Role of DNA Methyltransferases in Regulation of Human Ribosomal RNA Gene Transcription

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We have previously demonstrated that the expression of DNA methylation, histone modifications, and chromatin remodeling mediate epigenetic regulation of gene expression. These mechanisms can modify the expression of genes in a cell cycle-dependent manner (8). 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 expression (Refs. 1–7). Most studies on this unique process have focused on the rDNA promoter (for review, see Refs. 1–7). In this study, we report that methylation of the rDNA promoter is associated with the inactive rDNA gene, whereas the unmethylated rDNA promoter is associated with active rDNA genes. The authors stand by the reproducibility of the experimental data and the conclusions of the paper. The paper, with confirmatory data supporting the results, can be obtained by contacting the authors.

This article has been withdrawn by the authors. In June 2017, the Journal questioned whether there were duplications in Figs. 1E and 6C and questioned the absence of data in one field of Fig. 5B. The original data and originally submitted figures were not available for evaluation. The authors are not convinced there are any duplications in Figs. 1E and 6C. Regarding Fig. 5B, the authors agree that the data for “Vector” was inadvertently omitted from the final figure. The authors were able to locate replicate data performed at the time of the original work, which they state shows an absence of expression in the Vector panel. The authors offered to publish a correction based on this repeat experiment and, alternatively, offered to repeat the experiment. However, the Journal declined both these offers, a decision with which the authors disagree. The authors stand by the reproducibility of the experimental data and the conclusions of the paper. The paper, with confirmatory data supporting the results, can be obtained by contacting the authors.

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3 The abbreviations used are: pol II, RNA polymerase II; pol I, RNA polymerase I; DNMT, DNA methyltransferase; MT1KO, DNMT1 knockout; 38KO, DNMT3B knockout; DKO, DNMT1/DNMT3B knockout; MBD, methyl CpG domain-binding protein; CHIP, chromatin immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase; DKO, double knockout; TRITC, tetramethylrhodamine isothiocyanate; HDAC, histone deacetylase; TSA, trichostatin A.

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Although the factors involved in the epigenetic regulation of pol II-directed genes have been well studied, such an 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 with 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 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 NH2-terminal domain differs significantly between DNMT1 and DNMT3A/3B. The unique NH2-terminal domain of mammalian DNMTs harbor several regulatory domains that mediate both protein-protein and protein-DNA interactions (24–27). The NH2-terminal domains of DNMTs can also mediate transcriptional repression of genes independent of their methyltransferase activity (19). Here, we focused on the role of DNMTs in regulating rDNA promoter activity following the unmethylated as well as the unmethylated state, and identified a subset of the transcriptional repressor domains of these enzymes in suppressing rDNA promoter.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**

**Human rDNA-Luciferase Vector (pHrD-IRES-Luc)—** Construction of plasmid pHrD-IRE-Luc has been described earlier (9). In brief, human rDNA promoter spanning −410 to +81 bp (accession number K01105) with respect to the transcription initiation site was amplified from −2-kb fragments of HeLa genomic DNA digested with EcoRI and cloned into pGL3-IRES (9) to generate pHrD-IRE-Luc.

**Expression Vectors for Mouse DNMT1, -3A, and -3B—** These plasmids were constructed in mammalian expression vector pcDNA3.1 (+/−) (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 EcoRI and the resultant ∼5.2-kb fragment was cloned into the same site in pcDNA3.1 (+) to generate pc-DNMT1. PCR with the vector- and insert-specific primers determined the correct orientation of DNMT1. To generate DNMT1/ΔCAT, a 1.5-kb fragment from the COOH-terminal of DNMT1 was excised and the rest of the plasmid was religated. The DNMT1/ΔNLS plasmid is described earlier (28). Similarly, mouse DNMT3A cDNA from pSX137 (a generous gift from Dr. Tim Bestor) was excised with BamHI and XbaI and cloned in the same sites of pcDNA3.1 (+/−) vector. The mouse DNMT3B was subcloned from the antisense clone (26) in pcDNA3.1(−), to generate the sense clone.

**Cell Culture and Transfection Assays**

Wild type HCT116 cells, DNMT1−/−, DNMT3B−/−, and DNMT1−/−/DNMT3B−/− (a generous gift from Dr. Bert Vogelstein) were all grown in α-minimal essential medium with 10% fetal bovine serum. HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The transfection studies were performed as described (9).

**In Vitro DNA Methylation**

M.HhaI methylation of pHrD-IRES-Luc was performed as described (9).

**Isolation of Nucleoli**

Nucleoli were isolated from HeLa cells following the protocol described (29).

**Western Blot Analysis**

Human rDNA promoter spanning 410 bp to H11011 was amplified from genomic DNA digested with EcoRI and cloned into pGL3-IRES/Luc. The resultant plasmid was treated with HindIII and the resultant plasmid was digested with M.HhaI and MspI, and the digests along with an equal amount of mock DNA was treated with RNase A and proteinase K, and purified as described (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 antigen-antibody immune complex was then captured by protein A beads, and immunoblotted with antibodies against nucleolin, DNMT1, DNMT3A and -3B (antibodies raised in our laboratory).

**Chromatin Immunoprecipitation**

Formaldehyde cross-linked chromatin was prepared as described (32). For chromatin immunoprecipitation (ChIP) analysis, antisera against UBF (Santa Cruz), DNMT1 (New England Biolabs), DNMT3A and -3B raised in our laboratory, were used (31). The chromatin was first pre-cleared with pre-immune 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, and washed successively with buffers as described (31, 32). Immunoprecipitated DNA-protein complex was eluted, un-cross-linked, treated with RNase A and proteinase K, and purified as described (31). ChIP and the input DNA were digested with HpaII or MspI and the digests along with an equal amount of the undigested immunoprecipitated DNA were subjected to
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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 anti-nucleolin 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 4',6-diamidino-2-phenylindole in the mounting fluid.

DNA Isolation and Southern Blot Analysis

Genomic DNA isolation, HpaII/MspI 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 HpaII/MspI-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'-TCCATTTCCTCCTCATTCTCTGC-3'; hALB-PR, 5'-ATGTTCACATTCTGCTGTCG-3', with an annealing temperature of 52.5 °C.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated using TRIzol (Invitrogen) following the manufacturer's protocol. Total RNA (10 μg) was separated on a formaldehyde-agarose (1.0%) gel and transferred to a nylon membrane. The membrane was hybridized with a random-primed [α-32P]dCTP-labeled rRNA probe in rapid hybridization buffer (GE Healthcare) followed by the manufacturer’s protocol.

Reverse Transcription and Real Time PCR Analysis

Reverse transcription was carried out with random hexamers and Moloney murine leukemia virus reverse transcriptase from 3 μg of total RNA in 20 μl 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 were carried out using the Mx3000 Multiplex Quantitative PCR System (Stratagene). The optimum primer concentration was 150 nM. All PCR amplifications were performed using Brilliant® SYBR® Green QPCR Master Mix (Stratagene) with ROX as a reference dye in a 10-μl reaction volume. A standard curve for each cDNA was first generated using 10-fold serial dilutions (10^8 to 10^2 copies) of the respective cDNAs as template. The copy number of each cDNA 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 30 s, the annealing temperature for the specific primer pair, for 30 s, and a dissociation cycle of 95 °C for 60 s, 55 °C for 30 s (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 92 bp): 47S-F, 5'-CCTGTCGAGGAGTTGG-3', 47S-R, 5'-ACCCCA-CGGCTTCCACACAC-3', an annealing temperature of 60 °C; 18S rRNA (product size: 492 bp): 18S-F, 5'-TCAAGAAGC-GAAAGTGCGAGG-3', 18S-R, 5'-GGACATCTAACAG-GGCATCACA-3', an annealing temperature of 57 °C.

RESULTS

DNMT1 and DNMT3B Synergistically Maintain the Methylation Profile of the 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), DNMT3B (DNMT3B−/− or 3BKO cells), or both alleles of DNMT1/DNMT3B (DKO cells, double knockout) (33). Western blot analysis confirmed the absence of the 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 the rDNA promoter occurred in DNMT1 or DNMT3B null cells. Hybridization of the rDNA probe with methylated and unmethylated DNA (homoduplex and homoduplex-digested DNA) was carried out to Southern blot analysis using rDNA promoter-specific probe. The probe hybridized significantly reduced methylation compared with MT1KO cells (Fig. 1D, lanes 2 and 5), whereas 3BKO cells did not reveal any noticeable hypomethylation (Fig. 1D, lanes 2 and 8). Comparable hybridization profile of DNA digested with methylation-insensitive isoschizomeric MspI in all four cells indicate that different profiles in the HpaII digest are because of differential methylation of the promoter (Fig. 1D, lanes 3, 6, 9, and 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), HpaII-digested (H), and MspI-digested (M) genomic DNA using rDNA specific primers (Fig. 1B). This data showed (Fig. 1F, lanes 1 and 2) that 28% of the rDNA promoter in HCT116 cells is unmethylated. DNMT1 disruption resulted in an additional 22% demethylation (Fig. 1F, compare lanes 2 and 5). DNMT3B deletion alone resulted in an additional 10% demethylation (compare lanes 2 and 8). In DKO cells 76% of rDNA promoter is demethylated, demonstrating an additional 48% demethylation (76–28%) in these cell lines following disruption of DNMT1 and DNMT3B. To confirm complete restriction enzyme digestion, the 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 a comparable
amount of DNA in PCR (Fig. 1E).
This data further authenticates the
Southern blot analysis showing syn-
ergism between DNMT1 and -3B in
maintaining the 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 the 47 S precursor rRNA, the pri-
mary transcript, revealed at least a
2-fold increase in its level in both
MT1KO and 3BKO cells compared
with the parental or DKO cells (Fig.
1G). Quantitative analysis of the
transcript by real time RT-
PCR showed a 2- and 3.4-fold
increase in the primary transcript in
MT1KO and 3BKO cells, respec-
tively (Fig. 1H). A significant in-
crease in precursor rRNA level in
MT1KO cells correlated with
hypomethylation of its promoter
(Fig. 1, D and E). Striking elevation
of the rRNA transcript in 3BKO
cells suggests that DNMT3B regu-
lates rDNA transcription by a meth-
ylation independent mechanism,
because the rDNA promoter did not
undergo significant hypomethyla-
tion in 3BKO cells (Fig. 1, D and
E).

No significant change in the 47 S
rRNA level in DKO (Fig. 1H) cells
despite remarkable hypometh-
ylation of the promoter (Fig. 1,
D and E) could be attributed to the detri-
mental effect of global hypometh-
ylation on growth regulatory genes
that significantly compromised cell
growth (33). Increased rRNA levels
in MT1KO and 3BKO cells impli-
cates their role in regulating its
expression in vivo.

**DNMT1 and DNMT3B Localize
in the Nucleolus and Associate with
rDNA Promoter**—Immunofluores-
cence analysis and biochemical
fractionation demonstrated localized
localization of DNMTs in the nucleolus
(Fig. 2A). These proteins also co-lo-

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**FIGURE 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 HpaII (H) or MspI (M). The digested products were separated on a 0.8% agarose gel and stained with ethidium bromide. D, DNA was transferred to nylon mem-
brane and probed with a 32P-labeled rDNA promoter probe. E, rDNA, GAPDH, and albumin promoters were
amplified from mock-digested (U, Uncut), HpaII (H), or MspI (M) digested genomic DNA from WT HCT116,
MT1KO, 3BKO, and DKO cells and resolved on 1.5% agarose gel, and probed with the 91-bp pre-RNA probe.
F, total RNA isolated from the cells was resolved on a 1.0% agarose/formaldehyde gel, transferred to nylon membrane, and probed with the 41-bp pre-RNA probe. G, total RNA from the cells was treated
with RNase-free DNase I, reverse transcribed, and subjected to real time PCR using 47 S rRNA-specific primers.
Data presented was normalized to 18 S rRNA. RNA without reverse transcription did not generate PCR product.
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calized with nucleolin, a nucleolar marker, because the two stains (fluorescein isothiocyanate and TRITC) merged (panels e, j, and o). Furthermore, 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. Furthermore, biochemical fractionation coupled with Western blot analysis demonstrated the presence of DNMT1,
-3A, and -3B as well as nucleolin in the purified nucleolar fraction of HeLa cells (Fig. 2B). As expected, DNMTs are also present in the nucleolar 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 the 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 HpaII, MspI, or mock digested prior to amplification and analysis (fragment shown in Fig. 1B). This amplified promoter region harbors four HpaII/MspI sites. DNA pulled down was quantified from the standard curve generated using serial dilution of the input DNA. The HpaII-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 the HpaII-resistant product represents the level of unmethylated promoter (U). PCR product was not generated from MspI-digested DNA because it is a methylation insensitive enzyme. Real time PCR data revealed that all 4 HpaII sites of only 20% of the total input DNA are methylated in HeLa cells. DNMT1 association with the methylated promoter was 30-fold higher than the rest of the promoter (Fig. 2C). On the contrary, DNMT3A was equally associated with both unmethylated and methylated rDNA promoters (Fig. 2C, lanes 5 and 6). DNMT3A associate predominantly with the methylated promoter (lanes 7 and 8). As a control, we performed the ChIP assay with anti-UBF antibody, which showed that there are a large fraction of unmethylated rDNA promoters demonstrating the stringency of the ChIP-CHOP assay. Precipitated chromatin in the absence of specific antibodies (control) showed minimal amplification of rDNA promoter (lanes 1–3). Thus, all three DNMTs associate with the methylated rDNA population, which confirms localization of DNMTs with the inactive rDNA at the perinuclear 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 is a large protein comprising 1620 amino acids with a COOH-terminal catalytic domain (Fig. 4A). The NH2-terminal domain harbors a region that interacts with various proteins (24, 36), and different targeting motifs (37–39) (Fig. 4A). The NH2-terminal domain is a large protein comprising 1620 amino acids with a large COOH-terminal catalytic domain (Fig. 4A). The NH2-terminal domain harbors a region that interacts with various proteins (24, 36), and different targeting motifs (37–39) (Fig. 4A). The NH2-terminal domain harbors a region that interacts with various proteins (24, 36), and different targeting motifs (37–39) (Fig. 4A). The NH2-terminal domain harbors a region that interacts with various proteins (24, 36), and different targeting motifs (37–39) (Fig. 4A).

![FIGURE 2. A] A, all three DNMTs localize in the nucleolus. HeLa cells were stained with TRITC-tagged mouse monoclonal antibody against nucleolin and with fluorescein isothiocyanate-tagged rabbit polyclonal antibody against DNMT1, -3A, -3B, or UBF (panels a–i). In a separate set, cells were stained with fluorescein isothiocyanate-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 4',6-diamidino-2-phenylindole and visualized under a 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 (250 μg) 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 pre-cleared and immunoprecipitated overnight with antisera specific for DNMT1, DNMT3A, DNMT3B, UBF, or preimmune sera. The immune complexes were precipitated by protein A/G beads, washed with different buffers (detailed under “Experimental Procedures”), eluted, and un-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 HpaII or MspI. An aliquot of each digestion product was subjected to real time PCR with primers specific for rDNA promoter. Association of different DNMTs with the rDNA promoter was analyzed using a standard curve generated by serial dilution of the undigested input DNA. Association with methylated promoter equals HpaII signal in ChIP DNA/HpaII signal in input (1:300 dilution). Association with unmethylated promoter corresponds to the signal in undigested minus signal in HpaII-digested ChIP DNA/undigested signal in HpaII-digested DNA (1:300 dilution). **U** and **M** indicate methylated and unmethylated rDNA promoters, respectively.

![FIGURE 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.HhaI-methylated pHrD-IRES-Luc (Methylated) along with 4 μg of the empty vector or pcDNMT1, pcDNMT3A, or pcDNMT3B. After 48 h, 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.**
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 (DNMT1/CS) (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 HpaII or MspI and subjected to amplification of the rDNA promoter fragment. A 10-fold increase in the HpaII-resistant PCR product from cells expressing ectopic DNMT1 compared with that from the vector-transfected cells suggests significant methylation of the rDNA promoter by DNMT1 (Fig. 4C, lanes 2 and 5). Because MspI cleaved the plasmid irrespective of its methylation status, there was no PCR product obtained upon MspI digestion. This data demonstrates that DNMT1-mediated promoter methylation is necessary to suppress promoter activity, which is in accordance with other reports on concomitant with its promoter activity of the DNMT1 transfected cells (Fig. 1, D and E).

Promoter in the Absence of the Catalytic Domain — We previously demonstrated significant inhibition of the rDNA promoter activity when M.HhaI-methylated CpG dinucleotides were present in the upstream control element and in the further upstream region (9). Interestingly, ectopic expression of DNMT1 further suppressed the methylated promoter (Figs. 3B and 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 a 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 NH2-terminal truncation retained the protein in the cytoplasm (28). This data reveals the role of the DNMT1 transcriptional repressor domain in impeding methylated rDNA expression.

The NH2-terminal domain of DNMT1 interacts with various co-repressors that include histone deacetylase (HDAC) 1/2. To determine whether 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. 5, A, and B, bottom panel; promoter activity in vector or DNMT1 transfected controls). However, treatment of the cells with 100 nM TSA for 24 h resulted in a 4-fold increase in the promoter activity even in DNMT1 overexpressing cells (Fig. 5B). DNMT1 overexpressing cells showed comparable DNMT1 expression in the presence and absence of TSA (Fig. 5B). This data suggests that DNMT1-me-
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DNMT3B-mediated Suppression of rDNA Promoter Does Not Require Its Catalytic Activity—Disruption of DNMT1, DNMT3B, or both in HCT cells displayed a differential effect on the rDNA transcription and methylation profile of the promoter. The ribosomal RNA transcript level in DNMT3BKO cells was significantly elevated without noticeable demethylation of its promoter (Fig. 1, C and D). To address the underlying mechanism, HeLa cells were transfected with wild type and catalytic site mutants of DNMT3B (CS mutant) along with mock or M.HhaI-methylated pHrD-IRES-Luc. Both the wild type and mutant DNMT3B significantly inhibited the promoter activity irrespective of its methylation status (average 50%, Fig. 6A). The expression of DNMT3B was comparable (Fig. 6A, C, and D). This data suggests that DNMT3B does not repress both the endogenous rDNA promoter in the chromatin context (Fig. 1D) and the transfected promoter independent of its catalytic activity.

The NH2-terminal domain of the 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. 6A). To identify the domain(s) of DNMT3B involved in the transcriptional repression of the rDNA promoter, HeLa cells were co-transfected with pHrD-IRES-Luc and different deletion mutants of mouse DNMT3B (ΔC-terminal, ΔN-terminalsegment, 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 a 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.

Because DNMT3B interacts with HDACs through the ATRX domain we investigated whether the HDAC inhibitor can alle...
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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 corepressors recruited by DNMT3B to inhibit rDNA promoter activity.

Decitabine and Trichostatin A

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

Because 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 versus 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 mock methylated pHrD-IRES-Luc and expression vector for different deletion mutants of DNMT3B (ΔPWPP, ΔATRX, ΔN-term, and ΔCAT represent PWPP, ATRX, ATRX/PWPP, 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 methylated pHrD-IRES-Luc and expression vector for different deletion mutants of DNMT3B. Cells were harvested 48 h 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 transduced cells were split 24 h post-transfection, treated with 100 nm TSA for an additional 24 h, 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.
Regulation of Ribosomal RNA Expression by DNMTs

Recent studies (8–10) have provided important insights into the role of promoter methylation in organisms ranging from Drosophila, mice, and humans. A comprehensive study in our laboratory showed reduced methylation of the rDNA promoter in the primary human hepatocellular carcinoma relative to matching control liver tissue. This is consistent with the elevated levels of rRNA synthesis required for the increased demand of ribosomes in tumors. A comprehensive study on the role of different methyl CpG-binding proteins in rDNA promoter methylation showed that one of the MBDs, 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). Because the 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 the 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 methyl C-binding proteins (9) in the nucleolus further attests to functional significance of the DNA methylation machinery in this organelle.

Of the three functional DNMTs in higher organisms (DNMT 1, -3A, and -3B), only DNMT1 and -3B are involved in the methylation mediated suppression of the human rDNA promoter. An interesting observation is the significant synergy between the two proteins in maintaining the CpG methylation of the promoter in colon cancer cell line HCT116. Southern blot analysis showed low level hypomethylation only in MT1KO cells. An increase in the expression of the 47 S rRNA in MT1KO cells without significant variation in promoter methylation indicates transcriptional repressor function of DNMT3B in rDNA regulation. A recent study in our laboratory has further shown that methyltransferase agents responded differently to a combination of HDAC inhibitors.

FIGURE 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 50 nM Decitabine for 24 h followed by 100 nM TSA for an additional 12 h. Total RNA was treated with DNase I before cDNA synthesis. An aliquot of cDNA was subjected to real time PCR analysis with primers specific for 47 S rRNA and 18 S rRNA. RNA without reverse transcription did not generate PCR product. B, HeLa cells were transfected with mock methylated or methylated pHrD-IRES-Luc and split into four dishes 24 h after transfection. The cells were either left untreated or treated with 1 µM Decitabine (D) for 12 h followed by 100 nM TSA for 12 h (D/T) or TSA alone (T) for 12 h. Cells were harvested and luciferase activity was measured in the cell lysate. Results are represented as hours of DNA promoter activity/µg of protein.

DISCUSSION

The synergistic role of DNMT1 and DNMT3B on rDNA promoter methylation is also consistent with the occurrence of these enzymes 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 with 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 co-operators with endogenous DNMT3B to initiate methylation of the 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 a similar effect of TSA on human rDNA promoter acti-
Regulation of Ribosomal RNA Expression by DNMTs

Furthermore, combined treatment of cells with the DNA hypomethylating agent Decitabine, a potent inhibitor of DNMT1 (28, 49–51) and TSA synergistically activated only the transiently transfected methylated rDNA promoter. Because mammalian cells harbor DNA methyltransferase 1 (DNMT1) (28, 49–51) and TSA synergistically activated only the DNA hypomethylating agent Decitabine, a potent inhibitor of DNA methylation. Because mammalian cells harbor DNA methyltransferase 1 (DNMT1) and co-repressors such as histone deacetylases (HDACs), which can selectively degrade DNMT1 that could lead to a 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 the mouse system that contains a single CpG residue in the promoter region, human promoter contains 19 CpG residues. Because the single CpG residue in the mouse promoter is in the promoter region, human promoter contains 19 CpG residues. 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 HDACs may be dislodged following Decitabine-mediated degradation of DNMT1 that could lead to a decrease in HDAC recruitment and the observed synergistic activation of the rDNA promoter.

The repressor complex that includes DNMT1 and co-repressors such as HDACs may be dislodged following Decitabine-mediated degradation of DNMT1 that could lead to a decrease in HDAC recruitment and the observed synergistic activation of the rDNA promoter.
Role of DNA Methyltransferases in Regulation of Human Ribosomal RNA Gene Transcription
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