1,N^6-Ethenoadenine Is Preferred over 3-Methyladenine as Substrate by a Cloned Human *N*-Methylpurine–DNA Glycosylase (3-Methyladenine–DNA Glycosylase)†

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ABSTRACT: A lethal DNA adduct induced by methylating agents, 3-methyladenine (m^3A), is removed by both the constitutive (Tag) and inducible (AlkA) bacterial m^3A–DNA glycosylases. The human 3-methyladenine–DNA glycosylase also releases m^3A as well as other methylated bases. The rate of release of m^3A from alkylated DNA by the purified or recombinant human m^3A glycosylase is much higher than that of the other methylated bases. We now find that a partially purified recombinant human m^3A–DNA glycosylase, expressed in *Escherichia coli*, releases at least 10-fold more 1,N^6-ethenoadenine (ϵA) than m^3A from DNA. ϵA is completely unrelated to m^3A since it is a heterocyclic adduct produced by the carcinogen vinyl chloride. The rates of release of ϵA and m^3A were both dependent on protein concentration and time. The differential release of ϵA and m^3A occurs regardless of whether DNA containing each adduct is assayed separately or is assayed in a mixed substrate containing both DNAs. This result raises the question of what structural features are involved in recognition and excision by the human m^3A–DNA glycosylase and what may be its primary substrate.

Release of m^3A from methylated DNA was originally described in 1976 by Lindahl, who found this activity in *Escherichia coli*. The necessity for organisms requiring this glycosylase, termed 3-methyladenine–DNA glycosylase (m^3A–DNA glycosylase), was attributed to the need to remove this derivative, which was potentially lethal. The evolutionary basis for such a specific enzyme was attributed to the fact that m^3A (and other methylated bases) were continuously formed endogenously by the nonenzymatic alkylation of DNA by *S*-adenosylmethionine (SAM) (Lindahl *et al.*, 1981). There did not appear to be measurable removal of any other methylated bases by this particular enzyme.

A second m^3A–DNA glycosylase was later found, termed m^3A–DNA glycosylase II or AlkA protein (Karran *et al.*, 1980). This inducible enzyme had a much broader substrate specificity and, to varying extents, removed N-3 and N-7 methylpurines (Yamamoto & Sekiguchi, 1979; Laval *et al.*, 1981), as well as O^2-methylthymine and O^2-methylcytosine (Ahmmed & Laval, 1984). At about the same time, it was reported that human and rodent cells and tissues also contain an m^3A–DNA glycosylase which resembles the bacterial Tag protein in its ability to release a number of alkyl bases (Singer & Brent, 1981; Pegg & Margison, 1981; Laval *et al.*, 1981). The substrate specificity for the bacterial AlkA protein was recently extended to include one of the (2-haloalkyl)nitrosourea products, N^2,3-ethanoguanine (Habraken *et al.*, 1991), and N^2,3-ethenoguanine (Matijasevic *et al.*, 1992), a product of vinyl chloride and related compounds *in vivo* (Laib *et al.*, 1985).

In 1991, human cell extracts were found to contain a binding activity toward an oligonucleotide with a single 1,N^6-ethenoadenine (ϵA) site-specifically incorporated (Rydberg *et al.*, 1991). This compound is formed *in vivo* by vinyl chloride and related carcinogens metabolized to 1-halooxiranes (Guengerich, 1991). Its repair *in vivo* had not been previously demonstrated. In addition, this binding protein also had glycosylase activity since it was found to release ϵA from both a defined oligomer and DNA (Rydberg *et al.*, 1992; Singer *et al.*, 1992). Previously characterized eukaryotic glycosylases were examined for a possible activity toward ϵA. Surprisingly, it was found that the cloned truncated human m^3A–DNA glycosylase (O'Connor & Laval, 1991) both bound and released ϵA (Singer *et al.*, 1992). Similarly, a purified m^3A–DNA glycosylase from human lymphoblasts (Gallagher & Brent, 1982) also released both ϵA and m^3A (Singer *et al.*, 1992). In addition, a glycosylase from human placenta or cells released all four cyclic etheno adducts (Dosanjh *et al.*, 1994).

Having found this unexpected dual activity for both the ϵA-binding protein and the human m^3A–DNA glycosylases, a study was initiated to compare, in detail, the rates of release of the two completely unrelated compounds, ϵA and m^3A (Figure 1), by a human recombinant m^3A–DNA glycosylase, the cDNA of which was one of the three independently isolated clones (Chakravarti *et al.*, 1991; Samson *et al.*, 1991; O'Connor & Laval, 1991).

Our well-characterized cloned enzyme (Chakravarti *et al.*, 1991; Mitra *et al.*, 1993) enabled us to quantitate the removal of ϵA and m^3A individually and of ϵA and m^3A from a mixed substrate. Since we find that ϵA is greatly preferred to m^3A by this glycosylase, it will be important and interesting to further explore the substrate specificity for heterocyclic compounds, particularly those formed through endogenous processes.

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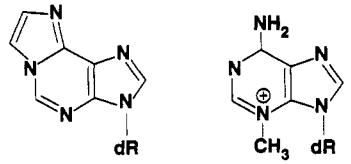

εdA m³dA

FIGURE 1: Structures of 1,N^6-ethenodeoxyadenosine (εdA) and 3-methyldeoxyadenosine (m^3dA). εdA is highly fluorescent (Sattsangi *et al.*, 1980). The base, εA, also fluorescent, can be detected and measured at picomole amounts. m^3dA is a quaternary base and thus has an extremely labile glycosyl bond (Singer & Grunberger, 1983).

MATERIALS AND METHODS

Materials. The 25-base oligonucleotide 5′-CCGCT(εA)-GCGGGTACCGAGCTCGAAT-3′, where εA stands for 1,N^6-ethenoadenine, was a gift from Drs. A. K. Basu and J. M. Essigmann. The same oligonucleotide with A in place of εA, its complementary 25-mer oligonucleotide with T opposite the εA adduct, was synthesized using standard phosphoramidite chemistry. The A-8 cation-exchange column for HPLC was from Bio-Rad.

Substrates. The εA-containing oligonucleotide was ^{32}P-labeled at the 5′ end by T4 polynucleotide kinase and annealed to the complementary 25-mer with T opposite εA as described (Rydberg *et al.*, 1991).

Globally modified DNA containing εA was prepared by reaction with chloroacetaldehyde (CAA). Calf thymus DNA (0.5 mg) was incubated with 0.69 M CAA in 0.5 mL of 0.5 M sodium cacodylate buffer (pH 7.25) for 48 h at 37 °C. The DNA was then purified by repeated ethanol precipitations at 0 °C and redissolved in 10 mM Tris-HCl and 1 mM EDTA, pH 7.8.

Globally modified DNA containing ^{3}H-labeled bases including m^3A was prepared by treating sonicated calf thymus DNA (50 mg) with [^{3}H]dimethyl sulfate (100 μCi, 4.5 Ci/mmol) in 5.0 mL containing 200 mM sodium cacodylate, pH 8.7, buffer for 16 h at room temperature. DNA was then purified by repeated ethanol precipitations at 0 °C and dissolved in 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. The specific activity of radiolabeled DNA was 2.4 Ci/mmol, and m^3A comprised 13% of the total ^{3}H-methylated bases.

Cell-Free Extracts. E. coli alkA⁻ tag⁻ (MV1932) cells harboring human MPG cDNA plasmid (pDG23) (Chakravarti *et al.*, 1991) or the control empty vector (pUC19) were grown in LB broth containing ampicillin (100 μg/mL) with vigorous shaking at 37 °C to $A_{600} \approx 0.3$. After addition of isopropyl β-D-thiogalactoside to 0.5 mM, incubation with shaking was continued for an additional 3 h. The cells were harvested, resuspended in a glycosylase storage buffer [20 mM Tris-HCl, pH 8.0/1 mM EDTA/1 mM dithiothreitol/10% (v/v) glycerol] supplemented with 0.4 M NaCl, and disrupted in a Branson sofifier at low power for six 30-s bursts on ice. The cell lysates were then centrifuged at 38000*g* for 30 min and poly(ethylenimine) was added to the supernatant at 18 μg per A_{260} unit of the extract to precipitate nucleic acids. After centrifugation at 12000*g* for 15 min, the supernatants were brought to 55% saturation of ammonium sulfate. The precipitates containing MPG activity were dissolved in glycosylase storage buffer supplemented with 0.1 M NaCl and dialyzed against the same buffer. The dialysate was passed through a column containing single-stranded DNA–cellulose (Sigma) equilibrated with the same buffer. After the column was washed with six column volumes of buffer, the bound proteins were eluted using glycosylase storage buffer containing 0.4 M NaCl. Fractions having MPG activity were pooled, dialyzed against the storage buffer, but containing only 0.1 M NaCl, and concentrated by Centriprep-10 (Amicon). This 80–100-fold purified protein preparation was directly used for subsequent assays for εA and m^3A releasing activity.

Band-Shift Assay. Oligonucleotides were ^{32}P-5′-end-labeled using polynucleotide kinase and then annealed to the complementary strand in 150 mM Tris-HCl (pH 7.8) and 30 mM $MgCl_2$ (Rydberg *et al.*, 1991). The binding reaction was carried out in an assay buffer containing 25 mM Hepes–KOH (pH 7.8), 0.5 mM EDTA, 0.5 mM dithiothreitol, 150 mM NaCl, 10% glycerol, 1 μg of poly(dI-dC) and up to 5 μL of cell-free extract in a final volume of 20 μL. Unlabeled double-stranded competitor oligonucleotides were added when necessary, and 40 fmol of the ^{32}P-labeled oligonucleotide was added last. After 30 min at room temperature (unless otherwise stated), 5–10 μL of the reaction was electrophoresed on a 6% nondenaturing polyacrylamide gel using TBE buffer (0.09 M Tris–borate, pH 8.0, and 2 mM EDTA) at 10 V/cm for 75 min. After drying, the gel was autoradiographed. Quantitation of the relative amount of oligonucleotide bound to the binding protein was obtained by densitometer scanning.

Supershift Assay. The gel retardation experiment, in the presence of anti-human MPG polyclonal antibody, was carried out using purified antiserum raised in a rabbit against a C-terminal synthetic peptide [20 amino acid peptide from position 256 to position 275 at the C-terminal end of the cloned human MPG (Chakravarti *et al.*, 1991)]. Forty femtomoles of ^{32}P-labeled εA-containing oligonucleotide was incubated with 580 ng of partially purified recombinant human MPG in the band-shift assay buffer (see Band-Shift Assay) for 30 min at 25 °C, followed by the addition of 6.5 μg (2 μL) of ammonium sulfate antibody or 100-fold-diluted nonimmune rabbit serum for further incubation for an additional 60 min at 25 °C in a total volume of 20 μL. For control experiments, the radiolabeled probe was incubated either with *E. coli* control protein for 30 min or with anti-MPG peptide antibody alone for 60 min at 25 °C. Reaction mixtures (3–5 μL) were electrophoresed on a 6% nondenaturing polyacrylamide gel using TBE buffer at 10 V/cm for 90 min. After drying, the gel was autoradiographed.

Glycosylase Assay. DNA glycosylase activity was measured by incubation of partially purified cloned human MPG glycosylase extracts with the εA-containing double-stranded oligonucleotide or with modified DNA in 35 mM Hepes–KOH, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 40 mM KCl (pH 7.2). Analysis of εA released from the 25-mer oligonucleotide or from CAA-treated DNA used an Aminex A-8 cation-exchange column eluted with 0.4 M, pH 7 ammonium formate at 45 °C. Detection was by fluorescence (Rydberg *et al.*, 1992; Singer *et al.*, 1992).

Release of m^3A from DNA treated by [^{3}H]DMS was measured using the same HPLC cation-exchange system. The eluate was simultaneously monitored for fluorescence and UV at 260 nm. This HPLC system allowed separation of m^3A from other methylated derivatives. Fractions corresponding to the m^3A peak were collected and analyzed for ^{3}H counts by liquid scintillation. In addition, in some experiments, all fractions were so analyzed, including those containing [^{3}H]-7-methylguanine (m^7G) and [^{3}H]-3-methylguanine (m^3G). The latter is the peak eluting at about 13 min in Figure 2. The position of εA fluorescence is indicated by an arrow.

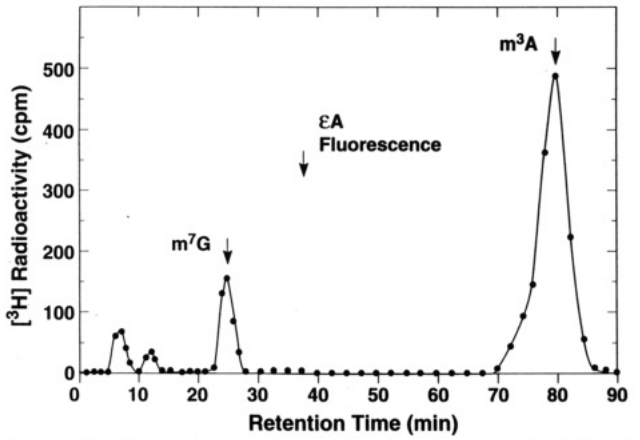

FIGURE 2: Simultaneous release of 7-methylguanine (m^7G) and 3-methyladenine (m^3A) from [^{3}H]dimethyl sulfate-treated DNA and of ϵA from CAA-treated DNA. Ten micrograms of the partially purified MPG protein was incubated with the two modified DNA samples for 30 min at 37 °C. Released low molecular weight products were analyzed by HPLC (see Materials and Methods). ϵA was quantitated by fluorescence (Singer *et al.*, 1992), and methylated products were quantitated by scintillation counting. The individual HPLC fractions counted are shown by closed circles. The position, in this experiment, of ϵA fluorescence is indicated. The small peak eluting at about 13 min is 3-methylguanine.

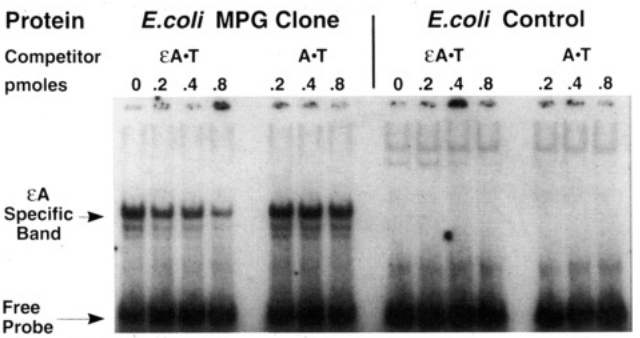

FIGURE 3: Gel retardation assay using the protein from recombinant human MPG cloned in *E. coli* MV1932 cells (left side) and the control *E. coli* protein preparation lacking the MPG (right side). The ϵA specific binding band is indicated by an arrow, as is the position of the unbound ^{32}P-5′-labeled 25-mer double-stranded probe with ϵA paired with T. No ϵA specific band is formed with the control *E. coli* protein(s). This indicates that the binding of ϵA to protein results from the MPG protein only. The first four lanes have increasing amounts of unlabeled ϵA·T oligomer as the competitor. As can be seen, this band decreases with increasing competitor. In contrast, the next three lanes show no diminishment of the ^{32}P intensity when competed with unmodified, unlabeled A·T competitor. This comparison of competitors shows that the binding is MPG and ϵA specific.

RESULTS

Binding Assay. The cloned partially purified protein bound the ϵA probe as can be seen in Figure 3 (left panel). This complex, indicated as the ϵA specific band, was competed by increasing amounts of unlabeled ϵA·T probe but not by the unmodified A·T probe. This is indicative of specific binding for ϵA by the MPG protein. On the right side of Figure 3, it can be seen that the *E. coli* control protein(s), lacking the recombinant human MPG, does (do) not show specific binding to an ϵA-containing oligonucleotide binding under identical conditions.

Supershift Assay. To confirm the specific binding of partially purified MPG protein by the ϵA probe, a gel retardation experiment using MPG was performed in the presence of polyclonal anti-human MPG peptide antibody. The antibody interacts with recombinant human MPG and

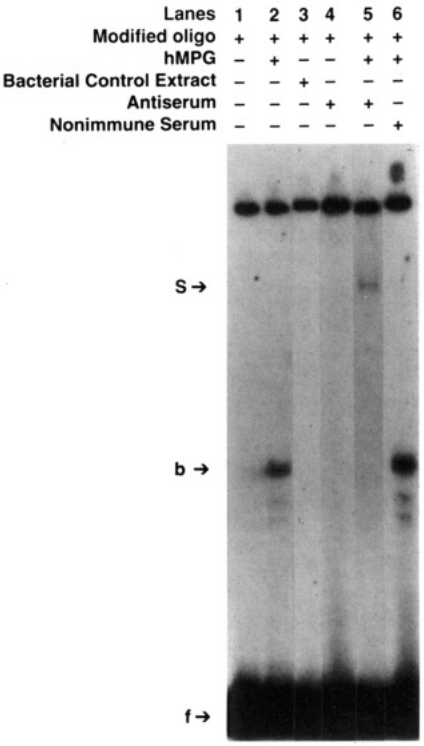

FIGURE 4: Supershift assay of recombinant human MPG protein. The proteins were incubated with a ^{32}P-labeled ϵA oligonucleotide, paired with T opposite ϵA and with either anti-human MPG peptide antibody or a nonimmune rabbit serum, and electrophoresed under conditions described in Materials and Methods. The positions of the free DNA probe (f), enzyme-bound DNA (b), and the antibody-bound DNA–protein complex, termed supershift (S), are indicated.

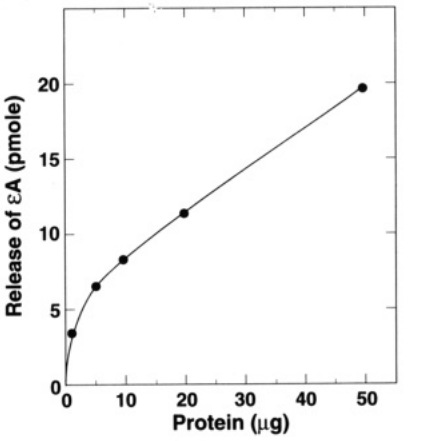

FIGURE 5: Protein-dependent release of ϵA from chloroacetaldehyde-treated DNA. Increasing amounts of partially purified human MPG were incubated with excess ϵA-containing DNA for 1 h at 37 °C. Quantitation of ϵA is by separation using HPLC, followed by fluorescence determination.

forms a complex with a slower migration on a nondenaturing polyacrylamide gel. In contrast, either the nonimmune rabbit serum does not interfere with complex formation or the polyclonal antibody, in the absence of MPG, does not result in a slower migrating complex on the gel (indicated as S in Figure 4). This confirms the specific binding of the human MPG with the ϵA-containing oligonucleotide.

Quantitation of m^3A and ϵA Release. The release of ϵA from CAA-treated DNA was found to be both protein concentration (Figure 5) and time dependent (Figure 6). The amount of initial ϵA release using 1 μg of protein was 30 fmol of ϵA (μg of protein)$^{-1}$ min^{-1}, compared to m^3A release, which was 2.4 fmol (μg of protein)$^{-1}$ min^{-1} (Table 1). Figure 6

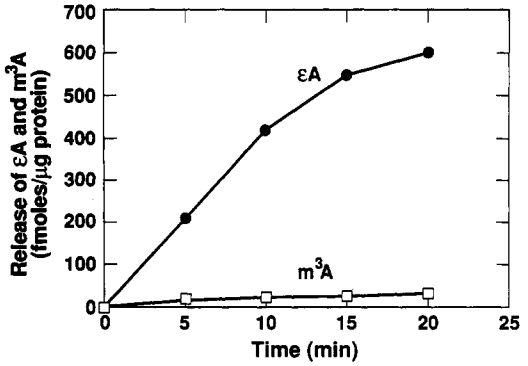

FIGURE 6: Release of m^3A (□) and εA (●) from [^{3}H]dimethyl sulfate- and chloroacetaldehyde-treated DNA, respectively, by partially purified human m^3A–DNA glycosylase (MPG). The incubations were carried out using 20 μg of MPG protein and either excess m^3A- or εA-containing DNA substrate at 37 °C. Aliquots were taken at indicated times for HPLC separation, followed by quantitation of m^3A by radioactivity measurement and of εA by fluorescence. The results are expressed as femtomoles released per microgram of protein.

Table 1: Release of m^3A and εA by the Cloned MPG Protein[a]

substrate	fmol (μg of protein)$^{-1}$ min^{-1}	
	m^3A[b]	εA[c]
(1) [^{3}H]DMS-treated DNA	2.4	
(2) CAA-treated DNA[d]		30[e]
(3) mixed DMS and CAA DNA[f]	1.9	25
(4) site-specifically placed εA·oligo[g]		20

[a] Data shown are averaged from two to four independent experiments. [b] [^{3}H]m^3A release was determined by scintillation counting. [c] Determined by fluorescence. Excitation was at 290 nm and emission at 410 nm. [d] Contains all four etheno adducts (as well as the hydrated intermediate of εC; Kuśmierek & Singer, 1982) which are all substrates for the human m^3A–DNA glycosylase (Dosanjh *et al.*, 1994). [e] Derived from data using both time course experiments (Figure 6) and protein concentration dependence (Figure 5). [f] Mixed substrates (1 and 2) containing a >5-fold excess of the m^3A and εA released under the experimental conditions used. [g] 25-mer synthetic oligonucleotide containing a single εA at the sixth position from the 5′ end (Rydberg *et al*, 1991).

shows a time course of m^3A release from [^{3}H]DNA or εA release from the εA-containing oligomer. Using the mixed DNA substrates containing excess m^3A and εA (as well as all other expected methylated bases and etheno bases), the release of m^3A was 1.9 and of εA was 25 fmol (μg of protein)$^{-1}$ min^{-1} (Table 1, Figure 2). In all types of comparative experiments, εA release was always >10-fold that of m^3A. In some cases, εA release was as much as 25-fold that of m^3A (Figure 6).

DISCUSSION

It was unexpected that the well-characterized repair enzyme 3-methyladenine–DNA glycosylase was also able to remove the unrelated vinyl chloride derived adduct 1,N^6-ethenoadenine (Singer *et al.*, 1992) (Figure 1). This led us to investigate quantitation of the relative activity of the cloned human enzyme 3-methyladenine–DNA glycosylase, termed MPG (Mitra *et al.*, 1993), for these two substrates, m^3A and εA.

The data presented show that εA is preferred to m^3A by more than 10-fold (Table 1, Figure 6), regardless of whether the release is measured in separate parallel experiments or using a mixture of the two DNAs, containing either m^3A or εA. It should be noted that these DNAs also contained the other expected methylation products (Singer & Grunberger, 1983) and those formed by CAA treatment (Leonard, 1984, 1992). An earlier report suggested that both adducts are released with similar efficiency (Singer *et al.*, 1992). However, the enzyme used in this previous study was purified from human placenta. There is a possibility that these cells may contain a slightly altered glycosylase since Vickers *et al.* (1993) suggest altered splicing to generate different MPG messages which may have somewhat different activities. For the recombinant enzyme used in the present study, the presence of other possible substrates does not interfere with the large differential in release of the two adducts quantitated.

Historically, the existence of a mammalian m^3A–DNA glycosylase was postulated since m^3A was known to be rapidly lost from DNA of animals administered carcinogenic methylating agents, such as methyl methanesulfonate, dimethylnitrosamine, 1,2-dimethylhydrazine, and methylnitrosourea [reviewed by Singer and Grunberger (1983)]. When the substrate range of isolated mammalian m^3A–DNA glycosylases was examined, it was found to include other methyl derivatives (Laval, 1981; Singer & Brent, 1981; Margison & Pegg, 1981; Gallagher & Brent, 1984). Similarly, it was found that N-3 and N-7 ethylpurines were also, though less efficiently, released by this glycosylase (Singer & Brent, 1981). These findings of a broad alkyl substrate range suggested that the mammalian m^3A–DNA glycosylase appeared to correspond to the inducible bacterial m^3A–glycosylase II (AlkA), rather than the constitutive m^3A–glycosylase I (Tag).

The substrate specificity of mammalian 3-methyladenine–DNA glycosylase was not studied extensively until the cloning and expression of their cDNAs were achieved recently. In view of the observation that the recombinant glycosylases of humans and mice can efficiently release other *N*-alkylpurines in DNA, the mammalian protein was renamed *N*-methylpurine–DNA glycosylase (MPG) (Mitra *et al.*, 1993). More recently, it was observed that the mammalian MPGs show some release of 8-hydroxyguanine from DNA (Bessho *et al.*, 1993).

These studies did not preclude that other types of modified bases could be excised from carcinogen-exposed DNA. The preferential release of εA by MPG in the present work raises the issue of (1) the enzyme's substrate range and (2) the nature of the primary substrate.

Inasmuch as m^3A is formed endogenously (Lindahl *et al.*, 1982), it is reasonable that a repair mechanism for such an adduct should have evolved. Similarly, vinyl chloride represents one of a class of 2-carbon compounds which are metabolically converted by the human liver P-450 system to an intermediate which results in etheno derivatives (Guengerich *et al.*, 1991; Guengerich & Kim, 1991). This endogenous presence of varied adducts may be the biological rationale for the evolution of the enzyme which is known as m^3A–DNA glycosylase for its primary substrate.

In view of the observation that the DNA repair genes, including MPG, are regulated in mammalian tissues (Mitra & Kaina, 1992), the reaction rate and substrate preference of the glycosylase could be critical in ensuring removal of potentially lethal or mutagenic adducts. Our present studies suggest that 1,N^6-ethenoadenine is likely to be removed from DNA much more efficiently than 3-methyladenine *in vivo*. The *in vivo* function of the enzyme can be studied by using cells deficient in this glycosylase activity. Production of such mutants by gene targeting is currently under way.

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