Transcription Coupled DNA Methylation Mediated by RNA Pol II and DNMT1

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Abstract: Transcriptional gene silencing is mediated by various epigenetic modifications including DNA methylation, histone modifications and recruitment of binding proteins that reads the methyl and other modification marks. The order in which these modifications occur followed by repressor protein recruitment remains contentious. Here, using purified protein components, we show that mammalian RNA polymerase II (RNA Pol II) is involved in DNA methylation control. DNA (cytosine-5) methyltransferase 1 (DNMT1) colocalizes, directly interacts and binds to the phosphorylated C-terminal repeat domain (CTD) of Rpb1, a major structural subunit of RNA Pol II. The association of RNA Pol II with DNMT1 during transcription enhances DNA methylation, and methylated DNA doesn't affect *in vitro* transcription. Addition of methyl CpG binding protein 2 (MeCP2), inhibited *in vitro* transcription of DNA in a methylation dependent manner, suggesting a possible mechanism for RNA Pol II coupled transcriptional silencing mediated by DNMT1 and MeCP2.

Keywords: DNA methylation, DNMT1, RNA Pol II, transcription, MeCP2.

INTRODUCTION

DNA methylation in mammals occurs by covalent modification of the carbon-5 (C^5) in the cytosine base and the majority of these modifications are located at symmetrical CpG dinucleotides sequences of the genome [1]. Methylated cytosine in the genome is essential for mammalian development. DNA methylation is maintained by several factors including three active DNA (cytosine-5) methyltransferases (DNMT1, DNMT3A and DNMT3B) and a methyltransferase like protein, DNMT3L [2-6]. Methylated cytosine (m^5C) accounts for about 1 % of total DNA bases and estimated to represent ~70-80 % of all CpG in the genome [7]. The CpGs are distributed unevenly across the human genome, but are concentrated in dense pockets known as CpG islands (CGIs), typically found at the promoter of the housekeeping genes. These CGIs are prone to aberrant DNA methylation leading to silencing of the corresponding genes during diseases, specifically in cancer. Thus maintaining CGIs free from DNA methylation is vital for normal cell development and survival. Conversely, genetic disruption of methyltransferase gene/(s) in mammalian cells also leads to aberrant methylation, chromosome aberration and activation of the G2/M checkpoint, resulting in cell cycle arrest at the G2 phase and apoptosis [8, 9]. Therefore, level of DNA methylation in mammalian genome and their distribution is a crucial parameter of cell survival.

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The dynamic nature of DNA methylation of the mammalian genome, particularly during cellular differentiation and proliferation was demonstrated using high-throughput reduced representation bisulfite sequencing [10]. In the same study, DNA methylation patterns were found to be correlated with histone methylation patterns of the chromatin, demonstrating crosstalk between DNA and histone marks. This hypothesis was further supported by previous observations of functional and physical association between epigenetic factors such as DNMT1 and histone lysine methyltransferase, G9a, during cellular processes [11, 12]. Similar genome-wide analysis of DNA methylation using bisulfite conversion and sequencing in the wild-type plant genome, particularly in gene context, demonstrated elevated CG methylation in the body of the transcribed protein coding gene and complete abolition of this methylation profile in a *met1* (a mammalian *DNMT1* homologue) null *Arabidopsis* genome [13]. Furthermore, similar distributions of 5-methylcytosine were also found in mammalian genome, linking DNA methylation with gene body.

However, maintenance methylation of the mammalian genome during successive cell division occurs during DNA replication [14]. The detailed mechanisms of inheritance of cytosine methylation are not fully understood. It was demonstrated that DNMT1 with the help of replication loading factor, proliferative cell nuclear antigen (PCNA), facilitates methylation marks on the newly synthesized daughter strands [15]. Another nuclear protein factor, UHRF1 (Ubiquitin-like, containing PHD and RING finger domains, 1), was shown to bind hemimethylated DNA and it

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was hypothesized to be a facilitator of genomic DNA methylation, since *Uhrf1* null mouse ES cells displayed impaired DNA methylation [16, 17]. Although, association of DNMT1 with the replication machinery enhances methylation efficiency, it is not strictly required for maintaining global methylation, because a DNMT1 mutant with abolished PCNA binding activity exhibited modest change in DNA methylation [18, 19]. These observations led to the hypothesis of replication independent DNA methylation, possibly through alternate mechanisms including protein-protein interaction. Previous studies demonstrated variety of transcription factors and repressor proteins including RNA Pol II binding to DNMT1 [20, 21]. Hyperphosphorylated C-terminal repeats domain (CTD) of RNA Pol II is shown to be the interacting region for DNMT1. In another study, DNA methyltransferase was also found in the peroxisome proliferator-activated receptor α (PPARα) pull-down complex from rat liver nuclear extracts. Other pull-down proteins identified in the PPARα complex included variety of coactivators and corepressor proteins that interact with PPARα and RNA Pol II to bring about the formation of preinitiation complex. Similar to multiple interacting partners of DNMT1, several histone methyltransferases, including Set1 and Set2, that catalyze methylation of histone H3 lysines Lys-4 and Lys-36, marks of transcriptionally active chromatin, are also found to be associated with phosphorylated CTD during transcription [23, 24]. Mammalian mediator complex that play a central role in RNA Pol II transcription is shown to be associated with another histone methyltransferase G9a, to mediate neuronal gene silencing in non-neuronal cells [25]. Indeed, most of the protein coding genes including the ones that are transcriptionally inactive, experience transcriptional initiation by RNA Pol II [26]. Therefore, it is plausible that RNA Pol II may play a significant role in gene expression and regulation by modulating epigenetic marks and its inheritance. In this study we demonstrate a direct interaction between DNMT1 and RNA Pol II, and further studied the implication of this association in context to DNA methylation and transcriptional gene silencing.

MATERIALS AND METHODS

Cell Culture

All cell lines (Jurkat, HeLa and COS-7) were obtained from the American Type Culture Collection (ATCC) and were grown as per recommendations. HeLa cells were treated with 0 to 0.125 μM of RNA polymerase inhibitor, α-amanitin (Sigma-Aldrich) for immunofluorescence.

Cloning of Rpb1 Subunit of Human RNA Polymerase II (hRNA Pol II) as GST Fusions

For Rpb1 cloning, cDNA were amplified from Jurkat cells and cloned at *Bam*HI–*Eco*RI sites. First strand cDNA synthesis kit (New England Biolabs (NEB), Ipswich, MA) was used for cDNA, and sub-clones were ligated to pGEX-5X-1 (GE Healthcare Life Sciences). For phosphorylation studies of CTD repeats, above fragment was cloned into pET-28a (Novagen). All GST-DNMT1 constructs have been described previously [27].

Immunoprecipitation, GST Pull-Down, and Western Blot Analysis

Nuclear extracts were made from Jurkat cells (ATCC TIB-152) as described previously [28]. Antibodies used were anti-DNMT1 (NEB), anti-RNA polymerase II (clone 8WG16, Upstate), anti-phospho-Rpb1 CTD (Ser2/5) and IgG were from Cell Signaling Technology (CST). Co-immunoprecipitation (Co-IP) and GST pull-down assay were performed as described previously [11]. Briefly, GST fusions of Rpb1 fragments and DNMT1 fragments were expressed and purified by using glutathione sepharose beads (GE Healthcare Life Sciences). Ni-sepharose (GE Healthcare Life Sciences) column was used to purify (His)6- Rbp1-CTD and glycogen synthase kinase 3 beta (GSK-3β, NEB) was used for *in vitro* phosphorylation of (His)6- Rpb1-CTD. DNMT1 was expressed and purified from baculovirus-infected Sf9 cells [29]. Purified human RNA Pol II (native complex) was purchased from Protein One.

GST pull-down assays were performed by incubating the GST or GST fusion protein beads with purified DNMT1 or RNA polymerase II complexes. Similarly, (His)6- Rpb1-CTD/ (His)6- Rpb1-phosphoCTD was used as bait in the binding buffer (50 mM Tris pH 7.5, 28 μM ZnCl2, 0.4% Triton X-100, 150 mM NaCl, 10 % glycerol) at 4 °C for 2 h. The beads were washed 3 times with the binding buffer containing 0.5 M NaCl and the protein complex was eluted with SDS sample buffer at 95°C for 5 min. Interacting proteins were visualized by western blot using anti-DNMT1, anti-RNA Pol II, clone 8WG16 and anti-phospho-Rpb1 CTD (Ser2/5) (CST).

Co-immunoprecipitation was performed with nuclear extracts from Jurkat cells. For each antibody 500 μg of nuclear extract in 1x PBS (pH 7.4) was used. Two microgram of antibody specific to DNMT1 (NEB) and phosphorylated form of RNA polymerase II (CST) were added and the mix was incubated 1 h at 4˚C. Two microgram of normal mouse IgG (Jackson lab) was used as control. Thirty μl of protein G agarose beads (NEB) washed twice with IP buffer were added to samples and mixed for further 2 h at 4°C. After three washes with cold PBS the protein complex was eluted with SDS loading buffer (NEB) at 95°C for 5 min. Western blots were performed with anti-DNMT1 (NEB) and anti-phospho-Rpb1 CTD (Ser2/5) (CST).

Gel Filtration Chromatography

Gel filtration of nuclear extract was performed using a HiPrep 26/60 Sephacryl S-200 HR (GE Healthcare Life Sciences) equilibrated in 10 mM Tris (pH. 7.5), 0.1 mM EDTA, and 500 mM NaCl using a FPLC (GE Healthcare Life Sciences). Three ml of nuclear extracts (60 mg) were loaded onto the column, 3 ml fractions collected at a flow rate of 0.5 ml/ min. HMW Gel Filtration Calibration Kit (GE Healthcare Life Sciences) was used to determine the molecular weight of the eluted peaks. Blue dextran was used for determination of the void volume of the column. Ferritin (449 kDa) and aldolase (158 kDa) were used for gel filtration profile monitoring.

Immunocytochemistry

COS-7 cells were cultured on cover slips and transfected with a mixture of plasmids and Transpass D2 reagent (NEB) at a ratio of 1 µg/3 µl. After 48 h, cells were treated with α-amanitin (0 or 10 µg/ml, Sigma-Aldrich) for 5 h. The cells were visualized using a Zeiss 200M Axiovert microscope with a 63x oil objective lens at 568 nm for DsRed-DNMT1 detection; and 460 nm for nuclear staining using Hoechst 33342. For labeling of endogenous RNA polymerase II, cells were fixed in 4% paraformaldehyde, permeabilized with 1x PBS, 1% Triton X-100 and incubated with anti-RNA polymerase II (Upstate) or anti-phospho-Rpb1 CTD (Ser2/5) (CST) antibody for overnight after blocking the slides with 5% BSA in 1x PBS-Tween 0.1%. After three washes with 1x PBS, RNA Pol II or phosphorylated RNA Pol II were revealed by using anti-mouse IgG Alexa Fluor 594 nm or anti-rabbit IgG conjugated with Alexa Fluor 594 nm (Molecular Probes) respectively.

In Vitro Transcription Assay

Total reaction mixture of 25 µl was used, containing 100 ng of C-tailed oligonucleotide template (Table **1**, [30]) 6.4 µM AdoMet, 500 µM ATP, CTP, GTP, 10 µM UTP and 10 µCi α^{33}P-UTP, with various combination of 500 ng/ 160 ng DNMT1/ DNMT1$^{\Delta 580}$, 100 ng RNA polymerase II, 5 mM/ 10 mM $MgCl_2$ in transcription buffer (20 mM HEPES, 50 mM NaCl, 10% glycerol, 5 mM DTT, 0.5% NP-40, 25U RNase inhibitor, 50 µg/ ml BSA). Details of which combination of factors added in each experiment are described in figure legends. Reactions were pre-incubated at RT for 10 min and then transferred to 30°C for 1 h. Reactions were stopped by transferring to dry ice-ethanol bath. The ^{33}P-RNA was fractionated on 15% TBE-urea gels and exposed to a Bio-Rad phosphorimager. For hemimethylated and unmethylated IVT substrate (Table **1**), the DNA strands were synthesized without 5-methylcytosine.

DNA Methyltransferase Assay and Immunodepletion

Conditions were similar to *in vitro* transcription assay except 6.4 µM S-Adenosyl-L-(methyl-^{3}H) methionine was added to the reaction [29]. Reactions that were processed for bisulfite sequencing were methylated using cold AdoMet and duplex linker ligation (linker top sense/bottom sense; linker top anti-sense/bottom anti-sense; Table **1**) was performed before they were subjected to bisulfite conversion. C-tailed duplex substrate sequence and linker sequences are shown in Table **1**, and are identical to the transcription template.

For immunodepletion, reaction mixtures were pre-incubated with anti-phospho-Rpb1 CTD (Ser2/5) (CST) and/or a carboxyterminal antibody of DNMT1 for 1 h at 4 °C in transcription buffer. 30 µl of agarose G beads (NEB) were added and incubated for 1 h at 4 °C. Bound protein-antibody complex was removed by centrifugation and the supernatant was used for methyltransferase assay.

Bisulfite Sequencing

Two hundred ng of purified duplex oligonucleotide DNA (from *in vitro* transcription/ methylation reaction) were treated with bisulfite using the epiTect Bisulfite kit according to manufacturer's instructions (Qiagen). About 20 ng of bisulfite converted DNA was used to amplify top or bottom strand of *in vitro* mock methylated and methylated oligonucleotide substrate, using primers. Amplified regions were cloned into pCR2.1-TOPO (Invitrogen) and sequenced using M13 primer. The sequencing primers are in Table **2**.

RESULTS

Direct Interaction and Colocalization of DNMT1 and RNA Pol II

Transcription coupled events in mammalian cells generally depend on the CTD of the Rpb1 subunit of RNA Pol II, which binds to multiple nuclear factors including histone methyltransferases. H3K9 methyltransferase, G9a, is shown to bind RNA Pol II in non-neuronal cells for neuronal gene silencing [25] and also interacts with human DNMT1 during cell cycle [11]. To determine if there is an interaction between DNMT1 and RNA Pol II machinery, we performed a reciprocal co-immunoprecipitation of either DNMT1 or RNA Pol II using specific antibodies along with an anti-IgG control. The immunoprecipitated complexes were western blotted and probed with either anti-DNMT1 or anti-phospho CTD/Rpb1. Indeed, anti-DNMT1 antibody was able to immunoprecipitate RNA Pol II with phosphorylated CTD and phosphorylated RNA Pol II was able to immunoprecipitate DNMT1 (Fig. **1A**). This observation speculated the presence of complex between DNMT1 and RNA Pol II. We further confirm the presence of DNMT1 and RNA Pol II in the same complex by fractionating a Jurkat nuclear extract over a gel filtration column and

Table 1. ***In vitro*** **transcription/ methylation substrate and linker sequences.**

Name	Sequence
IVT-substrate Top	GACCMGGAGTACTGTCCTCMGCTCTTTTTTTCCCTTTTTTATGCCCMGMGGMGMGCCATTAACMGCCA
IVT-substrate Bottom	TGGMGGTTAATGGMGMGCMGMGGGGCATAAAAAAGGGAAAAAAAGAGMGGAGGACAGTACTCMGGGTCAA AAAAAATTA
Linker Top sense	GGGATCGCGTAATTTTTTTT
Linker Top anti-sense	7CCCAAACGATCG ATCGAGGG
Linker Bottom sense	7CGCGATCCC
Linker Bottom anti-sense	CCCTCGATC GATCGTTTGGGGCTA

M in the sequence is either cytosine or 5-methylcytosine. Number 7 represents phosphorylated residue at 5' end.

Table 2. Bisulfite sequencing primers for top and bottom strand DNA.

Name	Sequence
IVT oligo top- sense	GGGATTGTGTAATTTTTTTTGATT
IVT oligo top- anti-sense	CCCTCAATCAATCATTTAAAACTATAA
IVT oligo bottom- sense	AAAATCACATAATTTTTTTTATCC
IVT oligo bottom- anti-sense	TTTTTGATTGATTGTTTGGGGTT

probing individual fractions with anti-DNMT1 and anti-phospho CTD Rpb1 antibody. The elution peak of DNMT1 and Rpb1 was around 450 kDa, the additive molecular weight of both the proteins (Fig. **1B**). This binding result was consistent with the co-immunoprecipitation observation. Since DNMT1 and RNA Pol II are both nuclear proteins, and are physically associated, this would suggest the possibility of their nuclear interaction and colocalization. RNA Pol II gets recruited onto the DNA in a dynamic manner for transcription initiation and the enzyme could be stalled in the presence of the α-amanitin. Therefore, we transfected mammalian cells (COS-7) with DsRed-DNMT1 fusion expression construct and examined the presence of both DNMT1 and RNA Pol II using antibody specific for Rpb1 phospho CTD. Indeed the DsRed-DNMT1 fusion protein and phosphorylated Rpb1 co-localized in the nucleus as observed by distinctive yellow spots (Fig. **1C**, merge panel).

To determine whether DNMT1 directly interacts with the RNA Pol II complex, we used purified proteins for pull-down assays. Fragments of DNMT1 encompassing the entire 1616 amino acids of DNMT1 were fused to GST and immobilized on glutathione beads. The beads bound to GST-DNMT1 fusion fragments were incubated in the presence of the active RNA Pol II complex purified from HeLa cells.

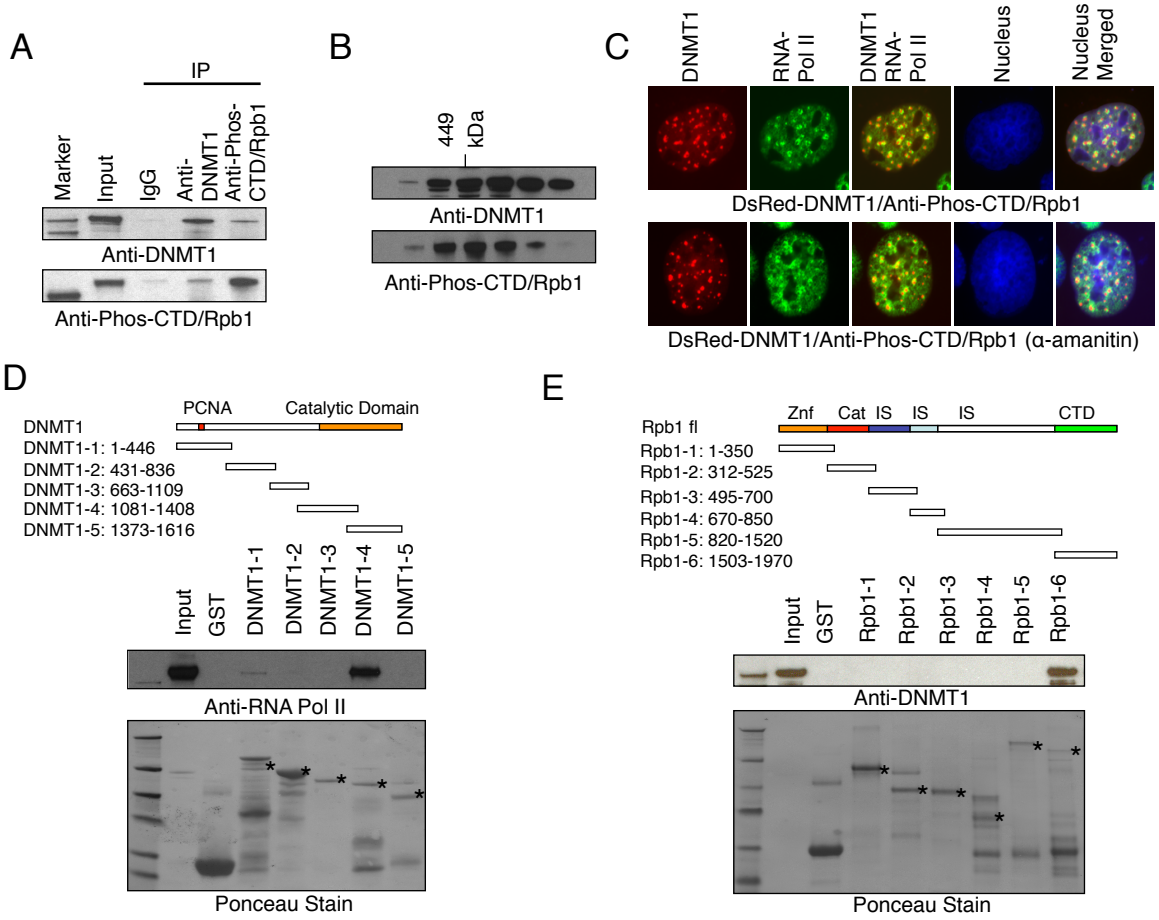

Fig. (1). DNMT1 associates with RNA Pol II in cells. (**A**) Immunoprecipitation of DNMT1 and RNA Pol II from nuclear extract of Jurkat cells. The input amount is 1/100th of the IP extract. (**B**) Gel filtration profile of DNMT1 and RNA Pol II along with the molecular weight markers at the top. (**C**) Colocalization of DNMT1 (DsRed-DNMT1) and RNA Pol II. DNMT1 shown in red and RNA Pol II in green in the nucleus of a COS-7 cell along with the yellow merged patterns. Hoechst 33342 stained nucleus in blue. (**D**) Direct interaction between DNMT1 and RNA Pol II by GST pull-down assay. (**E**) Similar to **D**, direct interaction between RNA Pol II and DNMT1. Both in **D** and **E** the target proteins are marked with asterisks, as determined by ponceau stain.

The catalytic region of DNMT1 encompassing amino acids 1081-1408 bound tightly to the Rpb1 subunit of RNA Pol II complex (Fig. **1D**). Neither of the fragments containing amino acids 663-1109 or 1373-1616 bound to Rpb1, demonstrating that the DNMT1 interaction region lies between amino acids 1109-1373. In a reciprocal pull-down experiment, GST-Rpb1 fragments_encompassing the entire 1970 amino acids were incubated with baculovirus purified catalytically active DNMT1. Indeed, DNMT1 remain bound to the CTD (1502-1970 amino acids) of Rpb1 (Fig. **1E**). Based on the above experiments we concluded that RNA Pol II and DNMT1 interact directly *in vitro* and form complexes in mammalian cells.

Phospho CTD of Rpb1 Binds Strongly with DNMT1 and RNA Pol II Complex Activates DNMT1

Structurally mammalian RNA Pol II consists of four mobile modules consisting of the core, jaw-lobe, clamp and self-module along with unstructured CTD domain [31]. Transcription coupled events generally depend on the CTD of the RNA Pol II, which recruits several transcription factors during elongation. The CTD region is a disordered structure and forms a mobile extension from the core structure of RNA Pol II and consists of heptapeptide (YSPTSPS) consensus repeats. These repeats get phosphorylated at Ser residues which creates platform for recruitment of chromatin remodeling proteins such as Set2, and this was shown to modulate H3 methylation in yeast [32, 33]. Since DNMT1 binds to CTD, it is plausible that phosphorylation may play a role in RNA Pol II-DNMT1 interaction. To validate this hypothesis, we immobilized GST fused CTD and phosphorylated it with glycogen synthase kinase 3 beta (GSK3-β). The *in vitro* phosphorylated GST-CTD of Rpb1 by GSK3-β mimics the *in vivo* phosphorylation pattern (YSPTpSPS, pS=phosphoserine) and a monoclonal antibody specific for phospho-CTD was able to recognize it validating the phosphorylation state (Fig. **2A**). The mock phosphorylated and GST-phosphorylated CTD immobilized beads were washed to remove the GSK3-β, then incubated with an increasing amount of purified DNMT1. Indeed the phosphorylated CTD bound stronger to DNMT1 than the mock-phosphorylated CTD indicating direct interaction and stronger affinity between DNMT1 and phosphorylated Rpb1 (Fig. **2B**).

To elucidate if this interaction can affect DNA methylation we performed methyltransferase assays on human genomic DNA in the presence of both DNMT1 and RNA Pol II complex (Rpb1 was phosphorylated as determined by western blot using phospho-CTD specific antibody). Approximately, a two-fold increase in DNA methylation was observed when both RNA Pol II complex containing Rpb1 and DNMT1 were present in the reaction (Fig. **2C**, lane 1 *vs* 3). Immunodepletion of either DNMT1 or RNA Pol II resulted in significant loss of methylation activity (Fig. **2C**, lane 2 and 4). Some background activity in the DNMT1 immunodepleted samples was observed possibly due to initial loading of the enzyme followed by subsequent turnover (Fig. **2C**, lane 4).

RNA Pol II Enhances DNA Methylation Independent of Transcription

Mammalian genes are transcribed from promoter elements to give rise to mature transcripts. Studies on human ES cells suggest transcription initiation from both active and inactive genes, although only the active genes get elongated to a full-length transcript [26]. Since phosphorylated RNA Pol II is involved in transcription and binds to DNMT1, we next examined if RNA Pol II mediated activation of DNMT1 is transcription dependent. To address our hypothesis we made an *in vitro* transcription template based on previous

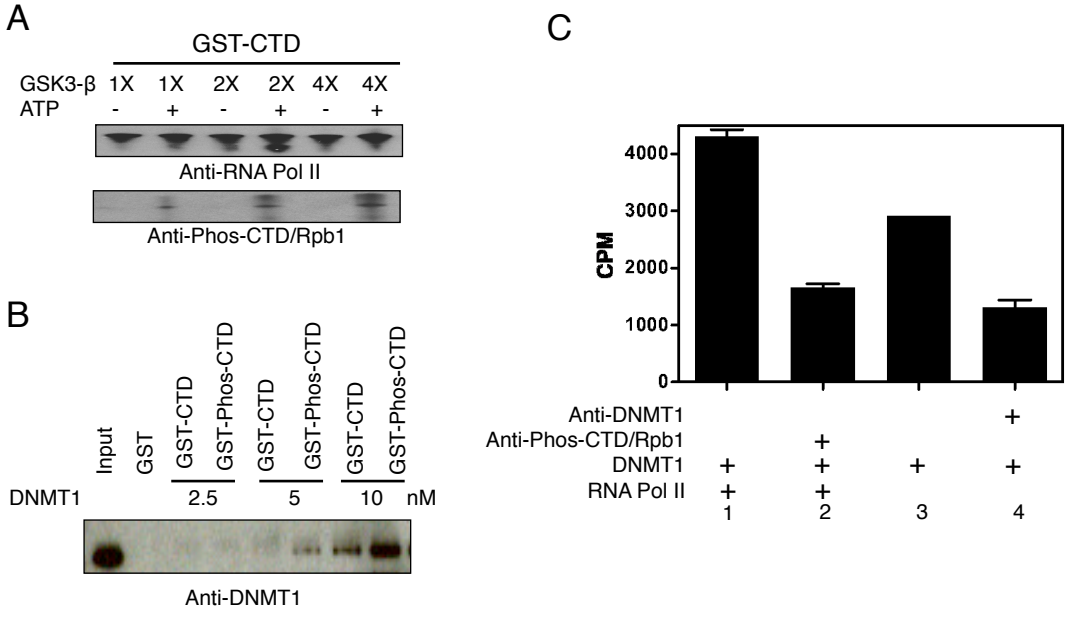

Fig. (2). Phosphorylation of CTD affects DNMT1 binding, and RNA Pol II activates DNA methylation. (**A**) GSK3-β phosphorylates CTD in the presence of ATP. (**B**) Stronger binding between phosphorylated CTD and DNMT1 full-length enzyme. (**C**) RNA Pol II activates DNA methylation by DNMT1 on human genomic DNA substrate. Immunodepletion of either DNMT1 (anti-DNMT1) or RNA Pol II (anti-phos-CTD/Rpb1) complex negate DNA methylation activation.

work of Tantin *et al.* [30], that included a stretch of CpG rich sequences, representing a portion of a CpG rich region of the human genome (Fig. **3A**, Table **1**, IVT substrate). For methyltransferase assay, we used an amino terminus deletion mutant DNMT1 ($DNMT1^{\Delta 580}$), which retained the Rbp1 binding domain and was less sensitive to the presence of Mg^{2+}, an essential ingredient for *in vitro* transcription. Indeed, we observed a four-fold increase of DNA methylation in the presence of RNA Pol II in the absence of transcription. In the presence of transcription (by addition of Mg^{2+}, Fig. **3B**, upper panel) methylation increase was about five fold as compared to DNMT1 alone. To determine if this methylation is DNA strand-specific, we purified DNA from the reactions, ligated duplex adaptor (Table **1**), bisulfite converted, PCR amplified, cloned and sequenced. In the presence of DNMT1 alone, a small percentage of CpGs were methylated. However, after addition of RNA Pol II there was a 4.4 fold increase in DNA methylation, similarly in the presence of transcription methylation increased 5.7 fold (Fig. **3C**), which correlates with the results of methylation assay (Fig. **3B**). These results would suggest that RNA Pol II enhances DNA methylation in a substrate specific manner. A similar activation of ~ 2-fold increase in DNA methylation was observed for full-length DNMT1 in the presence of RNA Pol II complex, as determined by bisulfite sequencing of the reaction products, although the efficiency of m5C incorporation was reduced due to the presence of Mg^{2+}.

DNMT1, RNA Pol II and MeCP2 Participate in Transcriptional Gene Silencing

It has been documented that the methyl-CpG binding protein, MeCP2, interacts directly with DNMT1 and forms stable complexes on hemimethylated and fully-methylated DNA [34]. Thus, one would speculate a role for a DNMT1-MeCP2 binary complex to be catalytically active on hemimethylated DNA, thereby making it fully methylated. However, this hypothesis does not imply if such complexes to be transcriptionally active, although recent data suggests RNA Pol II initiates transcription at both active and inactive gene loci [26, 35]. To determine the role of RNA Pol II, DNMT1 and MeCP2 in transcription, we performed an *in vitro* transcription assay using IVT substrate that was unmethylated, hemimethylated and fully methylated DNA in the presence or absence of the methyl donor AdoMet (Table **1**). Surprisingly, the methylated DNA was transcriptionally active and the transcription efficiency was comparable in unmethylated or hemimethylated DNA by RNA Pol II in the presence of DNMT1 (Fig. **4A**, lane 1). However, addition of MeCP2 to RNA Pol II and DNMT1 (without AdoMet) imposed gene silencing on the methylated DNA but not on unmethylated DNA, suggesting MeCP2 mediated gene silencing requires methylated DNA (Fig. **4A**, lane 2 and 3). To support MeCP2 mediated methylated DNA dependent gene silencing, we added AdoMet to unmethylated or hemimethylated DNA in the presence of DNMT1, RNA Pol

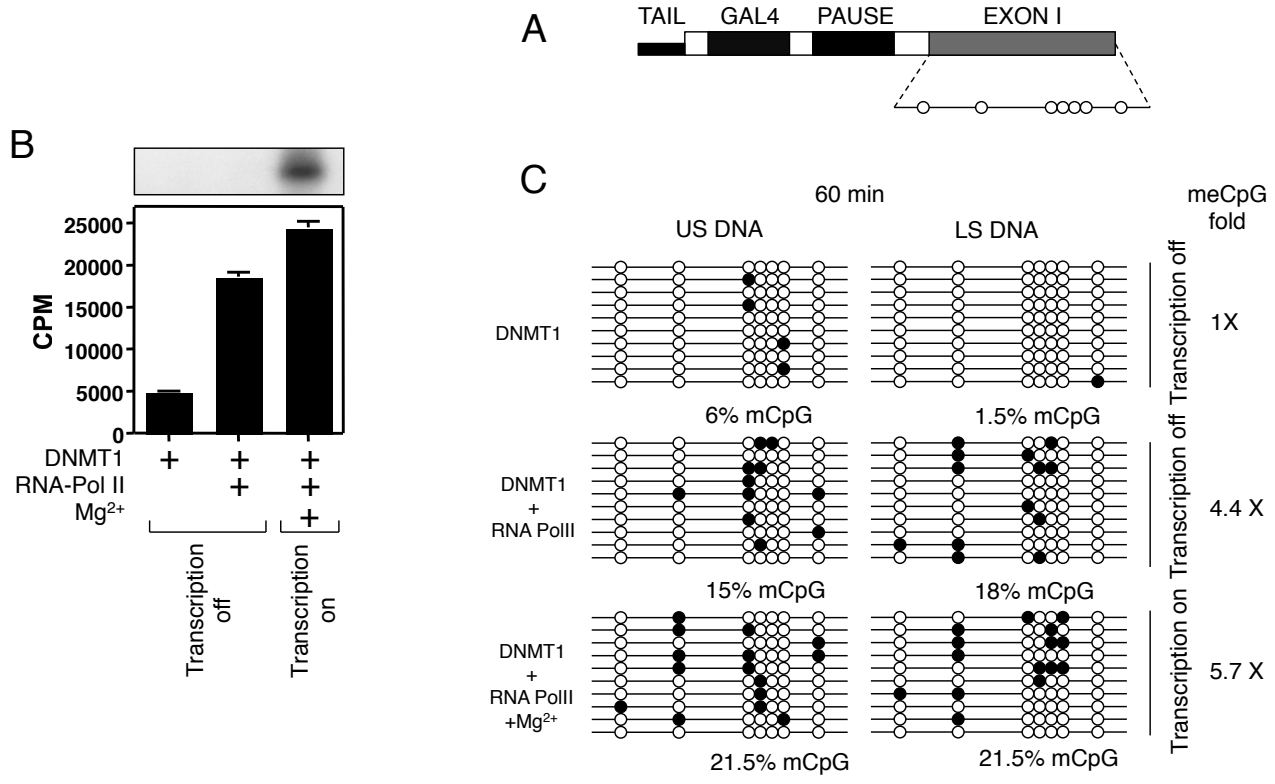

Fig. (3). DNA methylation activation by RNA Pol II is transcription independent. (**A**) A schematic diagram of the *in vitro* transcribed DNA template showing functional regions. (**B**) DNA methyltransferase activity in the presence of RNA Pol II on synthetic substrate DNA. Transcription was initiated by addition of Mg^{2+} as shown on top. (**C**) Bisulfite sequencing of both strand of the substrate DNA in the presence of DNMT1 (top), DNMT1 and RNA Pol II (middle) and DNMT1, RNA Pol II and Mg^{2+} (bottom). Folds increased in methylated CpGs are indicated at the right. US and LS are abbreviation for upper and lower strand. Rpb1 domain scheme: IS means Interaction with other Rpb Subunits.

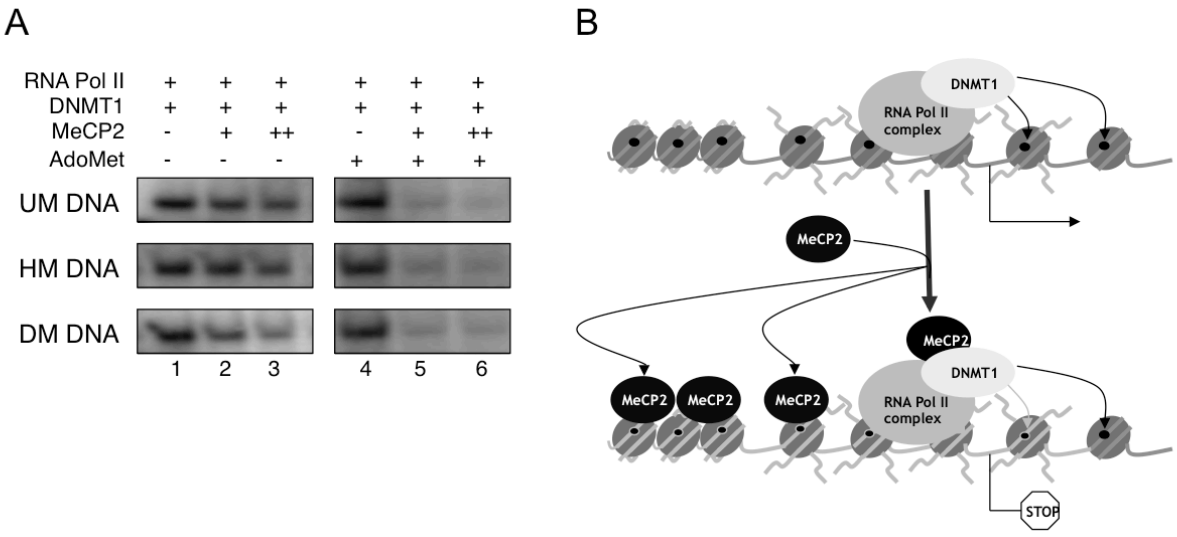

Fig. (4). DNA methylation and transcription *in vitro*. (**A**) *In vitro* transcription assay on unmethylated (UM), hemimethylated (HM) and fully methylated (DM) substrate DNA in the presence (+) or absence (-) of AdoMet. RNA Pol II and DNMT1 quantity remained constant and MeCP2 was added in an increasing concentration. (**B**) A model for RNA Pol II mediated transcriptional repression. RNA Pol II complex, DNMT1 and MeCP2 are shown. MeCp2 can bind to methylated DNA *via* recruitment through DNMT1 for accessibility, and blocks transcription.

II and MeCP2 to mimic DNA methylation and transcription. Indeed, in the presence of catalytically competent DNMT1 and active methylation both unmethylated and hemimethylated DNAs were transcriptionally silenced, demonstrating RNA Pol II mediated gene silencing may involve active DNA methylation and MeCP2 (Fig. **4A**, lane 5 and 6).

DISCUSSION

Transcriptional activation or repression in mammals occurs at many different levels, and the regulatory mechanisms governing these processes involve chromatin structure and its epigenetic status. Transcriptional activation followed by elongation is associated with specific post translational modification of the histone molecules, including the histone H3 lysine 4 trimethylation mark at the promoter [35] and the histone H3 lysine 36 trimethylation in the body of the gene [36, 37], along with generally unmethylated DNA. Experimental evidence indicates that phosphorylated RNA Pol II recruits the corresponding histone methyltransferases, such as Set2, to the target gene for modifications [32]. These observations raise an interesting question on how a transcriptionally activated gene gets silenced upon certain cellular stimuli or signaling. Our *in vitro* results suggests that elongating RNA polymerase II, that is phosphorylated, can offer a platform for DNA methyltransferase I to be recruited to the transcriptionally active genes. In our model RNA Pol II-DNMT1 complex is catalytically competent and can perform *de novo* methylation of the transcribed DNA. Indeed DNMT1 is shown to propagate robust *de novo* methylation of the CpGs, although it is still commonly classified as a maintenance methyltransferase [38]. Surprisingly, in our experiments the methylated DNA remained transcriptionally active till MeCP2 proteins were added (Fig. **4A**, **B**). This model suggests transcriptional silencing is not only a DNA methylation event per se; it also needs protein that can read these modifications and bind it tightly to prevent RNA Pol II activity. However, all the *in vitro* observations of transcriptional silencing may be interpreted with caution.

Recently, a large number of such proteins that could read methylation signatures on the chromatin have been discussed. Among them the methyl DNA binding proteins, MBD1, MBD2, MBD3, MBD4 and MeCP2 share a well-conserved methyl CpG binding domain (MBD). There are also some other proteins, such as UHRF1 that can bind to methylated DNA. Thus, methylated DNA binding proteins may provide the key to interpret the connection between DNA methylation and gene silencing. Similar to DNA methylation, lysine methylation on histone molecules, particularly H3K9 can serve as a determinant for DNA methylation, thereby inducing gene silencing. H3K9 trimethylation by SUV39H1 attracts binding of heterochromatic protein 1 (HP1). In turn, HP1 serves as an adaptor molecule to recruitment of various DNA methyltransferases. Such observation establishes HP1 as a lysine methylation monitor and DNA methylation enforcer, thereby inducing gene silencing [12]. Similar lysine methylation dependent DNA methylation is also observed in Neurospora and Arabidopsis [39, 40].

Another observation from our results is the dual role that might be played by RNA Pol II, as a transcriptional initiator and recruiter of repressor proteins. There are several previous studies that have detected RNA Pol II being associated with both transcriptionally active as well as inactive genes. Studies using genome-wide ChIP-on chip studies have revealed RNA Pol II accumulation at transcriptionally silent genes more commonly than was originally thought. Although, these sets of repressed genes do not make the full-length transcript, they make small "unstable" transcripts. It is not known if DNMT1 remain associated with RNA Pol II on these silenced genes to be an

enforcer of transcriptional repression. Indeed, transcription is shown to be a prerequisite in establishment of germline methylation marks on imprinted genes, although the extent of DNMT1's role is yet to be established [41]. Therefore, a plausible hypothesis for a DNMT1-RNA Pol II interaction may be one of the requirements for imprinting establishment, genome maintenance and methylation surveillance, that relies on DNA methylation signature in mammals (Fig. **4B**). Furthermore, transcriptional gene regulation may harbor components of DNA methylation machinery and methyl DNA readers during cell development and differentiation.

ABBREVIATIONS

DNMT1 = DNA (cytosine-5) Methyltransferase 1

RNA Pol II = RNA polymerase II

MeCP2 = Methyl CpG binding protein 2

m^5C = 5-methylcytosine

CTD = C-terminal repeat domain

CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest.

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