1,N^6-Ethenodeoxyadenosine, a DNA Adduct Highly Mutagenic in Mammalian Cells†

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ABSTRACT: 1,N^6-Ethenodeoxyadenosine (ϵdA) is one of four exocyclic DNA adducts produced by chloroethylene oxide and chloroacetaldehyde, reactive metabolites of vinyl chloride, a human carcinogen. ϵdA has also been detected in DNA of the liver of humans and untreated animals, suggesting its formation from endogenous sources. The mutagenic potential of ϵdA was studied using a single-stranded shuttle vector system in several *E. coli* strains and in simian kidney cells (COS7). This vector system enables quantitative analysis of translesional synthesis past a site-specifically placed DNA adduct in both hosts owing to the lack of the complementary strand. In experiments with five strains of *E. coli*, a very limited number of targeted mutations (one ϵdA→T, one ϵdA→dC, and two ϵdA→single base deletion) were observed among 756 transformants in hosts preirradiated with UV; no targeted mutations were observed among 563 transformants in nonirradiated hosts. These results indicate that nonmutagenic base pairings of ϵdA:T are the almost exclusive events in *E. coli*. In COS7 cells, the frequency of targeted mutations was 70%, consisting of ϵdA→dG (63%), ϵdA→T (6%), and ϵdA→dC (1%), indicating that the insertion of dCMP opposite the adduct is predominant. When compared with the results for 3,N^4-ethenodeoxycytidine (ϵdC), which was studied previously in the same system [Moriya et al. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11899−11903], the results of this study indicate that the intrinsic mutagenic potency of ϵdA is comparable to that of ϵdC in mammalian cells.

1,N^6-Ethenodeoxyadenosine (ϵdA)[1] is one of four exocyclic DNA adducts [1,N^2-ethenodeoxyguanosine, N^2,3-ethenodeoxyguanosine (ϵdG), 3,N^4-ethenodeoxycytidine (ϵdC), and ϵdA] produced by chloroethylene oxide and chloroacetaldehyde, reactive metabolites of the human carcinogen, vinyl chloride [reviewed by Bolt (1994) and Persmark et al. (1994)]. ϵdA and ϵdC have also been identified in mice treated with vinyl carbamate or ethyl carbamate (Leithauser et al., 1990). Recently, these two adducts were shown to exist in liver DNA of untreated rats and humans (Nair et al., 1995), suggesting their formation from certain endogenous sources. In fact, Ghissassi et al. (1995) have shown that lipid peroxidation products cause the formation of ϵdA and ϵdC. Even though these cyclic adducts are removed from DNA by specific DNA glycosylases (Dosanjh et al., 1994; Saparbaev et al., 1995), they are suspected to have a significant impact on carcinogenesis because of their miscoding capability *in vitro* (Revich & Beattie, 1986; Simha et al., 1991; Singer et al., 1984, 1991; Zhang et al., 1995) and *in vivo* (Cheng et al., 1991; Palejwala et al., 1991; Basu et al., 1993; Moriya et al., 1994). Primer extension studies *in vitro* have revealed that dAMP or TMP is incorporated opposite ϵdC (Simha et al., 1991; Zhang et al., 1995), suggesting the *in vivo* mutational specificity of C→T and C→A (Palejwala et al., 1991; Basu et al., 1993; Moriya et al., 1994); TMP is inserted opposite ϵdG (Singer et al., 1991), causing G→A transitions (Cheng et al., 1991); and it has been suggested that ϵdA directs the insertion of dGMP (Singer et al., 1984). Some incorporation of ϵdA monophosphate opposite T, G, and C of template DNA has also been reported (Revich & Beattie, 1986). ϵdA shows very weak mutagenicity when this adduct, site-specifically placed in viral genome, is replicated in *Escherichia coli* (Basu et al., 1993).

The single-stranded (ss) vectors have been successfully used in mutation studies of chemically defined and site-specifically placed DNA adducts in bacteria (Loechler et al., 1984; Banerjee et al., 1988; LeClerc et al., 1991) and have been further extended to yeast (Gibbs et al., 1993) and mammalian cells (Moriya, 1993). In ss vectors, the effects of DNA repair mechanisms such as nucleotide and base excision repairs are minimized, and complication through preferential replication of the unadducted complementary strand is obviated, thereby allowing quantitative analysis of *in vivo* translesional synthesis. Circular ssDNA is believed to be copied by the replicative DNA polymerase (pol), pol III, in bacteria [edited by Denhardt et al. (1978)], whereas the mechanism in mammalian cells is not well-known. Chen et al. (1995) have shown that conversion of ssDNA to double-stranded DNA requires proliferating cell nuclear antigen (PCNA), a processivity factor for the replicative DNA polymerases, which is essential for chromosomal DNA replication [Waga and Stillman (1994) and papers therein]. This finding suggests that the replicative polymerase, pol δ, also catalyzes DNA synthesis on ssDNA.

Recent studies with ss shuttle vectors have revealed that the fidelity of translesional synthesis past several DNA adducts is very different between *E. coli* and eukaryotic cells (Gibbs et al., 1993; Moriya et al., 1994), emphasizing the

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[1] Abbreviations: ϵdA, 1,N^6-ethenodeoxyadenosine; ϵdC, 3,N^4-ethenodeoxycytidine; ϵdG, N^2,3-ethenodeoxyguanosine; exo⁻, 3′→5′ exonuclease-deficient; PCNA, proliferating cell nuclear antigen; pol, DNA polymerase; ss, single-strand(ed).

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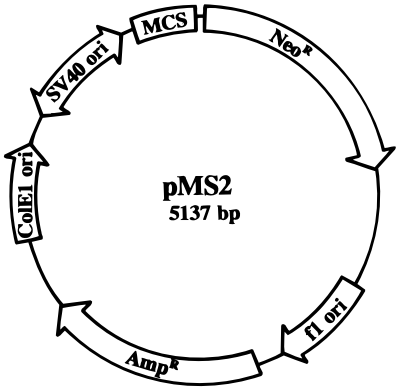

FIGURE 1: Structure of pMS2. MCS represents a multiple cloning site where a sequence for the hairpin structure (Figure 3A) has been introduced.

importance of mutagenicity studies in eukaryotic cells. We report here that ϵdA is a strongly mutagenic DNA adduct when replicated in mammalian cells.

EXPERIMENTAL PROCEDURES

Bacteria, Mammalian Cells, and Plasmid. *E. coli* CSH62, KH1229 (CSH62 *dnaQ49*), and KH1269 (CSH62 *mutD5*) (Maruyama et al., 1983) were obtained from T. Horiuchi (Kyushu University, Japan). AB1157 (DeWitt & Adelberg, 1962) and NR9232 (*mutD5*) (Schaaper, 1988) were provided by B. Bachmann (Yale University, CT) and R. M. Schaaper (NIEHS, NC), respectively. The simian kidney cell line, COS7 (Gluzman, 1981), was obtained from the tissue culture facility of SUNY at Stony Brook, NY. The construction of pMS2 (Figure 1) was described previously (Moriya, 1993). Single-stranded DNA was prepared from JM109 harboring this plasmid with VCSM13 (Stratagene) as a helper phage.

Oligodeoxyribonucleotide. The oligodeoxyribonucleotide ($^{5'}$CCATAXGTACTTC, where X is ϵdA or A) was synthesized at Oligos Etc. (Wilsonville, OR) and supplied to us in the form attached to the controlled pore glass support. Following cleavage and deprotection with concentrated NH_4OH, the DNA, carrying a dimethoxytrityl group at the 5′ end, was separated from incomplete products by HPLC. The dimethoxytrityl group was removed upon incubation in 80% glacial acetic acid for 30 min at room temperature. The 13-mer oligodeoxynucleotide was purified by denaturing 20% polyacrylamide gel and HPLC as described (Moriya et al., 1991). Purified 13-mer was analyzed for base composition as described by Li and Swann (1989).

Construction of Single-Stranded pMS2 DNA Containing a Single ϵdA Residue. Details of the procedure were published (Moriya, 1993; Moriya et al., 1994). A schematic illustration of the experimental strategy is shown in Figure 2. Briefly, ss pMS2 was hybridized with a 59-mer (Figure 3A) overnight at 9 °C and digested with *Eco*RV to yield gapped ssDNA (Figure 3B). Purified 13-mer was phosphorylated at its 5′ end, hybridized to the gap, and then ligated to the vector overnight at 4 °C. The ligation mixture was washed with H_2O in Centricon-100 (Amicon), an ultrafiltration device, to remove unligated 13-mer. DNA was precipitated with ethanol and dissolved in H_2O. A portion of the ligation mixture was removed and used to confirm ligation of the 13-mer insert as described below. The rest of the mixture was treated with exonuclease III and T4 DNA polymerase to digest hybridized 59-mer and was then extracted with a phenol/chloroform mixture. DNA was precipitated with ethanol. This final construct was dissolved in 1 mM Tris-HCl−0.1 mM EDTA, pH 8.

To confirm ligation of the 13-mer insert, the intermediate DNA construct hybridized with 59-mer (saved as described above) was digested with *Ban*I and *Hae*III (Figure 3B). The 5′-end phosphates of DNA fragments were replaced with ^{32}P by exchange reaction (Sambrook et al., 1989). Following ethanol precipitation, DNA fragments were separated in denaturing 12% polyacrylamide gel (Figure 5). To determine the concentration of the ssDNA construct, an aliquot of the final DNA sample was subjected to electrophoresis in 0.9% agarose gel. Known amounts of ssDNA were also subjected to electrophoresis in parallel to establish a standard dose−response curve. After electrophoresis, the DNA was transferred to nylon membrane and hybridized to ^{32}P-labeled oligonucleotide SR14 (Figure 3B). This probe hybridizes to DNA that contains the sequence of the 13-mer but does not hybridize to the parental DNA, ss pMS2. Radioactivity in each band was measured by a Phosphorimager (Bio-Rad), and the concentration of ssDNA construct was determined.

Transformation of E. coli Strains. Electrocompetent *E. coli* cells were prepared by washing bacteria in logarithmic-phase growth three times with sterile H_2O at 4 °C and making a suspension in 1/100 volume of a 10% glycerol solution. To induce SOS functions, bacteria were suspended in 5 mL of sterile H_2O, irradiated with UV at 20 or 40 J/m^2, and resuspended in the glycerol solution. Electrocompetent cells (50 μL) were electroporated with 50 ng of the ssDNA construct in a 2-mm gap cuvette by ECM600 (BTX, San Diego). Electroporation conditions were as follows: resistance; 129 ohm; maximum voltage range/timing mode, 2500 V/resistance, charging voltage, 2450 V. Immediately thereafter, 0.95 mL of YT (2×) medium was added to 50 μL of the electroporation mixture and incubated for 10 min at 37 °C. An aliquot (10 μL) was plated onto a YT (1×) agar plate containing ampicillin (100 μg/ml) to determine the number of transformants in the mixture. The remaining mixture was cultured for another 50 min to fully express the ampicillin-resistant gene. Ten milliliters of YT (2×) containing ampicillin was then added, and the culture was incubated overnight. Plasmid was prepared from an overnight culture to transform *E. coli* DH10B (GIBCO/BRL). Transformants were analyzed for mutations as described below.

Transfection of COS Cells. COS7 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. The cells were seeded at 5×10^5 cells per 60-mm plate. After overnight incubation, the cells were transfected with 170 ng of ssDNA with the aid of Lipofectin (GIBCO/BRL) overnight, and then transfection medium was replaced with fresh medium. This culture was maintained for 2 days, and progeny plasmid was then recovered by the method of Hirt (1967). This plasmid was used to transform *E. coli* DH10B, and transformants were analyzed for mutations as described below.

Analysis of Transformants for Mutations. *E. coli* transformants were inoculated individually to a 96-well plate, cultured for several hours, and then transferred onto filter paper (Whatman 3MM) that had been placed on an agar plate (YT, 1×) containing ampicillin. The filter was incubated at 37 °C overnight, soaked in 0.5 N NaOH to lyse bacteria, then neutralized in 0.5 M Tris-HCl, pH 7.4, and washed with SSC (1×) and ethanol. After drying, the filter was baked at 80 °C for 2 h. Hybridization was carried out with probes

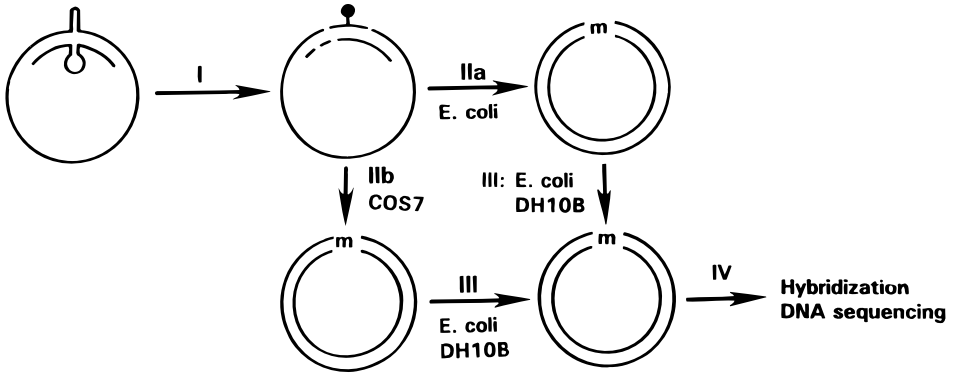

FIGURE 2: Experimental strategy. Following the construction of modified ssDNA (I), DNA is introduced into *E. coli* (IIa) or COS7 (IIb), in which translesional DNA synthesis is carried out. Progeny plasmid is prepared and introduced into the second host, *E. coli* DH10B (III). Transformants are analyzed for targeted events by differential oligonucleotide hybridization and DNA sequencing (IV). See text for details. "m" is a targeted event.

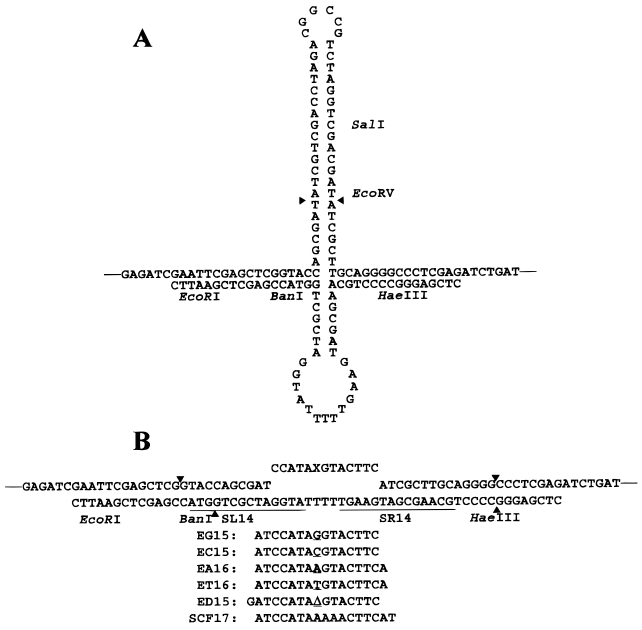

FIGURE 3: (A) Sequence of hairpin region. The upper strand is a part of ss pMS2 DNA, and the lower strand is a 59-mer scaffold. Filled triangles show the sites of cleavage by *Eco*RV. (B) Ligation of oligodeoxynucleotide into the gap generated by *Eco*RV digestion of ss pMS2 hybridized with 59-mer. X in the 13-mer insert represents modified base (ϵdA). Note two base mismatches ($^{5'}$XGT/$^{3'}$TTT) which serve as a genetic marker to identify products of DNA replication. Filled triangles show the sites of cleavage by *Ban*I and *Hae*III. SL14, SR14, EG15, EC15, EA16, ET16, ED15, and SCF17 are the probes used in oligonucleotide hybridization.

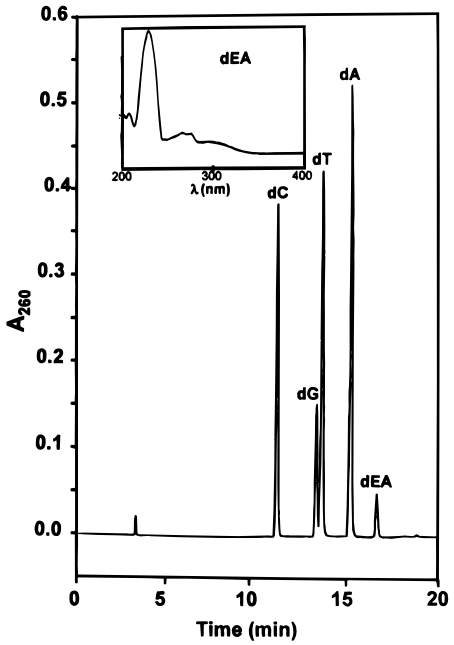

FIGURE 4: HPLC profile of the products obtained by the treatment of ϵdA-containing 13-mer with snake venom phosphodiesterase and alkaline phosphatase. Chromatographic conditions: μBondapak C18, 0−30% acetonitrile in 0.1 M ammonium acetate, pH 5.8, over 15 min with a flow rate of 1 mL/min. Inset shows the absorption spectrum of the peak at 16.5 min. dEA represents ϵdA.

shown in Figure 3B. EG15, EC15, EA16, ET16, and ED15 were designed to determine the base replacing ϵdA; SL14 and SR14 were used to screen for plasmids having a correct insert; and SCF17 was employed to detect progeny plasmid derived from the 59-mer scaffold. SCF17 contains two base mismatches of $^{5'}$AA at the positions of $^{5'}$GT (Figure 3B) which serve as a genetic marker to identify the products derived from the 59-mer scaffold. Hybridization temperature (HT) was calculated by the formula: HT= (G + C) × 4 + (A + T) × 2 − 4. At this temperature, only the perfectly matched probe hybridizes with DNA. Examples of hybridization are seen elsewhere (Moriya & Grollman, 1993).

RESULTS

Analysis of ϵdA-Containing Oligodeoxynucleotide. The purified ϵdA-containing 13-mer showed a single band and peak on analytical polyacrylamide gel electrophoresis and HPLC, respectively (data not shown). HPLC analysis of the products generated by treatment with snake venom phosphodiesterase and alkaline phosphatase showed five peaks (Figure 4). These correspond exactly to the peaks obtained from an artificial mixture of dC, dG, dT, dA, and ϵdA. The UV spectrum (Figure 4, inset) of the last peak was identical to that of authentic ϵdA (Sigma). Relative amounts (molar ratios) of nucleosides are shown in Table 1.

Table 1: Relative Nucleoside Content of ϵdA-Containing 13-mer[a]

	rel nucleoside content			rel nucleoside content	
nucleoside	theor	obsd	nucleoside	theor	obsd
dC	1.00	1.00	dA	0.75	0.89
dG	0.25	0.28	ϵdA	0.25	0.23
dT	1.00	1.14			

[a] Relative contents (molar ratio) of nucleosides were calculated by dividing the integrated HPLC peak area for each nucleoside by the relevant extinction coefficient at 260 nm and normalizing to dC.

Analysis of ssDNA Construct. Digestion with *Ban*I and *Hae*III in the presence of the hybridized 59-mer releases a

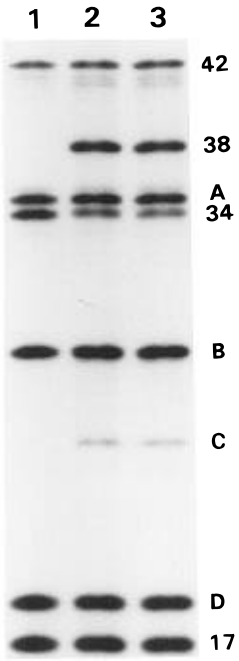

FIGURE 5: Analysis of ligation products by digesting with *Ban*I and *Hae*III in the presence of hybridized 59-mer. Lane 1, ligation mixture in the absence of 13-mer; lanes 2 and 3, ligation mixture in the presence of control (lane 2) or modified (lane 3) 13-mer.

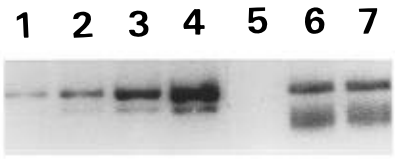

FIGURE 6: Southern blot analysis of ssDNA construct. ssDNA was electrophoresed in 0.9% agarose, transferred to nylon membrane, and probed with ^{32}P-labeled SR14 (Figure 3B). Lanes 1−4, ssDNA of 5, 10, 25, and 50 ng, respectively; lane 5, ligation mixture in the absence of 13-mer; and lanes 6 and 7, ligation mixture in the presence of control (lane 6) or modified (lane 7) 13-mer. Upper and lower bands correspond to circular and linear ssDNA, respectively.

38-mer from the ssDNA construct when a 13-mer is ligated to the vector (Figure 3B). A 34-mer is generated from the 59-mer scaffold. The 38-mer was observed (Figure 5) when the ligation mixture contained unmodified (lane 2) or modified (lane 3) 13-mer but was not observed in the control mixture lacking the 13-mer (lane 1). A 17-mer and a 42-mer are generated from the 59-mer by digestion with *Ban*I. Band A is generated by digestion of ss pMS2 alone with *Ban*I. Similarly, bands B and D are generated by digestion of the vector alone with *Hae*III. Band C (lanes 2 and 3) is probably derived from ssDNA ligated with a 13-mer at its 3′ end; a 24-mer is generated from this construct by digestion with *Ban*I. We did not observe a 27-mer, which would be expected from *Hae*III digestion of ssDNA ligated with a 13-mer at its 5′ end. Presumably, this product comigrates with band B. Radioactivity in band D and in the 38-mer was measured. Band D served as an internal standard for quantitative assessment of the ssDNA loaded in each lane. The ligation efficiency of the modified 13-mer was 92% of the unmodified 13-mer, indicating that ϵdA did not significantly inhibit ligation of the 13-mer to the vector.

The final ssDNA construct, from which the 59-mer scaffold had been removed, was analyzed by Southern blot hybridization to determine its concentration (Figure 6). The SR14 probe covers the ligation site (Figure 3B) and hybridizes to the construct containing the 13-mer, but not to pMS2. This probe revealed bands in lanes 1−4, which contained known amounts of ssDNA that had the 13-mer sequence and was prepared from *E. coli* transformants, and in lanes 6 and 7, which contained unmodified and modified 13-mer, respectively. Lane 5, which contained no 13-mer, showed no bands. The radioactivity associated with circular ssDNA (upper band) in lanes 6 and 7 was measured and plotted on a standard dose−response curve established with the results for lanes 1−4. This analysis showed the final yields for the control and modified constructs to be 22.5% and 25.0%, respectively.

Table 2: Transformation of *E. coli* Strains with ssDNA Constructs

hosts	UV dose (J/m^2)	no. of colonies ($\times 10^5$) per transformation: control construct	ϵdA construct
AB1157	0	17.8	10.9 (61)[a]
	20	7.80	4.45 (57)
	40	1.04	0.81 (78)
CSH62	0	1.50	2.46 (164)
	20	0.49	0.48 (98)
	40	0.29	0.40 (138)
KH1229 (*dnaQ49*)	0	3.67	2.69 (73)
	20	0.88	0.93 (106)
	40	0.29	0.26 (90)
KH1269 (*mutD5*)	0	1.94	
	20	0.64	0.94 (147)
	40	0.14	0.39 (278)
NR9232 (*mutD5*)	0	1.51	2.21 (146)

[a] % to corresponding control.

Transformation of E. coli. Table 2 shows the number of *E. coli* transformants obtained with 50 ng of ssDNA. AB1157 showed higher transformation efficiencies than the other four strains. The irradiation of host cells with UV light reduced their transformation efficiencies. The modified construct yielded 61%, 164%, 73%, and 146% of corresponding control values for AB1157, CSH62, KH1229, and NR9232, respectively, in the absence of UV irradiation, indicating that the ϵdA adduct is not a strong inhibitor, if at all, of DNA synthesis in *E. coli*. The UV irradiation of hosts (induction of SOS functions) did not significantly affect the number of transformants relative to the corresponding control treated with the same dose of UV. Under the same condition, benzo[*a*]pyrene-modified ss pMS2 (Moriya et al., submitted for publication), a positive control for SOS induction, yielded only 0.28% of the control value in nonirradiated AB1157; this percentage increased 5-fold (to 1.38%) and 20-fold (to 7.5%) when the host cells were preirradiated at 20 and 40 J/m^2, respectively.

Mutational Analysis. In the experiments using five strains (AB1157, CSH62, KH1269, KH1229, and NR9232) of *E. coli*, the analysis of transformants by oligonucleotide hybridization with SL14 or SR14 showed that 3.6% (49/1369) did not hybridize to both probes. One transformant hybridized with SCF17, indicating that this progeny was derived from the 59-mer scaffold. These 50 transformants were omitted from the analysis. The results indicate that 96.4% of transformants were derived from correctly ligated constructs. Each strain, except NR9232, was irradiated with UV at 0, 20, or 40 J/m^2 as shown in Table 2. We analyzed between 70 and 150 transformants per UV dose per strain. ϵdA was, at most, marginally mutagenic in *E. coli*: only four mutants were observed in SOS-induced hosts. These were targeted to the site of the adduct: one ϵdA→T in

Table 3: Mutational Specificity of ϵdA in COS7 Cells

	no. of targeted events (ϵdA → A G, T, or C)			
transfection plate no.	A	G	T	C
1	22	57	4[a]	0
2	27	56[b]	2[c]	1
3	28[d]	43	3	1
4	32	49[e]	3	0
5	19	49[f]	12	0
6	24	54	5	1
(Total)	152 (31)[g]	308 (63)	29 (6)	3 (1)

Single-stranded modified DNA (170 ng) was transfected into COS7 cells by the Lipofection method. Progeny plasmid was recovered 48 h later by the method of Hirt (1967) and was used to transform *E. coli* DH10B. Transformants were analyzed for a targeted event by differential oligonucleotide hybridization. See Experimental Procedures for details. [a]−[f]A single untargeted mutation was observed; it is listed below together with a targeted event. X in the first line (the sequence of 13-mer) represents ϵdA. [g] The number in parentheses is %.

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    CCATAXGTACTTC
a ———TA———————
b ——A–G———————
c ———T——T——————
d ——A–A———————
e ———G–A———————
f ———GA ———————
```

AB1157 irradiated at 20 J/m^2, one ϵdA→dC in KH1269 at 20 J/m^2, and two targeted single-base deletions in KH1229 at 40 J/m^2. Therefore, the mutation frequencies are 0.53% (4/756) for UV-irradiated hosts and <0.18% (0/563) for nonirradiated hosts when the results for the five strains are combined.

In the experiments with COS7 cells, 8.7% (47/540) of *E. coli* transformants, obtained from extracts of transfected COS7 cells, did not hybridize with SL14 and SR14, and one transformant hybridized with SCF17; these 48 transformants were omitted from further analysis. Mutation analysis was carried out independently on 6 transfection plates (Table 3) and gave consistent results. In contrast to the results for *E. coli*, ϵdA was highly miscoding in COS cells. The combined results show the frequency of targeted mutations to be 70%, consisting of ϵdA→dG, 63%; ϵdA→T, 6%; and ϵdA→dC, 1%. We observed six untargeted mutations which are also listed in Table 3. Five of these mutations were accompanied by targeted mutations.

Analyses of the respective 192 DH10B transformants obtained from SOS-induced AB1157 and COS7 cells for the control construct revealed no mutations.

DISCUSSION

The intrinsic mutagenic potential of ϵdA was studied in *E. coli* and mammalian cells. This adduct did not block DNA synthesis strongly and was very marginally mutagenic in *E. coli*. In previous studies (Moriya, 1993; Moriya & Grollman, 1993; Moriya et al., 1994; Moriya et al., submitted for publication), we analyzed more than 1500 transformants in total obtained from various *E. coli* strains that had been transformed with control constructs ligated to oligonucleotides with different DNA sequences. We have found no mutations at sites corresponding to the sites of modified bases: the cumulative frequency of mutations for control constructs is <0.07% when the data for different constructs are combined. In this study, we observed four mutations that were targeted only to the site of ϵdA and did not find any untargeted mutations in *E. coli*. Therefore, the four targeted mutations are likely induced by the ϵdA adducts. The low mutagenicity of ϵdA *in E. coli* is consistent with that reported by Basu et al. (1993). The almost exclusive ϵdA→dA nonmutagenic events were observed in the two 3′→5′ exonuclease-deficient (exo$^-$) strains, KH1269 (*mutD5*) and KH1229 (*dnaQ49*), and confirmed by the third exo$^-$ strain, NR9232 (*mutD5*). These results contrast with those for another exocyclic adduct, ϵdC. This adduct is weakly mutagenic in AB1157 and substantially mutagenic in NR9232 (Moriya et al., 1994), indicating that the exonuclease activity modifies the mutagenicity of ϵdC. From these results, we conclude that the *E. coli* replicative DNA polymerase inserts TMP (nonmutagenic) almost exclusively (<99%) opposite ϵdA.

In COS7 cells, on the other hand, ϵdA is highly mutagenic (Table 3). The predominant event is ϵdA→dG followed by ϵdA→dA, ϵdA→T, and ϵdA→dC in decreasing order, suggesting that mammalian DNA polymerase preferentially inserts dCMP opposite ϵdA, which was not at all observed in *E. coli*. Our finding is consistent with the results obtained in the experiments in human cells with shuttle plasmid randomly modified with 2-chloroacetaldehyde, a reactive metabolite of vinyl chloride; A:T→G:C transitions are prominent among mutations involving A:T pairs (Matsuda et al., 1995).

Solution structure studies of duplex oligodeoxyribonucleotide containing a base pair of ϵdA:T or ϵdA:dG have been conducted by Kouchakdjian et al. (1991) and by de los Santos et al. (1991). These studies have revealed that the ϵdA(*syn*):dG (*anti*) pairing, which results in the rare ϵdA→dC transversions, is stabilized by two hydrogen bonds, whereas the nonmutagenic ϵdA(*anti*):T(*anti*) pairing forms a nonplanar alignment without any hydrogen bonds. These findings indicate that the nucleotide incorporation opposite ϵdA *in vivo* is not simply determined by the ability to form stable hydrogen bonds and that the lack of hydrogen bonding does not necessarily result in a strong inhibition of DNA synthesis. The structure of ϵdA:dC base pairing, the major event in COS cells, remains to be determined.

Thus far, five DNA adducts, 8-oxoguanine (Moriya, 1993), 1,N^2-(1,3-propano)guanine, ϵdC (Moriya et al., 1994), and (+)- and (−)-*trans*-*anti*-benzo[*a*]pyrene diol epoxide-N^2-dG (Moriya et al., submitted for publication), have been studied in this pMS2 system; all, except 8-oxoguanine, have shown a marked difference in the fidelity of translesional synthesis between the two hosts. Understanding the mechanism of DNA synthesis in the ssDNA is important to the evaluation of these results. While this synthesis is known to be catalyzed by the replicative DNA polymerase, pol III, in *E. coli* [edited by Denhardt et al. (1978)], the mechanism in mammalian cells is not well-known. Nonetheless, several seminal studies allow us to propose a model: first, the studies using porcine circovirus, a mammalian virus, which contains circular ssDNA as the genome, showed that DNA polymerase α-primase synthesizes primers at several sites on the ssDNA (Gassmann et al., 1988). Another study (Méchali & Harland, 1982) suggests that the mechanism is similar to that for the lagging strand synthesis. Chen et al. (1995) have shown that PCNA, which is a processivity factor for the eukaryotic DNA polymerases and is essential for DNA replication [Waga and Stillman (1994) and papers therein],

is also required for the DNA synthesis on ssDNA. This suggests that the synthesis is also catalyzed by the replicative DNA polymerase, possibly pol δ. Although both pol δ and pol ϵ are dependent on PCNA, pol ϵ is suggested to be involved in nucleotide excision repair (Shivji et al., 1995). Upon synthesis of primers, pol δ replaces pol α-primase and synthesizes DNA in a processive manner in the presence of PCNA. The primers are removed by RNase H1 and MF1 (maturating factor) containing 5′→3′ exonuclease activity (Turchi et al., 1994; Waga & Stillman, 1994); small gaps are filled by pol β (Jenkins et al., 1992) and nicks are sealed by DNA ligase. If this model is correct, the mammalian PCNA-dependent replicative DNA polymerase inserts nucleotides opposite DNA adducts in a manner that is very different from the *E. coli* counterpart, pol III.

We studied the mutagenicity of ϵdA in the same sequence context as that employed for ϵdC (Moriya et al., 1994), allowing the direct comparison of the results. ϵdC is a stronger blocking lesion than ϵdA in *E. coli*; the former reduced the number of transformants to 20−30% of the control value while the latter did not show substantial inhibitory effects. These results are basically consistent with those described by Basu et al. (1993) although they reported some inhibitory effects by ϵdA. In *E. coli*, ϵdA is, at most, marginally mutagenic even in the presence of the induced SOS functions, whereas the weak mutagenicity of ϵdC is markedly enhanced upon induction of induced SOS functions. In mammalian cells, both adducts are equally highly mutagenic. ϵdA induces primarily ϵdA→dG transitions. ϵdC causes both ϵdC→T transitions and ϵdC→dA transversions.

We conclude from this study that ϵdA, like ϵdC, has a strong miscoding capability when replicated in mammalian cells. However, it should be emphasized that information about the formation of these adducts and the rate of their removal (DNA repair) must be considered for an overall evaluation of the contribution of these adducts to genotoxicity *in vivo*.

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