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Protection against chloroethylnitrosourea cytotoxicity by eukaryotic 3-methyladenine DNA glycosylase

(environmental exposure/DNA alkylation/DNA repair/chemotherapy/tumor resistance)

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ABSTRACT **A eukaryotic 3-methyladenine DNA glycosylase gene, the *Saccharomyces cerevisiae MAG* gene, was shown to prevent *N*-(2-chloroethyl)-*N*-nitrosourea toxicity. Disruption of the *MAG* gene by insertion of the *URA3* gene increased the sensitivity of *S. cerevisiae* cells to *N*-(2-chloroethyl)-*N*-nitrosourea, and the expression of *MAG* in glycosylase-deficient *Escherichia coli* cells protected against the cytotoxic effects of *N*-(2-chloroethyl)-*N*-nitrosourea. Extracts of *E. coli* cells that contain and express the *MAG* gene released 7-hydroxyethylguanine and 7-chloroethylguanine from *N*-(2-chloroethyl)-*N*-nitrosourea-modified DNA in a protein- and time-dependent manner. The ability of a eukaryotic glycosylase to protect cells from the cytotoxic effects of a haloethylnitrosourea and to release *N*-(2-chloroethyl)-*N*-nitrosourea-induced DNA modifications suggests that mammalian glycosylases may play a role in the resistance of tumor cells to the antitumor effects of the haloethylnitrosoureas.**

The toxicity associated with exposure to DNA-damaging agents is of particular concern because it poses a serious risk of genetic damage. As a consequence, cellular mechanisms that protect against this damage are also important and are of current interest.

Escherichia coli cells exposed to low concentrations of methylating agents respond with a well-characterized adaptive response that increases their resistance to the toxic and mutagenic effects of DNA methylation (1, 2). This response is initiated by the repair of methyl phosphotriesters in DNA by the *E. coli ada* gene product; transfer of methyl groups from the phosphotriesters to cysteine-69 in the N-terminal domain of the polypeptide converts the Ada protein into a transcriptional activator of the *ada* operon (3).

The products of *ada* and *alkA*, two genes within the *ada* operon, contribute to cellular resistance to methylation. The Ada protein repairs methyl phosphotriesters, O^6-methylguanine and O^4-methylthymine. The product of the *alkA* gene, 3-methyladenine DNA glycosylase II, releases 3- and 7-methylpurines and O^2-methylpyrimidines from methylated DNA. These enzymes recognize other types of DNA damage besides simple methylation; for example, both enzymes recognize ethylated, hydroxyethylated, and haloethylated bases; the *alkA* gene product releases 3-ethylthioethyladenine, 7-ethylthioethylguanine, 1,2-bis(7-guanyl)ethane, N^2,3-ethanoguanine, and N^2,3-ethenoguanine from DNA (4–8). By contrast, a constitutive *E. coli* DNA glycosylase, 3-methyladenine DNA glycosylase I, has a more narrow specificity, releasing only 3-methyladenine from methylated DNA (9) and 3-ethylthioethyladenine, but not 7-ethylthioethylguanine, from chloroethyl ethyl sulfide-treated DNA (10).

Because of the wide variety of toxic compounds that higher organisms may encounter, it is important to determine whether eukaryotic homologues of these enzymes recognize DNA modifications besides those resulting from simple methylation and whether these enzymes offer protection against cell killing by other alkylating agents. These questions are important in relation to environmental exposure and to cancer chemotherapy as well. For example, methyltransferase in eukaryotic cells repairs some of the DNA modifications introduced by the haloethylnitrosoureas and causes resistance to treatment (5, 11). Some haloethylnitrosourea-resistant tumor cells also have enhanced glycosylase levels, but it is not yet known whether the presence of this enzymatic activity contributes directly to the resistance phenomenon (12).

Recently, a 3-methyladenine DNA glycosylase (*MAG*) gene was isolated from *Saccharomyces cerevisiae*, and the gene product was shown to have significant amino acid homology with *E. coli* 3-methyladenine DNA glycosylase II (13, 14). *MAG* mRNA levels are induced by exposure to methylating agents as are *alkA* levels in adapted *E. coli* (14). However, *MAG* expression is not regulated by the yeast O^6-methylguanine DNA methyltransferase, and *MAG* mRNA is induced by other kinds of DNA damage in addition to methylation (15). Expression of the *MAG* gene protects glycosylase-deficient mutants of *S. cerevisiae* and *E. coli* from cell killing by methylating agents (14).

In this study, we show that the *MAG* gene also protects these cells from *N*-(2-chloroethyl)-*N*-nitrosourea (CNU)-induced cell-killing. To investigate the basis for this protection, we assayed the ability of the *MAG* glycosylase to release modified bases from a *N*-(2-[^{3}H]chloroethyl)-*N*-nitrosourea-modified DNA ([^{3}H]CNU DNA) substrate. Extracts of *E. coli* that contain a plasmid expressing the *MAG* gene do release CNU-modified bases from DNA, and the two most prevalent CNU-modified bases, 7-hydroxyethylguanine (HEG) and 7-chloroethylguanine (CEG), are released in a protein- and time-dependent manner.

MATERIALS AND METHODS

Materials. [^{3}H]CNU (7.1 Ci/mmol; 1 Ci = 37 GBq), custom synthesized by Moravek Biochemicals (La Brea, CA), was a gift of W. J. Bodell (University of California, San Francisco). Calf thymus DNA was purchased from Sigma; the UV absorbance markers HEG and CEG were prepared as described (8); RNase A, proteinase K, and purified glycogen were obtained from Boehringer Mannheim; unlabeled CNU was obtained from the Division of Cancer Treatment, Na-

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Abbreviations: CNU, *N*-(2-chloroethyl)-*N*-nitrosourea; CEG, 7-(2-chloroethyl)guanine; HEG, 7-(2-hydroxyethyl)guanine.

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tional Cancer Institute (Bethesda, MD), and other chemicals were reagent grade materials.

Yeast and Bacterial Strains. Yeast survival studies were performed with the *S. cerevisiae* haploid strain DBY747 (*MAT* **a**, *his-Δ-1, leu2-3, leu2-112, trp1-289, ura3-52*) and its alkylation-sensitive derivative JC9005 (*mag-2::URA3*) in which the *MAG* gene is disrupted by insertion of the *URA3* gene (14). Bacterial survival studies were performed with *E. coli* strains MV1932, MB1900, and their derivatives carrying the pUC-2.1 plasmid. The pUC-2.1 plasmid contains a yeast genomic fragment encoding the *MAG* gene, which is efficiently expressed in *E. coli* (13). MV1932 (a gift from M. Volkert, University of Massachusetts Medical School) carries the *alkA1* and *tag-1* point mutations. MB1900 was generated by transducing the *tag* mutation (plus the closely linked *zhb*::Tn*5* marker) from strain GC4800 into MV1902 (both strains also gifts from M. Volkert), an *alkA105*::λ*pSG1* derivative of AB1157 (16). Experiments with MV1932 and MB1900 gave essentially the same results.

Survival Studies. Cell survival was measured as described (13). Yeast cells were grown in YPD (1% yeast extract/2% peptone/2% dextrose) to a density of 10^7 cells per ml and bacterial cells were grown in LB medium to a density of 10^8 cells per ml. CNU freshly dissolved in absolute alcohol was added to the cultures to the appropriate concentrations. Aliquots were taken from the cultures at the indicated times, diluted, and plated on YPD or LB plates for determination of survival.

[^{3}H]CNU DNA Substrate. Calf thymus DNA was dissolved in NaCl/sodium citrate buffer, pH 7.0 (8 mg/ml) and purified by treatment with RNase A (100 μg/ml) and proteinase K (50 μg/ml) followed by two subsequent extractions with chloroform/isoamyl alcohol (10:1). Finally, the DNA was precipitated with ethanol and redissolved in 10 mM sodium cacodylate (pH 7).

Purified DNA (8 mg/ml) was reacted for 6 h at 37°C with [^{3}H]CNU [specific activity, 7.14 Ci/mmol (0.1 mCi per mg of DNA)]. Noncovalently bound radioactivity was removed from DNA by repeated ethanol precipitation and redissolution in 20 mM Tris·HCl buffer (pH 7). Typically, the specific activity of alkylated DNA was 1.3×10^7 cpm/mg. The substrate was depurinated in 0.1 M HCl for 18 h at 37°C to determine its content of acid-labile purines. The distribution of modified bases was determined by high-performance liquid chromatography (HPLC) as described below; 64% of the total radioactivity was eluted from a C_{18} column (purine fraction) and the two most prevalent modified bases, HEG and CEG, accounted for 53% and 21%, respectively, of the radioactivity in the HPLC profile (see Fig. 2*A*).

DNA Glycosylase Assays. Bacterial cell extracts were prepared as described (13, 17). Briefly, cells in logarithmic growth were harvested by centrifugation and resuspended in L buffer (10 mM Tris·HCl, pH 7.4/10 mM NaCl/5 mM $MgCl_2$/0.5 mM $CaCl_2$/0.2% Nonidet P-40/1 mM dithiothreitol) (18). The cells were disrupted by sonication and, after centrifugation at 9000 × *g* for 15 min, the supernatant was frozen in liquid nitrogen and stored at −70°C until assayed.

Assay mixtures contained 9 μg (120,000 cpm) of [^{3}H]CNU DNA and the indicated amounts of protein extract in 150 μl of L buffer. Mixtures were incubated at 25°C for various lengths of time and then the DNA was precipitated with ethanol in the presence of glycogen as carrier. The supernatant was dried under vacuum, residues were redissolved in water, and, after passage through a DEAE-Sephadex A25 column (1-ml bed volume) to remove any oligonucleotides that might be present, aliquots equal to 71% of the incubation mixture were analyzed by HPLC.

HPLC Analysis. Modified purines were separated on an Alltech Spherisorb ODS-2 5 μm (4.6 × 250 mm) C_{18} column and eluted at 0.76 ml/min with increasing concentrations of acetonitrile in 25 mM KH_2PO_4 (pH 4.5) as follows: 0.5% acetonitrile for 36 min, 0.5–10% acetonitrile for 20 min, 10% acetonitrile for 10 min, 10–50% acetonitrile for 10 min, and 50% acetonitrile for 20 min. The UV absorbance of the markers was monitored during the chromatographic separations at 270 nm with a Perkin–Elmer LC-55 spectrophotometer. One-minute fractions were collected and dissolved in Ultima Gold (Packard), and their radioactivities were measured in a Beckman LS-1800 scintillation counter. The radioactive content of each fraction was plotted versus elution time, and the total activity in each peak was calculated by a computer program that automatically subtracts background.

RESULTS

Previous studies have shown that the yeast strain JC9005 (*mag-2::URA3*), which has been made glycosylase deficient by disruption of the *MAG* gene, is more sensitive to cell killing by methylating agents than is the wild-type DBY747 (14). As shown in Fig. 1*A*, JC9005 is also more sensitive to cell killing by CNU. For example, after 60 min of exposure to 4.5 mM CNU, ≈10% of the wild-type yeast survive while <0.1% of JC9005 survive. The ratio of DBY747 to JC9005 survival increases logarithmically with exposure time and is significantly different from 1 ($P < 0.01$; Student's *t* test).

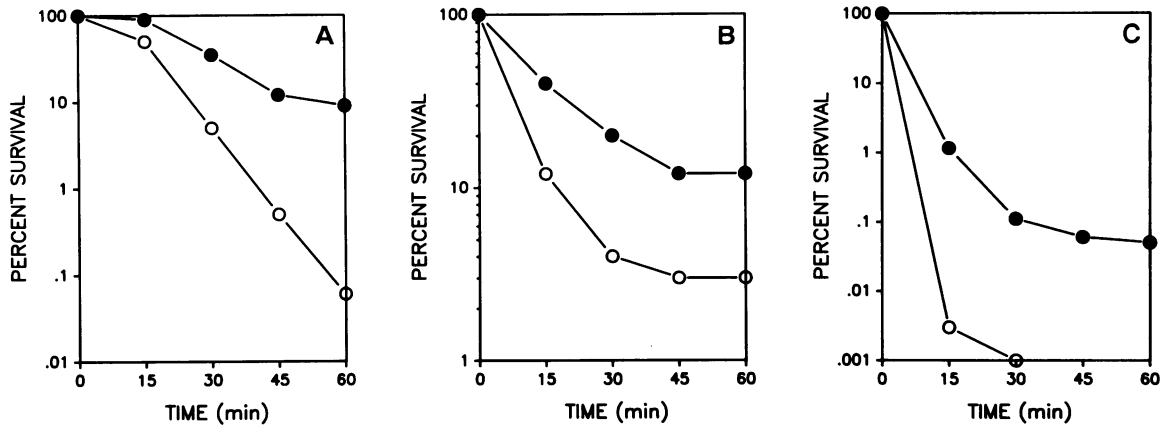

FIG. 1. CNU-induced cell killing. (*A*) Colony-forming abilities of yeast strains DBY747 (wild type) (●) and JC9005 (*mag-2::URA3*) (○) after treatment with 4.5 mM CNU for the indicated times. (*B*) Colony-forming abilities of *E. coli* strains MV1932 (*alkA tag*)/pUC-2.1 (●) and MV1932 (*alkA tag*)/pUC-19 (○) after treatment with 1 mM CNU for the indicated times. (*C*) Colony-forming abilities of the same strains of *E. coli* after treatment with 3 mM CNU.

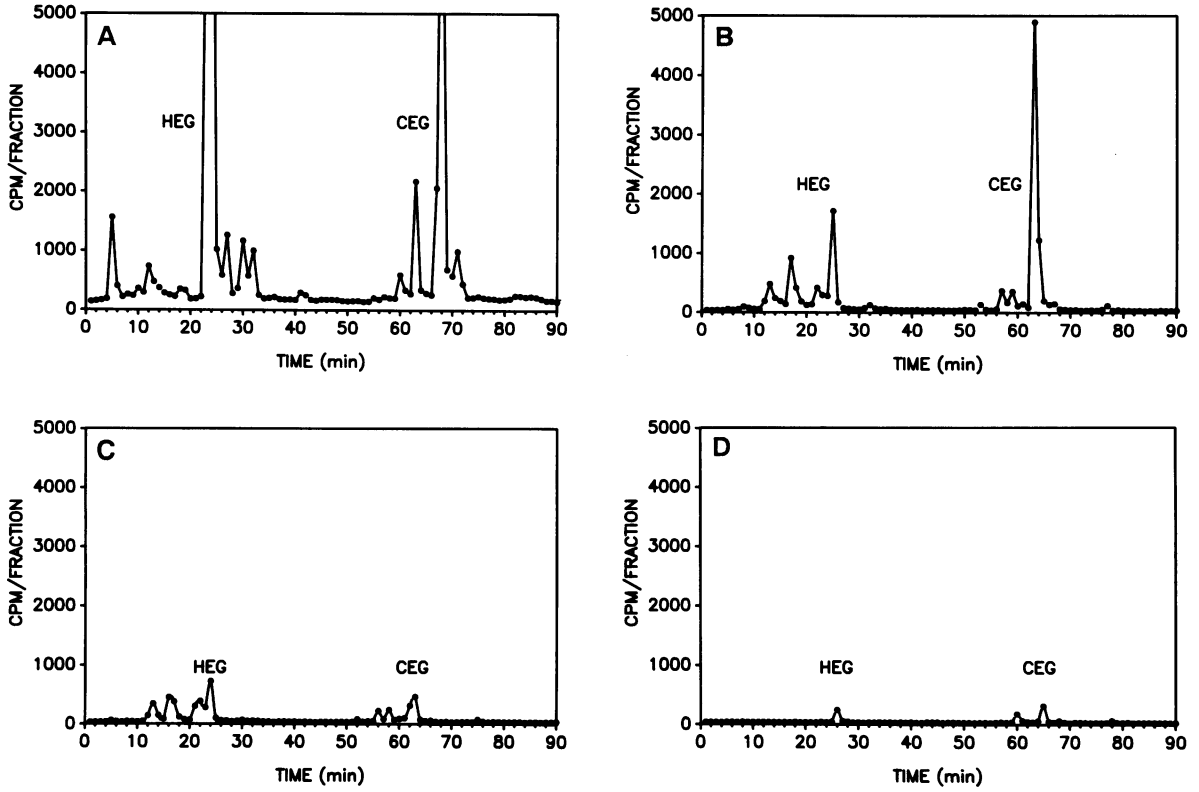

FIG. 2. HPLC profiles of [^{3}H]CNU-modified bases released from DNA by acid treatment (*A*), 1 h of incubation with 67 μg of extract from *E. coli* MB1900 (*alkA tag*) cells that contained the pUC-2.1 plasmid expressing yeast glycosylase (*B*), 1 h of incubation with 76 μg of extract from *E. coli* cells that did not contain the plasmid (*C*), incubation with buffer control (*D*). Radioactivity in the major peaks in *A* and *B* coeluted with optical markers for HEG and CEG.

When the plasmid encoding the *MAG* gene is introduced into the glycosylase-deficient *alkA tag E. coli* strain MV1932, the cells become more resistant to cell killing by CNU than cells bearing the plasmid vector that does not contain the *MAG* gene. The survival of cells exposed to 1 and 3 mM CNU is shown in Fig. 1 *B* and *C*, respectively. Again, the increased survival is significant by Student's *t* test ($P < 0.01$ for the exposure at 1 mM and $P < 0.05$ for the exposure at 3 mM).

To determine whether the *MAG* glycosylase releases modified bases from CNU-treated DNA, we incubated [^{3}H]CNU DNA with extracts of *E. coli* cells that did or did not contain the *MAG* glycosylase. Fig. 2*A* shows the HPLC profile of radiolabeled bases released from [^{3}H]CNU DNA by acid treatment. The positions of the two most prevalent DNA modifications, HEG and CEG, are indicated by optical markers. Fig. 2*B* shows the profile of bases released by 67 μg of an extract from *E. coli* cells containing the glycosylase; Fig. 2*C* shows the profile of bases released by 76 μg of an extract from *E. coli* cells that did not contain the plasmid; Fig. 2*D* shows the profile of bases released in a buffer control. It is clear that the extract containing the *MAG* glycosylase releases significant amounts of HEG and CEG in comparison with the controls.

Fig. 3 shows that release of these modified bases is protein dependent. As shown in Fig. 3*A*, the total release of radioactivity into the supernatant increases with protein content but gradually plateaus at higher concentrations. Fig. 3 *B* and *C* shows that the release of HEG and CEG individually, as determined by HPLC analysis of the supernatant, also increases with protein concentration. Under the conditions of this assay, 31% of the total purine fraction, 10% of the HEG, and 62% of the CEG were released by incubation with 120 μg

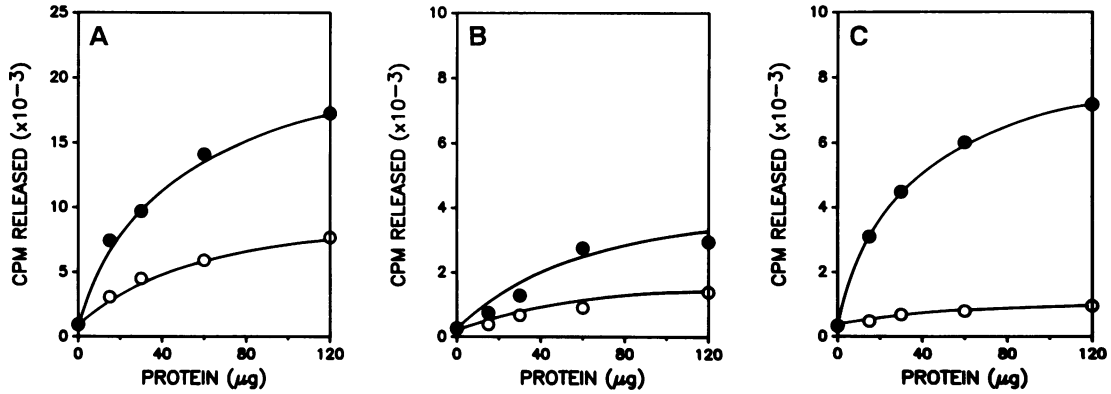

FIG. 3. Protein-dependent release of [^{3}H]CNU-modified bases from DNA by extracts of *E. coli* MB1900 (*alkA tag*)/pUC-2.1 (●) and MB1900 (○) cells after incubation for 1 h at 25°C. (*A*) Total bases released. (*B*) HEG. (*C*) CEG.

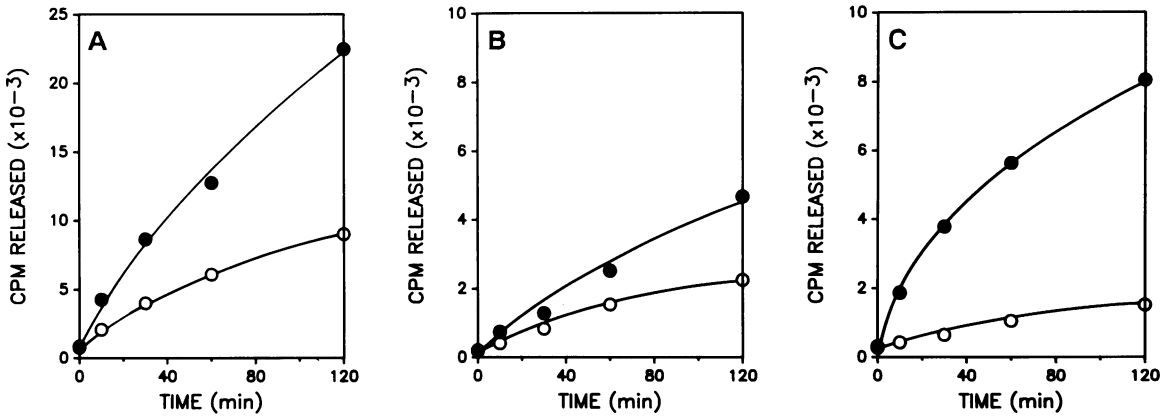

FIG. 4. Time-dependent release of [^{3}H]CNU-modified bases from DNA after incubation with 60 μg of extracts of *E. coli* MB1900 (*alkA tag*)/pUC-2.1 (●) and MB1900 (○) cells at 25°C. (*A*) Total bases released. (*B*) HEG. (*C*) CEG.

of extract containing the *MAG* glycosylase. In comparison, 14% of the purine fraction, 5% of HEG, and 8% of CEG were released by 120 μg of cell extract that did not contain the glycosylase.

Fig. 4 shows the time-dependent release of modified bases from [^{3}H]CNU DNA. Under the conditions of this assay, there is a gradual release of radioactivity that extends over 2 h. At the end of this period, 40% of total acid-labile radioactivity, 16% of the HEG, and 70% of the CEG have been released. By comparison, the extract of cells that did not contain the plasmid with the *MAG* gene released only 16% of the total acid-labile radioactivity, 7% of the HEG, and 13% of the CEG, respectively.

Again, these results are highly significant by Student's *t* test. For example, the release of total radioactivity, HEG, and CEG by extracts from the glycosylase-containing strain is significantly higher than the release by extracts from the glycosylase-deficient strain at the 60-min, 60-μg point with P values < 0.02 in all cases.

DISCUSSION

The results presented above show that the *MAG* gene product can protect both prokaryotic and eukaryotic cells from the lethal action of CNU. Evidence for this conclusion is 2-fold. First, when the *MAG* gene is disrupted in *S. cerevisiae*, sensitivity to cell killing is greatly increased. Second, when the intact *MAG* gene is introduced into glycosylase-deficient *E. coli*, these cells are protected against the lethal action of CNU.

Accordingly, we determined whether the eukaryotic *MAG* glycosylase can release modified bases from CNU-treated DNA. Cell extracts containing the *MAG* glycosylase released significant amounts of HEG and CEG. It is also apparent that the enzyme releases CEG far more readily than it releases HEG. Thus, as shown in Fig. 3, ≈6200 cpm of CEG (24% of the total CEG) but only 1600 cpm of HEG (2.5% of the total HEG) are released in excess of the control by 120 μg of extract, and, as shown in Fig. 4, ≈6600 cpm of CEG (26% of the total CEG) but only 2400 cpm of HEG (3.8% of the total HEG) are released in excess of the control after 2 h of incubation.

A preference for CEG over HEG was also observed with bacterial 3-methyladenine DNA glycosylase II (8). Since the acid stability of 7-hydroxyethyldeoxyguanosine is approximately equal to that of 7-chloroethyldeoxyguanosine, this preference is probably based on the recognition of the substrate by the enzyme rather than on the strength of the glycosyl bond (8).

The protein- and time-dependent nature of this activity is shown in Figs. 3 and 4, respectively. Release of total acid-labile radioactivity, and of HEG and CEG individually, all increase in a protein- and time-dependent fashion. The preference for CEG over HEG is emphasized by these results. In both the protein- and time-dependent studies, approximately twice as much CEG was released as HEG in spite of the fact that HEG was ≈2.5 times as abundant.

These data show that the *MAG* gene product not only protects cells from the lethal action of CNU, but that its gene product releases two CNU-modified bases, CEG and HEG, from substrate DNA. It seems likely that release of CNU-modified bases is followed by endonuclease, polymerase, and ligase action to restore the integrity of the DNA. This may mean that one or both of these modifications are lethal, perhaps because they block replication or cause mutations in essential genes. Another possibility is that CEG, which contains a reactive group, leads to the formation of 1,2-bis(7-guanyl)ethane and other DNA modifications that may be lethal (19). By analogy with bacterial 3-methyladenine DNA glycosylase II, it is likely that the *MAG* glycosylase recognizes other minor base modifications that might result in lethality. In this regard, it is interesting to note that a human 3-methyladenine DNA glycosylase releases not only 3-methyladenine, but also 1,N^6-ethenoadenine (20).

In summary, our data indicate that the eukaryotic *MAG* gene protects cells from the lethal action of CNU and releases modified bases from CNU-treated DNA. If the eukaryotic glycosylases found in tumor cells share these activities, they could play an important role in tumor resistance to alkylating agents used in chemotherapy.

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