ROLE OF DNA METHYLTRANSFERASES IN REGULATION OF HUMAN RIBOSOMAL RNA GENE TRANSCRIPTION

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Running title: Regulation of ribosomal RNA expression by DNMTs

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We have previously demonstrated that the expression of human ribosomal RNA genes (rDNA) in normal and cancer cells is differentially regulated by methylation of the promoter CpG islands. Further, we showed that the methyl CpG binding protein MBD2 plays a selective role in the methylation-mediated block in rDNA expression. Here, we analyzed the role of three functional mammalian DNA methyltransferases (DNMTs) in regulating rDNA promoter activity. Immunofluorescence analysis and biochemical fractionation showed that all three DNMTs (DNMT1, DNMT3A and DNMT3B) are associated with the inactive rDNA in the nucleolus. Although DNMTs associate with both methylated and unmethylated rDNA promoters, DNMT1 preferentially associates with the methylated gene. The rDNA primary transcript level was significantly elevated in DNMT1−/− or DNMT3B−/− human colon carcinoma (HCT116) cells. Southern blot analysis demonstrated moderate level of rDNA promoter hypermethylation in DNMT1−/− cells and dramatic loss of rDNA promoter methylation in double knock out cells. Transient overexpression of DNMT1 or DNMT3B suppressed the luciferase expression from both methylated and unmethylated pHrD-IRES-Luc, a reporter plasmid where rDNA promoter drives luciferase expression. DNMT1-mediated suppression of the unmethylated promoter involves de novo methylation of the promoter whereas histone deacetylase 2 (HDAC2) cooperates with DNMT1 to inhibit the methylated rDNA promoter. Unlike DNMT1, both wild type and catalytically inactive DNMT3B mutant can suppress rDNA promoter irrespective of its methylation status. DNMT3B-mediated suppression of rDNA promoter also involves histone deacetylation. Treatment of HCT116 cells with decitabine (DNMT inhibitor) or trichostatin A (HDAC inhibitor) upregulated endogenous rDNA expression. These inhibitors synergistically activated methylated pHrD-IRES-Luc whereas they exhibited additive effects on the unmethylated promoter. These results demonstrate localization of DNMTs with the inactive rDNA in the nucleolus, the specific role of DNMT1 and DNMT3B in rDNA expression and the differential regulation of rDNA expression from the methylated and unmethylated promoters.

DNA methylation, histone modifications and chromatin remodeling mediate epigenetic regulation of gene expression (for review, see (1-7)). Most studies on this unique process have focused on genes transcribed by RNA polymerase II (pol II)1. Recent studies from a few laboratories including our own have shown that epigenetic mechanisms also regulate RNA polymerase I (pol I)-directed ribosomal RNA gene (rDNA) expression (8-10). Only a fraction of the multiple copies of these genes is, however, transcriptionally active at any given time (11). The promoters of active ribosomal RNA genes are devoid of CpG methylation and associate with acetylated histones (12-14) whereas epigenetically suppressed genes has reverse pattern (9,15). These studies suggest that epigenetic
mechanisms can modify the rDNA chromatin structure and control the ratio of active to silent gene (8).

Studies on the methylation profiles of rDNA in rodents and humans have resulted in unique observations. The human gene contains 19 CpGs in the upstream promoter element (UPE) and 6 CpGs in the core promoter region, whereas the mouse and rat promoters contain only one and five CpGs, respectively (9). Unlike numerous pol II-directed genes silenced in response to methylation of short CpG regions, designated CpG islands (CGI); methylation of the single CpG located at -133 (with respect to initiation site) suppresses mouse DNA expression. Methylation at this site inhibits access of the key transcription factor UBF to the upstream control region of the mouse promoter when packaged into nucleosomes. Although human rDNA promoter methylated at a single site can significantly impede promoter activity when transfected into human cells, methylation of multiple sites in the promoter region resulted in complete inhibition of the promoter activity. This observation suggests an inverse relation between promoter activity and the density of methylation (9). Further, analysis of the methylation profile of human hepatocellular carcinomas and matching normal liver tissue by bisulfite genomic sequencing showed significant hypomethylation of the rDNA promoter in tumors compared to the corresponding matching normal tissues. This is consistent with the relatively high level of ribosomal RNA (rRNA) synthetic activity of the rapidly proliferating tumor tissue (9).

Although the factors involved in the epigenetic regulation of pol II-directed genes have been well studied, such approach has not been fully used in deciphering their role in Pol I-directed ribosomal gene expression. The existence of CGI in the human rDNA promoter compared to only a few CpGs in the rodents (9), particularly in the mouse promoter (16), suggests distinct mechanism of transcriptional regulation in the two systems. Methylation at C-5 of CpG by DNA methyltransferases (DNMTs) results in recruitment of proteins, designated MBDS (methyl CpG domain binding proteins), followed by histone modifications and association of distinct chromatin remodeling factors (17,18). We have shown specific association of one of the MBDS, namely MBD2, with the endogenous methylated human rDNA promoter and suppression of this promoter by MBD following transfection (9).

Three distinct DNMTs, namely DNMT1, 3A and 3B encoded by different genes direct DNA methylation in mammalian cells (19,20). DNMT1 generally utilizes hemimethylated DNA as the substrate and is involved in the maintenance methylation. Recent studies have associated DNMT1 with methylation of unmethylated human CGIs in cancer cells (21). DNMT3A and 3B participate predominantly in the de novo methylation of unmethylated DNA (22). All DNMTs share a common catalytic domain in the carboxyl terminus, but the N-terminal domain differs significantly between DNMT1 and DNMT3A/3B. The unique N-terminal domains of mammalian DNMTs harbor several regulatory domains that mediates both protein-protein and protein-DNA interactions (for review, see (19,23)). Relatively large N-terminal domains of DNMTs can also mediate transcriptional repression of genes independent of their methyltransferase activity (24-27). The N-terminal domains of DNMT3A and 3B exhibit some homology but are distinct from that of DNMT1 (19). Here, we focused on the role of DNA methyltransferases in regulating rDNA promoter activity in both methylated as well as unmethylated state, and the involvement of the transcriptional repressor domains of these enzymes in suppressing rDNA promoter.

**EXPERIMENTAL PROCEDURES**

**Construction of plasmids:** (a) Human rDNA-luciferase vector (pHRD-IRES-Luc): Construction of the plasmid pHrD-IRES-Luc has been described earlier (9). In brief, human rDNA promoter spanning -410 to +81bp (accession number K01105) with respect to transcription initiation site was amplified from ~2kb fragments of HeLa genomic DNA digested with EcoR I and cloned into pGL3-IRES (9) to generate pHrD-IRES-Luc.
Expression vectors for mouse DNMT1, 3A and 3B: These plasmids were constructed in mammalian expression vectors pcDNA 3.1(+) (Invitrogen) to obtain pc-DNMT1, pc-DNMT3A, and pc-DNMT3B. Briefly mouse DNMT1 cDNA in pBlueScript-SK(+) (a generous gift from Dr. Tim Bestor) was digested with EcoR I and the resultant ~5.2 kb fragment was cloned into the same site in pcDNA3.1(+) to generate pcDNMT1. PCR with the vector- and insert-specific primers determined the correct orientation of DNMT1. To generate the DNMT1/ΔCAT a 1.5 kb fragment from the C-terminal of DNMT1 was excised and rest of the plasmid was religated. The DNMT1/ΔNLS plasmid is described earlier (28). Similarly, mouse DNMT3A cDNA from pSX137 (a generous gift from En Li) was excised with BamH I and Xba I and cloned in the same sites of pcDNA3.1(+) vector. The mouse DNMT3B was subcloned from the antisense clone (26) in pcDNA 3.1(-), to generate the sense clone.

Cell culture and transfection assays: Wild type HCT116 cells, DNMT1−/−, DNMT3B−/− and DNMT1−/−/DNMT3B−/− (generous gift from Dr. Bert Vogelstein) were all grown in MEM-alpha with 10% fetal bovine serum FBS). HeLa cells were grown in DMEM with 10% FBS. The transfection studies were performed as described (9).

In vitro DNA methylation: M.Hha I methylation of pHrD-IRES-Luc was performed as described (9).

Isolation of nucleoli: Nucleoli were isolated from HeLa cells following the protocol of Muramatsu et.al. (29).

Western blot analysis: Whole cell extract (200 µg) prepared from HeLa cells overexpressing different DNMTs was separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, blocked in 3% milk in Tris buffered (pH 7.5) saline (TBS). For the detection of the overexpressed proteins the membrane was subjected to immunoblot analysis with anti-Flag M2 antibody (Sigma) (30,31) and HRP-conjugated anti-mouse IgG as the secondary antibody. The antigen-antibody complex was detected using ECL™ kit (Amersham), following the manufacturer’s protocol.

Chromatin Immunoprecipitation: Formaldehyde cross-linked chromatin was prepared as described (32). For ChIP analysis, antisera against UBF (Santa Cruz), DNMT1 (NEB), DNMT3A and 3B raised in our laboratory, were used (31). The chromatin was first pre-cleared with preimmune sera-coupled to protein A/G beads followed by overnight incubation with preimmune or immune sera. The immune complex was then captured by protein A beads, washed successively with buffers as described (31,32). Immunoprecipitated DNA-protein complex was eluted, de-crosslinked, treated with RNase A and proteinase K and purified as described (31). ChIP and the input DNA were digested with Hpa II or Msp I and the digests along with equal amount of the undigested immunoprecipitated DNA were subjected to real time PCR with human rDNA promoter specific primers (9). The results are expressed as the ratio of methylated or unmethylated DNA precipitated with the antibodies to the respective input.

Indirect Immunofluorescence analysis: Immunofluorescence analysis was performed of antinucleolin monoclonal antibody (C23), anti-UBF antibody (both from Santa Cruz) and antibodies raised against recombinant DNMT3A and 3B in our laboratory or DNMT1 (New England Biolabs) as described (32). Nuclei were stained using DAPI in the mounting fluid.

DNA isolation and Southern blot analysis: Genomic DNA isolation, Hpa II / Msp I digestion and Southern blot hybridization were performed as described (26). For quantitative analysis of the Southern blot data, rDNA promoter was amplified from undigested and Hpa II/Msp I digested genomic DNA. GAPDH promoter and albumin promoter were amplified as control for complete restriction enzyme digestion and equal input DNA, respectively. The primer sequences for rDNA and GAPDH promoters
are as mentioned (9), and primer sequences for albumin promoter are as follows:

hALB-PF: 5'-TCCATTTTCTCTCCATCTCTGC-3'
hALB-PR: 5'-ATGTTCCCATTCCTGCTGTGGC-3'

Annealing temperature 52.5°C

**RNA Isolation and Northern blot analysis:** Total RNA was isolated using Trizol (Invitrogen) following manufacturer’s protocol. Total RNA (10µg) was separated on formaldehyde-agarose (1.0%) gel and transferred to nylon membrane. The membrane was then hybridized to human random-primed [α-32P]dCTP-labeled pre 47S rRNA or 18S rRNA probe in rapid hybridization buffer (GE Biotech) following manufacturer’s protocol.

**Reverse transcription and Real time RT-PCR analysis:** Reverse transcription was carried out with random hexamers and M-MuLV reverse transcriptase from 3µg of total RNA in 20µl of total volume following the manufacturer’s protocol. An aliquot of the cDNA (equivalent to 100 ng of RNA for 47S rRNA, and 10 pg for 18S rRNA) was used for real-time PCR analysis. All real-time PCR reactions were carried out using the Mx3000 Multiplex Quantitative PCR System (Stratagene). The optimum primer concentration was 150nM. All PCR amplifications were performed using Brilliant® SYBR® Green QPCR Master Mix (Stratagene) with ROX as a reference dye in a 10µl reaction volume. A standard curve for each cDNA was first generated using ten fold serial dilutions (10^8 to 10^2 copies) of the respective cDNAs as template. The copy number of each cDNA expressed was calculated from the standard curve and normalized to that of 18S rRNA. PCR cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 30s, annealing temperature for specific primer pair, 30s, and a dissociation cycle of 95°C for 60s, 55°C for 30s (to check the formation of primer dimer). Dissociation profile of the amplified products indicated that none of the primer pairs generated dimers. The primer sequences are the following:

47S (product size 92bp)
47S-F: 5'-CCTGTCGTCGGAGAGGTTGG-3'
47S-R: 5'-ACCCCACGCGCTTCCCCACAC-3'

Annealing temperature 60°C

18S rRNA (product size: 492 bp)
18S rRNA-F: 5'-TCAAGAACGAAAGTCGGAGG-3'
18S rRNA-R: 5' -GGACATCTAAGGGCATCACA-3'

Annealing temperature 57°C

**RESULTS**

DNMT1 and DNMT3B synergistically maintain the methylation profile of human rDNA promoter and regulate its expression

To identify the DNMTs involved in methylation and regulation of rDNA we took advantage of the human colon cancer cell line HCT116 in which homologous recombination disrupted both alleles of DNMT1 (DNMT1/ or MT1KO cells) or of DNMT3B (DNMT3B/ or 3BKO cells) or both alleles of DNMT1/DNMT3B (DKO cells, Double Knock Out) (33). Western blot analysis confirmed absence of respective proteins in null cells, whereas the third functional enzyme DNMT3A was detectable in all three cell lines (Fig. 1A).

Next we addressed whether any alteration in methylation status of rDNA promoter occurred in DNMT1 or DNMT3B null cells. Hpa II (methylation sensitive) or Msp I (methylation insensitive) digested genomic DNA from the wild type and knock out HCT116 cells were subjected to Southern blot analysis with rDNA promoter specific probe. The probe harbors 4 Hpa II/Msp I sites (Fig.1B). DKO cell genome exhibited significantly reduced methylation compared to MT1KO or 3BKO as evident from ethidium bromide staining of Hpa II digested DNA (Fig.1C, compare lanes 2,5,8&11), and is consistent with the earlier report (33). Extensive hybridization of rDNA probe to low molecular weight DNA fragments in Hpa II digested DNA in DKO cells showed dramatic hypomethylation of rDNA in these cells compared to the parental cells (Fig.1D, lanes 11 and 2). In contrast, small but significant hypomethylation of the rDNA promoter was observed in MT1 KO cells (Fig.1D, lanes 5 and 2) whereas 3BKO cells did not reveal any noticeable hypomethylation (Fig.1D, lanes 8 and 2). Comparable hybridization profile of DNA digested with methylation-insensitive
isoschizomeric Msp I in all four cells indicate that different profile in Hpa II digest is because of differential methylation of the promoter (Fig.1D, lanes 3, 6, 9, 12). Significantly reduced rDNA promoter methylation in DKO cells relative to other cell lines suggests that both DNMT1 and DNMT3B are essential to maintain its methylation profile. For quantitative analysis of this co-operative effect, rDNA promoter was PCR amplified from undigested (U), Hpa II digested (H) and Msp I digested (M) genomic DNA using rDNA specific primers (Fig.1B). This data showed (Fig.1F, lanes 1 & 2) that 28% of the rDNA promoter in HCT116 cells is unmethylated. DNMT1 disruption resulted in additional 22% demethylation (Fig 1F, compare lanes 2 & 5). DNMT3B deletion alone resulted in additional 10% demethylation (compare lanes 2 and 8). In DKO cells 76% of rDNA promoter is demethylated, demonstrating additional 48% demethylation (76%-28%) in these cell lines following disruption of DNMT1 and DNMT3B. To confirm complete restriction enzyme digestion, GAPDH promoter harboring 2 HpaII/MspI sites was amplified from the same digest (9). GAPDH was amplified only from the undigested DNA from each cell line. Albumin promoter fragment that does not span any HpaII/MspI site was amplified equally well from each DNA confirming comparable amount of DNA in PCR reactions (Fig 1E). This data further authenticates the Southern blot analysis showing synergism between DNMT1 and 3B in maintaining rDNA promoter methylation profile.

We then determined whether DNMT gene disruption alone or in combination had any effect on rDNA expression in HCT116 cells. Northern blot analysis of the total RNA with a probe complimentary to 47S precursor rRNA, the primary transcript, revealed at least two-fold increase in its level in both MT1KO and 3BKO cells compared to the parental or DKO cells (Fig.1G). Quantitative analysis of the rRNA transcript by real-time RT-PCR showed a 2- and 3.4-fold increase in the primary transcript in MT1KO and 3BKO cells respectively (Fig.1H). Significant increase in precursor rRNA level in MT1KO cells correlated with hypomethylation of its promoter (Fig.1D,E). Striking elevation of rRNA transcript in 3BKO cells suggests that DNMT3B regulate rDNA transcription by a methylation independent mechanism, since rDNA promoter did not undergo significant hypomethylation in 3BKO cells (Fig.1D,E). No significant change in 47S rRNA level in DKO (Fig.1H) cells despite remarkable hypomethylation of the promoter (Fig.1D,E) could be attributed to the detrimental effect of global hypomethylation on growth regulatory genes that significantly compromised cell growth (33). Increased rRNA level in MT1KO and 3BKO cells implicates their role in regulating its expression in vivo.

DNMT1 and DNMT3B localize in the nucleolus and associate with rDNA promoter

Immunofluorescence (IF) analysis and biochemical fractionation demonstrated localization of DNMTs in the nucleolus (Fig. 2A). These proteins also colocalized with nucleolin, a nucleolar marker, since the two stains (FITC and TRITC) merged (panels e, j and o). Further, double staining of cells with anti-UBF and anti-nucleolin antibodies (panels p-t), showed UBF localization at the center of the nucleoli, and peripheral nucleolin staining. Similarly, dual staining of cells with DNMT1 and UBF antibodies demonstrated distinct localization of these two proteins within the nucleolus (panels u-y). This is probably due to association of UBF primarily with the active promoter and predominant localization of DNMTs with the inactive rDNA promoter. Further, biochemical fractionation coupled with Western blot analysis demonstrated presence of DNMT1, 3A, 3B as well as nucleolin in the purified nucleolar fraction of HeLa cells (Fig. 2B). As expected, DNMTs are also present in the nuclear fraction. The absence of RNA polymerase II in the nucleolar fraction ruled out possible contamination of this fraction with nucleoplasmic proteins (Fig.2B). These observations support the notion that DNMTs are present in the nucleoli and associated with inactive rDNA.

Mammalian cells harbor ~400 copies of rDNA some of which are methylated in somatic cells (34,35). To determine the nature of association of DNMTs with the rDNA promoter we performed ChIP-CHOP assay. HeLa cells express all three
DNMTs (28). DNA pulled down by specific antibodies as well as the input DNA was digested with Hpa II, Msp I or mock-digested prior to amplification and analysis (fragment shown in Fig.1B). This amplified promoter region harbors four Hpa II/Msp I sites. DNA pulled down was quantified from the standard curve generated using serial dilution of the input DNA. The Hpa II resistant PCR product reflects the level of the rDNA promoter, methylated at all four sites (M). The PCR product from the undigested DNA minus that from Hpa II resistant product represents the level of unmethylated promoter (U). PCR product was not generated from Msp I digested DNA since it is a methylation insensitive enzyme. Real-time PCR data revealed that all 4 Hpa II sites of only 20% percent of the total input DNA are methylated in HeLa cells. DNMT1 association with the methylated promoter was 30-fold higher than rest of the promoter (Fig.2C). On the contrary, DNMT3B was almost equally associated with both unmethylated and methylated rDNA promoters (Fig.2C, lanes 5,6). Like DNMT1, DNMT3A associate predominantly with methylated promoter (lanes 7,8). As a control, we performed ChIP assay with anti-UBF antibody, which showed exclusive association of UBF with large fraction of unmethylated promoter (lanes 9,10), demonstrating the stringency of the ChIP assay. Immunoprecipitated chromatin in absence of the antibody (control) showed minimal amplification of rDNA promoter (lanes 1-3). Thus, all three DNMTs associate with methylated rDNA population, which confirms localization of DNMTs with the inactive rDNA at perinucleolar heterochromatin (Fig. 2A).

**DNMT1 and DNMT3B suppress rDNA promoter activity**

To determine the role of DNMTs in regulating rDNA transcription, pHrD-IRES-Luc and mammalian expression vectors for Flag–tagged DNMT1, 3A or 3B were transiently transfected in HeLa cells (9). Western blot analysis demonstrated comparable levels of ectopic DNMT3A and 3B expression, whereas DNMT1 expression was significantly less (~50%) than that of DNMT3A/3B (Fig.3A). Expression DNMT1 or DNMT3B resulted in significant downregulation of the rDNA promoter activity (50 and 60% respectively, Fig.3B). Under this condition, DNMT3A did not exhibit any inhibitory effect on the promoter. Similarly, the Hha I methylated rDNA promoter was significantly inhibited by DNMT1 and DNMT3B (60 and 72% respectively, Fig.3B) while DNMT3A did not have any effect.

**Methyltransferase activity of DNMT1 is essential for inhibition of unmethylated rDNA promoter**

To decipher the mechanism of DNMT1 mediated repression of rDNA promoter several deletion mutants of DNMT1 were generated (Fig.4A). DNMT1 is a large protein comprising of 1620 amino acids with a large N-terminal regulatory domain and relatively smaller C-terminal catalytic domain (Fig.4A). The N-terminal domain harbors a region that interacts with various proteins (24,36), and different targeting motifs (37-39) (Fig.4A). A bromo homology domain containing two BAH (Bromo Adjacent Homology) motifs is located adjacent to the Zn binding domain which is probably involved in protein–protein interaction (for review see (40)). Recently we have shown that nuclear localization of DNMT1 requires both NLS and BAH domains (28). We generated the catalytic domain deletion mutant (DNMT1/ΔCAT) where ~400 amino acids from the C-terminal catalytic domain was eliminated. In a second deletion mutant the N-terminal 350 amino acids were deleted, which removed the nuclear localization signal with PCNA and DMAP1 interacting regions (DNMT1/ΔNLS).

HeLa cells were transfected with mock-methylated rDNA promoter along with expression vectors for different DNMT1 deletion mutants. The luciferase assay revealed that only the wild type DNMT1, but not DNMT1/ΔCAT or DNMT1/ΔNLS was able to impede activity of the mock-methylated promoter (Fig. 4B). This data suggests that the catalytic activity of DNMT1 is critical for inhibiting unmethylated promoter activity. Inability of a catalytically inactive point mutant (DNMT1CS) (Fig.4A) (28) to impede pHrD-IRES-Luc activity reinforces the notion that DNMT1 inhibits the promoter activity by catalyzing its methylation
(Fig.4B). To confirm that the exogenous promoter was indeed methylated by ectopic DNMT1, pHrD-IRES-Luc purified from the transfected cells was digested with Hpa II or Msp I and subjected to amplification of the rDNA promoter fragment. A 10-fold increase in the Hpa II resistant PCR product from cells expressing ectopic DNMT1 compared to that from the vector-transfected cells suggests significant methylation of the rDNA promoter by DNMT1 (Fig.4C, lanes 5 and 2). Since Msp I cleaved the plasmid irrespective of its methylation status, there was no PCR product obtained after Msp I digestion. This data demonstrates that DNMT1-mediated rDNA promoter methylation is necessary to suppress the promoter activity which is in accordance with increased rDNA transcription concomitant with its promoter hypomethylation in MT1KO cells (Fig.1D & E).

**DNMT1 suppresses the activity of the methylated rDNA promoter in absence of the catalytic domain**

We previously demonstrated significant inhibition of the rDNA promoter activity when M.Hha I methylated CpG dinucleotides are present in upstream control element (UCE) and in the further upstream region (9). Interestingly, ectopic expression of DNMT1 further suppressed the methylated promoter (Fig.3B & 5A). To get mechanistic insight of this process HeLa cells were co-transfected with methylated pHrD-IRES-Luc along with the expression vectors for different DNMT1 deletion mutants. The results showed that DNMT1/ΔCAT and DNMT1/CS exhibited similar repressive effect (52% and 58%) as the wild type DNMT1 (56%, Fig.5A). No significant repression was, however, noted upon DNMT1/ΔNLS expression, as the N-terminal truncation retained the protein in the cytoplasm (28). This data reveals the role of DNMT1 transcriptional repressor domain in impeding methylated rDNA expression.

The N-terminal domain of DNMT1 interacts with various co-repressors that include HDAC1/2. To determine if HDAC plays any role in the DNMT1-mediated suppression of human rDNA gene transcription, HeLa cells were first transfected with methylated pHrD-IRES-Luc with or without DNMT1 expression vector. The cells were then treated with the HDAC inhibitor trichostatin A (TSA). As observed earlier, ectopic expression of DNMT1 inhibited rDNA promoter activity (Fig.5A, Fig. 5B, Bottom panel: promoter activity in vector or Dnmt1 transfected controls). However, treatment of the cells with 100nM TSA for 24 hours resulted in ~ 4-fold increase in the promoter activity even in DNMT1 overexpressing cells (Fig.5B). Dnmt1 overexpressing cells showed comparable Dnmt1 expression in presence and absence of TSA (Fig5B). This data suggests that DNMT1 mediated suppression of the rDNA promoter involves HDACs.

Next, HDAC1 or HDAC2 was expressed alone or co-expressed with DNMT1 along with methylated pHrD-IRES-Luc in HeLa cells. To determine if there is any synergy between DNMT1 and HDACs, expression of DNMT1 was titrated to a level where repression of the promoter by DNMT1 alone was < 35%. HDAC2 inhibited the methylated promoter by 25% whereas in combination with DNMT1 the activity was suppressed by 70% (Fig.5C). This data suggests that HDAC2 cooperate with DNMT1 to downregulate rDNA promoter. On the other hand, HDAC1 did not modulate the effect of DNMT1 on the promoter (Fig.5C).

**DNMT3B mediated suppression of rDNA promoter does not require its catalytic activity**

Disruption of DNMT1, DNMT3B or both in HCT cells displayed differential effect on rDNA transcription and methylation profile of the promoter. Ribosomal RNA transcript level in DNMT3BKO cells was significantly elevated without noticeable demethylation of its promoter (Fig.1C, D). To address the underlying mechanism, HeLa cells were transfected with wild type and catalytic site mutant of DNMT3B (CS mutant) along with mock- or M. Hha I-methylated pHrD-IRES-Luc. Both the wild type and mutant DNMT3B significantly inhibited the promoter activity irrespective of its methylation status (average 50%, See Fig.6A). The expression of ectopic wild type and mutant DNMT3B was comparable (Fig.6A, lower panel). This data suggests DNMT3B repress both the
endogenous rDNA promoter in the chromatin context (Fig.1D) and the transfected promoter independent of its catalytic activity.

The N-terminal domain of DNMT3B protein does not exhibit any similarity with that of DNMT1 (41). Besides a nuclear localization signal, DNMT3B has a well-defined PWWP domain involved in DNA binding (42) and an ATRX homology domain that mediates protein-protein interactions (25,43) (Fig.6B). To identify the domain(s) of DNMT3B involved in the transcriptional repression of rDNA promoter HeLa cells were co-transfected with pHrD-IRES-Luc and different deletion mutants of mouse DNMT3B (ΔC-terminal, ΔN-terminal, ΔPWWP and ΔATRX) (26). Expression of the truncated and wild type proteins was monitored by Western blot analysis (Fig.6C). As observed earlier, both the wild type and ΔC-terminal DNMT3B inhibited the unmethylated rDNA promoter to comparable extent (~50%). However, ΔPWWP, ΔATRX or ΔN-DNMT3B could not suppress the promoter activity (Fig.6D). Similar inhibitory effect of the DNMT3B mutants was also noted with the methylated promoter (data not shown). Thus both PWWP and ATRX domains of DNMT3B are essential to suppress the rDNA promoter.

Since DNMT3B interacts with HDACs through ATRX domain we investigated whether HDAC inhibitor can alleviate its repression on rDNA promoter. Treatment of HeLa cells co-transfected with pHrD-IRES-Luc and DNMT3B empty vector with 100nM TSA for 24 hours resulted in ~5-fold increase in both mock- and methylated-promoter activities (Fig.6E). The unmethylated and methylated promoter activities increased 3.7 and 4-fold respectively when DNMT3B expressing cells were treated with TSA. This data implicates that HDAC(s) is one of the co-repressors recruited by DNMT3B to inhibit rDNA promoter activity.

Decitabine and Trichostatin A synergistically activate the methylated rDNA promoter
Inhibitors of DNMTs and HDACs act synergistically to upregulate some pol II-driven promoters that are methylated and silenced (31,44). It was, therefore, of interest to explore whether these two inhibitors exhibit similar effect on the rDNA promoter. To test this possibility, HCT116 cells were treated with decitabine (50nM) alone or a combination of decitabine and TSA (100nM). Decitabine at higher dosage displayed toxic effect on HCT116 cells. Real time RT-PCR analysis demonstrated increased expression of 47S rRNA in decitabine treated cells, which was further upregulated by TSA exposure (Fig.7A). Similar result was obtained when HeLa cells were treated with decitabine (1µM) and TSA (100nM) alone or in combination (data not shown).

Since the proportion of methylated rDNA promoter varies among 300-400 copies of the genes, it is difficult to decipher the effect of decitabine and TSA on methylated vs. the unmethylated promoters. The data presented in Fig.7A, therefore, represents the cumulative effect of the inhibitors on both promoters. To analyze the effect of these two inhibitors on methylated and unmethylated promoters separately, we took advantage of the transient transfection system. Treatment of HeLa cells transfected with the unmethylated promoter resulted in 5.2–fold activation by decitabine (1µM) and TSA (100nM), which is essentially additive effect of decitabine (1.7-fold) and TSA (3.1-fold) treatment alone (Fig.7B). However, a synergistic activation (9.2-fold) of the promoter was observed following transfection of methylated (vs. unmethylated) promoter and treatment with both inhibitors (Fig.7B). Exposure of the methylated promoter separately to decitabine and TSA resulted in 1.9-fold and 3.4 fold activation, respectively. This data suggests that the methylated and unmethylated rDNA promoters respond differently to a combination of these two agents.

**DISCUSSION**

Recent studies (8-10) have provided important insights into the role of promoter methylation in rDNA expression in arabidopsis, mouse and humans. Previous study from our laboratory showed reduced methylation of rDNA promoter in the primary human hepatocellulararcarcinoma relative to matching control liver tissue. This is consistent with elevated
levels of rRNA synthesis required for the increased demand of ribosomes in tumors. A comprehensive study on the role of different methylCpG-binding proteins in rDNA promoter methylation showed that one of the methylC-binding proteins, MBD2, is associated with the methylated promoter and suppressed its activity (9). The present study explored the role of DNA methyltransferases in the epigenetic regulation of human rRNA synthesis. The presence of DNMTs in the nucleolus and extensive demethylation of the rDNA promoter in DNMT1 and 3B knockout cells is consistent with their role in its methylation. To our knowledge, this is the first demonstration of the localization of DNMTs in the nucleolus in addition to the nucleoplasmic region and their role in rDNA expression. Both DNMT3A and 3B harbor conserved PWWP domains that are involved in localization of these two proteins at pericentric heterochromatin (45). Since PWWP domain can bind DNA in vitro (42) interaction of DNMT3B with the rDNA promoter might involve the same domain. The present study also revealed that PWWP domain of DNMT3B along with the ATRX homology domain is involved in DNMT3B-mediated suppression of rDNA promoter activity. The occurrence of these enzymes as well as the methylC-binding proteins (9) in the nucleolus further attests to functional significance of the DNA methylation machinery in this organelle.

Out of the three functional DNMTs in higher organisms (DNMT 1, 3A, 3B), only DNMT1 and 3B are involved in the methylation-mediated suppression of human rDNA promoter. An interesting observation is the significant synergy between the two proteins in maintaining the CpG methylation of the promoter in the colon cancer cell line HCT116. Southern blot analysis showed low level hypomethylation only in MT1KO cells. An increase in the expression of 47S rRNA in 3BKO cells without significant alteration in promoter methylation suggests transcriptional repressor function of DNMT3B in rDNA regulation. In this context, a recent study in our laboratory has shown that DNMT3B is involved in the NGF-mediated differentiation of PC12 cells and that this process is independent of the catalytic activity of DNMT3B (26). Although ChIP analysis revealed association of DNMT3A with methylated rDNA promoter in HeLa cells; current studies did not reveal its direct involvement in regulating rDNA promoter function. Generation of DNMT3A null cells may address the role of this enzyme in rDNA expression.

The synergistic role of DNMT1 and DNMT3B on rDNA promoter methylation is also consistent with the occurrence of these proteins in a complex following extensive biochemical fractionation (46) as well as the known functional interaction of these two enzymes (33). Although the association of ectopic DNMT1 and 3B with transiently transfected reporter plasmid driven by mouse pol I promoter has been demonstrated earlier (14), its role in rDNA expression has not been studied. The present study has revealed in vivo association of both DNMT1 and 3B with unmethylated as well as methylated rDNA promoters, and suggests that the promoter is methylated first and subsequently suppressed by DNMT1. Increased demethylation of the promoter in MT1KO cells compared to the wild type HCT116 cells further validates this observation. In this context, it should be noted that DNMT1 could also function as de novo cytosine methyltransferase in addition to its role in maintenance methylation of DNA (47). Alternatively, ectopic DNMT1 cooperates with endogenous DNMT3B to initiate methylation of rDNA promoter.

The effect of HDAC inhibition on rDNA promoter function merits discussion. Previous study has shown that rodent rDNA promoter activity is significantly elevated upon treatment with TSA, the HDAC inhibitor (48). The present study demonstrated similar effect of TSA on human rDNA promoter activation. Further, combined treatment of cells with DNA hypomethylating agent Decitabine, a potent inhibitor of DNMT1 (28,49-51) and TSA synergistically activated only the transiently transfected methylated rDNA promoter. These two drugs probably exhibit the same synergistic effect on the endogenous methylated promoter. Because mammalian cells harbor multiple copies of rDNA with differential methylation state, it is not possible to distinguish the effect of these drugs on methylated
vs. unmethylated promoter in vivo. In this context, pol I promoter behaves like pol II promoter where strong synergy between the two agents was observed for several methylated pol II promoters (31,44). We have recently demonstrated that the DNA demethylating agent Decitabine can selectively degrade DNMT1 by a proteasomal pathway (28). The repressor complex that includes DNMT1 and co-repressors such as HDAC may be dislodged following Decitabine-mediated degradation of DNMT1 that could lead to decrease in HDAC recruitment and the observed synergistic activation of the rDNA promoter.

A unique aspect of epigenetic modulation of rDNA expression is that unlike mouse system that contains a single CpG in the promoter region, human promoter contains 19 CpG residues. Since the single CpG residue in the mouse promoter is located in the region where the key pol I transcription factor UBF binds, methylation of the cytosine of this dinucleotide prevents mouse rDNA gene transcription. It is likely that the role of three DNMTs, particularly the proteins associated with these enzymes (27,46,52-54) in the methylation of rodent rDNA gene may be distinct from that of the human gene. It is logical to conceive that a distinct set of DNMT-associated proteins participate in the methylation of pol I and II promoters.
REFERENCES


FOOTNOTES
We thank Drs. Bert Vogelstein for DNMT knockout HCT116 cells, Chin Lin Heish for DNMT3A and 3B expression vectors, Edward Seto and Stuart Schriber for HDAC2 and HDAC1 expression vectors. This study was supported in parts by grants ES10874 and CA86978 from the National Institutes of Health.

1 The abbreviations used are: pol II, RNA polymerase II; pol I, RNA polymerase I; rDNA, ribosomal DNA; rRNA, ribosomal RNA; DNMT, DNA methyltransferase; MT1KO, Dnmt1 knock out; 3BKO, DNMT3B knock out; DKO, DNMT1/Dnmt3B knock out; CGI, CpG island; MBD, methyl CpG domain binding protein;
FIGURE LEGENDS

Fig. 1. DNMT1 and 3B synergistically maintain rDNA promoter methylation. A. Whole cell extracts from HCT116 (WT), DNMT1−/− (MT1KO), DNMT3B−/− (3BKO) and DNMT1−/−/DNMT3B−/− (DKO) cells were separated by SDS-PAGE and Western blot analysis was performed with specific antibodies. B. Schematic presentation of the rDNA promoter region used as probe for Southern blot analysis. C. Genomic DNA from the cells were either mock-digested (U, Uncut), and digested with Hpa II (H) or Msp I(M). The digested products were separated on a 0.8% agarose gel and stained with ethidium bromide. D. DNA was transferred to nylon membrane and probed with 32P-labeled rDNA promoter probe. E. rDNA, GAPDH and albumin promoters were amplified from mock-digested (U, Uncut), Hpa II (H) or Msp I(M) digested genomic DNA from WT HCT116, MT1KO, 3BKO and DKO cells and resolved on 1.5% agarose gel. F. Quantitative analysis of the PCR amplified rDNA products. G. Total RNA isolated from the cells was resolved on a 1.0% agarose/formaldehyde gel, transferred to nylon membrane and probed with 91bp preRNA probe. H. Total RNA from the cells was treated with RNase-free DNase 1, reverse transcribed and subjected to real time PCR using 47S rRNA specific primers. Data presented was normalized to 18S rRNA. RNA without reverse transcription did not generate PCR product.

Fig. 2. A. All three DNMTs localize in the nucleolus. HeLa cells were stained with TRITC tagged mouse monoclonal antibody against nucleolin and with FITC-tagged rabbit polyclonal antibody against DNMT1, 3A, 3B or UBF (panels a-t). In a separate set, cells were stained with FITC-tagged rabbit polyclonal antibody against UBF and TRITC tagged mouse monoclonal antibody against DNMT1 (panels u-y). All five sets of cells were also stained with DAPI and visualized under fluorescence microscope. B. All three DNMTS co-fractionated with nucleolin in the nucleolar fraction: Nucleolar extract and nuclear extract (nucleolus and nucleoplasm) from HeLa cells (250µg) were subjected to Western blot analysis with antibodies against nucleolin, RNA polymerase II, DNMT1, 3A and 3B. C. DNMT1, 3B and 3A are associated with methylated rDNA promoter. Formaldehyde cross-linked chromatin was precleared and immunoprecipitated overnight with antisera specific for DNMT1, DNMT3A, DNMT 3B, UBF or preimmune sera. The immune complexes were precipitated by protein A/G beads, washed with different buffers (detail in the Method Section), eluted and de-cross-linked. DNAs pulled down by different antibodies as well as input DNA were divided into three identical fractions that were either mock-digested or digested with Hpa II or Msp I. An aliquot of each digestion product was subjected to real-time PCR with primer specific for rDNA promoter. Association of different DNMTs and UBF with rDNA promoter was analysed using a standard curve generated by serial dilution of the undigested input DNA. Association with methylated promoter equals Hpa II signal in ChIP DNA/Hpa II signal in input (1:300 dilution). Association with unmethylated promoter corresponds to the signal in undigested minus signal in Hpa II digested ChIP DNA/Input signal in undigested minus Hpa II digested DNA (1:300 dilution). U and M indicate methylated and unmethylated rDNA promoters, respectively.

Fig. 3. Ectopic DNMT1 and DNMT3B, but not DNMT3A, inhibit rDNA promoter activity irrespective of its methylation status. A. HeLa cells were transiently transfected with DNMT1, DNMT3A or DNMT3B expression vectors. Whole cell extracts from these cells were subjected to Western blot analysis with anti-Flag M2 antibody. B. HeLa cells were transfected with either mock methylated pHrD-IRES-Luc (Unmethylated) or M. Hha I methylated pHrD-IRES-Luc (Methylated) along with 4µg of the empty vector or pcDNMT1, pcDNMT3A or pcDNMT3B. After 48 hr firefly
Luciferase activity was measured in the cell extracts. Results are represented as rDNA promoter activity upon overexpression of different DNMTs with empty vector transfected pHrD-IRES-Luc activity as 100.

**Fig. 4. Catalytic domain of DNMT1 is necessary to inhibit unmethylated rDNA promoter that is de novo methylated by ectopic DNMT1.** A. Schematic diagram of different deletion/point mutants of DNMT1 used in transient transfection studies. B. HeLa cells were transfected with mock methylated pHrD-IRES-Luc and empty or expression vector for different deletion mutants of DNMT1 (ΔCAT: catalytic domain deleted, CS: Cysteine to serine mutation at PCQ motif, ΔNLS: N-terminal domain deleted including nuclear localization signal). After 48 hr firefly luciferase activity was measured in the cell extracts. Results are represented as rDNA promoter activity upon overexpression of different DNMT1 mutants. One hundred percent activity represents empty vector transfected pHrD-IRES-Luc activity. C. HeLa cells were cotransfected with mock-methylated pHrD-IRES-Luc and either empty vector or DNMT1 expression vector. The pHrD-IRES-Luc plasmid was isolated from the transfected cells 48 hr post transfection and digested with Hpa II or MspI. The rDNA promoter was amplified from mock digested (U), Hpa II (H) or MspI (M) digested plasmid using a vector specific and a rDNA promoter specific primer and resolved on 1% agarose gel.

**Fig. 5. Catalytic activity of DNMT1 is not essential to inhibit methylated human rDNA promoter activity.** A. HeLa cells were transfected with M.Hha I methylated pHrD-IRES-Luc and empty or expression vector for different deletion/point mutants of DNMT1 (ΔCat, CS and ΔNLS). After 48 hr firefly luciferase activity was measured in the cell extracts. Results are represented as rDNA promoter activity upon overexpression of different DNMT1 mutants with activity of the empty vector transfected cells as 100. B. HeLa cells co-transfected with M.Hha I methylated pHrD-IRES-Luc and empty vector or expression vector for DNMT1 (1µg) were splitted 24 hr post transfection and treated with 100nM TSA for an additional 24 hr followed by luciferase activity assay. Results are represented as fold increase in rDNA promoter activity upon TSA treatment with empty vector or DNMT1 expression vector transfected pHrD-IRES-Luc activity as 1. Panels at the bottom shows i)DNMT1 protein levels in the overexpressing cells in presence and absence of TSA, ii) promoter activity/µg protein under different conditions. C. HeLa cells were co-transfected with HDAC2 expression vector alone or in combination with DNMT1 along with M.Hha I methylated pHrD-IRES-Luc. The luciferase activity was measured 48 hr post transfection. Results are represented as residual rDNA promoter activity upon overexpression of different proteins (as indicated) with empty vector transfected pHrD-IRES-Luc activity as 100.

**Fig. 6. A. Catalytic activity of DNMT3b is not involved in DNMT3B mediated inhibition of rDNA promoter activity.** HeLa cells were transiently transfected with mock or M.Hha I methylated pHrD-IRES-Luc along with empty vector (Vec), expression vector for wild type DNMT3B (3B) or its catalytic site mutant (3Bmut). Cells were harvested 48 hr later and luciferase activity was measured. Results are represented as residual rDNA promoter activity upon overexpression of wild type and mutant DNMT3B, with activity of the empty vector transfected cells as 100. Western blot analysis of the DNMT3B, wild type and CS mutant is presented in the lower panel. B. Schematic diagram of DNMT3B. C. HeLa cells were transiently transfected with different deletion mutants of Flag-tagged DNMT3B (ΔPWWP, ΔATRX, ΔN-terminal and ΔCAT represent PWWP, ATRX, ATRX/PWWP and catalytic domain deleted mutants respectively). Whole cell extracts from these cells were subjected to Western blot analysis with anti-Flag M2 antibody. D. HeLa cells were transiently transfected with mock- or M.Hha I-methylated pHrD-IRES-Luc and expression vector for different deletion mutants of DNMT3B. Cells
were harvested 48 hr later and luciferase activity was measured. Results are represented as residual rDNA promoter activity upon overexpression of DNMT3B variants with activity of the empty vector transfected cells as 100. E. HeLa cells were transiently transfected with pHrD-IRES-Luc and expression vector for wild type (DNMT3B). Each set of transfected cells were splitted 24 hr post transfection, treated with 100nM TSA for an additional 24hr and luciferase activity was measured. Results are represented as fold increase in rDNA promoter activity upon TSA treatment with empty vector or DNMT3B expression vector transfected pHrD-IRES-Luc activity as 1.

Fig. 7. Decitabine and Trichostatin A synergistically activate transiently transfected methylated rDNA promoter, but exhibit an additive effect on the unmethylated promoter. A. HCT cells were treated with 50nM Decitabine for 24 hr followed by 100nM TSA for additional 12 hr. Total RNA was treated with DNaseI before cDNA synthesis. An aliquot of cDNA was subjected to real time PCR analysis with primers specific for 47S rRNA and 18S rRNA. RNA without reverse transcription did not generate PCR product. B. HeLa cells were transfected with mock-methylated or methylated pHrD-IRES-Luc and splitted into four dishes 24 hr after transfection. The cells were either left untreated or treated with 1µM Decitabine (D) for 12 hr followed by treatment with 100nM TSA for 12 hr (D/T) or TSA alone (T) for 12hr. Cells were harvested and luciferase activity measured in the cell lysate. Results are represented as hrDNA promoter activity/µg of protein.
Copy No. of 47S rRNA normalized to 18S rRNA $\times 10^5$

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**Fig. 1**

**E**

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- rDNA
- Albumin
- GAPDH

**F**

Fold activation

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**G**

47S rRNA

18S rRNA

**H**

Copy No. of 47S rRNA normalized to 18S rRNA $\times 10^5$
Fig. 2 A

DNMT1  Nucleolin  DAPI  MERGE

DNMT3A  Nucleolin  DAPI  MERGE

DNMT3B  Nucleolin  DAPI  MERGE

UBF  Nucleolin  DAPI  MERGE

UBF  Dnmt1  DAPI  MERGE
Antibody    No Ab     DNMT1  DNMT3B  DNMT3A      UBF

UnMeth 2.35E+00 2.15E+00 2.09E+02 2.40E+00
Meth 4.81E+00 6.85E+01 1.44E+02 5.84E+01
Fig. 3

A

B

HrDNA promoter activity

Unmethylated  Methyalted

pcDNA 3.1  DNMT1  DNMT3A  DNMT3B

None  DNMT1  DNMT3A  DNMT3B

None  DNMT1  DNMT3A  DNMT3B
Fig. 4

A

DNMT1/FL

Charge rich region
PCNA
Nuclear Localization signal
Replication Foci targeting sequence
Zinc binding domain
Bromo adjacent homology domain
Catalytic domain

DNMT1/ΔCAT

DNMT1/ΔNLS

DNMT1/CS

1 350 613 748 1124 1620

1 350 1620

1 1620

1 1620

B

Unmethylated

HrDNA Promoter Activity

Vector DNMT1 DNMT1/ΔCAT DNMT1/CS DNMT1/ΔNLS

%inhibition — 48 — 12 —

C

Vector | DNMT1
--- | ---
U | H | M | U | H | M

rDNA promoter

M 1 2 3 4 5 6
Fig. 5

A

Methylated

hrDNA Promoter Activity

% inhibition

Vector | DNMT1 | DNMT1 ΔCAT | DNMT1 CS | DNMT1 ΔNLS

| % inhibition 56 52 58 11 |
Fig. 5

B

Methylated

Fold increase in HrDNA promoter activity

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Plasmid

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C

Methylated

HrDNA Promoter Activity

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**Fig. 6**

A. Bar graph showing HrDNA Promoter Activity for unmethylated and methylated conditions. The graph includes data for Plasmid Expression with % inhibition values of 0, 47, 41, 0, 53, and 56.

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B. Diagram illustrating the Catalytic domain of the protein with PWNP, NLS, and ATRX regions.

C. Western blot image showing expression levels of different constructs with labels Vec, WT, ΔCat, ΔPWNP, ΔATRX, and ΔN-term.

D. Bar graph depicting HrDNA Promoter Activity with % inhibition values of 0, 20, 40, 60, 80, and 100. The graph includes data for DNMT3B expression with constructs Vec, WT, ΔCAT, ΔN, ΔA, and ΔP.
Fig. 6

HrDNA Promoter Activity

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Fig. 7

A

Fold Increase in 47S rRNA transcript

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B

HrRNA Promoter Activity/μg protein X 10^3

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Fold increase – 1.9 3.4 9.2
Role of DNA methyltransferases in regulation of human ribosomal RNA gene transcription
Sarmila Majumder, Kalpana Ghoshal, Jharna Datta, David Spencer Smith, Shoumei Bai and Samson T. Jacob

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