Epigenetic Regulation of the MGMT and hMSH6 DNA Repair Genes in Cells Resistant to Methylating Agents

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ABSTRACT

We investigated the relationship between DNA cytosine methylation and the expression of two genes associated with resistance to DNA methylation damage. Variants of RajiMex cells acquired resistance to N-methyl-N-nitrosourea by either reactivating a previously silent O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT) gene or by repressing the hMSH6 mismatch repair gene. DNA sequencing and measurements of mRNA and enzyme levels revealed that MGMT activity was not correlated with methylation of the core MGMT promoter. Treatment with the demethylating agent 5-azadeoxycytidine reduced MGMT mRNA and enzyme levels, indicating that methylation of some nonpromoter sequences may be required for MGMT gene expression. In contrast, both hMSH6 mRNA and protein levels were increased by 5-aza deoxycytidine treatment of an N-methyl-N-nitrosourea-resistant variant that did not express detectable hMSH6, which implies that this gene was transcriptionally silenced by cytosine methylation. This could be substantiated by in vitro modification of the CpG sites in the hMSH6 promoter with restriction methylase M.SssI, which abolished the transcription of a reporter gene under its control in a transient transfection assay. Taken together, our data show that treatment with chemical methylating agents alters gene expression patterns through increased CpG methylation of genomic DNA, and thereby permits the emergence and selection of clones that are resistant to these agents due to increased repair or tolerance of O\textsuperscript{6}-methylguanine.

INTRODUCTION

Methylation agents such as MNU\textsuperscript{1} and N-methyl-N\textsuperscript{-}nitro-N\textsuperscript{-}nitrosoguanidine are powerful mutagens and carcinogens. They are also highly cytotoxic, and analogous methylating compounds, temozolomide and dacarbazine, which have similar reactivity toward DNA, are used in chemotherapy. Cells can avoid most of the lethal and mutagenic effects of these compounds by reversing methylation of the O\textsuperscript{6} atom of DNA guanine. This demethylation reaction is carried out by a specific DNA repair enzyme, MGMT, which is encoded by the human MGMT gene. Repair by MGMT is stoichiometric, and the MGMT molecule inactivates itself by irreversibly transferring the methyl group to one of its own cysteine residues. The capacity of the cell for O\textsuperscript{6}-methylguanine repair is, therefore, determined by the number of its active MGMT molecules at the time of DNA methylation (for reviews see Refs. 1 and 2).

MGMT expression varies widely among human cells (3, 4). In extreme cases, cells produce no MGMT protein despite retaining an intact MGMT gene. These Mex\textsuperscript{-} cell lines comprise approximately 25% of those transformed in vitro or cultured from tumors (for review see Ref. 1). It has been known for some time that the MGMT gene is susceptible to epigenetic regulation associated with an altered frequency of DNA 5-meC residues in CpG dinucleotides (5–8). This “biological” methylation (for a recent review see Ref. 9) is distinct from “chemical” methylation mediated by agents such as MNU or temozolomide, which modify the nitrogen and oxygen atoms of DNA. Early studies indicated that regulation of MGMT expression was complex and the MGMT gene was highly methylated in expressing Mex\textsuperscript{+} cells (5, 6, 10). The most recent studies using the bisulfite DNA sequencing technique (11) have suggested that the level of MGMT is inversely related to the density of CpG methylation in the MGMT promoter (12–14).

Resistance to methylating agents may also develop via tolerance of DNA O\textsuperscript{6}-methylguanine. Methylation tolerance is a consequence of an abrogated long patch MMR pathway (for review see Ref. 15). Inactivation of one of the components of two essential MMR complexes, hMutS\textalpha{} (a heterodimer comprising the hMSH2 and hMSH6 proteins (16) or hMutL\textalpha{} (hMLH1 and hPMS2; Ref. 17), is sufficient to confer a high level of methylating agent resistance in a Mex\textsuperscript{-} cell. MMR defective variants have been isolated from several Mex\textsuperscript{-} human cell lines by a simple selection for resistance to MNU or N-methyl-N\textsuperscript{-}nitro-N-nitrosoguanidine.

A number of independent MNU-resistant variants of the Burkitt’s lymphoma cell line Raji were recently assigned to two categories (18). Some had regained expression of the MGMT gene, whereas the predominant fraction became methylation tolerant through the impairment of hMutS\textalpha{} function—most likely of the hMSH6 subunit. Because the MGMT gene was known to be epigenetically regulated, the latter findings suggested that hMSH6 might also be susceptible to suppression through methylation. The ability of identical treatment to elicit two distinct types of phenotypic change, MGMT reactivation and hMSH6 loss, in a single cell type offered a unique opportunity to examine the factors that might regulate the expression of these genes. Here, we report that, although MGMT regulation in Raji cells is influenced by CpG methylation, the relationship between the methylation status of the MGMT promoter and its transcriptional activity is complex, inasmuch as a high level of methylation failed to attenuate the expression of the gene. In contrast, expression of hMSH6 seems to be regulated by cytosine methylation in the more conventional, inverse fashion. Treatment with a demethylating agent could reactivate the silenced hMSH6 gene, and a high density of CpG promoter methylation efficiently reduced its expression. hMSH6, therefore, joins the hMLH1 MMR gene (19–21) as a potential candidate for suppression by this mutation-free mechanism in human tumors.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma Chemical Co., except where otherwise stated.

Cell Lines. The Mex\textsuperscript{-} and Mex\textsuperscript{+} cell lines and the MNU-resistant variants were maintained as described previously (18). The HT29 colorectal cancer cell line was obtained from C. Dixon (Cancer Genetics Laboratory, Imperial
Cancer Research Fund) and cultured as described previously (22). HeLa cells were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% FCS. Human embryonic kidney 293 cells were maintained in DMEM D5671 (Sigma Chemical Co.) supplemented with 2% inactivated horse serum. Both media contained 2 mm l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. For starvation medium, the inactivated horse serum concentration was reduced to 0.2%. Cells were synchronized at the G1-S boundary by transfer to starvation medium for 48 h, followed by return to complete medium supplemented with 100 μM l-mimosine for a further 14 h.

**DNA Isolation and Bisulfite Treatment.** DNA was isolated essentially following the method of Wu et al. (23). Bisulfite treatment was carried out following the modified (24) procedure of Frommer et al. (11). Briefly, 5 μg of genomic DNA were digested with EcoRI (New England Biolabs) and denatured with 0.3 M NaOH for 15 min at 37°C. A freshly prepared solution of sodium metabsulfite (2.5 M, pH 5.0) and hydroquinone (100 mm) was added to the denatured DNA, and the mixture was incubated at 55°C for 5 h. After desalting (Wizard Clean Up System; Promega), the DNA was desuphontated with 0.3 M NaOH for 15 min at 37°C. The solution was neutralized with 5 M ammonium acetate (pH 7.0), and the DNA was ethanol precipitated and resuspended in Tris-EDTA (pH 7.5).

**PCR Amplification, DNA Cloning, and Sequencing.** Part of the MGMT 5′ CpG island was amplified as two PCR products, using primers established by Qian and Brent (12). First-round PCR was carried out using the sense primer 1 (TGGATATTGATAGTTTTAGGCGGAAGTTGG, positions −284/−255) and the antisense primer 2 (AAACGAAAGCAGACACAT-CACCAAAAT, positions +156/+185). This was followed by a second round of amplification using primer 1 and the nested antisense primer 3 (AAAC-GAAACCCGAAGCAAACATTTTCCCT) for fragment A and primer 2 and the nested sense primer 4 (TGTAGGTAGTGGTGAAGTTGG, positions −159/−127) for fragment B. The amplifications were carried out starting from 0.5 μg of bisulfite-treated DNA under the following conditions: 95°C for 3 min; then 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and finally 1 cycle of 72°C for 7 min. Amplified DNA was ligated into the pBS-SK+ vector (Stratagene) and transformed into Escherichia coli. Several clones for each DNA were sequenced using a Sequenase version 2.0 DNA sequencing kit (Amersham).

**5-Aza-2′-Deoxycytidine Treatment.** Raji cells seeded in exponential growth were treated with 5-aza-2′-deoxycytidine for four cycles of 24 h each during a 5-week period. The drug was removed, and the cells were maintained in fresh medium until normal growth resumed. At this point, we were either harvested for analysis or retreated with drug. Initial treatments were carried out at a concentration of 1 μM. This was increased to 3 μM for subsequent treatments.

**Western Blot Analysis.** Cells were lysed on ice for 1 h using a buffer containing 1% NP40, 10 mm NaF, 1 mm NaH₂PO₄, 10 mm NaHPO₄, 1 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mm AEBSF in PBS and centrifuged at 15,000 × g for 30 min. Fifty micrograms of each extract were separated on 8% SDS-polyacrylamide gels. The proteins were transferred to polyvinylidene difluoride membranes (Millipore) using a semidry electrophoretic transfer apparatus (Bio-Rad) at room temperature. The membranes were blocked for 1 h in PBS-T (containing 0.1% Tween 20) plus 5% powdered skim milk and incubated overnight at 4°C with rabbit anti-MSH6 or mouse anti-PCNA antibodies diluted in PBS-T containing 5% skim milk and 0.1% Tween 20. The membranes were washed in PBS-T and then incubated with horseradish peroxidase-labeled secondary antibody (Bio-Rad) in PBS-T/5% BSA solution. Bound antibody was detected using the enhanced chemiluminescence detection kit (Amersham International).

**RNA Isolation and RT-PCR.** Extraction of total cellular RNA was carried out using Trizol reagent (Life Technologies, Inc.) according to manufacturer’s instructions. Approximately 300 ng of RNA were reverse-transcribed with random hexamers (Perkin-Elmer Corp.). Amplification of all cDNAs was performed under the following PCR conditions: 5 min at 95°C for 1 cycle; then 29 cycles of 95°C for 1 min, 50°C for 30 s, and 72°C for 1 min; and a final elongation step at 72°C for 7 min. The primers used for the amplification were as follows:

- **β Actin:** (25)
  - Sense: ACACATGGCCCCTACTCACGAGG
  - Antisense: AGGGGCGCGACTTCTCATCT

- **MGMT:** (26)
  - Sense: TGTTGGACGGAGACATGAGGG
  - Antisense: TGGGGCGCGGCGCTGGCAAG

The amplified β-actin fragment spans several exon-intron boundaries and was used as a control for DNA contamination. The RT-PCR products were separated by electrophoresis on a 3% agarose gel, stained with ethidium bromide, and visualized by UV light.

**MGMT Measurements.** The MGMT activity in cell extracts was determined as described previously (27).

**Isolation and Characterization of Clones Containing the hMSH6 5′ Flanking Region.** The P1 clone 6261 (kindly provided by C. Lengauer and B. Vogelstein) containing the 5′ flanking region and part of the coding region of hMSH6 was randomly digested with HindIII and subcloned into pUC18. The promoter region was identified by screening this DNA library using a primer, M1AS, designed from the sequence of the 5′-flanking region of hMSH6: 5′-CCAGTGGCCAATCAACAGGCG-3′ (positions −125 to −146). The hMSH6 genomic sequence and a portion of the 5′ untranslated sequence are available; GenBank accession no. AH005068.

The nucleotide sequence of the 5′-flanking region and part of the exon/intron boundary were determined using the ABI Prism dye terminator sequencing kit (Perkin-Elmer Corp.) and a PE 310 sequencer (Perkin-Elmer Corp.).

**Reporter Gene Plasmid Constructs.** Two hMSH6 upstream fragments were tested for the ability to drive the test to activate the firefly luciferase reporter gene in the promoterless plasmid pGL3-Basic (Promega). A 4.8-kb KpnI-NgoMIV fragment was inserted between the KpnI and XmnI sites upstream from the firefly luciferase reporter gene in pGL3-Basic. This construct was designated pGL3 M6US-3558. For the construction of the other deletion mutant, pGL3 M6US-3558 was digested with MluI, rendered blunt-ended with T4 DNA polymerase, and self-ligated. The resulting construct was designated “pGL3 M6US-143.” The orientation and sequence of the inserts were verified by sequencing through the insert-vector junctions. Plasmid DNA purifications were carried out using the Qiagen Midi- or Maxi-prep kit (Qiagen, Inc.).

**Transient Transfections and Dual Luciferase Activity Assays.** HeLa cells (3 × 10⁴) were plated in 35-mm dishes. Following a 24-h incubation, they were transfected with the nonliposomal formulation FuGENE 6 transfection reagent (Boehringer Mannheim), according to manufacturer’s instructions. Each deletion construct (0.5 pmol) was combined with 55 fmol of the pRL-TK vector (Promega) as an internal control in 100 μl of serum-free DMEM containing FuGENE 6 (5 μl). (pRL-TK encodes the Renilla luciferase and its activity can be distinguished from that of the firefly luciferase encoded in the pGL3-Basic in the Dual-Luciferase Assay System, Promega). The FuGENE 6 Reagent/DNA complex was allowed to stand for 15 min at room temperature and was then added dropwise to the cell cultures and evenly dispersed. The cell extracts were prepared 40 h after transfection, using reporter lysis buffer (300 μl; Promega). The dual luciferase reporter assays were carried out on each lysate (5 ml), as recommended by the manufacturer (Promega). Luciferase activities were measured in a luminometer. The transactivation activity of each construct was normalized to the Renilla luciferase internal control. All transfections were carried out in duplicate and repeated at least twice.

**Promoter Methylation Studies.** Plasmid pGL3 M6US-3558, containing the complete promoter region, and the deletion construct pGL3 M6US-143 were methylated by M.SssI, or M.HhaI methyltransferases (New England Biolabs). Methylation by M.HhaI (4 units) was performed for 1 h at 37°C in 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 5 mM β-mercaptoethanol, and 80 μM S-adenosylmethionine. M.SssI methylation was carried out for 1 h at 37°C with 10 units of M.SssI in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, and 80 μM S-adenosylmethionine. The reactions were terminated by heating at 65°C for 10 min. Complete methylation was defined by resistance to cleavage by HhaI and HpaII restriction endonucleases, as determined by agarose gel electrophoresis. Only plasmids that were completely resistant to digestion were used in the transfection experiments aimed at determining the effects of CpG methylation on hMSH6 expression in vivo.

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RESULTS

Methylation-resistant Raji Cell Lines. RajiMex is a Mex− and TK− subline of the Raji Burkitt’s lymphoma. RajiMex− cells are MMR proficient and are sensitive to MNU. Several independent variants of RajiMex−, which exhibit stable resistance to MNU, have been described (18). Three, including Raji 101 and Raji 105, regained expression of MGMT. MGMT activity in extracts of Raji 101 and Raji 105 is 0.33 units/mg protein, which is comparable with that of the RajiMex+ cell line from which the Mex− subline was originally derived. The remaining clones all remained Mex− and are tolerant to DNA methylation damage due to a defect in MMR. Biochemical complementations using cell extracts and purified MMR proteins indicated that the majority, including Raji 104, Raji 9, and the previously described RajiF12, are defective in hMutSα, most likely in hMSH6. All of the variants share a similar 40-fold resistance to MNU, and the MMR-deficient clones also exhibit a modestly increased rate of spontaneous mutation in microsatellites and at the HPRT locus.

Promoter Methylation and MGMT Expression. The methylation status of the MGMT promoter was analyzed by direct DNA sequencing following bisulfite-induced deamination of cytosine residues. DNA isolated from the Mex+ “revertants” Raji 101, Raji 105, and the methylation tolerant Mex− Raji 9 was compared with that of HT29, RajiMex+, and RajiMex−, which served as controls. The DNA samples were digested with EcoRI, denatured, and deaminated with sodium bisulfite. The region −258 to +148, which includes the minimal MGMT promoter, the putative SP1 binding sites, and most of the untranslated exon 1 (Fig. 1a), was then amplified by PCR and cloned. At least 10 clones were sequenced for each cell line. Inspection confirmed that cytosines in non-CpG elements had been quantitatively converted to uracil by the bisulfite treatment. Fig. 1b is an example of this analysis for the −133 to −53 region of the Mex+ HT29 and the RajiMex− cell lines.

Fig. 2 summarizes the distribution of 5-meC in the MGMT promoter of each of the cell lines. MGMT expression was not correlated
with the extent or the pattern of cytosine methylation in the MGMT promoter of RajiMex\textsuperscript{1} and RajiMex\textsuperscript{2} cells. The −258 to +153 region of both cell lines was heavily methylated. Many CpG positions were methylated in every MGMT allele examined. The exceptions were the relatively methylation-free regions around +10 to +70 and −75. In contrast, and in agreement with published data (12), all of the MGMT promoter alleles of the Mex\textsuperscript{+} HT29 cells were almost entirely free of 5-methylcytosine. The high frequency of methylated CpG dinucleotides and the infrequently methylated areas in the Raji MGMT promoter closely resembles the pattern previously reported for Mex\textsuperscript{+} BE tumor cells (12). In Raji cells, however, this pattern of CpG methylation is not associated with the absence of MGMT expression. Although we cannot formally exclude MGMT expression from rare unmethylated MGMT alleles in RajiMex\textsuperscript{+} cells, it seems more likely that extensive methylation of the MGMT promoter is not incompatible with MGMT gene expression.

Reexpression of MGMT in the revertant Raji 101 was not accompanied by reduced cytosine methylation in the −258 to +153 region. The relatively methylation-free region between +10 and +70 was retained, although overall, methylation-free stretches of DNA were less apparent in the Mex\textsuperscript{+} Raji 101 cells. A less extensive analysis of a second Mex\textsuperscript{+} variant, Raji 105, indicated a similar methylation pattern to RajiTK\textsuperscript{−} and Raji 101 in the region −130 to +153 of (Fig. 2). Thus, reactivation of the MGMT gene is not accompanied by a global CpG demethylation in the MGMT promoter of two independent Mex\textsuperscript{+} revertants of Raji Mex\textsuperscript{−} cells. As an additional control, we analyzed the MGMT promoter of Raji 9 cells, which had undergone MNU selection but remained Mex\textsuperscript{−}. As expected, the MGMT pro-

Fig. 2. Methylation patterns in the −258 to +150 MGMT promoter region of Raji and HT29 cells. Each vertical bar represents a CpG site. The fraction of clones with methylated cytosine (% methylation) is shown. Each value is derived from at least 10 clones.
moter in Raji 9 was heavily methylated. Comparison of the promoter sequences of Raji 101, Raji 105, and Raji 9 reinforced the impression of a tendency toward more extensive methylation in cells that had undergone MNU selection.

In summary, a high density of methylation within the core promoter region is not incompatible with MGMT gene expression. The almost complete absence of cytosine methylation in the MGMT promoter of the Mex- human colorectal tumor cell line HT29 was confirmed. The similar methylation patterns of the Raji Mex- and Raji Mex+ MGMT promoters and the retention of extensive promoter methylation in the Mex- Raji 101 or Raji 105 variants indicate that MGMT can be expressed from a methylated promoter. The tendency toward increased CpG methylation in MNU-treated cells suggests that MNU may induce, or select, cells with higher levels of CpG methylation.

MGMT Expression following Azadeoxycytidine Exposure. The Mex- phenotype is associated with a transcriptional silencing of the MGMT gene. RT-PCR of MGMT cDNA confirmed that RajiMex- cells do not express detectable MGMT mRNA (Fig. 3a). MGMT cDNA from RajiMex- and Raji 101 cells was, however, readily amplified. We investigated whether MGMT expression in Raji cells could be altered by modulating the level of 5-meC in their DNA. RajiMex- and Raji 101 cells were treated with the methylation inhibitor AzadC. MGMT mRNA levels in RajiMex- and Raji 101 cells were reduced by AzadC treatment. As determined by semiquantitative RT-PCR, we estimate that MGMT expression was reduced by about 5–10-fold in Raji 101 cells and by approximately 2–3-fold in RajiMex- cells.

The reduced levels of MGMT mRNA in AzadC-treated RajiMex+ and Raji 101 cells were reflected in reduced MGMT activity. MGMT assays from two independent experiments are shown in Fig. 3, b and c. In agreement with its more pronounced effect on mRNA expression in Raji 101, AzadC treatment reduced MGMT activity to a greater extent in these cells than in RajiMex-. Nevertheless, AzadC treatment consistently reduced MGMT enzyme levels in both cell lines. Reactivation of MGMT activity in RajiMex- or Raji 9 was not observed in several experiments in which these cells were treated with AzadC under these or other conditions, which resulted in reexpression of hMSH6 or hMLH1 genes (see below).4

Overall, the observations are consistent with a positive correlation between DNA 5-meC and MGMT expression in Raji cells. Promoter methylation is clearly not the only determinant of MGMT expression. Demethylation is associated with reduced MGMT mRNA and protein levels. These data imply that Raji MGMT is expressed from a methylated gene and that some level of cytosine methylation is a prerequisite for its expression. This essential methylation is presumably outside of the promoter regions.

Loss of hMSH6 Expression in Methylation-tolerant Raji Cells. The Raji 104 clone was selected at the same time and using the same protocol as Raji 101 and Raji 105. RajiF12 was isolated by a similar protocol and has been in culture for several years (28). Both Raji 104 and RajiF12 exhibit stable defects in the hMSH6 function of MMR and are unable to correct single base mispairs in an in vitro assay (18). RajiF12 is deficient in mismatch binding. The hMSH6 function is also defective in Raji 9, which was selected by acute MNU exposure. We analyzed hMSH6 protein levels in these cells by immunoblotting (Fig. 4). The hMSH6 protein was easily detectable in extracts of parental RajiMex- cells and in extracts of three other MMR defective variants (Raji 7, Raji 19, and Raji 10). In contrast, hMSH6 was significantly less abundant or undetectable in extracts of the RajiF12, Raji 9, and

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Cytosine Methylation and hMSH6 Expression. The promoter region of the hMSH6 gene contains a CpG island (see Fig. 5a). Extensive attempts to analyze the methylation pattern of this region of the hMSH6 promoter were unsuccessful. Several different protocols of increasing severity were evaluated in attempts to ensure a complete deamination of the DNA. These included decreasing the size of fragments to be denatured, increasing the time of denaturation, and bisulfite treatment in the presence of urea at temperatures ranging from 50–65°C with periodic increases to 95°C. In all cases, one stretch of DNA was found to be refractory to deamination (data not shown). Although we cannot exclude the possibility that non-CpG sequences are methylated, it seems more likely that this region withstands the prolonged denaturation required for reaction with bisulfite. Whatever the reason for the resistance to deamination, it was not confined to any particular cell line or related in any simple way to hMSH6 expression. Identical regions in DNA from HT29, RajiMex+, RajiMex−, and Raji 9 cells were also resistant to deamination.

In an alternative approach, we examined the effect of in vitro CpG methylation on the expression of a reporter gene controlled by the hMSH6 promoter. To this end, we inserted a 3.9-kb fragment containing the entire hMSH6 promoter and its flanking sequences, or a fragment containing only the hMSH6 CpG island, upstream from the transcription start site of the luciferase gene in the plasmid pMnd (Invitrogen). These vectors (Fig. 5a) were methylated in vitro either with M.HhaI or M.SssI and then transfected into HeLa cells. Gene expression was quantified by the measurement of luciferase levels in the lysates 3 days after transfection. Expression of Renilla luciferase from a cotransfected vector was used as an internal control. Methylation by M.SssI resulted in a significant down-regulation of expression from either the complete hMSH6 promoter or solely from the CpG island fragment (Fig. 5b). In contrast, methylation by M.HhaI did not detectably alter luciferase levels. These data indicate that the hMSH6 promoter is sensitive to CpG methylation. Because M.SssI produces more dense methylation, they further suggest that transcriptional silencing is dependent either on a particular pattern or a critical density of CpG methylation.

hMSH6 Reactivation by Azadeoxycytosine. hMSH6 gene expression was analyzed by RT-PCR before and after AzadC treatment. hMSH6 mRNA was amplified to similar extents from RajiMex− and RajiMex+ cells. In contrast, little or no amplification was observed for Raji 9 cells (Fig. 6a). This observation suggests that the much reduced level of hMSH6 in Raji 9 cells is a consequence of either transcriptional down-regulation or production of unstable mRNA.

The same protocol of AzadC exposure that resulted in reduction of MGMT expression enhanced the level of hMSH6 mRNA as determined by RT-PCR. hMSH6 mRNA was amplified to similar extents in the AzadC-treated Raji 9 and parental RajiMex− cells (Fig. 6a). Immunoblotting indicated that the reactivation of hMSH6 expression following AzadC treatment was accompanied by the reappearance of the hMSH6 protein to approximately half of wild-type levels (Fig. 6b). Thus, restoration of hMSH6 expression by AzadC treatment was observed at both the mRNA and protein levels. These data are consistent with silencing of hMSH6 in Raji 9 by increased cytosine methylation.

In summary, methylation tolerance is associated with loss of hMSH6 function in Raji 9 and other Raji cell variants. This, in turn, is correlated with a significant reduction in the levels of hMSH6 protein. Dense methylation in vitro of its CpG island reduces the effectiveness of the hMSH6 promoter in a reporter construct. In vivo, hMSH6 expression was restored following exposure of Raji 9 to AzadC. This reactivation was evident at both the mRNA and protein levels. All these observations are consistent with a role for increased DNA cytosine methylation in down-regulating hMSH6 expression.

DISCUSSION

The relationship between promoter CpG methylation and MGMT expression has been somewhat controversial (13, 29–32). Apparent discrepancies may have arisen partly through comparisons between unrelated Mex− and Mex+ tumor cell lines and the limitations of restriction enzyme analysis. The MGMT promoter is amenable to bisulfite sequencing. The minimal promoter region and the untranslated exon 1 have previously been shown to be almost methylation free in the Mex− HT29 tumor cell line but heavily methylated in the unrelated Mex− BE cells (12). We confirmed the almost complete absence of 5-mec from the HT29 MGMT core promoter. The methylation patterns of both the Mex− and Mex+ Raji promoters resembled those of BE cells, however. These observations, together with similar data from the closely isogenic Mex− revertants, clearly indicate that a methylation-free promoter is not a prerequisite for MGMT expression.

The properties of the Raji variants were nevertheless consistent with epigenetic control of MGMT. Demethylation by 5-AzadC diminished MGMT activity by reducing the level of MGMT transcripts. A similar reduction in MGMT protein expression has been noted in the HT29 (5) and IMR-90 (30) cell lines exposed to a demethylating agent. A simple explanation for these observations is that a degree of methylation is required for MGMT gene expression and that the modified sequences lie outside of the region containing the promoter and the first exon. We have not identified these putative controlling elements, but there are two likely candidate regions. Methylation of an EcoRI restriction site within the first intron has been positively correlated with MGMT expression (10). In addition, exon 3 is highly CpG rich and meets many of the criteria of a CpG island. However, whereas most of the data on MGMT expression are consistent with the above model, there are exceptions. Spontaneous switching from a Mex+ to a Mex− phenotype in the human lymphoblastoid cell line GM1953 is accompanied by a significant increase in methylation of MGMT sequences (6), whereas azadC-induced reactivation of MGMT in HeLa cells is accompanied by promoter demethylation (12, 30). This suggests that additional or alternative factors may influence MGMT expression in long-term cultured tumor cells. These might include alterations in methylation density within extensive regions of chromatin, which would override the effects within a specific gene (33).

Promoter methylation has been correlated with the absence of MGMT expression in tumors (13). The same immunohistochemical analysis also suggested that up to 40% of certain tumor types may be
Mex\textsuperscript{−}. The fact that this value is about 2-fold higher than that estimated previously from the analysis of MGMT activity in similar tumors (34–36) might simply reflect a difference in the immunohistochemical scoring of tumors that resemble Raji cells, with methylated promoters but low or intermediate MGMT levels, as Mex\textsuperscript{−}. This would incorrectly imply a correlation between promoter methylation and the Mex\textsuperscript{−} phenotype, and overestimate the fraction of true Mex\textsuperscript{−} tumors. In this regard, it is noteworthy that although high levels of MGMT expression were associated with methylation-free promoter sequences in a series of colorectal carcinoma biopsies, promoter methylation status was not predictive for medium or low MGMT expression (37). We emphasize that our data also indicate that MGMT promoter methylation is not diagnostic for the Mex\textsuperscript{−} phenotype. A highly methylated MGMT promoter is fully compatible with a significant level of MGMT expression.

The hMSH6 protein is a component of the hMutS\textsubscript{a} mismatch recognition complex, which is essential for correction of most replication errors. hMSH6-defective cells exhibit increased rates of spontaneous mutation (for review see Ref. 38) and transgenic MSH6-deficient mice are cancer prone (39). Although bisulfite sequencing analysis of the hMSH6 promoter was unsuccessful, other approaches provided evidence for epigenetic down-regulation of this key MMR gene. hMSH6 expression was compatible with the conventional inverse relationship between gene expression and methylation, and the hMSH6 promoter could be shown to be inactivated by CpG methylation \textit{in vitro}. The hMSH6 gene, therefore, joins \textit{hMLH1} as a target for epigenetic regulation in tumors (19–21). Silencing of \textit{hMLH1} is common in sporadic colorectal carcinomas, which are defective in MMR. Indeed, it has been suggested that this mechanism may account for the majority of MMR defects associated with the nonfamilial forms of this disease (40). It is important to note that the strategy used in this study to select the Raji variants has also yielded Raji (18) or A2780 ovarian carcinoma cells with transcriptionally silenced \textit{hMLH1} genes.\textsuperscript{5}

The diagnosis of MMR deficiency in colorectal carcinoma has, to date, depended heavily on the demonstration of dinucleotide repeat microsatellite instability. Although this approach successfully detects \textit{hMLH1}-mutated tumors, the microsatellite instability associated with hMSH6-defective tumors is likely to be more subtle and to be largely

\textsuperscript{5} M. Masson, G. Aquilina, M. Bignami, P. Karran, unpublished data.

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**Fig. 5.** The \textit{hMSH6} promoter. \textit{a}, schematic representation of the \textit{hMSH6} promoter region. The distribution of the major restriction sites, the CpG and \textit{HhaI} sites, are shown. The test plasmids pGL3 M6US-3558, carrying the full-length promoter, and pGL3 M6US-143, bearing the 120 nucleotides upstream of the putative transcription start site, are shown. The reference \textit{Renilla} luciferase construct, which uses the \textit{Herpes simplex} virus type 1 thymidine kinase promoter, is also shown. \textit{b}, luciferase activities of the methylated or mock-methylated \textit{chimeric} \textit{hMSH6} promoter constructs transfected into HeLa cells. Activity is expressed relative to the expression of \textit{Renilla} luciferase from a cotransfected pRL-TK control vector. The experiments were performed as described in “Materials and Methods.” Luciferase activity is expressed as relative light units (RLU). Expression from methylated constructs is shown as filled columns. Hatched columns show the promoter activity of the unmethylated constructs. Bars represent the SD.

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confined to mononucleotide repeats. It is possible that a more thorough analysis of mononucleotide repeat instability in sporadic colorectal carcinoma will uncover a subset of tumors in which hMSH6 has been silenced epigenetically.

Epigenetic alteration of MGMT expression is associated with a modified chromatin configuration (33, 41). One important implication of the present study is that treatment of cells with simple methylating agents might trigger (or select for) CpG-related chromatin remodeling. These changes promote the emergence of resistance to methylating agents either through increased repair, by up-regulation of MGMT, or through tolerance, by loss of hMSH6 expression. A similar situation might exist in tumors. The causes and the consequences of the widespread alterations in CpG methylation levels in tumors are poorly understood, but it would seem that both endogenous and exogenous agents might be involved in this process. Our data raise the possibility that cytosine methylation-related chromatin remodeling might be triggered through chemotherapy. The interrelationships among chemotherapeutic agents, the levels of cytosine methylation, histone acetylation, and chromatin remodeling are of paramount importance to human cancer and merit closer investigation.

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REFERENCES


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