-1.1: RB causes a double stranded break in the U6 template DNA in vitro. (A) Ethidium bromide staining of DNA that was treated with either no NE, NE, NE+RB or NE+GST separated on an agarose gel, showing the loss of supercoiled plasmid DNA and appearance of linear DNA (lane 3) in an RB-dependent manner. Open circular DNA is also indicated. Lower panel shows DNA after Hpa II digestion of the U6 reporter plasmid that was treated with either no NE, NE, NE+RB or NE+GST indicating the presence of equal amounts of total DNA for each of the four treatments. B. Southern blot analysis showing the loss of supercoiled plasmid DNA and appearance of linear DNA (lane 3). Lower panel shows Southern blot probing after Hpa II digestion.


A
no NE
NE
NE + RB
NE + GST
Open Circular
no RE
Supercoiled
Linear
1
2
3
4
+ HpaII
5
6
7
8
B
no NE
NE
NE+RB
NE+GST
no RE
+ HpaII
1
2
3
4

However, following restriction digestion by Hpa II, these DNA fragments of different sizes get resolved into fragments of discrete sizes such that the mass of DNA for a given size is above the threshold for detection by ethidium bromide.

One explanation for RB-induced double strand break in the template DNA can be that RB leads to inhibition of Topoisomerase (topo) II function. Topoisomerases are enzymes participating in many key cellular processes such as transcription, DNA replication, chromosome segregation and may be DNA repair (1, 3). DNA Topoisomerases are classified into two broad categories-type I and type II. Topo I catalyses the breakage and passage of one strand of DNA and subsequent re-ligation (22). In the case of Topo II, both strands in the DNA helix are broken, followed by passage of one helix over another and re-ligation (1, 22). Inhibition of topo II re-ligation activity leads to double stranded breaks (6).

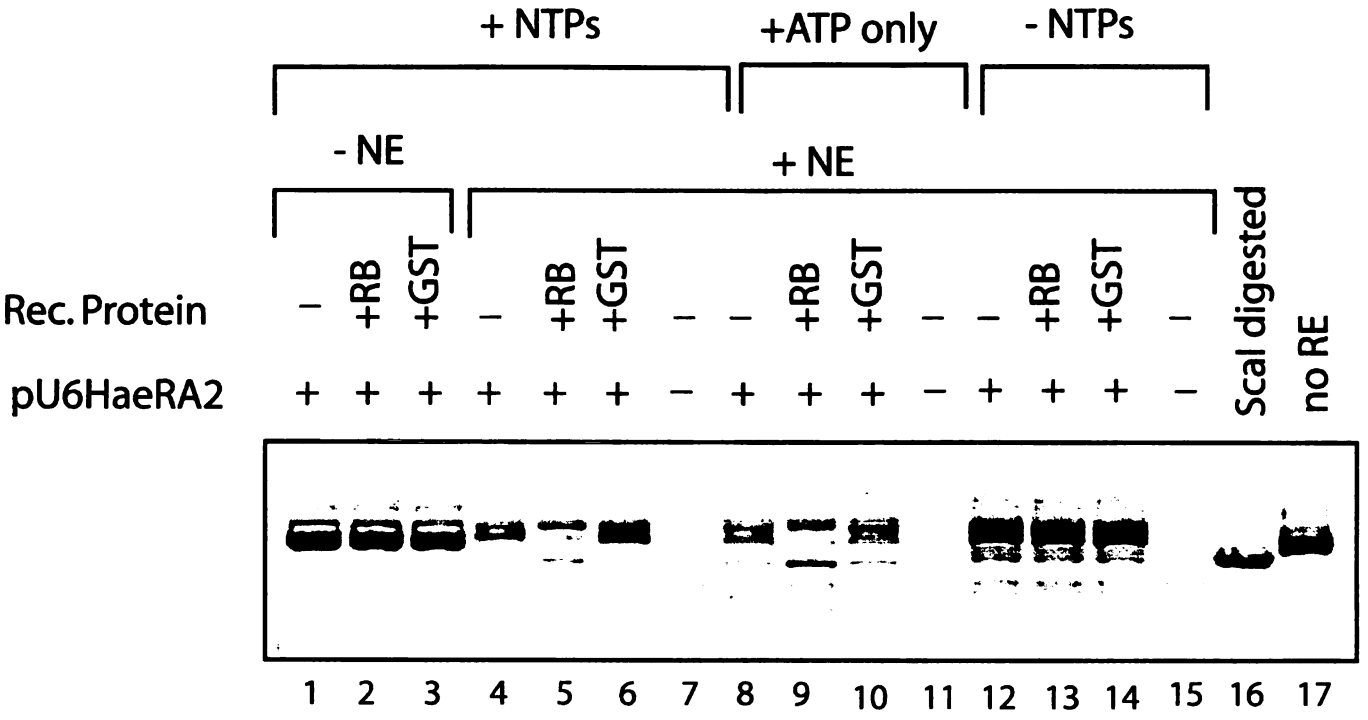
DNA topoisomerase II exists as two isoforms IIα and IIβ, (170 and 180 KDa respectively) encoded by different genes (1, 21). The action of Topo II is dependent on the presence of ATP (1, 23). Studies done by Bhat et al., (1) have indicated a physical association between RB and the human Topo IIα. In their study, Topo IIα was found to bind to the A/B pocket domain of the under-phosphorylated form of RB. Bhat et al., have also presented evidence for inhibition of Topo IIα activity by RB (1).

To further analyze the involvement of Topo II activity in the occurrence of the double stranded break in an RB-dependent manner, in U6 transcription reactions in vitro, I examined the requirement for ATP for the appearance of the linear species. Results shown in Figure AP-1.2 indicate that RB-directed double

strand DNA breakage occurred in an ATP dependent manner. In RB-containing transcription reactions carried out in the absence of NTP (lane 13, Figure AP-1.2) there was no linear DNA formation, indicating that there was no double strand break induction as seen with reaction carried out in the presence of NTPs (lane 5, Figure AP-1.2). Furthermore, the addition of ATP alone was sufficient to restore the double strand break formation (lane 9, Figure AP-1.2) suggesting that the formation of the double strand break was an ATP-dependent process. This result is suggestive of potential involvement of Topo II function in double strand break formation because Topo II enzymes function in an ATP-dependent manner (22). Furthermore, RB induced the double stranded break in a nuclear extract dependent manner. RB-containing reactions carried out in the absence of nuclear extract did not show the presence of the linear species. This suggests the possibility that RB requires the function of an additional factor present in the nuclear extract, probably topoisomerase activity. Reactions done in the absence of recombinant protein or containing GST are control reactions to demonstrate that the DNA double strand break formation occurs in an RB-dependent manner.

DNA topoisomerases II function by catalyzing a double-strand breakage reaction where a tyrosyl oxygen of the enzyme attacks the DNA phosphate backbone, resulting in the formation of a covalent phosphotyrosine link and breakage of the DNA phosphate backbone. This results in a topo-DNA complex referred to as the cleavable complex. After passage of the intact DNA strand through the break, the oxygen of the hydroxyl group that is generated in the first reaction attacks the phosphorus of the phosphotyrosine link, resulting in the

Figure AP-1.2: RB-induced double strand break is dependent on ATP. The appearance of linear DNA in an RB-mediated manner is dependent on the presence of nuclear extract and NTPs (lane 5). Reaction carried out in the presence of RB without addition of nuclear extract did not show the appearance of linear DNA (lane 2). Also reaction carried out with RB in the absence of NTPs did not show the appearance of linear DNA (lane 13). Adding only ATP was sufficient to cause the RB-mediated linearization of plasmid DNA (lane 9). Lane 16 shows linearized plasmid DNA after cutting with Sca I restriction enzyme. Lane 17 contains plasmid DNA not subject to restriction digestion. Lane 1 to 7 contain reactions carried out in the presence of NTPs. Lane 8 to 11 contain reactions carried out in the presence of ATP only (GTP, CTP and UTP are absent). Lane 12 to 15 contain reactions carried out in the absence of NTPs. Lane 1 to 3 contain reactions carried out in the absence of NE and lanes 4 to 15 contain reaction carried out in the presence of NE. Presence of recombinant protein (GST-RB or GST) is indicated. Lanes 7, 11 and 15 are reactions carried out without pU6HaeRA2 plasmid DNA.



Ethidium Bromide staining-Agarose gel electrophoresis

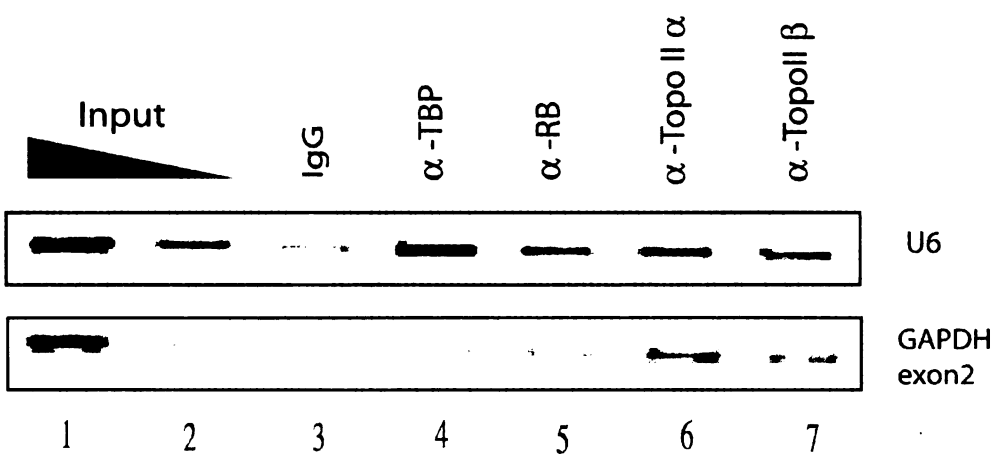
breakage of the covalent bond between topo and DNA. In this way topoisomerases unwind DNA and release torsional stress that is generated in DNA during replication or transcription (22).

Topoisomerase inhibitors fall under two categories, based on the mechanism of inhibition (22). The first type of inhibitors block enzyme catalysis without the formation of DNA breaks (eg., ICRF 193) (12). The other type of topo inhibitors work by stabilizing the topo-DNA complex (eg., Etoposide), resulting in DNA strand breaks after protein removal (12).

Results presented in Fig AP-1.1 and AP-1.2, are indicative of a scenario wherein RB inhibits topo II function by stabilizing the enzyme-DNA covalent complex which explains the appearance of a double stranded break. The observations that the RB-induced appearance of the linear DNA species is dependent on the presence of ATP and nuclear extract further implicates the role of Topo II function in facilitating the DNA breakage event. It is possible that RB inhibits Topo II function by stabilizing the enzyme-DNA complex resulting in the appearance of a double stranded break in the DNA upon protein removal.

Additional evidence suggesting that Topo II can be involved in U6 transcriptional regulation is observed in RB positive cells which demonstrate the presence of Topo IIα (lane 5, Figure AP-1.3) and Topo IIβ (lane 6, Figure AP-1.3) on the U6 promoter. Comparable enrichment of Topo IIα and moderate enrichment of Topo IIβ was also observed in GAPDH exon 2 region, probably due the widespread prevalence of topoisomerases on genomic regions possibly

Figure AP-1.3: Topoisomerases IIα and IIβ occupy the endogenous U6 promoter. (A) Chromatin immunoprecipitation was done from chromatin harvested from U2OS cells with antibodies raised against RB (lane 5), Topo IIα (lane 6) and Topo IIβ (lane 7). Immunoprecipitations done with antibodies against TBP (lane 4) and IgG (lane 3) serve as positive and negative controls respectively. PCR amplification of the GAPDH exon 2 region is also shown. Lane 1 contains 0.1% input.



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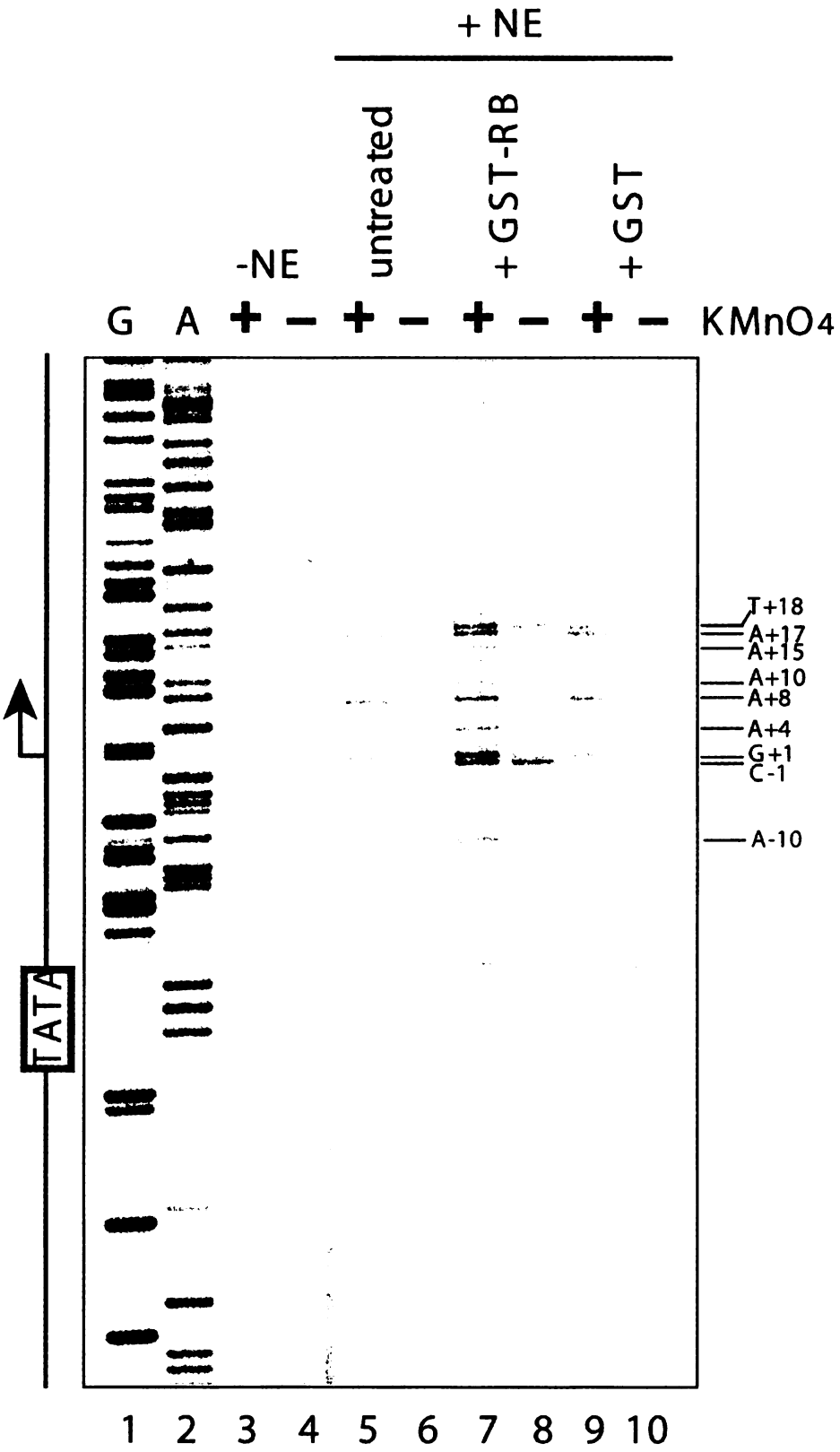
because of the involvement of topoisomerases in various cellular processes such as replication, transcription etc.

Considering the above mentioned results, it is possible that RB inhibition of topoisomerase II function contributes to repression of U6 transcription. This can occur because of an inability to release torsional stress created in DNA, due to positive supercoiling ahead of the transcription bubble as a result of DNA strand opening during transcription. It is possible that the inhibition of Topo II function can lead to resultant blocking of polymerase translocation into the transcribed region.

Indeed, studies done to detect single stranded-propensity at the U6 promoter as a measure of inhibition of promoter escape, showed evidence for RB-dependent enhancement in single-strandedness near the U6 start site region (Figure AP-1.4). $KMnO_4$ preferentially modifies thymines in single-stranded than double-stranded DNA. Therefore, $KMnO_4$ treatment of DNA involved in transcription leads to modification of thymines in open complex region (where the DNA is locally single-stranded). Following DNA recovery, primer extension with Taq DNA polymerase with the modified DNA as template results in stalling of the extending DNA polymerase at the modified thymines. On running the primer extension products on a sequencing gel, alongside dideoxy sequencing reactions, the signal corresponding to open complex can be observed. As seen in lane 5, Figure AP-1.4, the addition of nuclear extract led to $KMnO_4$ sensitivity at the U6 start site region, resulting in primer extension stall points at the indicated bases, suggestive of an 'open complex'. In the presence of RB, $KMnO_4$ sensitivity

Figure AP-1.4: $KMnO_4$ probing to detect open complex formation. Primer extension pattern from $KMnO_4$ modification of the bottom strand of the U6 promoter. 75ng of pU6Hae.RA2 was incubated with HeLa nuclear extract (NE) only (Lanes 5-6) or with NE in the presence of GST-RB (1 μg) (lanes 7-8) or GST (0.3 μg) (lanes 9-10, negative control). Reactions carried out in the absence of nuclear extract are in lanes 3-4. Transcription complex assembly and open complex formation was allowed to occur by incubating at 30° C for 30 minutes. $KMnO_4$ was then added to a final concentration of 22.2 mM and the modification reaction was allowed to proceed for 3 minutes at 30° C. Reaction was stopped by adding β-mercaptoethanol and DNA recovered by phenol extraction and ethanol precipitation. The template DNA was subjected to alkali denaturation at 80° C for 2 minutes. Primer extension reaction was then done with ^{32}P end-labeled primer DNA which anneals at ~100 bps from the start site of transcription. The extension products were then analyzed by fractionating on a 6% polyacrylamide-urea gel and the gel was then exposed to phosphor imager screen. Lanes 1-2: G, A: Dideoxy sequencing ladders. Reactions that were not treated with $KMnO_4$ served as negative controls (lanes 4, 6, 8, 10).


+ NE
untreated
+ GST-RB
+ GST
-NE
G A + − + − + − + − KMnO4
T+18
A+17
A+15
A+10
A+8
A+4
G+1
C-1
A-10
TATA
1 2 3 4 5 6 7 8 9 10

at the start site region was enhanced (lane 7, Figure AP-1.4) indicating enhanced single-stranded nature of the DNA at the U6 start site region. Reaction done with GST alone served as the negative control (lane 9, Figure AP-1.4). Another interesting observation was the presence of an RB-induced stall point at the start site in a $KMnO_4$ independent manner (indicated as C-1) (lane 8, Figure AP-1.4). One possible explanation for this observation can be the presence of a double stranded break at the start site, possibly caused due to RB-mediated inhibition of Topo II activity. This result led to a model wherein RB inhibition of topoisomerase function leads to an inability to relieve torsional stress required for transcription to proceed resulting in blocking promoter escape by the polymerase, thereby contributing to transcriptional repression. Functional interplay between other enzymatic functions such as DNA methylation, histone deacetylation, chromatin remodeling etc with Topo II activity at the U6 gene will be an interesting area for future research.

AP-2. HDACs 1 and 2 associate with the U6 promoter in RB positive cells

Earlier studies done by other groups have suggested a cooperative role for HDACs in RB repression of target genes (16, 25). Recruitment of HDACs to RB target genes in an RB-directed manner correlated with hypoacetylation and transcriptional repression (16). HDAC activity was found to be important for RB repression of cyclin E and DHFR genes (25). RB interacts with HDACs 1-3 (2, 4, 11, 16, 17). Furthermore, a link between DNMT1 and HDAC1 and RB in repressing RB target genes was reported in studies by Robertson et al., (18).

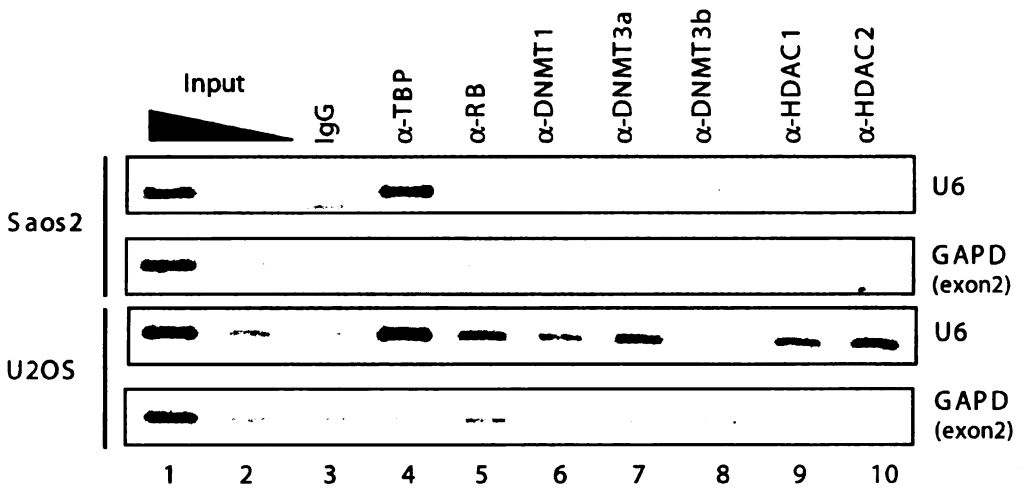
Considering this demonstrated cooperative link between DNMT and HDAC activity in RB repression and following up on studies presented in Chapter 2 where the involvement of DNMTs in RB repression of target genes was shown, I examined the potential involvement of HDACs in RB regulation of U6 transcription.

Comparative analyses using SAOS2 and U2OS cells to look for RB cofactor occupancy on the U6 promoter revealed the presence of HDAC1 (lane 9, Figure AP-2) and more prominently HDAC2 (lane 10, Figure AP-2) on the U6 promoter in RB positive cells (U2OS) and not in RB negative cells (SAOS2). Consistent with results presented in Chapter 2 (Figure 2-8A) DNMT1 (lane 6, Figure AP-2) and DNMT3A (lane 7, Figure AP- 2) occupied the U6 promoter in U2OS but not SOAS2 cells. GAPDH exon 2 serves as the negative control gene for PCR amplification. This result suggests a potential cooperative role for HDACs in RB regulation of U6 transcription. The inter-relationship between DNMT function and HDAC activity in RB repression of U6 transcription needs to be explored further.

AP-3. The SWI/SNF component BRG1 associates with the U6 promoter in RB positive cells

RB interacts with BRG1 and BRM (10, 20). The interaction between RB and BRG1 was found to be important for induction of growth arrest by RB (10). In cells lacking RB, BRG1 and BRM, induction of growth arrest by ectopically expressed RB required the presence of BRG1 expression, indicating that BRG1

Figure AP-2: DNMTs 1 and 3A and HDACs 1 and 2 occupy the endogenous U6 promoter in U2OS but not SAOS2 cells. Chromatin immunoprecipitation was done from SAOS2 or U2OS chromatin as explained above with antibodies against TBP (lane 4), RB (lane 5), DNMT1 (lane 6), DNMT3A (lane 7), DNMT3B (lane 8), HDAC 1 (lane 9), HDAC2 (lane 10) or non-specific IgG (lane 3). PCR amplification for either U6 or GAPDH exon 2 region was done.

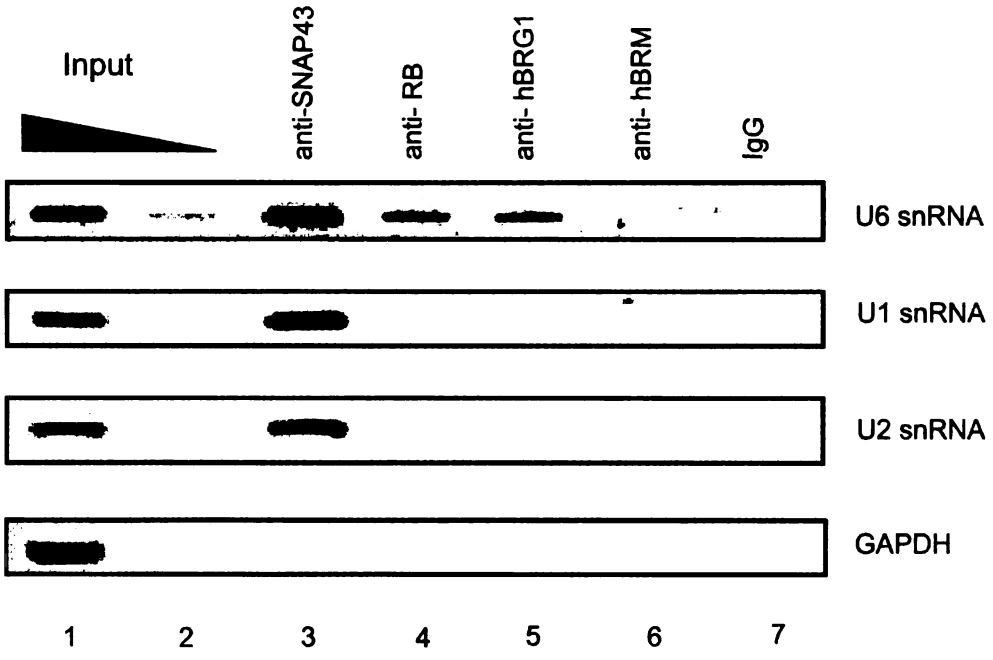


Chromatin Immunoprecipitation

co-operates in RB function (19, 25). These lines of evidence are indicative of a cooperative role for BRG1 in RB activity. Considering that RB regulates U6 transcription and is found to occupy the endogenous U6 promoter, I examined the occupancy status of BRG1 and BRM on the endogenous U6 promoter. The presence of either of these components on the U6 promoter would indicate a potential role for SWI/SNF components in U6 transcription, although further experiments need to be done to examine the functional link between RB and SWI/SNF on U6 transcriptional regulation.

Chromatin immunoprecipitation experiment performed in a normal fibroblast cell line (184B5) indicated that the ATPase subunit of the SWI/SNF chromatin remodeling complex – BRG1, occupies the endogenous U6 promoter (lane 5, Figure AP-3). As expected, RB occupied the U6 promoter (lane 4, Figure AP-3). In contrast, the BRM subunit did not occupy the U6 promoter (lane 6, Figure AP-3). BRG1 occupancy was specific to the RB targeted U6 snRNA gene only, as BRG1 did not occupy the U1 or U2 promoters. The positive correlation between RB occupancy and BRG1 occupancy on the U6 promoter is indicative of a possible co-operative role for BRG1 in RB function at the U6 promoter. Regarding the absence of BRM occupancy on the U6 promoter, I speculate that RB can preferentially recruit SWI/SNF containing BRG1 and not BRM to the U6 promoter. If so, the relevance of this selection needs to be studied further. However, there is also the possibility that the antibody raised against the BRM subunit is not efficient in detecting the factor at the U6 promoter, considering that I have not tested a positive control gene for BRM occupancy.

Figure AP-3: The SWI/SNF ATPase BRG1 occupies the endogenous U6 promoter: Chromatin immunoprecipitation was done from 184B5 cells which are normal fibroblast cells. Antibodies raised against RB (lane 4), BRG1 (lane 5), BRM (lane 6), anti-SNAP43 (lane 3) (as positive control) and IgG (lane 7) (negative control) were used for immunoprecipitations. PCR amplification was done for U6, U1, U2 promoter regions. GAPDH exon 2 serves as a negative control for factor occupancy.



Chromatin Immunoprecipitation

Immunoprecipitation performed with antibody against SNAP43 (lane 3, Figure AP-3) and IgG (lane 7, Figure AP-3) serve as positive and negative controls respectively. GAPDH exon 2 serves as the negative control for PCR amplification.

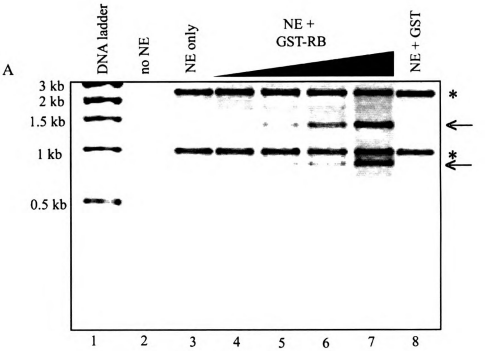
AP-4. GST-RB expressed in *E.coli* co-purifies with two predominant RNA species

Interestingly, in vitro U6 transcription experiments revealed that reactions containing GST-RB showed the presence of two RNA species, migrating at around 700 bases and 1400 bases in relation to double-stranded DNA markers (Figure AP-4A). Also, the amount of each RNA species increased with increasing amounts of GST-RB added. Additional experiments were done to confirm that these bands are indeed RNA and not DNA (data not shown). These results suggest that GST-RB co-purifies with RNA from *E.coli.*

Considering that the ratio of the size difference between these two species is two fold, it is possible that these RNA could be *E.coli* ribosomal RNA (23S and 16S). *E. coli* 23S, 16S and 5S rRNA are 2900, 1500 and 120 bases in length. The ratio of the molecular size difference between 23S and 16S rRNA is two fold suggesting the possibility that the RNA species that co-purifies with GST-RB are 23S and 16S rRNA from *E.Coli.* However, the two RNA species that co-purify with RB are observed to migrate at about 700 and 1400 bases. Possibly because of the comparison made between the migration pattern of double DNA and single stranded RNA, it is likely that these RNA species are observed to migrate at about one half their original size.

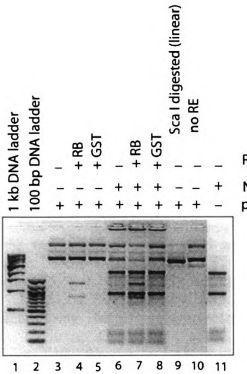
Figure AP-4: GST-RB expressed in *E. coli* co-purifies with two predominant RNA species. (A) Titration of GST-RB showing increase in the mass of the RNA species with increasing mass of GST-RB protein added. Lanes 4, 5, 6 and 7 are reactions carried out in the presence of 0.22μg, 0.67μg, 2 μg and 5.5 μg of GST-RB protein respectively. Lane 8 contains reaction carried out in the presence of 1.8 μg GST. Lane 1 contains double stranded DNA markers. Bands marked with arrows are RNA species associated with RB. Bands marked with asterisk are RNA species from nuclear extract. (B). Nucleic acids from in vitro transcription reactions under the indicated conditions were isolated by phenol extraction followed by ethanol precipitation and then separated on a 1% agarose gel. Nucleic acids were visualized by ethidium bromide staining. Lanes 1 and 2 are double stranded DNA markers. Lanes 3 to 5 contain reactions carried out in the absence of nuclear extract. Lanes 6 to 8 contain reactions carried out in the presence of nuclear extract. Lanes 4 and 7 that contain RB show the presence of two RNA species, the faster migrating band at about 700 bps of the double stranded DNA marker, and the slower migrating band at about 1400 bps.

A



Ethidium Bromide staining- Agarose gel Electrophoresis

B



Ethidium Bromide Staining-Agarose Gel Electrophoresis

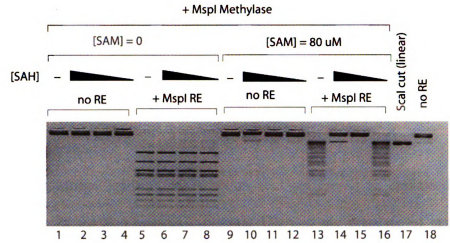
Furthermore, to confirm that the RNA species indeed originates from GST-RB, reaction containing pU6/Hae/RA.2 and GST-RB only (with no nuclear extract) were processed and analysed by ethidium bromide staining (Figure AP-4B). As seen in lane 4 of Figure AP-4B, the doublet RNA species is observed only in the reaction containing GST-RB and not in GST containing reaction (lane 5, Figure AP-4B) or reaction with no recombinant protein (lane 3, Figure AP-4B). Reactions carried out in the presence of nuclear extract are in lanes 6-8 of Figure AP-4B. Also shown are linear and supercoiled pU6/HAe/RA2 plasmid in lanes 9 and 10 respectively. Lane 11 contains reaction with nuclear extract only, showing the ribosomal RNA originating from Hela nuclear extract. Further experiments need to be done in detail towards this analysis to identify the RNA species and to examine whether mammalian RB can bind RNA. If mammalian RB is found to bind RNA (such as rRNA), it would be indicative of a novel aspect of RB function and will be interesting to analyze the role of this RNA binding property of RB in its transcription repression mechanism.

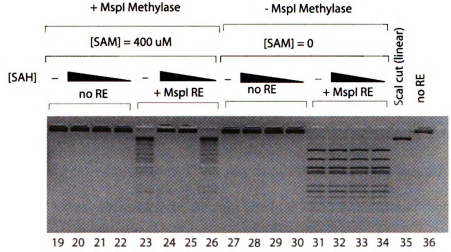
AP-5. Stimulatory effect of S-adenosyl homocysteine on Msp I methyltransferase function

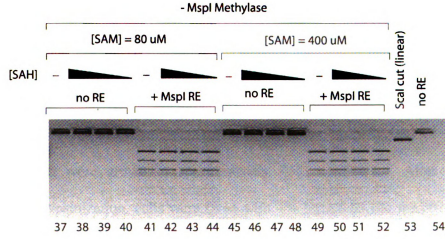
In contrast to the well-accepted idea of SAH acting as an inhibitor of methyltransferase function (5, 7-9, 14, 15, 24), surprisingly, in my in vitro methylation experiments using MspI methyltransferase, I observed an enhancement of MspI methylation function by SAH in a concentration-dependent manner. pU6 reporter plasmid was treated with MspI methyltransferase in the

presence of either 80 μM (vendor recommended amount) or 400 μM (5 fold excess than usually used for methylation) SAM. Following methylation, the plasmid DNA was recovered by phenol extraction and ethanol precipitation and then cut with MspI restriction endonuclease to analyze methylation efficiency. Methylation of the plasmid leads to impaired cutting by MspI restriction enzyme. Lane 5, Figure AP-5 shows that in the absence of SAM, methylation by MspI methylase did not occur, therefore, restriction by MspI restriction enzyme was efficient (compare to no restriction enzyme reaction lane (lane 1, Figure AP-5). In the presence of either 80 μM (lane 13, Figure AP-5) or 400 μM (lane 23, Figure AP-5) SAM, MspI methylation occurred as seen from impaired cutting by MspI restriction enzyme (compare to lane 5, Figure AP- 5). In the presence of increasing amounts (3.75 μM, 375 μM, 1 mM) of SAH (lanes 14-16 (SAM 80 μM) and lanes 24-26 (SAM 400 μM), Figure AP-5), methylation activity by MspI methylase seems to be enhanced as seen from nearly complete inhibition of restriction by MspI restriction enzyme. This effect induced by SAH is dependent on the presence of SAM and MspI methylase, as seen from lanes 6-8 (no SAM), and reactions that had no MspI methylase enzyme (lanes 42-44 (SAM 80 μM), lanes 50-52 (SAM 400 μM)). Linearized pU6/Hae/RA.2 after cutting with Sca I is also shown. This result suggests an SAH-dependent enhancement in MspI methyltransferase activity. This observation is in stark contrast to the well accepted notion of SAH acting as an inhibitor of methylation by product inhibition mechanism, as an excess of SAH, which is the byproduct of methyl transfer from SAM to cytosine by DNMTs, can prevent progress of the

Figure AP-5: S-adenosyl homocysteine (SAH) has a stimulatory effect on methyltransferase activity of MspI. Methylation reactions were done followed by DNA recovery and restriction digestion with MspI to examine methylation activity. Methylation reactions were setup in the presence of no SAM (lanes 1-8), or with 80 μM (lanes 9-16) or 400μM SAM (lanes 19-26). Reactions that included increasing amounts of SAH (3.75 μM, 375 μM, 1 mM) in the methylation reactions done with the various amounts of SAM are in lanes 6-8 (no SAM), lanes 14-16 (SAM 80 μM), lanes 24-26 (400 μM). Following methylation, DNA was recovered and restriction enzyme analysis was done with Msp I restriction enzyme or no restriction enzyme (negative control). Reactions set up in the absence of Msp I methylases serve as negative control for methylation (lanes 27-52). Linearized DNA (lanes 17, 35, 53) and untreated plasmid (18, 36, 54) are also run alongside in each lane.


+ MspI Methylase
[SAM] = 0
[SAM] = 80 uM
[SAH]
no RE
+ MspI RE
no RE
+ MspI RE
ScaI cut (linear)
no RE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18


+ MspI Methylase
- MspI Methylase
[SAM] = 400 uM
[SAM] = 0
[SAH]
no RE
+ MspI RE
no RE
+ MspI RE
ScaI cut (linear)
no RE
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36


- MspI Methylase
[SAM] = 80 uM
[SAM] = 400 uM
[SAH]
no RE
+ MspI RE
no RE
+ MspI RE
ScaI cut (linear)
no RE
37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54

methylation reaction (5). In this experiment, however, SAH seems to have a stimulatory effect on bacterially expressed and purified MspI methylase. The mechanism by which this can occur is unclear and needs further exploration.

Materials and Methods

$KMnO_4$ footprinting: Transcription reaction was performed as explained in Chapter II with 75 ng pU6/Hae/RA.2 plasmid DNA in the presence of carrier DNA (100 ng per reaction) but in the absence of rNTPs with only dATP. At the end of the 30 minutes incubation period, DNA was treated with 22 mM final concentration KMnO4 for 3 minutes at 30° C followed by quenching with beta-mercaptoethanol. DNA recovery was done by phenol extraction and ethanol precipitation. Approximately half of the recovered DNA was denatured briefly with 1 mM final concentration NaOH at 80° C for 2 mins followed by primer extension with a 5'-P^{32}-end labeled DNA oligonucleotide (16 nt in length) that anneals at ~ 100 bases upstream from the start site to the anti-sense strand (bottom strand). The primer extension products are then analysed by fractionating on a 6% Urea-polyacrylamide gel and then exposed to phosphorimager screen and then images scanned and recorded with a phosphorimager scanner (Molecular Dynamics).

Chromatin immunoprecipitation: Chromatin immunoprecipitations were done as described earlier (13). Chromatin was harvested from SAOS2 or U2OS cells that were grown to approximately 75% confluency. After harvesting by trypsinization, cells were fixed with 1% formaldehyde for 30

mins followed by washing with PBS, buffer I (10 mM HEPES pH 6.5, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100) and buffer II (10 mM HEPES ph 6.5, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl). Cells were then suspended in 1 ml lysis buffer per 10^8 cells (Lysis Buffer composition - 50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS, 0.5 μM phenylmethylsulfonyl fluoride (PMSF), 1 μM pepstatin A, 1 mM sodium bisulfite, 1 mM benzamidine, 1 mM DTT) followed by sonication to obtain soluble chromatin. Immunoprecipitation reactions were set up in dilution buffer (20 mM Tris pH 8.0, 2 mM EDTA, 1% Triton X-100, 0.5 μM PMSF, 1 mM DTT) with chromatin equivalent of 10^7 cells using 1μg antibody in a total volume of 1 ml. Immunocomplexes were recovered using Protein-G agarose beads. The beads were washed once with TSE (20 mM Tris pH 8.0, 0.1% SDS, 2 mM EDTA, 1% Triton X-100), TSE with 250 mM NaCl, TSE with 500 mM NaCl, Buffer III (10 mM Tris pH 8.0, 1 mM EDTA, 0.25 M LiCl, 1% NP-40, 1% deoxycholate) and TE (20 mM Tris pH 8.0, 2 mM EDTA). Complexes were eluted from beads with elution buffer (0.1 M NaHCO3 + 1% SDS) followed by reverse crosslinking at 65° C overnight. DNA was then recovered after phenol-chloroform extraction followed by ethanol precipitation. PCR analysis was done with primers as described previously (13). PCR products were then separated on a 2% 0.5X TBE agarose gel and images recorded with Kodak imaging software.

In vitro transcription: In vitro transcription reactions were performed as described previously (13). 250 ng of pU6/Hae/RA.2 plasmid DNA was incubated with HeLa nuclear extract with appropriate recombinant proteins. Transcription was done at 30° C for 30 mins and was stopped by adding stop mix (0.3 M sodium acetate pH 7.0, 0.5% SDS and 2.5 mM EDTA). Proteinase K digestion (20 μg/ml) was then done at 37° C for 1 hr. Nucleic acids were recovered by phenol extraction and ethanol precipitation. The nucleic acid pellet was then resuspended in 10 μl water and separated on a 1% agarose gel and visualized by ethidium bromide staining.

Southern Blot: Southern blot was done based on protocols listed in Bioprotocols (www.bio.com) contributed by Jasper Rine, University of California, Berkeley.

DNA samples from in vitro transcription reactions were recovered by phenol extraction and ethanol precipitation. The recovered DNA samples were separated on an agarose gel and visualized by ethidium bromide staining. The agarose was then prepared or transfer. Depurination of the gel was done in 0.2 M HCl for 10 mins. Denaturation was done for 30 mins in Chloride/Hydroxide solution (1.5M NaCl, 0.5 M NaOH) followed by neutralization for 45 mins in Tris/Sodium Chloride buffer (1.5M NaCl pH 7.4, 1M Tris) DNA from the agarose gel was then transferred overnight onto a hybond N membrane in 10XSSC pH 7.2 (3M NaCl, 0.3 M sodium citrate). DNA was UV cross-linked o he membrane. Pre-hybridization was done for 1 hr at 50° C in hybridization buffer (0.5% w/v) SDS, 5X SSC, 1X Denhardt solution, 0.1 mg/ml E. coli genomic DNA). P^{32} end-

labeled oligonucleotide that binds 80 bp upstream from the U6 start site was added to the hybridization buffer, and hybridization was done overnight at 50° C. After hybridization, the membrane was washed in SSC/SDS containing solution, covered with saran wrap, and then exposed to phosphor imager screen (Molecular Dynamics).

In vitro methylation: Methylation of pU6/Hae/RA2 plasmid DNA with MspI methylase (NEB) was done with indicated amounts of SAH and SAM (Figure AP-5) and methylase buffer in a total volume of 10 μl. Methylation was done overnight at 37° C. DNA was recovered by phenol extraction and ethanol precipitation and resuspended in water. DNA was then restriction digested with MspI restriction enzyme at 37° C for 2 hours. DNA was recovered by phenol extraction and ethanol precipitation and separated on a 2% agarose gel and visualized by ethidium bromide staining.

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