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Inactive O^6-methylguanine-DNA methyltransferase in human cells

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ABSTRACT

A plasmid encoding a recombinant human O^6-methylguanine-DNA methyltransferase (MGMT) fused to a fragment of the bacteriophage λN protein has been constructed. The fusion protein retained methyltransferase activity when expressed at high levels in *E.coli* and was purified to essential homogeneity by a simple procedure. Antisera raised against the purified fusion protein recognized MGMT in western blots of extracts of human cells. For most cell lines, there was a quantitative relation between the amount of immunologically detectable MGMT protein and enzyme activity. However, four cell lines contained detectable MGMT protein despite having no measurable methyltransferase activity. Additionally, a HeLa line contained considerably more immunoreactive MGMT protein than could be accounted for by its methyltransferase activity. Thus, some cells contain significant amounts of inactive MGMT. Preliminary characterization of the inactive protein in HeLaS3 cells indicated that it has some properties in common with MGMT methylated at the active cysteine residue.

INTRODUCTION

The DNA repair enzyme O^6-methylguanine (O^6-MeGua)-DNA methyltransferase (MGMT) protects human cells against the genotoxic effects of a number of important alkylating agents (For reviews see 1,2). In particular, loss of its expression predisposes the cell to killing, and to the induction of mutation and sister chromatid exchanges by methylating agents such as N-methyl-N-nitrosourea (MNU) and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). Unrepaired O^6-MeGua in DNA causes G:C to A:T transition mutations as a consequence of its ability to mispair with thymine during DNA replication. G:C to A:T transitions are a predominant feature of some spontaneous mutation spectra (3) and there is some evidence that MGMT acts to prevent this type of spontaneous mutation (Aquilina et al, In press). A possible explanation for the observed protection is that O^6-MeGua is introduced into DNA, perhaps by an endogenous methylating agent, and that in cells of the MGMT-defective or Mex^- (Mer^-) phenotype it is a major contributor to spontaneous mutagenesis.

Loss of MGMT activity is commonly associated with cellular transformation. In particular, cell lines derived from tumor biopsies or transformed *in vitro* frequently exhibit the Mex^- phenotype. Comparison of enzyme activities indicates that human cell lines can express a wide range of MGMT activity between the extreme Mex^+ and Mex^- states. In this regard, cell lines in culture reflect the considerable interindividual and intertissue variation in human MGMT activity (4,5). Among cultured cells, MGMT gene expression can exhibit a spontaneous instability and consequently the level of MGMT activity for any particular cell population may not be fixed (6,7). This instability introduces variability in methyltransferase determinations even in initially clonal cell populations.

An important contributary factor to the loss of MGMT activity is apparently a transcriptional silencing of the MGMT gene since MGMT mRNA is present in Mex^+ cells but normally undetectable by northern blotting in Mex^- cells (8,9). Alterations in the pattern of cytosine methylation of the MGMT gene may play a part in this regulation although the relation between cytosine methylation and MGMT gene silencing is complex: in most Mex^- cell lines the gene is hypomethylated relative to Mex^+ lines whereas some others exhibit the more usual relationship and MGMT sequences are hypermethylated (10,11, S.Cairns-Smith and P.K., In press). The role of MGMT in protecting against transformation by common alkylating agents, indicates the importance of understanding the factors that control the variability of expression of MGMT activity within cell populations.

The MGMT acts by an unusual mechanism in which a unique activated cysteine residue accepts the methyl group transferred from the methylated base. Methylation of the cysteine residue inactivates the enzyme and active molecules cannot be regenerated. The fate of methylated molecules is not known although there is some evidence to suggest that they might turn over more rapidly than their active counterparts (12). The suicidal nature of the MGMT reaction indicates that the repair of spontaneously occurring O^6-MeGua could contribute to the observed variations in active MGMT between cell types. In order to test this possibility, we have used immunoblotting with rabbit antisera raised against a purified recombinant human MGMT to investigate the steady-state level of human MGMT protein in cell

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extracts. Comparison of MGMT protein and methyltransferase activity in eight Mex$^+$ human cell lines indicated a quantitative relation between these two parameters. Both enzyme activity and MGMT protein were undetectable in three Mex$^-$ lines. In contrast, four cell lines were found that exhibited an excess of MGMT protein over methyltransferase activity. Three lymphoid lines and a HeLa line that expressed no detectable MGMT activity and had been designated Mex$^-$, contained significant levels of MGMT protein. In addition, a Mex$^+$ HeLaS3 line contained an excess of MGMT protein over methyltransferase activity. These data indicate that inactive MGMT protein may be present in each of these cell types. The active and inactive forms of MGMT in HeLaS3 cells could be resolved by chromatography on phosphocellulose and in this respect the chromatographic behaviour of the inactive protein resembled that of MGMT methylated at the active cysteine.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from BDH and were Analar grade or better. Biochemicals were obtained from Sigma except where specifically stated otherwise. Radiochemicals were from Amersham International. Restriction enzymes (BRL), calf intestinal phosphatase (Boehringer Mannheim), T4 DNA ligase (BRL) and S1 nuclease (Boehringer Mannheim) were used in accordance with suppliers' instructions. Protein standards were obtained from Biorad.

Bacterial strains and plasmids

The *E.coli* strain UT5600recA, and the plasmid pHE6 (13) were kindly provided by Professor L.Grossman. The human MGMT cDNA clone pHM14 (8) was maintained in *E.coli* JM101. Propagation of bacteria, purification of plasmids, preparation of competent *E.coli* and manipulation of recombinant DNA were carried out by standard techniques (14).

Human cell lines

The human lymphoblastoid cell lines GM2634, GM2485, GM2344, GM2345, GM2781, GM1646, GM2248, GM0892, GM0621, GM2498 and GM1953 were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ, USA. GM1953(S) is a Mex$^-$ subline of GM1953 that arose spontaneously in our laboratory (6). The Burkitt's lymphoma lines Raji and Daudi and the HeLaS3 cell line were obtained from the American Type Culture Collection, Rockville, MD, USA. The Mex$^-$ HeLa line, HeLaMR, was kindly provided Dr. M.Bignami.

Methyltransferase assay

Crude extracts of human cells were prepared by extracting washed cells (10^8/ml) in ice-cold Triton Extraction Buffer (50mM Tris·HCl, pH 7.5, 1mM EDTA, 10mM DTT, 0.2% Triton X-100). Debris were removed by centrifugation (5min, 14000rpm) and the supernatant, which contained 10–20mg protein/ml used for assay. In the standard assay, 0–200μg extract was added to 100μl reaction mixture (70mM Hepes·KOH, 1mM EDTA, 10mM DTT) containing partially depurinated [^{3}H]MNU-treated *M. luteus* DNA. The removal of [^{3}H]-methyl groups from this substrate was monitored as previously described (15). The [^{3}H]MNU had a specific activity of 16Ci/mmole and each assay contained 0.15pmole [^{3}H] O^6-methylguanine (1500cpm). One unit of methyltransferase activity demethylates 1 pmol O^6-methylguanine in the standard assay.

Construction of recombinant plasmids encoding a human MGMT-λN fusion protein

The human MGMT cDNA was excised from plasmid pHM14 by digestion with EcoRI and purified by agarose gel electrophoresis. 30ng purified EcoRI fragment were digested with 3 units S1 nuclease for 10min at 37°C in 50mM Na Acetate pH 4.6, 1mM $ZnCl_2$, 250mM NaCl, 50μg/ml bovine serum albumin. The reaction was terminated by addition of EDTA (5mM) and the digested cDNA recovered by ethanol precipitation.

Purified pHE6 DNA (700ng) was digested with SmaI and dephosphorylated (37°C, 30min) with calf intestinal phosphatase (0.3 units). Dephosphorylation was terminated by addition of an equal volume of SDS buffer (100mM NaCl, 10mM Tris·HCl, pH8.0, 1mM EDTA, 0.1% SDS) and heating at 70°C for 15min. After phenol extraction, the S1 nuclease digested cDNA and dephosphorylated vector were ligated overnight at 20°C in ligation buffer (66mM Tris·HCl, pH 7.5, 5mM $MgCl_2$, 5mM DTT, 1mM ATP) containing 400 units T4 DNA ligase.

The ligated DNA was transformed into competent UT5600recA which were plated on LB plates containing ampicillin (100μg/ml). After growth overnight at 30°C, colonies were transferred to nitrocellulose filters and screened by hybridization using [^{32}P]-labelled MGMT cDNA as probe. A total of nine positively hybridizing colonies was obtained. Partial restriction analysis based on the known map of pHM14 cDNA indicated that three contained the insert in the correct orientation. The three correctly oriented plasmids were designated pNMT1–3. pNMT1 was used for further characterization and purification of the recombinant protein.

Characterization of human MGMT-λN fusion protein

10ml cultures of UT5600recA:pNMT1 in exponential growth were induced by the addition of an equal volume of L broth pre-warmed to 54°C and transferred to 42°C. Incubation at this temperature was continued with shaking for a further 60min. Cells were harvested by centrifugation, washed in 50mM Tris·HCl, pH 8.0 and disrupted by sonication on ice in 200μl extraction buffer (50mM Tris·HCl, pH 8.0, 1mM EDTA, 10mM DTT). Debris were removed by centrifugation, protein concentrations were determined and the supernatant was used to determine methyltransferase activity and for analysis by SDS-PAGE.

SDS PAGE analysis was performed in a Biorad Miniprotean gel apparatus using 12% gels with 5% stacking gels. 30μg of each cell extract was applied to the gel. Fixed gels were stained with Coomassie blue.

Purification of human MGMT-λN fusion protein

A 20l exponentially growing fermentor culture of UT5600recA:pNMT1 was induced by rapid temperature shift to 42°C and growth continued for 60min. Harvested cells (20g) were washed and disrupted by grinding with 50g acid-washed sand. This step and all subsequent operations were carried out at 0–4°C. All buffers contained 1mM EDTA and 14mM β-mercaptoethanol. Disrupted cells were extracted with 100ml extraction buffer (50mM Tris·HCl, pH 8.0, 100mM NaCl) by stirring on ice for 30min.

Sand and cell debris were removed by centrifugation at 2k rpm, 5min and subsequently 7k rpm, 10min. The supernatant was brought to 20% saturation by the slow addition of solid

ammonium sulfate (Ultra Pure, BRL Laboratories). Precipitated material was removed by centrifugation (9k rpm, 15min) and the supernatant brought to 60% ammonium sulfate saturation. Precipitated material was recovered by centrifugation (9k rpm, 15min), dissolved in 30ml DNA Cellulose Buffer (20mM Tris·HCl, pH 8.0) and dialysed for 8−12 hours against 3 changes each of 2l of the same buffer. A small precipitate was removed by centrifugation (10k rpm, 20min), and the supernatant (40ml) was applied to a column of single-stranded DNA cellulose (Sigma) (10cm×4cm dia. equilibrated in DNA Cellulose Buffer) at a flow-rate of 10ml/hr. Fractions (12ml) were collected. After loading, the column was washed with DNA Cellulose Buffer (400ml) followed by the same buffer containing 0.1M and 0.3M NaCl. Washing was continued in each case until the A_{280} of column fractions returned to background. Methyltransferase activity was eluted in a subsequent wash with DNA Cellulose Buffer containing 0.5M NaCl. Occasionally, methyltransferase was eluted in both 0.3M NaCl and 0.5M NaCl washes. Since there were no apparent differences between the two activities, they were pooled for subsequent steps.

Active fractions from the DNA cellulose column were pooled (80ml) and concentrated by ultrafiltration through an Amicon YM10 membrane. The concentrated material (6ml) was applied to a pre-calibrated column (100cm×2cm dia.) of AcA54 (LKB) equilibrated in AcA54 Buffer (50mM Tris·HCl, pH 8.0, 200mM NaCl, 5% glycerol). Methyltransferase activity eluted as a single symmetrical peak with an elution volume characteristic of a protein of $M_r = 2.9.10^4$. Active fractions were pooled, concentrated by ultrafiltration and stored at −20°C. The purification procedure typically yielded approximately 600μg purified protein with a specific activity of 10^4 methyltransferase units per mg protein.

Partial purification of human MGMT

Approximately 2.10^9 Daudi or GM2498 cells were washed with PBSA. All subsequent steps were performed at 0−4°C. Extracts were prepared by resuspension of cells in 50ml Triton Extraction Buffer containing: 50mM Tris·HCl, pH 7.5, 100mM NaCl, 1mM EDTA, 14mM β-mercaptoethanol, 1mM PMSF and leupeptin, chymostatin and pepstatin (all 0.5μg/ml). Debris were removed by centrifugation (10^4 rpm, 30min). 0.25 volumes of a concentrated slurry of DE52 (Whatman) equilibrated in DE52 Buffer (20mM Tris·HCl, pH 7.5, 50mM NaCl, 1mM K_2HPO_4, 1mM EDTA, 1mM DTT, 14mM β-mercaptoethanol) was added to the supernatant. After thorough mixing, the DE52 was removed by centrifugation (10^3 rpm, 5min). The supernatant was retained and the DE52 washed by resuspension in 20ml DE52 Buffer. Following recentrifugation, the two supernatants were combined and 0.5 volumes of a concentrated slurry of Phosphocellulose P11 (Whatman) equilibrated in DE52 Buffer was added. The resulting slurry was stirred gently for 60min. and then poured into a column. The column was washed with DE52 Buffer until the A_{280} returned to background and was then eluted with DE52 Buffer supplemented with 0.5M NaCl. For the Daudi cell extract, fractions were assayed for methyltransferase activity. Active fractions were pooled (P11+ pool), dialysed overnight against 3 changes of 1l 1mM KPO_4, pH 7.5, 1mM EDTA, 10% glycerol and stored at −20°C. For the Mex^- line GM4298, equivalent fractions of the P11 eluate were pooled, dialysed and stored in an identical fashion (P11-pool). Analysis of MGMT in HeLaS3 cells was performed in a similar fashion.

Production of antisera

New Zealand White rabbits were immunized at multiple sites by subcutaneous injection of a total of 100μg purified MGMT-λN fusion protein in complete Freund's adjuvant. A total of six subsequent immunizations using the same antigen in incomplete Freund's adjuvant were performed at four-weekly intervals. Antibody titers were determined by ELISA using 96-well microtiter plates coated in MGMT-λN fusion protein (16).

Immunoblotting

Freshly-prepared crude cell extracts were resoved on 12% SDS-polyacrylamide gels in a Biorad Miniprotean apparatus. Immunoblotting was carried out essentially as described by Harlow and Lane (16). Briefly, transfer to nitrocellulose filters was performed at 4° overnight at 20V. After blocking with 10% horse serum in D Buffer (50mM Tris·HCl pH 7.5, 200mM NaCl, 0.05% NP40) for 2−3 hours at room temperature, filters were exposed to the primary antibody (anti-human MGMT-λN fusion protein antisera) at a dilution of 1:500 in D Buffer containing 10% horse serum for a further 2hr. After washing 5 times with 25ml D Buffer (5−10min each wash), filters were subsequently probed with 25ml secondary antibody, 1μCi [^{125}I]-labelled donkey anti-rabbit IgG (12−14 μCi/μg, Amersham International) in D Buffer containing 10% horse serum. Unbound radioactivity was removed by 6−8 changes of 25ml PBSA. Washed filters were exposed to X-ray film. Densitometric analysis was performed on a Molecular Dynamics densitometer.

PCR analysis of MGMT mRNA expression

Whole RNA was prepared by published procedures (14). First strand cDNA was synthesized from 4μg RNA with the cDNA Synthesis System Plus (Amersham International) in a reaction volume of 20μl using random hexamer priming. To amplify MGMT sequences, 10μl cDNA was incubated with 90μl PCR buffer (10mM Tris·HCl, pH 8.3, 50mM KCl, 0.01% gelatin, 2.5mM $MgCl_2$, 0.5mM dNTPs, 10% DMSO) 50pmol oligonucleotide primers and 2.5units Amplitaq (Perkin-Elmer Cetus). Primers corresponding to positions 1−23 and 438−464 of the published MGMT cDNA sequence (8) were used and amplification was carried out for 40 cycles in a DNA thermal cycler (Perkin Elmer). Each cycle was: 91°C for 1min, primer annealing for 2min at 60°C, and polymerization at 72°C for 2min. PCR products were separated on 1% agarose gels and MGMT species were identified by Southern analysis using MGMT cDNA as a probe.

RESULTS

Construction and purification of a human MGMT-λN fusion protein

The full-length human MGMT cDNA excised from the plasmid pHM14 with EcoRI and made blunt-ended by digestion with S1 nuclease, was ligated into the SmaI site of the expression vector pHE6 bringing the MGMT sequences under the control of the bacteriophage λP_L promoter and the temperature-sensitive λcI857 repressor. After transformation into *E.coli* UT5600recA and selection for resistance to ampicillin, colonies containing MGMT sequences were identified by hybridization to the cDNA. Partial restriction mapping of 9 positive plasmids indicated that 3 contained an apparently unrearranged, full-length MGMT cDNA inserted in the correct orientation. After induction at 42°C,

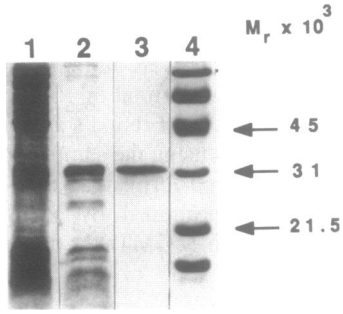

Figure 1. Purification of MGMT-λN fusion protein SDS-PAGE analysis. MGMT-λN fusion protein was purified from 20g induced UT5600recA:pNMT1 cells as described in Materials and Methods. Proteins were separated on 12% SDS-polyacrylamide gels and stained with Coomassie Blue. Lane 1: Crude Extract (30μg). Lane 2: DNA Cellulose Pool (1μg). Lane 3: AcA 54 Pool (0.5μg). Lane 4: Molecular Weight Standards.

a novel protein of approximate M_r = 31000 was observed in crude extracts of the positive clones analysed by SDS-PAGE and Coomassie blue staining. Induction of this protein was dependent on both heat and the presence of the cDNA insert in the correct orientation. The induced protein comprised an estimated 0.5−1% of the total in crude extracts (data not shown). Its size was consistent with that expected for a human MGMT molecule fused to 40 aminoacids of the λN protein. One positive recombinant plasmid, designated pNMT1, was used to characterize the fusion protein.

The protein encoded by pNMT1 was a functional methyltransferase. Extracts from non-induced UT5600recA: pNMT1 cells contained O^6-methylguanine-DNA methyltransferase activity of approximately 2 units/mg protein. This value represents the combined activities of the endogenous Ada and Ogt proteins of wild-type *E.coli* (17). Following heat induction, methyltransferase activity increased approximately 100-fold to 220 units/mg protein. Thus the induced MGMT-λN fusion protein is a protein of approximate M_r = 31000 and is an active methyltransferase.

The MGMT-λN fusion protein was purified from thermally induced UT5600recA:pNMT1 cells in three steps. A 20−60% ammonium sulfate fraction of a crude cell extract was dialysed and applied to single-stranded DNA cellulose. The pNMT1-encoded methyltransferase was retained and could be eluted with buffers containing 0.3−0.5M NaCl. Further purification was effected by size fractionation by chromatography on AcA54. This procedure resulted in an essentially homogeneous preparation of MGMT-λN fusion protein purified approximately 200-fold in about 15% yield (Figure 1).

Antibodies against the MGMT-λN fusion protein

Polyclonal antisera were raised against the purified MGMT-λN fusion protein by repeated immunization of rabbits. Western blotting using the immune serum at a dilution of 1:500 and [^{125}I]-labelled anti-IgG was used to quantitate MGMT. Under the standard conditions, the limit of detection of the purified MGMT-λN fusion protein was 0.1−0.2ng (Figure 2a). This detection limit represents approximately 3.10^9 MGMT-λN fusion protein molecules.

In order to investigate whether the antibodies against the fusion protein recognized the unmodified human protein, MGMT was partially purified from the Burkitt's lymphoma line Daudi. Nucleic acid and some protein was removed from a crude cell

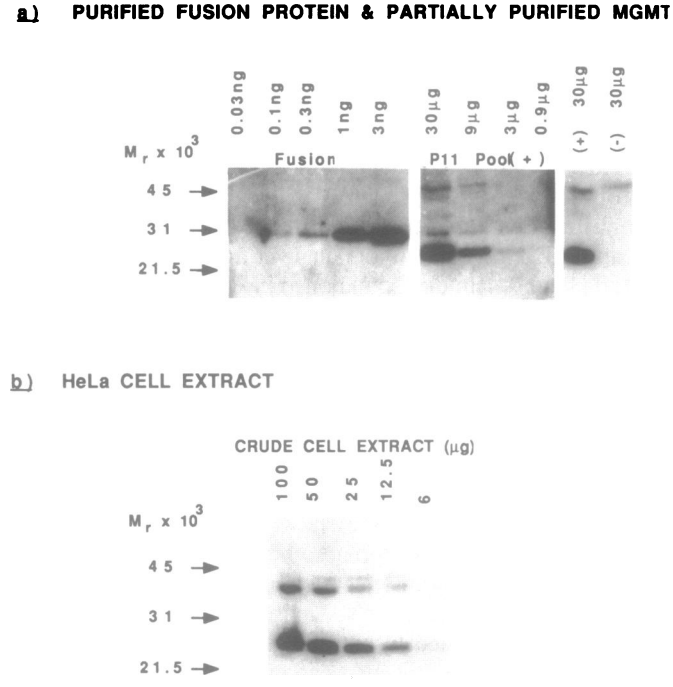

Figure 2. Detection of MGMT protein by immunoblotting. a) Purified MGMT-λN fusion protein and partially purified MGMT. Purified MGMT-λN fusion protein (0.03−3ng), partially purified Daudi MGMT (P11 Pool (+); 0.9−30μg) or a P11 eluate from the Mex$^-$ line GM2498 ((−); 30μg) were separated on 12% SDS-polyacrylamide gels, electroblotted and probed with anti-MGMT-λN serum. Immunoreactive proteins were visualized with [^{125}I]-labelled donkey anti-rabbit IgG. b) Crude cell extracts. An unfractionated extract of HeLaS3 cells (6−100μg) was analysed as above.

extract by adsorption to DE52. Unbound proteins were applied to phosphocellulose P11. MGMT activity was retained by the P11 and eluted at high ionic strength (0.5M NaCl). The eluted MGMT was about 5-fold purified. As a negative control, a crude extract of the Mex$^-$ human lymphoblastoid cell line GM2498 which contains ≤0.05 units/mg MGMT activity, was chromatographed on P11 in an identical fashion. As expected, no MGMT activity was observed in column fractions. The 0.5M NaCl eluate of the column was retained and concentrated.

When the high salt P11 eluate from the Mex$^+$ Daudi (P11+) and the negative GM2498 cell line (P11-) were analysed by immunoblotting, a major band of approximate M_r = 24000 and additional well-resolved minor bands of higher molecular weight were detected in the MGMT-enriched material from Daudi cells. Minor bands were also present in the (P11-) fraction from GM2498 whereas the M_r = 24000 band was absent (Figure 2a). The M_r of the human MGMT is 21700 but it normally migrates with an apparent M_r = 24000 (8). Thus the antiserum directed against the MGMT-λN fusion protein recognises the normal human MGMT and the MGMT protein is absent from the Mex$^-$ line GM2498. Under standard conditions, MGMT protein was detectable when a minimum of 2μg of the P11+ pool was applied to the gel. From the specific activity of this partially purified MGMT, we calculate that the minimum detectable level of MGMT is around 5.10^9 -10^{10} molecules (assuming all MGMT molecules retain activity). This value is consistent with the detection limit for the MGMT-λN fusion protein and indicates that normal MGMT and fusion protein molecules are recognized with similar efficiencies. The antisera also recognized a protein

Table 1. MGMT activity and protein in cell extracts

Cell Line	MGMT Activity[1] (units)	MGMT Protein[2] (arbitrary units)	MGMT Protein[3]	Activity:Protein[4]
EXPERIMENT 1				
HeLaS3	0.14	62	1.8	0.5
Raji(+)	0.07	23	0.7	0.6
GM1953(+)	0.07	12	0.3	1.2
GM2781	0.14	26	0.7	1.1
GM1646	0.12	19	0.5	1.3
GM2634	0.10	16	0.5	1.3
GM2485	0.09	14	0.4	1.3
GM2344	0.14	26	0.7	1.1
GM2345	0.14	21	0.6	1.4
GM1953(−)	<0.001	4	0.1	
Raji(−)	<0.001	6	0.2	
HeLaA6	<0.001	9	0.3	
EXPERIMENT 2				
HeLaS3	0.14	80	1.3	0.6
Raji(+)	0.07	45	0.7	0.5
GM1953(+)	0.07	35	0.6	0.7
GM2781	0.14	65	1.1	0.8
GM1646	0.12	66	0.9	0.8
GM2634	0.10	48	0.6	1.0
GM2485	0.09	51	0.7	0.8
GM2344	0.14	65	0.9	0.9
GM2345	0.14	70	0.9	0.9
Daudi	0.15	84	1.1	0.8
GM1953(−)	<0.001	7	0.1	
Raji(−)	<0.001	8	0.1	
HeLaA6	<0.001	11	0.2	
GM0621	<0.001	5	0.1	
GM2248	<0.001	≤1	≤0.01	
GM2498	<0.001	≤1	≤0.01	
GM0892A	<0.001	≤1	≤0.01	

[1]MGMT activity was determined in the standard assay. The values shown here represent the amount of methyltransferase activity in 100μg cell extract protein applied to the gel.
[2]Determined by densitometric scanning of autoradiograms of western blots. The values of MGMT activity and protein shown here were determined from the same cell extract.
[3,4]To correct for different exposure times and variations in activity of [^{125}]I-labelled secondary antibody, these values are normalized to the value for the Daudi P11(+) sample included in each blot and set to unity.

of M_r = 24000 in crude extracts of HeLaS3 cells (Figure 2b). In this case, MGMT protein was detectable in 6μg crude cell extract. From the specific enzymatic activity, we calculate that this represents approximately 3.10^9 active MGMT molecules. These detection limits are similar to the limit of sensitivity of the standard enzymatic assay.

Immunoprecipitation and enzyme inhibition

Preincubation of homogeneous MGMT-λN fusion protein, partially purified MGMT or crude human cell extracts with serum dilutions in the range 1:10^5 to 1:100 did not inactivate the methyltransferase. Similarly, the anti-MGMT antisera in conjunction with protein A did not precipitate the unmethylated or methylated forms of either the fusion protein or normal human MGMT (data not shown). The inability of protein A to precipitate immune complexes between MGMT and the anti-MGMT antibodies may reflect the relatively small size of the MGMT protein. Alternatively, the antibodies may recognize the protein in an unfolded form but not in its native conformation.

MGMT levels in extracts of human cell lines

Estimates of the steady-state levels of methyltransferase in Mex$^+$ human cell lines range from several thousand to ≥10^5 active molecules per cell (15,18). Average values for Mex$^-$ cells range from undetectable to several hundred active MGMT molecules per cell. We analysed the steady-state levels of total MGMT protein in extracts of a number of Mex$^+$ and Mex$^-$ human cell lines and compared these to the levels of active methyltransferase determined by enzymatic assay. Crude cell extracts prepared from approximately 10^7 exponentially growing human lymphoblastoid, Burkitt's lymphoma and HeLa cells were assayed for MGMT activity. The data from 9 Mex$^+$ and 7 Mex$^-$ lines are presented in Table 1. Values of methyltransferase activity in extracts of Mex$^+$ cells ranged from 0.7 to 1.5 unit/mg protein. These values correspond respectively to approximately 6.10^4 and $1.3.10^5$ active MGMT molecules per cell, in good agreement with previous estimates. Under the standard assay conditions (up to 200μg extract), enzyme activity was undetectable in extracts of the Mex$^-$ cell lines (<0.01 units

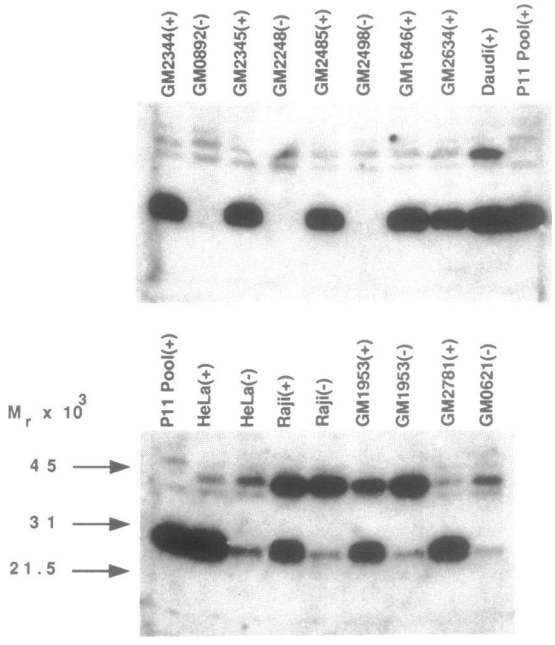

Figure 3. MGMT in crude extracts of Mex^+ and Mex^- cells. Crude extracts (100μg) of each cell line were resolved on 12% SDS-acrylamide gels and electroblotted. Blots were probed with anti-MGMT-λN serum and developed with [^{125}I]-labelled donkey anti-rabbit IgG. The $Mex^{+/-}$ status of each line is given in parenthesis.

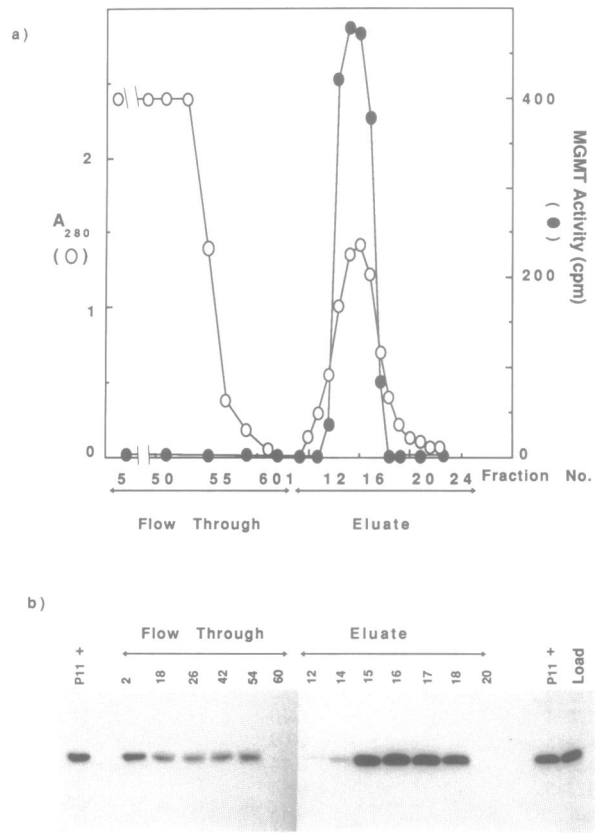

Figure 5. Fractionation of HeLaS3 MGMT on phosphocellulose P11. a) MGMT activity. An extract of 5.10^9 HeLaS3 cells from which nucleic acids had been removed by adsorption to DE52, was mixed with P11 at low ionic strength (0.05M NaCl) and poured into a column. The column was washed with low-salt buffer (Fractions 1−60; Flow-Through) and subsequently eluted with the same buffer containing 0.5M NaCl (Fractions 1−24; Eluate). The protein concentration of fractions was monitored by absorbance at 280nm. Each fraction was 5ml and 20μl of each was assayed for MGMT activity as shown. MGMT activity is expressed as the radioactivity removed from methylated DNA. (○) Absorbance: (●) MGMT activity. b) MGMT Protein. 30μl of the each of the fractions indicated was analysed by western blotting. Blots were probed with anti-MGMT-λN serum.

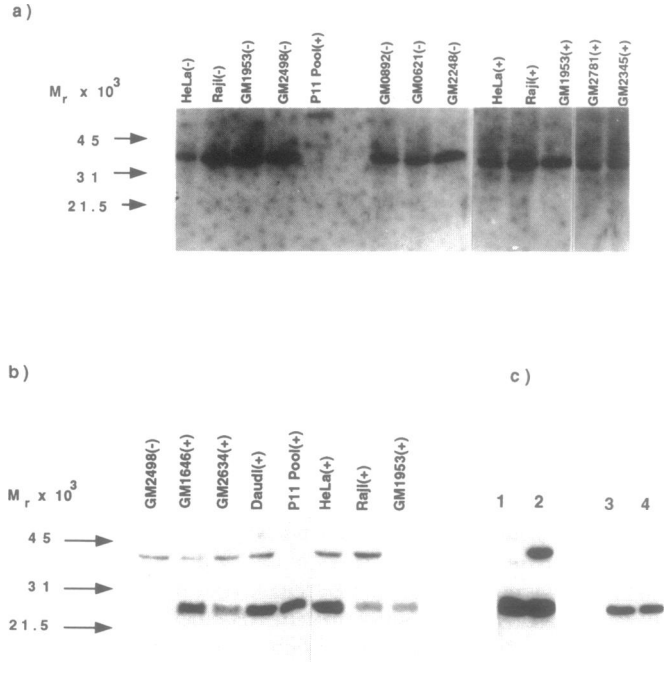

Figure 4. Non-specific staining by anti-MGMT antisera. Crude extracts (100μg) of Mex^+ and Mex^- cells were analysed by immunoblotting using preimmune serum (a) or anti-MGMT-λN serum (b,c) and [^{125}I]-labelled secondary antibody. c) Two pairs of identical samples (lanes 1 & 3: 30μg P11 Pool(+); lanes 2 & 4: 100μg Daudi(+) cell extract) were analysed with identical reagents on successive days. The blot for the autoradiograph in the left panel was performed one day earlier and the exposure to X-ray film was one day longer than the right panel.

activity/mg protein). These Mex^- cells contain, on average, fewer than 900 active MGMT molecules per cell.

An aliquot (100μg) of each cell extract was analysed by immunoblotting. The partially purified MGMT from Daudi cells (P11+) was included as a positive control. After probing with immune serum, intensely staining bands were observed at positions corresponding to proteins of M_r = 24000 and M_r = 40000 (Figure 3). The higher molecular weight band was not correlated with the Mex status of the cells and varied among cell lines with Raji and GM1953 exhibiting the highest intensities. This band was also prominent in blots probed with the pre-immune rabbit serum (Figure 4a) in which Raji and GM1953 extracts were again, in general, more intensely stained. Adsorbtion to DEAE cellulose followed by chromatography on phosphocellulose P11 completely removed the M_r = 40000 protein recognised by both pre-immune and immune sera (P11+, Figure 3). The proteins of approximate M_r = 40000 recognised by both pre-immune and immune sera are therefore probably identical. In numerous blots containing either Mex^- or Mex^+ extracts probed with the pre-immune serum, we never observed immunoreactive material migrating with an apparent M_r =

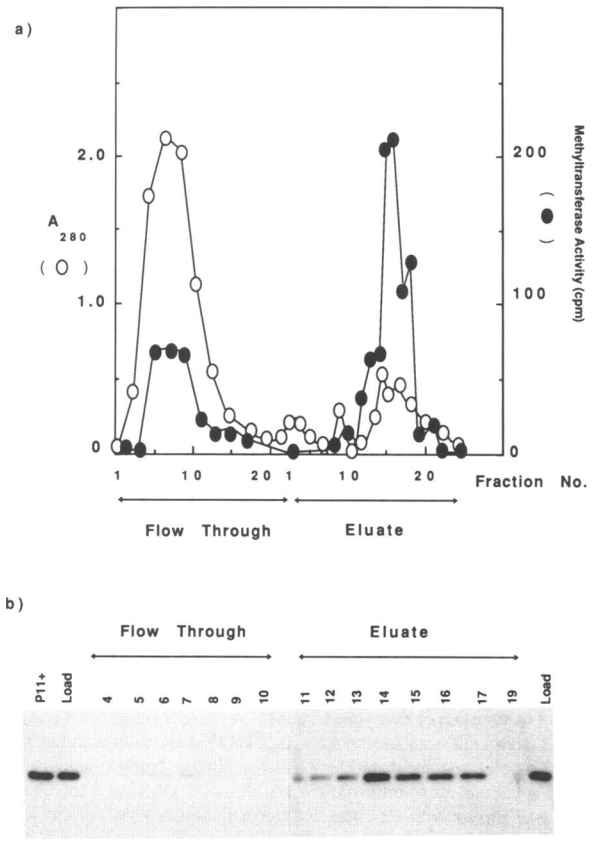

Figure 6. Fractionation of Daudi MGMT on phosphocellulose P11. a) MGMT Activity. An extract of 5.108 Daudi cells was mixed with DE52 and unbound material was adsorbed to phosphocellulose P11 as described in the legend to Figure 4. Unadsorbed protein was removed by washing at low ionic strength. Adsorbed material was eluted with buffer containing 0.5M NaCl. Aliquots (20μl) of each fraction (5ml) were assayed for MGMT activity. MGMT activity is expressed as the radioactivity removed from methylated DNA.(○) Absorbance: (●) MGMT activity. b) MGMT Protein. 30μl of the each of the fractions indicated was analysed by western blotting. Blots were probed with anti-MGMT-λN serum.

24000 (Figure 4a). These data suggest that it is unlikely that any M_r = 24000 material detected by the immune serum is derived by proteolysis from the cross-reacting M_r = 40000 material. In addition, in experiments carried out over several months, the intensity of the M_r = 40000 band was variable between blots probed with either pre-immune or immune serum (compare Figures 3 & 4a,b) and this band was occasionally absent (Figure 4b,c). Figure 4c shows a comparison of identical samples analysed on successive days using identical reagents in which the M_r = 40000 is apparent in the first but is not present in the second blot. In contrast, the M_r = 24000 band was only detected by immune serum and was always of a predictable intensity. We conclude that the M_r = 24000 protein is immunologically distinct and is not generated by proteolytic degradation of the M_r = 40000 protein.

The immunoreactive M_r = 24000 protein in crude cell extracts comigrated with the single intense band in the Daudi P11+ sample (Figure 3,4b,c). This band was present in all extracts from cell lines designated Mex$^+$ but was absent from crude extracts of the Mex$^-$ lymphoblastoid lines GM2248, GM0892 and GM2498 (Figure 3,4b). These properties serve to identify this protein as the MGMT. The amount of MGMT protein was quantitated by densitometric scanning and values from two independent experiments are presented in Table 1. Since the signal intensity is dependent on factors such as exposure time and radiochemical age of the secondary antibody, each gel included a sample of the Daudi P11+ pool and the values of specific activity are normalized to this standard. The intensities of the M_r = 24000 MGMT bands relative to the methyltransferase activities in the Mex$^+$ cell extracts were in good quantitative agreement with the value for the Daudi P11+ pool. In agreement with the absence of methyltransferase activity, MGMT protein was undetectable by densitometric scanning in extracts of GM2248, GM0892 and GM2498.

There were exceptions to the general correlation between methyltransferase activity and level of immunoreactive MGMT protein. Firstly, cell extracts of four designated Mex$^-$ lines: GM0621, HeLaMR, Raji Mex$^-$, GM1953(S) (which is a Mex$^-$ subline of the Mex$^+$ lymphoblastoid line GM1953) reproducibly contained immunoreactive protein that comigrated with authentic MGMT protein (Figure 3) despite the absence of measurable methyltransferase activity (<0.01units/mg protein). In addition, HeLaS3 and Raji cell extracts contained levels of MGMT protein that were disproportionately high relative to their enzymatic activity (Table 1). The ratio of methyltransferase activity to the level of immunreactive MGMT protein was approximately half the value of the Mex$^+$ lymphoblastoid cell lines indicating that as much as 50% of the MGMT protein may be present in an enzymatically inactive form.

Partial characterization of the inactive MGMT in HeLaS3 cells

The active and inactive forms of MGMT were resolved by fractionation of an extract of HeLaS3 cells on phosphocellulose P11. After removal of the nucleic acids from the crude extract by batchwise adsorption to DE52, the nonadsorbed material was mixed with P11 at low ionic strength (0.05M NaCl) and after washing, bound material was eluted with the same buffer containing 500mM NaCl. Fractions were assayed for MGMT activity. The elution profile is shown in Figure 5a. No MGMT activity was detected in the unbound material (Flow Through) and all active MGMT was eluted at high ionic strength (Eluate). Equal volumes of representative column fractions were analysed by western blotting (Figure 5b). The active methyltransferase in fractions 14−18 of the eluate was detected by the antiserum. In addition MGMT protein was present throughout the flow-through fractions 2−54. Since the column effectively concentrates the active protein into 5 fractions and the inactive protein is uniformly distributed throughout the flow-through fractions, we estimate that the inactive MGMT comprises ≥50% of the total MGMT protein applied to the column.

Since MGMT is inactivated by methylation, we examined the behaviour of the methylated MGMT protein on P11. A crude extract of Daudi cells was divided into two parts. One part was incubated with substrate DNA containing [^{3}H]-O^6-methylguanine in order to inactivate and radioactively label the MGMT while the other part remained untreated. Each sample was then separately adsorbed to DE52 and fractionated on P11 as described above. Fractions from the untreated sample were assayed for MGMT activity (Figure 6a) and analysed by western blotting (Figure 6b). As was observed for the HeLaS3 cells, >80% of the active MGMT was retained by the P11 column and required high ionic strength for elution. Western blotting analysis of Flow-Through and Eluate fractions is shown in Figure 6b. A small

amount of immunoreactive material, consistent with a low level of MGMT activity, was present in the Flow-Through fractions but ≥80% of MGMT protein was coincident with the MGMT activity in the Eluate fractions.

The radioactive methylated protein exhibited different chromatographic behaviour. Aliquots of the Flow-Through and Eluate fractions of the P11 column were incubated overnight with Proteinase K to digest the radioactive methylated MGMT to a TCA-soluble form. Figure 7 shows the elution profile of the methylated protein. A small amount of radioactive proteinase K-sensitive material bound to the column and was eluted at high ionic strength, but most of the radioactive material (>80%) eluted in the Flow-Through along with the majority of the cellular protein. It appears that methylation alters the MGMT protein in such a way as to prevent its binding to P11 and that this property is shared by the inactive MGMT in HeLaS3 cells.

MGMT mRNA in Mex$^+$ and Mex$^-$ lines

Whole or polyA$^+$ RNA from Mex$^+$ but not Mex$^-$ cells contains MGMT mRNA detectable by northern analysis. Using a more sensitive PCR-based technique, MGMT mRNA has been demonstrated in a number of Mex$^-$ human tumor cell lines (9). We analysed the MGMT mRNA in our Mex$^-$ lines by PCR amplification of MGMT cDNA followed by blotting and probing with the cloned MGMT cDNA. Figure 8 shows that all Mex$^-$ lines including HeLaMR, RajiMex$^-$ and GM1953(S) contain low but detectable levels of MGMT mRNA by this analysis. As expected, treatment of the RNA with RNase prior to PCR amplification abolished or severely reduced the signal from Mex$^+$ RNA and completely eliminated the signal from the Mex$^-$ RNA (Figure 8b).

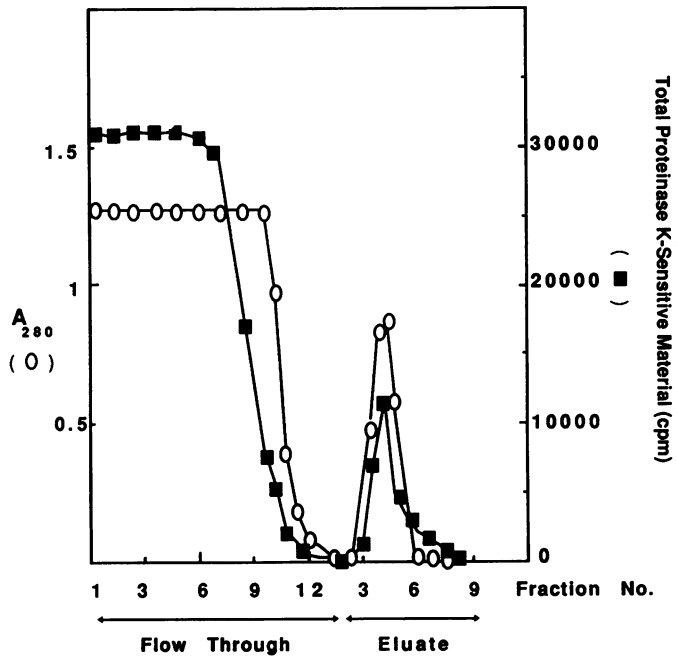

Figure 7. Fractionation of methylated MGMT on phosphocellulose P11. An extract of 5.10^8 Daudi cells was incubated with [^{3}H]-O^6-meGua-containing substrate DNA (50 pmole) and incubated at 37°C for 30min. Nucleic acids, including unreacted substrate, were removed by adsorbtion to DE52 and the unadsorbed material was mixed with P11 and fractionated as described in the legend to Figure 4. Aliquots of each fraction were digested with proteinase K (40 μg, 16 hrs) and the radioactivity of TCA-soluble material was determined by scintillation counting. (○) Absorbance: (■) [^{3}H]-MGMT.

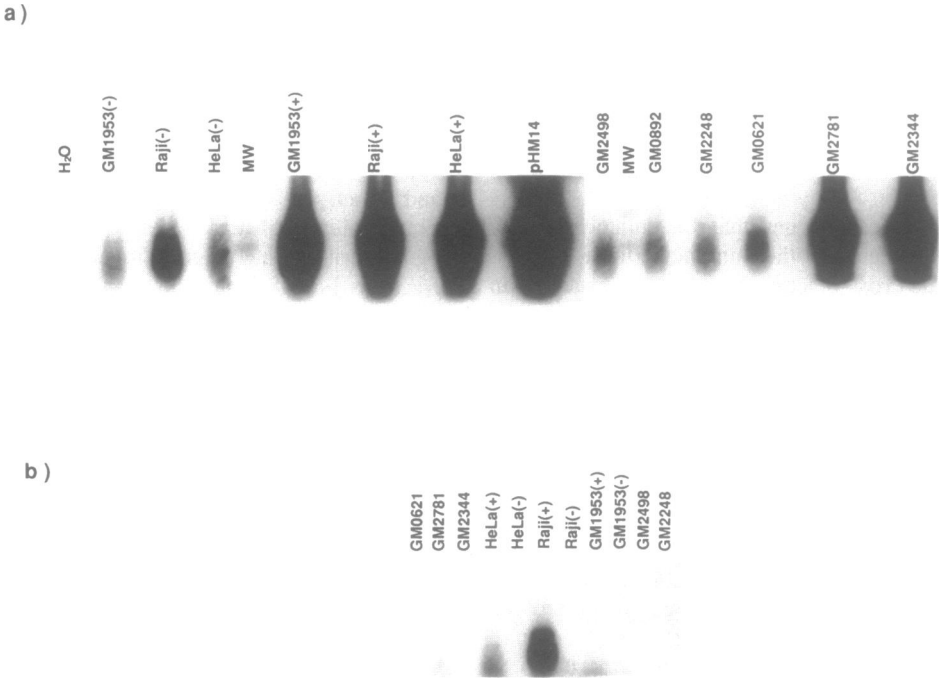

Figure 8. MGMT mRNA in Mex$^+$ and Mex$^-$ cells. 4μg total RNA was used to synthesize cDNA by random priming. MGMT cDNA was amplified through 40 cycles of the PCR as described in Methods. PCR products were separated on 1% agarose gels and MGMT sequences were detected by hybridization to [^{32}P]-labelled human MGMT cDNA. 50pg plasmid pHM14 which contains the human MGMT cDNA was included as a positive control for the PCR. A negative control (no cDNA; H_2O) was also included. MW designates the DNA molecular size markers (BRL; bacteriophage λ 1kb Ladder). b) The same RNA samples were treated with heat-treated RNase A (10 Kunitz units/ml; Sigma) for 30min, at 37°C, before preparation of cDNA. Products were analysed as above.

DISCUSSION

Rabbit antisera raised against the fusion protein encoded by the recombinant plasmid pNMT1 recognise a protein of the M_r of MGMT in immunoblots of crude extracts of all the Mex$^+$ human cell lines tested. The immunoreactive material is absent from several Mex$^-$ lines. Thus, immunoblotting using these polyclonal antisera can be used to detect and quantify the human MGMT protein in crude extracts of human cells. Based on comparisons between the immunoreactivity and enzymatic activity of the human MGMT, we estimate that the limit of detection by immunoblotting is approximately $5.10^9 - 10^{10}$ MGMT molecules or about 0.2ng MGMT protein. This limit is similar to the detection limit of the standard methyltransferase assay employing [^{3}H]-labelled O^6-methylguanine-containing DNA and represents a sensitivity comparable to those reported for monoclonal or polyclonal antibodies raised against MGMT-specific peptides (19,20).

There is a good quantitative relation between the methyltransferase activity and the level of immunoreactive material for several Mex$^+$ and Mex$^-$ human lymphoblastoid cell lines. In three lines designated Mex$^-$, MGMT protein was not detectable by immunological staining. Under the conditions of the assay, this indicates a lower limit of 5000 MGMT molecules per cell although by increasing the amount of cell extract analysed on the gel, a more precise minimum value could be defined. Similar correlations between methyltransferase activity and MGMT immunoreactive protein have recently been observed in human tumor cell lines (19,20).

HeLaS3 cells (and also Raji although our data are less extensive for this line) were an exception to the general correlation between methyltransferase activity and level of MGMT protein. These cells contained high levels of both MGMT activity and immunoreactive MGMT protein. However, the level of MGMT protein was higher than could be accounted for by the enzyme activity, suggesting that a fraction of the MGMT protein of these cells is inactive. A comparison of the relative specific activities of the MGMT protein in the Mex$^+$ lymphoblastoid and HeLaS3 cell lines indicated that in the latter up to 50% of the MGMT protein may be enzymatically impotent.

MGMT protein was also detectable in four Mex$^-$ cell lines: HeLaMR, RajiMex$^-$, GM0621 and GM1953(S). These cell lines were otherwise typical of Mex$^-$ lines in that cell extracts contained ≤ 0.01 units of active MGMT per mg protein. The MGMT protein in these Mex$^-$ extracts was around 10–30% of the levels in Mex$^+$ cells whereas enzyme activity was $<5\%$ of Mex$^+$ values. The absence of immunoreactive material from crude extracts of three other Mex$^-$ lymphoblastoid cell lines suggests that the protein of M_r = 24000 in the HeLaMR, RajiMex$^-$ and GM1953(S) lines is indeed MGMT protein and not an abundant protein that cross-reacts with MGMT antisera. The absence of detectable enzyme activity indicates that the MGMT protein observed in immunoblots of these three cell lines is also in an inactive form.

Since the steady-state level of MGMT mRNA in Mex$^-$ cells, including GM1953(S) and Raij(Mex$^-$) (6,8), is below the limit of detection by northern blotting, it seems paradoxical that they contain MGMT protein. However, analysis by a more sensitive PCR-based technique has indicated that low but detectable levels of MGMT mRNA may be present in some Mex$^-$ cell lines (9,21). Our own experiments using PCR amplication of MGMT cDNA, although non-quantitative, confirm the presence of MGMT mRNA in a high proportion of Mex$^-$ lines including HeLaMR, GM1953(S) and Raij(Mex$^-$) in which MGMT protein is also detectable.

There are two possible sources of inactive MGMT in cells: inactive MGMT molecules may be synthesized in the non-functional state or functional protein may have become inactivated. In the first case, inoperative enzyme would be produced from a mutated MGMT allele, the other allele being perhaps deleted or maintained in a transcriptionally silent state. Some recent evidence indicating that Mex$^-$ HeLaMR cells contain a mutated copy of the MGMT gene and produce a slightly truncated MGMT mRNA (21) would be consistent with this idea. In addition, there is considerable evidence to suggest that although the relationship is complex, DNA cytosine methylation underlies the Mex$^-$phenotype (10,11, S.Cairns-Smith and P.K., In Press). It seems possible therefore that a type of allelic exclusion (22) is responsible for the Mex$^-$ phenotype in some cell lines.

A second source of inactive protein is *via* demethylation of endogenous DNA O^6-methylguanine by a functional MGMT. This alternative is supported by our observation that the inactive MGMT in HeLaS3 cells shares some of the altered biochemical characteristics of the self-methylated protein. The methyl group donor, S-adenosylmethionine (S-Adomet) can act as a weak alkylating agent (23,24). Some Mex$^-$ lines, including Raji(Mex$^-$) and GM1953(S), exhibit an unusual generalized hypermethylation of CpG sequences (S.Cairns-Smith and P.K., In Press) that may be associated with increased concentrations of this methyl group donor. Although at normal intracellular concentrations, the amount of O^6-MeGua introduced into DNA by S-Adomet should be low (23), it is possible that imbalances in the intracellular concentration of S-Adomet lead to increased non-enzymatic DNA methylation with consequent inactivation of MGMT. While a definitive answer to these questions requires investigation of the presence of S-methylcysteine in the inactive protein, our preliminary biochemical characterization is consistent with its being methylated. The steady-state levels of inactive MGMT protein are low. However, the observation (12) that inactivated MGMT molecules are lost from cells at elevated rates suggests that what we observe might underestimate the amount of inactive MGMT produced in the cell.

In summary, our data obtained using polyclonal antisera against the human MGMT-λN fusion protein extend the quantitative relationship between MGMT activity and protein levels previously observed in human tumor cell lines (19,20) to a number of *in vitro* transformed lymphoblastoid lines. In addition, they define a sub-group of cell lines that contain detectable amounts of inactive MGMT protein that has some of the properties of automethylated MGMT.

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