A Comparison of Human \(O^6\)-Methylguanine-DNA Methyltransferase Promoter Activity in Mer\(^+\) and Mer\(^-\) Cells

Linda C. Harris, Philip M. Potter, Joanna S. Remack, and Thomas P. Brent

Department of Biochemical and Clinical Pharmacology, St. Jude Children's Research Hospital, Memphis, Tennessee 38101-0318

Abstract

The activity of the human \(O^6\)-methylguanine-DNA methyltransferase (MGMT) gene promoter was determined in eight human cell lines by measuring chloramphenicol acetyltransferase activity in a reporter gene system. MGMT promoter activities in cells that do not express MGMT (Mer\(^-\)) fell within the range of activities seen in cells that do express MGMT (Mer\(^+\)). The promoter region contains 11 potential binding sites for the transcription factor Sp1, but no correlation was seen between cellular Sp1 protein and MGMT promoter chloramphenicol acetyltransferase activity. Because Mer\(^-\) cells are not deficient in the factors needed for transcription of MGMT, we suggest that at least two mechanisms regulate MGMT expression. One suppresses MGMT mRNA and protein in Mer\(^-\) cells, and another regulates the levels of constitutive expression in Mer\(^+\) cells. Sp1 is not a limiting factor in MGMT expression.

Introduction

The ubiquitous enzyme MGMT\(^3\) (EC 2.1.1.63) repairs \(O^6\)-alkylguanine and \(O^4\)-alkylthymine in DNA\(^1\) and can similarly repair the precursors to interstrand cross-links produced by aneuplastic chloroethylating agents such as 1,3-bis-(2-chloroethyl)-1-nitrosourea\(^2\). MGMT is constitutively expressed in normal cells and 80% of tumor cells (Mer\(^+\) phenotype) at levels characteristic of each cell type; the protein is undetectable in 20% of tumor cell lines (Mer\(^-\) phenotype)\(^1\). Investigation of the manner in which expression of this DNA repair enzyme is regulated may explain the mechanism of MGMT suppression in Mer\(^-\) cells and may eventually enable clinicians to modulate the levels of MGMT in drug-resistant tumors. Having reported the location and general characteristics of the MGMT gene promoter\(^3\), we now investigate promoter activity and transcription factor interactions. In the present study, we quantitate and compare the MGMT promoter activities in a variety of Mer\(^+\) and Mer\(^-\) human cell lines. We also examine the role of the transcription factor Sp1 in the regulation of MGMT gene expression by measuring the relative amounts of this protein in the cells tested.

Materials and Methods

Cell Lines. HeLa CCL2 and HeLa S3 (CCL2.2), obtained from the ATCC (Rockville, MD), were grown in Eagle’s MEM supplemented with 10% fetal calf serum. CEM-CCRF and Molt-4 T-lymphoblast cells were the gift of A. Fridland, SJCRH, Memphis, TN. CEM-CCRF cells were grown in Eagle’s MEM and Molt-4 cells in RPMI-1640, each containing 10% newborn calf serum. The \(\beta\)-lymphoblast cell lines Raji (ATCC) and TK6 (the gift of M. Fox, Paterson Institute for Cancer Research, Manchester, England) and the rhabdomyosarcoma cell lines Rh18 and Rh28 (the gift of P. Houghton, SJCRH, Memphis, TN) were grown in RPMI-1640 containing 10% fetal calf serum. All cells were grown at 37°C in 95% air and 5% CO\(_2\).

Plasmids. pCAT control (Promega, Madison, WI) and pCMV-ATCC (the gift of C. Rooney, SJCRH, Memphis, TN) contain SV40 and CMV promoters, respectively; both express the CAT gene when transfected into mammalian cells. pOCATI (the gift of D. Moore, Harvard Medical School, Boston, MA)\(^4\) is a promoterless negative control plasmid, and pTKBCAT is pOCAT1 containing a 1.2-kilobase BamHI fragment of the MGMT promoter sequence from pKT200\(^5\) (the gift of A. Fridland, SJCRH, Memphis, TN). The plasmid pSV-\(\beta\)-Gal (Promega, Madison, WI) expresses \(\beta\)-galactosidase under the control of the SV40 promoter, as does pCH110 (Pharmacia, Uppsala, Sweden) which was used for transfecting Rh28 and Rh18 cells.

Transient Transfections. Rh18 and Rh28 cells were transfected by a modified calcium phosphate cocipitation method\(^5\) (using a Stratagene (La Jolla, CA) kit according to manufacturer instructions. The other cell lines were electroporated using the BioRad (Richmond, CA) Gene Pulser apparatus under optimized conditions. HeLa cells (5 \(\times\) 10\(^6\)) were electroporated in 800 \(\mu\)l of N-(2-hydroxyethyl)piperazine-N’-(2’-ethanesulfonic acid) buffer, serum-free medium at 350 V, 500 \(\mu\)F under conditions as described by BioRad. Lymphoblast cells (1 \(\times\) 10\(^7\)) were electroporated (6) in 200 \(\mu\)l of phosphate-buffered saline at 960 \(\Omega\)F with the following optimized voltages: Molt 4 cells, 200 V; CEM and Raji, 220 V; and TK6 cells, 250 V. Cell lines were transfected independently with 20 \(\mu\)g of each CAT-reporter gene plasmid. Cotransfection with 5 \(\mu\)g of pSV-\(\beta\)-Gal or pCH110 allowed correction for transfection frequency following assays of cell extracts.

Enzyme Assays. CAT and \(\beta\)-galactosidase activities were measured in crude cell extracts 48 h after transfection. CAT activity was measured by liquid scintillation counting of \(\text{[\(\text{H}\)}]acectylchloramphenicol}\(^7\) following incubation with a \(\text{[\(\text{H}\)}]\)acetylcyanogen A substrate (New England Nuclear, Boston, MA; Amersham, Arlington Heights, IL). \(\beta\)-Galactosidase was assayed spectrophotometrically using either \(O\)-ni-trophenyl-\(\beta\)-D-galactopyranoside (Sigma, St. Louis, MO) or chlorophe-nol red \(\beta\)-D-galactopyranoside (Boehringer Mannheim, Indianapolis, IN) as substrates\(^8\). MGMT activity was measured as previously described using a DNA substrate treated with \(\text{[\(\text{H}\)}]\)methylnitrosourea (22 Ci/mmol; Amersham)\(^2\). CAT activity was calculated as cpm of \(\text{[\(\text{H}\]}\)acetlycholoramphenicol/h/mg of protein and was divided by the \(\beta\)-galactosidase activity to correct for differences in transfection frequency. The mean value for the promoterless control, pOCAT1, was subtracted from this figure.

Western Analysis. Cell extracts (40 \(\mu\)g) were electrophoresed on a 9% polyacrylamide gel using the BioRad Mini Protein II system, and proteins were electroblotted to polyvinylidene fluoride membranes (Immobilon, Millipore, Bedford, MA). Western analyses were performed using the anti-MGMT monoclonal antibody \(3.1\) (produced against MGMT protein purified from CCRF-CEM cells), an anti-\(\beta\)-tubulin monoclonal antibody (ICN, Lisle, IL) and anti-Spl polyclonal antibodies (the gift of S. Jackson, Cancer Research Campaign, Cambridge, England).
with each of the four CAT constructs and with a β-galactosidase-expressing plasmid. As shown in Fig. 1, promoter strengths were directly compared by expressing CMV and MGMT promoter activities in terms of the SV40 values for each cell line. The relative CMV promoter activity varied minimally (5-fold) across the cell lines, suggesting that the two viral promoters may be dependent on similar transacting factors for transcription. CMV promoter activity was 1.4- to 7-fold that of SV40 within the cell lines; this is consistent with the report of Foecking and Hofstetter (9) that CMV promoter activity was 3- to 18-fold that of SV40 in a variety of cell types from different species. In contrast, the MGMT promoter activity varied 2500-fold across the eight cell lines, suggesting that different transcription factors are necessary to control levels of MGMT expression in different cell types.

MGMT-CAT expression (corrected for transfection frequency) was 2.4- to 125.7-fold above background expression in all cell lines. Thus, although Mer+ cells do not efficiently transcribe MGMT mRNA (1) which is detectable only by multiple (30 to 40) polymerase chain reaction cycles (10), they appear to possess the necessary transcription factors. This suggests that MGMT is suppressed in Mer- cells by an alternative mechanism, such as cytosine methylation in MGMT promoter sequences, as we have previously suggested (11), or through posttranscriptional events that affect RNA processing, stability, or transport.

In most of the Mer+ cell lines, MGMT promoter activity appeared to correlate with cellular MGMT activity (Fig. 1); exceptions to this relationship suggest the presence of other regulatory elements not included in the 1.2-kilobase fragment used for the transfection. Surprisingly, MGMT promoter activity in the two leukemic T-lymphoblast cell lines (CEM and Molt-4) was 1.7- and 9.5-fold greater than that of CMV, despite the report of Boshart et al. (12) that the CMV promoter-enhancer unit is the strongest yet identified. This suggests that either T-lymphoblasts possess an abundance of specific transacting factors which do not interact with the viral promoters or that the CMV and SV40 promoters are inhibited in these cells. The CEM and Molt-4 cell lines expressed the highest levels of cellular MGMT, supporting the former possibility. This finding may reflect the binding of the T-cell-specific transcription factor TCF-1 to the MGMT promoter, which contains four potential binding sites (Fig. 2) (13). It also suggests that the MGMT promoter could be exploited for overexpression of other genes following transfection in this cell type.

The 2500-fold variation in MGMT promoter activity appears inconsistent with the 5-fold variation in cellular MGMT enzyme level in Mer+ cells. In Molt-4 cells, for example, the MGMT promoter is 30-fold stronger than in CEM, yet the cellular MGMT level is 50% lower. This disparity may reflect variable stability of MGMT mRNA or protein in the two cell lines, or it may result from suppressor sequences previously proposed to be 5' to the 1.2-kilobase BamHI promoter fragment (3). In any case, the variation among the cell lines in MGMT activity, plus the tentative correlation between promoter activity and cellular MGMT expression in Mer+ cells, suggests that transcription factors that bind to this 1.2-kilobase sequence participate in the regulation of MGMT expression.

The MGMT promoter fragment contains 11 potential binding sites (GC boxes) for the transcription factor Spl (3) (Fig. 2), suggesting that this DNA-binding protein may play a major regulatory role in MGMT transcription. Because similar GC boxes have been identified in a variety of mammalian gene promoters, including those of "housekeeping genes" (14, 15), Spl is generally thought to control basal-level gene expression. Saffer et al. (16), however, have demonstrated that gene expression can be increased by artificially elevating Spl levels; thus Spl can function as a transcription regulatory factor in mammalian cells. In addition, a 10-fold variation has been demonstrated in Spl levels among different cell types (17), reinforcing the likelihood of a regulatory function.

Spl protein was quantitated by Western analysis in the eight human cell lines described (Fig. 3). Human cells contain two Spl proteins (M, 95,000 and 105,000) resulting from different posttranslational modifications of a single gene product (18). The anti-β tubulin and anti-MGMT monoclonal antibodies recognized M, 55,000 and 25,000 proteins, respectively. Spl protein levels varied 2.3-fold among seven of the eight cell lines relative to tubulin, which acted as a control for sample loading.
Fig. 3. Western analysis of crude cell extracts from eight human cell lines. The membrane was trisected horizontally, and sections were hybridized with anti-Spl protein. A. CCRF-CEM; Lane 4, Molt-4; Lane 5, Raji; Lane 6, TK6; Lane 7, HeLa S3; Lane 9, Rhl8; and Lane 10, Rh28.

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References


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