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Both purified human 1,N^6-ethenoadenine-binding protein and purified human 3-methyladenine-DNA glycosylase act on 1,N^6-ethenoadenine and 3-methyladenine

(DNA repair/DNA alkylation/cyclic adduct/vinyl chloride)

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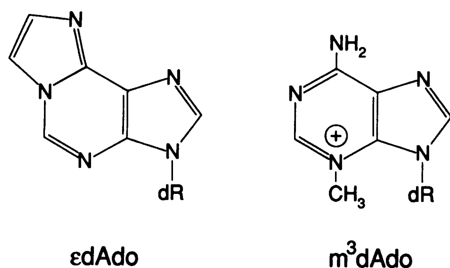
ABSTRACT We previously described a protein, isolated from human tissues and cells, that bound to a defined double-stranded oligonucleotide containing a single site-specifically placed 1,N^6-ethenoadenine. It was further demonstrated that this protein was a glycosylase and released 1,N^6-ethenoadenine. We now find that this enzyme also releases 3-methyladenine from methylated DNA and that 3-methyladenine-DNA glycosylase behaves in the same manner, binding to the ethenoadenine-containing oligonucleotide and cleaving both ethenoadenine and 3-methyladenine from DNA containing these adducts. The rate and extent of glycosylase activities toward the two adducts are similar.

Cyclic DNA adducts, such as 1,N^6-ethenoadenine (εA), are produced by a variety of industrial and environmental chemicals. These include vinyl chloride (1, 2), urethane (3), vinyl carbamate (4), and other two-carbon compounds (5) metabolized in liver microsomes (5, 6). 3-Methyladenine (m^3A) is a major product produced in DNA by methylating agents such as alkyl sulfates and nitrosoureas (7) and may also be formed in cells by nonenzymatic methylation by a normal constituent, S-adenosyl-L-methionine (8). The structures of the deoxyribonucleosides of εA and m^3A are shown below.



Repair of m^3A, initiated by DNA glycosylases, occurs in organisms as diverse as bacteria and mammals and is well characterized (9). In addition, the cDNA encoding human m^3A-DNA glycosylase has been cloned (10–12). This enzyme seems to have a fairly broad substrate range, including 3-alkyl- and 7-alkylpurines (13, 14). Repair of εA, on the other hand, is less well documented. Experiments with rats given vinyl chloride by inhalation have demonstrated a long-lived fraction of εA, apparently remaining unrepaired in rat liver (15). Oesch *et al.* (16) described release of εA and N^2,3-ethenoguanine from chloroacetaldehyde-treated DNA by extract from rat brain cells. However, only qualitative data were presented in that preliminary report. Recently, however, an εA-specific DNA-binding protein (17) and an εA-DNA glycosylase (18) have been characterized in human cell-free extracts. In the present work it is shown that the properties of these two glycosylases (human εA-DNA glycosylase and m^3A-DNA glycosylase) appear similar, including binding to an εA-containing probe, suggesting that they may be the same protein.

MATERIALS AND METHODS

Materials. *N*-[^{3}H]Methyl-*N*-nitrosourea ([^{3}H]MNU, 1.7 Ci/mmol, ethanol solution; 1 Ci = 37 GBq) was obtained from New England Nuclear. [^{14}C]Dimethyl sulfate ([^{14}C]DMS, 14.2 mCi/mmol) was from Sigma. Sephadex G-50 was from Pharmacia, double-stranded (ds) DNA cellulose was obtained from Sigma or was prepared as described (19). A 25-mer oligonucleotide with εA specifically incorporated at position 6 from the 5′ end has been described (17). *Escherichia coli* BH290 (*alk*$^-$, *tag*$^-$) expressing the cloned human m^3A-DNA glycosylase [alkyl-*N*-purine DNA glycosylase (ANPG) protein] (10) and *E. coli* BH290 harboring the pUC19 plasmid as a negative control were kindly made available to us by T. R. O'Connor and J. Laval (Institut Gustave–Roussy, Villejuif, France). A-8 cation-exchange columns for HPLC were 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 (17).

Globally modified DNA containing εA was prepared by reaction with chloroacetaldehyde. Salmon sperm DNA (0.8 mg) was incubated with 0.23 M chloroacetaldehyde in 0.6 ml of 0.33 M sodium cacodylate buffer (pH 7.25) for 48 hr at 37°C. The DNA was then purified by ethanol precipitation at 0°C and dissolved in 10 mM Tris·HCl/1 mM EDTA, pH 7.8.

Globally modified DNA containing radioactively labeled m^3A was prepared by reaction with [^{3}H]MNU or [^{14}C]DMS. [^{3}H]MNU (0.5 mCi) was dried with a stream of nitrogen at 0°C and dissolved in 200 μl of 0.5 M sodium cacodylate buffer (pH 7.25) containing 0.5 mg of salmon sperm DNA. The mixture was then incubated for 1 hr at 37°C. The DNA was purified by two ethanol precipitations or by Sephadex G-50 column chromatography followed by ethanol precipitation and dissolved in 10 mM Tris·HCl/1 mM EDTA, pH 7.8. Alternatively, [^{14}C]DMS (0.125 mCi) was incubated with 1 mg of calf

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Abbreviations: εA, 1,N^6-ethenoadenine; m^3A, 3-methyladenine; MNU, *N*-methyl-*N*-nitrosourea; DMS, dimethyl sulfate; ANPG, alkyl-*N*-purine-DNA glycosylase; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

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thymus DNA in 225 μl of 0.45 M sodium cacodylate buffer (pH 8.0) for up to 2 days at 37°C. The DNA was then purified by Sephadex G-50 column chromatography, concentrated by ethanol precipitation, and dissolved in 10 mM Tris·HCl/1 mM EDTA, pH 8.0. Part of the m^3A is lost under these conditions of temperature and time.

Cell-Free Extracts. A partial purification protocol for the εA-DNA-binding protein from a nuclear extract of human placenta has been described (18). Following this protocol through purification to step IV resulted in 75-fold purification (Table 1). A portion of this material was further purified by chromatography on a column (1.1 × 3.5 cm) of dsDNA-cellulose. Bound material was eluted with a gradient of 0–1.0 M KCl in 25 mM Tris·HCl, pH 7.8/1 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/1 mM benzamidine/2 mM 2-mercaptoethanol/10% (vol/vol) glycerol, and fractions with the highest specific activity were collected to give fraction V (Table 1).

Purification of a human lymphoblast m^3A-DNA glycosylase by successive chromatography on DEAE-cellulose, dsDNA-cellulose, and single-stranded (ss) DNA-Sepharose columns (i.e., up to stage IV) was carried out as described (20), with the following modifications. The source was cultured human lymphoblasts instead of human placental tissue, and commercially prepared dsDNA-cellulose was used in step III rather than a laboratory-prepared dsDNA-Sepharose resin (Table 2).

Crude extracts from *E. coli* expressing the human ANPG protein and *E. coli* control cells were prepared using a protocol similar to that for the human εA-binding protein (17). Exponentially growing bacteria in LB broth were harvested by centrifugation, suspended in 50 mM Tris·HCl (pH 7.8), recentrifuged, suspended in a small volume of 50 mM Tris·HCl, pH 7.8/5 mM EDTA/1 mM phenylmethylsulfonyl fluoride, and stored at −70°C. The thawed cells were disrupted by incubation in the same buffer supplemented with Nonidet P-40 (0.07%) and lysozyme (0.2 mg/ml) for 30 min at 0°C. After addition of dithiothreitol (2 mM), ammonium sulfate (0.4 M), glycerol (20%), spermine (0.1 mM), and spermidine (0.5 mM), the extracts were held for another 20 min at 0°C followed by centrifugation (0°C) for 2 hr at 50,000 rpm in a Beckman SW50.1 rotor. The supernatants were collected as crude extracts and stored at −70°C.

Assays. The DNA binding assay in 6% polyacrylamide gels, using a εA-containing 25-mer oligonucleotide annealed to the complementary oligonucleotide with T opposite εA, was carried out as described (17).

DNA glycosylase activity was measured by incubation of cell-free extracts with this double-stranded oligonucleotide or with DNA in 35 mM Hepes·KOH/0.5 mM EDTA/0.5 mM dithiothreitol/40 mM KCl at pH 7.2. εA released from the 25-mer oligonucleotide or from chloroacetaldehyde-treated DNA was analyzed by HPLC on A-8 cation-exchange columns with fluorescence detection (18).

Release of m^3A from DNA treated with either [^{3}H]MNU or [^{14}C]DMS was measured using the HPLC system described in the previous paragraph, which separated m^3A from other methylated derivatives. Fractions corresponding to the m^3A peak were collected and radioactivity was measured by liquid scintillation counting. In addition, in some experiments, radioactivity was measured in all fractions from HPLC, including those containing 7-methylguanine and 1-methyladenine.

Table 1. Purification of human placental εA-binding protein

Step*	Protein, mg	Binding activity, units†	Relative specific activity
I. Nuclear extract	4200		
II. Ammonium sulfate	1770	57	1
III. Phosphocellulose	80	44	17
IV. Blue Sepharose	13	31	75
V. dsDNA-cellulose	0.03	1.3	1300

*See *Materials and Methods*.
†Arbitrary units.

Table 2. Purification of human lymphoblast m^3A-DNA glycosylase

Step	Protein, mg	Enzyme activity,* units	Relative specific activity
I. Crude extract	800	1800	1
II. DEAE-cellulose	495	1560	1.4
III. dsDNA-cellulose	1.0	430	187
IV. ssDNA-Sepharose	0.13	370	1275

*Determined by the methods described in ref. 20.

RESULTS

Enzyme Extracts. A human εA-DNA-binding protein, which also has DNA glycosylase activity acting on εA (18), was purified from a nuclear extract from human placenta (Table 1). Starting with a nuclear extract, rather than a whole-cell extract, corresponded to an initial 5-fold purified starting material. Fraction IV, which was obtained after phosphocellulose and Blue Sepharose column chromatography as reported (18), was 75-fold purified. Most studies were carried out with this fraction. A further purification step, using a dsDNA-cellulose column, resulted in a 1300-fold overall purification (Table 1).

A human m^3A-DNA glycosylase was purified 1275-fold from human lymphoblasts by using DEAE-cellulose, dsDNA-cellulose, and ssDNA-Sepharose columns (Table 2). Similar preparations have been extensively studied and characterized (13, 20).

A cloned human m^3A-DNA glycosylase (ANPG protein) was a gift from T. R. O'Connor and J. Laval for comparative studies (10). This protein lacks 70 amino acids at the N-terminal end but is a functional glycosylase for m^3A. The enzyme was expressed in *E. coli* that lacked the bacterial m^3A-DNA glycosylases (*alk*$^-$, *tag*$^-$). Crude extracts from *E. coli* were used in the present study. Extract from the same *E. coli* strain lacking the human clone was used as negative control.

Binding to εA-Containing Oligonucleotide. All three enzyme preparations studied were found to contain a DNA-binding protein specific for εA. Fig. 1 compares the εA-binding protein and the m^3A-DNA glycosylase for their binding ability. Both preparations showed a major delayed εA-specific band at identical locations in the gel. Both preparations also had weaker bands slightly below the main band, which were more prominent in the m^3A-DNA glycosylase preparation. These lower bands are probably a result of limited proteolytic cleavage of the main protein. Fig. 2 shows comparative binding of the εA-binding protein and the cloned ANPG protein. A strong delayed εA-specific band is seen for the ANPG protein. Its migration in the gel is slightly faster, which is expected because this protein is truncated and thus of a lower molecular weight.

Release of εA Base from DNA or Oligonucleotide. Purified proteins were incubated with an εA-containing oligonucleotide or chloroacetaldehyde-treated DNA, and the liberated εA was identified after HPLC using fluorescence detection. Fig. 3 shows results derived from the lymphoblast m^3A-DNA glycosylase. Both chloroacetaldehyde-treated DNA and the εA-containing 25-mer oligonucleotide served as substrate, and near-complete removal of the εA base could be obtained. Similar results were obtained with the εA-binding protein and

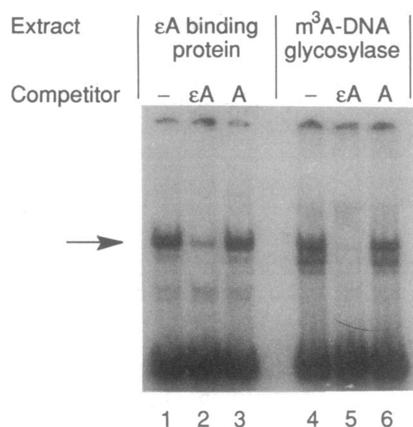

FIG. 1. Bandshift assay using an εA-containing 25-mer oligonucleotide. Cell-free extracts were incubated with the ^{32}P-labeled oligonucleotide annealed to the complement with T opposite εA, and the mixtures were analyzed by electrophoresis in a nondenaturing 6% polyacrylamide gel followed by autoradiography. The arrow points to the εA-specific delayed band due to protein binding. The incubations included competitor oligonucleotide as shown: –, no competitor; εA, a 20-fold excess of the unlabeled εA-containing probe; A, a 20-fold excess of the same probe lacking εA. The extracts were as follows: lanes 1–3, εA-binding protein, fraction IV (1 μg); lanes 4–6, m^3A-DNA glycosylase, fraction IV (0.4 μg).

with the crude extract of cloned ANPG protein (Tables 3 and 4).

Release of m^3A from DNA by the εA-DNA-Binding Protein. The εA binding protein (fraction V) was incubated with DNA containing radioactively labeled methyl groups. Low molecular weight products were then identified by HPLC, using comparison with standards. As shown in Fig. 4, the protein stimulated the release of m^3A and 7-methylguanine, indicative of a m^3A-DNA glycosylase.

Relative Rate of Release of εA and m^3A. The data shown above indicate that both εA and m^3A are efficient substrates for the two human enzymes. For quantitative comparison, mixed εA- and m^3A-containing substrates were used. These contained chloroacetaldehyde-treated DNA to give a total of 10 pmol of εA (in DNA), [^3H]MNU-treated DNA to give ^3H-labeled m^3A methyl groups, and [^{14}C]DMS-treated DNA to obtain a total m^3A concentration (in DNA) of 10 pmol. Most m^3A groups in this mixture were from the DMS-treated DNA, while nearly all radioactivity originated from the MNU-treated DNA. The enzymes were able to release m^3A from both substrates, although the [^{14}C]DMS was of low specific activity. However, using a mixture of the alkylated DNAs made it possible to have both the total amount of DNA and the total amount of m^3A (10 pmol) constant from experiment to experiment. After incubation with various extracts, release of εA was measured by fluorescence, whereas release of m^3A was measured by radioactivity coinciding with authentic marker. Table 3 shows that m^3A-DNA glycosylase activity had an approximately constant relation to εA-DNA glycosylase activity during the various purification steps of the εA-binding protein.

Comparisons between various extracts in a separate experiment are shown in Table 4. Within a factor of 2, both εA and m^3A were released at similar rates, showing that εA is as good a substrate as m^3A for these glycosylases. The small differences seen, however, were reproducible. The purified m^3A-DNA glycosylase had a slight preference for m^3A, while the crude extract from the cloned m^3A-DNA glycosylase had a slight preference for εA. The purified εA-binding protein (fraction IV) showed the same rate of release of both bases (Table 4).

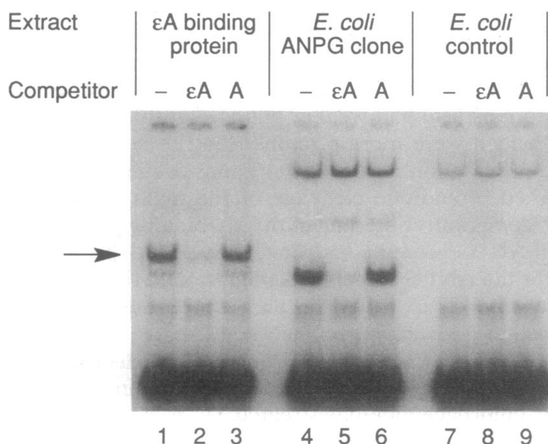

FIG. 2. Bandshift assay with the cloned human ANPG protein. Methods and symbols are as in Fig. 1. Lanes 1–3, εA-binding protein, fraction IV (1 μg); lanes 4–6, crude extract from *E. coli* (*alk*⁻, *tag*⁻) expressing the human ANPG clone (2 μg); lanes 7–9, crude extract from *E. coli* (*alk*⁻, *tag*⁻) control with the pUC19 vector alone (2 μg).

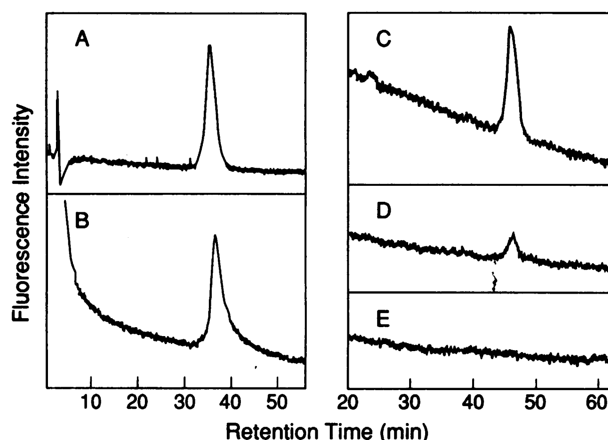

FIG. 3. Release of εA by partially purified m^3A-DNA glycosylase. The enzyme was incubated with εA-containing oligonucleotide or chloroacetaldehyde-treated DNA. Low molecular weight products were analyzed by HPLC with fluorescence detection. (*A* and *C*) Marker εA (10 pmol). (*B*) The εA-containing oligonucleotide (10 pmol) was incubated with m^3A-DNA glycosylase (8 μg of fraction IV) for 21 hr at 37°C. (*D*) Chloroacetaldehyde-treated DNA containing 10 pmol of εA adducts was incubated with m^3A-DNA glycosylase (16 μg of fraction IV) for 4 hr at 37°C; 2.4 pmol of εA was liberated as shown. (*E*) Same as *D*, except the extract was heat inactivated for 5 min at 50°C in 35 mM Hepes·KOH/0.5 mM EDTA/0.5 mM dithiothreitol/40 mM KCl at pH 7.2. The difference in retention time for εA in *A* and *B* compared with *C–E* is due to the use of two different A-8 cation-exchange columns.

Table 3. Activity releasing εA and m^3A from DNA at various stages of purification

εA-binding protein fraction	Amount, μg	Release of base, pmol	
		εA	m^3A
II	3000	3.1	1.7
III	200	5.0	3.4
IV	50	5.0	4.2
V	3	3.4	2.5

The indicated amount of protein extract was incubated for 4 hr at 37°C in 74 μl of assay buffer containing a mixture of DNA treated with chloroacetaldehyde, [^3H]MNU, and [^{14}C]DMS to give equimolar amounts of εA and m^3A in DNA (10 pmol). Total amount of DNA was 15 μg per assay. Spontaneous release of m^3A has been subtracted.

Table 4. Release of εA and m^3A from DNA by various extracts

Enzyme extract	Incubation time, hr at 37°C	Release of base, pmol	
		εA	m^3A
εA-binding protein fraction IV (48 μg)	2	1.1	0.95
	4	2.0	1.9
m^3A-DNA glycosylase fraction IV (16 μg)	2	1.6	2.7
	4	2.4*	4.5
ANPG, *E. coli* crude extract (480 μg)	2	4.0†	2.5†

See legend to Table 3 for details; 10 pmol of εA and 10 pmol of m^3A were present in DNA in each experiment.
*See Fig. 3*D* for HPLC analysis.
†No release was detected with *E. coli* control extract, lacking the human ANPG clone.

DISCUSSION

The two enzyme preparations (εA-DNA-binding protein and m^3A-DNA glycosylase) were each partially purified, using different chromatographic procedures. When compared, the two preparations were found to have similar properties with respect to (*i*) binding to an εA-containing probe, giving rise to a characteristic delayed band; (*ii*) glycosylase action toward εA in a 25-mer oligonucleotide or in chloroacetaldehyde-treated DNA; and (*iii*) glycosylase action toward m^3A in methylated DNA at a rate about equal to the rate of release of εA. The presence of these activities could be the result of copurification of two independent DNA glycosylases or be due to a single DNA glycosylase sharing activity toward both substrates. Data obtained by using the cloned truncated m^3A-DNA glycosylase suggest that all properties studied are present in a single human m^3A-DNA glycosylase. This enzyme is therefore likely to be the m^3A-DNA glycosylase purified in the placental and lymphoblast preparations, although there may be other enzymes with similar properties that cannot be ruled out.

In bandshift gels (as illustrated in Fig. 1) we typically see only one εA-specific delayed band at early stages of purification, provided that protease inhibitors are present. Without protease inhibitors, or at higher levels of purification, two additional bands below the main band are usually also present. Fractions containing mainly the lower bands are still active. Thus, heterogeneous populations of active enzyme are typically obtained during purification, apparently from proteolytic activity. Heterogeneity in the size of mammalian m^3A-DNA glycosylases has also been observed by others (21) and may explain slight differences between different extracts. Such phenomena could explain the differences seen (within a factor of 2) of the relative rate of removal of εA and m^3A in various extracts. Another factor could be the total amount of protein present, which will vary with the degree of purification.

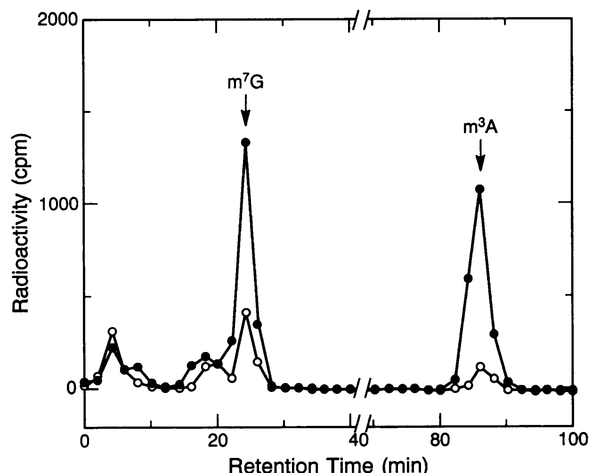

FIG. 4. Release of m^3A and 7-methylguanine (m^7G) from DNA by partially purified εA-binding protein. Enzyme extract (14 μg of fraction V) was incubated with [^3H]MNU-treated DNA for 4 hr at 37°C. Released low molecular weight products were analyzed by HPLC. •, With enzyme extract; ○, without enzyme extract. The released m^7G (6.7 pmol) corresponds to about 8% of the total amount present; the released m^3A (9.6 pmol) corresponds to nearly complete removal. The total amounts of each derivative were measured using acid depurination and the same HPLC system.

Previously it has been shown that purified εA-DNA-binding protein has εA-DNA glycosylase activity (18), suggesting that both activities are due to the same protein. This is supported by the m^3A-DNA glycosylase data, which show that this enzyme can act also as an εA-specific binding protein. The binding requires either high salt concentration (150 mM NaCl) or the presence of Mg^{2+}, spermine, or spermidine (17). It is noteworthy that the constituents found to promote εA-specific DNA binding also have been found to be inhibitors of m^3A-DNA glycosylase (20). For example, the enzyme is very sensitive to salt, and its activity is decreased by a factor of >10 at 150 mM NaCl in comparison with its optimal activity at 50 mM NaCl. However, moderate spermine or spermidine concentrations (0.2 mM) promote binding, whereas inhibition of glycosylase activity requires higher concentrations. This needs further investigation but suggests that the phenomenon of DNA binding is related to partial stabilization of the enzyme–substrate complex.

The glycosyl bond of m^3A is too unstable to allow a site-specific probe to be made for DNA binding studies (7). However, experiments using DMS-treated DNA as competitor in the binding assay, with or without limited hydrolysis to remove m^3A adducts, suggest that competitive binding to m^3A does not occur or is too transient to be detected. It should be noted that because the glycosyl bond in m^3A is unstable, the time of binding may be too short to detect the complex in our assay. On the other hand, the glycosyl bond in εA is as stable as that of the parent nucleoside and may require a longer interaction with the protein.

The present data suggest an extended substrate range for a human m^3A-DNA glycosylase to include εA, a cyclic DNA adduct. Since cyclic DNA adducts represent a large group of potentially carcinogenic and mutagenic DNA lesions produced by a large variety of environmental chemicals (22), this apparently extends the area of study of human DNA glycosylase activities to a new interesting group of substrates.

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