Methylation of CpG Island Transcription Factor Binding Sites Is Unnecessary for Aberrant Silencing of the Human MGMT Gene*

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Aberrant transcriptional inactivation of the non-X-linked human O-6-methylguanine DNA methyltransferase (MGMT) gene has been associated with loss of open chromatin structure and increases in cytosine methylation in the Sp1-binding region of the 5' CpG island of the gene. To examine the necessity of these events for gene silencing, we have isolated and characterized a subline of human MGMT+ T98G glioma cells. The subline, T98Gs, does not express MGMT activity or MGMT mRNA, and exhibits no in vivo DNA-protein interactions at Sp1-like binding sites in the MGMT 5' -CpG island. While the MGMT CpG island is less accessible to exogenously added restriction enzymes in T98Gs nuclei than in T98G nuclei, it is similarly methylated in both T98G and T98Gs cell lines 5' and 3' to the transcription factor binding sites, and similarly unmethylated in the region encompassing the binding sites. Inappropriate transcriptional inactivation of MGMT, therefore, does not require methylation of transcription factor binding sites within the 5'-CpG island. Rather, MGMT gene silencing and transcription factor exclusion from T98Gs MGMT CpG island binding sites is most closely associated with condensed chromatin structure, which is in turn indirectly influenced by distant sites of methylation.

Approximately 60% of all human genes contain at their 5' ends GC-rich regions of DNA known as CpG islands (1). CpG islands are frequently associated with the regulatory regions of genes, and are characterized by a high CpG dinucleotide content, an abundance of binding sites for ubiquitou transcription factors (such as Sp1), an open chromatin structure, and a lack of cytosine methylation (2). CpG island-containing genes are frequently expressed in all tissues in a "housekeeping" fashion, although they can also in two circumstances exist in a silenced state in normal tissue. Tissue-specific CpG island-containing genes are silenced in normal, non-expressing tissues by a methylation-independent change in chromatin structure. The CpG islands of such genes remain unmethylated, although their chromatin structure changes in such a way as to exclude transcription factor binding and gene expression (3). CpG island-containing genes on the inactive X chromosome can also be silenced in normal tissue. This silencing process appears to be more complex and involves cytosine methylation as well as alterations in chromatin structure. The relationship between, and necessity of, both methylation and changes in chromatin structure in the process of normal X-linked gene inactivation has been extensively examined, although not entirely resolved.

It was initially thought that the processes of gene inactivation, CpG island methylation, and chromatin condensation were intimately linked. In a number of X-linked gene CpG islands, however, the "closing" of chromatin structure and loss of gene expression were subsequently shown to precede methylation of all potential CpG sites (4, 5). In addition, recent studies have shown that complete methylation of the CpG island may not be necessary for normal X-linked gene silencing as critical regulatory regions of the hypoxanthine phosphoribosyltransferase CpG island are not methylated, yet remain inaccessible to transcription factors on the inactive X chromosome (6). These studies, as well as those with CpG island-containing tissue-specific genes, suggest that in the normal silencing of CpG island-containing genes, methylation plays at best an indirect role.

In addition to being silenced in a normal fashion, CpG island-containing genes can also be abnormally silenced. This process has taken on increasing importance with the realization that it occurs in primary tumors and allows for not only the inappropriate silencing of genes involved in growth control, but also potentially contributes to the clonal evolution of tumors (7-9). Despite its importance, relatively little is known about the abnormal silencing of somatic CpG island-containing genes. Aberrant silencing of somatic CpG island-containing genes has been shown to involve a closing of chromatin structure in the CpG island, as well as increases in cytosine methylation (10-13). Where studied in any detail, however, methylation and closed chromatin conformation appear to be uniformly distributed across inactivated CpG islands, including areas containing transcription factor binding sites (10). As such, and in contrast to normal X-linked gene silencing, it has been difficult to assess the influences of methylation and chromatin structure on aberrant somatic gene silencing.

Several possibilities have, however, been suggested as to how methylation and/or chromatin structure may influence the expression of CpG island-containing somatic genes. Methylation may play a primary role in CpG island-containing gene silencing by directly interfering with transcription factor binding (14, 15). Changes in chromatin structure of silenced genes would then be a consequence of loss of gene expression. This possibility, however, only seems applicable to transcription factors whose binding is methylation-sensitive, and not to those transcription factors such as Sp1, whose binding, at least in vitro, is methylation-insensitive (16, 17). Alternatively meth-
Methylation and Chromatin Structure in MGMT Silencing

The relative amount of MGMT mRNA and MGMT activity in each glioma cell line was determined by Northern (RNA) blot analysis, and by a restriction endonuclease assay, respectively, both as described previously (13, 32, 33).

The restriction endonuclease assay was performed using 10 μg of total cellular protein from each cell line, an amount which was determined to be in the linear range of the assay. For T98G and T98Gs cells, additional assays were performed using 0.5–10 and 10–100 μg of cellular protein, respectively.

In Vivo Dimethyl Sulfate Footprint Analysis of the MGMT Promoter

DNA-protein interactions in the MGMT promoter in living cells were identified by ligation-mediated PCR (LMPCR)-based amplification of methylated DNA from cells exposed to dimethyl sulfoxide (10, 34). Conditions and primers used were identical to those previously described for the analysis of region 2 of the MGMT promoter (10).

In Vivo Analysis of MGMT Promoter Accessibility to Restriction Endonucleases

Analysis of chromatin structure of the MGMT promoter was assessed by isolation of nuclei from SF 767, T98G, T98Gs, or CLA cells, incubation with MspI or AvAI, isolation of DNA, and amplification of the cleaved products by LMPCR. Conditions and primers used were identical to those previously described (10) except that the amount of nucleic digest was increased to that equivalent to 200 μg of DNA, and the amount of MspAI and AvAI used was 5–200 and 16 units, respectively.

Southern Blot Analysis of MGMT Promoter Methylation

For analysis of CpG sites in one SacI recognition sequence (nt 625 and 627) and one EagI recognition sequence (nt 723 and 727) (Fig. 1) DNA from SF 767, T98G, T98Gs, and CLA cells was isolated and cleaved with PstI (10 units/μg of DNA, 20 h) to release an 809-bp fragment. Following phenol:chloroform extraction and ethanol precipitation, the DNA was reconstituted with no enzyme. SacI (12 units) cleaved 120 μg of DNA, 37 °C, 24 h) or EagI (10 units/μg of DNA, 37 °C, 24 h), electrophoresed on a 1.5% agarose gel (30 V, 20 h, 20 μg/mlane), transferred to a nylon membrane, and hybridized to a uniformly32P-radiolabeled MGMT promoter probe spanning nt 384–1193 (28). The membrane was washed as described previously (13), and the amount of hybridized probe was quantitated on a PhosphorImager. The percent- age of molecules methylated at the SacI or EagI sites was determined by comparing the amount of probe hybridized to the 809-bp band in a given sample to that hybridized to the same band in the sample not exposed to SacI or EagI.

For methylation analysis of CpG sites in one Smal recognition sequence (nt 885) or two BssHI recognition sequences (nt 911 and 913, and nt 932 and 934) (Fig. 1), DNA from a variety of cell lines was isolated and cleaved sequentially with BglI and SstI (10 units/μg of DNA, 25 °C, 24 h), or BssHII (10 units/μg of DNA, 50 °C, 4 h), electrophoresed on a 1.5% agarose gel (30 V, 20 h, 15 μg/lane), transferred to a nylon membrane, and hybridized to a uniformly32P-radiolabeled MGMT promoter probe spanning nt 676–967. The membrane was washed as described previously (13), and the amount of hybridized probe was quantitated on a PhosphorImager. The percent- age of molecules methylated at one Smal site or all three BssHII sites, was determined by comparing the amount of probe hybridized to the 721-bp band in a given sample to that hybridized to the same band in the sample not exposed to Smal or BssHII.

Generation of Probes for Southern Blot Analysis of MGMT Promoter Methylation

The MGMT promoter probe used for analysis of methylation at BssHII and Smal recognition sequences was a 291-bp fragment of the MGMT promoter (bp 676–967) generated by PCR amplification using as a template genomic DNA from human 8226/S myeloma cells. PCR was performed on 1 μg of EcoRI-digested DNA in a reaction mixture comprised of 1 × PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl2), 100 μM each of dATP, dCTP, dTTP, and 75 μM 7-deaza-2'-dGTP, 25 μM GTP, 2.5 units of Taq polymerase, and 50 pmol each of primers corresponding to nt 676–694 and 949–967. PCR parameters were as follows: initial denaturation for 5 min at 95 °C, 35 cycles of 95 °C for 1

MATERIALS AND METHODS

Cell Culture

The glioma cell lines used in this study were established from grade III to IV human astrocytomas and glioblastomas. The glioma cell lines used were previously described (29), except for the Hs683 and T98G lines, respectively, used were previously described (29), except for the Hs683s and T98Gs

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The abbreviations used are: MGMT, O-6-methylguanine DNA methyltransferase; PCR, polymerase chain reaction; LMPCR, ligation-mediated polymerase chain reaction; nt, nucleotides; bp, base pair(s).
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min, 62 °C for 15 s, and 72 °C for 15 s, and a final extension for 5 min at 72 °C. The resultant PCR product was ligated into the plasmid pCRII (Invitrogen, San Diego, CA), and the ligation products were used to transform Escherichia coli INVeF’ cells (Invitrogen). Individual colonies were isolated and analyzed for the presence of the appropriate-sized insert, and the identity of the MGMT promoter insert was confirmed by dideoxy sequencing. The MGMT promoter probes used for analysis of methylation at SadI and Eagl recognition sequences was a 809-bp PstI fragment of the MGMT promoter (nt 384-1193) isolated from a genomic clone containing approximately 15 kilobases of 5’ sequence from the MGMT gene. Both probes were uniformly radiolabeled by random priming (35) using [a-32P]dCTP (specific activity 3000 Ci/mmol, Amersham Corp.).

**LMPCR Based Analysis of MGMT Promoter Methylation**

Analysis at Specific Restriction Enzyme Recognition Sequences—DNA from SF767, T98G, T98Gs, CLA, and normal human T cells was digested with Alul (10 units/μg of DNA, 37 °C, 20 h), and, following phenol:chloroform extraction and ethan alcohol precipitation, incubated with BssHII (0 or 10 units/μg of DNA, 50 °C, 4 h). The DNA (0.2 μg/group) was then subjected to Amplification by LMPCR using previously described conditions (10). Primers for these reactions were, for extension of an oligonucleotide complementary to nt 977-996 of the MGMT promoter, for initial amplification an oligonucleotide complementary to nt 954-976, and for the final cycles of amplification an oligonucleotide complementary to nt 950-976. Following amplification of cellular DNA digested with Alul and/or BssHII, the radiolabeled products were electrophoresed on a 8% denaturing polyacrylamide gel, and quantitated for the dried gel by phosphomager analysis. For each group the percentage of DNA molecules methylated at both BssHII sites was determined by comparing the amount of 103-bp product produced using Alul-digested DNA template versus that produced using BssHII-digested DNA template.

Analysis at All CpG Sites—Analysis of methylation at CpG sites regardless of sequence context was performed by LMPCR amplification of DNA subjected to the Maxam-Gilbert sequencing reaction (34, 36). LMPCR analysis of CpG methylation was carried out exactly as described previously (29) using three sets of primers. For analysis of nt 706–809, primers previously described (29) for analysis of MGMT promoter region 1 were used. For analysis of nt 892–934, primers described in the previous section for LMPCR analysis of methylation at specific restriction enzyme recognition sequences were used. For analysis of nt 1022–1150, a primer complementary to nt 1178–1195 was used for the extension reactions, a primer complementary to nt 1175–1155 was used for the initial amplification steps, and a primer complementary to nt 1175–1150 was used for the final cycles of amplification. Following amplification, oligos of the radiolabeled products were electrophoresed (60 watts, 1.5–5 h) on a 6% denaturing polyacrylamide gel to resolve the products of interest. Quantitation of the radioactive products of these reactions was performed using a Betascope analyzer. The intensity of bands representing potentially methylated cytosines, i.e. cytosines in CpG dinucleotides, was compared to that of bands representing non-CpG cytosines within each group. This ratio, which compensates for variance in loading of the gel, was then used to compare the degree of methylation at CpG sequences between groups.

**RESULTS**

Characterization of MGMT Expression in Glioma Cell Lines—The levels of MGMT mRNA and MGMT activity of various glioma cell lines relative to those in the T98G cell line are presented in Table I. MGMT expression at the mRNA and protein activity level in the T98G cell line was comparable to that seen in SF767 cells, and to that reported previously (10, 29). CLA cells, and cells of the T98G subline T98Gs were, given the limits of detection of the assays, devoid of MGMT mRNA and protein activity. MGMT activity could be detected in T98G cells using as little as 0.5 μg of total cellular protein, whereas no activity was detected in up to 100 μg of cellular protein from T98Gs cells.

**DNA-Protein Interactions in the MGMT Promoter in Select Glioma Cell Lines**—Transcriptional inactivation of the MGMT gene cannot be demonstrated by nuclear run-on assay because of the low rate of MGMT transcription (10, 37). MGMT—cells have, however, been shown by in vivo footprinting techniques, to lack in vivo DNA-protein interactions in the MGMT promoter, consistent with transcriptional inactivation of the gene (10). In the MGMT region spanning nt 890-1050, cells expressing MGMT (SF767, T98G) exhibited both protection of five Sp1-like binding sites from N-7 alkylation (Fig. 2, vertical dashed lines) and hypersensitivity of guanines 5’ to these sites (Fig. 2, arrows). A sixth site of Sp1-like protection (Sp1 site 6 in Fig. 1) was apparent upon longer exposure. The in vivo DNA protein interactions in the T98G cells were weaker than those seen in the SF767 and other MGMT-expressing (MGMT+) cells previously examined, although guanine hypersensitivity was still apparent. The non-MGMT expressing cell lines CLA and T98Gs did not exhibit protection of Sp1-like binding sites, nor did these cells exhibit hypersensitive guanines surrounding the sites of DNA-protein interactions. These results are consistent with MGMT silencing at the transcriptional level.

**Restriction Endonuclease Accessibility to the MGMT Promoter within Nuclei**—As a measure of accessibility of chromatin structure to specific DNA recognition proteins, nuclei from various cell lines were incubated with the restriction enzymes MspI or Avall, and the degree of DNA cleavage was monitored by LMPCR-based amplification of the digestion products. As shown in Fig. 3, recognition sequences for MspI (10 sites over nt 712–917) were readily accessible in SF767 cells, even at the lowest concentration of MspI used (5 units). Similar results were noted when MGMT+ T98G nuclei were analyzed. In contrast, none of the 10 recognition sites for MspI were cleaved in T98G or CLA nuclei incubated with 5 or 20 units of MspI, although the presence of these sites and their ability to be cleaved and to give rise to amplifiable products was demonstrated using template DNA from T98Gs or CLA nuclei digested with amounts of MspI (100–200 units) large enough to cause massive cleavage and loss of chromatin structure integrity (Fig. 3). Therefore, MspI sites analyzed in the MGMT promoter of T98Gs and CLA cells were at least 4-fold, and potentially greater than 20-fold, less accessible than the same sites in SF767 and T98G cells.

The 10 MspI sites analyzed for accessibility in nuclei primarily lie 5’ to the 6 regions of in vivo DNA-protein interactions verified in Fig. 2, and also all contain the CpG dinucleotide. To analyze chromatin structure at a site which could not be directly affected by methylation, and which was also within the region of DNA-protein interactions, similar restriction enzyme accessibility studies were carried out using the restriction enzyme Avall. In the region examined, there is only one Avall recognition sequence (GGTCC) at nt 953. As shown in the rightmost four lanes of Fig. 3, this site was also very accessible to low levels of Avall in nuclei from MGMT+ SF767 and T98G cells, but was inaccessible in MGMT-T98Gs and CLA nuclei. These results suggest that a greater than 200-bp region of the

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2 S. Patel and R. Pieper, unpublished data.
291-bp probe used for Southern blot analysis in Fig. 4 at consensus Sp1 binding sites, as determined in Ref. 10, are indicated by (Footnote 2). The location of the CpG island in the 5' region of the gene is indicated by the upper dashed line. In vivo DNA-protein interactions at consensus Sp1 binding sites, as determined in Ref. 10, are indicated by dashes numbered one through six above the map. The location of the 291-bp probe used for Southern blot analysis in Fig. 4B is indicated by the lower solid line.

Fig. 1. Map of relevant restriction enzyme recognition sites in the 5' end of the human MGMT gene. P, PstI; Bg, BglII; B, BssHII; Sc, ScaI; E, EagI; S, Smal; Al, AluI; A, AvalI; Ss, SstII. Numbering and location of all sites is as described in the published sequence (28) except PstI 193 (Footnote 2). The location of the CpG island in the 5' region of the gene is indicated by the upper dashed line. In vivo DNA-protein interactions at consensus Sp1 binding sites, as determined in Ref. 10, are indicated by dashes numbered one through six above the map. The location of the 291-bp probe used for Southern blot analysis in Fig. 4B is indicated by the lower solid line.

MGMT promoter spanning the known in vivo DNA-protein binding sites is in a relatively open chromatin conformation in MGMT+ cells, but is in a significantly more closed conformation in MGMT− cells.

Cytosine Methylation in the MGMT Promoter in SF767, T98G, T98Gs, and CLA Cells—To analyze CpG methylation in the glioma cell lines, and to relate this methylation to chromatin structure, three independent assays were performed. Initially, the methylation at ScaI, EagI, SmaI, and BssHII recognition sequences was measured by Southern blot analysis. In these studies, methylation of either of two CpG dinucleotides in the ScaI or EagI recognition sequence (nt 625 and 627, and nt 723 and 727, respectively) blocks cleavage of an 809-bp PstI fragment (Fig. 1). Southern blot analysis of these digests (shown in Fig. 4A and quantitated in Table II) indicates that one or both of the ScaI site CpGs is extensively methylated in all four cell lines examined. This ScaI site, therefore, may lie outside of the MGMT CpG island. In contrast, the EagI recognition sequence, which lies 99 bp downstream of the ScaI site, is essentially unmethylated in SF767 cells (6% methylation), completely methylated in T98Gs and CLA cells (95 and 99% methylated, respectively), and methylated to an intermediate degree (69%) in T98G cells.

Methylation of the cytosine in the single CpG dinucleotide of the Smal recognition sequence at nt 855, or at either of two CpG dinucleotides in each of three BssHII sites (nt 576 and 578, 911 and 913, 932 and 934), blocks Smal or BssHII cleavage of a 721-bp BglI-SstI fragment (Fig. 1). Southern blot analysis of these digests (shown in Fig. 4B and quantitated in Table II) indicates that while few, if any, molecules were methylated at the nt 855 CpG dinucleotide in a number of MGMT− cells, methylation was detectable in MGMT− cells, although the percentage of methylated molecules varied by cell line. Most significantly, however, while there was a large difference in the degree of methylation of this CpG site between MGMT+ SF767 and MGMT− CLA cells (4% versus 46% methylation respectively), there was little difference between MGMT+ T98G and MGMT− T98Gs cells (6% versus 3% methylation, respectively). Similarly in MGMT+ cells relatively few if any molecules were methylated in at least one CpG in all BssHII sites. In MGMT− cells there was a detectable number of molecules methylated at all three sites, the amount ranging from 68% in...
CLA cells to 90% in CRO cells. Again while there was a large difference in the degree of methylation of these sites between SF 767 and CLA cells (<0.1 versus 68% methylated at all sites, respectively, Table II), there were few molecules methylated at all three sites in both T90G and T90Gs cells (Table II).

To verify these results, as well as to develop a more sensitive and quantitative method of analyzing methylation at specific sites, a modified restriction enzyme methylation analysis employing LMPCR was used (38, 39). In this method glioma DNA cleaved with both Alu1 and BssHII was subjected to LMPCR analysis. Ligation of a common linker to BssHII-generated products allows for PCR amplification of these DNA fragments if present. In the event that BssHII cleavage is blocked by cytosine methylation, fragments generated by Alu1 (Alu1 does not contain a CpG dinucleotide in its recognition sequence and is unaffected by methylation) by cleavage at a site distal to the BssHII sites (Fig. 1) would be generated. Reactions using template DNA cleaved only with Alu1 yielded a 103-bp product (the distance from the 5' end of the most internal LMPCR primer to the Alu1 site at nt 899, plus 25 bp provided by the linker). Reactions using BssHII-cleaved, unmethylated normal human T cell DNA as template yielded a 69-bp product (the distance from the 5' end of the internal LMPCR primer to the proximal BssHII site at nt 931). The amount of each product produced was linear using 0.02–0.5 μg of DNA template (R² = 0.96). For an equal amount of template added (0.1 μg each) three times more of the 69-bp product than the 103-bp product was produced (3.1 ± 0.4), a ratio which was consistent across a 100-fold mixture range. Reactions using Alu1 + BssHII-digested template DNA from glioma cells yielded, depending on the cell line of origin of the DNA, varying amounts of both the 103- and 69-bp product, as well as a small amount of 90-bp product derived from BssHII cleavage at only the second BssHII site (nt 910). The lack of standard DNA cleaved at only one of the two BssHII sites prohibits quantitation of methylation at each BssHII site using this technique. Comparison of the amount of the 103-bp product generated using Alu1 cleaved DNA versus Alu1 + BssHII cleaved DNA from the same source does, however, allow for accurate determination of the percentage of molecules methylated at both BssHII recognition sequences (nt 910 and 931). As shown in Table II, and consistent with Southern blot analysis, there was a large difference in the degree of methylation of both BssHII sites between MGMT+ SF 767 and MGMT– CLA cells, but no statistical difference between T90G and T90Gs cells, neither of which contained statistically significant methylation at both sites.

As a final analysis of methylation of CpG dinucleotides throughout the MGMT promoter, LMPCR-based amplification of DNA subjected to Maxam-Gilbert sequencing reactions was employed. Using this technique 5-methylcytosines in the DNA are unreactive with hydrazine and will not serve as sites of piperidine cleavage in subsequent steps of the sequencing reaction (36). 5-Methylcytosines appear as gaps in the sequence ladder generated by amplification of products of the sequencing reactions, with the decrease in intensity corresponding directly to the degree of methylation of the nucleotide in the population (39). Representative autoradiographs from these studies are presented in Fig. 5, and results from triplicate analyses are summarized in Table III. There was no statistically significant methylation of CpG dinucleotides across a region of the SF 767 MGMT promoter encompassing the 444 bp analyzed. In contrast, and consistent with Southern blot and restriction enzyme-LMPCR data, there was extensive methylation of the MGMT promoter of CLA cells. This methylation was variable contrast, and consistent with Southern blot and restriction enzyme-LMPCR data, there was extensive methylation of the MGMT promoter of CLA cells. This methylation was variable depending on the region examined. The degree of methylation was greatest in regions 5' and 3' to the area containing the six sites of in vivo DNA-protein interactions, although low levels of methylation (average 24%) were present even in the transcription factor binding area. The MGMT+ T90G cells also exhibited methylation 5' and 3' to the area of DNA-protein interac-

| Gioma cell line | % Methylated at Sadl sitea | % Methylated at Eagl sitea |
|-----------------|--------------------------|--------------------------|
| SF 767          | 84.5                     | 6                        |
| T90G            | 100                      | 69                       |
| T90Gs           | 100                      | 95                       |
| CLA             | 96                       | 99                       |

Values were derived from Southern blot analysis (Fig. 4A) by comparing the amount of probe hybridized to a PsI-generated fragment from the MGMT promoter in a given sample to that hybridized to the same band in the sample additionally digested with either Sadl or Eagl. Values are the average of two experiments.

Values were derived from Southern blot analysis (Fig. 4B) by comparing the amount of probe hybridized to a BglI-SstI-generated fragment from the MGMT promoter in a given sample to that hybridized to the same band in the sample additionally digested with either Smal or BssHII. Values are the average of two experiments.

Values determined by LMPCR analysis and represent the mean ± S.D. of three experiments.
FIG. 5. Methylation analysis of the MGMT promoter in cells expressing (SF767 and T98G) and not expressing (T98Gs and CLA) MGMT. EcoRI-digested DNA from the four glioma cell lines was reacted with Maxam-Gilbert genomic sequencing chemicals. Linearized plasmid DNA containing a 1.2-kilobase BamHI-SstI fragment (panels A and B) or a 6-kilobase BamHI fragment (panel C) of the MGMT promoter were similarly treated. All nucleotides (G, guanine; C, cytosine; T, thymine; A, adenine) in the cloned DNA, guanines and cytosines in the glioma DNA spanning the nt 709–809 (panel A) and 1022–1150 (panel C), and cytosines in the glioma DNA spanning nt 892–934 (panel B) were analyzed by LMPCR. One-third of the reaction mixtures was electrophoresed on a 6% denaturing polyacrylamide gel and autoradiographed for 24–72 h. Arrows indicate sites of cytosines in CpG dinucleotides. Panel B displays only cytosines. All autoradiographs are representative of three analyses.
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TABLE III
Methylation of CpG sites in the MGMT promoter
Values are the means of three independent experiments and were determined by first comparing the amount of radioactivity in bands representing potentially methylated cytosines to that in bands representing non-methylated cytosines in the same lane and the same region of the gel. These values were then compared to those derived from plasmid DNA (0% methylation).

| CpG site | SF 767 | T98G | T98Gs |
|----------|--------|------|-------|
| 706      | -      | ++   | +     |
| 709      | -      | ++   | +     |
| 713      | -      | ++   | +     |
| 723      | -      | ++   | +     |
| 727      | -      | ++   | +     |
| 739      | -      | ++   | +     |
| 748      | -      | ++   | +     |
| 760      | -      | +    | -     |
| 763      | -      | +    | -     |
| 769      | -      | +    | -     |
| 783      | -      | +    | -     |
| 786      | -      | +    | -     |
| 801      | -      | +    | -     |
| 809      | -      | +    | -     |
| Spl-1 892| -      | -    | -     |
| Spl-2 903| -      | -    | -     |
| 909      | -      | -    | -     |
| 911      | NA     | NA   | NA    |
| 918      | +      | -    | -     |
| Spl-3 924| -      | -    | -     |
| 930      | -      | -    | -     |
| 932      | -      | -    | -     |
| 934      | -      | -    | -     |
| Spl-6 1022| -    | -   | -     |
| 1024     | -      | -    | -     |
| 1050     | -      | +    | +     |
| 1061     | +      | +    | +     |
| 1068     | -      | +    | +     |
| 1073     | +      | +    | +     |
| 1076     | +      | +    | +     |
| 1080     | -      | +    | +     |
| 1092     | -      | +    | +     |
| 1097     | -      | +    | +     |
| 1102     | +      | +    | +     |
| 1108     | +      | +    | +     |
| 1129     | -      | +    | +     |
| 1134     | -      | +    | +     |
| 1140     | +      | +    | +     |
| 1150     | +      | +    | +     |

A, 0–20% methylation; +, 20–40% methylation; ++, 40–60% methylation; +++ , 60–80% methylation; ++++, 80–100% methylation; NA, cannot be analyzed.

Significant difference (p < 0.05) between T98G and T98Gs values.

DISCUSSION

In the present study two cell lines with an identical genetic background but differing in MGMT expression were examined at high resolution for methylation and chromatin structure in the 5'-CpG island of the non-X-linked MGMT promoter. The cell lines were found to differ in chromatin structure across the entire MGMT 5'-promoter/CpG island region examined, although they differed in methylation in a very limited number of sites surrounding the region of transcription factor binding. The cell lines did not differ in the degree of methylation of the transcription factor binding region in this promoter, there being no significant methylation in either cell line. Some care must be taken, however, in interpretation of the methylation data. Information derived from Southern blot analysis is not easily quantitated at low levels of methylation. Restriction enzyme digestion in combination with LMPCR allows for more sensitive detection of low levels of methylation, but, like Southern blot analysis, can only study methylation at restriction enzyme recognition sequences. Analysis by LMPCR genomic sequencing is not limited to restriction enzyme recognition sequences, but the data derived are less reproducible, especially at highly methylated sites (39). Nonetheless, the data presented here using all three techniques are in good agreement, and with that previously published (29). The present data clearly indicate that there is no statistically significant difference in methylation between MGMT+ T98G and T98Gs cells at 37 of 41 sites both proximal to and distal to the transcription factor binding region, and no statistically significant difference in methylation between these two cell lines at any site within the transcription factor binding region. This lack of difference in methylation stands in contrast to the large difference in MspI accessibility of the same region of the MGMT 5'-CpG island in T98G and T98Gs nuclei. The results of MspI and AvalI accessibility studies together suggest that the chromatin structure of the MGMT promoter in the MGMT+ T98G cells differs from that in the MGMT- T98Gs cells over a region of at least 241 bp (nt 712–953) and more accurately 427 bp (nt 712–1139). In T98Gs cells, therefore, silencing of the MGMT gene is associated with changes in chromatin structure in the absence of methylation of the CpG island region which contains transcription factor binding sites. The silencing of the MGMT gene in T98Gs cells therefore does not involve direct effects of methylation on transcription factor binding, and is also not the result of indirect interference of transcription factor binding by recruitment of methylated-DNA binding proteins to transcription factor binding areas. This is to our knowledge the first such separation of these events noted within a 5'-CpG island of an abnormally silenced somatic gene.

Given the lack of involvement of direct methylation, the MGMT gene must be silenced in T98Gs cells by mechanisms involving indirect effects of methylation, or by methylation-independent mechanisms. The idea that MGMT silencing in T98Gs cells occurs by a methylation-independent mechanism is supported by the observation that the difference in methylation of the MGMT CpG island between MGMT+ T98G cells and MGMT- T98Gs cells is not large, and certainly not as large as that noted between other MGMT+ and MGMT- cells analyzed in this and other studies (29, 41). It may, however, be possible that very small increases in methylation of multiple sites proximal and distal to the transcription factor binding area of the T98Gs MGMT promoter could escape detection by LMPCR analysis, and could in turn be associated with, or trigger, changes in chromatin structure in the transcription factor binding area of the MGMT 5'-CpG island. Previous studies,
however, have suggested that small, widespread increases in CpG island methylation in the MGMT promoter are associated not with gene silencing, but rather with modest down-regulation (29). Alternatively, given that the T98G cells are significantly more methylated than T98G cells in four sites in the MGMT promoter, significant increases in methylation at only a few sites in the MGMT CpG island could be associated with, or could trigger, a global change in MGMT CpG island chromatin structure and methylation expression. The complete demethylation of select sites in the CpG island of the X-linked phosphoglycerate kinase gene has been correlated with global changes in chromatin structure and reactivation of the silenced gene (42). CpG island methylation has also been shown to cause chromatin condensation of promoter regions upstream of CpG islands in the 5’ region of the myoD gene, although the methylation changes noted in the myoD 5’ region were large and uniformly distributed throughout the CpG island rather than being focussed on specific CpG dinucleotides as noted in this study (22). If large differences in methylation of these four sites plays a role in MGMT gene silencing in T98G cells, however, there must exist a narrow threshold of methylation beyond which dramatic and global changes in chromatin structure and gene activation occur, as methylation is present at these sites in both T98G and T98G cells. The relevance of methylation in the silencing of the MGMT gene could in theory be evaluated by removing methylation from the MGMT CpG island and assessing the effect on MGMT CpG island chromatin structure or MGMT expression. The use of 5-azacytidine to cause such demethylation, however, would likely be confounding as MGMT expression has not only been shown to be associated with methylation of the 5’-CpG island, but also with methylation of the body of the gene (29). Where 5-azacytidine has been used in attempts to reactivate MGMT gene expression in MGMT cells containing methylated MGMT CpG islands, results have been variable (13, 41), likely due to the fact that the demethylating actions of 5-azacytidine would be expected to favor expression in the 5’ regions of the gene, yet favor gene inactivation in the body of the gene. As such, the characteristics of the MGMT gene hinder a complete definition of the role of CpG island methylation in chromatin structure and MGMT gene silencing.

The idea that silencing of the MGMT gene in T98G cells is independent of methylation, however, seems incompatible with the observation that the MGMT CpG island in both T98G and T98G cells is uniformly and significantly methylated relative to that in MGMT+ SF767 cells. We have, however, observed significant methylation of the MGMT CpG island in other MGMT- cells, and have correlated this methylation with the extent of MGMT expression (29). In this sense the MGMT CpG island in T98G cells may be very similar to, or perhaps only slightly more methylated than, the same CpG island in other MGMT+ cells, while the MGMT CpG island in T98G cells may differ primarily in the additional loss of open chromatin structure. If MGMT gene inactivation in T98G cells is primarily a chromatin structure-related event, it is unclear what triggers such change. It may be possible that the T98G cell line developed from a cell containing a mutation in a critical region of the MGMT gene which either directly resulted in a change in chromatin structure in the MGMT 5’-CpG island, or a loss of transcription factor binding which subsequently resulted in a loss of CpG island open chromatin structure. No C or G mutations were apparent in the sequencing of the MGMT 5’ region 706-1150, which includes the region containing transcription factor binding sites, although the entire gene (>150 kilobases) would likely need to be sequenced to rule out the possibility of mutation-induced gene silencing. Alternatively, aberrant silencing of somatic CpG island-associated genes may be the consequence of abnormal function of any number of a growing family of proteins thought to control chromatin structure (43).

Although a complete explanation of the silencing of the MGMT gene in T98G cells remains elusive, the present data demonstrate that direct methylation of the regions of 5’-MGMT CpG island containing all relevant transcription factor binding sites is unnecessary for MGMT silencing. Rather, MGMT gene silencing and transcription factor exclusion from T98 MGMT CpG island binding sites is most closely associated with condensed chromatin structure, which is in turn, and at best, indirectly influenced by distant sites of methylation. While it remains unclear whether the gene silencing event monitored in T98G cells is unique to these cells and/or to the MGMT gene, the processes that control chromatin structure, as well as those which allow for different degrees of linkage between methylation and chromatin structure within the same gene, will undoubtedly be of great importance in understanding regulation of expression of not only the MGMT gene, but also of the estimated 60% of all human genes potentially regulated by the same mechanism (1).

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