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THE ROLE OF METHYLATION AND CHROMATIN STRUCTURE IN THE REGULATION OF THE O^6-METHYLGUANINE-DNA METHYLTRANSFERASE (MGMT) GENE IN HUMAN GLIOMA CELLS

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENT

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

NEUROSCIENCE GRADUATE PROGRAM

BY

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JUNE, 1994

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THE ROLE OF METHYLATION AND CHROMATIN STRUCTURE IN THE

REGULATION OF THE 0^6-METHYLGUANINE DNA

METHYLTRANSFERASE GENE IN HUMAN GLIOMA CELLS

There is considerable interest in identifying factors responsible for expression of the O-6-methylguanine DNA methyltransferase (MGMT) gene as MGMT is a major determinant in the response of glioma cells to the chemotherapeutic agent 1,3 bis(2-chloroethyl)-1-nitrosourea (BCNU). Since the majority of glioma cells express the MGMT gene, understanding factors that regulate MGMT expression is critical for the design of therapeutic strategies to inhibit MGMT expression and thereby overcome BCNU resistance. MGMT expression is presumed, but not proven to be regulated at the transcriptional level. Consistent with a transcriptional level of regulation, preliminary studies suggested that cytosine methylation may be one factor that influences MGMT expression. This dissertation was therefore designed to address the role of cytosine methylation in MGMT gene expression in human glioma cells.

In order to develop the probes for examining methylation within the MGMT gene, a map of the coding sequences of the MGMT gene was generated by screening a genomic library (derived from a human fibroblast cell line) with an MGMT cDNA probe. Twenty MGMT genomic clones were isolated, mapped with restriction

enzymes (RE) and aligned, yielding a map of the gene containing four translated exons spread over >80 kb. Several regions of the cloned MGMT gene with an overabundance of potential methylation sites (CpG dinucleotides) were identified with RE that recognize and cleave DNA at various CpG containing sequences. These and other regions across the body of the MGMT gene were then used to analyze MGMT gene methylation in human glioma cells.

To determine if methylation in the body of the gene correlates with MGMT expression, the methylation status of CpGs throughout the body of the MGMT gene were examined in nine human glioma cell lines exhibiting a wide range of MGMT expression. Methylation in the body of the MGMT gene was analyzed by Southern blot analysis of glioma DNA digested with methylation sensitive REs. All of the MGMT gene regions tested were methylated in the MGMT expressing (MGMT+) glioma cells and relatively unmethylated in the nonexpressing cells (MGMT-). Quantitative analysis of methylation at an intron I CpG demonstrated graded methylation and a strong, positive correlation between methylation and glioma MGMT mRNA levels. These results suggest that methylation is uniform over the entire body of the MGMT gene and correlates in a direct, graded fashion with MGMT expression. This correlation is consistent with an indirect mechanism by which uniform methylation over the body of the MGMT gene may influence chromatin structure and hence MGMT expression.

To determine if the association between methylation in the body of the MGMT gene and MGMT expression is mediated by changes in chromatin structure,

the accessibility of RE to the MGMT gene within intact nuclei was assessed. The unmethylated body of the MGMT gene was clearly more accessible to RE in nuclei from all MGMT- glioma cell lines tested relative to the methylated gene in nuclei from MGMT+ cells. The only accessible sites within the MGMT+ nuclei corresponded exactly to the few sites that were also unmethylated. These experiments demonstrate that methylation and chromatin structure in the body of the MGMT gene are closely linked, and are likely involved in MGMT expression.

To determine if promoter methylation is also an important component of MGMT expression, this dissertation research addressed the complex interactions between methylation, chromatin structure, and in vivo transcription factor occupancy in the MGMT promoter of glioma cells. The level of MGMT promoter methylation, assessed by linker mediated PCR, was also graded across the cell lines (at 21 of 25 CpGs tested), but correlated in an inverse, rather than direct fashion with MGMT expression. The basal promoter in MGMT+ glioma cells was entirely accessible to RE, suggesting that this region may be free of nucleosomes. The basal promoter in MGMT- nuclei was entirely inaccessible to RE, supporting the idea that methylation and chromatin structure in the promoter are also associated. Despite the presence of the relevant transcription factors in all the cells examined, in vivo footprinting showed DNA-protein interactions at 6 Sp1 binding sites and one novel binding site in MGMT+ glioma cells but no such interactions in MGMT- cells. In contrast to previous in vitro studies, these data indicate that Sp1 is an important component of MGMT transcription. The data also strongly suggest that

methylation and chromatin structure, by determining whether Sp1 and other transcription factors can access the MGMT promoter, set the transcriptional state of the MGMT gene.

In summary, these data provide compelling evidence for the involvement of methylation and chromatin structure, both in the promoter and body of the MGMT gene, in the regulation of MGMT expression. If these factors are also critical in glioma cells within tumors, the "accessible" chromatin in the MGMT expressing, and hence BCNU resistant, glioma cells may be a good target for inhibition of MGMT expression, possibly through triple helix formation.

ACKNOWLEDGEMENTS

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A	adenine
bp	base pair
BPB	bromphenol blue
BCNU	1,3 bis(2-chloroethyl)-1-nitrosourea
C	cytosine
CpG	cytosine (phosphate) guanine dinucleotide
cDNA	complementary DNA
CENU	Chloroethylnitrosourea
Ci	Curie
CPM	counts per minute
DMS	dimethylsulfate
DTT	dithiothreitol
G	guanine
G^{me}	O^6-methylguanine
hnRNA	heteronuclear RNA
hr	hour(s)
kbp	kilobase pair
LMPCR	linker-mediated polymerase chain reaction

M	Molar
MeCP	methylated DNA binding protein
MGMT	O^6-methylguanine-DNA methyltransferase
MGMT+	MGMT expressing
MGMT-	MGMT nonexpressing
Ci	curie
g	gram
l	liter
M	molar
min	minute(s)
mRNA	messenger RNA
MTase	DNA cytosine methyltransferase
nt	nucleotide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
mol	moles
RE	restriction enzymes
SDS	sodium dodecyl sulfate
sec	second(s)
SSC	standard saline citrate
SSPE	standard saline phosphate-EDTA
T	thymine

TBE	**tris-borate-EDTA**
TE	**tris-EDTA**
vol	**volumes**
x g	**times gravity**

CHAPTER I

INTRODUCTION

Gliomas are the most prevalent form of primary brain cancer and include tumors arising primarily from astrocytes, but also less frequently, from oligodendrocytes and ependymal cells. All patients with gliomas eventually die from their tumors, often within months or a few years of diagnosis, unless other diseases predominate (Ransohoff, et al., 1991). Although the etiology of gliomas is unknown, late age of onset, poor neurological status and significant residual tumor volume following surgery are factors intimately associated with decreased length of survival (Ransohoff, et al., 1991). As the incidence of gliomas is increasing in the United States (Greig et al., 1990), there is considerable interest in elucidating the biochemical and genetic defects leading to glioma formation and malignant progression, and in developing effective therapies to treat gliomas.

Gliomas are classified by neuropathologic criteria into stages of increasing malignancy, from benign to anaplastic to glioblastoma multiforme (Ransohoff, et al., 1991). These stages represent a continuum of glioma progression within each patient, although many patients do not survive through the anaplastic stage. Increased malignancy is closely associated with decreased survival time. Precise molecular and cytogenetic changes occurring at each stage of glioma progression have recently been described and may eventually be used in conjunction with the

neuropathologic criteria for more accurate diagnosis of the degree of malignancy (Collins and James, 1993). Such accuracy in diagnosis is critical, as the malignancy stage strongly determines the response to and usefulness of various therapies currently used to treat gliomas (Weingart and Brem, 1993).

The initial treatment for gliomas is surgical removal of the tumor. However, because these tumors have very irregular shapes and ill-defined borders, residual tumor cells remain in the patient following surgery, and invariably, the tumor recurs (Ransohoff, et al., 1991). Surgery is therefore followed with radiation therapy and often chemotherapy in attempt to eliminate the residual tumor cells.

Gliomas are treated most effectively with the chemotherapeutic agent 1,3-bis(chloroethyl)-1-nitrosourea (BCNU)(Walker, et al., 1980). BCNU is useful in treating brain tumors for two reasons. First, the lipid soluble nature of BCNU molecules allows them to cross the relatively impermeable barrier between the blood vessel interior and brain tissue (the blood-brain barrier) and reach the brain tumor site (Rall, et al., 1962). Second, the chloroethyl group adducts at the O^6-position of guanine produced by BCNU rearrange to form DNA interstrand crosslinks that are cytotoxic to rapidly dividing cells (Kohn, 1977; Ewig and Kohn, 1978; Erickson, et al., 1978). Because the vast majority of normal brain cells are in a nondividing state, BCNU is much more toxic to the tumor cells. Initially, responses to BCNU (tumor stasis or partial tumor regression) are seen in approximately one-half of all glioma patients treated with BCNU (Walker, et al., 1980). At undefined points during or after recurrence however, the majority of these gliomas become resistant

to BCNU chemotherapy, and the tumor progresses rapidly (Weingart and Brem, 1993). Research designed at elucidating the mechanisms conferring BCNU resistance in glioma cells is paramount for improving the currently ineffectual treatment of resistant gliomas.

Seventy percent of human glioma cell lines and many human glial tumors are resistant to the cytotoxic effects of BCNU (Kornblith, et al. 1981; Sariban, et al., 1987; Day, et al. 1990; Ostrowski, et al., 1991). BCNU resistance is conferred by the DNA repair protein, O^6-methylguanine DNA methyltransferase (MGMT) (Pegg, et al., 1982; Tano, et al., 1990). MGMT removes the BCNU-induced chloroethyl group adducts from O^6-guanine in the glioma cell DNA, preventing the formation of cytotoxic interstrand crosslinks (Erickson, et al., 1980). Thirty percent of BCNU-sensitive brain tumor cell lines are devoid of the MGMT mRNA and MGMT protein despite the presence of an intact MGMT gene (Day, et al., 1990, Ostrowski, et al., 1991; Costello, et al., 1994). This and other observations suggest that the BCNU-sensitive phenotype of these brain tumor cells appears to result, at least in part, from suppression of MGMT gene transcription (Ostrowski, et al., 1991, Costello, et al., 1994a). Examining factors that may suppress MGMT gene transcription is essential for designing therapeutic strategies to overcome BCNU resistance in MGMT-expressing brain tumors.

Transcriptional control of gene expression is mediated by a variety of mechanisms including DNA cytosine methylation (reviewed in Jost and Saluz, 1993). In the DNA of all human cells, a subset of cytosines in the dinucleotide CpG are

modified by an endogenous methylating enzyme (Bestor, et al., 1988). Addition of a methyl group to cytosines of a CpG is a normal DNA modification that plays a central role in transcriptional regulation of gene expression (Jost and Saluz, 1993). Regions of DNA known as CpG islands contain clusters of CpG dinucleotides (relative to the majority of bulk DNA which is CpG-depleted) that are normally maintained in the unmethylated state (Bird, 1986). Because the MGMT cDNA has an overabundance of CpGs (Tano, et al., 1990), one or more of the MGMT exons comprising the cDNA sequence may be part of a CpG island. Processes which are associated with abnormal de novo CpG island methylation such as tumorigenesis (De Bustros, et al., 1988), viral transformation (Vertino, et al., 1993) and cell culture (Antequera, et al., 1990), are also frequently associated with loss of MGMT gene expression (Green, et al., 1990; Fornace, et al., 1990). Abnormal methylation of potential MGMT gene-associated CpG islands could therefore be one mechanism by which transcription of the MGMT gene is suppressed. Indeed, preliminary evidence suggests that the methylation status of MGMT exons is altered in virtually all MGMT nonexpressing (MGMT-) cell lines tested (Pieper, et al., 1991; Wang, et al., 1992). The purpose of my dissertation research is thus to determine the relationship between cytosine methylation and MGMT expression in human brain tumor cells. I have proposed three specific aims to examine this relationship:

Aim 1: To isolate regions of the MGMT gene that contain CpG island characteristics.

Aim 2: To determine the relationship between the methylation status of potential MGMT gene-associated CpG islands and MGMT gene expression in human glioma

cells.

Aim 3: To examine the mechanism by which methylation may influence MGMT expression in human glioma cells.

The hypothesis that this dissertation research attempts to validate is that glioma cells regulate expression of the MGMT gene by virtue of differential methylation of MGMT gene-associated CpG islands. Methylation of the CpG island regions, through methylated DNA binding proteins, alters the local chromatin structure such that accessibility of other proteins necessary for transcription of the MGMT gene is altered.

Understanding the relationship between cytosine methylation and MGMT gene expression is important because it may ultimately be useful in the development of therapies designed to inhibit MGMT expression. Inhibition of MGMT expression followed by BCNU administration might then enhance the antitumor activity of BCNU, significantly reduce the number of residual tumor cells following surgery and thereby improve chemotherapy of BCNU-resistant gliomas.

CHAPTER II

LITERATURE REVIEW

Primary Brain Cancer

Approximately 16,000 new cases of primary brain cancer occur each year and the incidence is increasing in the United States (Ransohoff, et al., 1991). The etiology of primary brain cancer is unknown. Primary brain cancer rarely, if ever, metastasizes outside the central nervous system, yet many forms of primary brain cancers are fatal (Weingart and Brem, 1993).

Gliomas are the most prevalent form of primary brain cancer. Gliomas include tumors arising predominantly from astrocytes, but also less frequently from oligodendrocytes or ependymal cells. All glioma patients die of their tumors within months or a few years of diagnosis. The most important prognostic criteria for increased length of survival for glioma patients are younger age of onset, better neurologic status, and decreased residual tumor volume following surgical resection of the tumor (Ransohoff, et al., 1991).

Gliomas are classified in grades of malignancy according to neuropathologic and genetic criteria. Gliomas often progress from benign to anaplastic to glioblastoma multiforme (GBM) within each patient, although many patients die before the most malignant stage (GBM) is reached. At diagnosis, all gliomas are

visible by magnetic resonance imaging (MRI). Neuropathologically, benign tumors are identified by the presence of microcysts and uniformly sized nuclei, while anaplastic gliomas are identified by hyperplasia of blood vessel endothelial cells within the tumor, and GBMs are identified by the presence of geographic necrosis (Kornblith, et al., 1985). Recently, identification of specific genetic abnormalities occurring at high frequencies within each of the neuropathologically-defined malignancy stages have yielded genetic definitions of glioma progression. The genetic changes identified in gliomas involve deletions, gene amplifications, and mutations, with many of the changes imparting a growth advantage in the tumor cell. Regions of chromosomal deletion are thought to involve the loss of tumor suppressor genes, whereas gene amplifications in gliomas are exclusively associated with oncogenes. Mutations within chromosomes may involve either tumor suppressor genes or oncogenes. Genetic changes found at similar frequencies in all stages, such as deletions within chromosome 13q, 17p and 22q (James, et al., 1988; Fults, et al., 1990), may be related to tumorigenesis and likely occur early in tumor progression. Progression from benign to malignant glioma is accompanied by additional deletions, within chromosome 9p (Miyakoshi, et al., 1990) and 19q (von-Deimling, et al., 1992), mutations in the tumor suppressor gene p53 (James, et al., 1989), and/or MDM2 gene amplification (Collins and James, 1993). Genetic changes found only in later stages (GBM), such as complete loss of one copy of chromosome 10, deletions with 10p and 10q (Karlbom, et al., 1993), and amplification of the epidermal growth factor receptor (in 40% of all

GBMs)(Libermann, et al., 1985), may be exclusively involved in malignant progression. The frequent loss of one copy of chromosome 10 in GBMs is especially relevant to this dissertation, as the MGMT gene resides on this chromosome (Rydberg, et al., 1990; Natarajan, et al., 1992). Genetic analysis of gliomas in conjunction with neuropathology may thus provide for more accurate diagnosis of malignancy stage. Such accuracy in diagnosis is critical, as the malignancy stage strongly determines the response to, and usefulness of, various therapies currently used to treat gliomas.

The standard therapy for gliomas involves surgical removal of the tumor, followed by radiation therapy, and often chemotherapy. While there are no curative therapies available, surgical excision of gliomas is the treatment most clearly associated with increased survival. However, gliomas are nonencapsulated tumors and grow in a highly irregular, multidirectional fashion making complete surgical resection impossible. Radiation and chemotherapy are therefore used to eliminate residual tumor cells following surgery. Radiation treatment of gliomas also has survival benefit, but recurrent gliomas and especially GBMs eventually become unresponsive to further radiation therapy (Weingart and Brem, 1993). A variety of chemotherapeutic agents have been tested for efficacy against gliomas, with initial responses (tumor stasis or partial tumor regression) to several agents observed in approximately 50% of glioma patients (Wilson, et al, 1970; McVie, 1993). As with radiation therapy, gliomas also become unresponsive or resistant to chemotherapy. Understanding and eventually inhibiting the mechanisms by which glioma cells

become resistant to chemotherapy is a current goal of cancer research. Understanding these resistance mechanisms relies first on a clear knowledge of the mechanisms by which the chemotherapeutic agents exert their cytotoxicity and antitumor activity.

Chloroethylnitrosoureas (CENU) are a class of chemotherapeutic agents, of which 1,3 bis(2-chloroethyl)-1-nitrosourea (BCNU) is the most commonly used and most effective antitumor agent against gliomas (Wilson, et al, 1970; Walker, et al., 1980). BCNU is a lipid soluble DNA alkylating agent, and when administered intravenously to glioma patients, can easily cross the blood-brain barrier to reach the tumor site (Rall and Zubrod, 1962). BCNU spontaneously decomposes under physiological conditions to form a chloroethylating moiety and a carbamoylating moiety (Tong, et al., 1982). The chloroethylating moiety forms several DNA adducts including frequent N^7-hydroxylethyl and N^7-chloroethyl adducts on guanines and much less frequent O^6-chloroethyl adducts on guanines (Tong, et al., 1982). A wealth of data indicates that the O^6-chloroethyl group adducts on guanine are the adducts most relevant to the cytotoxicity induced by BCNU (reviewed in Erickson, 1991). Over a period of several hours, the O^6-chloroethyl adducts undergo an intramolecular rearrangement to form a cyclized intermediate, O^6-N^1-ethanoguanine, and finally, through interaction with the cytosine on the complementary DNA strand, form a guanine-N^1-cytosine-N^3 DNA interstrand crosslink (Tong, et al., 1982). The frequency of DNA interstrand crosslinks correlates well with degree of BCNU-induced cytotoxicity, supporting the idea that the crosslink is a relevant

cytotoxic lesion (Erickson, et al., 1980). Few if any BCNU-induced DNA interstrand crosslinks are formed in BCNU-resistant human glioma cell lines and xenografts. Accordingly, a great deal of research has been directed towards elucidating the mechanism by which resistant cells prevent BCNU-induced crosslink formation.

O^6-Methylguanine DNA Methyltransferase (MGMT)

Initial investigations of mechanisms of CENU toxicity and CENU-resistance were significantly advanced by several studies in the late 1970s and early 1980s. After it was established that the alkylating moiety of CENUs was capable of forming DNA interstrand crosslinks (Kohn, 1977; Ewig and Kohn, 1978), high levels of CENU-induced crosslinks were observed in CENU-sensitive human colon carcinoma cells (BE) but few or no crosslinks were detected in a CENU-resistant colon tumor cell line (HT29) (Erickson et al., 1978). The relationship between response to CENU and presence of DNA interstrand crosslinks was demonstrated in a dose-dependent fashion with at least four different CENU (including BCNU) (Erickson, et al., 1980, 1980a). It was proposed that the lack of crosslinks in CENU-resistant cells was due to a DNA repair mechanism operating in these cells but absent from CENU-sensitive tumor cells (Ewig and Kohn, et al., 1978; Erickson et al., 1980a).

In 1980, the CENU studies converged with other studies examining repair of DNA damage produced by the DNA methylating carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)(Day, et al., 1980). Human tumor cell lines tested for their ability to repair, and hence reactivate, MNNG-damaged adenovirus fell into

two groups. One group of tumor cell lines reactivated the MNNG-damaged adenovirus and was designated methylation repair positive, or Mer+. The group that was incapable of adenovirus reactivation, and hence incapable of methylation repair was termed methylation repair negative, or Mer-. Soon after this report, it was demonstrated in blinded studies that the CENU-resistant cell lines, which were capable of preventing CENU-induced crosslinks, were also Mer+ and the CENU-sensitive cell lines were Mer- (Erickson, et al., 1980). Additional studies firmly defined the Mer phenotype on the basis of a cell's ability to reactivate MNNG-treated adenovirus, prevent CENU-induced DNA interstrand crosslinks, and repair O^6-methylguanine in DNA (Day, et al. 1980a, and Erickson, et al., 1980a). A DNA repair protein responsible for the CENU resistance, termed O^6-methylguanine DNA methyltransferase, was subsequently identified in mouse liver (Bogden, et al., 1981), rat liver (Pegg, et al.,1983), and normal human liver (Pegg, et al., 1982).

O^6-methylguanine DNA methyltransferase (MGMT) is a major determinant in the sensitivity of glioma cells to CENU. MGMT confers resistance to CENU (and other agents that alkylate the O^6-position of guanine) by transferring alkyl group adducts, including chloroethyl adducts, from the O^6-position of guanine in DNA to a cysteine acceptor site at cys-145 within the MGMT protein (reviewed in Pegg, 1990). MGMT can also form covalent interactions with the CENU-DNA cyclized intermediate (Gonzaga, et al., 1992). The transfer reaction restores guanine to its unmodified state, thus preventing crosslink formation, and inactivates one MGMT molecule per lesion. MGMT does not require any other proteins or

cofactors for the transferase activity in vitro. Although CENU resistance may involve mechanisms other than MGMT in a limited number of cases (Karran and Stephenson, 1990; Bronstein, et al., 1992; Branch, et al., 1993), the demonstration that specific pharmacologic inhibition of MGMT sensitizes CENU-resistant cells (Zlotogorski and Erickson, 1983 and 1984; Dolan, et al., 1990) clearly establishes a dominant role for MGMT in conferring CENU resistance. This and other studies (Futscher, et al., 1989; Marathi, et al., 1993) also demonstrate the feasibility and usefulness of inhibiting MGMT for therapeutic benefit.

The cDNA for the O^6-methylguanine-DNA methyltransferase has been cloned (Tano, et al., 1990; Hayakawa, et al., 1990; Rydberg, et al., 1990), the chromosomal location of the MGMT gene defined (Rydberg, et al., 1990; Natarajan, et al., 1992) and the presence of MGMT mRNA verified in Mer+ cells (Rydberg, et al., 1990). The human MGMT cDNA was first isolated by transforming repair deficient bacteria with a cDNA library made from a Mer+ cell line, followed by phenotypic rescue of repair proficient cells in MNNG-containing media (Tano, et al., 1990). The protein coding portion of the MGMT cDNA is 624 nt and is flanked on the 5' end by at least 96 nt and 224 nt on the 3' end. The cDNA sequence also contains a consensus sequence for poly-A tail addition. The predicted amino acid sequence does not contain any clear nuclear localization signals as would be expected for a DNA repair protein. The MGMT protein is, however, localized exclusively in the nucleus (Ayi et al., 1992).

The level of MGMT expression in normal human tissue is cell type specific,

tissue specific and varies among individuals. Normal human brain and lung are low in MGMT activity and mRNA relative to liver, which has the highest level of MGMT in any normal tissue tested (Pegg, 1978; Citron, et al., 1991). Different cell types within the liver (Swenberg, et al., 1982) and kidney (Wani, et al., 1992) have 10-fold variations in MGMT levels. For example, MGMT mRNA is highest in distal tubular and glomerular epithelial cells and low in the Bowman's capsule cells, collecting and proximal tubular cells. The MGMT expression levels in specific cells of the liver and kidney correlate well with variations in persistence of O^6-alkylguanine lesions in these cells following exposure to alkylating agents. It has not been determined whether the levels of MGMT vary in a cell type specific manner in normal human brain (eg. neurons versus glia). MGMT measurements in peripheral blood lymphocytes showed a 7-fold interindividual variance (D'Ambrosio, et al., 1990), although no such interindividual variation was seen in MGMT mRNA levels in human kidney cells (Wani, et al., 1992).

The level of MGMT expression in human glioma tissue is highly variable. A one hundred-fold variation in MGMT activity among 27 unrelated glioma samples, including 22% with no detectable MGMT activity, was reported in one study (Citron, et al., 1991). Another study of 60 glioma samples showed a similar frequency (27%) of MGMT deficient gliomas (Silber, et al., 1993). These studies are consistent with the reported 20-30% of MGMT deficient human glioma cell lines (Ostrowski, et al., 1991). The frequency of MGMT deficient gliomas is not a universal finding however (Wiestler, et al., 1984) and even the existence of such

tumors is debated by some researchers.

MGMT expression is presumed, but not proven, to be regulated at the transcriptional level. Although all the glioma cell lines tested have an intact MGMT gene, the glioma cell lines devoid of MGMT activity are also lacking the MGMT protein, as measured by western blot analysis, and MGMT mRNA, as measured by northern blot analysis (Ostrowski, et al., 1991). Using the more sensitive PCR technique however, small amounts of MGMT mRNA are detectable in populations of some Mer- cell lines, indicating that MGMT transcription may occur at very low levels, or at higher levels with a concomitant decrease in mRNA stability, in these cell lines (Pieper, et al., 1990). A defect at the transcriptional level appears more likely though, as there were no differences in MGMT mRNA stability in cell lines with vastly different levels of MGMT mRNA (Kroes and Erickson, 1992). Attempts to measure transcription rates by detection of MGMT heteronuclear RNA (hnRNA), either through nuclear run-on assays or PCR, have been unsuccessful, even in cells with high levels of MGMT mRNA (Kroes and Erickson, 1992; Russell Pieper, personal communication). This may indicate that in MGMT expressing cells, the rate of MGMT transcription is too slow to detect and/or that other events such as processing of MGMT hnRNA occur rapidly after, or even concurrent with, transcription. Consistent with transcriptional differences in MGMT expression, a recent study has demonstrated that the level of cytosine methylation in MGMT exons is consistently decreased in MGMT deficient tumor cells (Pieper et al., 1991).

Cytosine Methylation and Gene Expression

Methylation of the C^5-position of cytosine is a normal, covalent DNA modification found in all mammalian cells. Methylation occurs exclusively at the dinucleotide CpG and approximately 60% of all CpGs are methylated (Bestor, et al., 1984). Cytosine methylation is accomplished by enzymatic transfer of a methyl group from S-adenosylmethionine to a cytosine within a CpG. The preferred substrate for the enzyme, cytosine methyltransferase (MTase), is hemimethylated DNA (Gruenbaum, et al., 1982). The methylation reaction occurs during DNA replication (Leonhardt, et al., 1992), allowing methylation patterns to be transmitted in a stable manner through each cell generation, and is thus termed maintenance methylation. In mammalian development, establishment of methylation patterns occurs in the postimplantation embryo by de novo methylation. Although the mechanism responsible for de novo methylation is unknown, establishment of appropriate methylation levels is critical for normal development and survival in mammals (Li, et al., 1993).

The distribution of the potential methylation site, CpG, is nonrandom. The overall genome is depleted of CpGs 5-fold from the expected CpG frequency, derived from base composition alone (McClelland and Ivarie, 1982). The genome-wide CpG depletion is thought to result, at least in part, from the spontaneous deamination of methylated cytosines to thymine (Salser, 1977). This mechanism of CpG depletion is consistent with the observed overabundance of TpG and ApG dinucleotides in the genome. Accordingly, methylation and subsequent deamination

tend to deplete potential methylation sites, and conversely, CpGs are more likely to be preserved through many cell divisions by being maintained in the unmethylated state.

In contrast to the majority of the genome, short segments of DNA, together comprising approximately 1% of the genome, have the theoretically expected frequency of CpGs and are termed CpG islands. CpG islands are defined as regions of DNA characterized by the following three features: 1) 0.3 to 3.0 kb in length, 2) the expected frequency of CpG dinucleotides (compared to the 5-fold CpG depletion seen in the genome overall), and 3) a guanine + cytosine (GC) content of >50% (compared to <40% in the genome overall) (Gardiner-Garden and Frommer, 1987). Additionally, CpG islands are normally maintained in the nonmethylated state, thus increasing the likelihood that these CpGs will be preserved (Bird, 1986). Approximately 45,000 CpG islands are present in the human genome and, similar to CpGs, are distributed non-randomly throughout the genome (Antequera and Bird, 1993). The non-random distribution and maintenance of CpG islands in the unmethylated state implies that there is some functional importance associated with CpG islands.

Virtually all known CpG islands are associated with genes, predominantly in the 5' region of genes, but also less frequently in 3' gene regions (Gardiner-Garden and Frommer, 1987). The promoters for all known housekeeping genes (genes essential for cell survival) are within, or part of, a CpG island. Forty-five thousand of the estimated 80,000 genes in the human genome are likely associated with a

CpG island (Antequera and Bird, 1993). Because the MGMT gene is considered a housekeeping gene it is likely that the MGMT promoter is associated with a CpG island. Because the MGMT cDNA is very GC rich and has an overabundance of CpGs, it is possible that one or more of the MGMT exons may also be part of a CpG island.

A variety of evidence indicates that CpG islands have a critical role in the regulation of genes with which they are associated. CpG islands invariably contain nucleosome free regions that are very accessible to diffusible factors (Tazi and Bird, 1990). Such accessible DNA sequences are essential for interaction with transcription factors and maintaining a transcriptionally active state. Although the nucleotide sequence among individual CpG islands is highly variable, most CpG islands have consensus sites for a common set of transcription factors (eg. Sp1), and these sites are indeed essential for transcription of the associated gene (Pugh and Tjian, 1990). Although CpG islands are usually unmethylated, the de novo methylation of CpG islands does occur, concomitant with suppression of transcription from the associated gene. The methylation status of CpG islands is therefore a critical parameter of gene regulation.

Changes in the methylation status of CpGs within CpG islands and CpGs located in non-island sequences may occur as a result of one or several normal (X chromosome inactivation, imprinting, tissue specific gene expression) or abnormal (tumorigenesis, viral transformation, cell culture) processes (reviewed in Jost and Saluz, 1993). The aberrant methylation changes noted in many, but not all, tumor

cells are described as widespread hypomethylation (Goelz and Vogelstein, 1985) and regional hypermethylation (De Bustros, et al., 1988). Widespread hypomethylation refers to an overall decrease in the level of methylated CpGs in the genome and probably involves gene and non-gene sequences. Regional hypermethylation in tumor cells occurs at normally unmethylated CpG islands. The frequency of methylation changes in tumor cells is not clear, although one study of the calcitonin gene-associated CpG island demonstrated hypermethylation in only 2 of 31 chronic myelogenous leukemia (CML) patients in less advanced stages (Nelkin, et al., 1991). Hypermethylation was detected much more frequently (11 of 12 patients) in advanced stage CML. This study suggests that methylation changes may contribute to tumor cell progression. In contrast to CML, colon cancer patients have frequent abnormal CpG island methylation in the calcitonin gene and chromosome 17p (a region thought to harbor a tumor suppressor gene) in the early, benign stages (Silverman, et al., 1989), suggesting that methylation changes may also contribute to tumorigenesis. Gliomas have not been tested for similar changes in methylation. Tumorigenic transformation produced by viral infection or by transfection of oncogenes also alters methylation within affected cells (Vertino, et al., 1993). Methylation changes in cultured cell lines have also been noted, with an estimated one-half of all CpG islands being subject to aberrant hypermethylation (Antequera, et al., 1990). The mechanisms involved in, or leading to, abnormal changes in methylation are not defined, although increased expression of the MTase gene has been noted in some tumor cells (El-Deiry, et al., 1991). As the MTase is a

maintenance methylase, overexpression of MTase alone cannot account for the high level of de novo methylation in tumor cells or cell lines. In X chromosome inactivation, tumorigenesis, viral transformation, and cell culture, the documented methylation changes are also accompanied by changes in expression of the associated gene. Since the tumorigenic state, cell culture, and viral transformation are conditions also associated with frequent loss of MGMT gene expression, it seems likely that the methylation status of the MGMT gene, possibly including MGMT gene-associated CpG islands, is altered in at least some of the cells in these conditions.

In the majority of genes studied, the methylation status of CpGs in the promoter correlates in an inverse, all-or-none fashion with gene expression. Whether the methylation status of a given promoter is a result of normal or abnormal processes, methylated promoters are associated with inactive genes and unmethylated promoters are associated with active genes (reviewed in Saluz and Yost, 1993). During X-chromosome inactivation for example, the CpG island promoter for the phosphoglycerate kinase (PGK-1) gene becomes methylated at 119 of 121 CpGs on the transcriptionally inactive X chromosome but remains unmethylated at all 121 sites on the active X chromosome (Pfeifer, et al., 1990). The inactivation of the X-linked genes precedes the CpG island methylation however, indicating that for these genes, methylation may serve to "lock in" the transcriptionally inactive state rather than cause gene silencing. On the other hand, methylated, inactive genes (including PGK-1) can be reactivated by exposing cells

to the demethylating agent 5-azacytidine. Additionally, a large number of studies have demonstrated that in vitro methylated genes are transcriptionally inactive when transfected into cells, whereas the unmethylated, transfected genes are expressed (Stoger, et al., 1993; Boyes and Bird, 1992). In general, unmethylated promoters are necessary but not sufficient for gene expression.

As opposed to promoter methylation, the relationship between the methylation status of CpGs in the body of genes and gene expression is poorly defined. A direct, rather than inverse, correlation between body methylation and gene expression has been noted in several studies. For several X chromosome linked (Wolf, et al., 1984) and autosomal (Razin and Riggs, 1980; Stein, et al., 1983) genes, extensive methylation of over entire body sequences is associated with active transcription. In the transcriptionally inactive state, the body of these genes is variably methylated, but generally much less methylated than the body of active genes. The body of the gene encoding the developmentally regulated transplantation antigen H-2K is unmethylated in cells that do not express H-2K, but upon differentiation, the H-2K gene becomes methylated coincident with transcriptional activation (Tanaka, et al., 1983). The active H-2K gene can then be suppressed by 5-azacytidine-induced demethylation of the body of the gene, further strengthening the direct relationship between body methylation and gene expression. In other genes such as the maternally imprinted Igf2-r gene, methylation of the body of the gene correlates directly with expression, but only in a very specific region (2 kb of 130 kb analyzed)(Stoger, et al., 1993). In vitro methylation of CpGs in the body of genes,

without promoter methylation, influences gene activity (Keshet, et al., 1985). In yet other genes, no obvious relationship is detected between methylation and gene expression (McKeon, et al., 1982). Although a mechanism by which methylation in the body of genes influences gene expression has not been defined, the conserved methylation status of CpGs in the body and promoter of many genes suggests that there is functional importance associated with methylation of both of these regions. Additionally, the direct association between methylation and gene expression in the body of genes and inverse association in promoters suggests that body methylation and promoter methylation, by different mechanisms, may be involved in setting the expression state of genes as active or inactive.

Two models have been proposed to mediate the relationship between methylation and gene expression (Antequera, et al., 1989). The first, "direct model" postulates that cytosine methylation interferes directly with the binding of transcription factors to their specific recognition sequence in regulatory regions of DNA. This model is supported by experiments comparing the transcriptional activity of methylated to nonmethylated cyclic adenosine monophosphate (cAMP) response element (CRE) sequence in cloned DNA. Methylation of a single cytosine within the CRE was sufficient to abolish both binding of the CRE binding protein to CRE and transcriptional activation of genes linked to CREs (Sanae and Schaffner, 1989). This mechanism however, is obviously limited to CpG-containing binding sites and is irrelevant to transcription factors such as Sp1, whose binding is methylation independent (Harrington, et al., 1988). A second, "indirect model"

suggests that cytosine methylation interferes with transcription via proteins that bind specifically to methylated DNA (MeCPs) and alter the local DNA conformation such that these DNA regions become transcriptionally inactive (Antequera, et al., 1989, 1990). This model is supported by the identification of four such proteins that bind selectively to DNA containing methylated cytosines (Wang, et al., 1986; Antequera, et al., 1989; Jost and Hofsteenge, 1992; Lewis, et al., 1992). Additional evidence for the indirect model comes from experiments using methylation sensitive DNA restriction enzymes (17). The DNA recognition sequence of the restriction enzyme MspI (CCGG) contains a potential methylation site (CpG dinucleotide). While MspI cleaves naked DNA (no protein) irrespective of methylation, it does not cleave DNA at methylated CCGG sequences contained within isolated, intact nuclei (protein bound DNA), suggesting that MeCPs, if present, block MspI accessibility to the DNA. The interrelationship of MeCPs with other chromosomal proteins (eg. histones) in the formation or maintenance of the methylation-related inactive chromatin is not understood. Methylation-related chromatin structures could explain why many genes, including MGMT, are not expressed in cells that contain all the relevant transcription factors (Antequera, et al., 1989).

While methylation clearly can be involved in setting the state of gene activation/inactivation, a limited amount of studies suggest that graded methylation, both in promoter and body regions of genes may be associated with more graded levels of gene expression. One study used an artificial system in which the entire human α-globin gene was methylated to low, intermediate and high levels

in vitro and then transfected into HeLa cells (Boyes and Bird, 1992). In this study, both the level of methylation and binding of MeCPs correlated inversely with expression of the transfected α-globin gene. Similar results were seen with SV40 promoter containing constructs methylated in vitro and transfected into mouse cells (Levine, et al., 1992). These studies raise the possibility that methylation, in addition to being a mechanism associated with regulation of gene expression in an all or none fashion, may also be associated with subtle changes in the level of gene expression.

CHAPTER III

METHODS

Cell Culture

The ten glioma cell lines used were established from grade III-IV human astrocytomas and glioblastomas. The glioma cell lines used were A1235, CLA, CRO, NAT (L. Erickson, Loyola University Medical Center, Maywood, IL.) SF763, SF767 (Brain Tumor Research Center, University of California, San Francisco, CA), Hs683, T98, U138, and U373 (purchased from American Type Culture Collection). The cell lines were all grown in α-minimal essential medium (Hyclone Laboratories) with 10% bovine calf serum (Hyclone), glutamine, vitamin B_{12}/Biotin, sodium pyruvate, gentamycin and nonessential amino acids. The cell lines were maintained in log phase growth at 37°C in a 95% air;5% CO_2 atmosphere.

MGMT cDNA probe synthesis

- An MGMT cDNA was obtained by polymerase chain reaction (PCR) amplification of reverse transcribed T98 RNA using two MGMT-specific oligonucleotides. First strand synthesis was performed in a 20 μl reaction containing 1 μg of T98 mRNA, 1 X PCR buffer (10 mM Tris-Cl, pH 8.3/50 mM KCl/3.0 mM $MgCl_2$/0.01% gelatin), 1 mM each of dCTP, dATP, dGTP and dTTP (Perkin Elmer Cetus (PEC)), 0.32 μg oligo dT (Bethesda Research

Laboratories (BRL)) and 200 U Moloney murine leukemia virus reverse transcriptase (BRL). The reaction was incubated at 25°C for 10 min, 37°C for 1 hr, and 95°C for 10 min, followed by cooling on ice. 10 X PCR buffer (without magnesium), dNTP mix and ddH$_2$O were added to give a final volume of 100 μl, 200 μM each dNTP, and a 1 X PCR buffer concentration. Fifty pmol each of two primers complementary to the published MGMT cDNA (Tano, et al., 1990) were added to the reaction. The 5' primer was 5'-AAGGTACCGTTTGCGACTTGGTACTTG-3' and the 3' primer was 5'-TAGTCGACCATCCGATGCAGTGTTACACG-3'. Both primers contain 7 nonMGMT-complementary nt at the 5' end (for cloning purposes). Three units of Taq polymerase (Perkin, Elmer, Cetus) were added and the reaction was amplified by PCR (95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec and a final incubation at 72°C for 10 min). The products of the reaction were electrophoresed through a 1.5% agarose gel and a gel slice containing the single PCR product (730 bp) was removed. The DNA was isolated from the gel slice by centrifugation (5 min, 12000 RPM) through glass wool (Alltech, Deerfield, Il.), and was ethanol precipitated, and resuspended to 10 ng/μl in ddH$_2$O. Twenty-five ng of the purified amplification product was used to synthesize a radiolabelled MGMT cDNA probe by the random primer method (Feinberg, et al., 1984) with [α-^{32}P] dCTP (specific activity 3000 Ci/mmol, Amersham, Arlington Heights, IL.). The specific activity of the labelled MGMT cDNA was typically $>10^9$ cpm/μg. Human histone H3.3 cDNA was generated in the same manner as the

MGMT cDNA except with the following histone-specific primers: 5' primer, 5'-CCACTGAACTTCTGATTCGC-3' and 3' primer, 5'-GCGTGCTAGCTGGATGTCTT-3'.

Genomic Library Screening and Restriction Enzyme Mapping

A genomic library constructed from the DNA of a human fibroblast cell line (WI38) inserted in the phage vector Lambda FIX II (Stratagene, La Jolla, CA.) was screened according to the manufacturer's protocol. Approximately 1 X 10^6 plaque forming units (PFU) were screened with the MGMT cDNA probe as follows: In each of 20 tubes, approximately 50,000 PFU were mixed with 600 μl of $O.D._{600}$ = 0.5 PLK17 bacteria, incubated for 15 min at 37°C, and added to 3 ml of 48°C top agar (LB broth (Difco), 0.7 % agarose (FMC). This mixture was poured on room temperature LB plates and incubated for 6 - 14 hours at 37°C, and then at 4°C for 2 hr. Twenty bacterial plates each with 50,000 of these plaques were used in the primary library screening.

In order to test the DNA from each plaque for hybridization to the MGMT cDNA, two nitrocellulose membranes were sequentially laid on the plates for 60 sec and then removed. Each filter was then submerged sequentially in denaturing solution (1.5 M NaCl/0.5 M NaOH, 2 min), neutralizing solution (1.5 M NaCl/0.5 M Tris-Cl pH 8.0, 5 min) and rinsing solution (0.2 M Tris-Cl pH 7.5/2X SSC). The filters were air dried on Whatman 3MM paper and the DNA was crosslinked to the filter by U.V. irradiation (Stratalinker, Stratagene). These duplicate membranes

were hybridized in a standard hybridization buffer (50 % formamide/10 % dextran sulfate/5X SSPE/EDTA buffer/1% SDS/250 μg per ml of denatured salmon sperm DNA/1X Denhardts solution) with a uniformly ^{32}P-labelled MGMT cDNA. The filters were washed under stringent conditions [0.1 X standard saline phosphate (SSPE) (1X SSPE = 180 mM NaCl/10 mM sodium phosphate/1 mM EDTA, pH 7.0)/0.1% sodium dodecyl sulfate, 60°C for 5 min] in a Disc-Wisk washing system (Schleicher and Schuell, Keene, NH.), dried and autoradiographed (12 - 24 hr with intensifying screens).

The autoradiograph was lined up with the original bacterial plate to determine which phage plaque corresponded to the positive signal on the autoradiograph. Identical hybridization signal on the autoradiograph of duplicate filters confirmed the specificity of the hybridization. Twenty plaques corresponding to positive signals were removed from the LB plate with the wide end of a pasteur pipette. Bacteriophage particles were eluted (4°C, overnight) into 100 μl - 1 ml SM buffer (1 L = 0.1 M NaCl/8.1 mM MgSO$_4$/50 mM Tris-HCl pH 7.5/.01% gelatin). The 20 positive phage populations isolated in this way were then purified through secondary and tertiary screening carried out in the same manner as the primary screening.

The twenty clones were divided into four groups based on hybridization to one of four MGMT cDNA restriction fragments. The four cDNA fragments, listed 5' to 3', are as follows: An 154 bp PvuII fragment spanning nucleotides 1 - 154 of the PCR amplified cDNA; a 207 bp HinfI fragment spanning nucleotides 100 - 307; an

107 bp HinfI/HhaI fragment spanning nucleotides 307 - 414; and an 137 bp SstI fragment spanning nucleotides 584 - 721.

Within each genomic clone, the MGMT gene fragment is internal to 17 bp T3 and T7 promoter sequences which are in turn flanked by various restriction enzyme (RE) sites, including NotI sites. Cutting the entire recombinant DNA molecule with NotI therefore excises a DNA fragment containing the MGMT gene fragment flanked by the T3 and T7 promoter sequences. In order to arrange all of the isolated MGMT gene fragments into a continuous length of DNA covering the coding portions of the MGMT gene, the NotI-excised DNA from each different phage was analyzed by RE mapping.

RE (BamHI, HindIII and EcoRI,) maps of the twenty cloned MGMT gene fragments were generated as follows: The MGMT gene fragments were cut from phage vector DNA by complete digestion with NotI (5 U/μg DNA, Stratagene). The DNA products of the NotI digest were then partially digested in separate reactions with BamHI, HindIII, or EcoRI, (0 U, .05 U, 0.5 U, 5 U, and 50 U, for 15 min, 37°C, (BRL)) and analyzed by Southern blot (as described below) with a ^{32}P end-labelled T3 or T7 promoter primer (Stratagene). Rehybidization of these Southern blots with a ^{32}P-labelled MGMT cDNA identified MGMT exons within each genomic clone. The number and position of restriction sites within each clone (the restriction map) were used to determine if the DNA fragments were overlapping or discontinuous with respect to their positions in the genome. Eight genomic clones with the minimal amount of overlap were then aligned to generate a discontinuous

map of the coding sequences of the MGMT gene.

Analysis of MGMT Exons

Four exons were identified in the genomic clones by Southern blot analysis with radiolabelled MGMT cDNA fragments (listed above). To facilitate sequencing of two MGMT exons, the genomic DNA fragments containing the exons were subcloned into plasmid vectors. A 4.8 kb BamHI/HindIII fragment of genomic clone 1e containing the 5'-most exon (designated exon 2) of the cDNA used, was subcloned into the plasmid pGEM-3Zf (Promega, Madison, WI.), and a 2.8 kb NotI/NaeI fragment (from genomic clone 7c) containing a second exon (designated exon 3) was subcloned into pBluescriptII SK+ (Stratagene). The resultant subclones were designated p5'BH and p7cNN, respectively. The exon regions of these subclones were sequenced with MGMT cDNA primers using the dideoxy sequencing method (Taq Track Sequencing System, Promega). Hybridization of a ^{32}P-labelled HinfI/HhaI fragment of the MGMT cDNA (see above) to Southern blots of genomic clone 5b identified a third translated exon, designated exon 4. Nucleotides complementary to the MGMT cDNA within the 3'-most exon were identified in cloned genomic DNA by PCR amplification (as described above) using primers complementary to the 3' end of the MGMT cDNA (primer 1, complementary to nucleotides 590-610, primer 2, complementary to nucleotides 776-755). This region was designated exon 5.

Generation of Intron Specific Probes

An intron 1 region, 5 kb 5' of exon 2, was derived from an EcoRI digest of genomic clone 1d. Following electrophoresis of the digestion products through a 0.5% low-melt agarose (FMC) gel, a 3.8 kb EcoRI fragment was cut from the gel and recovered by centrifugation (5 min, 12,000 x g) through siliconized glass wool (Alltech, Deerfield, IL.). A 3.8 kb EcoRI fragment, 10 kb 3' from exon 3 was used as an intron 3-specific probe. An EagI fragment, 4 kb 3' of exon 5, was used as a probe to examine methylation at the 3' end of the MGMT gene. Approximately 25 ng of the intron fragments were used as templates for generating intron-specific probes by random priming.

Identification and Sequencing of CpG Islands within Genomic Clones

Potential CpG islands within each MGMT genomic clone were identified by RE mapping as described above except using CpG island-sensitive RE (BssHII, EagI, NaeI, NarI, NotI, SmaI, SacII). Restriction sites for CpG island-sensitive REs are rare in bulk DNA, but occur relatively frequently in CpG island DNA (Lindsay and Bird, 1987). 0.5-3.0 kb clusters of the CpG island-sensitive RE sites thus identified potential CpG islands within the 12-23 kb MGMT gene fragments. Three MGMT gene regions containing clusters of these RE sites (designated region 1, 2, and 3), all within one genomic clone (7c), were subcloned and sequenced to determine if these regions had CpG island characteristics. The 5'- most region, containing part of exon 3, was excised from the genomic clone 7c by digestion with

NotI and NaeI. The 2.6 kb NotI/NaeI fragment was then ligated into NotI/SmaI-cut pBluescriptIISK+ plasmid (Stratagene) in a 10 μl ligation reaction containing 0.7 units of T4 DNA ligase, 1X ligase buffer, and an approximately 3:1 molar ratio of insert to vector DNA. Following incubation at 14°C for 12 hr, the reaction was diluted 1:5 with 10 mM Tris-HCL/1 mM EDTA pH 8.0, and 1 and 3 μl aliquots of the diluted reaction were used to transform competent bacteria (DH5α) according to the manufacturer's protocol (BRL). 9, 90, and 900 μl aliquots of transformed bacteria were then spread onto separate LB bacterial plates containing 0.5 mM IPTG (Fisher), 40 μg/ml X-Galactose (Promega), and 100 μg/ml ampicillin (Sigma). Several white bacterial colonies were removed from the plates and used to inoculate LB broth containing ampicillin. The inoculated LB broth was subsequently placed in a 37°C shaking incubator (250 cycles/min.) for 8 -12 hr. 50 - 100 μg of the recombinant plasmid DNA (p7cNN) was recovered from the transformed bacteria (Quiagen minicolumn). To facilitate sequencing of relevant areas, p7cNN was digested with EagI, which removed a 1.2 kb NotI/EagI fragment, and religated to generate the 1.6 kb EagI/NaeI insert in p7cEN.

A 4.7 kb EcoRI fragment (middle of 7c) and 3.8 kb EcoRI fragment (3' in 7c) of genomic clone 7C, which also contain clusters of the recognition sites for CpG island sensitive restriction enzymes, were subcloned in a similar manner into EcoRI digested pBluescript plasmid. To facilitate sequencing of regions of interest, these subclones were further subcloned as 3.1 kb and 1.6 kb SmaI/EcoRI fragments (for the 4.7 kb middle subclone) and as 2.4 kb BssHII/NotI fragment (for the 3.8 kb subclone). A nucleotide sequence for each subclone was determined using the

dideoxy sequencing method (Taq Track, Promega).

Northern Blot Analysis of MGMT mRNA

Total cellular RNA was isolated from each glioma cell line by a guanidinium isothiocyanate lysis procedure (Chirgwin, et al., 1979). Twenty μg of RNA from each glioma cell line was denatured by incubation at 50°C for 1 hr in a 21 μl reaction composed of 3 μl of 7 M glyoxyl, 10.5 μl DMSO, 2.1 μl 0.1 M NaH_2PO_4 pH 6.8 and 5.4 μl RNA. The denaturing reaction was then briefly cooled on ice before addition of 4 μl loading buffer (40% sucrose/0.25% bromophenol blue (BPB)). Following electrophoresis (100 V, 4.5 hr) in a 1% agarose gel (1.4 g agarose/140 ml 10 mM $NaPO_4$ pH 6.8) the RNA was transferred to a nylon membrane by capillary blotting. The membrane was then soaked sequentially in 50 mM NaOH for 15 sec and 1X standard saline citrate (1X SSC = 0.15 M NaCl/15 mM sodium citrate, pH 7.0), 0.2 M Tris-HCl pH 7.5 for 30 sec. The RNA was fixed to the membrane by UV crosslinking (Stratalinker) once the membrane had air dried. To confirm that the RNA was intact, the 28s and 18s rRNA bands were visualized on the filter under UV light. The membrane was prehybridized in standard prehybridization solution (same solution as for library screening) for 4 hr at 42°C. Following addition of ^{32}P-labelled MGMT cDNA probe, the membrane was washed initially in 1X SSPE/0.1% SDS, 25°C for 15 min and then at 60°C, 0.1X SSPE/0.1% SDS for 6 min in a Disc Wisk apparatus. Relative hybridization of the probe to glioma RNA was determined with a Betascope 603 blot analyzer (Betagen). Additionally, the membranes were

autoradiographed for 4-8 days.

The membranes were subsequently stripped of bound probe by submersion in a boiling solution composed of 1 % SDS, 10 mM Tris pH 8, 1 mM EDTA for 30 min. After drying, the membranes were prehybridized and hybridized with a radiolabelled histone H3.3 cDNA probe. Hybridization of the probe to histone H3.3 mRNA was visualized by autoradiography.

MGMT Protein Assay

The relative amount of MGMT DNA repair activity in each glioma cell line was determined by an in vitro assay which measures the extent to which glioma cell sonicates repair methyl group adducts at 0^6-guanine within a ^{32}P-end-labelled 18 bp DNA substrate (Wu, et al., 1987; Futscher, et al., 1989).

2-5X10^6 cells from each glioma cell line were centrifuged and the cell pellet was washed twice with cold 1X phosphate buffered saline (1X PBS = 0.137 M NaCl/2.68 mM KCl/10.14 mM Na_2HPO_4/1.76 mM KH_2PO_4, pH 7.4). The cells were resuspended in 400 μl ice cold assay buffer (50 mM Tris pH 8.0/1 mM EDTA/5 mM dithiothreitol/5% glycerol) and sonicated on ice in 12-5 sec bursts (with 5 sec off time between each burst to prevent the sonicator probe from heating the cell sonicates) of 0.3 relative output with a Virsonic cell disrupter (Virsonic). Following centrifugation (12,000 RPM, 30 min) the protein concentrations of the supernatants were determined spectrophotometrically. The extent of absorption of 595 nm light in a 1 ml solution composed of 1 or 2 μl aliquots of the supernatant

from each glioma cell line, 200 μl protein assay dye reagent (Biorad, Richmond, CA), and ddH$_2$0 was measured spectrophotometrically and then converted to μg of protein/μl of glioma sonicate by a Bradford protein assay program. The μl amounts of sonicate equaling 5, 10, and 25 μg protein for each cell line were then incubated separately in 150 μl reactions with 0.2 pmol of radiolabelled 18 bp MGMT substrate at 37°C for 2 hr. The labelled DNA substrate was then extracted three times with phenol/chloroform:isoamyl alcohol (24:1), once with chloroform:isoamyl alcohol and then precipitated (-70°C, 1 hr) with 1/10 vol. 3M sodium acetate pH 5.2, 3 vol 100% ethanol. After centrifugation (12,000 RPM, 30 min) the DNA was washed with 70% ethanol and lyophilized. The lyophilized DNA was resuspended in 17 μl ddH$_2$0 and incubated with 10 units of PvuII (BRL) for 1 hr at 37°C. Three unreacted control samples were incubated with no RE, PvuII, or HaeIII. The digestion reactions were then terminated by adding 9 μl of 96% formamide dye (96% formamide/1mM EDTA/0.1% BPB/0.1% xylene cyanol). After a 5 min incubation at 95°C and a quick cooling on ice, 10 μl of each sample was electrophoresed on a 20% denaturing polyacrylamide gel for 1.5 hr. The relative amount of radiolabelled, cleaved to uncleaved DNA substrate was then determined with a Betascope blot analyzer. Additionally, the gels were directly autoradiographed for 2-3 hr.

DNA Isolation

Genomic DNA was isolated from 10 - 20 X 10^6 cells of each glioma cell line.

The cells were centrifuged (2200 RPM, 5 min), washed twice with cold 1X PBS pH 7.4, and lysed in cell lysis buffer [17 mM $NaHCO_3$/27.6 mM Na_2CO_3/1 mM EDTA pH 8.0/0.4% N-lauroylsarcosine/.024% proteinase K (Merck)]. Following incubation at 37°C for 4 - 7 hr, DNA was recovered by three phenol/chloroform:isoamyl alcohol (24:1) extractions, one chloroform:isoamyl alcohol extraction, and an ethanol precipitation (0.2 volumes 11 M ammonium acetate/2.5 volumes 95% ethanol). The DNA pellet was washed twice in 70% ethanol, resuspended in 4 ml of ddH_2O, and treated with 40 μg of RNAse for 30 min at 37°C. The extraction, precipitation and ethanol washing steps were repeated, followed by DNA resuspension in 400-800 μl 10 mM Tris-HCl/1 mM EDTA, pH 8.0.

Analysis of the Methylation Status of the Body of the MGMT Gene

Analysis of the methylation status of the body of the MGMT gene in the glioma cell lines was accomplished by Southern blot analysis of genomic DNA digested with the methylation sensitive and insensitive isoschizomers HpaII and MspI, respectively (BRL). Ten μg of genomic DNA from each cell line was incubated (37°C, 6-8 h) in a 200 μl reaction containing 1X RE buffer and HpaII (7-10 U/μg DNA) or MspI (7-10 U/μg DNA). To control for completion of digestion, aliquots (20 μl) of the final reaction mixture were removed from each digestion reaction and incubated with a uniformly ^{32}P-labelled MGMT cDNA. Generation of the expected restriction fragment pattern of the labelled MGMT cDNA, as assessed by autoradiography of these control samples following electrophoresis and transfer

to a nylon membrane, reflected complete digestion in the primary digests. DNA from the primary digests was then extracted, precipitated, and resuspended in 20 μl ddH$_2$0. For analysis of intron 1 and the 3' end of the gene, the DNA samples were further digested with EcoRI (10 U/μg) for 6 hr. Equal amounts (10 μg) of DNA from each digest were electrophoresed (15-20 V, 24 hr) through 0.7 % agarose gels. The DNA was then depurinated by soaking the gel in 0.25 M HCl for 15 min. After rinsing the gel in dH$_2$0, the DNA was denatured by soaking the gel in 0.5 M NaOH/1.5 M NaCl for 30 min. Following a brief dH$_2$0 rinse, the gel was submerged in neutralization solution (0.5 M Tris pH 7.5/3 M NaCl) for 45 min. DNA was then transferred from the gel to a nylon membrane by capillary blotting in 1X SSC. Following the transfer, the membrane was submerged in 0.4 N NaOH for 45 sec, and then in 0.2 M Tris/2X SSC for 90 sec. After allowing the membrane to dry at room temperature, the DNA was covalently bound to the membrane by UV crosslinking (UV Stratalinker). The membrane was prehybridized and then hybridized to various MGMT intron specific probes in a manner analogous to that described for northern blots.

Quantitation of Methylation at an MGMT Intron 1 Site

The percent of MGMT gene alleles within each glioma cell line that are methylated at an intron 1 site, located 5 kb 5' of the translation start site, was determined by Southern blot analysis of EcoRI digested glioma cell line DNA hybridized with the 3.8 kb EcoRI intron I probe described above. DNA (10 - 20 μg)

from each glioma cell line was digested with 75 U EcoRI for 6 hr at 37^0C and then overnight with an additional 100 U EcoRI. Relative hybridization of the radiolabelled intron I fragment to the 3.8 kb (unmethylated) and 4.5 kb (methylated) EcoRI digestion products was assessed with a Betascope 603 blot analyzer. The percent methylation of the EcoRI site was calculated as the ratio of 4.5 kb fragments to 3.8 + 4.5 kb fragments multiplied by 100. The relationship between percent methylation and MGMT mRNA levels was examined by linear regression analysis (SigmaPlot5).

Linker-Mediated PCR Analysis (LMPCR) of MGMT Promoter Methylation

For methylation analysis by LMPCR, nuclei were isolated from the glioma cell lines according to a method described by Wijnholds, et al., 1988. $1X10^7$ - $1X10^8$ glioma cells were washed with ice-cold 1X PBS, centrifuged (2300 RPM, 5 min) and resuspended in 5 ml ice-cold buffer A (0.3 M sucrose/60 mM KCl/15 mM NaCl/60 mM Tris-Cl pH 8.0/0.5 mM spermidine/0.15 mM spermine/2 mM EDTA). 5 ml cold buffer A with 1% Nonidet P40 (NP-40, Sigma) was added and the samples were incubated on ice for 5 min. The sample was centrifuged (1000Xg, 5 min) and the nuclei pellet was washed with 15 ml of cold buffer A. DNA was isolated from the nuclei according to Saluz, et al., 1987 by resuspending the nuclei in 5 ml of buffer B (150 mM NaCl/5 mM EDTA pH 7.8), adding 5 ml of room temperature buffer C [20 mM Tris-Cl/20 mM NaCl/20 mM EDTA/1% SDS/600 μg/ml proteinase K (Sigma)] and incubating the samples at 37°C for 3 hr. RNase was added (100

μg/ml) and the samples were incubated at 37°C for 1 hr. DNA was extracted and precipitated as described above and then cut with EcoR I (5 U/μg) to reduce viscosity. Following extraction and precipitation, the DNA was dialyzed for 20 hr in 3 changes (at 4 hr, 18 hr and 19 hr) of 4 L ddH$_2$O. Forty μg of DNA was cleaved with genomic sequencing chemicals as described by Maxam and Gilbert, 1980. For sequencing of plasmid DNA, 1 μl of BamH I cut plasmid (containing the 1.2 kb MGMT promoter) was added to 40 μg of dialyzed, sheared salmon sperm DNA.

The LMPCR protocol was based on the method described by Pfeifer, et al., 1989 and consisted of extension, ligation and amplification steps. All DNA primers for LMPCR were gel purified except an 11 nt linker primer (see below). For extension reactions, a 15 μl reaction containing 5.0 μg of cleaved genomic DNA, 0.5 pmol of the extension primer (for promoter region I; 5'-CGGGCCATTTGGCAAACTAAG-3', corresponding to MGMT promoter nt 655-675, for region II; 5'-AGGCACAGAGCCTCAGGCGGAAGCT-3', corresponding to nt 805-823), and 1X sequenase buffer [United States Biochemical (USB)] was incubated at 95°C for 3 min, and then 60°C for 30 min. The reaction was cooled on ice and 7.5 μl of dNTP mix [final concentration in mix was 0.062 mM dGTP/0.188 mM 7-deaza dGTP/0.2 mM each of dCTP, dATP, dTTP (Pharmacia)], 0.5 μl of 0.5 M MgCl$_2$, 0.95 μl of 1 M dithiothreitol and 1.5 μl of a 1:4 dilution (in TE pH 8.0) of Sequenase version 2.0 (USB) were added. Following primer extension (48°C, 15 min), the reactions were cooled on ice, 6 μl of cold 300 mM Tris, pH 7.7 was added,

and the Sequenase was heat inactivated (67°C, 15 min). The reaction was cooled on ice. In ligation steps, a double stranded DNA linker (see below) was ligated to the extension products by addition of 45 μl of a ligation mix (13.33 mM MgCl$_2$/30 mM DTT/1.66 mM ATP/83.3 μg BSA/100 pmoles linker DNA and 3 U/reaction T4 DNA ligase (Promega)) to each reaction. After ligation (18°C, 12-16 hr) the reaction was heated (70°C, 10 min) and then cooled on ice. The DNA was precipitated (along with 10 μg yeast tRNA), washed with 70% ethanol, lyophilized and resuspended in 67 μl ddH$_2$O. The ligated DNA was then incubated in a 100 μl reaction containing 10 μl of dNTP mix (0.067 mM dGTP/0.133 mM 7-deaza dGTP/0.2 mM each of dATP, dCTP, dTTP), 1X Stoffel fragment buffer, 2.5 mM MgCl$_2$, 10 U Stoffel fragment of Taq Polymerase (Perkin Elmer Cetus), and 10 pmol each of the longer (25 mer) linker primer and a nested gene-specific primer (for promoter region 1; 5'-AGGCACAGAGCCTCAGGCGGAAGCT-3', nt 674-698, for promoter region 2; 5'-TGGGCATGCGCCGACCCGGTC-3', nt 841-861)(13) and amplified by PCR (5 min, 95°C followed by 18 cycles of 95°C for 1 min, 66°C for 2 min and 76°C for 3 min with a 5 sec extension of the 76°C step after each cycle and 10 min at 76°C after cycle 18). [32]P labelled PCR products were generated through two additional PCR cycles with a second nested end-labelled primer (promoter region I; 5'-AGGCACAGAGCCTCAGGCGGAAGCTGGGA-3', nt 674-702, promoter region 2; 5'-TGGGCATGCGCCGACCCGGTCGGG-3', nt 841-864). Seven μl of a mix containing 1X Stoffel buffer, 2.5 mM MgCl$_2$, 0.1 U Stoffel fragment/μl and 4.0 pmol of the ^{32}P labelled primer (see below) was added to the amplification reaction.

Following two cycles of PCR (same parameters as above except annealing was at 67°C and extension at 77°C), the DNA was extracted, precipitated, and resuspended in 10 μl of LMPCR dye (80% formamide/45 mM Tris base/45 mM boric acid/1 mM EDTA/.05% BPB/.05% xylene cyanol). Five μl of the sample was electrophoresed (55 Watts, 2 - 3 hr) through a 6 % denaturing polyacrylamide gel. Radiolabelled amplification products were detected by autoradiography (6-18 hr exposure).

In Vivo Dimethylsulfate (DMS) Footprint Analysis of the MGMT Promoter

Glioma cells were treated with 0.1 % DMS in fresh media (37°C, 2 min) and then washed three times with 1X PBS. DNA was then isolated, resuspended in 1M piperidine and heated for 30 min at 95°C. Following precipitation, the DNA was washed twice with 80 % ethanol and lyophilized overnight. The DNA was resuspended in ddH$_2$O and 5 μg was analyzed by linker-mediated PCR as described.

Gel Purification and Radiolabeling of LMPCR Primers

DNA Primers were gel purified according to published protocols (Ausubel, et al., 1992). 100-120 μg of each primer was diluted 2-fold in 2X formamide loading buffer [1X TBE (1X TBE = 89 mM Boric Acid/89 mM Tris base - 2.0mM EDTA)/90% deionized formamide/0.5% BPB], heated to 95°C for 5 min and placed on ice. The entire sample (80 - 120 μl) was loaded on a 15 % denaturing polyacrylamide gel using 1X TBE for running buffer. The samples were electrophoresed (300-500 V, 2-3 hr) after which the gel was removed from the

apparatus and wrapped in saran wrap. The gel was placed over a thin-layer chromatography plate with flourescent indicator and the DNA was visualized with a U.V. lamp (U.V. shadowing). A gel slice containing the appropriately sized primer DNA was removed, crushed, and the DNA was eluted (37°C, overnight, shaking incubator) from the acrylamide in 0.3 M sodium acetate, pH 7.5. The primer DNA was extracted once with phenol and once with chloroform before ethanol precipitation. The primers were end-labelled in a reaction containing 25 - 35 pmol primer (1 pmol/μl), 300 - 500 μCi gamma-^{32}P-ATP (>5000 Ci/mmol), 1X T4 polynucleotide kinase buffer (Promega) and 0.05 U polynucleotide kinase/μl. The reaction was incubated for 30 - 60 min at 37°C, 2 min at 95°C and cooled on ice. Unincorporated gamma-^{32}P-ATP was removed from the reaction by centrifugation (4 min, 3800RPM) through a G-25 sephadex column (5 Prime - 3 Prime) equilibrated with 3 ml of ddH$_2$O.

Linker Annealing

The linker primer sequences and annealing reactions were as described by Mueller and Wold, 1989. The 25 nt linker primer was 5' - GCGGTGACCCGGGAGATCTGAATTC - 3' and the 11 nt linker primer was 5' - GAATTCAGATC - 3'. The primers were annealed in a 300 μl reaction containing 6000 pmoles of each primer and 250 mM Tris-Cl pH 7.7. The reaction was heated to 95°C for 5 min, centrifuged briefly and incubated at 70°C for 10 min. The reaction was cooled to 25°C over 2 hr (2°C decrease every 5 min) and incubated at

25°C for 1 hr. After cooling to 4°C (2°C decrease every 5.5 min), the reaction was incubated at 4°C for 12 hr and stored at -20°C.

Analysis of Restriction Enzyme Accessibility to the MGMT Gene within Nuclei

Glioma cells were washed twice with cold 1X PBS and harvested by scraping into 8ml of fresh 1X PBS. The cells were centrifuged (5 min, 3500 RPM) and then resuspended in 1.0 ml of cold RSB buffer (10 mM Tris,pH 8.0/10 mM NaCl/3 mM MgCl$_2$) and .05 % NP-40 to disrupt the cell membranes. Nuclei were pelleted by centrifugation (12,000RPM, 4 sec), washed twice in 1X RE buffer and resuspended in 350 μl fresh 1X RE buffer. Nuclei equivalent to 30 μg of DNA were incubated (10 min, 37°C) with either MspI (20 - 400 U), AvaII (16 U), AluI (6 - 40 U), or DNAseI (0.6 - 60 U). DNA was then isolated from the nuclei, precipitated, and resuspended in ddH$_2$O (1.0 μg DNA/μl). For the promoter studies, 5.0 μg of DNA from the nuclei digests was analyzed by LMPCR as described above, except autoradiograph exposures were 2-5 hr, with intensifying screens. For analysis of RE accessibility to the body of the gene, DNA was isolated from the nuclei, precipitated, and resuspended in 16 μl dH$_2$0 + 2 μl 10X EcoR I buffer. All DNA samples were digested with 100 U EcoRI (2 hr, 37°C), one sample with EcoRI and MspI, (10 U/μg) and one with EcoRI and HpaII (10 U/μg). 10 μg of each digest was then analyzed by Southern blot as described above.

Gel Shift

To prepare protein extract for gel shift analysis 5 - 10X10^6 cells (glioma cells and for control reactions, HeLa cells) were centrifuged (12,000 RPM, 5 sec), the supernatant was discarded and the cells were quickly frozen in liquid nitrogen. The cells were resuspended in 5 volumes buffer C (25 % glycerol/420 mM NaCl/1.5 mM MgCl$_2$/0.2 mM EDTA/up to 100 ml with ddH$_2$O/20 mM Hepes pH 7.9/0.5 mM DTT/0.5 mM PMSF), centrifuged (100,000xg, 5 min), and the supernatant pipetted into a new tube. Ten μg cell extract (measured spectrophotometrically, as described for MGMT assay) was incubated with 0.1 ng (1 ng/μg) of a double stranded, ^{32}P-end-labelled, 22 bp DNA fragment (10,000 - 50,000 CPM/μl containing a single Sp1 binding site (Sp1 oligonucleotide, Promega) in a 25 μl reaction containing 1.0 μl poly(dI)poly(dC)(0.5 μg/μl), 1.0 μl of BSA (10 mg/ml), and 1X binding buffer (1X = 10 mM Tris pH 7.5/50 mM NaCl/1 mM EDTA/5% glycerol/1 mM DTT). For the specific competition experiment, an 100-fold excess unlabelled Sp1 oligonucleotide was added 20 min prior to addition of HeLa cell extract. A 100-fold excess unlabelled heat shock factor oligonucleotide (non-specific competitor)(5'-CTAGAAGCTTCTAGAAGCTTCTAG-3') was added to a separate tube prior to addition of HeLa cell protein extract. Each reaction (HeLa controls and glioma samples) was incubated for 20 min at 25°C, 2.5 μl dye (50 % glycerol - 0.2 % BPB - 0.2 % xylene cyanol) was added and the sample was electrophoresed (120 - 160 V, 2 - 3 hr) through a 4 % polyacrylamide gel. The gel was autoradiographed for 1 - 5 hr.

CHAPTER IV

RESULTS

Analysis of the MGMT Gene Coding Sequences

To examine the organization of the human MGMT gene coding sequences, a genomic library was screened with an MGMT cDNA probe. Twenty positive clones isolated after primary screening were purified to homogeneity through secondary and tertiary screening. The primary, secondary and tertiary screenings of a representative clone (7c) are shown in fig. 1. The clones were divided into four groups based on hybridization to one of four cDNA restriction fragment probes (fig.2). Two MGMT genomic clones (1b/2e) hybridized to cDNA fragments 3 and 4, suggesting that these clones may contain two or more exons, one common to group 3 clones and one common to group 4. Each clone was analysed for BamHI, HindIII, and EcoRI restriction sites, and then aligned with the other clones. Eight of these clones with minimal overlap were used to generate a preliminary map of the coding sequences of the MGMT gene consisting of four translated exons and spanning > 80 kb (fig.3)(exon 1, not shown here, is nontranslated (Harris, et al., 1991). Comparison of the nucleotide sequence of the 5' most exon, derived from sequencing genomic subclone p5'BH (fig.3), to the published cDNA sequences (Tano, et al., 1990) demonstrated that the 135 bp comprising this exon was contiguous with
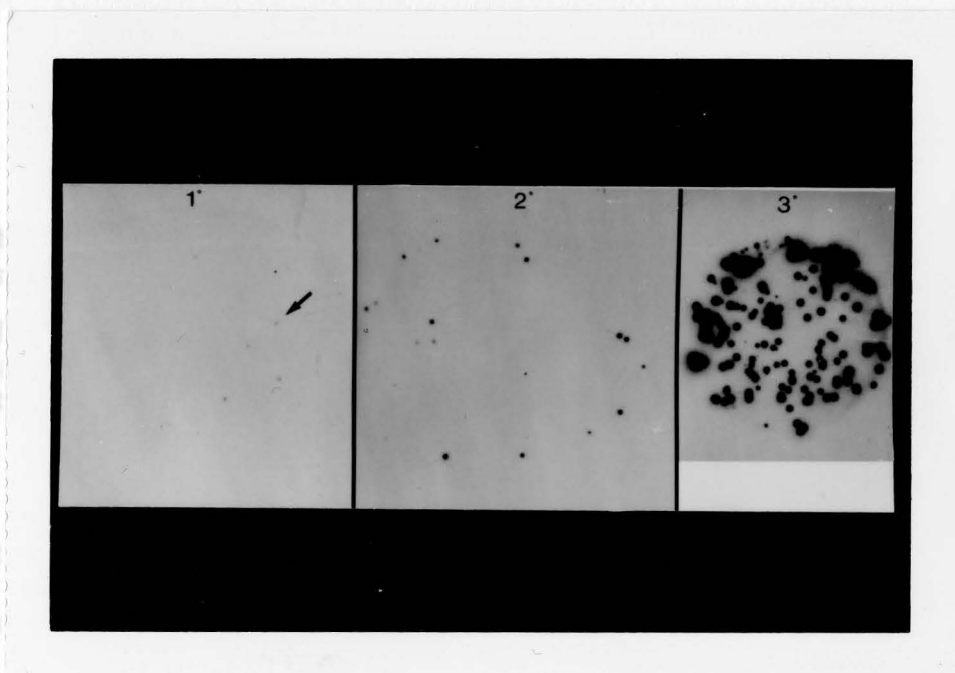
Figure 1. Primary, secondary and tertiary screening of a human genomic library with a radiolabeled MGMT cDNA. Approximately 1×10^6 clones were tested for hybridization to the 730 bp MGMT cDNA probe (which includes all the protein coding portions). Twenty positive clones were identified by the presence and identical location of positive signal on duplicate filters (from the same LB plate). A LB plug containing each of these clones corresponding to a positive signal (eg. arrow in 1° panel) was removed from the plate, eluted in 1 M SM buffer, and then purified through secondary (2°) and tertiary (3°) screening. Every phage plaque present on the tertiary screening plates hybridized to the MGMT cDNA probe, indicating that these phage populations contained MGMT gene fragments and were purified to homogeneity.

Figure 2 Relative placement of genomic clones into one of four groups based on hybridization to MGMT cDNA fragment probes. Two μg of each genomic clone were cleaved with NotI (10 U for 1 hr) and analyzed by Southern blot, as described, with sequential hybridization to each of the four cDNA fragment probes (open bars with vertical lines). Positive hybridization to genomic clones 2e and 1b was seen with the two 3' probes.
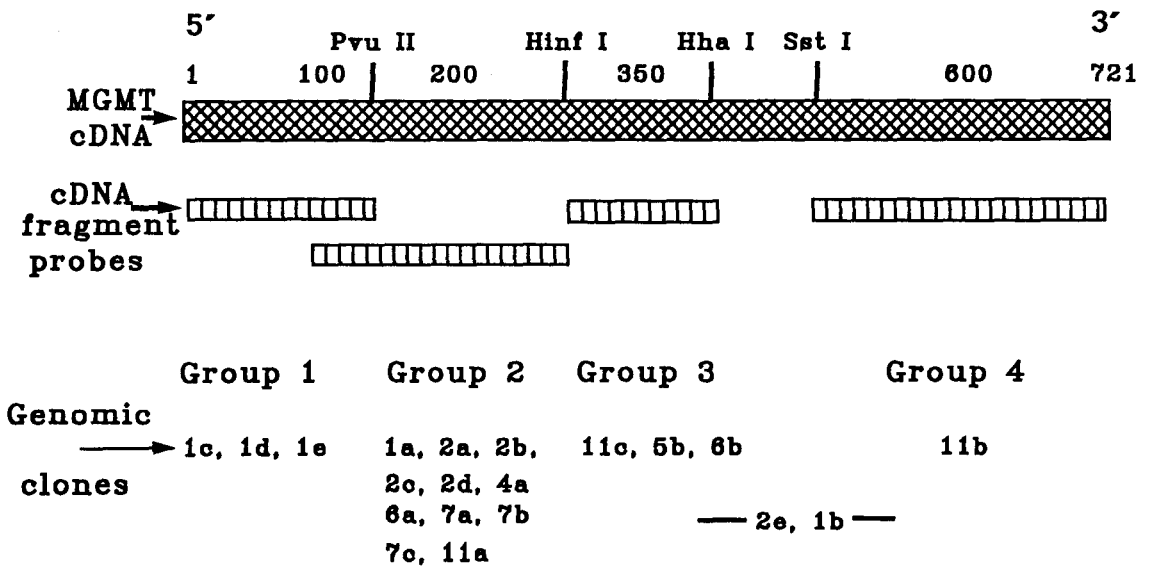
Figure 3. Restriction enzyme map of the coding sequences of the human MGMT gene. A genomic library derived from a human fibroblast cell line DNA was screened with a ^{32}P-labelled MGMT cDNA that spanned nucleotides 70-777 (which includes the entire protein coding region). Eight positive clones analyzed by restriction enzyme mapping with BamHI(B), EcoRI(E), and HindIII(H), were aligned to form a discontinuous map of the gene. Exons (open triangles) within two genomic subclones, p5'BH and p7cNN, were sequenced and compared to the corresponding cDNA nucleotides. Nucleotides complementary to cDNA sequences 590-776 were identified within genomic clone 11b (exon 5) by PCR amplification with primers defining this cDNA region. Gaps in the map represent intron regions of unknown size. The positions and length of the intron probes used for methylation analysis are indicated by solid lines located below the subclones. The dashed line immediately 5' of the intron 3 probe indicates the position of a 4.7 kb EcoRI probe also used to examine methylation.

TENTATIVE EXON ASSIGNMENT   ⟶   EXON 2   EXON 3   EXON 4   EXON 5

cDNA NUCLEOTIDES   ⟶   85 – 222   223 – 345+   −590 −776+

5' E   EE   BB E   EEH   E   E   E   E   EEEE   B   B   BBB   3'

H   HH   H   H   H   H   HH   H   HH

GENOMIC CLONES ⟶   1d   2c   5b
                 1e   7c   11b
                                                  1b/2e

SUBCLONES   ⟶   p5'BH   p7cNN

3 kb

INTRON PROBES ⟶

48

exon 1 in the MGMT cDNA. This region was therefore designated exon 2. Exon 2 contains approximately 12 nontranslated nucleotides followed by the translation start site and nucleotides encoding amino acids 1 through 41. Exon 3 encodes > 41 amino acids, beginning with amino acid 42, and is followed by two 3' exons which were separated by an intron of less than 7 kb. The first of these two exons (exon 4) was identified by hybridization of a 3' MGMT cDNA fragment to genomic clone 5b DNA. The 3'-most exon (exon 5), analysed by PCR amplification of genomic clone 11b DNA with 3' MGMT cDNA specific primers, spans > 185 bp and extends into the 3' nontranslated region. These genomic clones include all translated exons and span the body of the MGMT gene (Nakatsu, et al., 1993).

Mapping of CpG Island Sensitive RE Sites within MGMT Genomic Clones

Because the MGMT cDNA is very GC rich and contains and overabundance of CpGs, it is possible that one or more of the exons which comprise the cDNA sequence may be part of a CpG island. In order to identify potential CpG islands within the MGMT gene, the sites for CpG island-sensitive RE were mapped within genomic clones from each MGMT gene region. The recognition sites for CpG island sensitive RE are entirely composed of cytosines and guanines and contain one or more CpG dinucleotide. These sites are therefore rare in bulk DNA, but occur relatively frequently in CpG island DNA (Lindsay and Bird, 1987). Clusters of three or more of these sites within 0.5 - 3.0 kb indicate the location of potential CpG islands. MGMT genomic clones from each of the four groups were analyzed
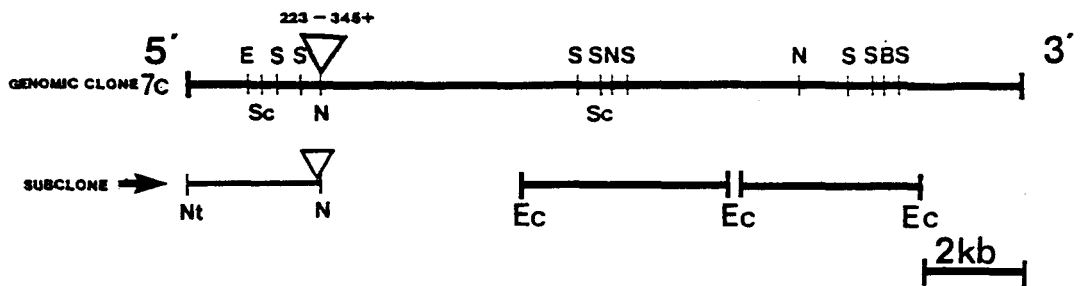
Figure 4. Map of sites for CpG island-sensitive RE in genomic clone 7c. The position of sites for BssHII (B), EagI (E), NaeI (N), NotI (Nt) SacII (Sc) and SmaI (S) were determined within clone 7c DNA by RE mapping. Subclones containing each of the three clusters of the CpG island-sensitive RE sites are shown directly below the region of 7c from which they were derived. Ec, EcoRI site, and (open triangles), position of exon 3. Sequence data from each of the subcloned regions is shown in table 1.

for potential CpG islands by mapping the restriction sites for the CpG island-sensitive REs BssHII, EagI, NarI, NaeI, NotI, SmaI, and SacII. Although most of the MGMT genomic clones contained only a few of these sites and generally not in clusters, three obvious clusters of these sites were found within genomic clone 7c (fig. 4). The 5' most cluster was associated with exon III and the middle and 3' clusters are contained in intron 3. DNA containing each cluster of sites was subcloned and partially sequenced to determine if these regions have the high GC content (>50%) and overabundance of the dinucleotide CpG [a ratio >0.6, calculated from the equation ((#CpG/#G x #C) x #nucleotides analyzed)] characteristic of CpG islands (Gardiner-Garden and Frommer, 1987). The results of the sequence analysis are shown in table 1. All three regions analyzed did have an overabundance of CpGs relative to bulk DNA and a GC content between 40-60% The overabundance of CpGs however, was clearly below the frequency found in CpG islands. Analysis of these sequences in segments of 125 - 565 nt, rather than as a whole, revealed small but CpG-rich regions of the MGMT gene. The significance of small CpG-rich segments of DNA is unknown, but because of the high content of CpGs the methylation status of these regions may be relevant in a potentially methylation-mediated establishment or maintenance of glioma MGMT gene expression.

Analysis of MGMT Expression in 10 Human Glioma Cell Lines

MGMT expression in each glioma cell line was determined at the mRNA and

Table 1. Analysis of GC content and CpG ratios in regions of the MGMT gene that contain a cluster of recognition sites for CpG island sensitive restriction enzymes

DNA Region	Nucleotides Analyzed	%GC	CpG ratio*
CpG island DNA	500-2000	>50%	>0.60
Region 1- exon 3 1.6kb Eag-Nae	1600 (1-1600)	54.0%	0.41
	565 (1035-1600)	57.2	0.53
	536 (5' 1-536)	50.7%	0.35
Region 2- intron 3 4.7kb Eco-Eco	160 (5' 1-160)	58.0	0.48
	994 (3 '1-994)	53.0%	0.32
	150 (3' 1-150)	58.4	0.53
	880 (5' 1-180)	43.0%	0.30
Region 3- intron 3 3.8kb Eco-Eco	490 (5' 390-880)	49.3	0.45
	765 (3' 1-765)	53.9%	0.29
	150 (3' 1-150)	63.0%	0.46
Bulk DNA	-	<40.0%	0.20

* CpG ratio calculated from the equation: CpG ratio =[#CpG dinucleotides/ (#C X #G)] X # nucleotides analyzed.
Note: Region 2 and 3 were sequenced inward from both ends of the subclones. In the Nucleotides Analyzed column the sequences are therefore denoted as 5' or 3' with respect to their ends within the MGMT gene.
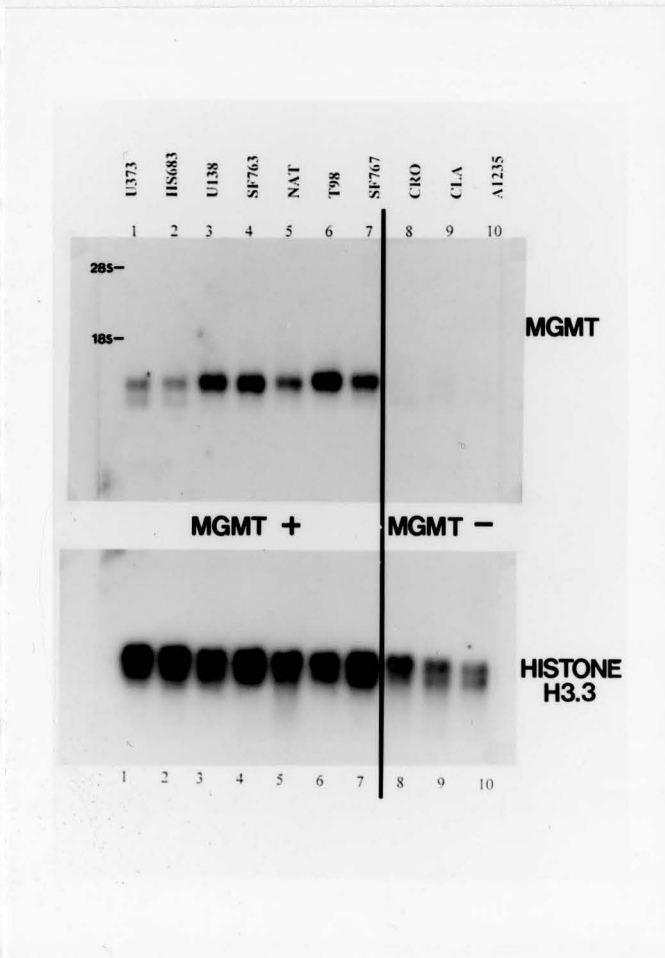
Figure 5. Northern blot analysis of MGMT mRNA levels in 10 glioma cell lines. Twenty μg of total cellular RNA from MGMT+ and MGMT- glioma cell lines was denatured, electrophoresed, transferred to a nylon membrane and hybridized with a ^{32}P-labelled MGMT cDNA (top panel). Following MGMT probe removal, the membrane was hybridized with a histone H3.3 cDNA probe (bottom panel). The membranes were washed under stringent conditions (60°C, 7 min, 0.1 % SDS/0.1X SSPE in a circulating water bath) and exposed to x-ray film (4-7 days) in the presence of intensifying screens.

protein levels.

The relative amount of MGMT mRNA in each glioma cell line was measured by northern blot analysis with an MGMT cDNA probe. The results of a representative northern blot, seen in figure 5, demonstrated that MGMT mRNA was present in T98, U138, SF763, SF767, NAT, U373, and HS683 but was not detectable in A1235, CLA, or CRO (top panel). The amount of MGMT mRNA within each cell line was quantitated and then expressed as the average of two independent northern blots (Table 2). In order to ensure that the lack of detectable MGMT mRNA in A1235, CLA and CRO was not simply due to degraded RNA in these samples, the northern blots were rehybridized with the cDNA complementary to the histone H3.3 gene. Histone H3.3 mRNA should be present in all the cell lines as histone H3.3 gene expression is constituitive and cell cycle independent (Wellman, et al., 1987). Figure 5 (bottom panel) shows that histone H3.3 mRNA was present in the RNA from all the cell lines indicating that the lack of detectable MGMT mRNA in A1235, CLA, and CRO was not due to degraded RNA in these samples.

The relative levels of MGMT activity were determined from an in vitro assay that measures the ability of glioma protein extracts to remove a methyl-group adduct from the O-6 position of guanine in an 18 bp DNA substrate (Wu, et al., 1987; Futscher, et al., 1989). Five, ten, or twenty-five μg of protein (lanes 1,2,3 of each triplet, respectively) from glioma cell sonicates were incubated with a radiolabelled 18 bp DNA fragment that contains a methyl group adduct at O^6-guanine within a PvuII RE site ($CAGCTG^{me}$). The amount of MGMT activity in
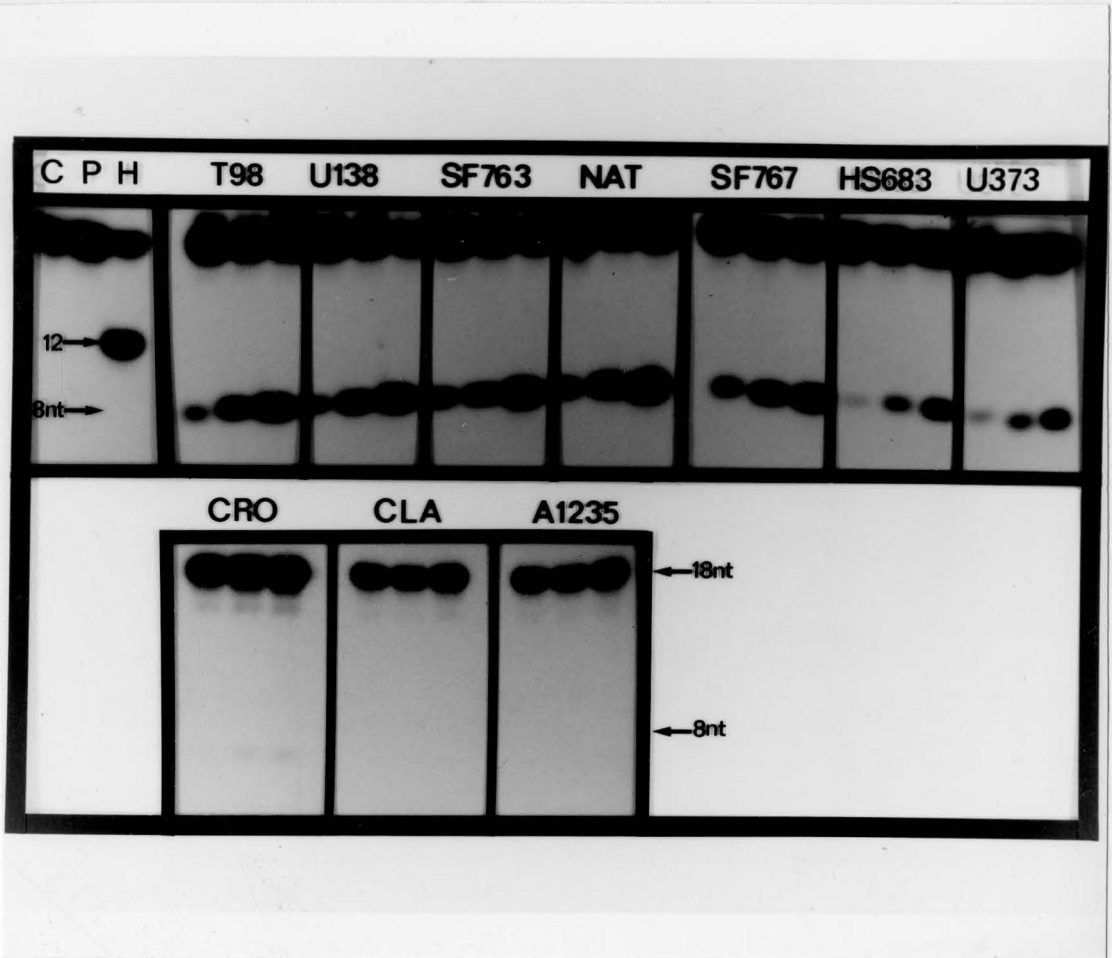
Figure 6. In vitro assay of MGMT activity within each glioma cell line. Five, ten or twenty-five µg of glioma cell sonicates were incubated (2 hr, 37°C) with a radiolabelled 18 bp DNA substrate containing a methyl-group adduct at the O-6 position of guanine within a PvuII recognition site. Unreacted 18 bp probe was incubated with PvuII (P), HaeIII (H) or no enzyme (C). The DNA from control and glioma cell sonicate-reacted samples was extracted, precipitated, and resuspended in 1X PvuII buffer (except the HaeIII control sample (H) which was resuspended in 1X HaeIII buffer). The DNA was digested with PvuII (1 hr, 37°C), 9 µl of 95% formamide dye was added and one third of the reaction was electrophoresed (1 - 2 hr, 26 mA) through a 20% denaturing polyacrylamide gel. The gel was autoradiographed for 1 - 3 hr in the presence of intensifying screens.

Table 2. Quantitation of MGMT mRNA and MGMT activity in
ten glioma cell lines

Glioma Cell Line	MGMT mRNA levels (% of T98)	MGMT Activity (% of T98)
T-98	100%	100%
SF-763	78.8	121 $\pm$ 15
U-138	76.8	96 $\pm$ 12
SF-767	61.6	110 $\pm$ 19
Nat	48.5	79 $\pm$ 12
U-373	38.4	38 $\pm$ 2
HS-683	33.3	38 $\pm$ 9
A1235	0	<1
Cla	0	<1
Cro	0	<1

Table 2. Quantitation of MGMT mRNA and MGMT activity in ten glioma cell lines. [a]MGMT mRNA levels in each cell line were quantitated (Betascope 603 blot analyzer) from two independent northern blots, averaged, and expressed as the percent of T98 MGMT mRNA level. Within each cell line the average difference between duplicate experiments was < 12%. [b]Values for MGMT activity, determined by an in vitro assay that measured the ability of glioma cell sonicates to remove a methyl group adduct from the O^6-position of guanine in a ^{32}P-labelled 18 bp DNA substrate, are the mean $\pm$ S.D. of three independent experiments expressed as percent of T98 MGMT activity.

glioma cell sonicates is thus proportional to the relative levels of repaired 18 bp

DNA substrate following incubation of the O^6-methylated 18 bp DNA with glioma cell

sonicates. After incubation, the methyl-adducted and repaired 18 bp DNA were

distinguished by digestion with PvuII. (PvuII is sensitive to methyl adducts at O^6-

guanine and therefore does not cut the adducted 18 bp DNA). The products of

PvuII digestion of the repaired 18 bp DNA were radiolabelled 8 bp and unlabelled

10 bp DNA. The 8 bp and 10 bp DNA were size separated from the adducted (and

therefore uncleaved) 18 bp DNA by electrophoresis through a 20 % denaturing

polyacrylamide gel. The gel was autoradiographed directly to detect the

radiolabelled 8 nt and 18 nt DNA. The relative proportion of 8 bp to 18 bp DNA

following incubation of the O^6-methylguanine-containing 18 bp DNA with glioma

cell sonicates is thus directly related to the amount of MGMT activity within the

cell sonicate.

Figure 6 shows an MGMT assay of glioma cell sonicates. There was no

detectable cleavage of the unreacted probe with (P) or without (C) PvuII digestion,

indicating that prior to incubation with glioma cell extracts the probe was fully

methylated at the O^6 position of guanine. Substantial (70-90%) digestion of the

probe to a 12 bp fragment by HaeIII (H) indicated that the probe DNA was not

inherently resistant to RE digestion. MGMT activity increased in a linear fashion

with increasing amounts of protein added. Quantitation of MGMT activity in each

cell line, expressed as a mean +/- standard deviation from three independent assay

samples, is shown in table 2. The rank order of the MGMT expressing glioma cell

lines according to MGMT activity, from highest to lowest, was SF763, SF767, T98, U138, NAT, HS683, and U373. MGMT activity is undetectable, in this assay system, in glioma cell lines A1235, CLA and CRO, consistent with the lack of detectable MGMT mRNA in these cell lines. Differences in the rank order of cell lines according to MGMT mRNA versus MGMT activity may reflect small differences in post-transcriptional events such as mRNA stability, translation efficiency or protein half-life.

Analysis of MGMT Gene Arrangement

The absence of MGMT expression in CLA, CRO, and A1235 could result from a deletion or rearrangement of the MGMT gene as gliomas frequently lose one copy of chromosome 10 (Collins and James, 1993) and/or have deletions of 10q (Karlbom, et al., 1993), the location of the MGMT gene (Natarajan, et al., 1992). The structure of the MGMT gene was therefore assessed in each glioma cell line by Southern blot analysis. Figure 7A and 7B represent Southern blots of MspI and EcoRI digested DNA, respectively, hybridized with a radiolabelled MGMT cDNA. The identity of hybridization patterns across all cell lines in each blot (except SF763 in the Msp blot) indicates that the lack of MGMT expression in the 3 MGMT- cell lines was not a result of deletion or gross rearrangement of the MGMT gene. The additional MGMT cDNA-recognized Msp fragment in the MGMT+ cell line SF763 is likely unrelated to (or not required for) MGMT expression as it is not detected in any of the other MGMT+ cell lines. The lack of MGMT mRNA and MGMT
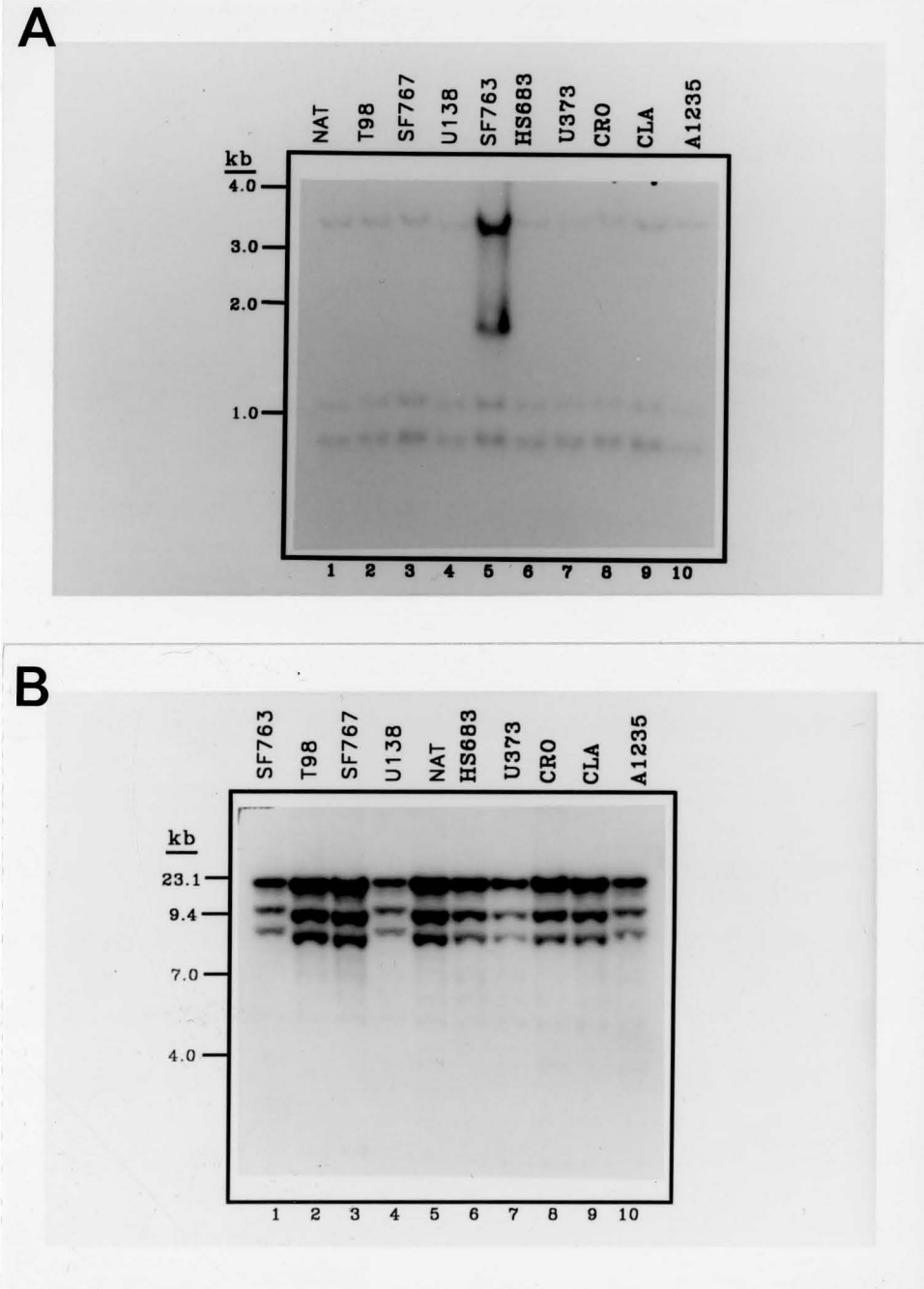
Figure 7. Southern blot analysis of MGMT gene structure in glioma cells. Ten μg of DNA from each glioma cell line was digested with MspI (10 U/μg DNA) or EcoRI (10 U/μg DNA). The DNA was extracted, precipitated and resuspended in ddH₂O and loading dye. The DNA was electrophoresed through a 1% agarose gel, transferred to a nylon membrane and hybridized to a 730 bp radiolabelled MGMT cDNA probe. The membranes were washed under stringent conditions (as described in fig.5), dried and autoradiographed for 2 - 4 days in the presence of intensifying screens.

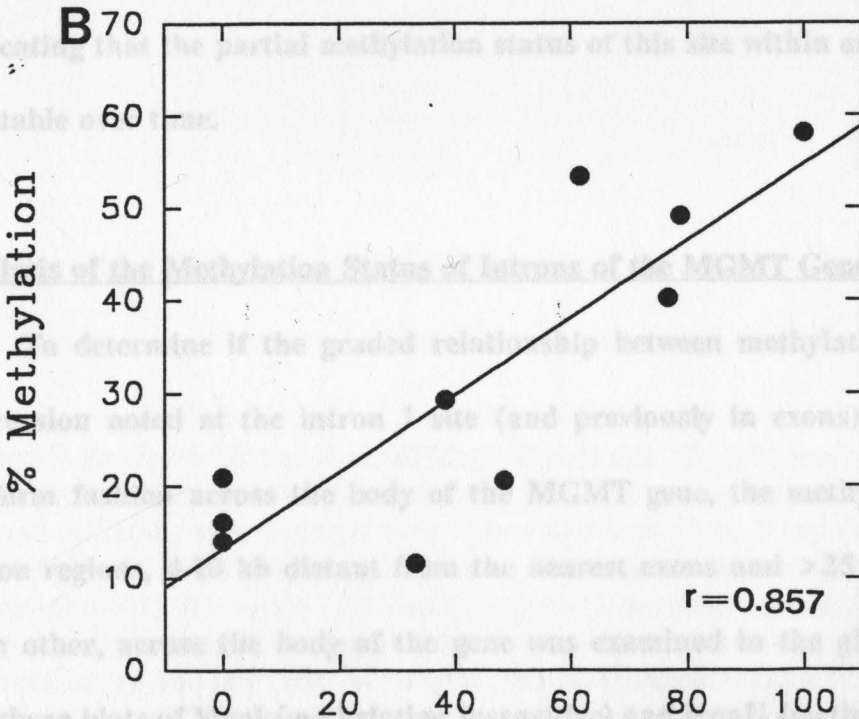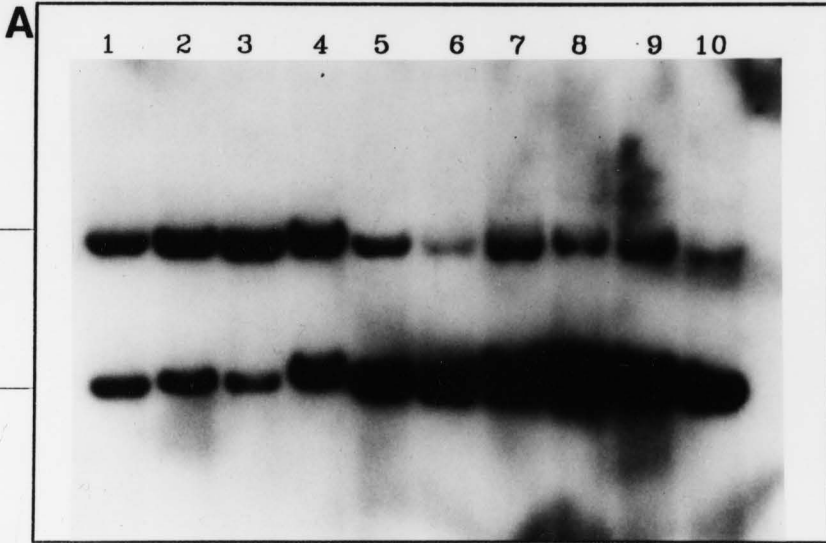activity, despite the presence of an intact MGMT gene suggests that the MGMT gene is regulated at the transcriptional level. The possibility that point mutations or very small deletions inactivate the MGMT gene cannot be excluded, but is considered unlikely, as the low frequency of point mutations in genes is incongruous with the high incidence of MGMT- cell lines (20 - 30%). Recent studies have suggested that other mechanisms of gene inactivation, such as changes in cytosine methylation, which occur frequently in tumor cells (Goelz and Vogelstein, 1985; De Bustros, et al., 1988), may influence MGMT gene expression (Pieper, et al., 1991).

Quantitative Analysis of MGMT Gene Methylation in Glioma Cell Lines

In order to examine the relationship between methylation and MGMT expression in a quantitative fashion, the percent of MGMT gene alleles that were methylated at an MGMT intron I site, 5kb upstream of the translation start site (* in fig. 3), within each glioma cell line was determined by Southern blot analysis with EcoRI digested glioma DNA (fig. 8A) and then compared to MGMT mRNA levels by linear regression analysis (fig.8B). As a result of the methylation sensitivity of EcoRI (EcoRI will not cleave at its recognition site, GAATTC, if the cytosine is followed by a guanine and the cytosine of this CG dinucleotide is methylated)(Nelson, et al., 1989), when the 3' EcoRI site flanking the 3.8 kb EcoRI intron 1 segment of the MGMT gene (see fig.3) was methylated in glioma DNA, the DNA was cut at the next available EcoRI site, approximately 0.7 kb downstream, generating a larger, 4.5 kb fragment. The proportion of 4.5 kb to 4.5 kb + 3.8 kb

Figure 8. Quantitative relationship between MGMT expression and methylation of an MGMT intron 1 site. A, Southern blot analysis of methylation at an EcoRI site located 5 kb upstream of the translation start site (see * in fig.3). DNA from the glioma cell lines was digested (37°C, overnight) with EcoRI (10 U/μg DNA), electrophoresed through a 1.0% agarose gel and transferred to a nylon membrane. DNA (10 μg/sample) from the EcoRI digests, arranged from left to right in order of decreasing MGMT expression in the cell line of origin, was hybridized with a ^{32}P-labelled 3.8 kb MGMT intron 1 fragment derived from genomic clone 1D and located 5 kb 5' of exon 2 (see fig.3). Hybridized membranes were washed under stringent conditions and then autoradiographed (4 days) in the presence of intensifying screens. The 3.8 kb fragments are unmethylated (at the EcoRI site) while the 4.7 kb fragments are methylated. B, Correlation of MGMT mRNA levels with percent methylation of an MGMT intron 1 site. Values for MGMT mRNA levels were taken from table 2. The percent methylation at the EcoRI site was determined from the Southern blot in fig.8A, and is expressed as the ratio of 4.5 kb fragments to 4.5 kb + 3.8 kb fragments (as assessed with a Betascope 603 blot analyzer) multiplied by 100. The values for percent methylation are the average of two independent experiments with an average difference of < 20%. The line of best fit and correlation coefficient (r=.857) were determined by least squares linear regression analysis (SigmaPlot5).

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20 percent between two experiments (which were done four months apart)

indicating that the partial methylation status of this site within each cell line may

be stable over time.

Analysis of the Methylation Status of Introns of the MGMT Gene

To determine if the graded relationship between methylation and MGMT

expression noted at the intron 1 site (and previously in exons) is present in a

uniform fashion across the body of the MGMT gene, the methylation status of

intron regions >10 kb distant from the nearest exons and >25 kb distant from

each other, across the body of the gene was examined in the glioma cells lines.

Southern blots of MspI (methylation insensitive) and HpaII (methylation sensitive)

digested glioma DNA were hybridized separately with intron probes. The probes

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fragments in the DNA of each cell line was therefore a measure of the percent of methylated (at this site) MGMT gene alleles within a population of glioma cells. In figure 8A, the cell lines are arranged from left to right in order of decreasing MGMT expression. The results of this analysis demonstrate that methylation was graded across the cell lines at the intron 1 EcoRI site and increased with increasing MGMT expression (fig.8). The methylation status of this intron 1 site correlates (r= 0.857, p<.001, fig.8B) with MGMT mRNA levels in a similar, positive fashion as previously seen in MGMT exons (Pieper, et al., 1991). The two minimally MGMT+ cell lines had methylation levels similar to those in MGMT- cell lines. The percent methylation at this site within each cell line varied on average less than 20 percent between two experiments (which were done four months apart) indicating that the partial methylation status of this site within each cell line may be stable over time.

Analysis of the Methylation Status of Introns of the MGMT Gene

To determine if the graded relationship between methylation and MGMT expression noted at the intron 1 site (and previously in exons) is present in a uniform fashion across the body of the MGMT gene, the methylation status of intron regions, 4-10 kb distant from the nearest exons and >25 kb distant from each other, across the body of the gene was examined in the glioma cells lines. Southern blots of MspI (methylation insensitive) and HpaII (methylation sensitive) digested glioma DNA were hybridized separately with 3 intron probes. The probes
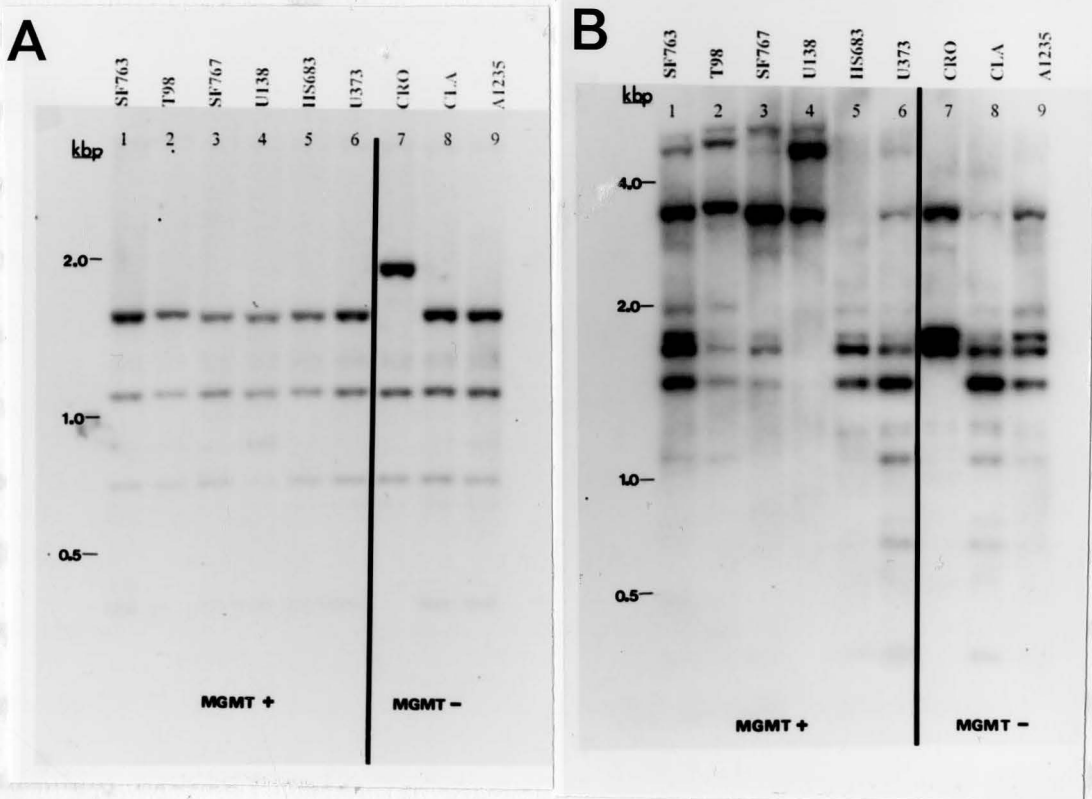
Figure 9. Southern blot analysis of MspI/HpaII site (CCGG) methylation in MGMT intron 1. DNA from MGMT expressing (MGMT+) and nonexpressing (MGMT-) glioma cell lines was digested with either A) MspI (7-10 U/μg DNA) or B) HpaII (7-10 U/μg DNA), followed by digestion with EcoRI (10 U/μg DNA). Following electrophoresis through a 1.0% agarose gel and transfer to a nylon membrane the DNA (10 μg/sample), arranged from left to right in order of decreasing MGMT expression in the cell line of origin, was hybridized with a ^{32}P-labelled 3.8 kb MGMT intron 1 fragment derived from genomic clone 1d and located 5 kb 5' of exon 2 (see fig.3). Hybridized membranes were washed under stringent conditions (as described in fig.5) and then autoradiographed (4 days) in the presence of intensifying screens.

were derived from 5', middle, and 3' intron regions of the MGMT gene. The

locations of each intron segment within the MGMT gene are depicted in fig. 3.

Southern blot analysis of the methylation status of a 3.8 kb MGMT intron 1

region, located approximately 5 kb 5' of exon 1 and 45 kb 3' of the MGMT

promoter (see fig.3, intron probes), in the glioma cell lines is shown in figure 9. The

pattern of intron 1 probe-hybridization to MspI digested DNA from the glioma cell

lines (fig.9A) was the same in all lanes (with the exception the MGMT gene in CRO,

lane 7, which has a point mutation or very small deletion in all alleles)), indicating

that the 3.8 kb intron 1 region was identical, with respect to the number and

location of MspI/HpaII sites. Southern blot analysis of HpaII digested DNA from

glioma cell lines (fig.9B), arranged from left to right in order of decreasing MGMT

expression, indicates that the intron 1 segment of the MGMT gene was relatively

unmethylated (lower molecular weight, greater degree of HpaII digestion) in the

minimally MGMT+ and MGMT- cell lines and methylated (higher molecular

weight, less HpaII digestion) in the MGMT+ cell lines, consistent with the

quantitative analysis of methylation of the EcoRI CpG bordering this region.

Additionally, the similarity in percent methylation of the intron 1 EcoRI site in

HS683 and U373 (both minimally MGMT+ cell lines) compared to MGMT- cell

lines was also detected in the extent of HpaII site methylation in intron 1 (fig.9B).

The presence of both the methylated and unmethylated fragments in HpaII digested

DNA from the MGMT+ glioma cell lines (fig. 9B, lanes 1-4) suggests the existence

of allelic differences (within a single cell) and/or population differences (within
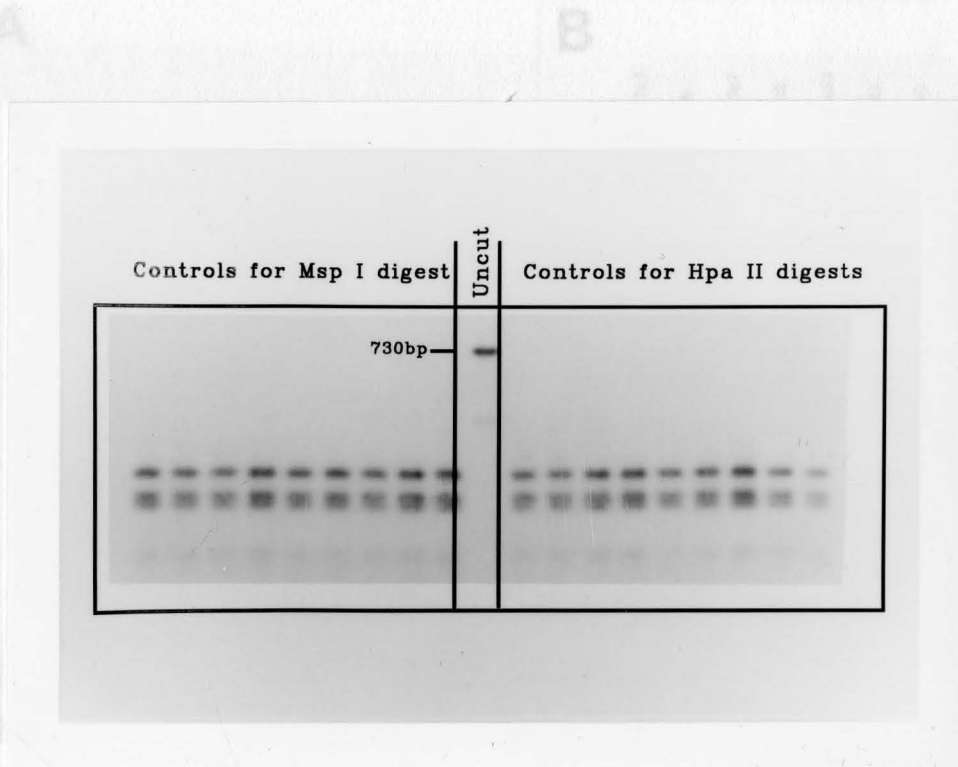
Figure 10. Analysis of the extent of MspI and HpaII digestion of the samples described in fig.9. Aliquots (20 μl) of each digestion reaction were removed from the primary digests (MspI and HpaII digests, fig.9) and incubated with a uniformly ^{32}P-labelled MGMT cDNA. The samples were electrophoresed through a 1.5 % agarose gel, transferred to a nylon membrane and autoradiographed (1 - 4 hr) in the presence of intensifying screens.

Figure 11. Southern blot analysis of MspI/HpaII site methylation in MGMT intron 3. A) MspI and B) HpaII digested DNA from each glioma cell line was analyzed by Southern blot analysis as described in fig.9 except that hybridization was with a ^{32}P-labelled MGMT intron 3 fragment derived from genomic clone 7c and located 10 kb 3' of exon III (see fig.3). Complete digestion of all samples was determined as described in fig.10.

each cell line) in MGMT gene methylation patterns. The variable hybridization intensities within each lane and across lanes likely reflect the degree of heterogeneity in methylation at various MGMT intron sites, i.e., a greater degree of heterogeneity in MGMT 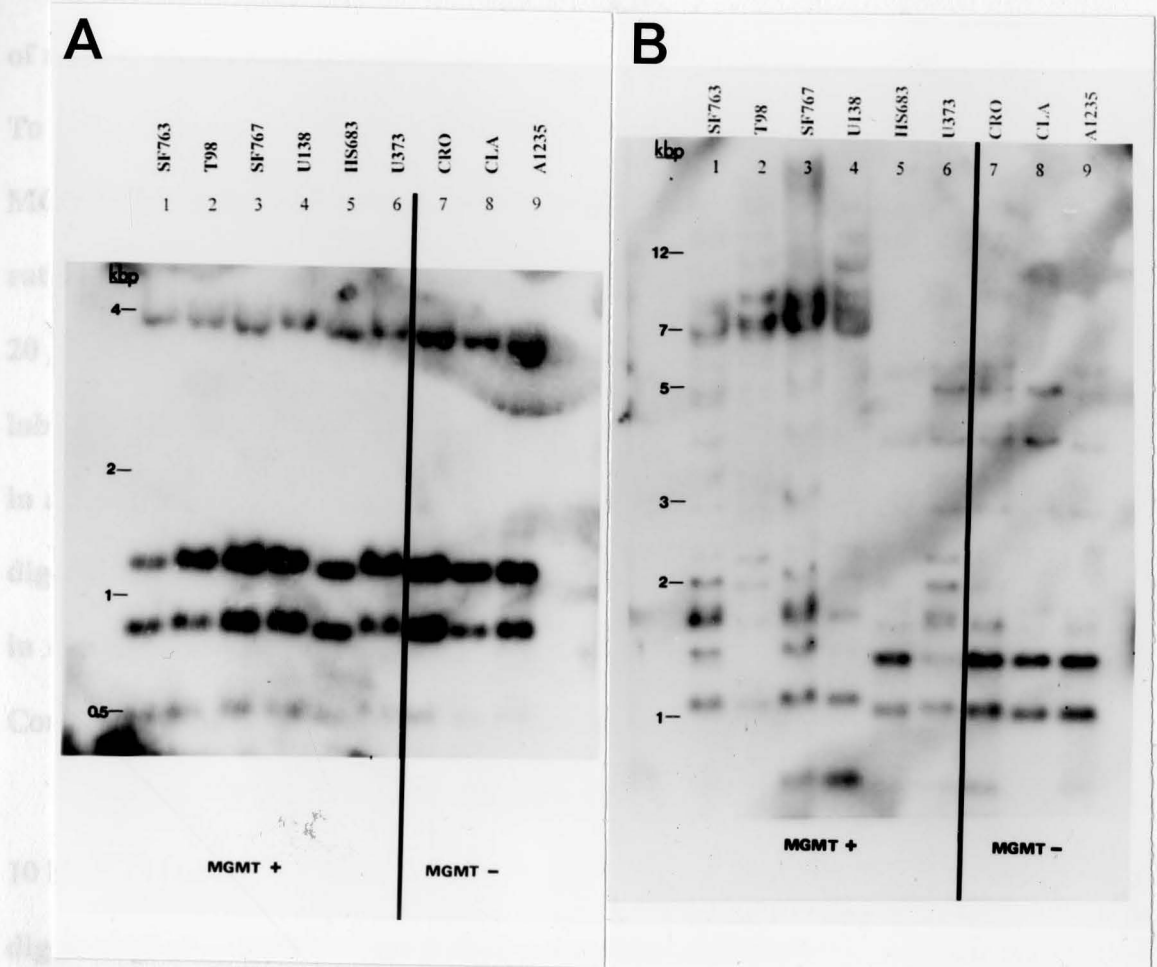gene methylation within a cell line results in fewer copies of a given HpaII fragment and thus weaker hybridization signal for that fragment. To exclude the possibility that the differences in the extent of HpaII digestion of the MGMT gene from each glioma cell line might be due to incomplete HpaII digestion, rather than methylation differences, control digests were performed. Incubation of 20 μl of each primary HpaII digest (as well as MspI digests) with a uniformly ^{32}P-labelled MGMT cDNA, followed by electrophoresis and autoradiography, resulted in an identical digestion pattern in all the samples (fig.10). This suggests that the digestion was complete in the primary HpaII and MspI digests, and the differences in extent of digestion in the primary samples can be attributed to methylation. Control digests were performed for each independent experiment.

Southern blot analysis of the methylation status of a 3.8 kb intron 3 region, 10 kb 3' of exon 3 (see fig.3, intron probes) is shown in figure 11. Analysis of MspI digested glioma DNA indicated that the number and position of MspI/HpaII sites in this intron 3 region was the same in all cell lines, regardless of MGMT expression (fig.11A). Figure 11B demonstrates that HpaII sites throughout this intron 3 region (6 kb through 14 kb 3' of exon 3) were methylated in MGMT+ glioma cell lines and relatively unmethylated in minimally MGMT+ and MGMT-glioma cell lines. The presence of a 1.1 kb fragment in all lanes (fig.11B) indicates

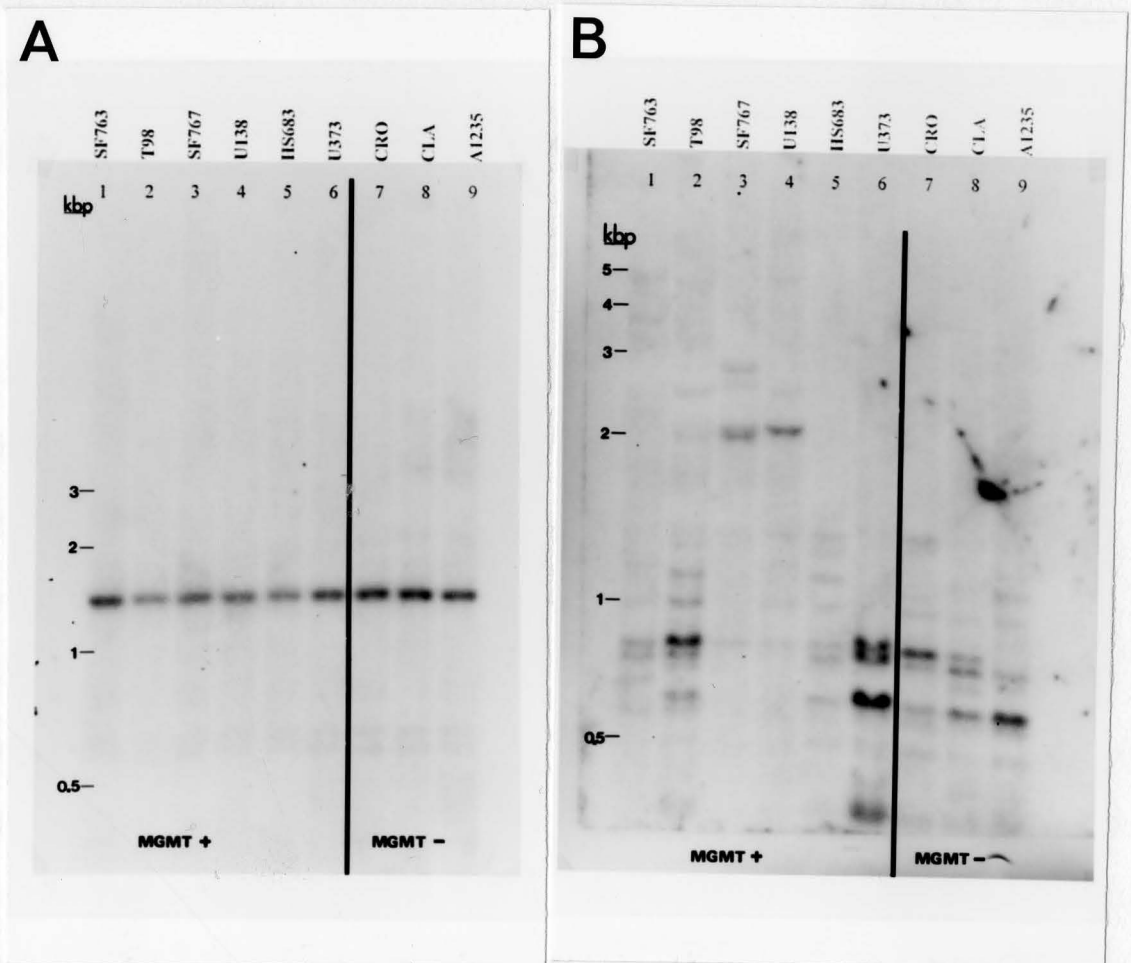Figure 12. Southern blot analysis of MspI/HpaII site methylation in the 3' end of the MGMT gene. A) MspI and B) HpaII digested DNA samples from each glioma cell line were analyzed by Southern blot analysis as described in fig.9 except hybridization was with a ^{32}P-labelled 3.5 kb EagI fragment derived from genomic clone 11b and located 4 kb 3' of exon 5. Complete digestion of all samples was determined as described in fig. 10.
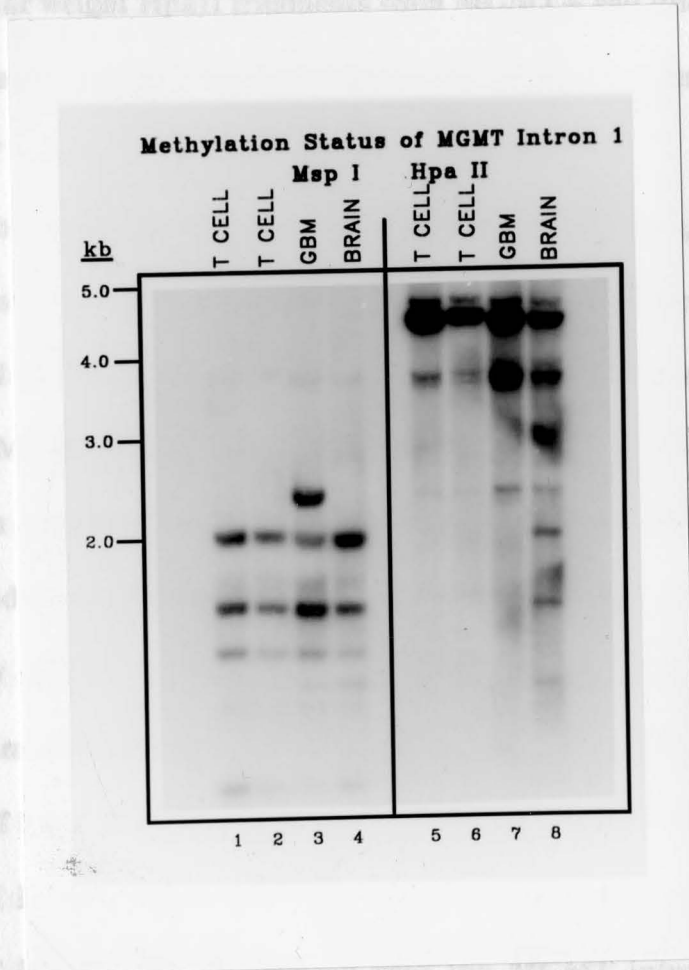
Figure 13. Southern blot analysis of MspI/HpaII site methylation in normal human T lymphocytes (T cells) and brain, and in a brain tumor sample (GBM). MspI (lanes 1 - 4) and HpaII (lanes 5 - 8) digested DNA from the human normal and tumor cells was analyzed by Southern blot analysis as described in fig.9 (including use of the same intron 1 probe).

that the two HpaII sites flanking this fragment were unmethylated to an extent, estimated by hybridization intensity, in all cell lines, with the greater number of unmethylated alleles in the MGMT- cell lines (fig. 11B). The strong hybridization to high molecular weight HpaII fragments from MGMT+ cell lines (fig.11, lanes 1-4) indicates that in a large number of these cells all HpaII sites were methylated over 7-12 kb of intron 3.

Analysis of methylation in the 3' end of the MGMT gene, shown in fig.12 yielded results similar to those in intron 1, intron 3 and exons, i.e. the MGMT gene was methylated in MGMT+ cell lines and relatively unmethylated in minimally MGMT+ and MGMT- cell lines. These results suggest that, within each cell line, the methylation status of the MGMT gene is uniform over >80 kb defined by the probes used and correlates in a positive, graded fashion with MGMT expression.

The body of the MGMT gene was also heavily methylated in several noncultured human cells that express the MGMT gene. Figure 13 shows a Southern blot analysis of MspI/EcoRI (lanes 1-4) and HpaII/EcoRI (lanes 5-8) cut DNA from a normal human brain sample, a glioblastoma multiforme tumor sample (GBM), and T lymphocytes, hybridized with the MGMT intron 1 probe. The number and position of MspI sites in this region was the same in T cells and normal brain, but an additional band of 2.4 kb was present only in the tumor sample. The 2.4 kb band corresponds exactly to the MspI fragment seen in the intron I from CLA cells (fig.9A) and may therefore be derived from a point mutation, common to CLA cells and >50 % of the cells in the tumor sample, that

destroys an MspI site flanking the 2.0 kb Msp I fragment. The MGMT gene was methylated at virtually all the HpaII sites in intron 1 of T lymphocytes, which have relatively high levels of MGMT activity (approximately 3-4 times the level in the T98 cell line). The MGMT gene in the tumor and normal human brain samples was also methylated to a level similar to the MGMT+ glioma cell lines, which express similar levels of MGMT, but to a lesser extent than in T lymphocytes. The somewhat decreased level of methylation in the brain sample compared to the tumor was consistent with the slightly lower level of MGMT activity in the brain sample. The apparent heterogeneity of MGMT methylation in the normal brain sample might be explained by differences in MGMT gene methylation patterns in the different cell types present in this sample.

RE Accessibility to the Body of the MGMT Gene within Intact Nuclei

The observation that methylation of the body of the MGMT gene correlated in a direct, graded fashion with MGMT expression suggested that there may be methylation related differences in chromatin structure of the body of the gene. The chromatin structure of the body of the MGMT gene was examined by determining the relative accessibility of MspI, AluI, and DNaseI to the body of the MGMT gene within isolated nuclei from MGMT+ and MGMT- glioma cells. Since both histones and methylated DNA binding proteins (MeCps) block RE digestion in nuclei (Tazi and Bird, 1990; Antequera, et al., 1989)), this analysis assesses, at least, the combined effect of these proteins on chromatin structure.
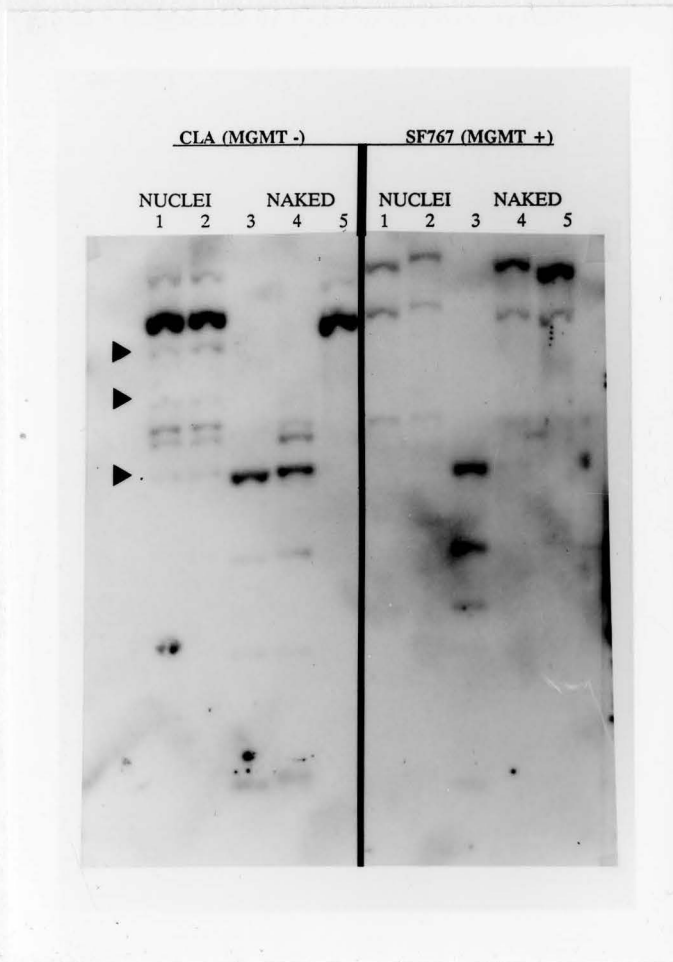
Figure 14. Southern blot analysis of MspI accessibility to and methylation of an MGMT intron 1 region. For analysis of MspI accessibility to the MGMT gene, nuclei from MGMT+ (SF767) and MGMT- (CLA) glioma cells were incubated with MspI (lanes 1,6; 20 U MspI for 10 min, lanes 2,7; 150 U MspI for 2 hr), the DNA was isolated and further cleaved with EcoRI. For analysis of intron 1 methylation, DNA was also isolated from untreated nuclei and digested with MspI/EcoRI (lanes 3,8), HpaII/EcoRI (lanes 4,9) or EcoRI only (lanes 5,10). The DNA from all the samples was analyzed by Southern blot as described in fig.9 (including use of the same intron 1 probe).

Figure 15. Southern blot analysis of MspI accessibility to and methylation of an MGMT intron 1 region in MGMT+ (T98) and MGMT- (A1235) glioma cells. Analysis of MspI accessibility to the MGMT intron 1 region within intact nuclei and methylation analysis of naked DNA were performed exactly as described in fig.14. Lanes 1,6; nuclei incubated with 20 U MspI for 10 min, lanes 2,7; nuclei incubated with 150 U MspI for 2 hr, lanes 3,8; MspI/EcoRI cut naked DNA, lanes 4,9; HpaII/EcoRI cut naked DNA, lanes 5,10; EcoRI cut naked DNA.
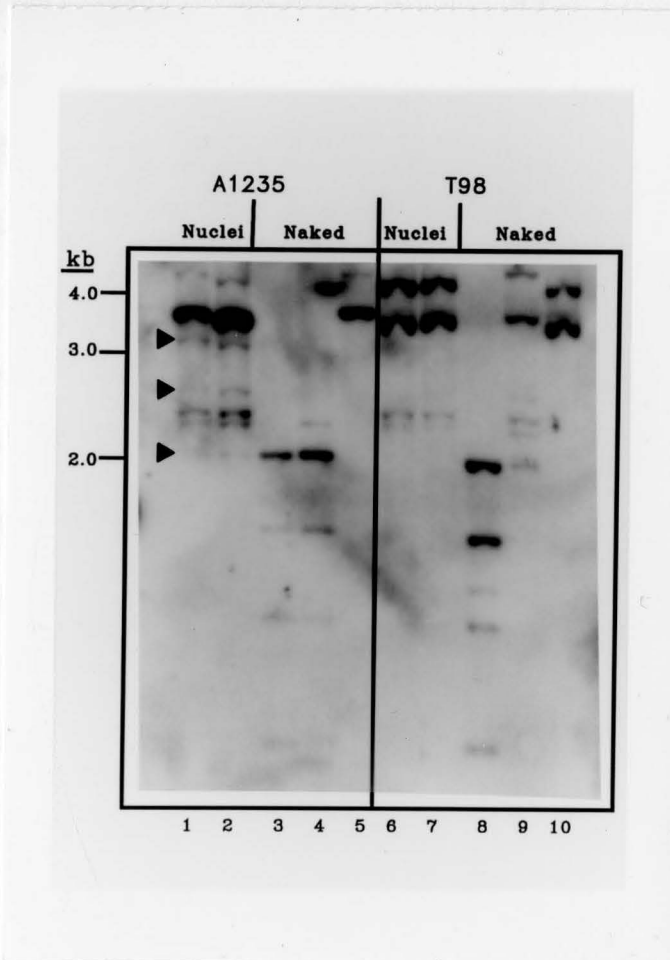
Figure 16. Southern blot analysis of MspI accessibility to and methylation of an MGMT intron 1 region in MGMT+ (T98) and MGMT- (Colo) cells. Analysis of MspI accessibility to the MGMT intron 1 region within intact nuclei and methylation analysis of naked DNA were performed exactly as described in fig.14. Lanes 1,6; nuclei incubated with 20 U MspI for 10 min, lanes 2,7; nuclei incubated with 150 U MspI for 2 hr, lanes 3,8; MspI/EcoRI cut naked DNA, lanes 4,9; HpaII/EcoRI cut naked DNA, lanes 5,10; EcoRI cut naked DNA.
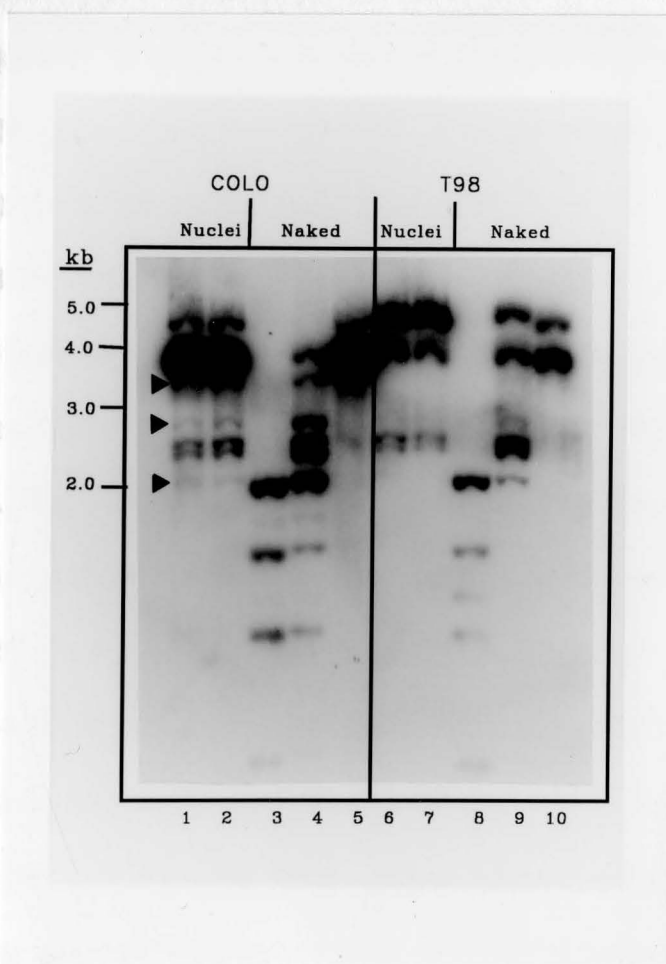
The recognition site for MspI (CCGG) contains a CpG and MspI accessibility to DNA in nuclei may therefore be directly influenced by MeCps (Antequera, et al., 1989). Comparison of the relative accessibility of MspI to MGMT gene sequences within nuclei as well as confirmation of the methylation status of the MGMT gene was accomplished by Southern blot analysis using the 3.8 kb EcoRI intron 1 probe described above. As a result of the methylation sensitivity of EcoRI, the 3.8 kb intron 1 probe recognized two fragments in the EcoRI only digests of naked DNA (fig.14-16 lanes 5, each panel). The larger, 4.5 kb fragment was generated from MGMT alleles methylated at the EcoRI site that defines the 3' end of this probe. The MGMT expressing cell lines had a greater proportion of the 4.5 kb fragments (fig.14-16, lanes 5) indicating a greater degree of methylation at this site in MGMT+ cells compared to MGMT- cells (lanes 10). Consistent with the EcoRI digests, the EcoRI/HpaII (fig. 14-16, lanes 4,9) digests also confirm that the intron 1 region was methylated in the MGMT+ cell lines and relatively unmethylated in the MGMT- cell lines. There was no difference in digestion patterns when the glioma DNA was incubated with the methylation insensitive enzyme, MspI (fig.14-16, lanes 3,7). Comparison of the Msp digests of DNA in nuclei demonstrates that the unmethylated MGMT gene-intron 1 region in MGMT- cells is more accessible to MspI relative to the methylated intron 1 in MGMT expressing cells, indicated by the presence of three additional probe-recognized fragments in the DNA from MGMT- cells (fig. 14-16, ▸, lanes 1-2) not seen in the digests of DNA from MGMT+ cells (SF767, lanes 6-7). Incubation of the nuclei

with 20 U MspI for 10 min (fig. 14-16, lanes 1,6) yielded the same degree of digestion as incubation with 150 U MspI for 2 hrs (lanes 2,7). The differential Msp accessibility in the intron 1 region is consistent across all 5 cell lines tested (fig. 14-16), suggesting that the methylation-related chromatin structure in the body of the gene may be a relevant component of MGMT transcription. Furthermore, the positions of the accessible MspI sites were identical in the nuclei from all three unrelated MGMT- cell lines tested, and two minimally MGMT+ cell lines (which are also relatively unmethylated) supporting the idea that methylation and chromatin structure in the body of the MGMT gene are closely linked, possibly through MeCps.

As opposed to MspI, enzymes with non-CpG-containing recognition sites are only blocked by MeCps if their sites are near a CpG (Antequera, et al., 1989). To determine if the differences in chromatin structure can be detected with enzymes that do not contain CpGs in their recognition sequence, the accessibility of AluI to the MGMT gene within nuclei was tested. Nuclei from 2 MGMT+ and 2 MGMT- glioma cell lines were incubated with increasing amounts of AluI, the DNA was isolated and cleaved with EcoRI and analyzed by Southern blot with the 3.8 kb intron 1 probe (fig.17-18). Increasing the amount of AluI in the reaction resulted in increased cleavage primarily in the MGMT- nuclei, but also, to a much lesser degree in one of the two MGMT+ nuclei (fig.17, SF767). The extent of AluI digestion of the MGMT gene was nevertheless significantly greater in MGMT- nuclei compared to MGMT+ nuclei, especially in nuclei incubated with 60 U AluI

Figure 17. Southern blot analysis of AluI accessibility to an MGMT intron 1 region within intact nuclei from MGMT+ (SF767) and MGMT- (CLA) glioma cells. Nuclei were incubated with AluI (0 - 60 U for 10 min), the DNA was isolated and further cleaved with EcoRI. Ten μg of DNA from each digest was analyzed by Southern blot with the intron 1 probe as described in fig.9.
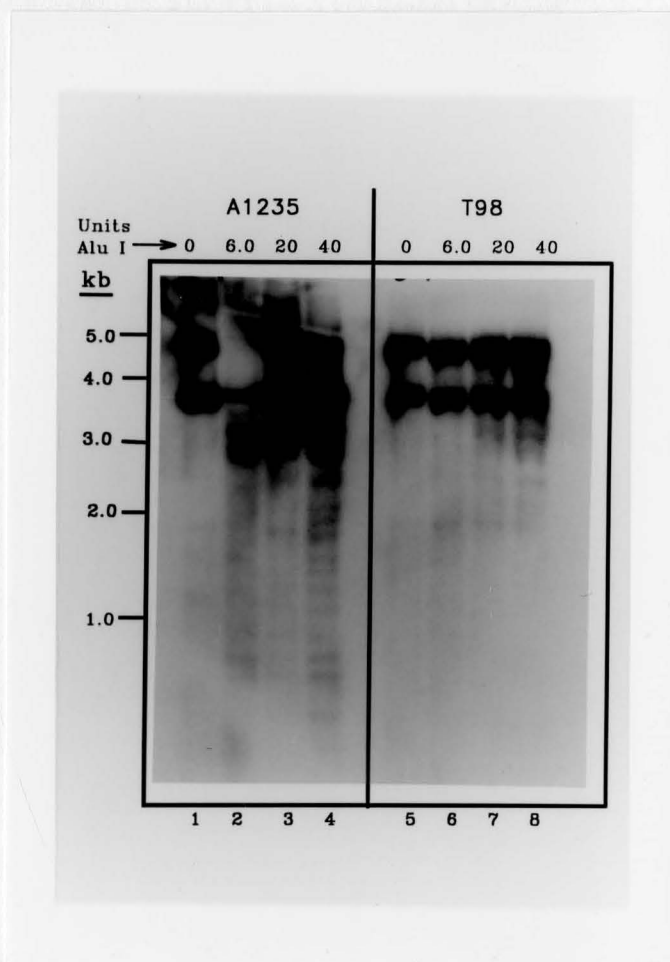
Figure 18. Southern blot analysis of AluI accessibility to an MGMT intron 1 region within intact nuclei from MGMT+ (T98) and MGMT- (A1235) glioma cells. Nuclei were incubated with AluI (0 - 60 U for 10 min), the DNA was isolated and further cleaved with EcoRI. Ten μg of DNA from each digest was analyzed by Southern blot with the MGMT intron 1 probe as described in fig.9.
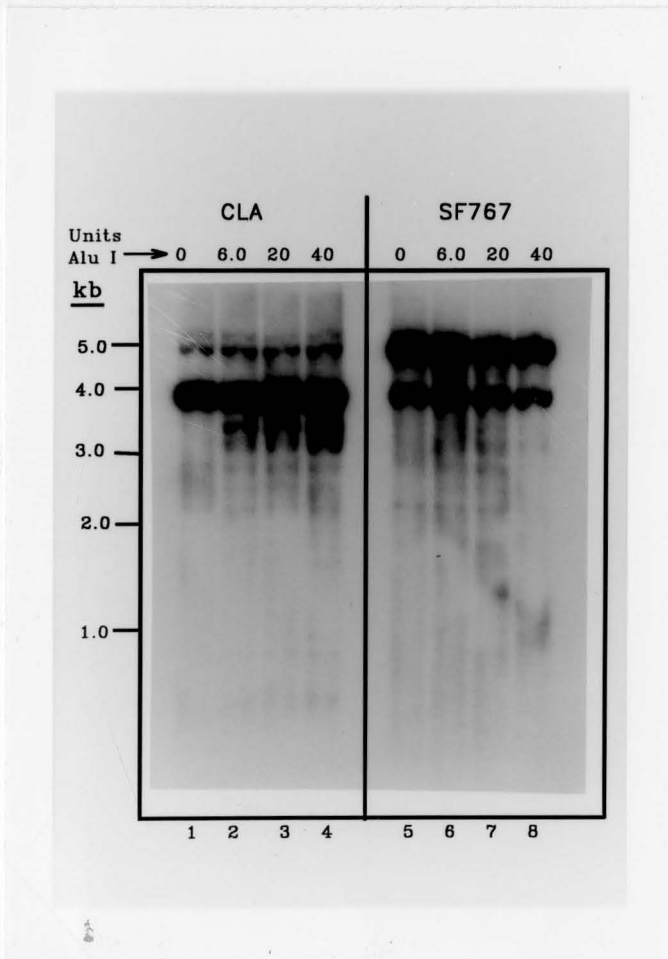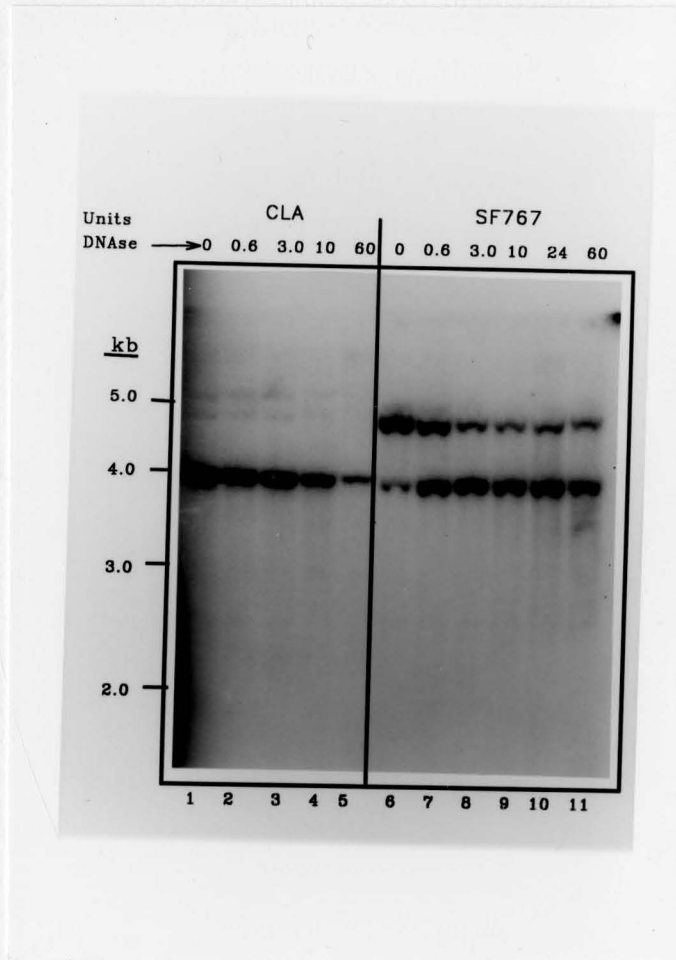
Figure 19. Southern blot analysis of DNaseI accessibility to an MGMT intron 1 region within intact nuclei from MGMT+ (SF767) and MGMT- (CLA) glioma cells. Nuclei were incubated with DNaseI (0 - 60 U for 10 min), the DNA was isolated and further cleaved with EcoRI. Ten μg of DNA from each digest was analyzed by Southern blot with the intron 1 probe as described in fig.9.

Figure 20. Southern blot analysis of DNaseI accessibility to an MGMT intron 1 region within intact nuclei from MGMT+ (T98) and MGMT- (A1235) glioma cells. Nuclei were incubated with DNaseI (0 - 60 U for 10 min), the DNA was isolated and further cleaved with EcoRI. Ten μg of DNA from each digest was analyzed by Southern blot with the intron 1 probe as described in fig.9.
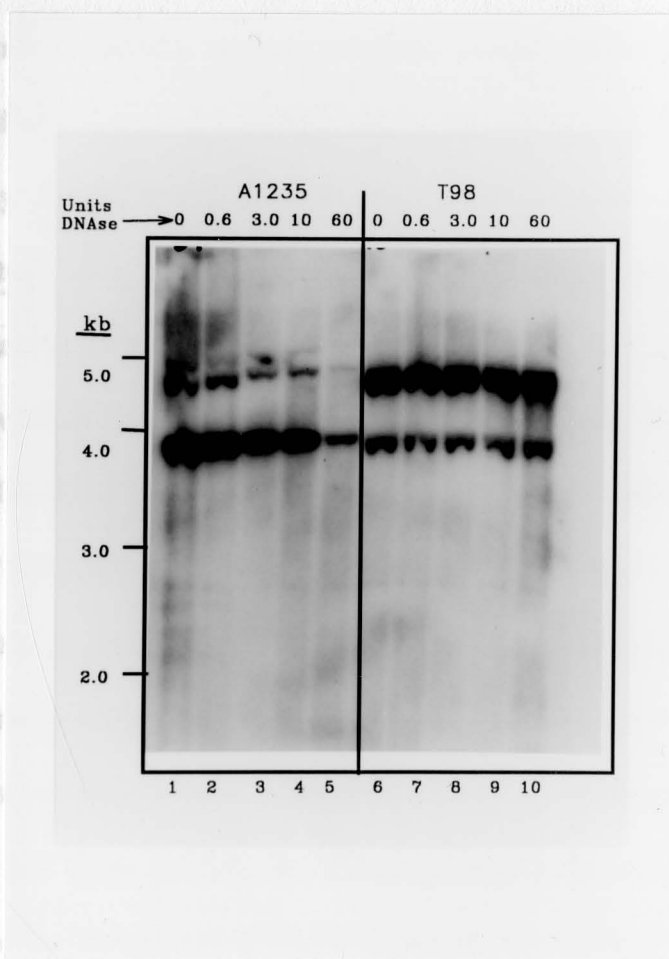
(fig.17,18, compare lanes 4 and 8). Thus, the different chromatin structures of the MGMT gene within MGMT- and MGMT+ nuclei can be distinguished at CpG-containing and nonCpG-containing sites.

The chromatin structure of the body of the MGMT gene was also analysed with a non-sequence specific enzyme, DNAseI, which cleaves DNA in the minor groove (reviewed in Gross and Garrard, 1988). DNAseI cleaves at nucleosome free regions (often synonymous with DNAseI hypersensitive sites) when added at low concentrations to nuclei, and at internucleosomal and intranucleosomal DNA at much higher concentrations. Nuclei from two MGMT+ and two MGMT- glioma cell lines were incubated with increasing amounts of DNAseI, after which DNA was isolated, cleaved with EcoRI and analyzed by Southern blot with the MGMT intron 1 probe (fig.19, 20). DNaseI did not cleave intron 1 at any of the concentrations shown in these experiments (fig.19,20). The average sized DNA fragment from these digests, as determined by visual inspection of the agarose gels prior to transfer, decreased with increasing DNaseI, indicating that much of the genome was cleaved by DNase, yet the body of the MGMT gene was very resistant to cleavage. Incubation of the nuclei with 120 U DNase resulted in complete loss of hybridizable DNA fragments in each cell line. Thus, there were no detectable differences in the presence or absence of nucleosomes in the body of the MGMT gene in nuclei from MGMT+ and MGMT- glioma cell lines.

In summary, MspI and AluI accessibility to the body of the MGMT gene was greater in nuclei from MGMT- cells relative to MGMT+ cells but MspI

accessibility was independent of MspI concentration whereas AluI accessibility increased with increasing AluI added. The greater MspI and AluI accessibility in MGMT- nuclei was likely not a result of the absence of nucleosomes in these cells since no differences in DNaseI digestion were seen at any of the DNaseI amounts tested, but may involve MeCps. Binding of MeCps to the methylated MGMT gene could be relevant in mediating the relationship between methylation and MGMT expression by maintaining the chromatin structure of the MGMT gene in a manner that facilitates transcription. These data suggest that alterations in methylation and chromatin structure in the body of the MGMT gene may be a common feature associated with loss of MGMT gene expression.

Analysis of Methylation in the MGMT Promoter

The methylation status of the MGMT promoter was determined by linker-mediated PCR (LMPCR) analysis of hydrazine treated glioma DNA. As hydrazine, in the presence of 1.5 M NaCl, reacts preferentially with cytosine but not 5-methylcytosine (Ohmori, et al., 1978), methylated cytosines decrease the intensity, or cause the disappearance of cytosine bands in sequenced DNA. Methylation was analyzed in two regions of the MGMT promoter that together likely contain all the basal promoter elements and approximately 60 percent of full promoter activity (Harris, et al., 1991). Region I includes nucleotides 703-800 and region II, which includes the transcription start site (nt 955), spans the basal promoter elements (nucleotides 865-1020) (Harris, et al., 1991). The full nucleotide sequence of these

Figure 21. Methylation analysis of the MGMT promoter in MGMT+ (SF767), minimally MGMT+ (Hs683) and MGMT- (CLA) glioma cells by LMPCR. DNA from the glioma cell lines and plasmid DNA containing a 1.2 kb BamH I/Sst I fragment of the MGMT promoter was reacted with genomic sequencing chemicals. All nucleotides (G, guanine, A, adenine, T, thymine, C, cytosine) in the cloned DNA and guanines and cytosines in the glioma DNA spanning promoter nt 703-800 (A, region I) and 865-1020 (B, region II) were analyzed by LMPCR. A MGMT promoter-specific primer (region I, nt 655-675 and region II, nt 805-823), was annealed to the cleaved DNA and extended (48°C, 15 min) with Sequenase. Extension products were ligated to a double stranded linker and then amplified by PCR (5 min, 95°C followed by 18 cycles of 95°C for 1 min, 66°C for 2 min and 76°C for 3 min with a 5 sec extension of the 76°C step after each cycle and 10 min at 76°C after cycle 18) with the longer (25 mer) linker primer and a nested gene-specific primer (for promoter region 1, nt 674-698, for promoter region 2, nt 841-861). ^{32}P labelled PCR products were generated through two additional PCR cycles with a second nested end-labelled primer (for promoter region I, nt 674-702, for promoter region 2, nt 841-864). Following two cycles of PCR (same parameters as above except annealing was at 67°C and extension at 77°C), the DNA was extracted, precipitated, and resuspended in 10 μl. 3 - 5 μl of the sample was electrophoresed through a 6 % denaturing polyacrylamide gel and then detected by autoradiography (6-18 h exposure). (▲) indicate cytosines within a CpG.
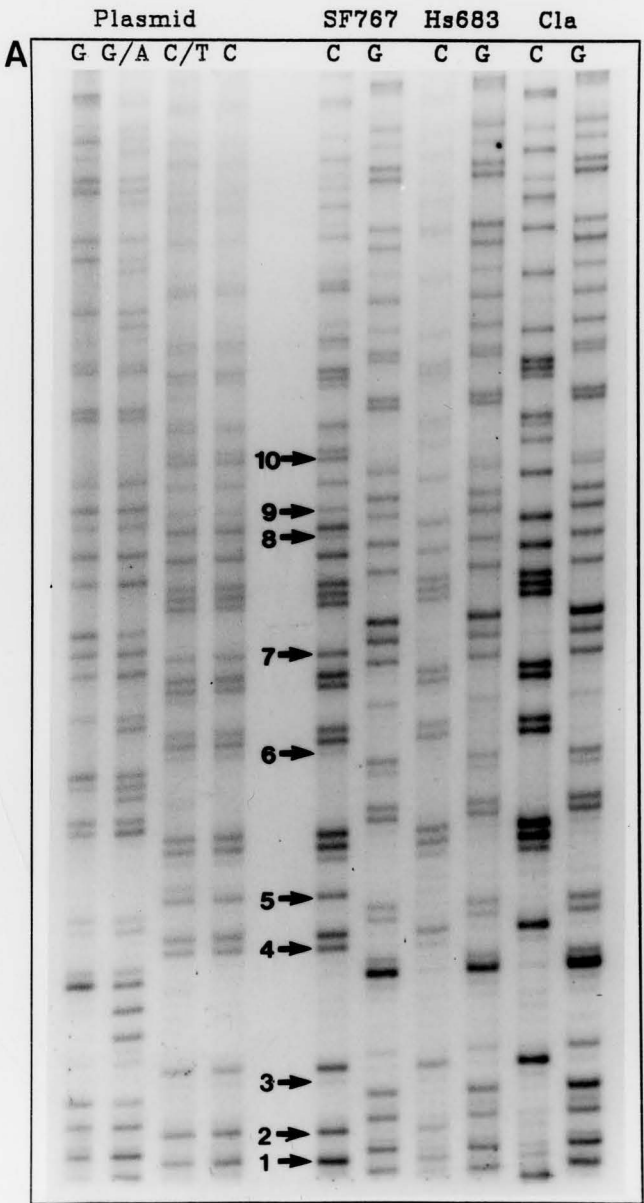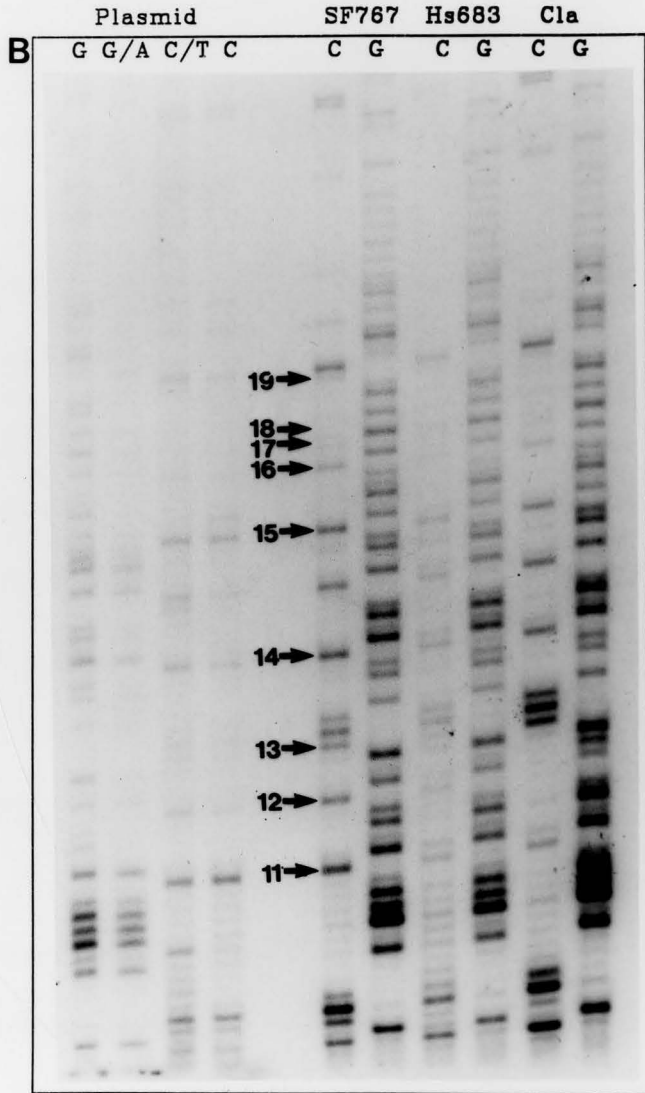
regions in the cloned promoter as well as the positions of guanines in the glioma DNA were also confirmed using genomic sequencing chemicals and LMPCR.

Figure 21A and 21B represent autoradiographs of the promoter methylation analysis in region I and region II, respectively. In both promoter regions, the relative intensity of most bands representing potentially methylated cytosines (numbered arrows) decreases, indicating fewer unmethylated cytosines, from SF767 (MGMT+) to Hs683 (minimally MGMT+) and CLA (MGMT-) amplified DNA. Methylation in these MGMT promoter regions appears uniform within each cell line and correlates in an inverse, rather than direct, fashion with MGMT expression.

The radioactive signal from 25 of the cytosine bands (of a CpG) was quantitated directly from the polyacrylamide gels and compared to a nearby non CpG cytosine to obtain a relative ratio (CpG cytosine/non-CpG cytosine) that reflects the level of methylation at that site. Determining relative values in this manner eliminates value variations potentially due to unequal ligation, amplification, or sample loading. The relative ratios from glioma cell DNAs were compared to plasmid DNA containing the MGMT promoter (100 percent unmethylated at all sites)(region I, fig.22A) or to the SF767 cell line DNA (region II, fig.22B). The results are expressed as percent of MGMT promoter alleles within each cell line that were unmethylated at a particular site, as the signal measured was a result of unmethylated cytosines. Methylation appears uniform within each cell line and graded across the cell lines at eight of ten sites (exceptions are sites 3 and 6) in region I and thirteen of fifteen sites (exceptions are sites 11 and 13) in

Figure 22. Quantitation of promoter methylation in MGMT+ (SF767), minimally MGMT+ (Hs683) and MGMT- (CLA) glioma cell lines. Promoter methylation was quantitated by measuring radioactive signal from the LMPCR products in polyacrylamide gels using a betascope 603 blot analyzer. Methylation values of each CpG were calculated as the ratio of unmethylated cytosine (in a CpG) to neighboring non-CpG cytosine. A, For promoter region I (nt 703-800), methylation from each cell line was expressed relative to the MGMT promoter in plasmid DNA (100 % unmethylated), and was the average of two independent experiments (average difference of 8.6 % between experiments). B, Methylation of CpG's in region II (nt 865-1020) was expressed relative to the SF767 cell line.

A

B
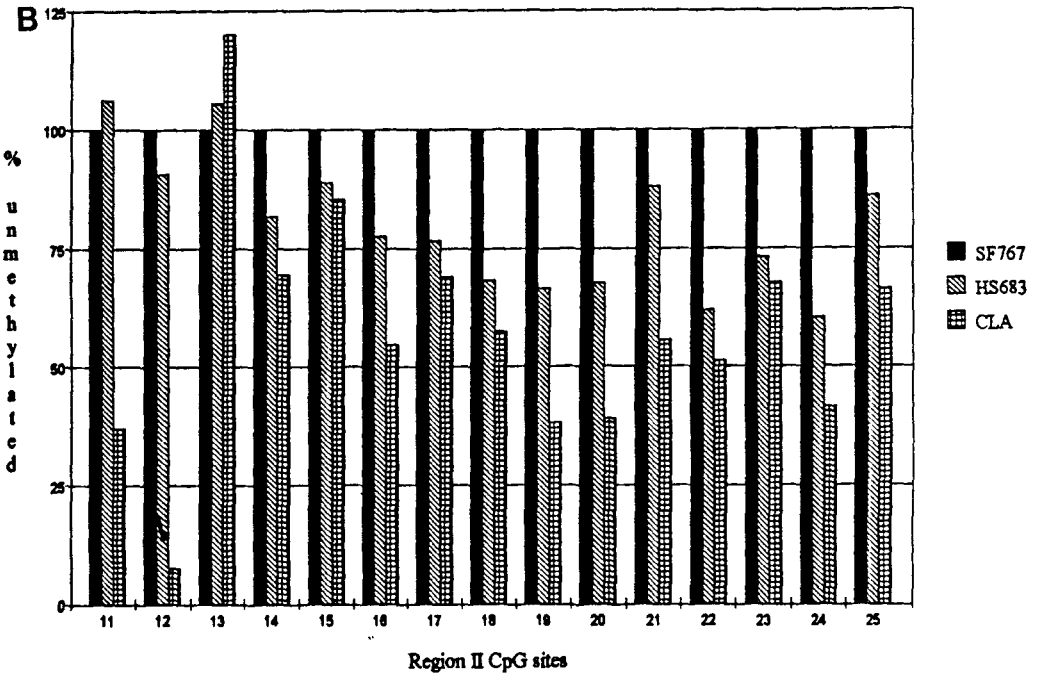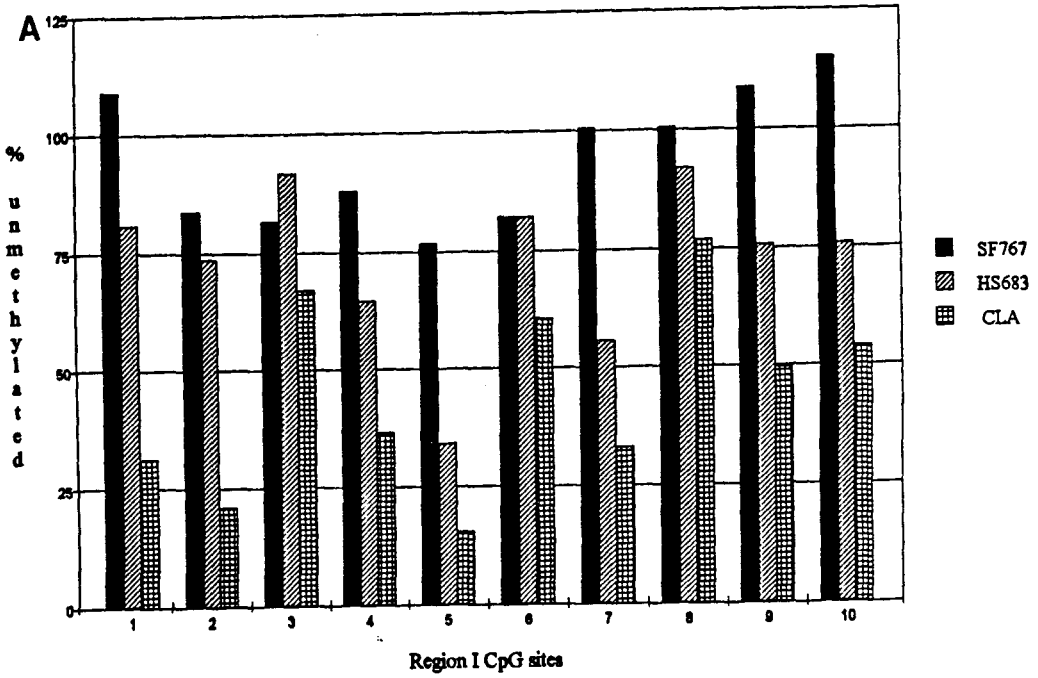
region II, and correlates in an inverse, rather than direct fashion with MGMT expression. Of the four apparently non-graded sites, three (sites 3,6,11) are still methylated to a greater extent in the nonexpressing cell lines compared to expressing cell lines, and one (site 13) is unmethylated in all cell lines. The graded methylation patterns at the majority of sites across the cell lines implies that there is a close, inverse relationship between methylation of the promoter and MGMT expression.

Restriction Enzyme Accessibility to the MGMT Promoter within Nuclei

Because the methylation status of the MGMT promoter was associated with MGMT expression in a graded, inverse fashion, the possibility that methylation may influence the chromatin structure of the MGMT promoter, and thus MGMT transcription, was investigated. The chromatin structure of the MGMT promoter was analyzed by incubation of nuclei from MGMT expressing and nonexpressing cells with AvaII or MspI followed by LMPCR analysis of the DNA.

Figure 23A and 23B show the results of LMPCR analysis of the AvaII accessibility to promoter region I and region II, respectively. The LMPCR products of 127 nt (fig.23A) and 140 nt (fig.23B) correspond exactly to the distance between the labelled LMPCR primers and the AvaII sites at nucleotides 773 and 953 of the MGMT promoter, respectively. The large amount of LMPCR product generated from DNA of the SF767 nuclei digests and virtual absence from equal amounts of Hs683 and CLA DNA indicates that the MGMT promoter is much more accessible

Figure 23. LMPCR analysis of AvaII accessibility to the MGMT promoter within intact nuclei. Nuclei isolated from MGMT+ (SF767), minimally MGMT+ (Hs683) and MGMT- (CLA) glioma cells were incubated with 16 U AvaII for 10 min at 37°C. DNA was isolated from the nuclei and 5 μg was analyzed by LMPCR as described in fig. 21, except only one-fifth of the final reaction was analyzed, and autoradiograph exposures were 2-5 h with intensifying screens. The LMPCR products of 127 nt (arrow in A, promoter region I) and 140 nt (arrow in B, promoter region II) are derived from glioma DNA cut at the AvaII site at promoter nt 776 and nt 956, respectively.

at both AvaII sites, and possibly in a more open chromatin conformation, in nuclei from MGMT+ cells (SF767) relative to minimally MGMT+ (Hs683) and MGMT- cells (CLA). The difference in AvaII accessibility between the three cell lines was reproducible in independent experiments. It is interesting to note that the differentially accessible AvaII site at nucleotide 953 falls in the immediate vicinity of the transcription start site (955) and several potential Sp1 binding sites (Harris,et al., 1991), where chromatin structure might directly influence MGMT transcription.

Figure 24 represents the LMPCR analysis of MspI accessibility to the MGMT promoter in nuclei from MGMT+ (SF767), minimally MGMT+ (Hs683) and MGMT- (CLA) glioma cell lines. Incubation of the nuclei with 20 U MspI (lanes 1- 3) resulted in a much greater cleavage at all promoter MspI sites tested in SF767 nuclei compared to Hs683 and CLA. The difference in MspI accessibility was also reproducible in independent experiments. Together, all the MspI and AvaII sites are accessible in nuclei from SF767 cells and encompass 372 nt (nt 712-1084, including an additional MspI site (nt 884) and an AvaII site (nt 1084)), a region of accessible DNA much larger than would be expected if normal nucleosomal phasing was present (McGhee, et al., 1980). Incubation of nuclei (from the same isolation) with an excess of MspI (lanes 4-9) resulted in measurable cleavage at all sites, indicating that the absence of LMPCR products is likely not a result of contaminants from the nuclei isolation that inhibit MspI digestion or contaminants that inhibit enzymes used in LMPCR. The MspI sites at 712, 722 and 738 (lanes
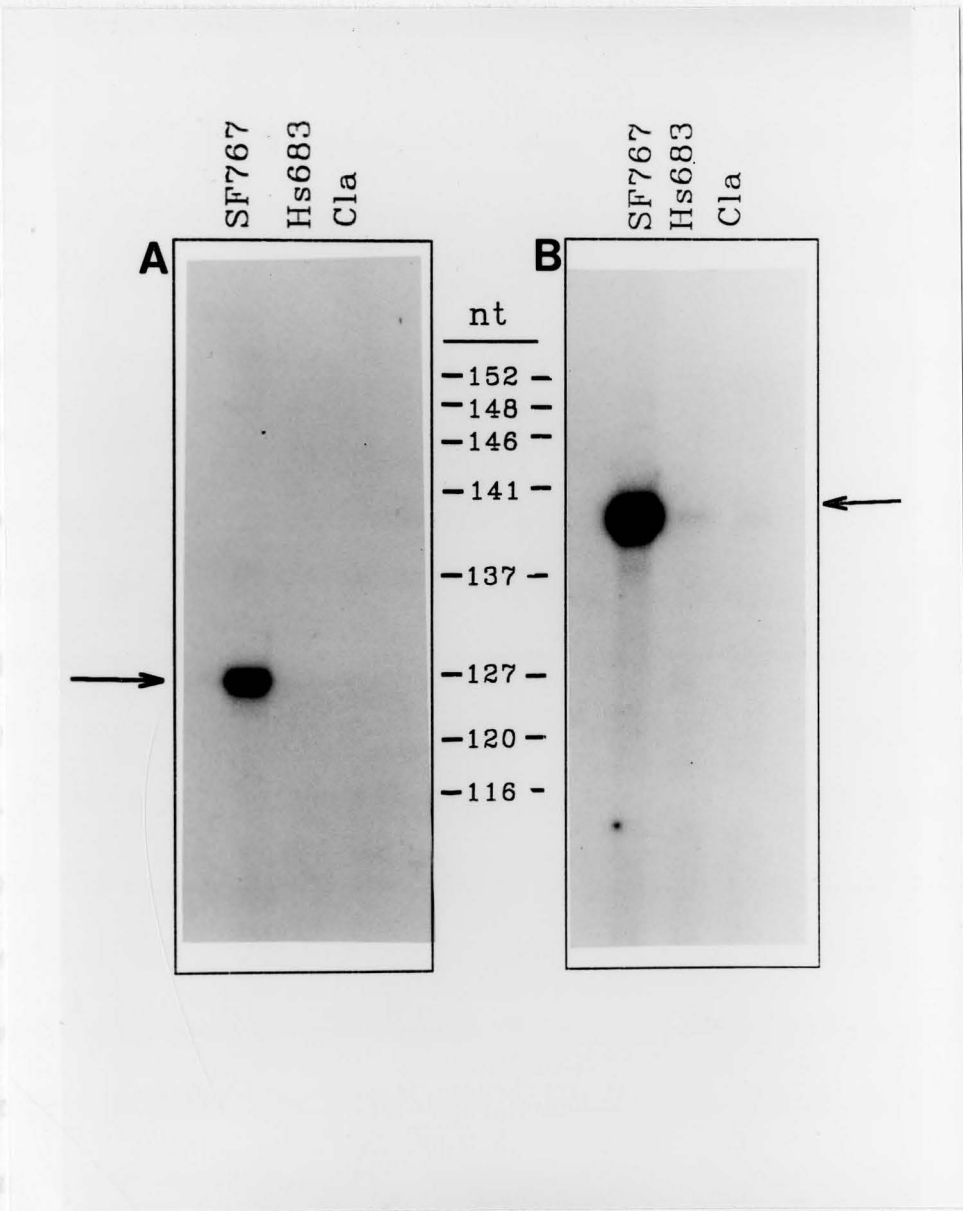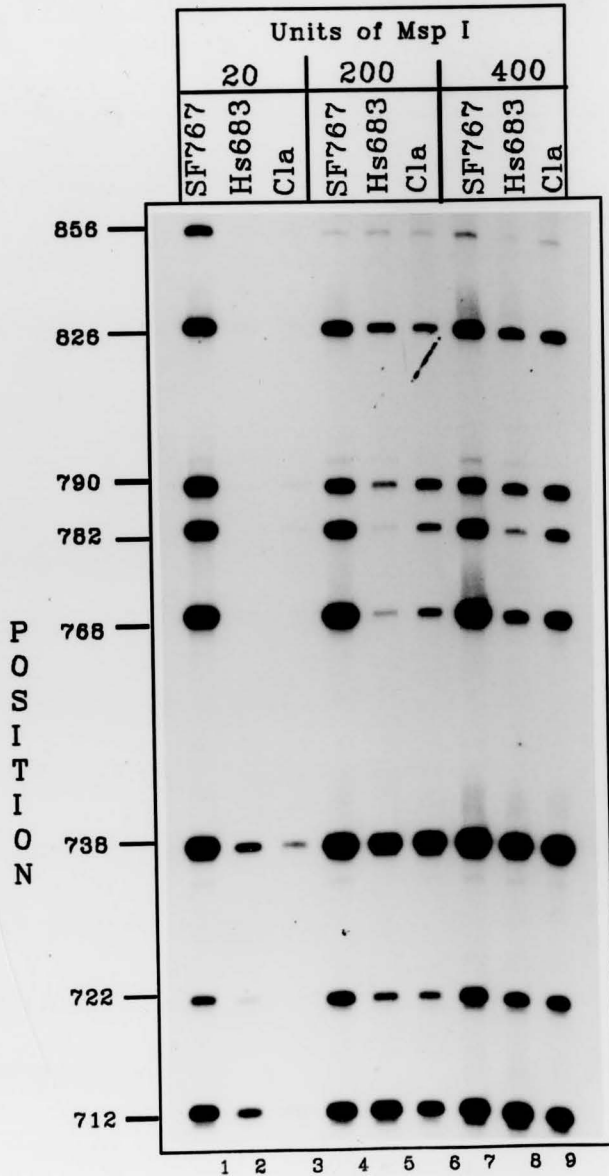
Figure 24. LMPCR analysis of MspI accessibility to the MGMT promoter within intact nuclei. Nuclei from MGMT+ cells (SF767), minimally MGMT+ cells (Hs683) and MGMT- cells (CLA) were incubated with either 20 U (lanes 1-3), 200 U (lanes 4-6) or 400 U (lanes 7-9) MspI, the DNA was isolated and 5 μg was analyzed by LMPCR. One fifth of each reaction was separated on a 6 % denaturing polyacrylamide gel and autoradiographed for 2-5 hr in the presence of intensifying screens.

1-3) appear to have graded degrees of accessibility across the cell lines that parallel, in an inverse fashion, the graded promoter methylation, suggesting that methylation and chromatin structure are closely linked in the MGMT promoter. The restriction enzyme inaccessible promoter in cells with little or no MGMT expression may also be inaccessible to endogenous transcription factors. The chromatin structure of the promoter may therefore determine, in part, the expression state of the MGMT gene.

In Vivo Footprinting of the MGMT Promoter

Specific in vivo protein-DNA interactions in the MGMT promoter were examined by linker-mediated PCR of DNA from glioma cells that were exposed to dimethylsulfate (DMS). As DMS reacts preferentially with the N7 position of guanines, and subsequent piperidine treatment of the isolated DNA results in cleavage of the DNA at DMS-methylated guanines (Mattes, et al., 1986), the LMPCR products generated from this DNA represent the guanines that are accessible to DMS within intact cells. As DMS is not blocked by nucleosomes or MeCPs but DNA is protected from DMS by many transcription factors (Pfeifer, et al., 1989a), the DMS footprinted protein-DNA interactions are qualitatively different from those assessed by MspI accessibility.

Figure 25 shows the in vivo footprint analysis of the MGMT promoter, in MGMT expressing and nonexpressing cells, from nucleotides 700-865 (fig.25A) and 865-1050 (fig.25B), which together include the transcription start site (nt 955) and likely all the basal promoter elements. There are no detectable footprints in any of

Figure 25. <u>In vivo</u> footprint analysis of DNA-protein interactions in the MGMT promoter. Normal human T lymphocytes (lane 1) and two glioma cell lines all with high levels of MGMT expression (lanes 2-3), one minimally MGMT+ glioma cell line (lane 4), and three MGMT- glioma cell lines (lanes 5-7) were incubated with 0.1 % DMS (2 min, 37°C). DNA was isolated from the cells, cleaved with piperidine, and 5 μg analyzed by LMPCR. One half of the reaction was separated on a 6 % denaturing polyacrylamide gel and autoradiographed for 6-18 hrs. A, Analysis of promoter nt 700-865; B, analysis of promoter nt 865-1050. Open rectangles are Sp1 sites unoccupied in all cells tested. Filled rectangle (■) indicates site that is footprinted in lanes 1-3 but not in lanes 4-7. Except for the footprint at nt 845-855 (in A, nt sequence given vertically), which is an as yet uncharacterized site, all footprinted regions are Sp1 consensus sequences (GGGCGG). → in B indicates transcription start site.
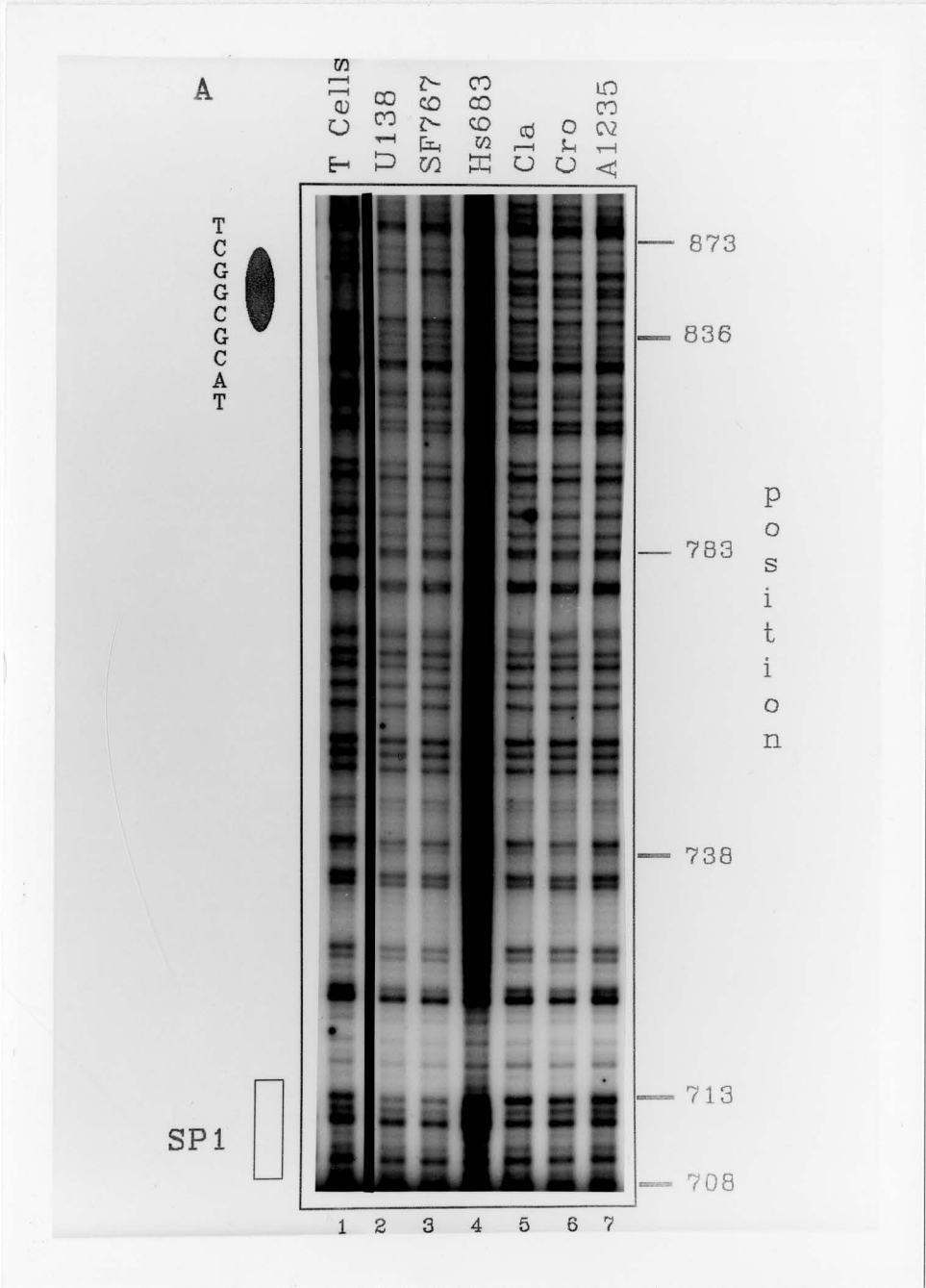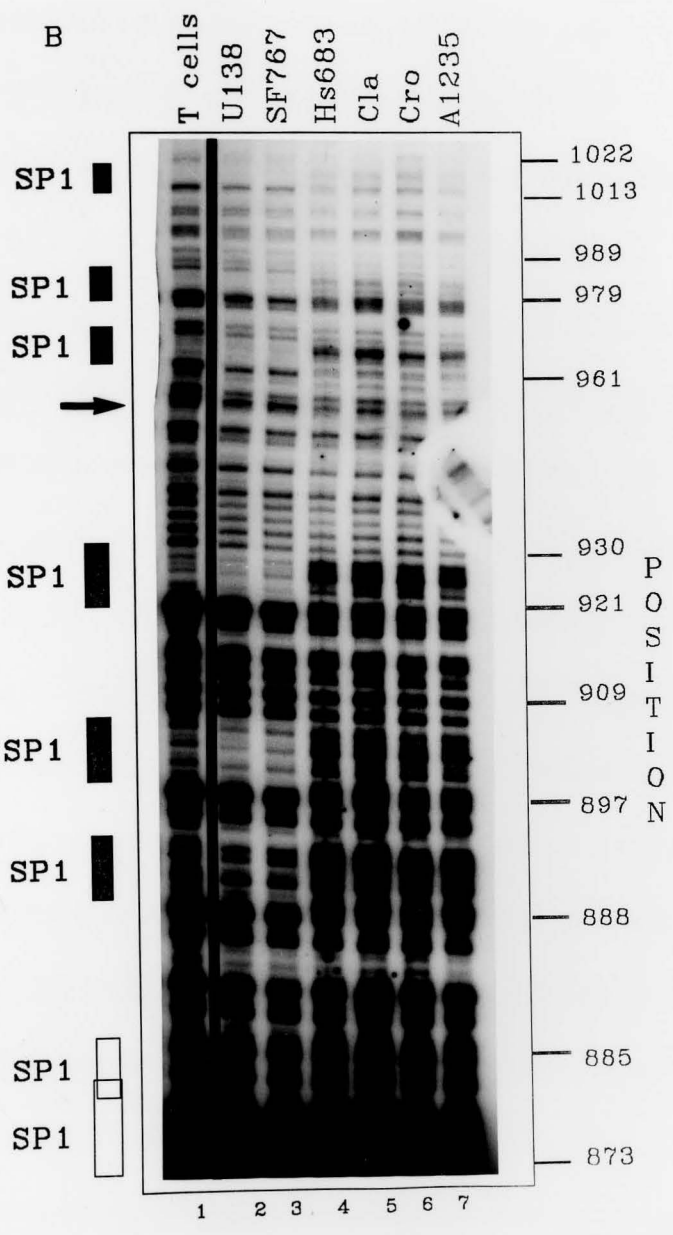
the cells in the region spanning nucleotide 700-845 (fig.25A), which includes a putative Sp1 recognition site (nt 708-713). The guanines at positions 849,851, and 852 however, are protected from DMS in the MGMT+ cells (lanes 1-3) but not in the minimally MGMT+ (lane 4) and MGMT- cells (lanes 5-7), indicating the presence of an MGMT expression-associated DNA-protein interaction. The protected sequence does not match with any known consensus sequence for transcription factor binding and may thus represent a novel protein binding site. In the promoter region spanning nt 865-1050 there are 6 footprints in the MGMT+ cells but none in the minimally MGMT+ or MGMT- cells. All six of these footprints correspond to Sp1 recognition sequences and have 5' hypersensitive guanines followed by at least five protected guanines, indicating that in MGMT+ cells, these sequences are likely protected by Sp1. There are no footprints at the two overlapping Sp1-like sequences spanning nt 875-885, or at the putative CCAAT box (nt 870-876) in any of the cells tested (fig.25B). Normal human T cells, which have high levels of MGMT expression, have an identical pattern of footprints as the MGMT+ glioma cell lines, indicating that these DNA-protein interactions are not confined to cell lines. Sp1 appears to be a common feature of and possibly critical for efficient basal level MGMT transcription. These data strongly suggest that the chromatin structure of the MGMT promoter determines whether Sp1 and other transcription factors can interact with the promoter in vivo, and likely influences the expression state of the MGMT gene.

Figure 26. Gel mobility shift analysis of basal Sp1 binding activity. Ten μg of protein extract from each cell line were incubated with a double-stranded, ^{32}P-labeled oligonucleotide containing Sp1 consensus sequences. The protein-bound and unbound (free) oligonucleotides were separated by electrophoresis through a 4% non-denaturing polyacrylamide gel and then detected by autoradiography. Lane 1: no protein, lane 2; 10 μg HeLa protein extract, lane 3; Hela extract preincubated with excess cold Sp1 oligonucleotide (specific competitor), lane 4; HeLa extract preincubated with excess cold HSE oligonucleotide (nonspecific competitor), lane 5-14; 10 μg protein extract from various cell lines, as noted.

Analysis of Sp1 Binding Activity

To ensure that the absence of Sp1 footprints in MGMT- was not simply due to a lack of Sp1 protein in these cells, Sp1 binding activity was measured in protein extracts from all the glioma cell lines by gel shift analysis. Ten μg of protein extract from each glioma cell line was incubated with a radiolabelled 22 bp DNA probe that contains a single Sp1 binding site. Sp1 binding activity was detected by separating the free probe from Sp1-bound probe (migrates much slower) by electrophoresis through a 6% nondenaturing polyacrylamide gel and autoradiography.

The results of the Sp1 gel shift analysis are shown in fig. 26. Probe alone (no protein extract) migrates near the bottom of the gel (lane 1). Three control reactions were performed to demonstrate that the protein bound to the Sp1 probe is in fact Sp1. Incubation of the probe with HeLa cell extract (positive control for Sp1) resulted in a slower migration of the probe through the gel because it is bound by Sp1 (a "gel shift," lane 2). Complete elimination of the HeLa cell Sp1 gel shift by preincubation of the HeLa extract with an excess of unlabelled Sp1 probe (self competition, lane 3) but no such effect by preincubation with an excess with an unlabelled 22 bp heat shock DNA (nonself competition, lane 4) indicated that the protein responsible for the gel shift was specific for the Sp1 probe and likely was Sp1. Gel shifts identical to that seen with HeLa cell extract were easily detectable in all the glioma cell lines tested (lanes 4-13). All the glioma cell lines therefore have functional Sp1 protein, regardless of MGMT expression. These data clearly

demonstrate that the lack of Sp1 footprints <u>in vivo</u> in MGMT- cells was not due to a lack of Sp1 in these cells and coupled with the data described above, provides compelling evidence for the involvement of methylation and chromatin structure in MGMT expression.

CHAPTER V

DISCUSSION

This dissertation was designed to examine the role of cytosine methylation in the regulation of MGMT gene expression in human glioma cells. The results of these experiments strongly support the hypothesis that methylation and chromatin structure are important components of MGMT gene expression in human glioma cells. The interpretation, significance, and potential limitations of the results leading to this conclusion are discussed below.

From the outset it should be acknowledged that regulation of MGMT gene expression has not been proven to occur at the level of transcription. The protein coding portions of the MGMT gene are spread over greater than 80 kb (Costello, et al., 1994) and the entire gene spans more than 170 kb (Nakatsu, et al., 1993), yet the MGMT mRNA is only 1 kb. The standard explanation for generation of the mature 1 kb MGMT mRNA involves transcription of all 170 kb of the MGMT gene, followed by or concurrent with accurate removal (splicing) of 99.4% of the primary transcript (MGMT hnRNA). In a simplistic sense, the process of generating a mature MGMT mRNA seems inefficient, and if the RNA polymerase transcribing the MGMT gene proceeds at a rate similar to other eukaryotic RNA polymerases, generation of a single MGMT hnRNA or mRNA would take at least 17 minutes. It

is expected that multiple MGMT transcripts initiated in this time period would increase the number of transcripts generated in a given time period. The high stability of the MGMT mRNA (half-life >12 hr (Kroes and Erickson, 1992)), may also counterbalance the presumably slow MGMT transcription and thereby maintain higher levels of MGMT expression. Currently, it is unknown whether the MGMT gene is transcribed in a fashion similar to most other genes. The only evidence suggesting that the MGMT gene transcription may be atypical is based on negative data. As opposed to the measurable transcription rate of the majority of genes, MGMT transcription has not been detected by either nuclear run-on analysis or PCR amplification of reverse transcribed hnRNA (Russell Pieper, personal communication; Kroes and Erickson, 1992). It is unclear if these negative results reflect extremely slow transcription, immediate processing of hnRNA, technical artifacts, or some combination of these phenomena. MGMT transcription, and thus transcriptional regulation of MGMT expression, cannot be directly assessed in either MGMT+ or MGMT- cells.

Several experiments described in this dissertation and elsewhere (Ostrowski, et al., 1991) provide indirect evidence for transcriptional regulation of MGMT expression. The absence of MGMT mRNA in three of the glioma cell lines, despite the presence of a relatively intact MGMT gene suggests that there is a defect in transcription in these cells. Additionally, the similarity in the rank order of glioma cell lines according to MGMT mRNA levels and MGMT activity indicates that post-transcriptional regulatory events play little, if any, role in determining the level of

MGMT expression (Costello, et al., 1994). The consistently different methylation status of the MGMT gene, in both the body and promoter, in MGMT+ cells relative to MGMT- cells is also indirect evidence for transcriptional regulation of MGMT expression, since transcriptional regulation of gene expression is thought to be influenced by cytosine methylation. Additionally, the transcriptionally inactive chromatin and absence of bound transcription factors in the promoter of MGMT-glioma cells strongly supports the idea that the MGMT gene is not transcribed in these cells. Thus, MGMT expression is presumed, but not proven, to be regulated at the transcriptional level.

Recently, studies of MGMT gene regulation were advanced by the isolation and characterization of the human MGMT promoter (Harris et al., 1991; Nakatsu, et al., 1993). The promoter for the MGMT gene has CpG island characteristics and lacks TATA and CAAT boxes, similar to promoters for many housekeeping genes. MGMT promoter elements required for basal promoter activity (41% of full activity) are located between nucleotides 886 and 1157 of the 1157 bp promoter, and contain the transcription start site (nt 955) and six putative Sp1 recognition sites (Harris, et al., 1991). In human tumor cells transfected with chloramphenicol acetyltransferase (CAT) constructs containing the MGMT promoter, both MGMT expressing and nonexpressing cell lines contained the factors necessary for transcription initiation from the MGMT promoter (Harris, et al., 1992), implying that differences between the transfected and endogenous MGMT promoters such as methylation status and/or chromatin structure may be important. Changes in the

body of the gene may also however, be relevant in MGMT expression as several studies have shown that the cytosine methylation patterns in MGMT exons are altered in nonexpressing tumors cells relative to expressing cells (Pieper, et al., 1991; Wang, et al., 1992; Cairns-Smith and Karran, 1992). Analysis of the role of methylation and chromatin structure in transcriptional regulation of MGMT expression must therefore consider both the body and promoter of the MGMT gene.

In this dissertation, the graded methylation and correlations between methylation and MGMT expression suggest that in both the promoter and body of the MGMT gene, methylation may influence the level of MGMT expression. Several studies have attempted to analyze methylation in the MGMT promoter in cells with different levels of MGMT expression. One study examining the methylation status of HpaII sites in the MGMT promoter failed to show a clear association between promoter methylation and MGMT gene expression, although the close proximity of the fourteen HpaII sites in the promoter region precluded resolution, by Southern blot, of the methylation status of these sites (Von Wronski, et al., 1992). Analysis of the methylation status of a single site 70 bp upstream of the transcription start site demonstrated a negative, but not absolute association between methylation and MGMT expression, although MGMT promoter activity studies demonstrated that deletion of 76 bp 5' from, and including this site did not alter promoter strength (Harris, et al., 1992). Another study examining methylation at HpaII sites in the MGMT promoter concluded that the promoter was methylated to a greater extent in MGMT expressing cell lines compared to nonexpressors, although this study also

used Southern blot analysis and thus did not resolve the relevant HpaII fragments (Nakatsu, et al., 1993). It is unclear from these studies whether the methylation status of the MGMT promoter is associated with MGMT expression. Because of the single nucleotide resolution of polyacrylamide gels and the very sensitive nature of linker-mediated PCR, the analysis of methylation presented here overcame the inadequate resolution noted in previous studies. The graded methylation across the cell lines at 21 of 25 CpGs tested in the MGMT promoter indicates that the methylation status is uniform within each cell line and that there is a close, inverse association between overall promoter methylation and MGMT expression (Costello, et al., 1994). The overall promoter methylation, expressed as the average "percent unmethylated" of all CpG sites, was similar in region I and II. For region I, the level was 93 % in SF767, 73 % in Hs683 and 45 % in CLA. In region II, the promoter was 100% unmethylated in SF767, 74 % unmethylated in Hs683, 58% unmethylated in CLA. These measurements reflect the average methylation within a population of MGMT gene alleles. The region II values were expressed relative to SF767 because the intensity of cytosine bands corresponding to the more 3' cytosines in the plasmid DNA was somewhat diminished, and thus difficult to accurately quantitate. As the three 5' most cytosines of CpGs in the plasmid region II were similar in degree of unmethylation to those in the SF767 cell line however, and as most sites in SF767 region I were completely unmethylated (given the 8.6 % average experimental error), it seems reasonable to assume region II cytosines in SF767 DNA are nearly 100 % unmethylated. It should be noted that in cells that

do not express the MGMT gene, methylation of the promoter, while less than in expressing cells, was still only 50 %, rather than 100 % methylation noted in X-linked inactive genes (Pfeifer, et al., 1990). These data suggest that while inactive genes on the X chromosome may be 100 % methylated, complete methylation may not be necessary for processes involved in promoter inactivity. Using linker-mediated PCR with hydrazine-reacted DNA, methylated cytosines are identified by the absence of hydrazine reactivity and hence the disappearance of cytosine bands. Point mutations that change a cytosine to any other base could also account for the disappearance of cytosine bands and would be erroneously interpreted as methylated cytosines. This possibility was completely eliminated in the MGMT promoter studies by the demonstration that the nucleotide present on the complementary DNA strand, and base paired with the base in question, was always guanine (unpublished data). These results suggest that an unmethylated promoter is required for efficient MGMT expression and that the level of promoter methylation is closely associated with the level of MGMT gene expression.

In contrast to promoter methylation, the relative contribution of methylation in the body of genes to the control of gene expression is less understood. However, the conserved methylation status in the body of many genes implies that these sites are important, at least in genes in which the body methylation correlates with gene expression. While methylation in the MGMT promoter correlates inversely with MGMT expression, the uniform methylation over the body of the MGMT gene and graded methylation at the intron 1 EcoRI site correlated directly with MGMT

expression, suggesting that methylation in the body may also influence MGMT expression in a graded fashion. Although the intron 1 EcoR I site analysis is consistent with the methylation analysis at HpaII sites in the same region, the possibility that the inhibition of EcoRI digestion at the internal EcoRI site in MGMT intron 1 is a result of a point mutation or small deletion that destroys the EcoRI site cannot be excluded. This possibility is unlikely, however, as the probability of this specific mutation occurring independently in all 10 of the unrelated glioma cell lines tested is exceptionally low. Two limitations of the HpaII site methylation analysis should also be acknowledged. First, CpGs within HpaII sites (CCGG) account for only 1/16 of all CpGs in a given region of DNA. The possibility that the non-HpaII CpGs are not differentially methylated in MGMT+ cells relative to MGMT- cells exists but is highly unlikely, as HhaI sites (GCGC)(unpublished data) and the EcoRI site in the intron 1 region were also differentially methylated. Second, for technical reasons, the analysis of HpaII fragments by Southern blot is limited to DNA fragments greater than 300 bp. If <300 bp clusters of HpaII sites exist within the analyzed regions of the body of the MGMT gene, many of these sites, if unmethylated, would not be included in the methylation analysis. On the other hand, the presence of large probe-recognized fragments in the DNA of the MGMT+ cells indicates that all HpaII sites within those fragments are methylated, including any potentially clustered HpaII sites. Because the body of the MGMT gene is methylated in MGMT expressing normal human brain cells and T lymphocytes, the close association between

hypomethylation of the MGMT gene and lack of MGMT expression in glioma cell lines suggests that maintenance of appropriate levels of methylation in the body of the gene may be important for MGMT expression. The picture that emerges from the methylation data is that for efficient MGMT transcription, the MGMT promoter should be unmethylated and the body of the gene, heavily methylated. Conversely, increased methylation in the promoter and decreased body methylation are associated with decreased MGMT expression. The graded nature of this relationship suggests that methylation and MGMT gene expression are closely, and possibly causally, linked.

Several recent studies suggest that in general, methylation and gene expression are causally linked, possibly in both a direct and inverse fashion. A study of mutant mice deficient in cytosine methyltransferase (MTase) provided examples in which preventing normal methylation inhibited gene expression in some genes and, in other genes, caused activation of normally silent genes (Li, et al., 1993). For example, in normal mice, the methylated (in the promoter), paternal allele of the H19 gene is transcriptionally inactive (Bartolomei et al., 1991), but in MTase deficient mice, this H19 allele is unmethylated and expressed. In contrast, 2 kb of an intron of the transcriptionally active maternal Igf-2r gene is methylated in normal mice (Stoger, et al., 1993), but in MTase deficient mice, this intron region is unmethylated and the maternal Igf-2r gene is not expressed (Li, et al., 1993). These studies indicate that, at least for the H19 and Igf-2r genes, methylation and gene expression are causally linked. These studies also demonstrate that

methylation can have completely opposite effects on gene expression, apparently depending on the region of the gene involved. Methylation in the promoter of genes inhibits gene expression, whereas methylation in introns may facilitate gene expression. In other genes, a cause and effect relationship between methylation and gene expression has been tested with the methylation inhibitor 5-azacytidine. For example, the 5-azacytidine-induced reactivation of the inactive, methylated HPRT gene occurs only following significant demethylation of the 5' CpG island (Sasaki, et al., 1992). Several studies have attempted to establish a causal relationship between MGMT gene methylation and MGMT expression by treating MGMT+ and MGMT- tumor cells with 5-azacytidine. Treatment of a MGMT+ tumor cell line with 5-azacytidine decreased methylation in the body of the gene to a level comparable to a MGMT- cell line and significantly decreased MGMT mRNA levels (Pieper, et al., 1991). Additionally, two reports (Mitani, et al., 1989; Ishida and Takashi, 1988) have demonstrated that treatment of MGMT- human cell lines with 5-azacytidine did not restore MGMT expression. These studies suggest that the absence of MGMT expression is not solely due to a reversible promoter hypermethylation, as has been demonstrated in the X-linked HPRT gene, but may also involve methylation in the body. In contrast, an induction in MGMT expression was observed following exposure to 5-azacytidine in two additional MGMT nonexpressing cell lines, although these studies did not assess methylation in the body of the gene (von Wronski and Brent, 1994). In sum, these experiments suggest that 5-azacytidine-induced demethylation can increase or decrease MGMT

expression, depending on the pre-treatment methylation status and the extent of induced demethylation of the MGMT gene. The interpretation of these results is complicated by the fact that 5-azacytidine, in addition to altering gene expression through demethylation of DNA, can also lead to chromosomal damage and instability that also potentially alter gene expression. Other more specific methods of inhibiting methylation, such as inhibition of MTase expression through MTase antisense oligonucleotides, might be more appropriate in addressing a potentially causal relationship between methylation and MGMT gene expression. Recent in vitro studies indicate that methylation of an MGMT promoter-CAT construct with bacterial CpG methylases reduced CAT gene expression, although it should be noted that both the MGMT promoter and body of the CAT gene were methylated in these studies (Harris, et al., 1994). This in vitro study of methylated and unmethylated MGMT promoter-CAT constructs and the 5-azacytidine studies are inherently incapable of addressing the relative contribution of methylation in the body and methylation in the promoter to MGMT gene expression. Although the results of most of these MGMT gene methylation studies, aside from their limitations, seem to indicate that there is a cause/effect relationship between methylation and MGMT gene expression, the events leading to the changes in methylation have not been identified. Conclusive testing of the potential cause/effect relationship between methylation and MGMT expression relies first on the identification of the process(es) leading to aberrant MGMT gene methylation. The expectation is that methylation changes will precede, or occur concurrent with, MGMT gene

inactivation, if altered methylation actually causes suppression of MGMT transcription.

Although a temporal relationship between changes in methylation and MGMT expression has not been investigated in gliomas or glioma cell lines, deficits in maintenance of normal methylation during tumorigenesis or tumor progression could be one mechanism by which the MGMT promoter (CpG island) hypermethylation and body hypomethylation, and possibly loss of expression, of the MGMT gene occurs. Clearly, this issue needs to be addressed in human glioma samples, as the methylation status of the DNA in the glioma cells used in this dissertation may be influenced by cell culture conditions as well as tumorigenesis. Examining potential MGMT methylation changes in the well defined stages of glioma progression could yield information about the temporal relationship between alterations in methylation and MGMT expression and might eventually be useful in identifying MGMT deficient, BCNU-sensitive gliomas.

One potential mechanism mediating the relationship between methylation and MGMT expression involves alterations in chromatin structure. Analysis of AvaII and MspI accessibility demonstrated that chromatin structure and methylation in the MGMT promoter are closely linked. The MGMT promoter was much more accessible to RE in cells with a completely unmethylated promoter compared to cells with promoters that are, on average, only 74 % unmethylated (Hs683) or 50 % unmethylated (CLA). The close association between methylation and chromatin structure was demonstrated by the graded fashion in which accessibility of at least

3 MspI sites correlated with methylation. The accessibility of all sites tested over 372 nt (712-1084) of the basal promoter in the nuclei from SF767 cells (fig. 27) indicates that this may be a nucleosome-free region, as DNA wrapped in nucleosomes would be protected from restriction enzyme digestion (Tazi and Bird, 1990). Conversely, the inaccessibility of the promoter at all sites over 372 nt in the CLA cells (fig. 27) suggests that nucleosomes and/or methylated DNA binding proteins are present and possibly involved in setting the transcriptionally inactive state of the promoter in these cells. The results of the chromatin structure analysis of the MGMT promoter are consistent with the idea that chromatin structure is an important part of MGMT transcription. Similar to the promoter, methylation and accessibility in the body of the gene were also closely associated in the expected fashion. The unmethylated gene in nuclei from three MGMT- cell lines was more accessible at three MspI sites, relative to the methylated gene in nuclei from two MGMT+ cell lines. The only MspI accessible sites in MGMT+ nuclei corresponded exactly to the few unmethylated sites in this region within MGMT+ nuclei. These data demonstrate that methylation and chromatin structure in the body of the MGMT gene are intimately associated and suggest that MeCPs may be mediating this association. These differences in accessibility can also be detected with AluI, which cleaves DNA at non-CpG containing sites. This suggests that either the AluI sites are sufficiently close to a CpG and are blocked by a nearby MeCP, or that some other component of the chromatin structure, such as histones, is present in MGMT+ cells but is absent or displaced from the MGMT gene in

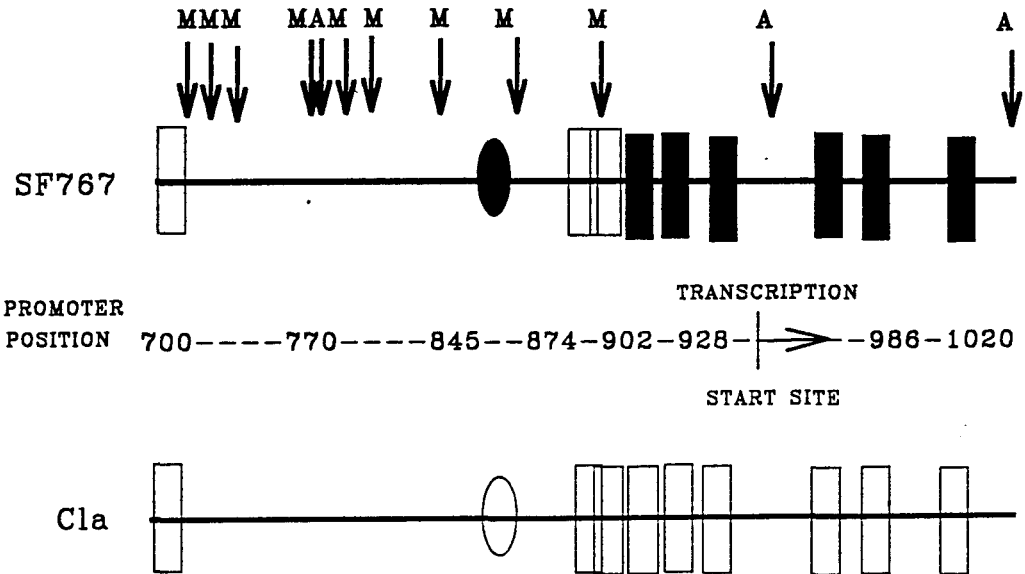Figure 27. Summary diagram of in vivo footprints in, and restriction enzyme accessibility to the MGMT promoter in MGMT+ (SF767) and MGMT- (CLA) glioma cells. Filled rectangle (▮) indicates footprinted Sp1 site, open rectangle indicates unoccupied Sp1 sites. Filled oval denotes footprinted site of undefined nature and the open oval is the same site unoccupied. ↓ indicates restriction enzyme accessible site; M, MspI, A, AvaII.

MGMT- cells. The latter possibility in unlikely though, as DNAseI, which readily cleaves histone-free DNA but not DNA within nucleosomes, does not cleave the MGMT gene in any of the nuclei. The association between decreased MspI/AluI accessibility and active transcription in these cells however, is surprising, as transcription is generally associated with a more open, accessible chromatin conformation. A mechanism by which the more "closed" chromatin conformation in the body of genes might facilitate transcription has not been defined. The identical nature of the closed conformation in all MGMT+ cell lines tested, and the absence from all three MGMT- cell lines however, suggests that there is some functional importance associated with the chromatin structure in the body of the MGMT gene. Additionally, the fact that more than 30 unrelated cell lines are differentially methylated in the body of the gene (Pieper, et al., 1991; Wang, et al., 1992) in the same manner described here, suggests that disruption of the methylation-related chromatin structure may be a common event contributing to loss of MGMT expression. The results of the chromatin structure analysis of the body and promoter are consistent with the idea that chromatin structure is an important part of MGMT transcription. The chromatin structure in the promoter of the gene may influence MGMT expression by allowing or excluding transcription factor access to relevant promoter sequences.

The methylation-related chromatin structure in the MGMT promoter is closely associated with in vivo transcription factor occupancy. In addition to the general association between accessible chromatin and transcription factor occupancy,

several footprints that are positioned closely to restriction enzyme accessible sites reveal specific examples of this association. For example, the protected guanine at nt 852 was only 3 nt away from an accessible MspI site and the presumably Sp1 protected guanine at 965 was only 7 nt away from an accessible AvaII site (fig. 27). Sp1 is the only detectable factor interacting with the minimal promoter elements (nt 886-955), indicating that Sp1 may be sufficient, in conjunction with protein-protein interactions, for basal level MGMT transcription. DNA-protein interactions were not detected on the opposite strand (nontranscribed strand) in the basal promoter (Russell Pieper, personal communication), which includes an imperfect Sp1 consensus sequence. The possibility that other transcription factor-promoter interactions relevant to basal expression occur at non-guanine residues cannot be excluded, although there are no other known transcription factor sites within the minimal promoter elements. In fact, no other DNA-protein interactions can be detected at guanines on either strand of the entire 1157 bp MGMT promoter (Russell Pieper, personal communication). It is not unexpected that there are no discernable footprints in the promoter of Hs683 cells, which express very low levels of MGMT, because the Hs683 promoter is inaccessible, relative to SF767. It is probable that Sp1 does nevertheless interact with the Hs683 promoter, but only too infrequently, as dictated by accessibility, to detect with DMS footprinting. The footprint at nt 845-852, while not within the proposed minimal promoter, is associated with MGMT transcription, as it is present in the expressing cell lines and T cells and absent from all three non-expressing glioma cell lines. This

presumably expression-related protein-promoter interaction is of note for three reasons. First, previous in vitro studies showed that addition of promoter nt 807-883, which includes the footprinted site, to the minimal promoter did not increase MGMT promoter-driven CAT expression (Harris, et al., 1991), suggesting that these sequences are inconsequential to MGMT promoter activity. As the effect of deletion of only these sequences from the full promoter was not tested, it remains possible that these sequences are important for promoter activity, but that their contribution is influenced by interactions with other promoter regions. Such interactions have been noted for synergistically functioning, yet distant Sp1 sites (Su, et al., 1991). Second, the DNA-protein interaction is novel as it involves nucleotides that do not correspond to any known transcription factor binding site. Although the footprinted sequence has minimal homology to an Sp1 site (7 of 10 nt), and several sequences that deviate from the canonical Sp1 site can bind Sp1 (Brown, et al., 1992), the pattern of the protected sequence is distinct from the canonical Sp1 protection, as seen at the other 6 footprints in the MGMT promoter. Third, in contrast to the single strand Sp1 footprints, the novel footprint is present on both DNA strands (Russell Pieper, personal communication). The data indicate that the protein interacting with promoter nucleotides 845-852 is not Sp1 and suggest that it may be a novel transcription factor, or a known transcription factor interacting with a novel binding site. Three additional sequences previously described as potential Sp1 sites based on sequence analysis (12), are not protected in any of the cells tested, suggesting that these sites do not function as Sp1 sites in vivo. Since these

sites have only marginal similarity to Sp1 sites (7 of 10 nt) it is possible that these sequences are incapable of binding Sp1. These in vivo results further support the idea that methylation-related differences in chromatin structure of the MGMT promoter determine whether Sp1, and possibly other factors necessary for MGMT expression, can interact with the MGMT promoter.

Given some latitude for speculation, the correlative data presented in this dissertation can be incorporated into a model of MGMT transcription. The MGMT promoter, like other CpG island-containing promoters, must be maintained in the unmethylated state for efficient transcription initiation. Maintenance of the unmethylated state could occur through binding of the 6 Sp1 molecules per promoter. If these sites are occupied by Sp1 shortly after DNA replication, Sp1 may block access to the basal promoter by cytosine MTase, and thus, maintain the unmethylated state. This part of the model is compatible with the fact that several CpGs 5' to the Sp1 sites are not protected by proteins, and are also methylated in MGMT+ and MGMT- cells (Qian, et al., 1994). Sp1 may therefore serve two purposes in MGMT expression. Sp1 may help maintain the unmethylated state of the basal promoter and likely also facilitates the assembly of the transcription complex. There is no obvious spatial pattern to the occupied Sp1 sites, although all 6 Sp1 molecules contact guanines which are likely on the same face of the double helix. This arrangement may be relevant to the known transcription activating function of Sp1. Sp1 is not bound to the methylated basal promoter possibly because MeCPs (or other chromatin structure proteins) bind these sites before or

more efficiently than Sp1. Alternatively, the methylated promoter, similar to other methylated sequences, may be replicated at a later stage when much of the Sp1 has been committed to other gene regulatory sequences. According to these suppositions, the affinity of Sp1 for each site in the unmethylated MGMT promoter would be much higher than the affinity of MeCPs (if MeCPs bind unmethylated DNA at all). As methylation increases to 25% (as in Hs683) the relative affinities may approximate each other and transcription may be initiated much less frequently, only when Sp1 can occupy a critical number of sites. These less frequent Sp1-promoter interactions would probably not be detectable by standard DMS footprinting. Between 25% and 50% basal promoter methylation, the promoter affinity shifts in favor of MeCP, resulting in exclusion of Sp1 and suppression of transcription initiation. Analysis of the Sp1 footprints at all stages of the cell cycle in synchronized cells might provide indirect evidence for this scheme. Any model of MGMT transcription must also incorporate the relative efficiency of transcription elongation, as the methylation-related chromatin structure in the body of the MGMT gene is different in MGMT+ compared to minimally MGMT+ cells (or MGMT+ cells treated with 5-azacytidine) and might facilitate or impede the transcription elongation complex. Since the changes in chromatin structure in the body of the gene are clearly related to methylation, MeCPs are possible candidates for mediating this relationship. Binding of MeCPs across the body of the gene could establish an efficiently transcribed DNA conformation by preventing binding of other proteins, such as histone H1, that are known to contribute to

transcriptionally inactive chromatin. Binding of MeCPs in the body of the gene would likely be weak because the CpG density is low relative to CpG islands. Thus, the MeCPs in the body of the gene could facilitate transcription by preventing the binding of histone H1-like proteins, while being easily displaced by RNA polymerase II during elongation. Alternatively, MeCPs may be bound to the body of the gene at specific sites to properly position the gene sequences within a transcriptionally active region of the nucleus. MeCPs might accomplish this through maintaining appropriate DNA attachment to the nuclear matrix, or orienting the exons near each other on adjacent chromatin loops to facilitate accurate and immediate splicing events. The conserved chromatin structure in the MGMT+ cells and disrupted chromatin structure at the same, specific sites in each of the minimally MGMT+ and MGMT- cells supports the idea that chromatin structure in the body of the gene is relevant to transcription.

The findings that the promoter in the MGMT expressing cells is unmethylated, accessible and occupied by transcription factors, suggest that MGMT expression is dependent on these factors. Since MGMT expression is a major determinant in sensitivity versus resistance to BCNU (Day, et al., 1980), methylation, chromatin structure and transcription factor occupancy of the promoter could be viewed as critical elements of the molecular mechanisms that determine the chemosensitivity of these glioma cells. In support of this, MGMT expressing SF767 cells are much more resistant to BCNU compared to the non-expressing CLA cells (Sariban, et al., 1987; Mitchell, et al., 1992). The fact that gliomas of oligodendrocytic origin are

especially sensitive to nitrosourea-based chemotherapy (MacDonald, et al., 1990)

implies that MGMT gene inactivation may be a frequent event, possibly mediated

by changes in methylation, chromatin structure and transcription factor access, in

these tumors. Consistent with this speculation, a recent study has demonstrated

that oligodendroglioma tumor samples are in fact very low in MGMT activity (Nutt,

et al., 1994). Furthermore, as inhibition of MGMT in tumor cells is a current goal

of BCNU-based chemotherapy, the "open" chromatin conformation of the promoter

in MGMT-expressing (and hence BCNU resistant) glioma cells may be a good

target for inhibition of MGMT expression, possibly through triple helix formation.

To be of therapeutic value, inhibition of MGMT through triple helix forming

oligonucleotides should be tumor specific. Since the MGMT promoter in normal

and BCNU-resistant tumor cells is likely identical, the tumor specificity of MGMT

inhibitors relies on tumor-specific delivery of these molecules. Direct delivery of

BCNU to glioma cells has been accomplished by implanting BCNU-saturated

anhydrous wafers directly into the tumor area following surgery. The results from

phase I clinical trials of this therapy in glioma patients indicates that the BCNU-

induced systemic side-effects are significantly reduced or absent (Brem, et al., 1991).

Incorporation of potential inhibitors of MGMT expression, such as triple helix

forming oligonucleotides, into the slow-release wafers might then also provide

tumor-specificity of MGMT inhibition and hence sensitization to BCNU. Obviously,

testing triple helix-forming oligonucleotides in MGMT inhibition is the next

preliminary step towards achieving these therapeutic goals.

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VITA

The author, Joseph F. Costello, was born in Oak Park, Illinois on November 30, 1965 to Joseph and Virginia Costello.

In September, 1983, Mr. Costello entered Marquette University in Milwaukee, Wisconsin, and received a Bachelor of Arts in Biology in 1988. Mr. Costello began his graduate studies in the Neuroscience Program, Loyola University Chicago, in August, 1989. He joined the laboratory of Russell O. Pieper, Ph.D., in November, 1990 where he studied the regulation of the gene, O-6-methylguanine-DNA methyltransferase, which confers resistance to chemotherapeutic agents used to treat primary brain cancer. Mr. Costello was a teaching assistant for Medical Neuroscience (1990-1992) and for the Molecular Biology Techniques laboratory course (1993). His participation in Graduate School activities also included positions as Neuroscience Representative, Vice President of the Student Council and Graduate School Representative on the Committee for Student Life. In 1993, Mr. Costello was awarded a fellowship by the Arthur J. Schmitt Foundation.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

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