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Methylation of Discrete Regions of the O^6-Methylguanine DNA Methyltransferase (MGMT) CpG Island Is Associated with Heterochromatinization of the MGMT Transcription Start Site and Silencing of the Gene

GEORGE S. WATTS,[1,2] RUSSELL O. PIEPER,[3] JOSEPH F. COSTELLO,[4] YEI-MEI PENG,[5] WILLIAM S. DALTON,[6] AND BERNARD W. FUTSCHER[1,2]*

Bone Marrow Transplant Program[1] and Department of Medicine and Section of Hematology/Oncology,[5] Arizona Cancer Center, and Department of Pharmacology,[2] University of Arizona, Tucson, Arizona 85724; Neuroscience Program and Division of Hematology/Oncology, Loyola University Chicago, Maywood, Illinois 60153[3]; Ludwig Institute for Cancer Research, La Jolla, California 92093[4]; and H. Lee Moffitt Cancer and Research Institute, University of South Florida, Tampa, Florida 33612[6]

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O^6-Methylguanine DNA methyltransferase (MGMT) repairs the mutagenic and cytotoxic O^6-alkylguanine lesions produced by environmental carcinogens and the chemotherapeutic nitrosoureas. As such, MGMT-mediated repair of O^6-alkylguanine lesions constitutes a major form of resistance to nitrosourea chemotherapy and makes control of MGMT expression of clinical interest. The variability of expression in cell lines and tissues, along with the ease with which the MGMT phenotype reverts under various conditions, suggests that MGMT is under epigenetic control. One such epigenetic mechanism, 5-methylation of cytosines, has been linked to MGMT expression. We have used an isogenic human multiple myeloma tumor cell line model composed of an MGMT-positive parent cell line, RPMI 8226/S, and its MGMT-negative variant, termed 8226/V, to study the control of MGMT expression. The loss of MGMT activity in 8226/V was found to be due to the loss of detectable MGMT gene expression. Bisulfite sequencing of the MGMT CpG island promoter revealed large increases in the levels of CpG methylation within discrete regions of the 8226/V MGMT CpG island compared to those in 8226/S. These changes in CpG methylation are associated with local heterochromatinization of the 8226/V MGMT transcription start site and provide a likely mechanism for the loss of MGMT transcription in 8226/V.

O^6-Methylguanine DNA methyltransferase (MGMT) is a DNA repair protein that removes alkyl adducts from the O^6 position of guanine in a reaction that inactivates one MGMT molecule for each lesion repaired (reviewed in reference 24). Cells deficient in or depleted of MGMT are therefore sensitive to the mutagenic and cytotoxic effects of carcinogens and chemotherapeutic agents that produce O^6-alkylguanine lesions (9, 12, 32). MGMT expression has been found to vary widely between normal and tumor tissues (3, 10, 30) and between individual cells within a tumor (21); additionally, 20% of human tumor cell lines lack MGMT activity altogether (8). Loss of MGMT expression is rarely, if ever, due to deletion, rearrangement, or mutation of the MGMT gene (27, 31). This observation, taken together with the relative ease with which MGMT gene expression can be lost in cell culture, suggests that the gene is under epigenetic control (2, 23).

The human MGMT gene possesses a CpG island, as defined by Gardiner-Garden and Frommer (13), which extends from approximately nucleotide (nt) 480 to 1480 of the 5′ end of the gene and spans approximately 500 bases 5′ and 3′ of the transcription start site at nt 956 (nucleotide numbering based on references 15 and 26). Inappropriate 5-methylation of CpG cytosines within the MGMT CpG island is a likely epigenetic mechanism of MGMT inactivation. The existence of this mechanism is supported by the demonstration that a cell line lacking MGMT activity ($MGMT^-$) was capable of transcribing a human MGMT promoter-chloramphenicol acetyltransferase construct, leading to the conclusion that all of the necessary transcription factors were present in the $MGMT^-$ cell line. Additionally, in vitro methylation of the same MGMT promoter-chloramphenicol acetyltransferase construct prevented its transcription in transient-transfection assays (16). Finally, analysis of the MGMT CpG island promoter in glioma cell lines has provided perhaps the strongest support for CpG methylation and inactivation of the MGMT gene. Linker-mediated PCR analysis of the methylation status of individual CpG cytosines in the MGMT promoter of glioma cell lines with variable levels of MGMT gene expression showed that increasing levels of methylation are associated with corresponding decreases in MGMT expression (6).

We have previously reported that the human multiple myeloma cell line 8226/S selected with verapamil and doxorubicin, either in parallel or serially, became phenotypically $MGMT^-$ (11). Reselection of 8226/S with verapamil alone led again to an $MGMT^-$ cell line, which we designated 8226/V. We have used this isogenic model to investigate the role of cytosine methylation in the control of MGMT expression. Analysis of the entire MGMT-associated CpG island, which includes the minimal promoter, the first untranslated exon, and the minimal enhancer (17), indicates that the loss of MGMT expression in 8226/V is associated with a marked increase in methylation of CpG cytosines within discrete regions that bracket the transcription start site. Furthermore, restriction enzyme accessibility assays showed that the $MGMT^+$ 8226/S

* Corresponding author. Mailing address: Arizona Cancer Center, 1515 N. Campbell Ave., Tucson, AZ, 85724. Phone: (520) 626-4646. Fax: (520) 626-2415. E-mail: bfutscher@azcc.arizona.edu.

cell line possesses a region of accessible chromatin around the MGMT transcription start site which has been lost in the methylated 8226/V MGMT promoter. These results indicate that the increase in cytosine methylation of the 8226/V MGMT promoter is associated with a local heterochromatinization of the MGMT transcription start site.

MATERIALS AND METHODS

Cell culture. 8226/S cells were obtained from the American Type Culture Collection (Rockville, Md.) and cultured in RPMI 1640 supplemented with 5% fetal bovine serum (Tissue Culture Biologicals, Tulare, Calif.), penicillin (100 U/ml) (GIBCO BRL, Grand Island, N.Y.), and glutamine (1%, vol/vol) (GIBCO BRL) in a 95% air–5% CO_2 atmosphere at 37°C. The 8226/V line was produced by verapamil selection of 8226/S with continuous exposure to the nontoxic concentration of 20 μM aqueous, racemic verapamil for 9 months. Both 8226/S and 8226/V were passaged once a week, with the addition of medium biweekly. No change in doubling time or viability was noted during verapamil selection. 8226/Vo.o.d. was produced by culturing 8226/V in the absence of verapamil for 1 year.

Reverse transcription-PCR (RT-PCR) analysis of MGMT mRNA expression. Total cellular RNA was isolated by guanidium isothiocyanate cell lysis followed by centrifugation through a 5.7 M CsCl gradient for 2.5 h at 205,000 × *g*. RNA was quantitated in a Beckman TL-100 spectrophotometer by measurements of absorbance at 260 nm. Total cellular RNA was reverse transcribed by incubating a 40-μl reaction mixture composed of 200 ng of RNA; 1× PCR buffer (10 mM Tris [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$); 1 mM each dATP, dCTP, dGTP, and dTTP; 200 pmol of random hexamer, 40 U of RNasin, and 24 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, Ind.) at 42°C for 60 min. The reaction was then stopped by incubation at 99°C for 10 min. MGMT-specific PCR was performed by adding 80 μl of amplification reaction buffer (1× PCR buffer, 25 pmol of MGMT-specific primers, and 2 U of *Taq* DNA polymerase) to 20 μl of the reverse transcription reaction mixture followed by incubation at 94°C for 5 min; 30 cycles of 94°C for 1 min, 60°C for 15 s, and 72°C for 1 min; a final extension at 72°C for 5 min; and a quick chill to 4°C. The upstream primer sequence was from exon 4 (nt 484 to 503), and the downstream primer was from exon 5 (nt 665 to 684) of the MGMT gene. Nucleotide positions were derived from the cDNA sequence (33). Histone 3.3 gene expression from the same cDNA reaction was analyzed as a control. PCR parameters and primer sequences for histone 3.3 were as described previously (26), except that 30 cycles of PCR were performed. For analysis, 10% of the respective PCR products were separated through a 3% agarose gel and visualized by ethidium bromide staining.

Determination of MGMT protein activity. MGMT protein activity was measured by using an oligomer repair assay (12, 36). Protein extracts were prepared by sonicating cells twice for 15 s on ice at a density of 10^6/100 μl in an MGMT assay buffer consisting of 50 mM Tris (pH 8), 5% glycerol, 1 mM dithiothreitol, and 1 mM EDTA, followed by centrifugation at 4°C and 15,000 × *g* for 15 min. The supernatant was collected, and protein was quantitated spectrophotometrically at 595 nm by the Bio-Rad (Hercules Calif.) Protein Assay. Twenty micrograms of protein was incubated for 2 h at 37°C in a total volume of 200 μl of MGMT assay buffer containing 0.1 pmol of an end-labeled 18-mer which contained an O^6-methylguanine lesion incorporated into a *Pvu*II restriction site. Following incubation, the 18-mer was phenol-chloroform extracted, ethanol precipitated with 10 μg of carrier tRNA, resuspended in appropriate restriction buffer, and digested with *Pvu*II. The digestion products were separated by electrophoresis through a 7 M urea–20% polyacrylamide gel at 25 mA for 1.5 h and visualized by autoradiography.

Bisulfite sequencing of the MGMT promoter. Genomic DNA was isolated by standard procedures (29) and digested at 37°C overnight with *Eco*RI at a concentration of 5 U/μg of DNA, followed by phenol-chloroform extraction, ethanol precipitation, and spectrophotometric quantitation at 260 nm. Five micrograms of the *Eco*RI-digested DNA was modified with sodium bisulfite under the conditions described by Clark et al. (4). Briefly, DNA was denatured with 0.3 M NaOH, reacted with 3.6 M sodium bisulfite (pH 5) at 55°C for 14 h, desalted by using a Wizard Prep kit (Promega, Madison Wis.), desulfonated with 0.3 M NaOH, and finally ethanol precipitated in preparation for PCR. The entire MGMT CpG island was amplified from the bisulfite-modified DNA as four PCR products. Each PCR product was obtained through two rounds of PCR with heminested primers specific to the bisulfite-modified sequence of the MGMT CpG island. The primers were as follows: primer 1 (nt 1 to 27), 5′-GGATTTTGTTTTTTTTGAAGGTTTTAG-3′; primer 2 (nt 690 to 667), 5′-CCTAAAACTCTATACCTTAATTTA-3′; primer 3 (nt 463 to 437), 5′-CCCCAAACCAACAAAAACCCTATCAAA-3′; primer 4 (nt 442 to 465), 5′-TAGGGTTTTTGTTGGTTTGGGGGT-3′; primer 5 (nt 1133 to 1109), 5′-AAACTACCCAAACACTCACCAAATC-3′; primer 6 (nt 667 to 690), 5′-TAAATTAAGGTATAGAGTTTTAGG-3′; primer 7 (nt 1034 to 1059), 5′-ATGTTGGGATAGTTAGAGTTTTTAGA-3′; primer 8 (nt 1484 to 1458), 5′-AAAAACTTCTAAAAACTTCCTAAAAC; and primer 9 (nt 1103 to 1127), 5′-GTTTGAGATTTGGTGAGTGTTTGGG-3′. Amplification conditions were as follows: 95°C for 1 min; followed by 45 cycles of 92°C for 1 min, a 0.3°C/s ramp to 56°C for 3 min, a 0.5°C/s ramp to 72°C, 72°C for 1 min, and a 0.5°C/s ramp back to 92°C; followed by a final extension of 72°C for 5 min and a quick chill to 4°C on a DNA Engine (MJ Research Inc. Watertown Mass.). One to two percent of the first-round PCR product was used for a second round of PCR under the same conditions as described above in order to obtain sufficient product for cloning into a TA vector according to the manufacturer's instructions (Original TA cloning kit [Invitrogen San Diego, Calif.] and pGEM-T Easy cloning kit [Promega]). The four MGMT CpG island PCR products were created as follows: product 1 (nt 1 to 460), first round of PCR with primers 1 and 2 followed by a second round with primers 1 and 3; product 2 (nt 442 to 690), first round of PCR with primers 4 and 5 followed by a second round with primers 4 and 2; product 3 (nt 667 to 1133), first round of PCR with primers 4 and 5 followed by a second round with primers 5 and 6; and product 4 (nt 1103 to 1484), first round of PCR with primers 7 and 8 followed by a second round with primers 8 and 9. Nucleotide positions are based on the published MGMT promoter region sequence (15, 26). Ten positive recombinants were isolated from each cell line by using a Qiaprep Spin Plasmid Miniprep kit (Qiagen, Chatsworth Calif.) according to manufacturer's instructions and sequenced on an ABI automated sequencer. The methylation status of individual CpG sites was determined by comparison of the sequence obtained with the known MGMT sequence. The number of methylated CpGs at a specific site was divided by the number of clones analyzed (n = 10 in all cases) to yield a percent methylation at each site. It should be noted that the cytosine at nt 924 was found to be a thymine in both cell lines in all clones sequenced, thus eliminating one CpG site from the CpG island compared to the published sequence.

Chromatin accessibility assay of the MGMT promoter. Twenty million cells from each cell line were washed in phosphate-buffered saline and resuspended in hypotonic solution (10 mM HEPES [pH 7.9 at 4°C], 1.5 mM $MgCl_2$, 10 mM KCl) for 20 min at 4°C. Cellular membranes were lysed with 16 strokes in a Dounce homogenizer at 4°C. Nuclei were pelleted at 500 × *g* for 10 min, resuspended in hypotonic solution, repelleted, resuspended in the appropriate 1× restriction buffer, and divided into four aliquots. Zero, 25, 75, or 225 U of either *Xba*I, *Asp*I, or *Eco*NI was then added to the nuclei and incubated for 15 min at 37°C. Five minutes into the in vivo restriction digest, 20 μl of a 20-mg/mL RNase solution was added to each sample. DNA was then isolated by using a QIAamp Tissue Kit (Qiagen) and was digested with appropriate restriction enzymes to yield DNA fragments of a predictable and informative size (see Fig. 4a and legend for restriction enzymes used in vitro). DNA was size separated on a 2% agarose gel and capillary transferred onto a 0.45-μm-pore-size Nytran Plus membrane (Schleicher and Schuell, Keene N.H.). A random-primed ^{32}P-labeled fragment from the MGMT promoter (see Fig. 4a for probe identity) was then hybridized to the DNA at 42°C overnight. Membranes were then washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate solution at room temperature for 15 min, followed by a 30-min wash at 55°C in 0.1× SSC–0.5% sodium dodecyl sulfate solution. Results were visualized by autoradiography.

RESULTS

Selection of 8226/S with verapamil eliminates MGMT DNA repair activity. MGMT protein activity was determined by using an oligomer repair assay. A ^{32}P-end-labeled 18-mer containing an O^6-methylguanine lesion within a *Pvu*II restriction site was incubated with protein extracts from 8226/S and 8226/V. If the O^6-methylguanine lesion was repaired, then the *Pvu*II site was restored and subsequent digestion of the oligomer by *Pvu*II released an end-labeled 8-nt fragment. If no repair occurred, then *Pvu*II digestion was inhibited, and the oligomer remained an 18-mer. Figure 1a shows that the 8226/S extract contained MGMT activity as evidenced by the production of an 8-nt *Pvu*II digestion product. The 8226/V extract, however, showed no detectable MGMT repair activity.

Loss of MGMT activity following selection with verapamil is due to loss of gene expression. MGMT mRNA was measured in the 8226 cell lines by RT-PCR. First-strand cDNA was generated with total RNA from each of the cell lines and amplified with primers specific to exon 4 and exon 5 of the MGMT gene. As shown in Fig. 1b, MGMT mRNA is detected from 8226/S; in contrast, 8226/V does not express detectable levels of MGMT mRNA. Additionally, 8226/V grown in the absence of verapamil for 1 year (8226/Vo.o.d.) remained $MGMT^-$, which demonstrates the stability of the $MGMT^-$ phenotype in the absence of the selection pressure from verapamil.

To ensure that the loss of detectable MGMT mRNA was not due to gross rearrangement or deletion of the gene in 8226/V,

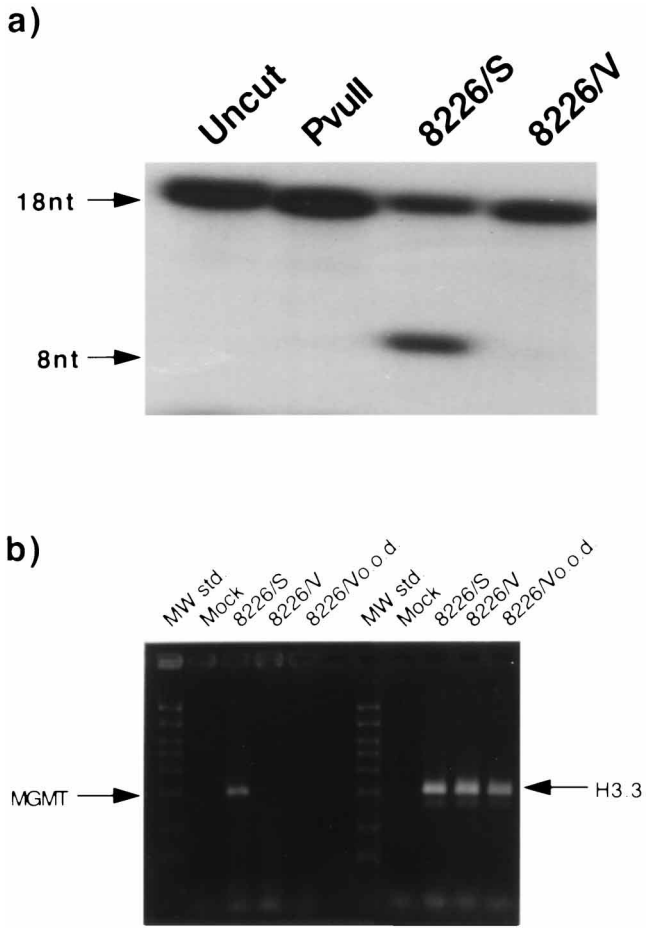

FIG. 1. (a) Analysis of MGMT protein activity in 8226/S and 8226/V. MGMT protein activity was determined by using an oligomer repair assay in which MGMT-mediated repair of an O^6-methylguanine lesion restores a *Pvu*II restriction site. The Uncut lane controls for oligomer integrity, while the *Pvu*II lane shows that without MGMT-mediated repair, the oligomer remains an 18-mer. The 8226/S protein extract displays MGMT activity as indicated by the presence of the 8-nt *Pvu*II restriction product; in contrast, 8226/V does not display any MGMT protein activity. (b) RT-PCR analysis of MGMT mRNA levels. mRNA for MGMT as detected by RT-PCR in the four left-hand lanes is indicated by the arrow at the left. While 8226/S expresses MGMT, neither 8226/V nor 8226/V.o.o.d. (removed from verapamil drug selection for 12 months) expresses detectable MGMT mRNA. RT-PCR for histone 3.3 (H3.3) mRNA was used as a positive control for the reaction and is shown in the four lanes at the right; the presence of product in all three cell lines is indicated by the arrow on the right. MW std., Boehringer Mannheim molecular size standard XI.

Southern analysis was performed on *Eco*RI-digested genomic DNAs from 8226/S and 8226/V. Comparison of the bands produced by *Eco*RI digestion of the two cell lines' genomic DNA showed that the MGMT-coding region appears to be normal in 8226/V (Fig. 2).

Cytosine methylation is found within discrete regions of the 8226/V MGMT promoter CpG island. As an explanation for the loss of detectable MGMT mRNA, we investigated the CpG methylation within the MGMT CpG islands of 8226/S and 8226/V. Initial data obtained by Southern analysis with methylation-sensitive restriction enzymes indicated that the MGMT promoter CpG island in 8226/V was methylated compared to the CpG island in 8226/S (data not shown). The information obtained by this Southern blot analysis was limited, however, because only CpGs within the *Hpa*II restriction enzyme site, 5′-CCGG-3′, were analyzed, and only fragments which coincided with the probe were detected.

To obtain a detailed quantitative analysis of MGMT CpG island methylation, bisulfite sequencing was used to determine the percent methylation at all 108 CpG sites within the MGMT promoter-associated CpG islands of 8226/S and 8226/V as well as nine CpGs in the promoter region 5′ to the CpG island. The results of the bisulfite sequencing showed that in 8226/S there were high levels of cytosine methylation in the nine CpG sites 5′ to the CpG island (nt 0 to 480) but that the CpG island was nearly devoid of cytosine methylation. Of the 108 CpG sites within the CpG island, only 13 were methylated, and then in only 10% of 8226/S cells (Fig. 3). In contrast, the MGMT CpG island in 8226/V was methylated both 5′ to and within the CpG island. The increased methylation within the 8226/V MGMT CpG island fell into three distinct regions. The furthest 5′ region of increased cytosine methylation occurred between nt 510 and 603, approximately 400 bp upstream of the transcription start site. The other two regions of increased methylation also spanned approximately 100 bp (nt 691 to 794 and nt 1068 to 1168) and flanked the transcription start site at a distance of approximately 150 bp. Surprisingly, this spacing of methylated regions around the 8226/V MGMT transcription start site created a relatively methylation-free region of approximately 300 bp (~nt 800 to 1100) that contained the transcription start site, the minimal promoter, and the minimal enhancer.

Increased methylation in discrete regions of the 8226/V MGMT CpG island is associated with heterochromatinization of the transcription start site. To determine if cytosine methylation was associated with a change in chromatin structure, chromatin accessibility experiments were used to probe the 8226/S and 8226/V MGMT CpG islands in vivo. Isolated nuclei

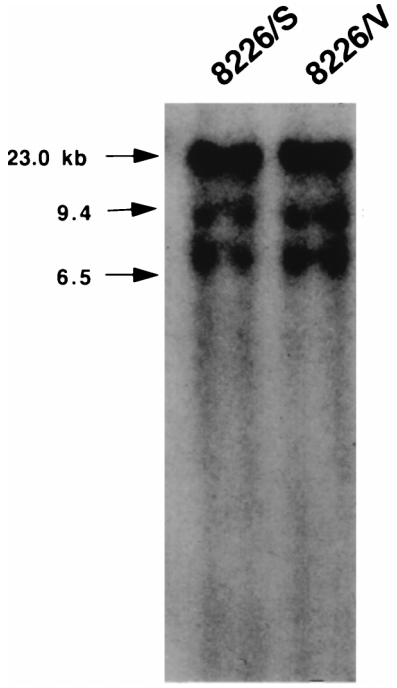

FIG. 2. Southern analysis of the MGMT-coding region. Genomic DNAs from 8226/S and 8226/V were digested with *Eco*RI, followed by Southern analysis with a probe specific for the entire MGMT-coding region. Three characteristic *Eco*RI restriction bands are seen in both cell lines; this indicates that the MGMT-coding region has not undergone any gross alteration. λ/*Hin*dIII size standards are indicated by arrows at the left.

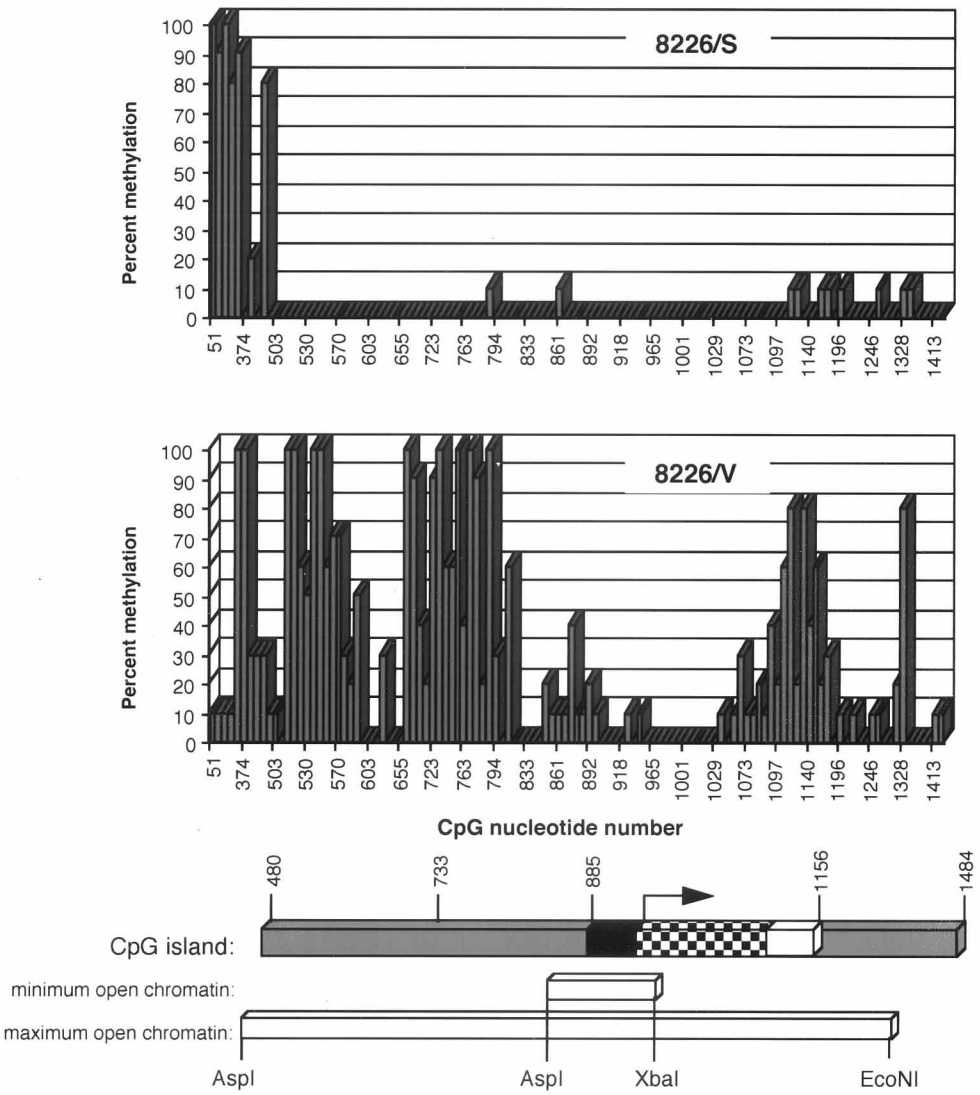

FIG. 3. Summary of results obtained from bisulfite sequencing of the CpG island MGMT promoter. Bisulfite-modified genomic DNAs from 8226/S and 8226/V were PCR amplified, cloned, and sequenced. Ten clones from each cell line were analyzed to obtain percent methylation at 117 CpG sites, including all 108 CpG sites within the CpG island. The minimal promoter, first exon, and minimal enhancer are shown as black, checkered, and white bars, respectively. The minimum and maximum areas of accessible chromatin in 8226/S as determined by restriction enzyme accessibility (Fig. 4 and text) are indicated. The transcription start site is indicated by the arrow. The CpG nucleotide numbering system is based on the published sequence of the MGMT 5′ region (15, 26).

from each cell line were exposed to various amounts of either *Xba*I, *Asp*I, or *Eco*NI, followed by DNA isolation and in vitro digestion with restriction enzymes to create MGMT promoter fragments of predictable sizes (Fig. 4a) for Southern analysis. Figure 4b shows that the *Xba*I site, which is only 17 bp 3′ of the transcription start site, was accessible to all concentrations of restriction enzyme in 8226/S (indicated by fragment 2). In contrast, the *Xba*I site in 8226/V was inaccessible to all concentrations of *Xba*I, which suggests that the MGMT transcription start site is in a functionally closed conformation.

To confirm the *Xba*I accessibility results and gain information about chromatin accessibility further 5′ of the transcription start site, *Asp*I was assayed for in vivo accessibility. *Asp*I has two informative restriction sites located 100 and 513 bp 5′ of the MGMT transcription start site. As seen in Fig. 4c (fragment 4), the *Asp*I recognition site 100 bp 5′ of the MGMT transcription start site was accessible in 8226/S but was inaccessible in 8226/V. Additionally, the *Asp*I site 513 bp 5′ of the transcription start site was largely inaccessible in 8226/S (accessibility seen only at 225 U of *Asp*I [fragments 5 and 6]) and completely inaccessible in 8226/V. This result indicates that the region of open chromatin in 8226/S extends at least 100 bp, and at most 513 bp, 5′ of the transcription start site and corroborates and extends the results of the *Xba*I accessibility experiment.

Finally, *Eco*NI was used to probe the region 3′ of the MGMT transcription start site. As seen in Fig. 4d (fragment 7), the *Eco*NI site 362 bp 3′ of the transcription start site was inaccessible in both 8226/S and 8226/V. Taken together, these results define a region of accessible chromatin around the MGMT transcription start site in 8226/S that is at least 117 bp long (Fig. 3, bottom) and at most 462 bp in length.

DISCUSSION

MGMT DNA repair activity is a major factor that protects cells from mutagenic and cytotoxic O^6-alkylguanine lesions

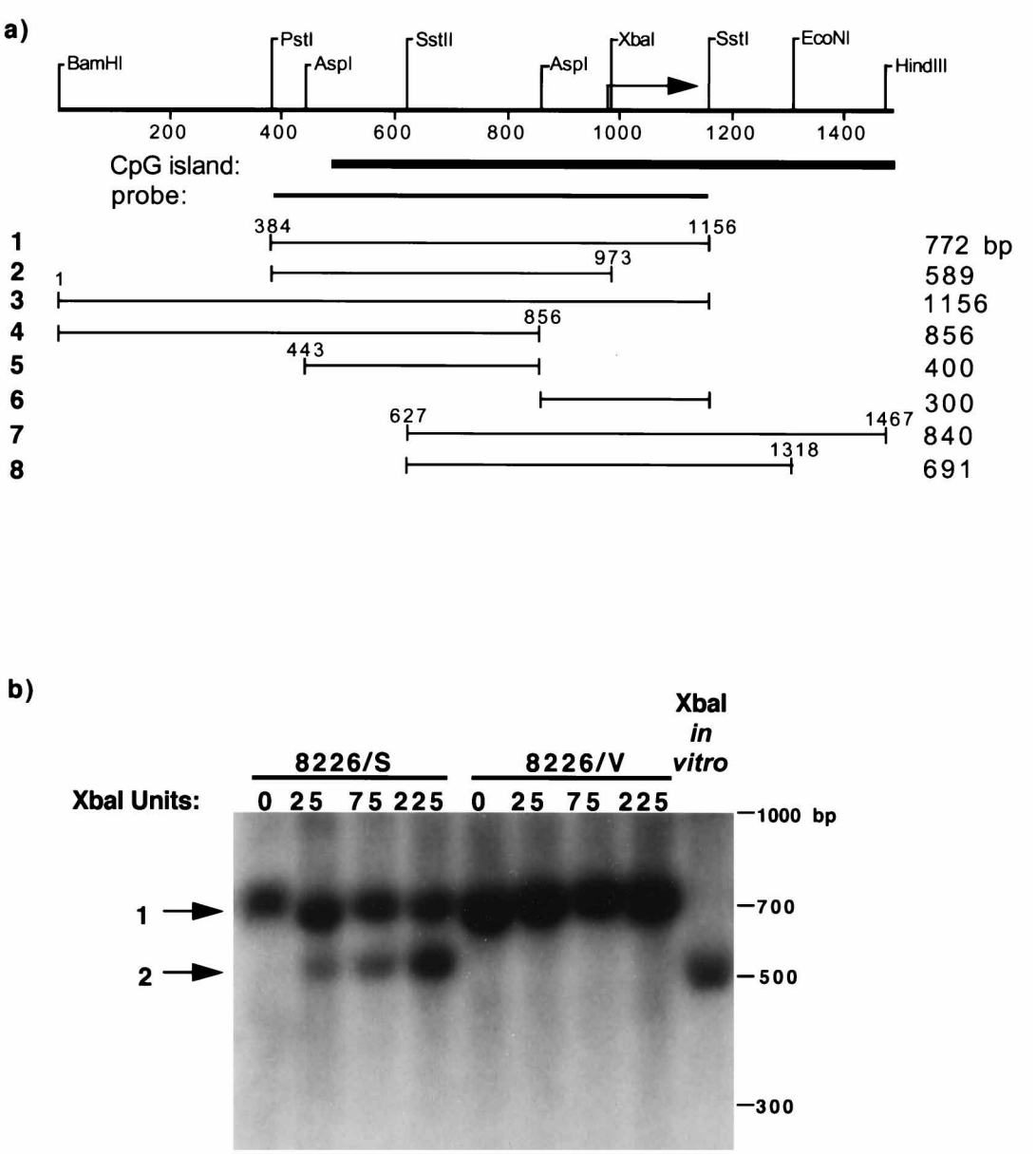

FIG. 4. Restriction enzyme access to the MGMT promoter as a probe of chromatin structure. (a) Nuclei from both cell lines were exposed to increasing amounts of *Xba*I, *Asp*I, or *Eco*NI in order to assay in vivo chromatin accessibility. The genomic DNA was isolated, in vitro digested with appropriate restriction enzymes to create predictable DNA fragments, and subjected to Southern analysis. DNA fragments detected by Southern analysis are shown with their predicted sizes and are numbered for reference with the autoradiographs in panels b, c, and d. Relevant restriction sites for the in vitro and in vivo digestions are shown. The arrow indicates the MGMT transcription start site. (b) DNA exposed to *Xba*I in vivo was isolated from nuclei of both cell lines. After in vitro digestion with *Pst*I and *Sst*I, the DNA was size separated on an agarose gel, capillary transferred to a nylon membrane, and hybridized to the probe indicated in panel a. A control digest with 8226/S DNA is shown in the far-right lane and represents the in vitro limit digest of *Pst*I, *Sst*I, and *Xba*I together. Size markers from a 100-bp ladder are indicated at the right. (c) DNA was isolated from nuclei of both cell lines exposed in vivo to *Asp*I. After in vitro digestion with *Bam*HI and *Sst*I, the DNA was size separated on an agarose gel, capillary transferred to a nylon membrane, and hybridized to the probe indicated in panel a. A control digest with 8226/S DNA is shown at the far right and represents the in vitro limit digest of *Bam*HI, *Sst*I, and *Asp*I together. Size markers from a 100-bp ladder are indicated at the right. (d) DNA was isolated from nuclei of both cell lines exposed in vivo to *Eco*NI. After in vitro digestion with *Hin*dIII and *Sst*II, the DNA was size separated on an agarose gel, capillary transferred to a nylon membrane, and hybridized to the probe indicated in panel a. A control digest with 8226/S DNA is shown at the far right and represents the in vitro limit digest of *Hin*dIII, *Sst*II, and *Eco*NI together. Size markers from a 100-bp ladder are indicated at right.

produced by environmental carcinogens; however, MGMT activity also limits the chemotherapeutic efficacy of nitrosoureas (25). We have developed a stable isogenic cell line model in which one cell line is $MGMT^+$ (8226/S) and one cell line is $MGMT^-$ (8226/V). This model has been used to study the association between cytosine methylation of the MGMT CpG island, chromatin structure, and silencing of MGMT gene expression.

Bisulfite sequencing was performed on both cell lines to obtain quantitative and complete information on the methylation status of CpGs within the entire MGMT CpG island. The results of bisulfite sequencing showed that cytosine methyl-

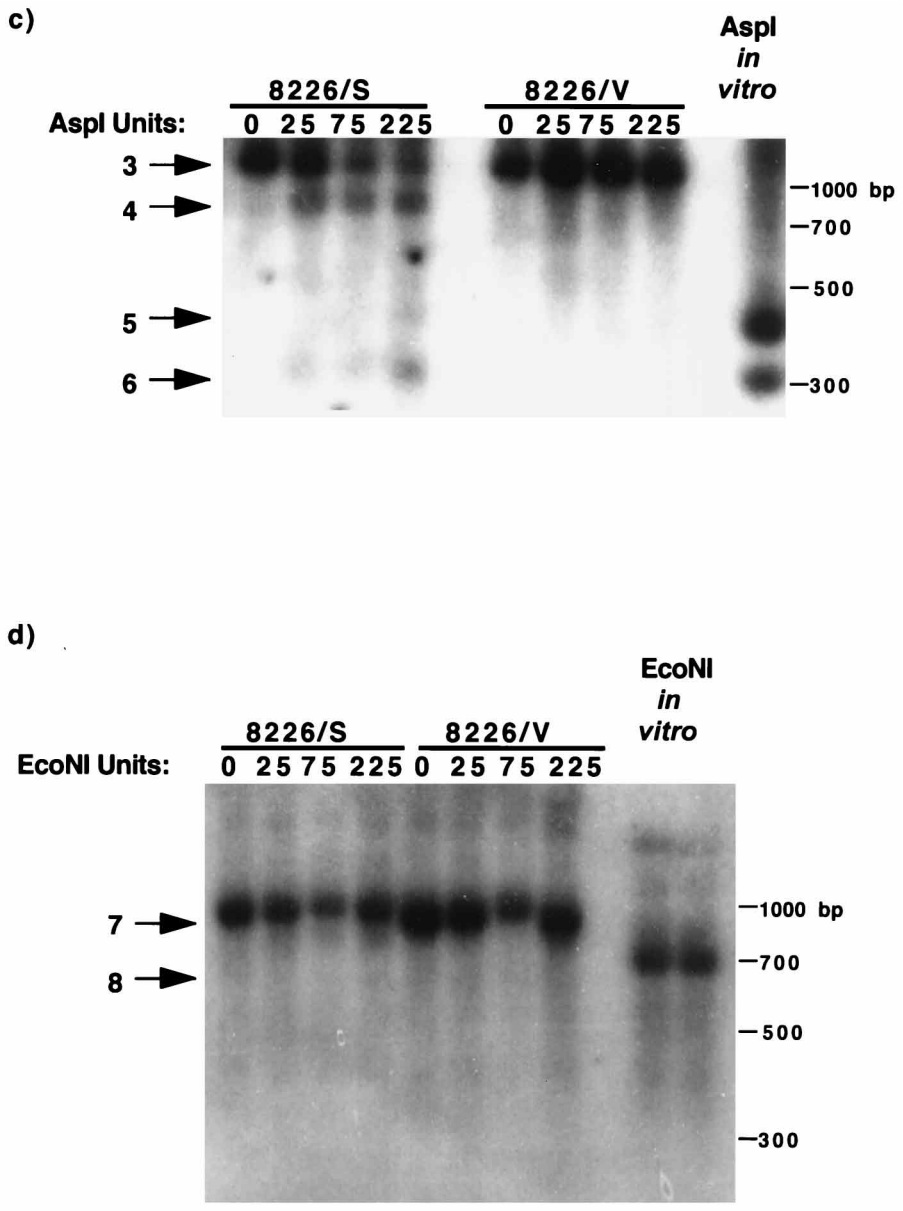

FIG. 4—*Continued.*

ation increased in three discrete regions of the CpG island in MGMT$^-$ 8226/V cells. Two of these regions closely bracketed a relatively methylation-free portion of the promoter which contained the minimal promoter, first untranslated exon, and minimal enhancer. Using in vivo restriction enzyme accessibility experiments, we analyzed this portion of the MGMT promoter in order to determine whether chromatin structure had been altered in association with the observed changes in cytosine methylation. While the minimal promoter region and the transcription start site were accessible to restriction enzymes in 8226/S, this same region was inaccessible in 8226/V. These results suggest that methylation of the MGMT CpG island may be involved in the functional inactivation of the MGMT transcription start site and transcriptional repression of the gene. A 5-methylcytosine-directed change in chromatin structure is an attractive explanation for the silencing of the MGMT gene transcription; however, the question of exactly how methylated cytosines are involved in the remodeling of chromatin structure remains.

Several mechanisms by which cytosine methylation could be involved in suppression of gene transcription have been proposed; these mechanisms may be classified as direct or indirect. In the direct mechanism, cytosine methylation in a protein binding sequence would prevent binding of the cognate protein. This type of direct interference by CpG methylation has been observed for several transcription factors, including AP-2 (5), the major late transcription factor (34), and an unidentified protein which binds the cyclic AMP-responsive element (18). The two regions of cytosine methylation 5′ to the transcription start site in the 8226/V MGMT promoter overlap with two AP-2 sites (nt 725 to 734 and nt 767 to 776) that contain CpG sites in their recognition sequences. These two

CpG sites are 100% methylated in 8226/V and have previously been shown to be methylated in other MGMT$^-$ human tumor cell lines (7, 28). The cytosine methylation at these two AP-2 sites could interfere with AP-2's ability to bind, which in turn may be important for transcriptional activation or maintaining a transcriptionally active chromatin structure.

One indirect mechanism proposes that the methylated cytosines within a promoter CpG island bind proteins which in turn inhibit transcription by occluding necessary promoter sequences. An example of this mechanism is methyl-CpG binding protein 1 (MeCP-1). This protein has been reported to bind methylated cytosines in a sequence-independent but methylation density-dependent manner and likely blocks the access of transcription factors to their recognition sequences (1). In the case of 8226/V, a large protein or protein complex such as MeCP-1 may bind to the regions of methylation which flank the transcription start site and occlude it. Whether such an interaction can explain the loss of restriction enzyme access to the MGMT promoter in 8226/V is unknown.

The lack of restriction enzyme access in the 8226/V MGMT promoter does, however, suggest another indirect mechanism. The loss of chromatin accessibility in 8226/V could be caused by a change in nucleosomal positioning within the MGMT promoter in a manner which inhibits both restriction enzyme accessibility and transcription (reviewed in reference 35). A possible connection between cytosine methylation and the formation of inaccessible, transcriptionally silenced chromatin structure is via nucleosomal interaction with histone H1 (22) or histone H1-like variants (19), which have been reported to bind preferentially to 5-methylcytosine-containing DNA. Such an interaction could stimulate formation of the transcriptionally silent, 30-nm solenoidal chromatin structure (14, 20). Thus, while significant CpG methylation correlates with an altered chromatin structure around the MGMT transcription start site in the MGMT$^-$ 8226/V cell line, a definitive cause-and-effect relationship cannot be determined without further information about the role of cytosine methylation in chromatin structure and packaging.

In summary, we have developed an isogenic human tumor cell line model to study the control of MGMT expression. Using this model, we showed that loss of MGMT expression is closely associated with methylation of discrete regions of the MGMT CpG island and a functional, yet limited, heterochromatinization of the MGMT minimal promoter and transcription start site. Further analysis of protein interactions in the MGMT CpG island in this isogenic model could be useful for elucidating the role of cytosine methylation in transcriptional regulation.

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