Suppression of Metallothionein Gene Expression in a Rat Hepatoma Because of Promoter-specific DNA Methylation*

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Metallothionein I can be induced in response to a variety of agents that include heavy metals and oxidative stress. On the contrary, its induction was suppressed in some lymphoid-derived cancer cells. The mechanism of this repression has not been elucidated. Here, we show silencing of MT-I gene in a solid transplanted rat tumor as a result of promoter methylation at all the 21 CpG dinucleotides that span the region from −225 bp to +1 bp. By contrast, none of these CpG dinucleotides were methylated in the livers from the rats bearing the tumor, which was consistent with the efficient induction of the gene in this tissue by zinc sulfate. Genomic footprinting revealed lack of access of the transcriptional activators to the respective cis-acting elements of the methylated MT-I promoter in the hepatoma. The absence of footprinting was not due to inactivation of the metal regulatory transcription factor MTF-1, because it was highly active in the hepatoma. Treatment of the hepatoma bearing rats with 5-azacytidine, a demethylating agent, induced basal as well as heavy metal-activated MT-I gene expression in the hepatoma, implying that methylation was indeed responsible for silencing the gene. Bisulfite genomic sequencing showed significant (>90%) demethylation of CpG dinucleotides spanning MT-I promoter in the hepatoma following treatment with 5-AzaC. The hypermethylation of MT-I promoter was probably caused by significantly higher (as much as 7-fold) level of DNA methyltransferase activity as well as enhanced expression of its gene in the hepatoma relative to the host liver. These data elucidated for the first time the molecular mechanism for the silencing of a highly inducible gene in a solid tumor transplanted in an animal, as compared with the robust induction in the corresponding parental tissue and have discussed the probable reasons for the suppression of this gene in some tumors.

Methylation at the 5 position of cytosine is a unique modification of eukaryotic genome that occurs within CpG dinucleotides of some genes (for recent reviews see Refs. 1–3). This DNA modification has evolved as an epigenetic mechanism in vertebrates, plants, and fungi but not in yeast, flies, and nematodes. Although CpG dinucleotides are present within the coding region, clusters of this sequence known as CpG islands occur frequently in the promoter regions of some genes. The CpG regions are usually devoid of methylation, but in some cancer cells, these sites appear to be susceptible to methylation. In normal cells, only a few genes on the inactive X chromosome and foreign DNA, e.g. transposable elements and provirus are silenced by this mechanism (4–6).

Many cancer cells have been shown to exhibit global hypomethylation of DNA compared with control cells (7, 8), which is consistent with the oncogenic potential of cells following treatment with 5-azacytidine (5-AzaC),3 a demethylating agent (9). On the contrary, the promoters of some tumor suppressor genes are methylated, resulting in their inactivation (1). Mutation in the coding region of the tumor suppressor genes has been generally considered the major mechanism of inactivation of the tumor suppressor genes. Recently, aberrant DNA methylation of the CpG islands in the promoter region has emerged as an alternative mechanism for the silencing of these growth regulatory genes and may be one of the earliest events in the neoplastic transformation of cells (10). Indeed, silencing of the tumor suppressor genes (e.g. p16, RB, e-cad, ER, VHL, APC, p53, WT1, and BRCA1) by promoter methylation is known to occur in many tumor types (1, 10–14). Promoter methylation is also responsible for some genetic diseases, e.g. fragile X, Prader-Wille, and Angelman syndromes (15, 16).

In recent years, there has been growing interest in the elucidation of the molecular mechanisms by which DNA methylation represses gene expression. Recent studies have identified two repressor proteins, MeCP1 and MeCP2, that bind specifically to methyl-CpG without apparent sequence specificity and repress methylated promoter activity in vitro as well as in vivo (17–19). Methylated genes can be transcribed efficiently in vitro in the absence of MeCPs. This finding suggests that CpG methylation does not by itself render these sites inaccessible to the basal transcriptional machinery or prevent interaction of the transcription factors with the promoters.

Our laboratory has been involved in studying the molecular mechanisms for the control of metallothionein (MT) expression (20–24). The rodents express four isoforms of MT, designated MT-I, MT-II, MT-III, and MT-IV. The first two isoforms are expressed in all tissues, whereas MT-III and MT-IV are expressed almost exclusively in the brain and the stratified epithelium of skin, tongue, etc., respectively (25, 26). The constitutive levels of MT-I and MT-II in most tissues are negligible but can be induced rather dramatically by a variety of agents that include heavy toxic metals, UV radiation, restraint stress, and agents that produce reactive oxygen species (for a review see Refs. 27–29). Overexpression of MT in cells can confer resistance to some of these agents (30–32), whereas disruption

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1 The abbreviations used are: 5-AzaC, 5-azacytidine; DNA-MTase, DNA methyltransferase; MT, metallothionein; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; bp, base pairs; MRE, metal regulatory element; LM, liglation-mediated.
of the MT genes renders the cells/tissues significantly more sensitive to these (33–35). The latter studies indicate a protective role for metallothionein against cellular damage and prompted us to explore the molecular mechanisms for MT expression.

Although heavy metals or other inducers up-regulate the expression of MT-I and MT-II genes in most tissues or cells in culture, there are a few exceptions. For example, none of these genes are induced in the lymphoid-derived tumor cells W7 and S49 by agents like heavy metals, glucocorticoids, etc. (36). The molecular mechanisms for the suppression of MT induction in these cells has not been elucidated. Further, the status of MT expression in the parental cells (e.g. thymus) has not been explored. It is also unknown whether repression of MT induction is unique to cancer cells of lymphoid origin and whether such repression is observed in solid tumors growing in animals, which are the prevalent forms of human cancer. The present study was undertaken to test this possibility. For this purpose, we studied the expression of MT-I in a rat hepatoma in response to heavy metals and compared it with that in the liver of the tumor-bearing rats. The data show that the hepatoma is incapable of expressing MT-I following treatment of the hepatoma-bearing rats with CdSO4 or ZnSO4, whereas it is induced in the host liver. Further, this investigation has elucidated the mechanism for the repression of MT gene expression in the tumor.

MATERIALS AND METHODS

Transplantation of Morris Hepatoma, Treatment with Heavy Metals and 5-AzaC

Morris hepatoma 3924A is a poorly differentiated, rapidly growing tumor with a mean doubling time of 4–5 days (37). It is grown by transplanting a thin slice (0.5 × 3–2 mm) of the solid tumor with a trocher in the hind leg of rats (ACI strain). The tumor on each leg grows to 15–20 g within 4–5 weeks. Most experiments were performed when the tumors attained this size. For heavy metal treatment, the tumor-bearing rats were injected intraperitoneally with CdSO4 or ZnSO4, or with saline alone (control) after 18 days of tumor pass (when tumor growth was obvious). After 2 weeks, the rats were used for DNA-MTase assay.

Total RNA was isolated from the liver and the hepatoma by guanidinium thiocyanate-acid phenol method (38). 30 μg of RNA was separated from formaldehyde-agarose (1.2%) gel electrophoresis and transcribed into cDNA. The DNA-MTase assay was performed following the protocol of Gorski et al. (42). For the preparation of competitive radiolabeled DNA probes, DNA (5 μg) was denatured from both liver and hepatoma nuclei followed by dimethyl sulfate treatment. Ligation-mediated PCR was carried out according to the procedure of Mueller and Wold (47), with modifications as described by Ping et al. (48). The MtI element and MLTF/ARE site were analyzed using one set of upper strand and one set of lower strand specific primers. Sequences of the primers used to read the lower and the upper strand, and the conditions for LM-PCR are described by Majumder et al. (49).

Isolation of RNA and Northern Blot Analysis

Total RNA was isolated from the liver and the hepatoma by guanidinium thiocyanate-acid phenol method (38). 30 μg of RNA was separated from formaldehyde-agarose (1.2%) gel electrophoresis and transferred to nylon membrane. The membrane was then hybridized to mouse random-primed, [γ-32P]dCTP-labeled MT-I minigene (39), mouse DNA methyltransferase (DNA-MTase)-1 (40), or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (41) cDNAs as probes in rapid hybridization buffer (Amersham Pharmacia Biotech) following the manufacturer's protocol.

Isolation of Nuclei from the Liver and Hepatoma and Preparation of Nuclear Extract

The liver and the hepatoma nuclei were isolated by homogenization in high density sucrose buffer following the protocol of Gorski et al. (42) and Rose et al. (43), respectively. The nuclei were then used for in vivo footprinting to make nuclear extract for assay of DNA-binding proteins and for DNA-MTase assay.

Extraction of DNA-binding Proteins from the Nuclei

The DNA-binding proteins were extracted with high salt (0.3 M KCl) from the isolated nuclei following the protocol of Wadzinski et al. (44). Protein in the extract was estimated according to Bradford's method with bovine serum albumin as standard using Bio-Rad reagent.

Electrophoretic Mobility Shift Assay

The DNA binding activities of MTF-1 and Sp 1 were measured by EMSA using specific oligonucleotide probes and 10 μg of the extract. These reagents were incubated with 0.1–0.5 ng of the 32P-labeled MRE-d oligo in the buffer containing 10 mM Hepes (pH 7.9), 60 mM KCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 10% glycerol, and 2 μg of poly(dI-dC) (23, 45). For competition, the extract was preincubated with 100-fold molar excess of the unlabeled competitor oligonucleotides (Sp 1, MRE-s) for 15 min on ice before addition of the labeled oligo. The reaction mixture was incubated on ice for 30 min, and the DNA-protein complexes were resolved by polyacrylamide gel electrophoresis (4% acrylamide; acrylamide/bisacrylamide = 38:7.1:3) in 0.25 × TBE running buffer. For antibody supershift assay, the reaction mixture at the end of the reaction was incubated with 1 μl of anti-MTF-1 antisera, a generous gift from Dr. Walter Schaffner, or antibodies against p70 subunit of Ku protein (Sagra Cruz Biotechnologies).

The sequences of the upper strand of the deoxyoxinucleotides used in the synthesis of probe for EMSA are as follows: (a) MRE-d oligo (for Sp 1 and MTF-1) 5′-GATCCAGGGAGCTCTGCACCTCCGGCCGAAAGT-GTA-3′; (b) MRE-s oligo (for MTF-1) 5′-GATCCAGGGAGCTCTGCAC-aaGCTCCGAAAAGTGA-3′ (the letters in lowercase denote the mutations that abolished Sp 1 binding); and (c) Sp 1 5′-ATTCGATCCGGGGCGGCGGCAGG-3′.

In Vivo Genomic Footprinting

Nuclei were isolated by sucrose density gradient centrifugation of both hepatoma and liver from the hepatoma bearing rats as described in the earlier section. In vivo methylation of nuclear DNA by dimethyl sulfate and the subsequent DNA preparation were performed following the protocol of Bossard et al. (46). For the preparation of control genomic DNA, hepatoma 3924A was isolated from both liver and hepatoma nuclei followed by dimethyl sulfate treatment. Ligation-mediated PCR was carried out according to the procedure of Mueller and Wold (47), with modifications as described by Ping et al. (48). The MRE elements and MLTF/ARE site were analyzed using one set of upper strand and one set of lower strand specific primers. Sequences of the primers used to read the lower and the upper strand, and the conditions for LM-PCR are described by Majumder et al. (49).

Bisulfite Genomic Sequencing

Preparation of Genomic DNA and Bisulfite Treatment—Genomic DNA from the hepatoma and the liver from rats bearing Morris hepatoma 3924A was isolated. DNA (5 μg) was denatured in 0.3 M NaOH for 30 min at 37 °C in 10 μl of volume, mixed with 100 μl of 2 M sodium metabisulfite (Sigma) containing 0.5 μM hydroxypoline (pH 5.0) and cycled in a thermal cycler at 50 °C for 30 min and 95 °C for 2 min for 20 cycles. The bisulfite-treated DNA was then desalted using Wizard DNA Clean-up Kit (Promega) and eluted in 100 μl of H2O. The DNA was then desulfonated in the presence of 0.3 M NaOH at 37 °C for 30 min. The solution was neutralized by addition of NaOAc (pH 4.5) to 0.2 M (final concentration). The bisulfite-converted DNA was then desalted again as stated before and eluted in 70 μl of H2O, and an aliquot (0.5–1 μl) was used for each PCR amplification.

PCR Amplifications of Bisulfite-treated DNA—The metallothyne promoter from −304 to +148 bp was amplified using two sets of primers from the bisulfite-treated liver and hepatoma DNA. The PCR protocol used is as follows: 50 μl of reaction mixture contains 200 μM dNTPs, 2 μM primers, 2.5 μl Taq polymerase (Life Technologies, Inc.). Hot start amplification was performed in a Perkin-Elmer Thermal Cycler under the following conditions: 94 °C/7 min × 1 cycle, 94 °C/2 min, 60 °C/1 cycle, 72 °C/2 min × 35 cycles; and 72 °C/10 min × 1 cycle. To avoid any nonspecific amplification, product from the first round of PCR was then subjected to amplification with a set of nested primers under the same PCR condition, with the exception that the annealing temperature was maintained at 59 °C. Sequences of the nested primers used to amplify the upper strand of MT-I promoter from the bisulfite-treated DNA and the annealing temperatures are described earlier (49).

Restriction Enzyme Analysis of the PCR Product to Confirm Completion of Bisulfite Treatment—To test the efficiency of bisulfite reaction, the amplified DNA (452 bp) was first digested with the restriction...
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Enzymes Apol (R \ $\Leftrightarrow$ AATT \ $\Uparrow$ Y) or Tsp5091 ( \ $\Leftrightarrow$ AATT \ $\Uparrow$ ) that can cut only the converted DNA but not the unconverted DNA. The restriction sites for these two enzymes are generated only when C residues are converted to T residues. The efficiency of bisulfite conversion was confirmed by complete restriction cut of the amplified DNA with Tsp5091 or Apol.

**DNA-MTase Assay**

The nuclei isolated from rat liver and hepatoma tissue were resuspended in 5 volumes of the following buffer: 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 1 mM diithiothreitol, 0.01% sodium azide, 0.2 mM phenylmethylsulfonyl fluoride, 10% glycerol, 1% Tween 20 and lysed as described by Fiala et al. (50). Briefly, the method consists of passing the nuclear suspension through a 25 gauge needle, freezing on dry ice, and subsequent thawing at 37 °C, with the freeze-thaw cycle repeated three times. The suspension was centrifuged at 800 × g for 5 min at 4 °C. The protein content was determined using Bio-Rad Protein Assay reagent with bovine serum albumin as the standard. The activity of DNA-MTase was determined essentially as described by Tollefsbol and with bovine serum albumin as the standard. The activity of DNA-mRNA level in the tumor is severalfold higher than that in the liver; 18S rRNA was used as control to show equal loading in each lane. Lanes 1–3 indicate RNA level in the livers, and lanes 4–6 denote that in the hepatoma from animals injected with saline, ZnSO₄ and CdSO₄, respectively.

**RESULTS**

**MT-I Gene Cannot Be Induced by Heavy Metals in a Rat Hepatoma but Is Expressed in the Liver of the Hepatoma-bearing Rats**—To compare the inducibility of MT-I in response to heavy metals in the liver and the hepatoma, the hepatoma-bearing rats were injected with zinc and cadmium salts, and MT-I mRNA level was measured 4 h post injection. Unexpectedly, the tumor was not responsive to either of the metals, whereas the liver expressed MT-I under these conditions (Fig. 1, compare lanes 1–3 with lanes 4–6, respectively). There was no detectable MT-I mRNA induction in the hepatoma even after 8 h and 24 h of zinc and cadmium treatment, respectively (data not shown). The MT-I induction in the liver was 10- and 20-fold after zinc and cadmium treatment, respectively. The reason for the relatively smaller extent of MT induction in this study is probably due to the higher basal level of MT mRNA in the host liver of the tumor bearing animals relative to the livers of normal ones (52). The higher basal level of MT-I mRNA in the livers of tumor-bearing rats appears to result from stress caused by the tumor burden. This observation is consistent with the dramatic induction of MT in the livers of normal rats exposed to simple stress (23). MT-II mRNA in the hepatoma also remained unaltered after heavy metal treatment (data not shown). Because MT-I and MT-II are coordinately induced in all tissues in response to heavy metals, it is not surprising that both these isoforms are not expressed in the hepatoma. Because the hepatoma contains a relatively higher level of GAPDH mRNA, 18 S ribosomal RNA profile in ethidium bromide-stained gel was compared with ascertain equal RNA loading. This experiment was repeated with several different tumor bearing rats, and the results were comparable.

The Key Transcription Factor MTF-1 That Is Required for Basal as Well as Heavy Metal-induced Expression of MT-I and the Factor Sp 1 Are Active in the Hepatoma—As a first step toward the elucidation of the mechanism of noninducibility of MT-I gene in the hepatoma, we measured the DNA binding activity of transacting factors by EMSA. The cis-acting elements and a few trans-acting factors that regulate MT gene expression are known. There are several copies (a–f) of metal regulatory element (MRE) that span the upstream promoter of mouse MT-I gene (28, 29). In addition to MREs, the MT-I promoter also contains the binding sites for Sp 1 and MLTF/USF (53, 54). MTF-1, a 69–86-kDa protein containing six zinc fingers, binds to the MREs in response to heavy metals and activates the genes (45, 58). This transcription factor is necessary for the basal and inducible expression of MT genes by heavy metals and oxygen free radicals (56, 57). EMSA was used to measure the DNA binding activity of MTF-1 in the liver and the hepatoma nuclear extracts. MRE-d was the oligo of choice in this assay, because MTF-1 exhibited strongest binding affinity for this MRE in vitro (45, 55). Because MRE-d has binding sites for both MTF-1 and Sp 1, two DNA-protein complexes were detected in the liver and hepatoma nuclear extracts (Fig. 2A, lanes 1 and 4). To identify the complexes, competitive EMSA was performed with 100-fold molar excess of Sp 1 consensus oligo and MRE-s oligo, a variant of MRE-d in which Sp 1 site is mutated to abolish its binding (45). The lower complex in both tissues was competed out with MRE-s oligo (lanes 2 and 5), and the upper complex disappeared with Sp 1 oligo (lanes 3 and 6). Because identical amounts of the protein were used in this assay, these data clearly show that both MTF-1 and Sp 1 are more active in the hepatoma than the host liver. The DNA binding activities of MTF-1 and Sp 1 were analyzed with several nuclear extracts prepared from different batches of hepatomas, and the results were reproducible. We also tested whether MTF-1 was activated in the hepatoma in response to zinc. For this purpose we made hepatoma nuclear extract from the animals injected intraperitoneal with ZnSO₄ (200 μmol/g) for 4 h and measured the MRE-d binding activity (Fig. 2B). The complexes formed are specific because their formation could be competed out with an excess of unlabeled MRE-d oligo (lane 3). The slower migrating complex was Sp 1, because its formation was competed by unlabeled Sp 1 consensus oligo (lane 4), and the faster migrating complex was MTF-1, because it could be supershifted with antibodies against p70 subunit of Ku protein (lane 6). Quantitation of the signal by Volume Analysis Program (Molecular Dynamics) showed that there was at least 4-fold activation of MTF-1 in the hepatoma after zinc treatment (lanes 1 and 2). These results indicate that the lack of MT-I inducibility in the hepatoma is not due to absence of specific transcription factors.

Constitutive or Inducible Binding of Transcription Factors Is Not Detected in the MT-I Promoter of the Hepatoma—To explore the possibility that the hepatoma MT-I promoter might

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2 K. Ghoshal, unpublished data.
have attained a refractory chromatin structure that does not allow the positive factors to access their cognate binding sites, we performed in vivo genomic footprinting. Fig. 3A depicts the sequence of the MT-I promoter highlighting the cis-elements relevant for the metal-induced MT-I expression. We designed MT-I gene specific primers for LM-PCR that would allow us to amplify and analyze −226 to −16 bp (with respect to transcription start site) of the promoter. This study would determine the state of promoter occupancy in the hepatoma and liver after zinc treatment. Nuclei from the liver and hepatoma isolated before and after treatment with zinc were exposed to dimethyl sulfate, genomic DNA was isolated and cleaved with piperidine, and MT-I promoter fragment was specifically amplified (see “Materials and Methods” for details). Naked genomic DNA from both liver and hepatoma was treated in a similar manner as intact nuclei and amplified to provide the G ladder. DNA-protein interaction protecting a G residue at a cis-element was visualized as less intense band, whereas more intense bands reveal G residues that are rendered hypersensitive because of factor binding in comparison with the untreated control or naked DNA ladder.

In control liver, constitutive foot printing was observed in the composite element MLTF/ARE, Sp1, and G box when compared with the naked G ladder. Comparison of lanes 1 and 2 in Fig. 3B shows strong protection of MT-I promoter element in the lower strand from control liver DNA. Constitutive footprinting of Sp1 was obvious in the upper strand where simultaneous protection as well as hypersensitivity of neighboring G residues was observed in control liver (Fig. 3C). The G box footprinting in the upper strand was identifiable by hypersensitivity of the G residues in control liver as compared with naked G ladder. Identical footprinting profile of MT-I promoter was obtained from the control and zinc-treated animals, whereas fewer G bases were observed with competition. The footprinting pattern of the different cis-elements in the liver before and after zinc treatment could be correlated with the state of MT-I promoter activation.

None of the classical constitutive or metal-induced footprinting observed in control liver was detectable in hepatoma MT-I promoter before (Fig. 3, B and C, compare lanes 2 and 5) and/or after zinc treatment of the tumor-bearing animals (compare lanes 3 and 6). This observation indicates that even though the positive factors were equally available in both liver and hepatoma, MT-I promoter was totally refractory to any DNA-protein interaction in the hepatoma (see Fig. 2). No unique footprinting appeared in the hepatoma compared with the liver in the region spanning from −226 to −16 bp. This would be possible only if a repressor would bind to a negative regulatory element of MT-I promoter in the hepatoma, creating a novel footprint, and as a consequence, positive factor binding would be inhibited. These data indicate that MT-I promoter is completely inaccessible to the functionally active trans-acting factors present in the tumor, which is most likely due to alteration in chromatin structure.

**MT-I Gene Is Silent in the Hepatoma Because of Methylation of Its Promoter at CpG Sequences**—To determine whether the lack of MT-I promoter occupancy in the hepatoma was due to methylation in the gene, we amplified this region from the hepatoma DNA with gene-specific primers. Sequence analysis of the PCR product established that there was no deletion or point mutation in the tumor (data not shown). Because several growth suppressor genes in many cancer cells can be suppressed as a result of promoter methylation (see Introduction for examples), we reasoned that MT-I promoter in the hepatoma also behaves in a similar manner. To explore such a possibility we treated the hepatoma-bearing rats after 3 weeks of tumor transplantation (when the growth of the tumor is obvious) with 5-AzaC. This cytosine analog demethylates DNA because of its incorporation into DNA that results in irreversible inactivation of DNA-MTase. After injecting the hepatoma-bearing rats with 5-AzaC for 2 weeks on alternate day, the animals were exposed to ZnSO4 (see “Materials and Methods” for details). 4 h later, the tissues (liver and hepatoma) were harvested, and RNA was analyzed by Northern blot analysis. This experiment was performed with four different tumor bearing animals, and a representative Northern blot is shown in
Fig. 4. The hepatoma from the animals pretreated with the cytosine analog showed significantly higher (4-fold) MT-I mRNA level compared with the animals injected with saline (compare lane 8 with lane 7), which was further increased (5-fold) after zinc treatment (lane 9). As compared with normal liver, the host liver from the tumor-bearing rats had 2–3-fold more MT-I mRNA level (compare lane 2 with lane 1). The higher constitutive level in the livers of the hepatoma-bearing...
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Fig. 4. Northern blot analysis of MT-I mRNA from the hepatoma and the host liver after 5-AzaC treatment of tumor-bearing rats. Hepatoma-bearing rats were injected with the cytosine analog or saline followed by injection of ZnSO₄ (200 μmol/kg body weight) for 4 h (see “Materials and Methods” for details). Total RNA (30 μg from hepatoma) isolated from the liver and the hepatoma were subjected to Northern blot analysis with 32P-labeled MT-I cDNA as described in the legend to Fig. 1. Lanes 2-5 represent the mRNA level in the liver from tumor-bearing rats (host liver), and lanes 6-9 indicate that in the hepatoma from the uninjected, saline injected, 5-AzaC injected and 5-AzaC plus zinc-injected hepatoma bearing animals, respectively. Lane 1c denotes mRNA level in the liver of saline-injected normal rat that does not carry the tumor. The lower panel shows the 18S rRNA level in each lane.

rings is probably due to the stress induced by the tumor load, because stress is known to induce MT expression (23, 59). The host livers from the saline- and 5-AzaC-injected rats had very similar MT-I mRNA level (lanes 3 and 4, respectively). There was further induction (10-fold) of MT-I mRNA in the liver when the 5-AzaC-treated animals were injected with zinc (lane 5).

Induction of MT-I mRNA in the hepatoma after 5-AzaC treatment was not unexpected because this tumor had higher MTF-1 activity (Fig. 2A), and the gene was turned on as soon as its promoter was demethylated. When these animals were treated with ZnSO₄, MT-I mRNA in the tumor was increased to a higher level because of further activation of MTF-1 (Fig. 2B). These results clearly indicate that hypermethylation of MT-I gene is primarily responsible for the lack of MT-I induction in the tumor after heavy metal treatment.

Most of the genes that are silent because of methylation contain CpG islands in their promoters (1, 10). Analysis of rat MT-I and MT-II promoter sequence revealed it has high GC content and the CpG to GpC ratio is ~0.6, characteristics of genes containing CpG island (1). Rat MT-I promoter region from −435 to +1 bp with respect to transcription initiation site contains 34 methylatable CpG base pairs. The extent of CpG methylation of MT-I promoter in the hepatoma and the liver was determined by bisulfite genomic sequencing, which allows one to determine the methylation state of each CpG dinucleotide in a DNA sequence (60, 61). The cytosine residues in the genomic DNA are converted to uracil upon bisulfite treatment, which are amplified and read as thymines during subsequent PCR and sequencing. The methylated cytosines, however, remain unaltered upon bisulfite treatment and are read as cytosines upon sequencing. Genomic DNA isolated from both liver and hepatoma were treated with sodium bisulfite (see “Materials and Methods” for details), and a 452-bp fragment of the MT-I promoter (from −304 to +148 bp) was amplified with gene specific primers designed to anneal to the upper strand of the bisulfite converted DNA. This region of the promoter selected for amplification encompasses 25 CpG base pairs and harbors most of the cis-elements, e.g., MREs, MLTF/ARE, that regulate MT-I expression in response to various inducers (Fig. 3A).

To confirm complete bisulfite conversion of the genomic DNA, the PCR-amplified DNA from each sample was digested with Apol and Tsp509I, the restriction sites of which are not expected. Fig. 6A represents a segment of the sequence of the amplified DNA from various samples. In the liver, all the cytosines (both CpGs and non-CpGs) were converted to thymines, whereas in the hepatoma, only the cytosine residues preceding guanosine were unconverted (denoted by the arrows) indicating methylation of these CpG dinucleotides. Out of the 13 CpG residues presented in Fig. 6A, cytosine residue of three CpGs (denoted by asterisks) were detected in both C and T lanes, indicating that among the hepatoma cell population these specific CpGs in the MT-I promoter existed both in methylated and unmethylated state. Careful analysis of the sequencing data revealed that out of the 21 CpG dinucleotides present in the MT-I promoter between −225 and +1 site, all except three CpG dinucleotides were methylated in the hepatoma, and the methylation status of the three CpG residues varied in the population. After treatment of the animals with 5-AzaC in the hepatoma, all methylated cytosines except one were demethylated as evidenced by their conversion to thymines. There was no difference in CpG demethylation pattern between hepatoma treated with 5-AzaC alone or with zinc. None of the corresponding cytosine residues was methylated in the liver from the same animal, and there was no change in methylation pattern in this tissue after 5-AzaC treatment, as expected. Fig. 6B is a schematic representation of the methylation state of CpG dinucleotides spanning this region. These results along with Northern blot analysis of the hepatoma RNA from 5-AzaC-treated animals indicate that methylation of CpG islands of MT-I promoter is responsible for silencing of the gene in the tumor.

Hepatoma Exhibits Significantly Higher DNA-MTase Activity and mRNA Level As Compared with the Liver—A probable
cause for the higher level of MT-I promoter methylation in the hepatoma may be the augmented activity of the enzyme DNA-MTase because of increased expression of DNA-MTase gene. To test this possibility, we first measured the activity of DNA-MTase in the nuclear extract prepared from the tumor and the host liver. This enzyme is known to methylate at the 5 position of cytosines of CpG base pairs. The substrate, poly(dI-dC) is commonly used to measure this enzyme activity because it does not require hemimethylation and is an excellent synthetic substrate of this enzyme. DNA-MTase activity was approximately 7-fold higher in the tumor than that in the liver (Fig. 7A). Further, Northern blot analysis showed a significant increase in the level of DNA-MTase mRNA in the hepatoma relative to the host liver (Fig. 7B). PhosphorImager analysis revealed that the mRNA level in the hepatoma was at least 10-fold higher than that in the liver. These results clearly showed that DNA-MTase was activated in the hepatoma and that the activation or de novo synthesis of this enzyme in the hepatoma probably explains the hypermethylation of some genes such as MT genes in this tumor.

**DISCUSSION**

The present study was undertaken to determine the molecular mechanism by which metallothionein gene is silent in a rat hepatoma. To our knowledge, this is the first direct demonstration that the lack of MT induction in a tissue is due to hypermethylation of its promoter. Recently, we have shown that the suppression of MT induction in the mouse lymphosarcoma cells, P1798, is also due to hypermethylation of its promoter. In the present study we have shown that inhibition of MT-I gene induction in the hepatoma is not due to lack of activation of the key transcription factor MTF-1 but due to methylation of the gene promoter. Activation of the gene after 5-AzaC treatment indicated that methylation was responsible for silencing the gene, which was further confirmed by bisulfite genomic sequencing.

It is not known why only certain genes are hypermethylated in some cancer cells. Although global hypomethylation of genes can occur during malignant transformation of cells, hypermethylation of a few critical genes such as tumor suppressor genes appears to be predominant in the transformed cells or tumors, probably as a result of localized exposure to activated or higher level of DNA methyltransferase (62). It appears that the structure of DNA (63) and the stage of DNA replication (62) may play an important role in determining the genes that undergo hypermethylation. As observed in many other tumors DNA-MTase 1 activity is significantly higher in the hepatoma. The higher level of DNA-MTase mRNA indicates that the increase in this enzyme is probably due to enhanced expression of its gene in the hepatoma. Transformation of cells following overexpression of DNA-MTase confirms the role of this unique enzyme in this process (64). Recently, DNA-MTase has been shown to function as a downstream signal for cell transformation by c-fos (65), SV40 T antigen (66), and ras (67). Further, this enzyme activity is cell cycle-regulated and is maximal at S phase when DNA replication occurs. The enhanced expression of DNA-MTase in the hepatoma may therefore be due to faster cell cycle or activation of some protooncogenes, e.g. c-fos etc. At present we are exploring the expression level of these genes in the tumor.

The suppression of MT gene expression in the hepatoma can be due to (a) inability of positive factors binding to the methylated cis-element, (b) steric hindrance or occlusion of the binding site of the activator by a repressor recruited upon CpG methylation, or (c) refractory chromatin structure that prevents access of basal transcription machinery to the promoter or binding of the positive factors to their cognate elements. It is known that methylation of the specific DNA-binding sites for the factors Sp 1 or MTF-1 cannot inhibit their binding to these elements in vitro and in vivo (68). Both these factors are involved in MT gene transcription, Sp 1 as a general Pol II transcription factor and MTF-1 as a factor for a few selected genes that include MT. Accordingly, we can rule out the possibility that direct inhibition of binding of at least two key factors to the methylated cis-elements of the promoter in hepatoma represses MT-I transcription. Genomic footprinting analysis of the hepatoma MT-I promoter did not identify a negative factor that interacts between −226 and −2 bp. We cannot, however, eliminate the possibility of interaction of a repressor at a site further upstream of the promoter. The absolute inaccessibility of the trans-acting factors to the promoter, as revealed by the in vivo genomic footprinting, suggests that alterations in the chro-

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3 K. Ghoshal and S. Majumder, unpublished data.
Methylation Silences MT-I Gene in Morris Hepatoma

Fig. 7. A. DNA methyltransferase activity in rat liver and hepatoma. Nuclear extracts (20 µg of protein) from the liver and hepatoma were used for each assay. Control reactions lacked the substrate poly(dI-dC)-poly(dI-dC). All assays were performed in triplicate. The DNA-MTase activity is defined as cpm/µg of total protein/h. Results represent the means ± S.E. of results from three independent experiments. B. Northern blot analysis for DNA-MTase mRNA level in the liver and the hepatoma. Total RNA (50 µg) from the liver (lane 1) and the hepatoma (lane 2) were transferred to nylon membrane and hybridized to 32P-labeled mouse DNA-MTase cDNA as probe (upper panel). The lower panel shows the amount of 18 S rRNA in each lane.

matin structure because of promoter hypermethylation is the probable mechanism for the silencing of MT gene in the hepatoma. This explains the lack of footprinting in the promoter region because MeCPs that bind to CpG base pairs cannot be footprinted (17, 18).

Recent studies have shown the existence of at least two major methyl C-binding proteins, namely MeCP1 and MeCP2 that are involved in repression of transcription from the methylated promoter (19). The efficient transcription of many fully methylated genes in the absence of MeCPs further attests to the notion that the CpG methylation by itself does not render these sites inaccessible to the basal transcriptional machinery or prevent interaction of the transcription factors with the promoters. MeCP2 is a well-characterized protein that is required for embryonic development (69). This protein not only interacts with methyl CpG elements but also recruits the repressor complex containing Sin 3A and histone deacetylase (HDAC1 and HDAC2) that represses transcription from the methylated promoter (70, 71). Several other MeCPs have been cloned by screening a human library with methyl-CpG-binding domain although most of them remain to be characterized (72). Among these methyl-CpG-binding domains, MBD3 has recently been identified as the DNA-binding protein of MeCP1 complex (73), which can recruit HDAC to methylated promoter by virtue of its interaction with nuclear remodeling complex NuRD (74). It would be of interest to identify methyl-CpG-binding proteins that bind to methylated MT-I promoter in the hepatoma and whether they are also differentially expressed in the tumor compared with the liver.

One can argue that the lack of MT-I expression may be compensated by the relatively higher level of MT-II expression. Our study has, however, shown that MT-II is also silent in the hepatoma, because Northern blot analysis failed to detect any MT message in hepatoma after heavy metal treatment (data not shown). This finding is not surprising, because MT-I and MT-II are known to be coordinately regulated (28). The mRNAs for two other isoforms, brain-specific MT-III (25) and tongue-specific MT-IV (26) are also not detectable in the tumor (data not shown). It is noteworthy that the tumor suppressor genes, p16, p53, and Rb, are expressed in the hepatoma (data not presented). The continued expression of these genes suggests that another tumor suppressor gene(s) may be inactivated in this tumor. In this context, it should be noted that repression of tumor suppressor genes is dependent upon the nature of the tumor. An obvious question is whether MT possesses potential growth-regulatory functions in some cell types under specific conditions. MT may function in concert with another known tumor suppressor or other proteins to suppress growth in specific cell types, particularly in rapidly dividing cancer cells. Indeed, overexpression of MT-I in transgenic mice has been shown to protect the animals from hepatic hyperplasia induced by hepatitis B viral antigen (75). Interestingly, the lack of MT induction is observed almost exclusively in specific cancer cells and not in normal cells at any stage of growth (76–78). It is also tempting to speculate that the lack of MT expression may promote the growth of some tumors. It is therefore logical to conceive that silencing of MT may be advantageous to at least some highly proliferating cells. Long term studies along these lines are now in progress.

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