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Mutagenic potency of exocyclic DNA adducts: Marked differences between *Escherichia coli* and simian kidney cells

(3,N^4-ethenocytosine/propanodeoxyguanosine/site-specific mutagenesis/shuttle vector)

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ABSTRACT **A single-stranded shuttle vector containing a single 3,N^4-etheno-2′-deoxycytidine (εdC) or 1,N^2-(1,3-propano)-2′-deoxyguanosine (PdG) DNA adduct was used to investigate translesional DNA synthesis in *Escherichia coli* and simian kidney (COS) cells. The presence of either exocyclic adduct was associated with reduced numbers of transformants. In *E. coli*, this inhibitory effect could be overcome partially by irradiating cells with UV light before transformation. Translesional synthesis past both exocyclic lesions was accompanied by targeted mutations. For PdG, the primary mutagenic events observed in both hosts were PdG → T transversions; in preirradiated *E. coli*, PdG → A transitions were also observed. The targeted mutation frequency for single-stranded DNA that contained PdG was 100% in nonirradiated *E. coli*, 68% in preirradiated cells, and 8% in COS cells. In contrast, the targeted mutation frequency for single-stranded DNA that contained εdC was 2% in nonirradiated *E. coli*, 32% in preirradiated cells, and 81% in COS cells. The primary mutations generated by εdC in both *E. coli* and COS cells were εdC → A and εdC → T base substitutions. These observations appear to reflect the variable specificity of DNA replication complexes in incorporating bases opposite certain adducts. We conclude that DNA synthesis past the same DNA adduct can have strikingly different consequences in bacteria and mammalian cells, underscoring the importance of establishing the intrinsic mutagenic potential of DNA adducts in mammalian cells.**

Many human carcinogens and environmental mutagens react with cellular DNA to produce adducts or other forms of genomic damage (1). Such unrepaired DNA damage leads to errors in replication, manifested by mutations that ultimately contribute to aging, cancer, and chronic disease processes (2, 3).

The frequency of mutations induced by chemical mutagens in bacteria and mammalian cells often differs, reflecting variable patterns of metabolism, DNA repair, and events involved in translesional synthesis (4–8). Recently, our laboratory developed a single strand (ss) shuttle vector system that allows us to determine the relative contribution of translesional synthesis to mutagenesis (9). In this system, a ss vector containing a single defined lesion is introduced into *Escherichia coli* or simian kidney (COS) cells. After replication, the DNA sequence corresponding to the adduct site in the progeny plasmid is analyzed. This forward mutation system, which generally is unaffected by DNA repair processes, permits quantitative analysis of mutagenic and nonmutagenic events. Although current knowledge regarding the mechanism of replication of circular ssDNA in eukaryotes is quite limited, studies using *Xenopus laevis* oocytes and their nuclear extract suggest that DNA polymerase α and β are involved in the synthesis of the complementary strand with polymerase β being involved at a later stage of the reaction (possibly, gap-filling synthesis after primer removal) (10). It also has been shown in a cell-free system that the enzymatic events responsible for complementary strand synthesis in extracts of *X. laevis* resemble those presumed to act at the lagging strand of the eukaryotic replication fork *in vivo* (11).

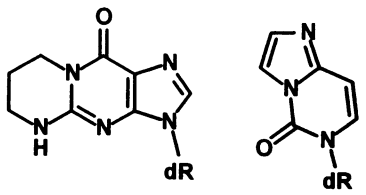

FIG. 1. Structure of PdG (*Left*) and εdC (*Right*).

In this study, this shuttle vector system was used to compare mutational events induced by exocyclic DNA adducts. Both N^2-(1,3-propano)-2′-deoxyguanosine (PdG) [an adduct produced by interaction of α, β-unsaturated aldehydes with DNA (12)] and 3,N^4-etheno-2′-deoxycytidine (εdC) [one of several exocyclic DNA adducts found in cells exposed to vinyl chloride (13)] were recently identified as components of cellular DNA, presumably generated by metabolic processes (Fig. 1; refs. 14 and 15).

εdC (16, 17) and a synthetic analog of PdG (18) were reported to induce point mutations in *E. coli*; their mutagenic effects have not yet been studied in mammalian cells. In this paper, we report that translesional synthesis past PdG and εdC results in strikingly different mutational events in bacteria and mammalian cells. Our observations may reflect, in part, the variable specificity of the DNA replication complexes in incorporating bases opposite certain adducts. Our results have important implications for risk assessment analysis, as they underscore the importance of establishing the intrinsic mutagenic potential of suspected chemical carcinogens and environmental agents in mammalian cells rather than in bacteria. The mutational specificity of PdG and εdC also may be useful in assessing the contribution of endogenous lesions to spontaneous mutagenesis and carcinogenesis and in relating occupational exposure to genotoxic agents with mutations in the p53 tumor suppressor gene (19).

MATERIALS AND METHODS

Cells and Vector. *E. coli* AB1157 (20), RK1517 (as AB1157, but *mutS*::Tn*5*) (21), NR9232 (*mutD5*) (22), simian virus 40-transformed simian kidney cell lines COS-7 (23) and COSts2 (24), and a ss shuttle vector pMS2 (9), which confers

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Abbreviations: ds, double strand(ed); εdC, 3,N^4-etheno-2′-deoxycytidine; Neo^R, neomycin resistance; PdG, 1,N^2-(1,3-propano)-2′-deoxyguanosine; ss, single strand(ed).

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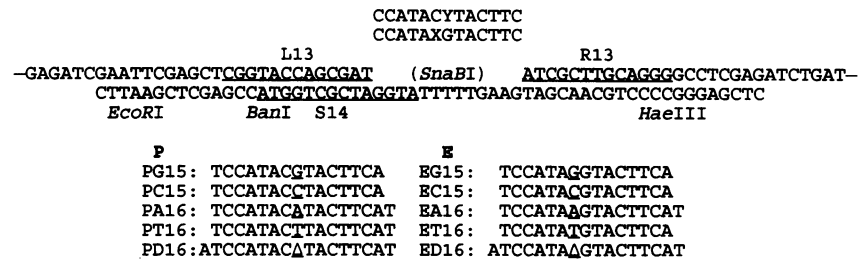

FIG. 2. Ligation of a 13-mer into the gap generated by *Eco*RV digestion of ss pMS2 (upper strand) hybridized to a 59-mer (lower strand). X represents dC or εdC; Y represents dG or PdG. A unique *Sna*BI site is created when X = dC or Y = dG. Three base mismatches within the *Sna*BI site serve as a genetic marker to identify products of DNA replication. P and E probes are used in the analysis of progeny from PdG- and εdC-containing DNA constructs, respectively; L13 and R13 probes for progeny of both constructs. The S14 probe is used to determine the amount of ligated ssDNA.

neomycin resistance (Neo^R) on mammalian cells and ampicillin resistance on bacteria, were used in this study.

Oligodeoxynucleotides. Methods for solid-state synthesis of oligodeoxynucleotides containing PdG have been described (25); synthesis of those containing εdC will be reported elsewhere. The unmodified (X = C, Y = G) and modified (X = εdC, Y = PdG) 13-mers, d(CCATAXGTACTTC) and d(CCATACYTACTTC), were purified by electrophoresis on a denaturing 20% polyacrylamide gel and by HPLC, as described (26). Purified 13-mers, labeled at the 5′ termini with ^{32}P, formed a single band on the gel. Modified oligodeoxynucleotides were digested with nuclease P1 and bacterial alkaline phosphatase (27). HPLC analysis revealed single peaks with UV spectra and retention times corresponding to authentic samples of PdG, εdC, and the natural DNA nucleosides. The 13-mer containing εdC was cleaved at the lesion site by treatment with hot 10% (wt/vol) piperidine (28), whereas the unmodified 13-mer remained intact.

Construction of Circular ssDNA Containing a Single Modified Nucleotide. The construction of circular ssDNA containing a single DNA adduct has been described in detail elsewhere (9). Briefly, ss pMS2 DNA was annealed to a 59-mer and then digested with *Eco*RV to create a 13-mer gap in the duplex region of the vector (Fig. 2). Modified or unmodified 13-mers complementary to the gapped region were ligated to the vector. A portion of the ligation mixture was used to establish ligation efficiency. After this step, the original procedure (9) was modified as follows: the ligation mixture was treated for 1 hr with exonuclease III (18 units/pmol of DNA) and T4 DNA polymerase (1 unit/pmol of DNA) to digest the hybridized 59-mer and then incubated with *Eco*RV and *Sal* I to cleave residual ss pMS2. The reaction mixture was extracted twice with phenol/chloroform, 1:1 (vol/vol), and twice with chloroform. After ethanol precipitation, DNA was dissolved in 1 mM Tris·HCl, pH 7.5/0.1 mM EDTA. A portion of the ligation mixture and known amounts of ss pMS2(CG) (defined below) were subjected to electrophoresis on a 0.9% agarose gel to separate closed circular and linear ssDNA. DNA was transferred to a nylon membrane and hybridized to a ^{32}P-labeled S14 probe (Fig. 2) complementary only to DNA that contained the 13-mer insert. The absolute amount of closed circular ssDNA was determined by comparing radioactivity in the sample with that in known amounts of ssDNA. DNA vectors containing unmodified and modified 13-mers were designated pMS2(CG), pMS2(εdC), and pMS2(PdG).

Mutagenesis Experiments. Unless indicated otherwise, all studies involving bacteria used *E. coli* AB1157 as the host cell. SOS functions (29) were induced by irradiating bacteria in logarithmic-growth phase with UV light (20 J/m^2) before rendering them competent. Competent cells, prepared by the method of Chung *et al.* (30), were transfected with 50 ng of ssDNA and immediately plated on ampicillin-containing plates to ensure independent transformation. Transformants were analyzed for mutated phagemids by oligonucleotide hybridization (31). Oligodeoxynucleotide probes used to analyze progeny phagemids are shown in Fig. 2. Probes L13 and R13 were used to select phagemids containing a correct insert. Two groups of probes (P and E) were used to identify the base replacing PdG and εdC, respectively. Transformants that failed to show a positive response to both L13 and R13 probes were omitted from the analysis. When L13/R13-positive transformants failed to hybridize to any of the probes designed to detect targeted events, double-strand (ds) DNA was prepared from these colonies and digested with *Sna*BI. DNA resistant to this treatment was subjected to dideoxynucleotide sequencing analysis (32). Phagemids carrying a mutation in the *Sna*BI site were classified as mutants.

Site-specific mutagenesis studies in mammalian cells were conducted as described (9). In brief, COS-7 cells were transfected with 100 fmol (170 ng) of ssDNA for 18 hr by the lipofection (GIBCO/BRL) method (33), after which cells were cultured for 2 days in Dulbecco's modified Eagle's medium/10% fetal calf serum. Progeny phagemids were recovered by the method of Hirt (34), treated with S1 nuclease to digest input ssDNA, and used to transform *E. coli* DH10B (GIBCO/BRL). Transformants were analyzed for mutated phagemid as described above. COSts2 cells were used to estimate efficiencies of transformation to a Neo^R phenotype (9).

RESULTS

In SOS-noninduced AB1157, the numbers of transformants recovered in each experiment were 2% and 4% of control values for pMS2(PdG) and 23% and 30% of control values for pMS2(εdC) (Table 1). The relative number of transformants obtained from modified vectors increased 2.6- to 4.5-fold in SOS-induced cells. In COSts2 cells (Table 2), Neo^R transformants represented 47–76% of control values for pMS2(PdG) and 69–95% of control values for pMS2(εdC);

Table 1. Transformation of *E. coli* AB1157 with ssDNA constructs

ssDNA*	SOS induction†	Transformants,‡ no. Exp. 1	Exp. 2
pMS2(CG)	−	8344 (100)	7077 (100)
	+	4872 (100)	2772 (100)
pMS2(PdG)	−	364 (4)	112 (2)
	+	539 (11)	249 (9)
pMS2(εdC)	−	1925 (23)	2128 (30)
	+	2863 (59)	2219 (80)

*Fifty nanograms of circularized ssDNA per transformation.
†Strain AB1157 in logarithmic-phase growth was irradiated with UV at 20 J/m^2.
‡Data are expressed per transformation; number in parentheses represents percentage of corresponding control value.

Table 2. Transformation of COSts2 cells with ssDNA constructs

ssDNA, fmol/ transfection	Neo^R colonies per transfection,* no.		
	pMS2(CG)	pMS2(PdG)	pMS2(εdC)
1	1090	510 (47)	1040 (95)
3	1770	1290 (73)	1510 (85)
10	2830	1780 (63)	2310 (82)
30	3720	2830 (76)	2560 (69)

*Number in parentheses represents percentage of control values.

these experiments were conducted over a range of 1–30 fmol of DNA per transfection.

More than 95% of the colonies derived from pMS2(CG), pMS2(PdG), and pMS2(εdC) under SOS-induced and noninduced conditions contained the 13-mer insert. Almost all mutations were targeted to the site of the lesion. In experiments using pMS2(PdG), all targeted events in SOS-noninduced AB1157 were PdG → T transversions (Table 3). In SOS-induced AB1157, the frequency of targeted mutations was 68%, and the frequency of nonmutagenic events (PdG → G) was 32%. A significant number of PdG → A transitions (17%) were also observed. In contrast to the results in *E. coli*, the mutation frequency in COS-7 cells was only 8%. Most mutations were PdG → T transversions; a small number of PdG → C transitions were observed. Five DH10B transformants failed to hybridize with any of the probes used to detect targeted events. Phagemids isolated from these transformants were cleaved by *Sna*BI, indicating that untargeted mutations were located outside the *Sna*BI site but within the region detected by the probes. These phagemids were classified as nonmutants.

In experiments using ssDNA that contained εdC, targeted mutations were mostly εdC → A and εdC → T base substitutions (Table 3). Frequencies of targeted mutations were 2% in SOS-noninduced AB1157 and 32% in SOS-induced cells. The mutation frequency in COS-7 averaged 81%, much higher than in bacteria. ss pMS2(εdC) also was introduced into NR9232, a *mutD5*-carrying strain of *E. coli* carrying an ε subunit in which 3′ → 5′ exonucleolytic proofreading activity is impaired (35). In this experiment, progeny phagemids were recovered from the transformation mixture and used to transform DH10B cells. The frequencies of targeted mutations were 33 and 55% in the absence and presence of induced SOS functions, respectively (Table 3). Both εdC → A transversions and εdC → T transitions were observed; the former occurred much more frequently. Because the NR9232 strain is reported to be defective in mismatch repair (22), the same construct was introduced into RK1517 (*mutS*::Tn*5*); results were similar to those obtained with strain AB1157 (data not shown).

Analysis of 96 control transformants obtained from each transformation of UV-irradiated and nonirradiated strains AB1157 and NR9232 with pMS2(CG) revealed no mutations; all colonies hybridized to probe EC15 (PG15). Similarly, no mutations were observed in 76 DH10B colonies transformed with progeny phagemids isolated from pMS2(CG)-transfected COS-7.

DISCUSSION

In this study, a ss shuttle vector was used to compare translesional synthesis past PdG and εdC in bacteria and mammalian cells. In contrast to a previous study of 8-oxoguanine (9), both adducts blocked DNA synthesis, as revealed by the reduced numbers of ampicillin- and neomycin-resistant colonies in *E. coli* and COS cells, respectively. In *E. coli*, the inhibitory effects were partially overcome by the SOS-inducing treatment. In COSts2 cells, as in *E. coli*, PdG is a more strongly blocking lesion than εdC. Apparently, COS cells tolerate the blocking effects of PdG and εdC better than *E. coli*, as judged by the number of Neo^R colonies recovered and yields of progeny plasmid (data not shown). However, the fact that a mammalian cell absorbs multiple plasmids makes the determination of the absolute efficiency of translesional synthesis difficult; even at the lowest dose of 1 fmol per transfection, 300 ssDNA molecules are available for transfection of each COSts2 cell.

The mutagenic potency of both PdG and εdC lesions differed strikingly in *E. coli* and COS cells, again contrasting with our study of 8-oxoguanine mutagenesis (9). PdG was highly mutagenic in *E. coli* but only weakly mutagenic in mammalian cells. For εdC, the converse relationship was established. Induction of SOS functions in *E. coli* increased the efficiency of translesional synthesis and altered the spectrum of targeted events. Because lesions were introduced into hosts by using a ssDNA vector, differences observed can be ascribed to DNA replication rather than DNA repair. The higher mutation frequency for εdC in the *mutD5*-carrying strain (NR9232), in which proofreading activity is impaired, supports this idea. Comparison of data for εdC in AB1157 and NR9232 strains suggests that the proofreading function, catalyzed by the ε subunit, is active in removing dT inserted opposite the lesion and that SOS functions do not represent simply an inactivation of proofreading activity (36).

Table 3. Mutagenicity of site-specifically placed PdG and εdC adducts in *E. coli* and COS-7 cells

ssDNA construct	Host*	Targeted events, no. [PdG or εdC → X (C, A, T, or G)]				Frequency of targeted mutations,† %
		C	A	T	G	
pMS2(PdG)	AB1157	0	0	261	0	100
	AB1157 + UV	1	46	138	87	68
	COS-7 → DH10B	3	0	24	319	8
pMS2(εdC)	AB1157	273	3	3	0	2
	AB1157 + UV	191	39	52	1	32
	COS-7 → DH10B	39	100‡	58	5	81
	NR9232 → DH10B	154	69	7	0	33
	NR9232 + UV → DH10B	100	112	12	0	55

*Transformation of *E. coli* was conducted as described. For COS cells, 170 ng (100 fmol) of adducted ssDNA was used to transfect COS-7; progeny phagemids were recovered after 48 hr and used to transform *E. coli* DH10B for mutation analysis.

†Combined data from at least two independent experiments using two different constructs. For PdG, [(no. of A + T + C)/total] × 100; and for εdC, [(no. of A + T + G)/total] × 100.

‡Two phagemids showed an additional mutation: CCAaAaGTACTTC, CCATAaGTACTTt (mutations indicated by lowercase type).

The exclusive formation of PdG → T transversions in SOS-noninduced *E. coli* and various patterns of targeted events in SOS-induced *E. coli* and in COS cells emphasize the interplay between DNA polymerase specificity and the conformational equilibrium between PdG(syn) and PdG(anti) in DNA. At physiological pH, approximately half of the molecules containing this adduct adopt PdG(syn)·dA(anti) pairing (37). The overwhelming predominance of PdG → T transversions appears to reflect preferential insertion of dAMP(anti) opposite PdG(syn) on template strand. In SOS-induced *E. coli* and in COS cells, the replication complex also promotes insertion of dCMP and/or dTMP opposite PdG in the DNA template. It is noteworthy that PdG·dA pairing predominates in bacteria, whereas PdG·C pairing occurs most often in COS cells. The precise conformation of PdG in duplex DNA, when dC or dT is the opposing base, remains to be determined.

εdC, either in syn or anti conformation, is structurally incapable of forming more than one hydrogen bond in B-DNA. Nevertheless, dGMP, dTMP, and dAMP are readily incorporated opposite εdC during DNA synthesis. Surprisingly, the apparent fidelity of DNA replication past this lesion in noninduced *E. coli* was much higher (98%) than in COS cells (19%). Because hydrogen bonding necessarily plays a limited role in coding by εdC, base selection must be determined by DNA polymerase and other components of the replication complex and, to a lesser extent, by base-stacking interactions.

Site-specific methods have been used by other investigators to establish the mutagenic potential of PdG and εdC in *E. coli* (16–18). Benamira *et al.* (18) reported that PdG induced targeted deletion mutations at a frequency of 2.5% in *E. coli*. The apparent discrepancy between their results and ours may be explained by differences in the experimental systems and the DNA sequences used. A dsDNA vector was used, in which the complementary strand contained uracil residues to promote replication of the strand having PdG, by Benamira *et al.* Recent studies have shown that 3-methyladenine DNA glycosylase removes several types of exocyclic DNA adducts from dsDNA (38–40). Thus, PdG may be removed by DNA repair mechanisms before the adduct encounters the DNA replication machinery, leading to a lower value for mutational frequency. Second, the experimental system used by Benamira *et al.* was designed for phenotypic detection of frameshift mutations. The target sequence used is a hot spot for deletion mutations in the *Salmonella* tester strain of Ames and Maron (41). The sequence context used in our study is less likely to lead to misalignment when dAMP is inserted opposite PdG (42).

There also are significant differences between our results and those of Palejwala *et al.* (16), who reported that εdC did not reduce survival in *E. coli* KH2 (*mutD*$^+$ as in AB1157) and induced targeted mutations at a frequency of 30% in the absence of SOS induction. The results of our experiments with noninduced *E. coli* AB1157 are consistent with those of Basu *et al.* (17), who also used ssDNA for their studies. The experimental systems used in the Essigmann laboratory and by Palejwala *et al.* (16) differ in one important aspect—namely, that in the latter, εdC was placed within a ss region of gapped dsDNA, whereas in experiments reported here and by Basu *et al.*, the adduct was incorporated in a ss vector. It is likely that the DNA polymerases involved in DNA synthesis on gapped and ssDNA templates, probably polymerase I and polymerase III, respectively, catalyze translesional synthesis past εdC in different ways.

It has been shown *in vitro* that the dNTP inserted opposite 8-oxoguanine varies, depending on the DNA polymerases used (43). Gibbs *et al.* (44) also reported different patterns of translesional synthesis past a T-T cyclobutane dimer in *E. coli* and yeast. Thus, intrinsic properties of the DNA replication apparatus may account for the observed differences in translesional events between *E. coli* and mammalian cells. Results of the present study emphasize the importance of establishing the mutagenic potential of suspected carcinogenic DNA adducts in mammalian cells.

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