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DNA Methyltransferase Levels and Altered CpG Methylation in the Total Genome and in the GSTP1 Gene in Human Glioma Cells Transfected With Sense and Antisense DNA Methyltransferase cDNA

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Abstract This study examines the efficacy of using plasmid expression vectors containing sense and antisense DNA MTase cDNA to both up- and downregulate intracellular DNA MTase levels in human glioma cells. The effects of the changes in MTase levels on global genomic DNA methylation and on the methylation status of CpG dinucleotides in the GSTP1 gene were determined in a glioma cell line that overexpresses the GSTP1 gene. In cells transfected with sense DNA MTase cDNA, MTase gene transcripts increased to a maximum of 2.5-fold at 24 h, while MTase activity increased to a maximum of 3.6-fold at 48 h. The effects of antisense MTase cDNA transfections were less pronounced, and levels of MTase gene transcripts and enzyme activity in transfectants were decreased to only, approximately, one-half the levels of controls. The alterations in DNA MTase expression were associated with corresponding changes in the level of global DNA methylation and in the methylation of the GSTP1 gene in the cells, however, with no detectable morphological or cytotoxic effects on the cells. No significant changes in GSTP1 gene expression were detected after the transfections, presumably because of the high levels of basal GSTP1 expression in the cells. Consequently, the p16 gene, known to be repressed transcriptionally by DNA methylation, was examined for the functional effects of the altered MTase levels. The results showed a 2-fold decrease in p16 gene transcripts with the sense MTase transfectants, while in the MTase antisense-transfected cells p16 transcript levels increased by 30%. Together, these results demonstrate the feasibility of using both sense and antisense DNA MTase expression vectors to regulate DNA MTase levels in glioma cells and that, over relatively short periods of time, the alterations in MTase activities are not deleterious to the cells. The system provides a model with which the role of DNA methylation in critical genes and DNA sequences can be investigated in glioma cells. J. Cell. Biochem. 77: 372–381, 2000.

Key words: DNA; methylation; CpG; glioma cells

5′-Cytosine methylation of CpG islands is an important mechanism involved in the control of the expression of a variety of critical cellular genes, including tumor suppressor genes [Ohtani-Fujital et al., 1993; Herman et al., 1994, 1995; Baylin et al., 1998], growth factor genes [Bender et al., 1998], genes encoding DNA repair proteins [Costello et al., 1994; Chu and Mayne, 1996], and genes involved in cellular metabolism [Keith et al., 1986; Toniolo et al., 1988; Lee et al., 1994; Antoun and Ali-Osman, 1996, 1999; Millar et al., 1999]. The methylated cytosines in CpG dinucleotides can also undergo enzymatic deamination to yield thymine, resulting in point mutations [Rideout et al., 1990].

The mechanism of CpG methylation involves transfer of the methyl group from AdoMet to the 5′-position of cytosine in the dinucleotide, a reaction catalyzed by the enzyme, 5′-cytosine-

Abbreviations used: AdoMet, S-adenosyl-L-methionine; DNA MTase, 5′-cytosine-DNA methyltransferase; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione S-transferase; MGMT, O^6-methylguanine DNA methyltransferase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

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DNA methyltransferase (DNA MTase). DNA MTase displays two methylation activities: (1) maintenance of methylation of the symmetrical cytosine in a hemimethylated CpG doublet, and (2) de novo methylation of unmethylated CpG dinucleotides during DNA replication [Adam et al., 1983; Bestor, 1992; Li et al., 1992]. The latter is a common cellular characteristic that ensures the restoration of the normal methylation status of newly synthesized genomic DNA [Vertino et al., 1994].

Normal tissues differ significantly with respect to their intracellular levels of DNA MTase, with the highest levels reported in placenta, brain, heart, and lung tissue [Yen et al., 1992]. In general, however, tumors exhibit higher DNA MTase activity than normal cells and tissues [Kautiainine and Jones, 1986; El-Deiry et al., 1991]. Thus, higher DNA MTase gene transcripts have been observed in colon carcinoma than in the normal colonal mucosa of the same patients [Schmutte et al., 1996]. In mouse fibroblasts, overexpression of DNA MTase has been shown to result in an increase in DNA methylation, accompanied by tumorigenic transformation [Wu et al., 1993].

The important role played by DNA methylation in the regulation of genes involved in critical events of the neoplastic process, such as tumor cell proliferation, tumor progression, and response to therapy, has led to significant interest in attaining improved understanding of the DNA methylation process in tumor cells. These include the role of DNA methylation in the regulation of genes encoding the DNA repair protein, MGMT [Costello et al., 1994; Von Wronski and Brent, 1994; Qian et al., 1995; Qian and Brent, 1997] and the drug metabolizing protein GST-pi [Lee et al., 1994; Antoun and Ali-Osman, 1996, Millar et al., 1999]. A common approach in several of these studies has been to use 5-aza-2′-deoxycytidine, an inhibitor of DNA MTase [Juttermann et al., 1994] to suppress the methylation process. In other strategies [MacLeod and Szy, 1995], modulation of DNA MTase actvity is accomplished through regulating DNA MTase gene expression. The results of these studies have shown that the cellular effects of DNA MTase modulation are heterogeneous. In some cell types, cell growth is inhibited after modest DNA MTase overexpression, while in others, proliferation is either unaffected, or, is even enhanced [Lee et al., 1996; Bender et al., 1998].

Methylation of a CpG dinucleotide in the *BssH*II site at −298 upstream of the GSTP1 gene has been shown to result in transcriptional silencing of the gene in prostate cancer cells [Lee et al., 1994]. In other tumor types, methylation of the GSTP1 promoter region has also been associated with decreased GSTP1 expression [Jhaveri and Morrow, 1998; Esteller et al., 1998]. In our laboratory, similar findings were made in a medulloblastoma cell line, with respect to the *BssH*II site; however, we observed that methylation of additional CpG sites, other than that contained in the *BssH*II motif, can also significantly alter GSTP1 promoter activity [Antoun and Ali-Osman, 1999]. Recently, extensive differential methylation of both the promoter region and the body of the GSTP1 gene have been reported in prostate cancer cell lines that differ in the levels of GSTP1 expression [Millar et al., 1999]. These reports clearly demonstrate the need for a better understanding of the relationship between DNA methylation and GSTP1 gene expression in tumors, such as, gliomas, in which a strong correlation between GSTP1 expression and both drug resistance and unfavorable prognosis has been observed.

In this study, we investigated the ability to over-express and to downregulate DNA MTase in human glioma cells using eukaryotic plasmid expression vectors containing sense and antisense DNA MTase cDNA, respectively. The results show that DNA MTase activity in glioma cells can be effectively up- and downregulated by this method, and that the changes in DNA MTase levels cause significant changes in global DNA methylation and in the methylation status of the GSTP1 gene in the cells. The system thus provides a model with which the role of DNA MTase and of the methylation process in glioma cells, and possibly, cells of other tumors can be studied.

MATERIALS AND METHODS

Cell Line

The MGR1 cell line used in this study was established in our laboratory from a primary surgical specimen of an anaplastic astrocytoma from a 68-year-old man. The specimen was dissociated enzymatically into single cells and the cells used to initiate primary monolayer cultures, as previously described [Ali-Osman, 1996a]. The cell types in the cultures ranged from spindle-shaped to stellate, with multiple

processes, and occasional giant epithelial-type cells. The cells were immunocytochemically positive for glial fibrillary acidic protein (GFAP) and vimentin and showed the typical pleomorphic morphology characteristic of malignant glial cells in culture [Ali-Osman et al., 1999]. The cell line was confirmed as human by karyotyping and isozyme analysis and was propagated routinely in monolayer cultures in Dulbecco's modified essential medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum (FCS) (HyClone Laboratories, Logan, UT), and 1 μg/ml gentamycin.

cDNA Probes

The full-length human GSTP1 cDNA [Ali-Osman et al., 1997b], the human DNA MTase cDNA [Vertino et al., 1996], and full-length human p16 cDNA amplified by reverse transcription-polymerase chain reaction (RT-PCR) [Srivenugopal and Ali-Osman, 1997] were used as probes in this study. A 1.1-kb of the human GAPDH cDNA (Clontech, Palo Alto, CA) was used as a control probe in all the northern analyses. All probes were ^{32}P-labeled by random priming to a specific activity of approximately, 5×10^8 cpm/μg and were purified on Bio-Spin 6 columns (Bio-Rad, Richmond, CA).

DNA MTase Sense and Antisense Expression Vectors

Two constructs, designated pCMV-HMT and pCMV-TMH, were used in these studies. pCMV-HMT contains the full-length DNA MTase cDNA in the sense orientation, while pCMV-TMH contains antisense DNA MTase cDNA. The cDNA were cloned into the pCMV neoBam vector to be under the control of the immediate early cytomegalovirus (CMV) promoter. To permit positive clone selection, the neomycin resistance gene was placed under the control of the HSV thymidine kinase promoter [Baker et al., 1990]. Details of the construction of these vectors have been described elsewhere [Vertino et al., 1996].

Transfection of Glioma Cells With Sense and Antisense DNA MTase cDNA

Transfection of glioma cells with sense and antisense DNA MTase cDNA was performed using the MBS mammalian transfection system (Stratagene, La Jolla, CA), with minor modifications of the manufacturer's recommended protocol. Two million exponentially growing MGR1 cells were seeded in triplicate in 75-cm^2 tissue culture flasks in DMEM containing 10% FCS; 24 h later, the cells were washed three times with phosphate-buffered saline (PBS), and 25 μg of pCMV-HMT or pCMV-TMH DNA was added to each set of cultures. Controls consisted of untransfected cells and cells transfected with pBK-CMV without the DNA MTase cDNA inserts. The flasks were incubated at 37°C and 5% CO_2 in a fully humidified atmosphere for 3 h, washed three times with PBS, and refed with fresh DMEM containing 10% FCS. The cells were then incubated for up to 48 h at 37°C in 5% CO_2 humidified atmosphere. At 24 and 48 h post-transfection, sets of cultures were harvested and analyzed for DNA MTase gene transcripts and DNA MTase enzymatic activity, as described below. The relative efficiency of the transfection was determined by transfecting replicate glioma cell cultures with a pCMV *lacZ* construct. Under the transfection conditions used, 68% of the tumor cells were positive for β-galactosidase.

Assay of DNA MTase Enzyme Activity

The enzyme activity of DNA MTase was assayed as previously described [Adams et al., 1991], with slight modifications. Exponentially growing MGR1 cells transfected with pCMV-HMT, pCMV-TMH or controls, were harvested by trypsinization, washed twice in PBS, and 3×10^5 cells were resuspended in 0.1 ml hypotonic lysis buffer containing 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM DTT, 0.01% NaN_3, 10% glycerol, 1% Tween 80, 60 μg/ml phenylmethanesulfonyl fluoride (PMSF), and 100 μg/ml RNase A. The cells were lysed by four cycles of freezing at −70°C and thawing at 37°C. Protein concentration of the supernatant, after centrifugation of the cell lysates at 15,000*g* for 20 min, was determined by the Bradford method (Bio-Rad, Richmond CA). Briefly, a 20-μl reaction mixture in lysis buffer was constituted containing 1 μg of cell extract protein, 1 μg poly[d(I-C),d(I-C)], and 3 μCi S-adenosylL[methyl-^{3}H]methionine (72 Ci/mmol; Du Pont NEN, Boston, MA). The mixture was incubated at 37°C for 2 h, and the reaction terminated by the addition of 300 μl of a solution containing 1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 3% 4-aminosalicylate, 5% butanol, 125 mM NaCl, 0.25 mg/ml salmon

sperm DNA, and 1 mg/ml proteinase K. After incubating for a further 30 min at 37°C, the reaction mixture was extracted with an equal volume of 88% phenol, 12% m-cresol, 0.1% 8-hydroxyquinoline. Two volumes of absolute ethanol were added to the aqueous phase and DNA was precipitated at −20°C. The DNA pellet was collected by centrifugation and dissolved in 0.3 M NaOH. Residual RNA was hydrolyzed by incubating the mixture at 37°C for 2 h. The DNA was collected on a glass fiber filter disc, previously saturated with 1 mM nonlabeled AdoMet, and washed with 5% TCA followed by 70% ethanol. The filter was air-dried and the DNA solubilized by heating in 0.5 M perchloric acid at 60°C for 1 h; 5 ml of scintillation fluid was added, and the radioactivity counted by β scintillation. After subtracting background, the radioactivity incorporated in the DNA, a measure of DNA MTase activity, was computed. Reactions were performed in triplicate and the results expressed as the mean ±1 SD.

Analysis of DNA MTase Transcript Levels by Northern Blotting

The alterations in DNA MTase gene transcripts after transfection of MGR1 cells with pHMT and pTMH were determined by Northern blot analysis. MGR1 cells were transfected with pCMV-HMT and pCMV-TMH, as described earlier, and harvested after 24 h and 48 h. Total cellular RNA was isolated by the acid guanidinium thiocyanate phenol-chloroform extraction method [Chomczynski and Sacchi, 1987]; 10 μg RNA from each sample was fractionated electrophoretically in 1.3% agarose gel containing formaldehyde, monitored by ultraviolet (UV) illumination after ethidium bromide staining, and capillary transferred to a nylon membrane (Magna NT, Micron Separation, Westborough, MA). The filters were baked at 80°C in vacuo for 2 h, prehybridized for 2 h, and hybridized for 16 h at 42°C with ^{32}P-labeled DNA MTase-specific probe as previously described [Ali-Osman et al., 1996b]. After washing, the filters were autoradiographed at −80°C on Kodak X-AR 5 X-ray films with intensifying screens and band intensities were aquatinted by densitometry. The filters were stripped, as previously described [Ali-Osman et al., 1996b], and rehybridized with a GAPDH cDNA probe. Band intensities were quantified by densitometry and the GAPDH mRNA band intensities were used to normalize DNA MTase mRNA levels in the cells.

Assay of Total Genomic DNA Methylation

The total global cellular DNA methylation status after transfection with vectors carrying sense and antisense DNA MTase cDNA was quantitated as the residual capacity of the DNA to undergo methylation, as determined by the extent of available methyl accepting sites. The assay was performed essentially as described by Wu et al. [1993]. MGR1 cells were transfected with either sense or antisense DNA MTase cDNA and after 72 h, DNA was isolated using the standard phenol-chloroform-isoamyl alcohol procedures; 200 ng of DNA was incubated with 4 U of M. *Sss*I CpG methylase (New England Biolabs, Beverly, MA), 3 μCi S-adenosylL[methyl-^{3}H]-methionine, and 1.5 μM nonradioactive AdoMet. Controls were similarly set up, but without the addition of methylase. The 20-μl reaction mixture was incubated at 37°C for 4 h, and the reaction was terminated by adding 5 μl of 2.5 mM nonradioactive AdoMet. The whole reaction mixture was spotted on 2.4-cm^2 Whatman glass fiber filter disc, and air-dried for 15 min. The filters were washed with 6 ml of 5% trichloroacetic acid (TCA), followed by 6 ml of 70% ethanol and radioactivity quantitated by liquid scintillation counting. After background radioactivity was subtracted, the residual radioactivity in the DNA was used to compute an index of total genomic DNA methylation, I_{TDM}, as follows:

$$I_{TDM} = 1/[\Delta A/A_{ctrl}]$$

where A_{ctrl} is the radioactivity in control cell DNA, and ΔA is the difference in radioactivity between the DNA of control cultures and that of cultures transfected with sense or antisense DNA MTase vectors. All data points were determined in triplicate and values were expressed as the mean ±1 SD. Each experiment was repeated to confirm results.

Analysis of GSTP1 Gene Methylation in Glioma Cells

The pattern of CpG methylation in the GSTP1 gene in MGR1 cells with and without transfection, with either sense or antisense DNA MTase cDNA, was determined by Southern blotting after genomic DNA digestion with methylation-sensitive restriction endonucleases. Ten μg of total genomic DNA extracted

from control and transfected MGR1 cells was digested simultaneously with *Eco*RI and *Hin*dIII (5 U/μg DNA) at 37°C for 6 h. After *Eco*R1/*Hin*dIII digestion, each DNA sample was digested sequentially with each of the following methylation-sensitive restriction enzymes: *Msp*I, *Hpa*II, and *Hha*I by incubation at 37°C for 16 h with 10 U enzyme per μg DNA. The digested DNA samples were electrophoresed in 1% agarose and transferred onto nylon membranes. After prehybridization, followed by hybridization with ^{32}P-labeled GSTP1 cDNA probe, as described previously, the membranes were washed and autoradiographed at −80°C on Kodak X-AR 5 X-ray films with intensifying screens. The restriction band patterns were compared with those of controls without transfections, to determine any changes in CpG methylation.

Northern Blot Analysis of p16 mRNA

5′-Cytosine methylation of CpG dinucleotides in the promoter region of p16 gene has been shown to result in a significant downregulation p16 gene transcription. We therefore examined changes in transcription of the p16 gene as a measure of the transcriptional effects of the alteration of DNA MTase activity in the glioma cells. Total RNA was isolated from MGR1 cells, after transfection with DNA MTase sense and antisense cDNA constructs, and analyzed for p16 gene transcripts by Northern blotting, using a ^{32}P-labeled full-length p16 cDNA probe. Hybridization and washing conditions were as described earlier. The p16 hybridization bands were quantitated densitometrically and expressed relative to GAPDH transcripts.

RESULTS

DNA Methyltransferase mRNA Levels After Transfection With Sense and Antisense DNA MTase cDNA

Figure 1 shows the results of the northern analysis of the effects of transfections of MGR1 cells with pCMV-HMT (sense) and pCMV-TMH (antisense) expression vectors on DNA MTase gene transcript levels. The ethidium bromide stained gels after electrophoresis are shown in Figure 1A. The Northern blots in Figure 1B show a single DNA MTase mRNA species, with an approximate size of 5.1 kb. In cells transfected with the sense DNA MTase cDNA, MTase mRNA levels increased by approximately 2.5-fold after 24 h and dropped to

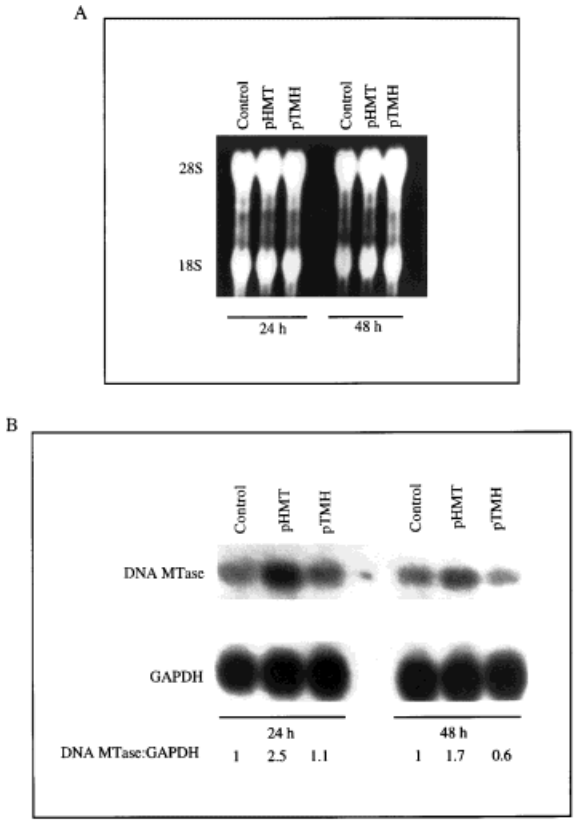

Fig. 1. Northern blot analysis of DNA MTase mRNA in MGR1 cells transfected with sense (pCMV-HMT) and antisense (pCMV-TMH) DNA MTase. **A:** Ethidium bromide-stained gel after electrophoresis of total cellular RNA extracted from cells at 24 h and 48 h posttransfection. **B:** Northern blot autoradiographs of sequential hybridizations with ^{32}P-labeled DNA MTase and GAPDH. MTase band intensities were normalized against GAPDH.

1.7 fold of control levels after 48 h. By contrast, in cells transfected with the antisense DNA MTase construct, DNA MTase gene transcripts levels were decreased to only 51% of control levels, even after 48 h. GAPDH mRNA levels did not change in both control and transfected cells over the course of 48 h.

DNA Methyltransferase Activity in Cells Transfected With Sense and Antisense DNA MTase cDNA

The results of these studies are summarized in Table I. DNA MTase enzyme activity in MGR1 cells transfected with sense DNA MTase cDNA increased by 1.9-fold after 24 h, reached a maximum of 3.6-fold at 48 h, and was reduced to about 2.5-fold of controls after 72 h. Conversely, in cells transfected with antisense DNA MTase cDNA, the effects on DNA MTase activity, although also significant, were

TABLE I. Relative DNA MTase Activity in MGR1 Cells Determined at Different Times After Transfection With Expression Vectors Containing Sense (pHMT) and Antisense (pTMH) DNA MTase cDNA*

	Relative DNA MTase activity (posttransfection time)		
	24 h	48 h	72 h
Control (vector)	1.00	1.00	1.00
pHMT (sense)	1.91 ± 0.7	3.57 ± 0.9	2.54 ± 0.11
pTMH (antisense)	0.84 ± 0.03	0.51 ± 0.07	0.56 ± 0.07

*Controls transfected with native vector were used to determine relative MTase activity in the transfected cells.

less dramatic. Relative to controls, MTase activity in the antisense MTase-transfected cells decreased by 16% after 24 h, 49% after 48 h, and by 44% at after 72 h.

Global Genomic DNA Methylation After Transfection With Sense and Antisense DNA MTase cDNA

Changes in global genomic DNA methylation after transfection with sense and antisense DNA MTase cDNA were examined using the DNA methyl acceptor assay, and the results are summarized in Table II. A direct correlation was observed between total genomic DNA methylation and the level of DNA MTase activity in MGR1 cells after transfection with sense DNA MTase cDNA. Global genomic DNA methylation index in cells transfected with sense DNA MTase cDNA was 1.33 at 24 h, 1.93 at 48 h, and 1.89 at 72 h, relative to controls (methylation index normalized to 1). By contrast, in the antisense MTase-transfected cells, there was a steady decrease in the global DNA methylation index, with indices of 0.81, 0.46 and 0.52 at 24, 48 and 72h posttransfection.

Changes in GSTP1 gene methylation after transfection with sense and antisense DNA MTase cDNA. Figure 2 shows the changes in the patterns of methylation of the GSTP1 gene in MGR1 cells, transfected with sense and antisense DNA MTase cDNA. The band patterns of DNA from control and transfected glioma cells digested with *Hha*I and *Msp*I were similar. This might indicate no significant change in the methylation of CpG dinucleotides in the *Hha*I/*Msp*I motifs of these endonucleases in the GSTP1 gene, or, that the changes are undetectable by the technique used. The latter is particularly likely because the CpG in the *Hha*I/*Msp*I motifs in the promoter region of the GSTP1 gene are very closely clustered together and, consequently, the restriction fragments will not differ significantly in electrophoretic mobility to be resolved under the conditions used. By contrast, the Southern blots of *Hpa*II digests showed a difference in the pattern of DNA methylation of the GSTP1 gene between control and transfected cells, as evidenced by the presence or absence of a 0.5-kb DNA fragment in the DNA of cells transfected with DNA MTase cDNA construct. Since *Hpa*II cleaves only when the cytosine in the CpG in motif is unmethylated, the absence of this fragment in the control DNA indicates that the *Hpa*II sites flanking this fragment were unmethylated de novo and became methylated only upon DNA MTase overexpression.

Changes in p16 Gene Transcript Levels

The results of the analysis of the functional effects of the transfections with sense and antisense MTase transfections were performed on the p16 gene, as summarized in the Northern blots presented in Figure 3. The data show that 24 h after transfection of MGR1 cells with the antisense MTase construct, p16 mRNA levels increased by 1.6-fold relative to controls. By contrast, in the sense DNA MTase-transfected cells, p16 transcript levels were reduced to 0.3 of controls.

Phenotypic Changes and Cytotoxicity in Transfected Cells

No significant changes in cellular morphology and in survival of MGR1 cells transfected with either sense or antisense DNA MTase vector constructs were observed over the 72-h period in which DNA MTase levels were modulated.

DISCUSSION

The role of DNA MTase in regulating the level and pattern of CpG methylation in the

TABLE II. Changes in Total Genomic DNA Methylation in MGR1 Cells Transfected With Sense and Antisense DNA MTase cDNA*

	DNA methylation index, I_{TDM} (posttransfection time)		
	24 h	48 h	72 h
Control (vector)	1.00	1.00	1.00
pHMT (sense)	1.33 ± 0.07	1.93 ± 0.03	1.89 ± 0.05
pTMH (antisense)	0.81 ± 0.004	0.46 ± 0.004	0.52 ± 0.09

*At the stipulated times after transfection, total genomic DNA methylation was determined using the DNA methyl acceptor assay, and a DNA methylation index, I_{TDM}, was computed as described under Materials and Methods.

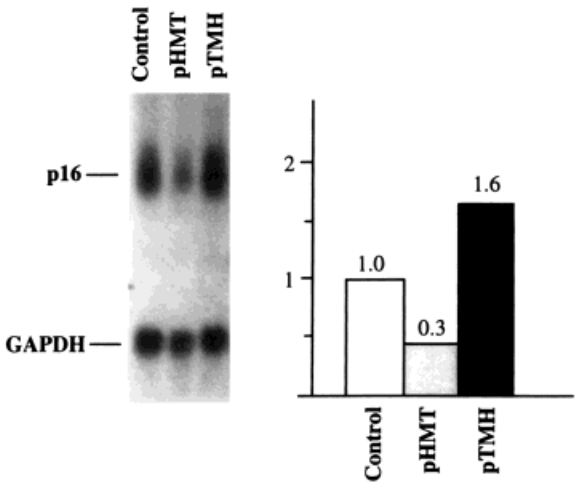

Fig. 3. Northern blot analysis of changes in p16 gene transcripts in MGR1 cells transfected with sense and antisense DNA MTase cDNA. The blot was sequentially probed with ^{32}P-labeled p16 and GAPDH cDNA. Densitometric analysis of the p16 mRNA band was normalized against GAPDH and the p16 mRNA was expressed relative to control.

cellular genome and the effects of alterations in intracellular DNA MTase levels on gene expression and/or cell survival are quite complex. Increases in DNA MTase are known to cause increased methylation of CpG islands that are already methylated under normal physiological conditions, but to have little effect on those that are normally unmethylated [Vertino et al., 1996]. Furthermore, several laboratories have reported significant heterogeneity in both the levels of basal DNA MTase expression, and the extent to which DNA MTase overexpression affects cell survival and/or gene expression in different tissues and cell types [Kautiainen and Jones, 1986; Yen et al., 1992, El-Deiry et al., 1991; Wu et al., 1993; Lee et al., 1994; Vertino et al., 1994].

In this study, we examined whether DNA MTase levels in human malignant glioma cells can be effectively up- and downregulated by transfection with plasmid expression vector constructs containing DNA MTase cDNA in the sense and the antisense orientations, respectively. The data showed increased DNA MTase expression achieved with the sense DNA MTase. The increase in DNA MTase was associated with alterations in the global methylation status of genomic DNA, without any detectable short-term adverse effects on the cells. These observations are particularly significant in light of previous reports showing that overexpression of the human DNA MTase was deleterious in some human and non-human cell lines [El-Deiry et al., 1991; Wu et al., 1993; Bender et al., 1998]. In murine fibroblasts and adrenocortical tumor cells, for example, a more than 3-fold overexpression of DNA MTase has been reported to result in cell death, whereas cell survival was unaffected by more modest increases in DNA MTase expression [Wu et al., 1993; MacLeod and Szy, 1995]. Interestingly, although the lower DNA MTase increases were not cytotoxic, they were sufficient to induce transformation in the fibroblasts [Wu et al., 1993]. This is consistent with the observed DNA MTase increases associated with tumor progression in colon carcinoma [Issa et al., 1993]. In the present study, we show that, in human malignant glioma cells, increasing DNA MTase levels up to 3.5-fold or, decreasing it to one-half of control levels, do not adversely affect cell survival or morphology, at least, over the relatively short 72-h period. Interestingly, the decrease in DNA MTase gene transcripts and enzyme activity observed in cells transfected with antisense DNA MTase cDNA was less than the increases achieved after transfection with sense DNA MTase cDNA. This might be due, in part, to a high turnover rate of DNA MTase and/or its higher stability in glioma cells, both of which could mask modest changes in basal DNA MTase transcript and protein

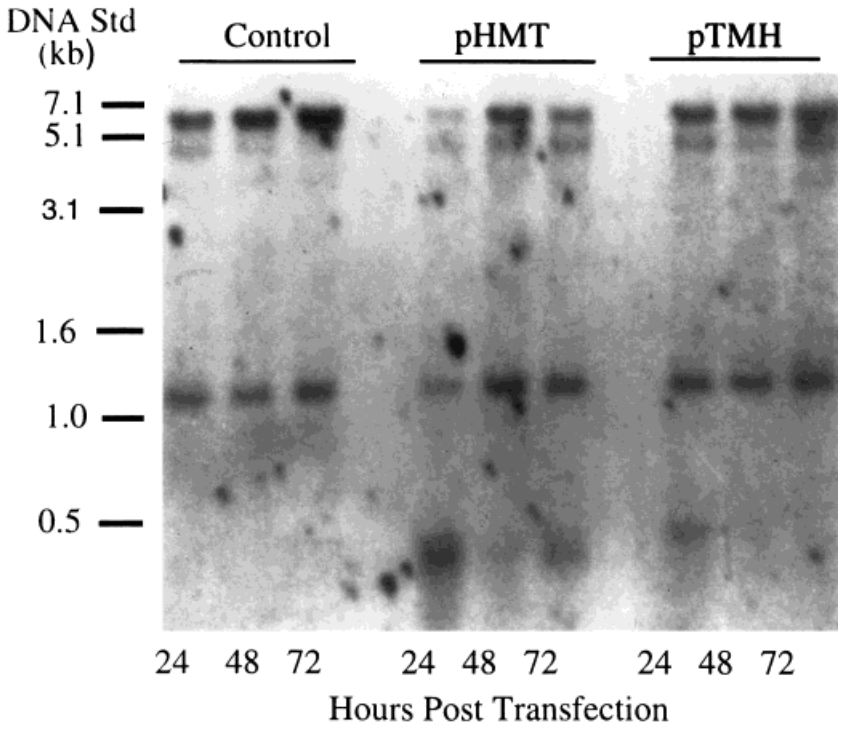

Fig. 2. Southern blot analysis showing the effects on the methylation of the GSTP1 gene in MGR1 cells after transfection with sense and antisense DNA MTase cDNA; 10 μg of total cellular DNA from transfected cells was digested with *Eco*RI and III, followed by digestion with *Hpa*II, as described under Materials and Methods. The digests were electrophoresed in 1% agarose and, after Southern blot transfer, were hybridized with $_{32}$P-labeled full length GSTP1 cDNA.

levels achieved by downregulating the DNA MTase gene.

The increased global methylation of genomic DNA and the altered methylation of the GSTP1 gene we observed in the cells of this glioma cell line after transfection with sense DNA MTase cDNA are particularly interesting. Although changes in GSTP1 gene methylation were not apparent after *Hpa*II digestion, this can be attributed to the high density of *Hpa*II sites in the 5′-region of the GSTP1 gene. Restriction of closely situated *Hpa*II motifs containing CpG dinucleotides would yield multiple small fragments that are not sufficiently different in size to be resolved under the conditions of agarose gel electrophoresis used in this study. Under our experimental conditions, we did not detect changes in GSTP1 expression in the cells after both sense and antisense MTase transfections. This is likely due to the extremely high levels of GSTP1 gene expression in the cell line used in these studies, which did not allow for the detection of relatively modest changes in GSTP1 gene transcripts or protein. It is also possible that factors other than CpG methylation may be contributing to the regulation of the GSTP1 gene in these cells. Another possibility is that significantly longer periods of altered MTase levels are required to appreciably affect GSTP1 gene expression in this cell line. Nonetheless, the association of increased DNA methylation and decrease in p16 gene transcripts observed with the sense cDNA transfectants clearly demonstrates that the increased DNA MTase expression while not toxic to the cells over the 3-day period can significantly affect gene expression. This is consistent with our previous findings with other brain tumor cell lines, showing that differences in de novo DNA methylation is associated with differences in basal GSTP1 expression and that DNA MTase inhibition with 5-azacytidine increased GSTP1 expression in the cells [Antoun and Ali-Osman, 1999]. In the present study, the decreased MTase levels and decreased DNA methylation observed in the antisense DNA MTase-transfected glioma cells are similar to those reported by MacLeod and Szyf [1995], who showed that, in syngeneic mice, expression of an antisense DNA MTase in adrenocortical carcinoma cells reduced the steady state levels of DNA MTase mRNA and protein, and of DNA MTase activity.

The effects of methylation of specific regions of the GSTP1 gene on its expression and/or on other GSTP1 associated phenotypes in glioma cells are not well understood and clearly will require further investigation. Even for prostate

cancer, in which methylation of a CpG dinucleotide in a *Bss*HII site near the GSTP1 promoter region has been shown to result in GSTP1 gene silencing, it was recently reported [Millar et. al., 1999] that differences in CpG methylation in different regions of the GSTP1 gene are associated with significant variations in GSTP1 expression in both normal and neoplastic prostate cells. Our findings in the present study demonstrate the feasibility of up- and down-regulation of DNA MTase expression in human glioma cells, using plasmid expression vectors. The system described should facilitate further elucidation of the role of DNA MTase in regulating DNA methylation and gene expression, not only of the GSTP1 gene, but of other critical genes involved in regulating cell growth, drug resistance, and other important phenotypes of tumor cells as well.

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