Role of Human Ribosomal RNA (rRNA) Promoter Methylation and of Methyl-CpG-binding Protein MBD2 in the Suppression of rRNA Gene Expression*

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The methylation status of the CpG island located within the ribosomal RNA (rRNA) promoter in human hepatocellular carcinomas and paired-matched liver tissues was analyzed by bisulfite genomic sequencing. Significant hypomethylation of methyl-CpGs in the rRNA promoter was observed in the tumor samples compared with matching normal tissues, which was consistent with the relatively high level of rRNA synthesis in rapidly proliferating tumors. To study the effect of CpG methylation on RNA polymerase I (pol I)-transcribed rRNA genes, we constructed pHrD-IRES-Luc (human rRNA promoter-luciferase reporter). In this plasmid, Kozak sequence of the pGL3-basic vector was replaced by the internal ribosome entry site (IRES) of encephalomyocarditis viral genome to optimize pol I-driven reporter gene expression. Transfection of this plasmid into HepG2 (human) cells revealed reduced pol I-driven luciferase activity with an increase in methylation density at the promoter. Markedly reduced luciferase activity in Hepa (mouse) cells compared with HepG2 (human) cells showed that pHrD-IRES-Luc is transcribed by pol I. Site-specific methylation of human rRNA promoter demonstrated that methylation of CpG at the complementary strands located in the promoter (−9, −102, −347 with respect to the +1 site) inhibited luciferase activity, whereas symmetrical methylation of a CpG in the transcribed region (+152) did not affect the promoter activity. Immunofluorescence studies showed that the methyl-CpG-binding proteins, MBD1, MBD2, MBD3, and MeCP2, are localized in the nuclei and nucleoli of HepG2 cells. Transient overexpression of MBD2 suppressed luciferase activity specifically from the methylated rRNA promoter, whereas MBD1 and MBD3 inhibited rRNA promoter activity irrespective of the methylation status. Chromatin immunoprecipitation analysis confirmed predominant association of MBD2 with the endogenous methylated rRNA promoter, which suggests a selective role for MBD2 in the methylation-mediated inhibition of ribosomal RNA gene expression.

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The transcriptional regulation of ribosomal RNA (rRNA) genes is a control point in the complex process of ribosome biogenesis. Diploid somatic cells harbor 300–400 copies of the rRNA genes that code for the most abundant cellular RNA. Only a fraction of these genes is transcribed, which depends on the growth stage of the cells and extracellular stimuli (for a review, see Refs. 1 and 2). In general, multiple copies of rRNA are found as repeated clusters, usually arranged in a head-to-tail fashion. The core promoter region spanning from −20 to +20 bp with respect to the initiation site is necessary and sufficient for the initiation of basal transcription in most species (for a review, see Refs. 3–6). Another key element is the upstream control element (UCE)3 that extends 150–200 bp upstream of the transcription start site. Apart from core promoter and UCE, upstream enhancers and terminator also play a critical role in rRNA transcription. Whereas the transcription machineries of RNA polymerase II (pol II) and RNA polymerase III (pol III) are often compatible with genes from widely different species, RNA polymerase I (pol I) exhibits stringent (7) but not absolute (8) species specificity. This could result from very little sequence similarity between rRNA promoters from different species despite the general conservation of functional transactivation domains of the transcription factors from mice to humans (6, 9).

Although considerable advances have been made in the identification and characterization of factors that up-regulate rRNA gene expression, the factors controlling its down-regulation have not been fully characterized. Methylation of DNA at the 5-position of cytosine of CpG base pairs, particularly in the promoter region is the predominant epigenetic modification of DNA in mammals and is known to suppress many RNA polymerase II (pol II) genes (10–12). DNA methylation is essential for development (13, 14). It regulates inactivation of X chromosome in females, genomic imprinting and spursious transcription from promoters of retroviruses and transposable elements integrated with the genome (15). In addition, aberrations in DNA methylation cause activation of oncogenes, genomic instability, and silencing of a variety of tumor suppressor genes (e.g. P16, P15, P21, E-CAD, VHL, etc.), leading to uncontrolled cell proliferation (for a review, see Refs. 11 and 15–17). This modification is initiated by de novo DNA methyltransferases (DNMT3A and DNMT3B) and is propagated in

1 The abbreviations used are: UCE, upstream control element; ETS1, external transcribed spacer region 1; pol I, II, and III, RNA polymerase I, II, and III, respectively; MBD, methyl-CpG-binding protein; DNMT, DNA methyltransferase; ChIP, chromatin immunoprecipitation; AdoMet, S-adenosyl-l-methionine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRES, internal ribosome entry site; TRITC, tetramethylrhodamine isothiocyanate; HCC, hepatocellular carcinoma.

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successive cell divisions by the maintenance methyltransferase (DNMT1). DNMT1 transfers methyl group from 5-adenosyl-l-methionine (AdoMet) to the newly replicated strand using hemimethylated strand as the template (for a review, see Refs. 18 and 19). Aberrations in DNA methylation lead to a variety of diseases. For example, ICF (immunodeficiency, centromeric instability, and facial anomaly) syndrome is caused by mutations in the DNMT3B gene (20–22). The drugs inhibiting DNMT, namely 5-deoxycytidylate, 5-fluorouracyle, and zebularine, alone or in combination with histone deacetylase inhibitors are used clinically in certain types of cancer to activate methylated tumor suppressor or differentiation-inducing genes (23, 24).

DNA methylation can impede the transcriptional activity of a pol II gene (25) directly by blocking the access of a transcription factor (e.g. AP-2, NF-κB, E2F, and c-MYC) to their cognate sites (19). Most of the methylated promoters are, however, recognized by a group of proteins called methyl-CpG-binding proteins (MBDs) by virtue of their conserved methyl-CpG binding domain. Five such MBDs with highly conserved DNA binding domains have been identified (26). Among these proteins, MeCP2, MBD1, MBD2, and MBD4 can bind to methylated DNA. MBD4 is a uracil-DNA glycosylase involved in G-T mismatch repair (12, 27). MBDs repress transcription by recruiting a variety of proteins such as Sin3a, histone deactylases, histone methyltransferases, and HP1a (heterochromatin protein 1a) as co-repressors (for reviews, see Refs. 10, 14, 28, and 29). Kaiso, a partner of β-catenin, lacking the signature methyl CpG binding domain can also bind to methyl-CpGs and repress methylated promoters (30). A defect in the functions of these proteins also leads to various abnormalities. Rett syndrome, a prevalent X-linked neurological disorder among Caucasian females, is caused by dominant negative mutations in the MECP2 gene (31). Adult MB1D1 knock-out mice, like MeCP2 null mice, also exhibit neurological abnormalities (32), whereas MBD4 null mice are susceptible to cancer because of enhanced CpG to TpG mutation in their genome (33).

Most of the studies to date have focused on the up-regulation of rRNA promoter activity. Since methylation of DNA, particularly the promoter region, is known to silence many pol II genes (23–26), it was of interest to investigate whether the methylation status of rRNA promoter modifies pol I-directed rRNA transcription. In the present study, we explored the methylation status of the CpG island that spans the rRNA promoter in human primary hepatocellular carcinomas and corresponding normal liver tissues from the same individuals were treated with sodium bisulfite according to the protocol optimized in our laboratory (36, 37). The rRNA promoter spanning 377–775 of the hRNA B1, 5′-GAGTGGGAGACGTTT-TTTGAG-3′). The annealing temperature used was 50 °C.

To confirm complete conversion of unmethylated cytosines to uracils, the PCR products were digested with TaqI, the restriction sites of which were as follows: hrRNA BF1, 5′-AATTTTGTGGGGATTTTTCTG-3′; hrRNA BF1, 5′-AGCTCAGGAGCCCTTTTCTGAG-3′. The annealing temperature used was 55 °C.

The nested primers were hrRNA BF2 (5′-GAGTGGGAGACGTTTTTTTGAG-3′) and hrRNA BR2 (5′-CATCAGAAAACCTCCCTCCTGAGAG-3′). The annealing temperature used was 55 °C.
methylated promoter fragment was then ligated to the same sites of pBRES-Luc. The ligated plasmid was separated on an agarose gel and purified using a gel extraction kit (Qiagen). Before transcription, the concentrations of methylated and mock-methylated plasmids were measured in a Beckman spectrophotometer at 260 nm.

Site-specific Methylation

Site-specific methylation was carried out following the methodology used for site-directed mutagenesis with some modifications. Single-stranded DNA was obtained by infection of XL-1-blue-MRF bacterial harboring HrDNA plasmid with helper phage (R408; Stratagene). In a typical reaction, positive strand Hr-D-Luc plasmid (~0.05 pmol) was annealed to 1.25 pmol of phosphorylated oligonucleotides (with a specific CpG oligonucleotide) spanning different regions of the oligonucleotide and a 1-h incubation at 66 °C. The annealed oligonucleotide was then extended and ligated in a reaction mixture composed of the oligonucleotide and a 1-h incubation at 66 °C. The reaction was carried out in a thermocycler (PerkinElmer Life Sciences) containing 100 mM glycine. Soluble chromatin with an average size of 600 bp was prepared following the protocol of Weinmann and amplicons were run on polyacrylamide gels (6% acrylamide), and the dried gels were subjected to autoradiography and PhosphorImager analysis. ^1P-labeled PCR products were quantified using ImageQuant software (Amersham Biosciences), and results were depicted as the ratio of methylated and unmethylated DNA precipitated with the antibodies to the input methylated and unmethylated DNA, respectively.

Indirect Immunofluorescence Assay

HepG2 cells were grown overnight on Labtek chamber slides (2 × 10^4 cells/chamber). Cells were fixed with 1:1 methanol and acetone mixture for 15 min at 4 °C, washed with PBS, and permeabilized with 0.3% Triton X-100 in PBS for 10 min at room temperature. Next the cells were incubated with 1% bovine serum albumin for 1 h to block nonspecific binding. Blocked chambers were subsequently washed and incubated overnight at 4 °C with a mixture of anti-nucleolin monoclonal antibody (anti-C23) and antibodies raised against recombinant MBDb, MBd3, and MCp2 in our laboratory or MBd2 (Upstate Biotechnology). Incubation overnight with MBD1, MBd3, and MCp2 antibodies were used at a dilution of 1:500 in PBS, whereas MBd2 antibody was used at a dilution of 1:20. Fluorescein isothiocyanate-conjugated anti-rabbit (for MBd1, MBd3, and MCp2) and anti-sheep (for MBd2) were used for green channel detection, whereas monoclonal anti-C23 antibody was detected by TRITC-conjugated monoclonal anti-mouse antibody (Sigma) for detection in the red channel. Nuclei were stained using 4′,6-diamidino-2-phenylindole in the mounting fluid.

RESULTS

Ribosomal RNA Promoter Is Hypomethylated in Human Hepatocellular Carcinomas—The sequence analysis of RNA transcriptional initiation region of the human rRNA gene showed that it is highly enriched in CpG and that the promoter harbors a CpG island encompassing 19 CpGs in the upstream control element (UCE) and six CpGs in the core promoter (CPE) (Table II). On the other hand, the rat rRNA promoters contain only one and five CpGs, respectively (Fig. 1A). Most of the CpG islands of housekeeping genes transcribed by RNA polymerase II are located in the promoter and exon 1 regions that are methylation-free in normal somatic cells. Although ribosomal RNA genes are housekeeping genes, they are highly reiterated, and only a fraction of these genes is transcribed by pol I. Therefore, we sought to investigate the methylation status of each CpG within different cis elements (spanning from −200 to −9 bp) in the rRNA promoter in human livers and their potential alterations in hepatocellular carcinomas (HCCs). To determine the methylation status of individual CpGs in the human rRNA promoter region, we performed bisulfite genomic sequencing (40) of DNA isolated from hepatocellular carcinomas and the pair-matched liver tissues. Treatment of DNA with bisulfite reagent converts unmethylated cytosine residues to uracils that are amplified as thymine during subsequent PCR. On the other hand, the methylated cytosine residues remain unconverted during bisulfite reaction and amplify as cytosines during subsequent PCR (41). The rRNA promoter region from bisulfite-treated DNA was amplified with nested primers and the PCR product was cloned (for details, see "Materials and Methods"). To analyze quantitatively the methylation status of each CpG spanning the core promoter and UCE, 8–10 randomly selected clones from each HCC as well as the matching liver were sequenced. Alignment and analysis of the methylation profiles of the rRNA promoter in HCC and liver from the same individual revealed a differential methylation profile of CpGs between the two tissues.
FIG. 1. A, schematic depiction of the CpG dinucleotides on rRNA promoters in humans, rats, and mice (not drawn to scale) and the location of CpG dinucleotides (represented as lollipops) spanning the promoter region of human ribosomal RNA. B, methylation status of each CpG base pair spanning −200 to −9 bp of human rRNA promoter in six human hepatocellular carcinomas and matching livers. Ribosomal RNA promoter region was amplified from bisulfite-treated genomic DNA and cloned in TA vector (Invitrogen), and 8–10 clones, randomly selected from each sample, were subjected to automated sequencing. Each row represents the sequence of an individual clone, whereas each column depicts the position of the CpG. The filled and open circles denote methylated and unmethylated CpGs, respectively. C, quantitative analysis of methylation density at −58, −66, and −98 bp with respect to the +1 site of the rRNA promoter in individual tumors and matching livers. The number of clones methylated and unmethylated at these positions among 10 clones (eight for sample 3) is represented in this bar diagram. N and T denote livers and hepatocellular carcinomas, whereas U and M indicate unmethylated and methylated CpGs, respectively.
Comparison of the methylation pattern of the six individual livers revealed variations among them, probably due to polymorphism. For example, the region spanning from $-100$ to $-148$ (with respect to the transcriptional start site) was poorly methylated in individuals 3 and 6 but heavily methylated in individuals 1 and 5. However, the overall methylation density at this region was comparable among the tumors and livers (Fig. 1B). Hypomethylation at UCE between positions $-136$ and $-58$ was more pronounced in all six tumors. Most CpGs within this region (e.g., $-136$, $-107$, $-102$, $-100$, $-98$, $-95$, $-86$, $-66$, and $-58$) were significantly hypomethylated in tumors. The quantitative analysis of methylated and unmethylated cytosine at positions $-58$, $-66$, and $-98$ (Fig. 1C) showed the differential level of methylation in the liver DNA among the individuals. Further, in five of six samples, clones containing unmethylated cytosines were more abundant in HCCs than in corresponding livers. These data demonstrate that the rRNA promoter, particularly in the UCE, is heavily methylated in human liver and is significantly hypomethylated in hepatocellular carcinomas compared with the matched liver tissues.

**rRNA Promoter Activity Inversely Correlates with the Density of Methylation**—To explore whether methylation of the CpG island in the human rRNA promoter indeed suppresses its expression, we cloned a region spanning from $-410$ to $+314$ bp comprising the upstream control element, core promoter, and part of ETS1 in pGL3-basic vector. The rRNA promoter-luciferase reporter (pHrD-IRES-Luc) was constructed by replacing the Kozak sequence of pGL3 vector with the internal ribosome entry site (IRES) (Fig. 2A) (for details, see “Materials and Methods”). This reporter plasmid was used in transient transfection studies. The rationale for eliminating the Kozak sequence was to minimize luciferase expression driven by spurious pol II promoters within the plasmid, since the 5'-trimethyl G-capped mRNAs (transcribed by pol II) exclusively require Kozak sequence for initial ribosome binding and translation (42). The IRES sequence was introduced immediately 5' of the...
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firefly luciferase cDNA for efficient translation of uncapped transcripts (pol I transcript and some viral RNAs), which requires IRES (43). To confirm further the authenticity of the pol I promoter activity in the transfection assay, we took advantage of the species specificity of pol I transcription (44). Accordingly, the human rRNA promoter will not be transcribed by mouse pol I transcription machinery and vice versa (7). For this purpose, we transfected both Hepa (mouse) and HepG2 (human) cells with either pIRES-Luc (promoterless) or pHrD-IRES-Luc vector. In HepG2 cells, the rRNA promoter-driven luciferase activity was 35-fold higher compared with pIRES-Luc, whereas only 3-fold higher activity of pHrD-IRES-Luc was observed in Hepa cells (Fig. 2, B and C). If the reporter gene was transcribed by pol II in HepG2 (human) cells transfected with pHrD-IRES-Luc, the luciferase activity would be comparable with that in Hepa (mouse) cells, since pol II transcription is not species-specific. Significantly higher (14-fold) activity of pHrD-IRES-Luc in HepG2 cells compared with that in Hepa cells clearly demonstrates that the rRNA promoter directs the luciferase expression from pHrD-IRES-Luc in HepG2 cells and is transcribed by pol I (Fig. 2C).

Next, we investigated the effect of methylation of the rRNA promoter on luciferase activity. To address this issue, HepG2 and Hepa cells were transfected with pHrD-IRES-Luc harboring methylated or mock-methylated (with M.HhaI) rRNA promoter or pIRES-Luc. Luciferase activity was measured 24 h post-transfection. The rRNA promoter activity was significantly inhibited in HepG2 cells when methylated by M.HhaI, compared with the mock-methylated control (Fig. 2, B, rows 5 and 6, and C). The inhibitory effect of methylation was also robust in HepG2 cells (10-fold) compared with only 2-fold reduction in Hepa cells (Fig. 2, B, rows 2 and 3, and C), attributing the minimal promoter activity observed in Hepa cells to some leaky expression. These results further confirmed that the rRNA promoter-driven luciferase activity in HepG2 cells was sensitive to methylation. These results also demonstrate that rRNA promoter activity in human system is sensitive to CpG methylation.

We next investigated whether the pol I-driven pHrD-IRES-Luc activity was sensitive to the density of methylation at the promoter. This was accomplished by methylating the promoter and the ETS1 region (–410 to +314) with bacterial methylases that methylate densely (M.SssI), moderately (M.HpaII), or sparsely (M.HhaI) (Fig. 3A). The promoter activity was indeed dependent on the extent of methylation (Fig. 3, B and C). Methylation of only seven sites (five in the upstream of UCE and two in UCE) with M.HhaI resulted in significant (78%) inhibition of the luciferase activity compared with the mock-methylated promoter (Fig. 3C). Methylation with HpaII (which methylates 13 CpGs in the promoter region) resulted in almost complete (94%) inhibition in rRNA transcription compared with the mock-methylated promoter. As expected, the promoter activity was abolished when all of the CpGs located in the region spanning –410 to +314 were methylated with M.SssI (Fig. 3C). These results were highly reproducible in different batches of transfected cells. These data clearly demonstrate that the methylation density at the CpG island plays an important role in repression of human rRNA transcription.

Methylation at Single Sites Located on the Promoter but Not on ETS1 of the Human rRNA Gene Down-regulates the Promoter Activity—The bacterial methylases can methylate specific sites not only in the promoter but also in ETS1. The effect of methylation in the ETS1 on rRNA promoter activity cannot be addressed by the previous experiment. To investigate whether methylation at specific sites in the promoter or the coding region had a differential effect on rRNA promoter activity, we developed site-specific methylation technique. For this purpose, we designed oligonucleotides containing either a methylated cytosine or unmethylated cytosine (as a control) at positions –347, –102, –9, and +152 (Fig. 4B) of the rRNA gene and generated mock-methylated, hemimethylated, and fully methylated circular pHrD-IRES-Luc plasmids (Fig. 4A; for details, see “Materials and Methods”). The promoter activity was measured in HepG2 cells 24 h post-transfection, and the result is expressed as the ratio of human rRNA promoter-directed firefly luciferase activity to the internal control, thymidine kinase promoter-driven Renilla luciferase activity (pRL-TK). When CpG at position –347 (upstream UCE) was symmetrically methylated on both strands (Meth M), the promoter activity was inhibited by ~50%, whereas methylation on individual strands (Meth C, Mock M) had no significant effect (Fig. 4, C, compare rows 2–4 with row 1 and D, lanes 2–4 with lane 1). A comparable level of inhibition of luciferase activity was attained when pHrD-IRES-Luc was methylated at –102 (located in UCE) or at –9 (located in the core promoter) on both strands (Fig. 4, C, rows 8 and 12, and D, lanes 8 and 12, respectively). In contrast, methylation at +152 located on ETS1 has no in-
hibitory effect on the promoter activity (Fig. 4, C rows 13–16, and D, lanes 13–16). These data clearly demonstrate that only symmetrical methylation of CpG at single sites located within the promoter but not in the ETS1 down-regulates human rRNA promoter activity.

Methyl-CpG-binding Protein MBD2 Specifically Represses Methylated rRNA Promoter Activity in HepG2 Cells, whereas MBD1, MBD3, and MeCP2 Inhibit the Promoter Activity Irrespective of Its Methylation Status—Methylation density-dependent inhibition of rRNA promoter activity implicates the role of MBDs in the repression of the methylated rRNA promoter. Transcription of rRNA precursor; its processing to mature 28 S, 18 S, and 5.8 S rRNAs; and their assembly into ribosomal subunits occur in the nucleolus. As a first step to explore the role of the MBDs in rRNA gene transcription in humans, we determined whether any of these MBDs are localized in the nucleoli. To address this issue, we studied co-localization of MBDs with nucleolin, a nucleolar marker protein (45). As expected, all of the MBDs tested are localized in the nucleus as demonstrated by their overlap with 4′,6-diamidino-2-phenylindole-stained nuclei (Fig. 5, panels 2 and 4).

Merging of MBD signals at two spots (panel 3) with that of nucleolin indicated that MBDs are localized also in the nucleolus. These results provided the impetus to explore the role of MBDs in rRNA expression.

Next, we co-transfected the methylated (M.HhaI) or mock-methylated rRNA-promoter in pIRES-Luc into HepG2 cells, along with the expression vectors encoding different MBDs. Overexpression of MBDs in HepG2 cells was confirmed by Western blot analysis with antibodies specific for MBD1 to -3 and MeCP2. These antibodies were raised against C-terminal recombinant proteins that lack highly homologous N-terminal MBD domains and do not cross-react (37, 38). The results showed that MBD2 is the most abundant among these MBDs in HepG2 cells, and its level increased 2–3-fold after overexpres-
In contrast, endogenous levels of MBD1, MBD3, and MeCP2 are low and increased at least 5–10-fold upon overexpression. Five different alternatively spliced variants of MBD1 have been identified in different mammalian cells (46). The endogenous variants of MBD1 expressed in HepG2 cells are 55 and 60 kDa, whereas that of the overexpressed MBD1 is ~65 kDa. Overexpressed MeCP2 generated two polypeptides of ~75 and 66 kDa detected by MeCP2-specific antibody. The lower polypeptide may arise either due to proteolysis or initiation from an internal ATG site. Overexpressed MBD3 co-migrated with the larger of the two closely migrating endogenous polypeptides.

We then determined the effect of MBDs on rRNA promoter activity 48 h post-transfection in HepG2 cells. MBD2 specifically suppressed the activity of the methylated (M.HhaI) promoter (75%) compared with unmethylated promoter (25%). In contrast, MBD1 had a profound repressive effect on the activity of both methylated (70%) and mock-methylated rRNA promoter (65%) (Fig. 6, B and C). A relatively small but reproducible repression (35–40%) of both promoters was observed in cells overexpressing MBD3. On the other hand, overexpression of MeCP2 had no significant effect on the rRNA promoter activity. This experiment was repeated at least three times with different batches of cells, and each transfection reaction was performed in triplicate. These results demonstrate that methyl-CpG-binding proteins indeed differentially modulate human rRNA promoter-driven reporter activity.

**ChIP Assay Demonstrates Association of MBD2 Specifically with the Endogenous Methylated rRNA Promoter in HepG2 Cells**—To confirm that the inhibitory effect of MBDs indeed occurs in vivo in the chromatin context, as observed in transient transfection assay, we analyzed the association of the MBDs with endogenous rRNA promoter by a ChIP assay. In the first step, formaldehyde cross-linked chromatin from growing HepG2 cells was immunoprecipitated with antibodies against specific MBDs. To distinguish between the methylated and unmethylated promoter, the input and ChIP-DNAs were digested with the isoschizomers MspI (methylation-insensitive) or HpaII (methylation-sensitive). The human rRNA promoter was then amplified from the undigested and digested DNAs. The region of the rRNA promoter amplified by the promoter-specific primers harbors four HpaII/MspI sites (Fig. 7A). The HpaII-resistant PCR product generated from the input DNA measures the level of the rRNA promoter methylated at all four sites in HepG2 cells, whereas the difference between mock-digested and HpaII-digested signal reflects the level of the rRNA promoters unmethylated in at least one of the four HpaII sites. Similarly, the HpaII-resistant PCR product obtained from the DNA immunoprecipitated by antibody for a specific MBD indicates its association with the rRNA promoter methylated at all four sites in HepG2 cells. The difference in the signal between the amplified product generated from the undigested and HpaII-digested DNAs. The region of the rRNA promoter amplified by the promoter-specific primers harbors four HpaII/MspI sites (Fig. 7A). The HpaII-resistant PCR product generated from the input DNA measures the level of the rRNA promoter methylated at all four sites in HepG2 cells, whereas the difference between mock-digested and HpaII-digested signal reflects the level of the rRNA promoters unmethylated in at least one of the four HpaII sites. Similarly, the HpaII-resistant PCR product obtained from the DNA immunoprecipitated by antibody for a specific MBD indicates its association with the rRNA promoter methylated at all four sites in HepG2 cells. The difference in the signal between the amplified product generated from the undigested and HpaII-digested DNA measures the association of the MBD with the rRNA promoter unmethylated at least in one of the four HpaII sites. As expected, the input and ChIP DNAs were completely digested with the methylation-insensitive enzyme MspI (3, 6, 9, 12, 15, 18, 21, 24). Quantification of the signal from the input DNA showed methylation of approxi-
immune complexes were pulled down by protein A/G beads, washed specific for MBDs, acetylated histone H3, or preimmune sera. The tin was precleared and immunoprecipitated overnight with antisera and the locations of HpaII sites.

FIG. 7. Analysis of methyl-binding protein association with methylated/unmethylated human rRNA promoter by ChIP analysis. A, schematic presentation of the rRNA promoter region amplified and the locations of HpaII sites. B, formaldehyde cross-linked chromatin was precleared and immunoprecipitated with antisera and the locations of HpaII sites. B, formaldehyde cross-linked chromatin was precleared and immunoprecipitated with antisera and the locations of HpaII sites.

The molecular mechanisms of the methylation-mediated alteration in gene expression associated with neoplastic transformation have not been completely elucidated. Multistage hepatocarcinogenesis is associated with regional hypermethylation of tumor suppressor genes (e.g. hMLH1 (48), RASSF1A (49), P16 (50), O-6-MGMT (51), and PTGPRO (52)) and genomewide hypomethylation of repetitive elements (53) and oncogenes with different buffers, 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 HpaII (H) or MspI (M). An aliquot of the product was subjected to semi-quantitative PCR with 32P-labeled forward primer specific for rRNA or GAPDH promoters. The reaction products were separated on polyacrylamide (8% for rRNA and 6% for GAPDH), and the dried gel was subjected to autoradiography (2-h exposure for rRNA and overnight exposure for GAPDH) and PhosphorImager analysis. C and D, quantitative analysis and graphical representation of the association of different MBDs with rDNA promoter. The Volume Analysis program of the ImageQuant software was used to quantify the 32P signal. Association with methylated promoter = signal in undigested minus signal in HpaII-digested ChiP DNA/input signal in undigested minus HpaII-digested DNA (1:500 dilution). U and M, methylated and unmethylated rRNA promoters, respectively.

C

| DNA            | UNMETH | METH |
|----------------|--------|------|
| INPUT(1:500)   | 1.1    | 1.1  |
| INPUT(1:250)   | 1.1    | 1.1  |
| Ac H4          | 1.2    | 0.4  |
| MECp2          | 0.4    | 0.4  |
| MBD3           | 0.4    | 0.4  |
| MBD2           | 0.1    | 0.4  |
| MBD1           | 0.5    | 0.7  |

**DISCUSSION**

The molecular mechanisms of the methylation-mediated alteration in gene expression associated with neoplastic transformation have not been completely elucidated. Multistage hepatocarcinogenesis is associated with regional hypermethylation of tumor suppressor genes (e.g. hMLH1 (48), RASSF1A (49), P16 (50), O-6-MGMT (51), and PTGPRO (52)) and genomewide hypomethylation of repetitive elements (53) and oncogenes with different buffers, 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 HpaII (H) or MspI (M). An aliquot of the product was subjected to semi-quantitative PCR with 32P-labeled forward primer specific for rRNA or GAPDH promoters. The reaction products were separated on polyacrylamide (8% for rRNA and 6% for GAPDH), and the dried gel was subjected to autoradiography (2-h exposure for rRNA and overnight exposure for GAPDH) and PhosphorImager analysis. C and D, quantitative analysis and graphical representation of the association of different MBDs with rDNA promoter. The Volume Analysis program of the ImageQuant software was used to quantify the 32P signal. Association with methylated promoter = signal in undigested minus signal in HpaII-digested ChiP DNA/input signal in undigested minus HpaII-digested DNA (1:500 dilution). U and M, methylated and unmethylated rRNA promoters, respectively.
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genes (e.g. RAS (54) and C-MYC (52)). Recent studies with
Dnmt1-deficient mice have shown that global hypomethylation
causes propensity toward tumorigenesis (55). A hallmark of
cancer cells is the augmented transcription of rRNA genes to
meet the demand for increased production of ribosomes and for
protein synthesis. We hypothesized that the higher level of
rRNA synthesis in the tumors could also result from hypom-
ethylation of rRNA promoters in addition to up-regulation of
pol I-specific transcription factor(s) (e.g. upstream binding fac-
tor (56)). To our knowledge, the present study is the first report
on the analysis of the methylation status of each CpG in the
promoter region of rRNA genes in human primary tumors and its
relationship to the rRNA promoter activity. Comparison of
the assembled genomic sequences from seven different species
has shown that the diversity among animals lies not in the
protein coding region but rather in the cis regulatory elements,
implying more complex regulation of gene expression in
higher organisms (57). The existence of CpG island in human,
but not in rodent, rRNA promoters suggested that the under-
lying molecular mechanism of methylation-mediated silencing
among these two species may be distinct. This prompted us to
explore whether the CpG island, located on the rRNA pro-
moter, is differentially methylated in human hepatocellular
carcinomas and the potential role of its methylation on rRNA
expression. Indeed, the present data have revealed an inverse
relationship between human rRNA promoter activity and CpG
methylation status. Bisulfite genomic sequencing also dem-on-
drated polymorphic variations in this epigenetic modification
in the liver DNA among the six individuals analyzed. We have
observed polymorphic variations in the liver DNA among the
erats of the same strain (Fisher 344) at the NotI site of a CpG
island on a protein-tyrosine phosphatase (PTPRO) promoter
(52). Like the rRNA promoter, the PTPRO promoter also
showed tumor-specific methylation (52).

The significant hypomethylation specifically in the UCE of
the rRNA promoter merits some explanation. This region is
probably less accessible to DNA methyltransferases during
replication in the rapidly proliferating tumor tissues. Alterna-
tively, CpGs in the UCE region may undergo active demeth-
ylation by MBD2b, a variant of MBD2 that has been reported to
probably less accessible to DNA methyltransferases during
the rRNA promoter merits some explanation. This region is

made up of a combination of a methylated oligonucleotide (in
place of a mutant oligonucleotide) and hemimethylase activity
of Dnmt1 generated a plasmid DNA with a single site methy-
lated in one or both strands (Fig. 4A). Transfection studies
using these plasmids have shown that symmetrical methyla-
tion at single sites located within the core promoter, UCE, or
sequence upstream of UCE, but not in the transcribed (ETS1)
region, represses rRNA promoter activity. Inhibition of pro-
moter activity upon methylation of the cytosine residue at
–347 indicates that methylation at sites outside of upstream
binding factor binding site can modulate rRNA promoter activity,
probably by recruiting MBDs. The unabated activity of the
hemimethylated rRNA promoter also indicates the involve-
ment of MBDs in the repression of rRNA genes, since recruit-
ment of MBDs to the DNA requires symmetrically methylated
CpGs (Fig. 4B). The technically demanding site-specific methyla-
tion analysis, used in the present study, provided the most
conclusive answer regarding the effect of methylation at a
single site on gene transcription.

Although all MBD proteins have highly homologous methyl-
CpG binding domains, immunolocalization studies demon-
strated that in vivo they are targeted to different regions of the
genome by yet unidentified mechanisms. Co-localization of
MBDs with nucleolin implicated their role in rRNA expression.
We used two approaches to identify the MBDs regulating the
rRNA promoter: (i) transient overexpression and its effect on
rRNA-luciferase activity and (ii) ChIP assay to identify their
interactions with the endogenous rRNA promoter. Among
these MBDs, MBD2 specifically repressed the methylated
rRNA promoter activity, whereas MeCP2 inhibited promoter
activity irrespective of its methylation status only when ex-
pressed at a high level (data not shown). MBD1 also repressed
the rRNA promoter irrespective of its methylation status.
This observation is in agreement with its ability to bind both
methylated and unmethylated DNA (29, 46). The ChIP data cor-
borate well the effect of MBDs on rRNA promoter activity in
transient transfection assays. MBDs associate with a variety of
corepressors that include mSin3a, histone deacetylases, and
histone methyltransferases (29, 62, 63). These repressor-core-
pressor complexes are responsible for the ultimate suppression
of pol II-directed gene expression. Identification of the core-
pressors that are recruited by different MBDs to repress rRNA
gene expression will be of considerable interest. Further stud-
ies are needed to address this issue. Nevertheless, the present
study has clearly demonstrated hypomethylation of the rRNA
promoter in human hepatocellular carcinomas and elucidated the
role of promoter methylation in the ribosomal RNA gene
expression and the potential role of the methyl-CpG-binding
protein MBD2 in the methylation-mediated silencing of the
rRNA promoter.

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