Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1024–1028, February 1994
Biochemistry

All four known cyclic adducts formed in DNA by the vinyl chloride metabolite chloroacetaldehyde are released by a human DNA glycosylase

(3-methyladenine-DNA glycosylase/1,N^6-ethenoadenine binding protein/1,N^2-ethenoguanine/N^2,3-ethenoguanine/3,N^4-ethenocytosine)

M. K. DOSANJH*, A. CHENNA*, E. KIM*, H. FRAENKEL-CONRAT*†, L. SAMSON‡, AND B. SINGER*§

*Life Sciences Division, Donner Laboratory, Lawrence Berkeley Laboratory, and †Department of Molecular and Cell Biology, Stanley Hall, University of California, Berkeley, CA 94720; and ‡Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, MA 02115

Contributed by H. Fraenkel-Conrat, October 5, 1993

ABSTRACT **We have previously reported that human cells and tissues contain a 1,N^6-ethenoadenine (εA) binding protein, which, through glycosylase activity, releases both 3-methyladenine (m^3A) and εA from DNA treated with methylating agents or the vinyl chloride metabolite chloroacetaldehyde, respectively. We now find that both the partially purified human εA-binding protein and cell-free extracts containing the cloned human m^3A-DNA glycosylase release all four cyclic etheno adducts—namely εA, 3,N^4-ethenocytosine (εC), N^2,3-ethenoguanine (N^2,3-εG), and 1,N^2-ethenoguanine (1,N^2-εG). Base release was both time and protein concentration dependent. Both εA and εC were excised at similar rates, while 1,N^2-εG and N^2,3-εG were released much more slowly under identical conditions. The cleavage of glycosyl bonds of several heterocyclic adducts as well as those of simple methylated adducts by the same human glycosylase appears unusual in enzymology. This raises the question of how such a multiple, divergent activity evolved in humans and what may be its primary substrate.**

Vinyl chloride is a known human and rodent carcinogen and causes hepatic angiosarcomas (1, 2). It is metabolized by the human liver P450 system (3) to the reactive unstable intermediate chloroethylene oxide, which rearranges rapidly to the more stable bifunctional carcinogen chloroacetaldehyde (CAA) (4). CAA reacts with nucleic acid bases to ultimately form four known cyclic adducts: 1,N^6-ethenoadenine (εA), 3,N^4-ethenocytosine (εC), N^2,3-ethenoguanine (N^2,3-εG), and 1,N^2-ethenoguanine (1,N^2-εG) (summarized in refs. 5 and 6). This type of adduct is formed in rodents by administration not only of vinyl chloride (7) but also of related compounds such as vinyl carbamate (8, 9), ethyl carbamate (8–10), acrylonitrile (11), and other two-carbon compounds that can be converted to 1-halooxiranes after oxidation by P450 enzymes (3). Lipid peroxidation has also been reported to form α,β-unsaturated aldehydes that react with deoxynucleosides yielding etheno adducts (12, 13). Adducts, including εA, εC, and 1,N^2-propano-G, have been detected in normal liver DNA from rodents (14, 15) and humans (15), suggesting that such cyclic adducts are formed endogenously.

The role of each of the known etheno adducts in causing toxic and carcinogenic effects has not been clearly defined in animals. The major modified base found in the liver of animals exposed to vinyl chloride is 7-(2-oxoethyl)guanine, originating from the first metabolite, chloroethylene oxide (16, 17). However, this modified base has not been shown to cause mispairing when replicated in a DNA template (18), and it is not likely to lead to biological effects unless deprinated (19). On the other hand, N^2,3-εG, εA, and εC have all been shown to cause mutations *in vitro* (20–22) and *in vivo* (23–25). Eukaryotic cells could be protected against the mutagenic effects of vinyl chloride and related compounds if they possessed a repair mechanism that removes etheno adducts from DNA.

In 1986, a preliminary report indicated that εA and N^2,3-εG were released from CAA-treated DNA by extracts from rat brain cells (26). However, this finding was qualitative and evidently not pursued. There is also evidence that cloned *Escherichia coli* 3-methyladenine (m^3A)-DNA glycosylase II [alkylated-DNA glycohydrolase (releasing methyladenine and methylguanine), EC 3.2.2.21; also called DNA-3-methyladenine glycosidase II] can excise N^2,3-εG from CAA-treated DNA (27). Recently, a εA-specific DNA-binding protein with εA-DNA glycosylase (glycosidase) activity has been characterized in human tissues and cell-free extracts in our laboratory (28–30). We later found that both the εA-DNA glycosylase and the human m^3A-DNA glycosylase, both partially purified, appeared to be similar in their ability to release m^3A and εA and in their ability to bind a εA-containing probe (30).

This raised the question as to the substrate range of the εA-DNA glycosylase purified from human cells and tissues and the cloned human m^3A-DNA glycosylase (31–34). We now report that all four known etheno adducts (including the most stable hydrated intermediate εC·H_2O; ref. 22) are released by both types of glycosylases (Fig. 1). It remains to be shown whether the εA-binding protein and any of the m^3A-DNA glycosylase clones (31–34) are the same enzyme or whether further investigation of substrate range will indicate divergence.

MATERIALS AND METHODS

Materials. CAA, 45% solution in water (≈6.9 M), was purchased from Merck. εA and 1,N^6-etheno-2′-deoxyadenosine (εdA) were purchased from Sigma, whereas εC and 3,N^4-etheno-2′-deoxycytidine (εdC) were prepared by the method of Barrio *et al.* (35). N^2,3-εG and 1,N^2-εG were synthesized as described by Sattsangi *et al.* (36) and Kuśmierek and coworkers (37, 38). [8-^{3}H]dGTP, [^{3}H]dCTP, and [^{3}H]dATP (20 Ci/mmol; 1 Ci = 37 GBq) were purchased from Amersham.

Preparation of Glycosylases. *Human cells.* Whole-cell extracts from HeLa cells (Cell Culture Center, Minneapolis) and nuclear extracts from human placenta were prepared and partially purified as described by Rydberg *et al.* (28, 29).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: εA, 1,N^6-ethenoadenine; εC, 3,N^4-ethenocytosine; εdA, 1,N^6-etheno-2′-deoxyadenosine; εdC, 3,N^4-etheno-2′-deoxycytidine; N^2,3-εG, N^2,3-ethenoguanine; 1,N^2-εG, 1,N^2-ethenoguanine; CAA, chloroacetaldehyde; m^3A, 3-methyladenine.

§To whom reprint requests should be addressed.

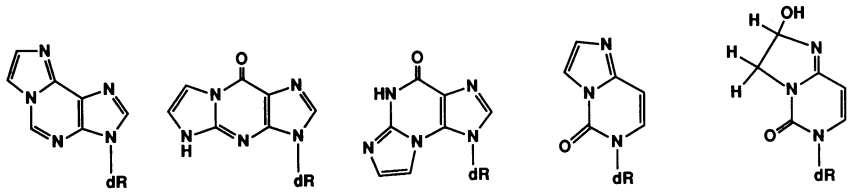

FIG. 1. Chemical structures of the cyclic products formed by reaction of vinyl chloride with DNA and one of the hydrated intermediates (εC·H_2O).

Cloned human m^3A-DNA glycosylase. Crude extracts from *E. coli* expressing human m^3A-DNA glycosylase (32) were prepared by using a protocol similar to that for HeLa cells (28, 29). (Note that the *E. coli* host strain was deficient in the endogenous AlkA and Tag I glycosylases.) Briefly, exponentially growing bacteria 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 with the same buffer containing 0.07% Nonidet P-40 and lysozyme at 0.2 mg/ml for 30 min on ice. After addition of 2 mM dithiothreitol, 0.4 M ammonium sulfate, 20% (vol/vol) glycerol, 0.1 mM spermine, and 0.5 mM spermidine, the extracts were kept on ice for a further 20 min followed by centrifugation for 2 hr at 50,000 rpm in a Beckman SW50.1 rotor. The supernatant was collected as crude extract (protein at ≈1 mg/ml) and stored at −70°C.

DNA Substrates. *Oligonucleotides.* A probe was constructed containing a single, site-specifically placed εC. This monomer was prepared as the deoxynucleoside (εdC) by using the methodology for the ribo derivative (35). The oligonucleotide was synthesized on an Applied Biosystem model 381 automated DNA synthesizer using standard phosphoramidite chemistry. The coupling time was prolonged to 15 min to improve the coupling efficiency, and the resulting oligomer was deprotected in 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) rather than ammonia to minimize possible ring opening. The oligonucleotide was then gel-purified and used for binding assays as described for the εA-containing probe, which was a gift from A. K. Basu (39). The εA probe was 5′-CCGCTεAGCGGGTACCGAGCTCGAAT-3′; the εC probe was 5′-CCGCTAGεCGGGTACCGAGCTCGAAT-3′. Each of the probes was ^{32}P-labeled at the 5′ end and hybridized to the complementary sequence in which T was opposite εA and G was opposite εC.

CAA-treated DNA. Calf thymus DNA (Sigma) was radiolabeled in either guanine, cytosine, or adenine with individual [^{3}H]dNTPs by using the nick-translation kit purchased from Amersham. The nick-translation reaction mixtures routinely contained 5 μg of DNA, 100 μCi of [^{3}H]dNTP, unlabeled dNTPs at 50 μM each, 25 units of DNA polymerase I, and 0.5 μg of DNase I.

The reaction mixture was nick translated by incubation for 2 hr at 15°C with DNA polymerase I and DNase I. The DNase I was then inactivated by heating at 65°C for 5 min. Ten units of DNA polymerase I was added, and the reaction was then continued for a further 15 min. Finally, replication was terminated by heating the mixture at 65°C for 5 min. Unincorporated dNTPs were removed by centrifugation with a Centricon 10 filter (Amicon). The resulting DNA was washed twice with 0.1 M sodium cacodylate buffer (pH 7.25) again with use of the Centricon 10 filters. The specific activity of the resulting DNA was found to be ≈1.8–2.6 × 10^6 cpm/μg of DNA for each of the [^{3}H]dNTP used. CAA modification of the labeled DNA was carried out in 0.1 M sodium cacodylate buffer (pH 7.25) containing 0.69 M CAA at room temperature for 18 hr to 3 days depending on the extent of modification desired. The CAA-treated DNA was purified by using Centricon 10 filters washed three times with 500 μl of glycosylase/nicking buffer (35 mM Hepes·KOH/0.5 M EDTA/0.5 M dithiothreitol/40 mM KCl at pH 7.2).

Analysis of CAA-Treated DNA. The percentage modification of the DNA by CAA was determined by HPLC after hydrolysis. DNA was hydrolyzed either by acid (for εA, N^2,3-εG, and 1,N^2-εG) or by enzymes (for εC) (see below). Authentic markers were used to identify the radiolabeled peak fractions, which were collected and assayed for radioactivity by liquid scintillation.

Acid hydrolysis. Purines were released from CAA-treated DNA with 0.1 M HCl for 3 hr at 37°C (for εA) and 12–18 hr at 37°C for the others. This depurination method prevents secondary reaction of εA in acid (data not shown).

Enzymatic hydrolysis. To a neutralized sample (after acid hydrolysis), 1 M Tris·HCl (pH 9) and 0.1 M $MgCl_2$ were added to a final concentration of 0.1 M and 0.01 M, respectively. DNA was incubated with 0.25 μg of DNase I per ml for 3 hr at 37°C, followed by the addition of 0.12 μg of snake venom phosphodiesterase, 0.4 μg of bacterial alkaline phosphatase, and 0.25 μg of nuclease P1 per ml. The mixture was incubated for a further 12 hr. Low molecular weight products (below the cutoff limit of M_r 10,000 of the Amicon 10 filters) were separated from the bulk of the proteins and any high molecular weight compounds by diluting the samples with 1 ml of H_2O followed by ultrafiltration. The filtrates were concentrated by evaporation under vacuum to 100 μl for HPLC analysis.

Binding and Nicking Assay. Prior to the use of the cell extracts for glycosylase reaction, all extracts were tested for their binding activity toward εA- and εC-containing oligonucleotide probes. The DNA binding assay in 6% polyacrylamide gels with either a εA- or a εC-containing 25-mer oligonucleotide hybridized to the complementary sequences with T opposite εA and G opposite εC was carried out as described (28). To test for nicking, the cell extracts were incubated with the ^{32}P-labeled probe for 1 hr at 37°C. The samples were then heat-denatured and electrophoresed on a 12% denaturing gel at 2500 V for ≈1 hr. These conditions separated the small cleaved fragments (five or seven bases) from the larger fragments. The size of these fragments resulting from cleavage of an abasic site was determined by the appropriate size markers.

DNA Glycosylase Assay. DNA glycosylase activity was measured by incubation of CAA-treated DNA with partially purified extracts from HeLa cells, nuclear extracts from human placenta, or crude *E. coli* extracts containing the cloned human m^3A-DNA glycosylase. The ethenobases enzymatically released from the CAA-treated [^{3}H]DNA were analyzed by HPLC after separation from proteins and high molecular weight DNA.

HPLC Analysis. For quantitating base modification after the hydrolysis of CAA-treated DNA and for measuring etheno adducts released by the protein extracts, samples were analyzed on a 250 × 4 mm Aminex A8 HPLC cation-

Table 1. HPLC retention times

Compound	Retention time, min pH 7	pH 9
2′-Deoxyadenosine	14	
Adenine	23	
εdA	30	
εA	46	
2′-Deoxycytidine	8	
εdC	15	
εC	23	
εC·H_2O	48	
2′-Deoxyguanosine	20	11
Guanine	24	21
1,N^2-εG	46	41
N^2,3-εG	23	16

HPLC analysis used an Aminex A-8 column with 0.4 M ammonium formate as the mobile phase and a flow rate of 0.5 ml/min at 45°C. εC·H_2O, 3,N^4-(N^4-α-hydroxyethene)cytidine. See Fig. 1 for structures.

exchange column (Bio-Rad) eluted with 0.4 M ammonium formate (pH 7 or 9) at 45°C (Table 1). Separation of guanine and the two εG adducts was only possible at pH 9. The eluate was simultaneously monitored for fluorescence and UV. Radioactivity was measured by liquid scintillation counting using 1-min fractions. Peaks were identified by subsequent coelution of a portion of relevant fractions with authentic markers. The coincidence of the UV marker with radioactivity was used to confirm the identity of the peak.

RESULTS

Human Binding Protein That Recognizes Both εA and εC. Partially purified HeLa extract was found to bind to both εA- and εC-containing probes. The binding was stronger with the εC oligonucleotide (Fig. 2), since the fraction of the probe that was retarded is greater and also the εC-containing oligonucleotides competed with the εA binding more effectively. Similar results for εC and εA binding were obtained when using the cloned m^3A-DNA glycosylase and placental extracts.

Specific Nicking at the 5′ Side of the εA and εC Adduct. To measure nicking by HeLa cell extracts as a result of the glycosylase action, binding reaction mixtures were incubated at 37°C for a further 1 hr and analyzed on a 12% denaturing polyacrylamide gel. A ^{32}P-labeled band that ran as a 5-base oligonucleotide was seen with the εA-containing probe, and a 7-base fragment was seen with the εC-containing probe, indicative of specific nicking of the abasic site resulting from glycosylase activity on the 5′ side of these two etheno adducts (Fig. 3). No additional endonuclease was required for cleavage, indicating that the partially purified extracts also contained an endonuclease.

Release of Etheno Bases from CAA-Treated DNA. Since we did not have site-specifically-placed probes for all four etheno adducts (εA, εC, 1,N^2-εG, and N^2,3-εG), we used CAA-treated nick-translated [^{3}H]DNA because these radiolabeled substrates made it possible to independently examine the release of all four etheno adducts. Nick-translation allows the incorporation of a specifically labeled dNTP with the radiolabel in the base, sugar, or the phosphate groups. The method is general in its application and helps in the identification of adducts, since only the moiety containing the label is detected by scintillation counting. In these assays the [^{3}H]dNTP contained the label in the base moiety, and [^{3}H]dNTPs were individually incorporated so that there were three DNA samples: [^{3}H]C-DNA, [^{3}H]G-DNA, and [^{3}H]A-DNA. The DNA was then modified with CAA as outlined in *Materials and Methods* and incubated with partially purified HeLa, placenta, or crude m^3A-DNA glycosylase. The resulting reaction mixture was analyzed by HPLC for the release of etheno bases. Fig. 4 shows the simultaneous release of εC and εA from a single sample in the presence of excess crude protein. The release of εC was quantitated by scintillation counting, and the release of εA was determined by fluorescence. The picomoles released for these adducts were similar.

By using DNA individually labeled with [^{3}H]A, [^{3}H]C, or [^{3}H]G it was found that all four known etheno adducts were released when samples were incubated with glycosylases in parallel under the same conditions. In the case of CAA-treated [^{3}H]C-DNA, both εC and εC·H_2O, the unsaturated hydrated intermediate (Fig. 1), were released. When the DNA sample was dehydrated (22) prior to the incubation with

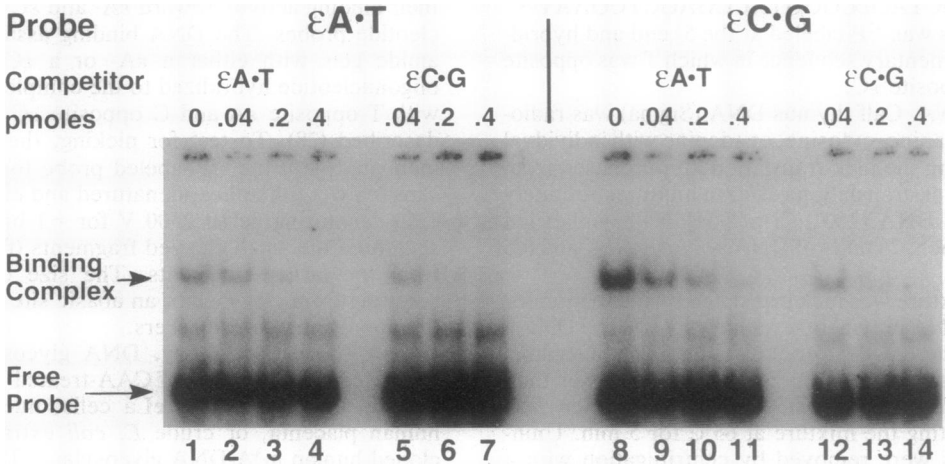

FIG. 2. A typical autoradiogram of a bandshift assay using 0.01 pmol of εA-containing (*Left*) or 0.01 pmol of εC-containing (*Right*) double-stranded 25-mer oligonucleotides. Partially purified HeLa extract was incubated with the 5′-^{32}P-labeled probes and increasing amounts of unlabeled competitor probe (0.04–0.4 pmol). An arrowhead indicates the resulting oligonucleotide–protein complexes. Lanes 1–7 contain the probe in which [^{32}P]εA is paired with thymine, and lanes 8–14 contain the probe in which [^{32}P]εC is paired with guanine. In the first four lanes in each group are increasing amounts of unlabeled εA-containing oligonucleotide competitor probe, and the following sets of three lanes contain increasing amounts of unlabeled εC oligonucleotide competitor. Note that the εC-containing oligonucleotide appears to both bind and compete more effectively than that with εA. Similar results were found with placental extracts and cloned m^3A-DNA glycosylase (data not shown).

the glycosylase, only one peak corresponding to εC was detected.

The identity of all released adducts was confirmed by collecting the appropriate fractions followed by coelution with authentic UV markers. Fig. 5 illustrates this technique for identification of the release of N^2,3-εG and 1,N^2-εG, using the crude human cloned m^{3}A·DNA glycosylase. Both placental and HeLa extracts also released the ethenoguanines.

Relative Rate of Release of the Etheno Adducts. The data in Fig. 4 and Table 2 show that both εA and εC are good substrates for the glycosylase and are released rapidly and at approximately the same rate. However, the two ethenoguanine adducts are released at a lower rate than εA or εC. This differential release of adducts was observed for both the HeLa and placenta extracts as well as the *E. coli* extracts containing the cloned glycosylase. The excision of all four etheno compounds is both time and protein dependent (data not shown).

DISCUSSION

In recent papers, we described the release of εA by a binding protein isolated from a variety of human cells and tissues (28, 29). Later, m^{3}A-DNA glycosylase from human lymphoblasts was also found to bind and release εA (30), in addition to its previously defined specificity for N-3- and N-7-alkylated bases. The unexpected glycosylase activity found for εA led us to address the question whether the other cyclic etheno derivatives could also be substrates for the εA-binding protein and the cloned human m^{3}A-DNA glycosylase (32).

Interestingly, both enzymes released all four known etheno bases (εA, εC, 1,N^2-εG, and N^2,3-εG) as well as the structurally different hydrated intermediate of εC (23) (Fig. 1). εC is released by both glycosylases at a rate similar to εA, while 1,N^2-εG and N^2,3-εG are released much more slowly. It should be noted that the planar 1,N^2-εG has a more stable glycosyl bond than that of deoxyguanosine (38), while angular N^2,3-εG is highly labile, even at pH 7.0 (37). εC is a pyrimidine, and both εdC and the parent nucleoside, deoxycitidine, are 2 orders of magnitude more stable than the purines, deoxyadenosine and deoxyguanosine (40). It thus appears that the rate of release of these adducts does not correlate with the strength of the individual glycosyl bonds.

In vivo, the extent of formation of each of these etheno derivatives may be influenced by the rate of formation of the reactive intermediate of the several carcinogens forming such adducts (41) as well as by the extent of DNA modification and repair of the resulting adducts. 1,N^2-εG is formed in low

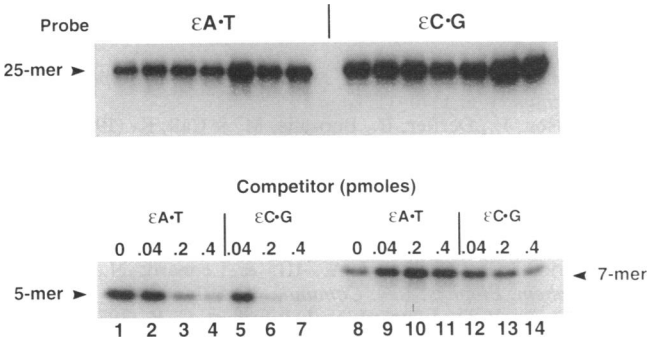

FIG. 3. A typical autoradiogram of a nicking assay of oligonucleotides containing εA or εC with and without competitors. The same reaction mixtures as used in Fig. 2 were incubated for an additional hour at 37°C, then denatured in formamide, and subjected to electrophoresis on a 12% denaturing gel (28). (*Left*) Experiments using the probe with εA paired with thymine. (*Right*) Experiments using the probe with εC paired with guanine. The mobility of the original 25-mers is indicated by an arrowhead, and the resulting 5-base fragment from the εA probe or the 7-base fragment from the εC probe is also indicated with an arrowhead. These fragment sizes are expected if the oligonucleotide is cleaved on the 5′ side of the etheno adduct (see *Materials and Methods* for the sequences used). Each lane corresponds to the same lane in the binding assay shown in Fig. 2. Note that the εC-containing oligonucleotide appears to be a better competitor than the εA-containing oligonucleotide for both probes. This conclusion is based on a number of additional experiments showing the same differential.

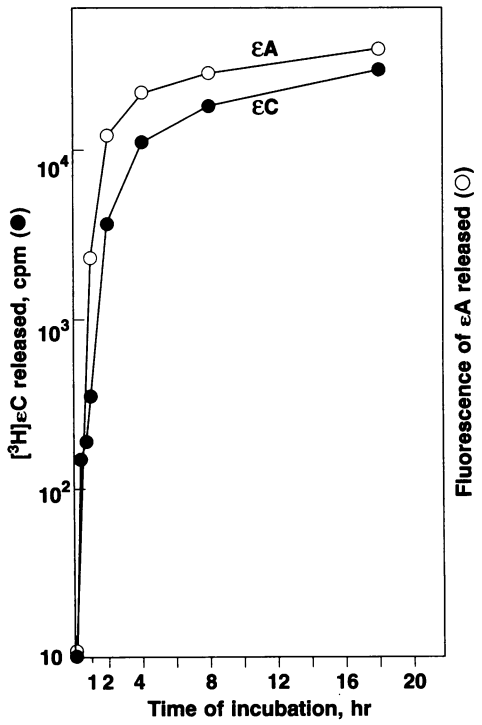

FIG. 4. Time-dependent release of εA and εC bases by m^{3}A-DNA glycosylase. Nick-translated DNA with [^{3}H]dCTP incorporated was modified with CAA as described in text and incubated with 500 μg of crude cell-free extract from bacteria expressing the cloned human m^{3}A-DNA glycosylase (30). The εA release was quantitated from a standard curve of fluorescence intensity of authentic εA base. This allowed quantitation of εA (pmol). Radioactivity assay via scintillation counting was the only method of determining εC, and the specific activity allowed a direct comparison of the two bases. With the amount of protein used, almost complete release of εA and εC was found after 8 hr of incubation.

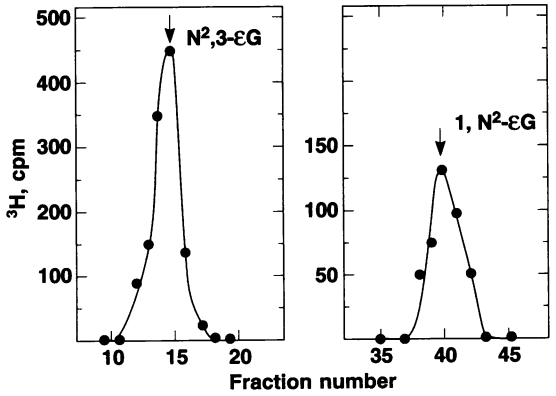

FIG. 5. Coelution of a portion of [^{3}H]εG adducts, originally collected at the retention time of authentic markers (Table 1). HPLC was performed in 0.4 M ammonium formate (pH 9). These samples were obtained after incubation of the CAA-treated [^{3}H]deoxyguanosine-inserted DNA with partially purified HeLa extracts. The arrows indicate the peak of authentic UV markers.

Table 2. Approximate extent of modification of DNA treated with CAA and release of the resulting etheno adducts using limiting amounts of protein

Adduct	% modification of parent base*	% of the modified base released by glycosylase†
εA	12	15
εC	14	20
N^2,3-εG	6	2
1,N^2-εG	0.4	4

Twenty-four-hour reaction of DNA with 0.69 M CAA at room temperature in 0.1 M sodium cacodylate (pH 7.25).

*Purine bases were determined by mild acid depurination (0.1 M HCl for 3 hr at 37°C) and pyrimidine deoxynucleosides were determined by enzyme hydrolysis.

†Eighteen-hour incubation with a limiting amount of crude human m^3A-DNA glycosylase protein (100 μg). Under the conditions used, the release of all four adducts was linear during the 18-hr period. Complete release of εA and εC by using excess protein is shown in Fig. 4.

amounts *in vitro* (38) but has not been detected *in vivo* (42), probably because of its low level of formation and the low affinity of the antibody used (42). This does not necessarily exclude it from being a possible initiator of malignant transformation.

N^2,3-εG is a major etheno adduct formed in liver, the target organ, of rats given vinyl chloride by inhalation (43). Similarly, when mice or rats were administered vinyl carbamate epoxide, the reactive metabolite of vinyl carbamate and ethyl carbamate, N^2,3-εG was again the major etheno adduct in the target organ (10). N^2,3-εG is formed *in vivo* in substantial amounts and is known to be highly mutagenic, producing G → A transitions exclusively (20, 23). Based on the present study, its repair is predicted to be slow. Thus, this adduct is likely to be primarily responsible for the observed carcinogenesis resulting from exposure to vinyl chloride and related compounds.

This work was supported by Grants CA 47723 and CA 42736 (B.S.) from the National Institutes of Health and administered by the Office of Health and Environmental Research, Office of Energy Research of the U.S. Department of Energy under Contract DE-AC03-76SF00098. L.S. is the recipient of the Burroughs Wellcome Toxicology Scholar Award.

1. Creech, J. L., Jr., & Johnson, M. N. (1974) *J. Occup. Med.* **16**, 150–151.
2. Purchase, I. F. H., Stafford, J. & Paddle, G. M. (1987) *Food Chem. Toxicol.* **25**, 187–202.
3. Guengerich, F. P., Kim, D.-H. & Iwasaki, M. (1991) *Chem. Res. Toxicol.* **4**, 168–179.
4. Laib, R. J. (1986) in *The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis*, eds. Singer, B. & Bartsch, G. (Oxford Univ. Press, London), IARC Sci. Publ. no. 70, pp. 101–108.
5. Leonard, N. J. (1984) *CRC Crit. Rev. Biochem.* **15**, 125–199.
6. Leonard, N. J. (1992) *Biochem. Mol. Biol. Chemtracts* **3**, 273–297.
7. Swenberg, J. A., Fedtke, N., Fennel, T. R. & Walker, V. E. (1990) in *Progress in Predictive Toxicology*, eds. Clayson, D. B., Munro, I. C., Shubik, P. & Swenberg, J. A. (Elsevier, New York), pp. 161–184.
8. Leithauser, M. T., Liem, A., Stewart, B. C., Miller, E. C. & Miller, J. A. (1990) *Carcinogenesis* **11**, 463–473.
9. Dahl, G. A., Miller, J. A. & Miller, E. C. (1978) *Cancer Res.* **38**, 3793–3804.
10. Park, K. K., Liem, A., Stewart, B. C. & Miller, J. A. (1993) *Carcinogenesis* **14**, 441–450.
11. Guengerich, F. P., Hogy, L. L., Inskeep, P. B. & Liebler, D. C. (1986) in *The Role of Cyclic and Nucleic Acid Adducts in Carcinogenesis and Mutagenesis*, eds. Singer, B. & Bartsch, H. (Oxford Univ. Press, London), IARC Sci. Publ. no 70, pp. 255–260.
12. Sodum, R. S. & Chung, F.-L. (1988) *Cancer Res.* **48**, 320–323.
13. Vaca, C. E., Wilhelm, J. & Harms-Ringdahl, M. (1988) *Mutat. Res.* **195**, 137–149.
14. Barbin, A., El Ghissassi, F., Nair, J. & Bartsch, H. (1993) *Proc. Am. Assoc. Cancer Res.* **34**, 136 (abstr.).
15. Nath, R. G. & Chung, F.-L. (1993) *Proc. Am. Assoc. Cancer Res.* **34**, 137 (abstr.).
16. Laib, R. J., Gwinner, L. M. & Bolt, H. M. (1981) *Chem.-Biol. Interact.* **37**, 219–231.
17. Osterman-Golkar, S., Hultmark, D., Segerbäck, D., Calleman, C. J., Göthe, R., Ehrenberg, L. & Wachtmeister, C. A. (1977) *Biochem. Biophys. Res. Commun.* **76**, 259–266.
18. Barbin, A., Laib, R. J. & Bartsch, H. (1985) *Cancer Res.* **45**, 2440–2444.
19. Loeb, L. A. (1985) *Cell* **40**, 483–484.
20. Singer, B., Kuśmierek, J. T., Folkman, W., Chavez, F. & Dosanjh, M. K. (1991) *Carcinogenesis* **12**, 745–747.
21. Barbin, A. & Bartsch, H. (1986) in *The Role of Cyclic and Nucleic Acid Adducts in Carcinogenesis and Mutagenesis*, eds. Singer, B. & Bartsch, H. (Oxford Univ. Press, London), IARC Sci. Publ. no. 70, pp. 345–358.
22. Singer, B., Kuśmierek, J. T. & Fraenkel-Conrat, H. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 969–972.
23. Cheng, K. C., Preston, B. D., Cahill, D. S., Dosanjh, M. K., Singer, B. & Loeb, L. A. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9974–9978.
24. Jacobsen, J. S. & Humayun, M. Z. (1990) *Biochemistry* **29**, 496–504.
25. Basu, A. K., Wood, M. L., Niedernhofer, L. J., Ramos, L. A. & Essigmann, J. M. (1993) *Biochemistry* **32**, 12793–12801.
26. Oesch, F., Adler, S., Rettelbach, R. & Doerjer, G. (1986) in *The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis*, eds. Singer, B. & Bartsch, H. (Oxford Univ. Press, London), IARC Sci. Publ. no. 70, pp. 373–379.
27. Matijasevic, Z., Sekiguchi, M. & Ludlum, D. B. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 9331–9334.
28. Rydberg, B., Dosanjh, M. K. & Singer, B. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6839–6842.
29. Rydberg, B., Qiu, Z.-H., Dosanjh, M. K. & Singer, B. (1992) *Cancer Res.* **52**, 1377–1379.
30. Singer, B., Antoccia, A., Basu, A. K., Dosanjh, M. K., Fraenkel-Conrat, H., Gallagher, P. E., Kuśmierek, J. T., Qiu, Z. H. & Rydberg, B. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 9386–9390.
31. O'Connor, T. R. & Laval, J. (1991) *Biochem. Biophys. Res. Commun.* **176**, 1170–1177.
32. Samson, L., Derfler, B., Boosalis, M. & Call, K. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9127–9131.
33. Chakravarti, D., Ibeanu, G. C., Tano, K. & Mitra, S. (1991) *J. Biol. Chem.* **266**, 15710–15715.
34. Vickers, M. A., Vyas, P., Harris, P. C., Simmons, D. L. & Higgs, D. R. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3437–3441.
35. Barrio, J. R., Secrist, J. A., III, & Leonard, N. J. (1972) *Biochem. Biophys. Res. Commun.* **46**, 597–604.
36. Sattsangi, P. D., Leonard, N. J. & Frihart, C. R. (1977) *J. Org. Chem.* **42**, 3292–3296.
37. Folkman, W., Kuśmierek, J. T. & Singer, B. (1990) *Chem. Res. Toxicol.* **3**, 536–539.
38. Kuśmierek, J. T. & Singer, B. (1992) *Chem. Res. Toxicol.* **5**, 634–638.
39. Basu, A. K., Niedernhofer, L. J. & Essigmann, J. M. (1987) *Biochemistry* **26**, 5626–5635.
40. Singer, B. & Grunberger, D. (1993) *Molecular Biology of Mutagens and Carcinogens* (Plenum, New York).
41. Guengerich, F. P. (1991) *Chem. Res. Toxicol.* **5**, 2–5.
42. Foiles, P. G., Miglietta, L. M., Nishikawa, A., Kuśmierek, J. T., Singer, B. & Chung, F.-L. (1993) *Carcinogenesis* **14**, 113–116.
43. Swenberg, J. A., Fedtke, N., Ciroussel, F., Barbin, A. & Bartsch, H. (1992) *Carcinogenesis* **13**, 727–729.