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Inducibility of the DNA Repair Gene Encoding O^6-Methylguanine-DNA Methyltransferase in Mammalian Cells by DNA-Damaging Treatments

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The inducibility of the mammalian O^6-methylguanine-DNA methyltransferase (MGMT) gene encoding the MGMT protein (EC 2.1.1.63) responsible for removal of the procarcinogenic and promutagenic lesion O^6-alkylguanine from DNA was examined by an analysis of transcription of the MGMT gene following exposure of repair-competent (Mex^+) and repair-deficient (Mex^-) cells to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). While human and rodent Mex^- cells (CHO-9, V79, HeLa MR) showed no detectable MGMT mRNA despite the presence of the gene in their genome, the amount of it in several Mex^+ lines (NIH 3T3, HeLa S3, HepG2) paralleled their MGMT activity. However, none of these cell lines showed an increase in the MGMT mRNA level after treatment with various concentrations of MNNG. In contrast, MNNG-treated rat hepatoma cells, H4IIE and FTO-2B, both Mex^+, had three- to fivefold more MGMT mRNA than the corresponding untreated controls as measured 12 to 72 h after alkylation. *N*-Methyl-*N*-nitrosourea, methyl methanesulfonate, *N*-hydroxyethyl-*N*-chloroethylnitrosourea, UV light, and X rays caused a similar accumulation of MGMT mRNA in rat hepatoma cells. Studies with inhibitors of RNA and protein synthesis indicate that the induced increase in the amount of MGMT mRNA was due to enhanced transcription of the gene. Furthermore, they revealed the turnover of the MGMT mRNA to be relatively low (half-life, >7 h). Mutagen-induced increase of transcription of MGMT mRNA in H4IIE cells was accompanied by elevation of MGMT repair activity and resulted in reduction of mutation frequency after a challenge dose of MNNG. Although induction of MGMT mRNA transcription has been observed in two rodent hepatoma cell lines so far, this appears to be the first demonstration of inducibility of a mammalian gene encoding a clearly defined DNA repair function. The transcriptional activation of the MGMT gene protects cells from the mutagenic effects of methylating agents.

Among the lesions generated by monofunctional alkylating agents in DNA, the minor alkylation product O^6-alkylguanine is responsible for induction of most of the mutations and malignant transformation of mammalian cells, because of its mispairing properties (34, 39, 48, 55). O^6-Alkylguanine is repaired by the O^6-methylguanine-DNA methyltransferase (MGMT) by transfer of the alkyl group to a cysteine residue of the repair protein itself, resulting in its inactivation and restoration of guanine in DNA (9, 11, 52).

In *Escherichia coli* there are two MGMT proteins of 39 and 19 kDa encoded by the *ada* and the *ogt* genes, respectively (33). The *ada* gene is inducible by alkylating agents, giving rise to adaptation of cells to the mutagenic and killing effects of alkylating compounds (49), and the Ada protein itself is involved in regulation of this adaptive response (33).

Mammalian cells show considerable variations of MGMT activity. Several established rodent lines (e.g., V79 and CHO) and 20 to 30% of human tumorigenic cell lines do not possess detectable MGMT activity; these lines are defined as Mex^- or Mer^- (8, 54, 61). In vivo, MGMT activity is dependent on the type of tissue studied and varies with species and developmental stage (19, 20, 60; for a recent review, see reference 40), indicating a cell- and tissue-type specific regulation of the gene. Whether the mammalian MGMT gene is inducible by alkylation and other stress factors has been a matter of controversy for years. Thus, there are reports both in favor of (24–26, 31, 50) and against (16, 30) an adaptive response in mammalian cells as measured by the end points of cell killing, mutagenesis, sister chromatid exchange, and aberration formation. At the protein level, most cell lines failed to respond with an increase of MGMT activity after alkylation (10).

An enhancement of MGMT activity has been observed in the rat hepatoma cell line H4 after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and other agents (18, 32) and for mouse C3H/10T1/2 cells after X irradiation (57). For human cells, reports are conflicting (10, 58). The most consistent increase of MGMT activity and O^6-methylguanine repair in DNA has been found in rat liver cells in vivo. The activity was elevated by treatment of the animals with a variety of hepatotoxins, including dialkylnitrosamines (5, 38, 41), 2-acetylaminofluorene (5), aflatoxin B_1 (4), and bleomycin (51) or by partial hepatectomy (42) and whole body X irradiation (37). However, the two- to fivefold increase in MGMT activity observed in these cases was smaller than that found in adapted *E. coli*. Thus, the possibility that this increase is due not to transcriptional activation of the MGMT gene but to stabilization of mRNA, posttranslational modifications, or cell-cycle changes cannot be excluded. Even the magnitude of induction after alkylation is uncertain because the inducing signal, the alkylation, may inactivate the MGMT protein; this may lead to underestimation of the

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amount of MGMT, because only the active protein was assayed.

The recent cloning of human MGMT cDNA (21, 47, 56) provides an opportunity for elucidating the genetic basis of the Mex^- phenotype and the mechanism of regulation of the gene, including its inducibility. In this report, we give evidence for the induction of the MGMT gene in certain rodent liver cell lines following exposure to genotoxic treatments.

MATERIALS AND METHODS

Cells and culture conditions. The cell lines V79 and CHO-9 (used in previous studies [26, 29]), HeLa MR (kindly provided by R. Goth-Goldstein, Lawrence Berkeley Laboratory, Berkeley, Calif.), HeLa S3, HeLa CCL2 (purchased from the American Type Culture Collection), VH44 (a diploid human fibroblast cell line), GM637 (a simian virus 40-transformed human fibroblast cell line), F9 (mouse teratocarcinoma cells) and FTO-2B (kindly provided by G. Ryffel, Institut für Zellbiologie, Essen, Germany) were cultivated in F10-Dulbecco's modified Eagle medium (1:1) containing 10% inactivated fetal bovine serum. NIH 3T3 and HepG2 cells (provided by G. Ryffel) were cultivated in Dulbecco's modified Eagle medium containing 10% inactivated fetal bovine serum. The H4IIE cell line (43) (the line is designated as H4 throughout this work) was obtained from K.-L. Lee (Oak Ridge National Laboratory). The cells were cultivated in alpha modified Eagle medium containing 5% inactivated fetal bovine serum. All media and the serum were purchased from GIBCO and were supplemented with 30 μg of gentamicin per ml.

Drug treatments and irradiation. Cells (5×10^5) were seeded in 2.5-cm dishes containing 2.5 ml of medium and treated 24 h later with MNNG by addition of the drug directly to the medium. MNNG and *N*-methyl-*N*-nitrosourea (MNU) were dissolved in dimethyl sulfoxide, diluted with distilled water, and stored in batches at −80°C. Methyl methanesulfonate (MMS), *N*-chloroethylnitrosourea (CNU), and *N*-hydroxyethyl-*N*-chloroethylnitrosourea (HeCNU) were dissolved in distilled water. After addition of the chemicals, the medium was not changed to avoid feeding effects. For UV irradiation (254 nm), the medium was removed from the plates, and the cells were irradiated and fed. Anisomycin and actinomycin D (Sigma) were added to the medium to final concentrations of 100 μM and 5 μg/ml, respectively.

DNA and RNA extraction. High-molecular-weight DNA was prepared as described previously (28). For total RNA extraction, the cells were rinsed with cold phosphate-buffered saline (PBS) and lysed on the dishes with 250 μl of 4 M guanidinium thiocyanate–25 mM sodium citrate (pH 7.0)–0.5% Sarkosyl–0.1 M 2-mercaptoethanol (3). The lysates of two dishes per treatment were combined and, after addition of 50 μl of 3 M sodium acetate (pH 4.0), were extracted with water-saturated phenol and chloroform-isoamyl alcohol (24:1). The RNA was precipitated with 2.5 volumes of cold ethanol. Poly(A)$^+$ mRNA was extracted by lysis of cells in sodium dodecyl sulfate (SDS) buffer (0.5% SDS, 100 mM NaCl, 10 mM EDTA, 20 mM Tris-Cl, pH 7.4), shearing of DNA by repeated pipetting, and digestion with proteinase K (37°C, 1 h). The solution was extracted with chloroform-isoamyl alcohol (24:1) and adjusted to 0.5 M NaCl, and mRNA was purified by binding to oligo(dT)-cellulose (type 7, Pharmacia; 100 mg per 10^7 cells) overnight. After washing (three times) with 0.1% SDS–300 mM NaCl–5 mM EDTA–10 mM Tris-Cl, pH 7.5, poly(A)$^+$ mRNA was eluted in sterile distilled water and ethanol precipitated.

DNA and RNA blot hybridizations. DNA was digested to completion with *Hin*dIII, separated on a 0.8% agarose gel in Tris-borate buffer, and immobilized on Hybond N$^+$ (Amersham) by transfer with 0.4 N NaOH. RNA was separated on 1.2% formaldehyde agarose gels, transferred overnight on Hybond N$^+$ with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and fixed onto the membrane by incubation in 50 mM NaOH (5 min). Prehybridization was performed for 2 h in 7% SDS (Bio-Rad)–1 mM EDTA–0.5 M phosphate buffer (pH 7.0). Hybridization occurred overnight in the same solution containing, in addition, 1% bovine serum albumin and the ^{32}P-labelled denatured probe (10^6 to 10^7 cpm/ml; specific activity, about 10^9 cpm/μg). The filters were washed twice in 2× SSC and once in 1× SSC (Northern [RNA] blot) or 0.5× SSC (Southern blot) containing 1% SDS and 1 mM EDTA. All steps were performed at 65°C. The MGMT probe consisted of the *Eco*RI fragment of the plasmid pKT100, comprising the 835-bp cDNA (56). In control hybridizations, a 1.3-kb *Pst*I fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (15), c-*myc* or ^{32}P-end-labelled 28S RNA was used. The DNA was labelled with [^{32}P]dCTP (3,000 Ci/mmol; Amersham) by using the multiprime T7 polymerase kit (Stratagene) according to the manufacturer's protocol. If not specified, total RNA was used for hybridization. mRNA was quantified by densitometric scanning of films that were exposed such that label intensity was in the linear range. Induction factors were calculated in relation to GAPDH. Previously, it was shown that this gene is not inducible by genotoxic treatments (45).

Determination of MGMT activity. About 10^6 exponentially growing cells were treated with the mutagen, cultured for 48 h, and harvested by trypsinization. Cells were rinsed with PBS, counted, and sonicated for 15 s in 50 mM Tris-Cl (pH 8.0)–1 mM EDTA–1 mM dithiotreitol–10% glycerol. Debris were removed by centrifugation. Cell extract (equivalent of 10^6 cells) was incubated with 1 pmol (2,800 cpm) of [8-^{3}H]m^{6}G in poly(dC, dG, m^{6}dG) for 4 h at 37°C. After treatment with proteinase K, the DNA was extracted with phenol-chloroform, hydrolyzed (40 min, 70°C, 0.1 N HCl), and subjected to high-pressure liquid chromatography with an Aminex A-6 column as described previously (10). The amounts of O^6-methylguanine and guanine in the samples were determined by scintillation counting. MGMT activity (expressed as molecules per cell) was calculated from the proportion of [^{3}H]guanine from the total radioactivity ([^{3}H]guanine plus O^6-methyl[^{3}H]guanine) recovered.

Survival and mutation assay. For determination of cell survival, 500 cells were seeded and treated 6 h later with different doses of MNNG. Colonies were fixed, stained, and counted 10 days later. Mutation induction was measured at the hypoxanthine-guanine phosphoribosyltransferase locus. Cells (2×10^5) were seeded and treated 1 day later with X rays or MNNG. Forty-eight hours thereafter, cells were treated with the MNNG challenge dose. Cells were cultured for a total expression period of 7 days (passage after day 4) and reseeded (2×10^5 cells per 10-cm dish) in medium containing 6-thioguanine (1 μg/ml) and in medium without the drug (10^3 cells) for determination of cell survival. Ten days thereafter, colonies were fixed, stained, and counted. Mutation frequency was given as number of 6-thioguanine-resistant cells/(number of cells seeded × plating efficiency).

RESULTS

Constitutive MGMT mRNA expression. To study the constitutive expression of the MGMT gene and to see whether cells lacking MGMT activity harbor the MGMT gene in their genome, DNA and RNA of various Mex$^-$ (CHO-9, V79, HeLa MR) and Mex$^+$ (HeLa S3, HeLa CCL2, VH44, NIH 3T3, F9, GM637) cell lines were analyzed in blot hybridization experiments. Although the MGMT restriction patterns of rodent and human cells differ, all the Mex$^-$ cell lines showed MGMT sequences in their genome. Interestingly, Chinese hamster, mouse, and human cells contain a 6.5-kb *Hin*dIII fragment that seems to be conserved among the species (Fig. 1A). No MGMT mRNA could be detected in the Mex$^-$ cells from lines CHO-9, V79, and HeLa MR. The amount of MGMT mRNA in Mex$^+$ cells varied considerably, with the highest levels in HeLa S3 and HeLa CCL2. The mRNA levels roughly paralleled the MGMT activity in these cell lines (Fig. 1B).

Induction of MGMT transcription after MNNG treatment. (i) Mex$^-$ cells. Considering the studies performed with V79 and CHO cells to explore the presence of an adaptive response in mammalian cells (for a review, see reference 17), it was of interest to see whether expression of the MGMT gene in Mex$^-$ cells can be induced by MNNG treatment. With a range of MNNG doses from 1 nM to 15 μM, in neither V79 nor HeLa MR cells could MGMT mRNA be detected 4 and 12 h after MNNG treatment (Fig. 2).

(ii) Mex$^+$ cells. For studies with Mex$^+$ cells, we initially chose cell lines expressing either low (NIH 3T3) or high (HeLa S3) MGMT activity. As shown in Fig. 2, there was no increase of MGMT mRNA expression in HeLa S3 and NIH 3T3 cells treated with various doses of MNNG, as measured 4 and 12 h after alkylation (both in terms of absolute amount and in relation of GAPDH; quantitation not shown). The MGMT mRNA levels in NIH 3T3 cells in the period up to 12 h after treatment with 5 μM MNNG showed no increase (see Fig. 2).

A cell line for which MGMT repair activity was demonstrated to increase upon alkylation is the rat hepatoma cell line H4 (32). We therefore investigated whether rat hepatoma cells show enhanced MGMT transcription after exposure to an alkylating agent. H4 cells did indeed respond to MNNG treatment with accumulation of MGMT mRNA (see Fig. 3 for Northern blots and Fig. 4A and B for the relative amount of MGMT mRNA as a function of MNNG dose and time after treatment). The earliest time point for which the effect was clearly observed was 12 h after treatment with 1 and 5 μM MNNG. MGMT mRNA overexpression in H4 cells (three- to fivefold, compared with the nontreated control) lasted up to 72 h after MNNG treatment (Fig. 3B and 4). The MGMT mRNA accumulation in H4 cells was inhibited completely by actinomycin D, given either immediately or 6 h after addition of MNNG to the medium (Fig. 3C). This strongly indicates that increased MGMT mRNA levels were due to stimulation of transcription of the MGMT gene after alkylation.

Induction of MGMT mRNA transcription and repair activity after exposure to various DNA-damaging treatments. Induction of MGMT transcription after MNNG treatment was accompanied by increase of O^6-methylguanine DNA repair capacity of the cells, as measured by the ability of cell extracts to convert O^6-methylguanine to guanine in a synthetic DNA template. As shown in Fig. 4D, maximal increase of MGMT activity was found with a dose of 10 μM MNNG. Although the amount of MGMT mRNA increased with 15 μM MNNG, no further enhancement of MGMT activity was observed. This is probably due to inactivation of MGMT by the high MNNG dose, either directly or indirectly because of the repair reaction performed. Inactivation of the MGMT protein is a reasonable explanation, in the case of alkylation by MNNG, for the difference in induction as measured on the protein (about twofold) and mRNA (about fivefold) levels.

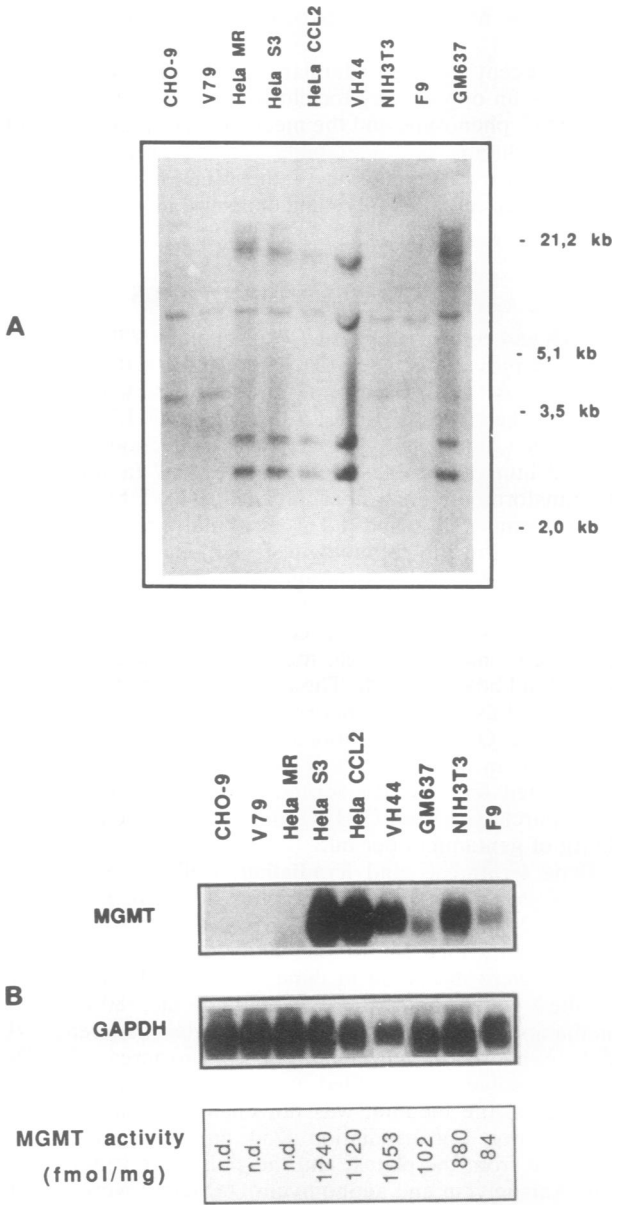

FIG. 1. Southern and Northern blot analyses of DNA and mRNA of various Mex$^-$ and Mex$^+$ cell lines. (A) DNA (10 μg) digested with *Hin*dIII and (B) poly(A)$^+$ mRNA (5 μg) separated by gel electrophoresis were hybridized with ^{32}P-labelled MGMT cDNA. Rehybridization of the RNA filter occurred with a ^{32}P-labelled GAPDH probe. MGMT activities were expressed as femtomoles per milligram of protein. n.d., not detectable.

Interestingly, the lowest MNNG doses that gave rise to MGMT gene induction exerted only slight reproductive cell death. Thus, treatment with 5 μM MNNG, which clearly induced MGMT, reduced cell survival (colony formation) by

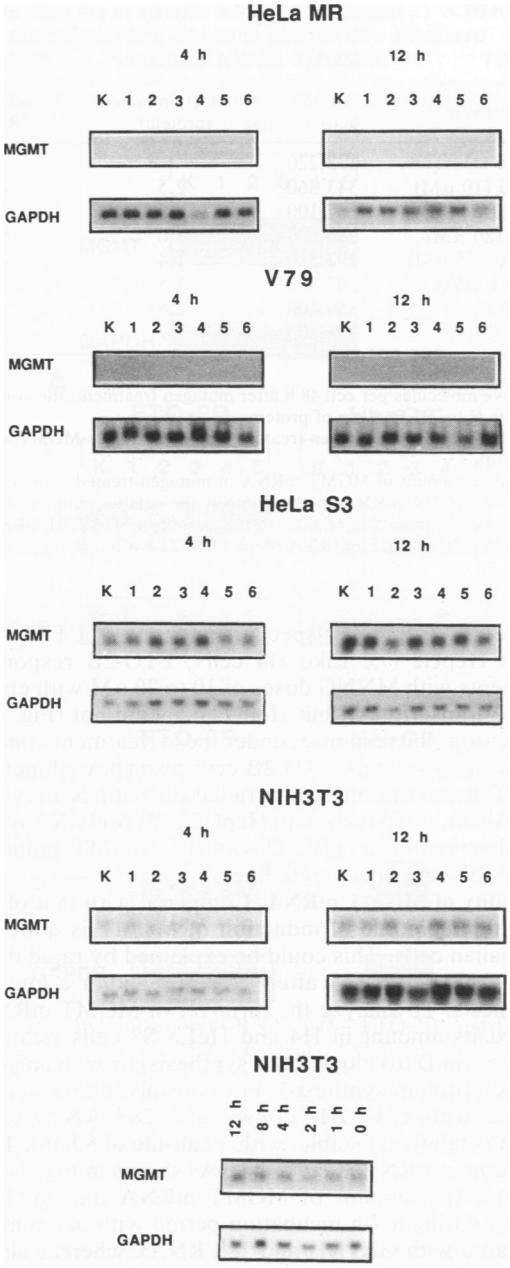

FIG. 2. Blot hybridization of RNA of HeLa MR, V79, HeLa S3, and NIH 3T3 cells with ^{32}P-labelled MGMT cDNA. RNA was extracted 4 and 12 h after addition of MNNG to the medium. MNNG concentrations for the lanes were as follows: K, control (no MNNG); 1, 1 nM; 2, 10 nM; 3, 100 nM; 4, 1 μM; 5, 5 μM; 6, 15 μM. About 10 μg of total RNA (extracted from 10^6 cells) was applied per lane. Filters were rehybridized with a ^{32}P-labelled GAPDH probe. Bottom panel: NIH 3T3 cells were exposed to 5 μM MNNG and lysed at the indicated time points.

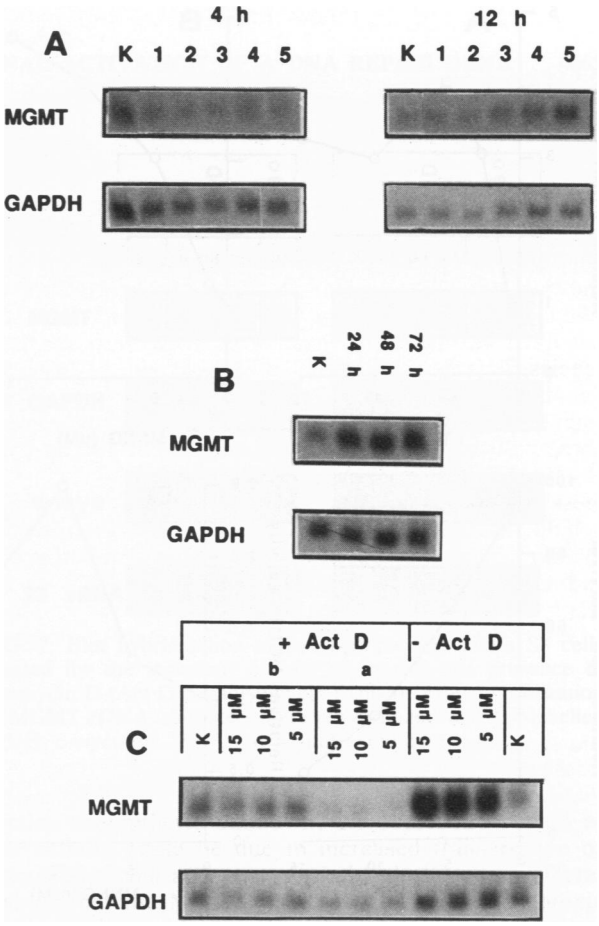

FIG. 3. Blot hybridization of RNA of control and MNNG-treated H4 cells with MGMT cDNA. (A) Dose dependence of MGMT mRNA levels 4 and 12 h after MNNG exposure (MNNG concentrations as in legend to Fig. 2). (B) Kinetics of MGMT mRNA accumulation of cells treated with 5 μM MNNG. (C) Inhibition of MNNG-induced MGMT transcription by actinomycin D. Cells were treated with the indicated MNNG concentrations (K, control) and incubated for 12 h. Actinomycin D was added immediately after MNNG addition (a) or 6 h later (b).

less than 40% (Fig. 4C). In the period of induction, up to 72 h after MNNG treatment, no toxic effects (morphologic and growth alterations of cells) were observed (data not shown).

To study whether MGMT induction is a specific response to MNNG treatment or a general genotoxic stress-induced phenomenon, we measured MGMT mRNA and MGMT repair activity after exposure of H4 cells to various alkylating and nonalkylating mutagens. As shown in Fig. 5 and Table 1, the amount of MGMT mRNA in H4 cells was enhanced not only upon treatment with MNNG, but also in response to MNU, MMS, and the chloroethylating compound HeCNU. There was also an increase of MGMT mRNA after UV irradiation or irradiation with X rays. The MGMT activity in H4 cells increased after alkylation or UV and X-ray exposure by a factor of 1.4 to 4.8, which roughly corresponds to the induction observed on the mRNA level (Table 1).

Transcriptional activation of the MGMT gene protects cells from alkylation-induced mutagenesis. To see whether induction of the MGMT gene is of biological significance (with respect to reduction of genotoxicity of alkylating agents), we measured the mutation frequencies of H4 cells not pretreated and those pretreated with X rays or MNNG (Table 2). Cells preexposed to doses that gave rise to stimulation of MGMT transcription and challenged 48 h later with MNNG showed reduction of the frequency of mutations induced by the challenge dose (no additive effect of pretreatment and challenge). Obviously, transcriptional activation of the MGMT gene by diverse agents such as X rays and MNNG

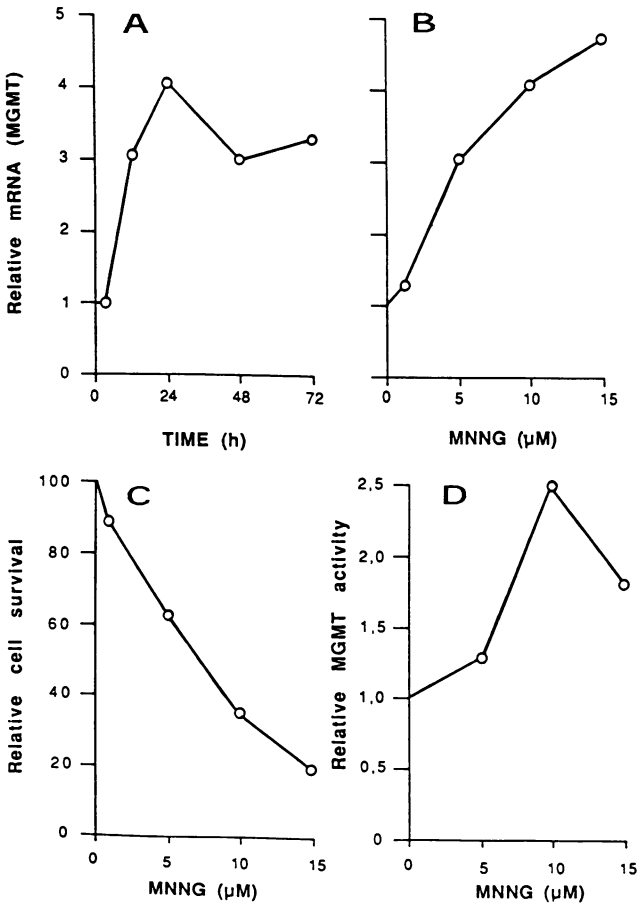

FIG. 4. MGMT mRNA level as a function of postincubation time (A) and MNNG dose (B) and effect of MNNG treatment on cell survival (C) and MGMT activity (D). The amounts of MGMT mRNA and MGMT activities were expressed relative to those of nontreated (control) cells. (A) Cells were treated with 5 μM MNNG. (B) Cells were postincubated for 12 h.

rendered cells more resistant to the mutagenic effects of a subsequent dose of an alkylating agent.

Comparison of MGMT induction in different hepatocyte lines. To elucidate whether stimulation of MGMT transcription is a general liver-specific response to mutagens, we measured MGMT mRNA expression in two other cell lines

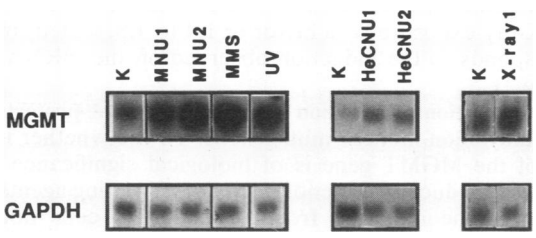

FIG. 5. Blot hybridization of RNA of H4 cells not exposed (K) and exposed to MNU, MMS, HeCNU, UV light, or X rays. Total RNA was extracted 24 h after mutagen treatment, hybridized with the MGMT cDNA, and rehybridized with a GAPDH probe. Treatments for the lanes were as follows: MNU1, 0.4 mM; MNU2, 1 mM; MMS, 0.2 mM; HeCNU1, 75 μM; HeCNU2, 150 μM; UV, 10 J/m^2; X-ray1, 2 Gy.

TABLE 1. Increase of MGMT activity in H4 cells after treatment with various mutagens and comparison to MGMT mRNA induction[a]

Treatment	MGMT activity[b]	Induction factor (protein)[c]	Induction factor (RNA)[d]
Control	138,220		
MNNG (10 μM)	343,360	2.5	4.2
MMS (0.2 mM)	667,100	4.8	3.8
CNU (120 μM)	280,500	2.0	ND
HeCNU (75 μM)	193,510	1.4	4.0
MNU (1 mM)	207,330	1.5	3.8
UV (10 J/m^2)	359,400	2.6	3.2
X rays (2 Gy)	373,200	2.7	2.5

[a] ND, not determined.
[b] Active molecules per cell 48 h after mutagen treatment; the control level corresponds to 747 fmol/mg of protein.
[c] MGMT activity in mutagen-treated cells in relation to MGMT activity of the control.
[d] Relative amount of MGMT mRNA in mutagen-treated cells per relative amount of MGMT mRNA in the control; the relative amount of MGMT mRNA was determined in relation to the quantity of GAPDH mRNA in the rehybridized Northern blot (RNA preparation 24 h after mutagen addition).

reported to exert liver-specific functions, rat FTO-2B and human HepG2 (7). Like H4 cells, FTO-2B responded to treatments with MNNG doses of 10 to 20 μM with enhanced MGMT transcription, but HepG2 cells did not (Fig. 6A; for comparison, the response, under these treatment conditions, of H4 cells is shown). FTO-2B cells also showed increase of MGMT transcription after irradiation with X rays or UV light. Again, no induction in HepG2 cells (only X-ray tested) was observed (Fig. 6B). Obviously, MGMT induction is limited to some hepatocyte lines.

Stability of MGMT mRNA. Compared with that of *E. coli* (33), the magnitude of induction of MGMT is quite low in mammalian cells. This could be explained by rapid degradation of MGMT mRNA after induction and/or a low rate of resynthesis. To analyze the turnover of MGMT mRNA, we assayed its amount in H4 and HeLa S3 cells treated with actinomycin D (to block RNA synthesis) or with anisomycin (to block protein synthesis). For controls, filters were rehybridized with GAPDH, c-*myc*, and 28S RNA. GAPDH mRNA is relatively stable, with a half-life of 8 h (6). The half life of c-*myc* mRNA is 0.5 h (44). As shown in Fig. 7, in both cell lines the amount of MGMT mRNA did not become reduced during a 7-h incubation period with actinomycin D (compared with GAPDH and 28S RNA), whereas already 1

TABLE 2. Mutation frequencies of H4 cells under MGMT-inducing and -noninducing conditions

Treatment	Mutation frequency (10^{-5})	Effect of challenge (corrected)[a]
Control (without treatment)	2.6 ± 0.4	
X-ray pretreatment (2 Gy)	7.6 ± 0.5	
MNNG pretreatment (5 μM)	17.6 ± 6.1	
MNNG challenge (15 μM)	33.6 ± 10.7	31.0
X-ray pretreatment + MNNG challenge	27.5 ± 9.1	19.9
MNNG pretreatment + MNNG challenge	33.0 ± 10.7	15.4

[a] The effect of an MNNG challenge dose was calculated by subtracting the control or pretreatment-induced mutation frequency from the challenge- or challenge-plus-pretreatment-induced mutation frequency (corrected effect of challenge). Data are the means of two to five independent experiments ± standard deviation. Mutation frequency is expressed as 6-thioguanine-resistant colonies per 10^5 survivors.

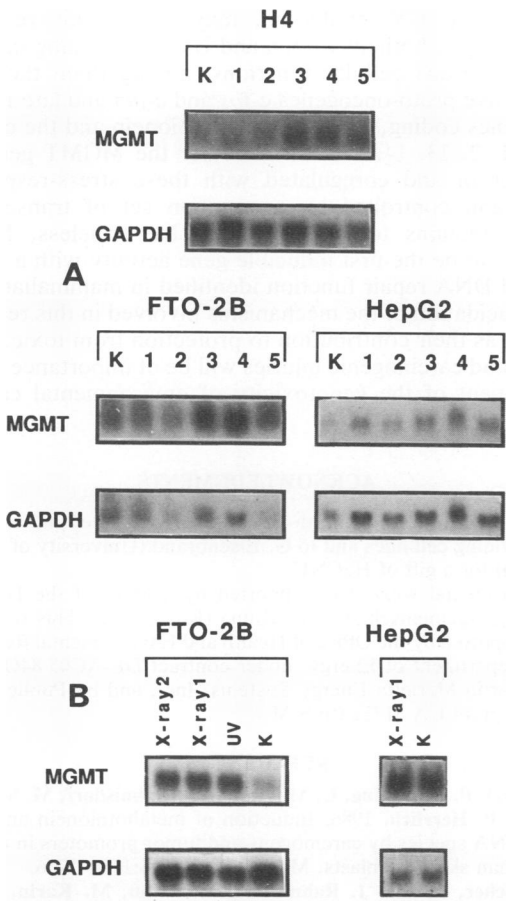

FIG. 6. Blot hybridization of RNA of H4, FTO-2B, and HepG2 cells exposed to various genotoxic agents. (A) Treatments of cells with MNNG for the lanes were as follows: K, control; 1, 1 μM; 2, 5 μM; 3, 10 μM; 4, 15 μM; 5, 20 μM. RNA was extracted 24 h after addition of MNNG to the medium. (B) Treatments of cells for the lanes were as follows: X-ray1, 2 Gy; X-ray2, 3 Gy; UV, 30 J/m^2; K, control. RNA was extracted 48 h after irradiation.

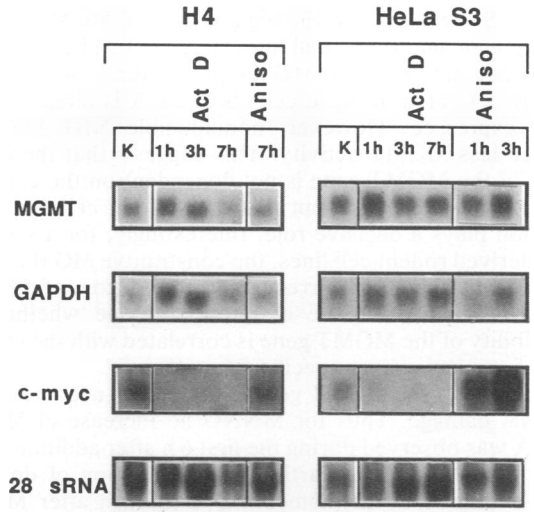

FIG. 7. Blot hybridization of RNA of H4 and HeLa S3 cells incubated for the indicated periods of time in the presence of actinomycin D (Act D) or anisomycin (Aniso). After hybridization with MGMT cDNA, the filters were rehybridized with ^{32}P-labelled GAPDH, c-*myc*, and 28S RNA. K, untreated control.

h after drug treatment, no c-*myc* mRNA could be detected. This indicates that the MGMT mRNA is quite stable, with a half-life longer than 7 h. Treatment with MNNG prior to actinomycin D gave essentially the same results as actinomycin D treatment alone (data not shown), suggesting that alkylation does not significantly reduce MGMT mRNA stability.

For several mRNAs, including c-*myc* and c-*fos* mRNA, anisomycin or cycloheximide was shown to give rise to mRNA accumulation due to prevention of mRNA degradation (36, 46). In our experiments with HeLa S3 cells, anisomycin (treatment of cells for 1 and 3 h, respectively) did not alter the amount of MGMT transcripts, although it induced a marked accumulation of c-*myc* mRNA (Fig. 7). This suggests that the MGMT gene is transcribed at a low constitutive level.

DISCUSSION

Increase of MGMT repair activity was observed both in vivo, in rat liver cells after genotoxic treatments of the animals (for a recent review, see reference 40) and in vitro, in rat hepatoma H4 and mouse C3H/10T1/2 cells exposed to alkylating agents and X rays (18, 32, 57). The mechanism of induction was hitherto unknown; the observed increase of repair activity could be due to increased transcription or posttranscriptional and posttranslational alterations. Here, we show that the MGMT gene is inducible by genotoxic treatments. Accumulation of MGMT mRNA was observed in two rat cell lines, H4 and FTO-2B, both of which have been reported to express liver-specific functions (7, 47a). We detected an increase of the amount of MGMT mRNA after alkylation with MNNG, MNU, MMS, and the chloroethylating compound HeCNU and, interestingly, also following X-ray treatment and UV irradiation. For those agents tested, induction of MGMT transcription was accompanied by increase of MGMT repair activity. Because the low-level O^6-methylguanine-inducing agent MMS, UV light, and X rays were effective inducers, we conclude that O^6-methylguanine is not the (only) signal giving rise to MGMT induction on rat hepatoma cells. We did not observe MGMT induction in the human hepatoma cell line HepG2. In this respect, it is interesting that no adaptive increase of MGMT activity was observed in human liver cells following alkylation (59). It might be that induction of the MGMT gene is a liver-specific response to genotoxic stress limited to rodent cells. To draw general conclusions, however, further studies on hepatocytes and nonhepatocytes of various species are needed.

The conclusion that the MGMT gene is inducible is based on inhibition experiments. In run-on experiments, we could not detect MGMT-specific transcripts in H4 cells, probably because of low constitutive expression of the gene. Although it is unlikely, we cannot completely exclude the possibility that MNNG and other DNA-damaging agents activate a gene, the product of which causes increase of MGMT mRNA stability.

Recently, a lack of MGMT induction has been reported for a human fibroblast and rodent cell line exposed to various genotoxic agents for 0.5 to 12 h (12). We also did not find transcriptional activation of the MGMT gene in diploid human fibroblasts, even if long postincubation periods were chosen (data not shown). Furthermore, there was no MGMT induction in HeLa S3, NIH 3T3, and HepG2 cells.

HeLa S3 cells express the highest level of MGMT activity of all human and rodent cell lines tested (10). Therefore, one could speculate that the MGMT gene cannot be transcriptionally activated in these cells because it is already maximally expressed. However, noninducible NIH 3T3 cells showed less MGMT activity. This suggests that the inducibility of the MGMT gene is not dependent on the constitutive level of expression but that the state of cellular differentiation plays a decisive role. Interestingly, for a series of liver-derived rodent cell lines, the constitutive MGMT repair activity was shown to correlate with the degree of differentiation (22). It would be of interest to see whether the inducibility of the MGMT gene is correlated with the expression of particular liver-specific functions.

Induction of the MGMT gene in H4 cells is a late response to DNA damage. Thus for MNNG no increase of MGMT mRNA was observed during the first 6 h after addition of the agent to the medium. Furthermore, inhibition of de novo RNA synthesis by actinomycin D 6 to 12 h after MNNG addition completely prevented MGMT mRNA accumulation. MGMT mRNA is relatively stable (comparable to GAPDH [6]), with a half-life longer than 7 h. In this respect, it differs from several DNA damage-inducible short-lived transcripts, e.g., c-*fos* and c-*jun* (46, 53). Another difference from these DNA damage-inducible genes (2, 23, 27, 53) is that transcriptional activation is maintained for long periods of time (up to 72 h after alkylation). It is tempting to speculate that, under conditions of continuous exposure to alkylating agents, it is advantageous for the cell to synthesize MGMT continuously at a slightly elevated level. This interpretation is supported by our finding that under conditions that induce the MGMT gene, the frequency of mutations induced by a challenge dose of MNNG was reduced in H4 cells that had been preexposed to MNNG or X rays.

In Mex^- HeLa MR, Chinese hamster V79, and CHO cells, we could detect MGMT transcripts neither under nonalkylated nor under alkylated conditions, although these cells appear to contain the MGMT gene. Obviously, the lack of MGMT activity in these cells is not due to synthesis of an inactive repair protein, but probably to down-regulation of the gene activity. Similar conclusions have recently been derived from studies of constitutive MGMT mRNA expression of other Mex^- cell lines (12, 47). Considering the lack of MGMT transcripts of V79 cells in Northern blots, the observation of an adaptive response in these cells is intriguing. Mutagenic and clastogenic adaptations were obtained after a single treatment of V79 cells with a very low nonmutagenic dose of MNNG (24, 26). By extrapolating dose-response curves for induction of O^6-methylguanine in Chinese hamster cells (29), the amount of O^6-methylguanine induced during the adaptation period in V79 cells is estimated to be less than 10^3 molecules per cell. Because a similar MGMT level is at the limit of detection, we cannot exclude the possibility that these cells possess a very low constitutive, and even inducible, expression of the MGMT gene undetectable by the methods employed. Other explanations should also be considered, such as activation or induction of repair functions other than MGMT that give rise to the adaptive phenomena observed.

Our finding of an increased transcription of the MGMT gene in H4 and FTO-2B cells after alkylation, accompanied by enhanced MGMT repair activity and reduced MNNG-induced mutation frequency, strongly indicates the existence of an adaptive response in some mammalian cell lines (rat hepatoma cells). In such cells, as opposed to *E. coli*, not only alkylation but also other DNA-damaging treatments, such as X rays and UV irradiation, may trigger this response. Interestingly, both alkylation and UV and ionizing radiation induce various cellular functions, among them the early-responsive proto-oncogenes c-*fos* and c-*jun* and late-responsive genes coding, e.g., for metallothionein and the collagenase (1, 2, 13, 14, 27, 35). Whether the MGMT gene is a member of and coregulated with these stress-responsive genes and controlled by a common set of transcription factors remains to be elucidated. Nevertheless, MGMT appears to be the first inducible gene activity with a clearly defined DNA repair function identified in mammalian cells. The elucidation of the mechanisms involved in this response as well as their contribution to protection from toxic, mutagenic, and carcinogenic injuries will be of importance for the assessment of the genotoxicity of environmental carcinogens.

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