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Mutagenesis Induced by a Single 1,N^6-Ethenodeoxyadenosine Adduct in Human Cells[1]

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ABSTRACT

To study the genotoxic properties of 1,N^6-ethenodeoxyadenosine (ϵdA) in human cells, a novel site-specific mutagenesis approach was developed, in which a single DNA adduct was uniquely placed in either strand of a shuttle plasmid vector. The analysis of progeny plasmid derived from the modified strand shows that ϵdA, when incorporated into the position of the second A of 5′-CAA (codon 61 of the *ras* gene), is mutagenic in human cells, inducing A→T, A→G, and A→C mutations. The efficient induction of A→T transversions in experiments using modified double- and single-stranded DNA substrates supports the hypothesis that A:T→T:A transversions in human and animal tumors induced by vinyl compounds reflect misinsertion of dAMP opposite this adduct. Mutagenic events were similar when the adduct was incorporated into either the leading or the lagging strand. ϵdA was more mutagenic than 8-oxodeoxyguanosine, which induced targeted G→T transversions in HeLa cells. In *Escherichia coli*, ϵdA did not significantly miscode (<0.27%) even in the presence of induced SOS functions.

INTRODUCTION

DNA damage is highly suspected of playing an important role in carcinogenesis and aging (1). ϵdA,[3] a member of a family of exocyclic DNA adducts, is produced by endogenous and exogenous agents and has been shown to be mutagenic *in vitro* (2, 3) and *in vivo* (4, 5). ϵdA forms when reactive metabolites of vinyl compounds, such as VC, vinyl carbamate, and urethane (reviewed in Ref. 6), and certain α,β-unsaturated aldehydes generated by lipid peroxidation (reviewed in Refs. 7 and 8) react with DNA. ϵdA was detected in human liver DNA at the level of ~20 adducts per 10^9 dA (9, 10). The level of ϵdA in DNA is increased by enhanced lipid peroxidation (10, 11). 1,N^6-Ethenoadenine was detected in the urine of untreated rats at the level of 21.6 pmol/ml (12).

In human tumors associated with exposure to VC and in animal tumors induced by administration of vinyl compounds, A:T→T:A transversions were observed in the *p53* tumor suppressor gene (13–15) and the *ras* oncogenes (codon 61; Refs. 16–18). In previous studies using ss plasmid DNA, ϵdA was strongly mutagenic in simian kidney cells (5). ϵdA→dG and ϵdA→dT base changes accounted for 63 and 6%, respectively, of translesional events. However, the strong dominance of ϵdA→dG in this experiment is inconsistent with the frequently observed A:T→T:A transversions in those tumors.

Among mutations at the second base of the c-Ha-*ras* codon 61 (CAA), A→T and A→G mutations show similar transforming activities (19, 20). A:T→T:A mutations in codons 179, 249, and 255 of the *p53* gene were observed in human liver angiosarcomas associated with exposure to VC (13, 14). Analysis of a database for human p53 mutations[4] reveals 17 and 52 examples of A→T and A→G mutations, respectively, at codon 179 (CAT), and 21 and 13 A→T and A→G mutations, respectively, at codon 249 (AGG). Taken together, these findings suggest that phenotypic selection does not account for the A:T→T:A mutations observed at these sites in the *p53* and *ras* genes in vinyl compound-associated tumors.

In this paper, we describe an experimental system that can be used with dsDNA and ssDNA substrates to study mutagenic events in human cells. With it, we found that ϵdA directs the incorporation of dAMP opposite the adduct when embedded in the sequence 5′-CAA, in which the modified base corresponds to the second base of codon 61 of the *ras* oncogene.

MATERIALS AND METHODS

Oligonucleotides and Enzymes. Restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were purchased from New England Bio-Labs (Beverly, MA). Unmodified and ϵdA-containing oligonucleotides were obtained from Oligos Etc. (Wilsonville, OR). An oligonucleotide containing 8-oxo dG was provided by F. Johnson (State University of New York at Stony Brook, Stony Brook, NY). Unmodified and modified oligonucleotides were purified by electrophoresis in a denaturing 20% polyacrylamide gel, followed by purification by high-performance liquid chromatography and then subjected to the analysis for base composition as described earlier (5). To examine the stability of ϵdA, the oligonucleotide was incubated in solutions used during construction and subjected to electrospray mass spectrometry. This analysis indicated that ϵdA is stable under the construction conditions.

Cell Lines and Bacteria. HeLa (cervical cancer), HCT116 (mismatch repair-defective colon cancer), and 293 (embryonal kidney epithelium) cells were obtained from American Type Culture Collection (Rockville, MD). They were free of *Mycoplasma*. Cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics. Electrocompetent *Escherichia coli* DH10B was purchased from Life Technologies, Inc. (Gaithersburg, MD). MM1991 (as AB1157, but *mutS201*::Tn*10*, *endA7*::*cm*) was constructed by P1 transduction.

Construction of Vectors. Three modifications were introduced into our pMS2 vector (Fig. 1; Ref. 21): (*a*) we introduced the human BK virus origin and BK large T antigen gene into the unique *Hin*dIII site of pMS2. These elements allowed the vector to replicate in human cells. For this modification, the BK shuttle vector (National Biosciences, Plymouth, MN) was digested with *Pvu*II, excising a 3.3-kb fragment containing the two elements. This fragment was ligated to the *Hin*dIII site of pMS2 in the orientation shown in Fig. 1, creating pBK20 (8.4 kb); (*b*) we transferred HP (*HP* in Fig. 1), the site for incorporation of a DNA adduct, downstream of the *neo* gene so that the DNA adduct was remote from the viral origins of replication. The transfer assured that TLS would be catalyzed during the elongation and not during the initiation stage. For this modification, pBK20 was digested with *Xho*I, and two fragments were isolated. The larger fragment (7.2 kb) was circularized by self-ligation creating pBK30, and the 1.2-kb smaller fragment, containing the *neo* gene, was ligated to *Eco*RI linkers at both ends. This *neo* gene fragment was then introduced into the unique *Eco*RI site located immediately upstream of HP (Fig. 1) in pBK30, creating pSBK; and (*c*) pSBK also contains the base change of GAT→CAT at codon 402 of the large T antigen gene. This change

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[3] The abbreviations used are: ϵdA, 1,N^6-ethenodeoxyadenosine; cc, closed circular; ds, double-stranded; ss, single-stranded; TLS, translesion DNA synthesis; VC, vinyl chloride; 8-oxo dG, 8-oxodeoxyguanosine; HP, hairpin sequence; pol, polymerase.

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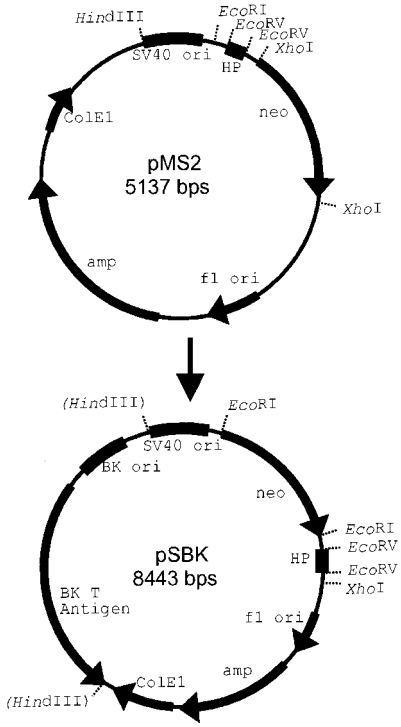

Fig. 1. Construction of pSBK. *HP*, represents the region containing a sequence for a HP structure. *(HindIII)*, this site was lost because of the insertion of the BK ori-T antigen sequence. Modified oligonucleotide is introduced between two *Eco*RV sites. See "Materials and Methods" for details.

substituted His for Asp, disrupting the interaction of T antigen with p53 protein (22, 23).

Strategy for Site-specific Mutagenesis. Our strategy for site-specific mutagenesis using dsDNA included: (*a*) incorporation of a single DNA adduct opposite a unique restriction enzyme site; (*b*) replication of the modified construct in host cells; (*c*) recovery of progeny plasmid from host cells; (*d*) removal by restriction enzyme digestion of progeny derived from the unmodified strand; and (*e*) analysis of TLS events by differential oligonucleotide hybridization. Detailed procedures for each step are described in the following sections.

Construction of DNA Containing a Single DNA Adduct. Fig. 2 shows the construction schematically. ds pSBK was digested with *Eco*RV to remove a 33-bp fragment from the HP region (Fig. 2, *step 1*). The digested vector was ligated to a blunt-ended 13-mer duplex [5′-d(AGGTACGTAGGAG)/3′-d(TCCATGCATCCTC)] containing a *Sna*BI site (5′-TACGTA; Fig. 2, *step 2*). Two constructs (pSBKG and pSBKD) were isolated, each having a single insert in the opposite orientation. For the leading strand construct, 60 pmol (162 μg) of ss pSBKD, prepared from JM109 harboring this plasmid, with the aid of the helper phage M13KO7 (Life Technologies, Inc.), was mixed with 20 pmol (108 μg) of *Eco*RV-digested ds pSBK. Gapped DNA was prepared according to the method of Horiuchi and Zinder (24). Annealing between circular ss pSBKD and its complementary strand, derived from *Eco*RV-digested ds pSBK, formed gapped DNA with a 13- nucleotide gap (Fig. 2, *step 4*). The formation of gapped DNA was confirmed by electrophoresis in a 0.8% agarose gel (Fig. 3). The DNA solution was desalted using a Centricon 30 Microconcentrator (Amicon, Beverly, MA). One hundred pmol of gel-purified control or adducted 13-mer [5′-d(CTCCTCXATACCT), where X is dA or ϵdA], were phosphorylated at the 5′ termini by T4 polynucleotide kinase and ATP, and ligated to the gapped DNA by T4 DNA ligase (100 units/μg DNA) at 10°C for 24 h (Fig. 2, *step 5*). After desalting in a Centricon 30, the ligation mixture was

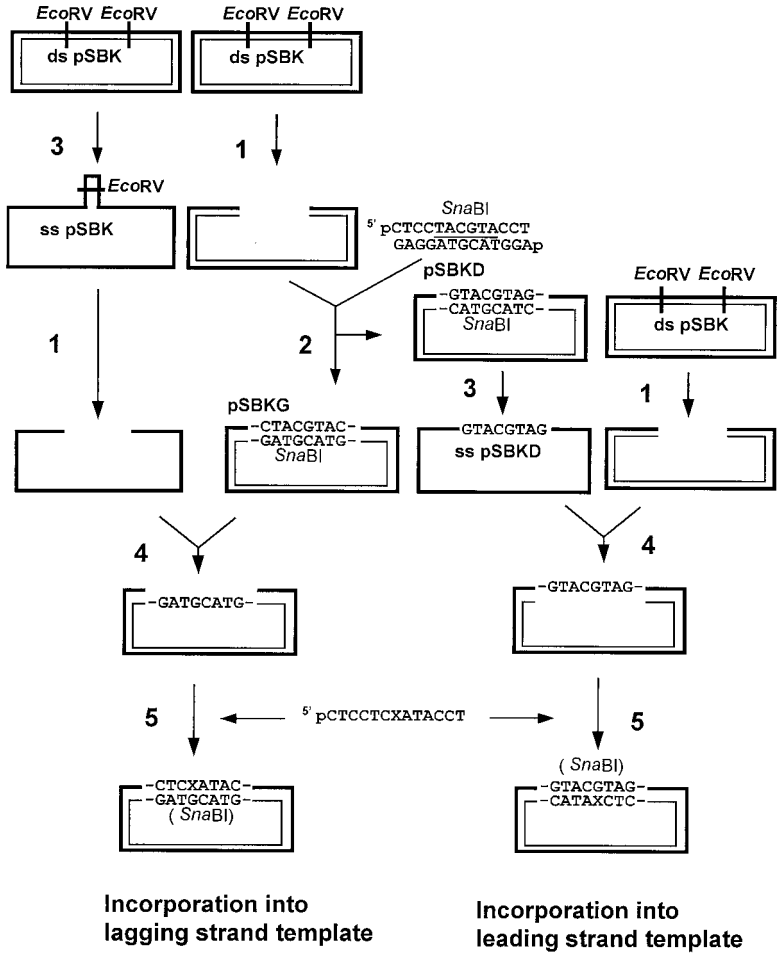

Fig. 2. Construction of heteroduplex DNA containing single ϵdA (X). *1*, *Eco*RV digestion. Two sites for *Eco*RV are located in HP (Fig. 1); *2*, ligation of a 13-mer duplex oligodeoxynucleotide containing a *Sna*BI site (5′-TACGTA); *3*, preparation of ssDNA; *4*, Preparation of gapped DNA; and *5*, ligation of modified oligodeoxynucleotide. There are three contiguous base mismatches in the *Sna*BI site, which exists only in the unmodified strand.

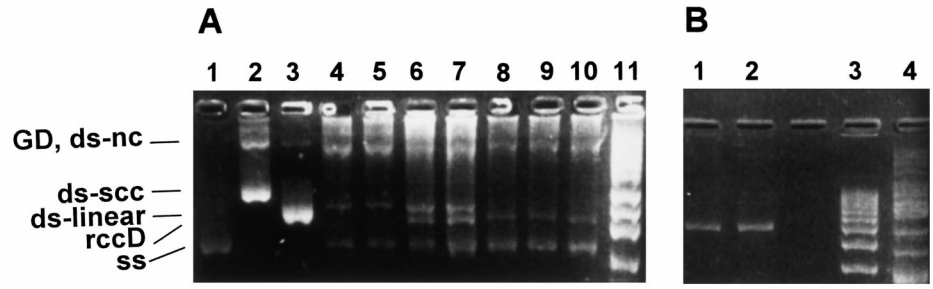

Fig. 3. Analysis of DNA construct by agarose gel electrophoresis. *A*, gapped DNA (*GD*) was converted to relaxed cc DNA (*rccD*) by ligation of 13-mer oligonucleotide into the gap. DNA was run in 0.9% agarose gel containing ethidium bromide (0.5 μg/ml) and Tris-borate buffer. rccD comigrates with supercoiled cc dsDNA (ds-scc) of 7 k bp. This rccD was resistant to *Eco*RV and *Sna*BI digestion. *Lane 1*, circular ss pSBK; *Lane 2*, supercoiled cc ds pSBK; *Lane 3*, *Eco*RV-digested linear ds pSBK (migrated faster than constructs in other lanes because of overloading.); *Lane 4*, gapped DNA before ligation; *Lane 5*, ligation mixture in the absence of 13-mer; *Lanes 6* and *7*, ligation mixture in the presence of control *(Lane 6*) or modified 13-mer *(Lane 7*); *Lanes 8* and *9*, ligation mixture containing unmodified *(Lane 8*) or modified *(Lane 9*) 13-mer was digested with *Sna*BI; *Lane 10*, ligation mixture containing modified 13-mer was digested with *Eco*RV; *Lane 11*, supercoiled cc dsDNA marker. *nc*, nicked circular. *B*, rccD was purified by ultracentrifugation in a CsCl-ethidium bromide solution. *Lane 1*, control rccD; *Lane 2*, ϵdA-containing rccD; *Lane 3*, linear dsDNA marker: *Lane 4*, supercoiled cc dsDNA marker.

treated with *Sna*BI and *Eco*RV to remove undesired products. The desired construct is resistant to these restriction enzymes. The formation of cc dsDNA was confirmed by agarose gel electrophoresis (Fig. 3), and the product was purified by ethidium bromide/CsCl density-gradient centrifugation (25). After the removal of ethidium bromide and CsCl, DNA was concentrated and washed with H_2O in a Centricon 30. The concentration of cc dsDNA construct was determined spectrophotometrically. The final yield ranged from 2 to 10% based on the initial amount of ds pSBK. Gapped DNA for lagging constructs were prepared similarly, using *Eco*RV-digested ss pSBK and ds pSBKG (Fig. 2). The construction of ssDNA bearing a single adduct has been described previously (5).

Transfection of Human Cells and Analysis of Progeny Plasmid. Three human cell lines, HeLa, HCT116, and 293, were plated at 1×10^6, 4×10^6, and 2×10^6 cells per 25-cm^2 flask, respectively. After overnight incubation, cells were transfected with 1–2 μg of an unmodified or modified construct, using Fugene6 Transfection Reagent (Boerhingher Manheim) as described by the manufacturer. After 24 h, cells were treated with trypsin and were divided as follows: 5% of the cells were used to determine the total number of transfected cells, and the remaining cells were used to amplify progeny DNA. Cells were grown in the presence of the neomycin analogue, G418 (Life Technologies, Inc.; 400–700 μg/ml medium) to select for transfected cells. After growing in the presence of G418 for 7 days, cells were replated in another flask. When the G418-resistant cells became confluent, cells were collected, and progeny plasmid was recovered. The plate containing 5% of the cells was maintained without replating in the presence of G418 for 12–14 days, at which time G418-resistant colonies were counted after staining. Progeny plasmid DNA was purified from 1×10^7 cells by the method of Hirt (26). After *Dpn*I treatment to remove input bacterial DNA, plasmid DNA was used to transform *E. coli* DH10B. For the analysis of mutational events, plasmid was digested with *Sna*BI. As described above, a unique *Sna*BI site is located opposite a DNA adduct in the unmodified complementary strand. This digestion removed progeny plasmid derived from the complementary strand and facilitated analysis of translesional events. To determine the base inserted opposite the adduct, differential oligonucleotide hybridization (5, 27, 28) was conducted on DH10B transformants using the probes shown in Fig. 4. This method allowed positive detection of sequences complementary to the probe because even a single base mismatch inhibits hybridization between probe and target DNA. Plasmids containing dA, dC, dG, or dT at the position of the adduct were included on the filter as positive controls. None of the probes cross-hybridized. Plasmids that contained mutations other than a targeted mutation did not hybridize to any of the AA, AT, AG, AC, or AD probes (Fig. 4). Their sequence changes were determined by DNA sequencing. Thus, all types of mutations were detected and determined by this technique.

Mutagenesis Studies in *E. coli*. An overnight culture of MM1991 was diluted 20-fold with 2× YT, cultured for 2 h at 37°C with shaking, and divided into two portions. Mitomycin C was added to one portion at a final concentration of 2 μg/ml. The culture was continued for another 30 min to induce SOS functions. Bacteria were made electrocompetent by washing twice with ice-cold H_2O. The final bacterial pellet was suspended in 0.005 of the original volume of 10% glycerol. Electro-competent MM1991 (50 μl) were mixed with 30 ng of a leading construct bearing ϵdA and electroporated using the *E. coli*-Pulser (Bio-Rad). After the addition of 950 μl of 2× YT, bacteria were cultured for 40 min. A small volume (1–10 μl) of transformation mixture was plated on 1× YT-ampicillin (100 μg/ml) plates to determine the number of transformants. After incubating the remaining transformation mixture for 20 min, the culture was added to 19 ml of 2× YT-ampicillin and cultured overnight. Plasmid DNA was prepared from an overnight culture by alkaline-lysis, was digested with *Sna*BI, and was used to transform *E. coli* DH10B. Transformants were analyzed for mutations as described above.

RESULTS

Characterization of DNA Constructs. One of the critical elements in this approach is to prepare large quantities of purified cc dsDNA. For this purpose, we used alkali denaturation-renaturation technique to prepare gapped DNA and ultracentrifugation in an ethidium bromide-CsCl solution to purify cc dsDNA. To confirm that the lower band that formed during ultracentrifugation contains only cc dsDNA, a control oligonucleotide was phosphorylated with γ-^{32}P]ATP at its 5′ end and was used to construct heteroduplex DNA. Upper and lower bands were collected separately and digested with *Xho*I. As shown in Fig. 5, two *Xho*I sites were located near the ligation sites. When the oligonucleotide was ligated to gapped DNA at its 5′ and 3′ ends, *Xho*I treatment generated a labeled 61-mer, whereas partially ligated products yielded a labeled 40-mer and 33-mer, re-

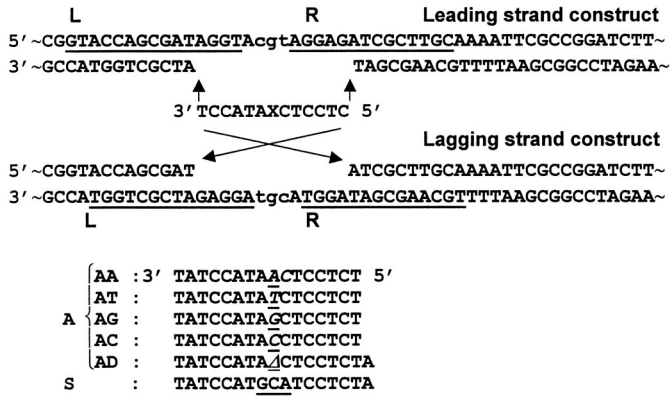

Fig. 4. DNA sequence of regions containing a DNA adduct and probes used for oligonucleotide hybridization. L and R probes (*underlined*) were used to identify progeny containing the sequence of inserted oligomer. *AA*, *AT*, *AG*, *AC*, and *AD*, probes used to determine the base that replaced ϵdA. *S*, probe detects progeny derived from the complementary strand, X = ϵdA. There are three base mismatches at, and adjacent to, ϵdA.

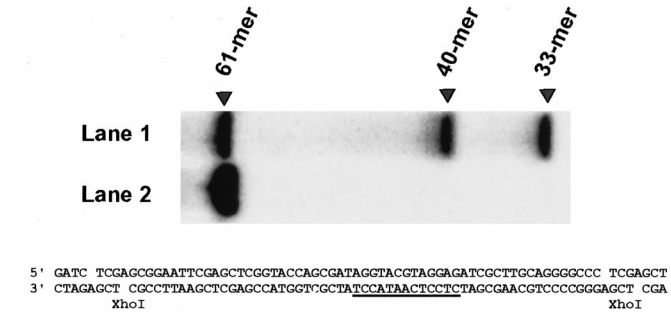

Fig. 5. Analysis of DNA construct by denaturing 20% PAGE. The unmodified 13-mer was labeled with ^{32}P at its 5′ end and ligated to gapped dsDNA. cc dsDNA and other forms of DNA were separated by ultracentrifugation. Recovered DNA was digested with *Xho*I and run in denaturing 20% polyacrylamide gel. *Underlined*, ligated 13-mer. *Lane 1*, DNA present in the upper band after ultracentrifugation; *Lane 2*, DNA present in the lower band.

Table 1 *Analysis of progeny*

Progeny from or with	Number of plasmids	
	dA[a]	ϵdA[a]
Adduct strand	216 (45)[b]	45[c] (9)
Complementary strand	258 (54)	423 (88)
Large deletions	6 (1.3)	12 (2.5)

[a] Leading constructs with dA (control) or ϵdA; HeLa cells were used as a host.
[b] Numbers in parentheses represent percent.
[c] This number consists of 34 ϵdA→A, 5 ϵdA→G, 3 ϵdA→C, 2 ϵdA→T and 1 mutant b (see Table 4 for sequence change).

spectively, when ligated to DNA at the 5′ or 3′ end of the insert. Only a 61-mer was observed for DNA recovered from the lower band (*Lane 2*), whereas 34-mer, 40-mer, and 61-mer were observed for DNA recovered from the upper band (*Lane 1*). These results established that the lower band contained fully ligated products.

Analysis of Progeny Plasmid. Progeny plasmid was purified from G418-resistant cells and was used to transform *E. coli* for analysis. There were three base mismatches in the adducted region; thus, progeny derived from the unmodified strand (*Sna*BI strand) hybridized to the S, L, and R probes (Fig. 4), whereas progeny derived from TLS hybridized to the L and R probes and to one of the A probes (Fig. 4). Progeny with deletions in the region of analysis did not hybridize to any of the probes. The analysis of progeny plasmid revealed that the ratio of progeny derived from the two strands was close to 1:1 (55 *versus* 45%) for the control construct (Table 1), which suggests that each strand replicated equally. In contrast, the fraction of progeny derived from the unmodified strand was much larger than that from the modified strand when the modified construct was used (88 *versus* 9%). This suggests that the ϵdA adduct blocked DNA synthesis. Another possibility was excision repair of ϵdA. Removal of this adduct and the 5′ and 3′ flanking mismatched bases, followed by the filling of the gap, created a *Sna*BI site in the modified strand. There was a 2-fold increase in the number of progeny containing large deletions in the modified construct, compared with that observed in the control (Table 1).

Incidence of Deletion Mutations in Human Cells. Our strategy for TLS analysis was to remove progeny derived from the unmodified strand by digesting with *Sna*BI. This enrichment facilitated our analysis because DNA adducts are likely to block DNA synthesis, in which case a major fraction of progeny is derived from the unmodified strand. Table 2 shows frequencies of progeny that contain deletions obtained after *Sna*BI enrichment. For both leading and lagging control constructs, deletion frequency was highest in HCT116 and lowest in HeLa. Deletions ranged from several hundred to 2000 bp. When modified constructs were used, the fractions of deletion mutants increased 4-fold for HeLa and 293 cells and 2-fold for HCT116 (Table 2). These increased frequencies resulted from a relative increase of a fraction of deletion mutants in progeny after *Sna*BI digestion. When data in Table 1 were recalculated, ignoring progeny derived from the *Sna*BI strand, the frequencies of deletions are 2.7% (6 of 222) for the control and 21% (12 of 57) for the modified construct. The results of experiments shown in Table 2 indicated that TLS analysis in HeLa cells was the least labor-intensive. Therefore, we used HeLa cells as the primary host for TLS analysis.

The incidence of plasmid that contained deletions was low in *E. coli*. Experiments with the unmodified leading construct yielded 0.5% (2 of 384) and <0.27% (0 of 384) in the absence and presence of induced SOS functions, respectively. The frequency of deletion mutants did not increase after *Sna*BI digestion. Thus, the plasmid was much more stable in *E. coli* than in transformed human cells.

Mutagenesis of ϵdA in Human Cells. To determine the number of G418-resistant cells per transfection, 5% of the transfected cells were plated separately and maintained in complete medium containing G418 for 12–14 days. G418-resistant colonies were counted after staining with Giemsa. The number of transfected cells ranged from 2000 to 6000 per μg of construct for the experiments shown in Table 3. The number of G418-resistant colonies does not represent the degree of TLS but shows simply the transfection efficiency.

To determine miscoding frequency and specificity, progeny plasmid was purified from pooled G418-resistant cells, digested with *Sna*BI, and used to transform *E. coli* DH10B. *E. coli* transformants were analyzed for targeted mutations by differential oligonucleotide hybridization using the probes shown in Fig. 4. This analysis showed 14 and 10% targeted miscoding frequencies in HeLa cells for the leading and lagging strand constructs, respectively. This indicated that dTMP was preferentially inserted opposite ϵdA at frequencies of 86 to 90% despite the lack of normal hydrogen bonding expected by the presence of the exocyclic ring. In another experiment, in which a leading strand construct was purified twice by ultracentrifugation, the frequency of miscoding was 14%. In an experiment with HCT116, the fraction of progeny derived from TLS was small as expected; mutation frequency was 7% for the leading strand construct (Table 3). Although the incidence varied, all types of base substitution mutations were observed. When the identical modified oligonucleotide was inserted into ssDNA and replicated in HeLa cells, results were similar to those observed for dsDNA (Table 3).

To validate this system, another DNA adduct, 8-oxo dG, was incorporated into the leading strand. This adduct induced G→T transversions in HeLa cells at a frequency of 1%. This result was consistent with that of a previous study in COS cells (21) and suggests that the mutational specificity observed for ϵdA reflects its intrinsic miscoding properties in human cells.

Three types of other sequence changes were observed when modified constructs were used (Table 4). A common feature of these

Table 2 *Incidence of large deletions in human cells*

DNA construct	Fraction (%) of mutants with large deletions[a]		
	HeLa	293	HCT116
Leading			
dA (Control)	4	11	31
ϵdA	16	44	63
Lagging			
dA (Control)	5	12	46
ϵdA	20	ND[b]	89

[a] This is not a frequency among all progeny inasmuch as progeny plasmid was enriched by *Sna*BI digestion, which removed progeny derived from unmodified complementary strand. For example, a fraction of 16% means that the remaining 84% progeny are derived from TLS-related events.
[b] ND, not determined.

Table 3 *Mutagenicity of a single ϵdA adduct*

Host	DNA construct	No. of plasmids ϵdA → A, C, G, or T				Targeted single mutations, frequency (%)	No. of plasmids with other sequence changes
		A	C	G	T		
Human cells	Leading						
HeLa	dA	224	0	0	0	<0.45	0
	ϵdA	151 (86)[a]	9 (5)	4 (2)	12 (7)	14	7 (7[b])
HCT116	dA	236	0	0	0	<0.43	0
	ϵdA	102 (93)	2 (2)	6 (5)	0	7	8 (8[b])
HeLa	dG	0	0	243	0	<0.41	0
	8-oxo dG	0	0	152 (99)	2 (1)	1	3 (2[b], 1[c])
	Lagging						
HeLa	dA	355	0	0	0	<0.28	0
	ϵdA	182 (89)	2 (1)	11 (5)	9 (4)	10	10 (7[b], 3[d])
	ssDNA						
HeLA	ϵdA	151 (89)	2 (1)	3 (2)	13 (8)	11	1?[e]
E. coli	Leading						
MM1991	ϵdA	371	0	0	0	<0.27	0
MM1991+mc[f]	ϵdA	380	0	0	0	<0.27	1 (1[b])

[a] Numbers in parentheses represent percent.
[b,c,d] See sequence changes (mutants b, c, and d) shown in Table 4.
[e] DNA sequencing failed repeatedly.
[f] *E. coli* was treated with mitomycin C to induce SOS functions. See "Materials and Methods" for conditions.

Table 4 *Other sequence changes observed*

Sequences flanking ϵdA (X) are shown; sequence changes are underlined.

Original construct (X = ϵdA)	Mutant b[a]	Mutant c[a]	Mutant d[a]
5′-AGGTACGTAGGAG	5′-AGGTATGTAGGAG	5′-AGGTACTTAGGAG	5′-AGGTATTTAGGAG
3′-TCCATAXCTCCTC	3′-TCCATACATCCTC	3′-TCCATGAATCCTC	3′-TCCATAAATCCTC

[a] See "number of plasmids with other sequence changes" (last column in Table 3) referencing Mutants b, c, and d.

mutations is a T:C→T:A change 5′ to the adduct site. Many of those were accompanied by an additional targeted base change of G:ϵA→G:C, *i.e.*, 5′-GT/3′-ϵAC→5′-GT/3′-CA ("mutant b" in Table 4). Mutants with this sequence change were observed reproducibly and accounted for 80% (24 of 30) of mutants with "other sequence changes" (Table 3).

Mutagenesis of ϵdA in *E. coli*. The leading strand construct containing ϵdA was used to transform MM1991. Progeny plasmid was prepared from a transformation mixture after overnight incubation in the presence of ampicillin and was used to transform *E. coli* DH10B. An analysis of DH10B transformants did not show any targeted point mutations; miscoding frequencies were <0.27% in the presence or absence of induced SOS functions. One plasmid obtained in the presence of induced SOS functions had the same sequence change as mutant b.

DISCUSSION

In this paper, we describe a site-specific approach that can be used to probe mutagenic mechanisms in human cells as well as in *E. coli* and is applicable to most DNA adducts. The analysis of progeny is facilitated by restriction enzyme digestion that inactivates progeny derived from the complementary strand.

While developing this approach, we found that plasmid that is maintained in human cells is associated with a high incidence of large deletions. The cause of these deletion mutations is not clear. They are not attributable to nicks in the constructs, inasmuch as we isolated cc dsDNA by ultracentrifugation, and in HeLa cells, even supercoiled dsDNA plasmid yielded deletion mutations at a similar frequency. Furthermore, the incidence of large deletions depends on host cells. It is not known what contributes to the difference in the spontaneous frequency of large deletions between transformed human cell lines. Among the three cell lines tested, plasmid was most unstable in HCT116. This may be ascribed to its mutator phenotype attributable to mismatch repair deficiency. For our purposes, HeLa cells proved to be the best host, showing the lowest incidence of deletions.

Our results show that ϵdA is substantially mutagenic in human cells. ϵdA did not miscode in *E. coli* when embedded in the same sequence. This confirms previous results obtained with ssDNA (5). Basu *et al.* (29) reported a limited number of A→G transitions when dsDNA containing ϵdA was replicated *in vitro* by HeLa cell extracts. Recently, *E. coli* UmuD′$_2$C complex (DNA damage-inducible proteins involved in error-prone TLS pathway) was discovered to have an error-prone DNA polymerase activity and designated DNA polymerase V (30). In *E. coli*, the ϵdA adduct inhibits DNA synthesis, and the induction of UmuD′ and UmuC proteins increases the level of TLS.[5] Our results in *E. coli* (Table 3) show that dTMP is the preferred nucleotide during TLS, which suggests that pol V may catalyze error-free DNA synthesis across this adduct.

Which DNA polymerase is responsible for the mutations observed in human cells? Human cells have at least two additional DNA polymerases, pol η and pol ζ (31–33). Both polymerases catalyze TLS across cyclobutane pyrimidine dimers. Whereas pol η catalyzes error-free TLS, pol ζ conducts error-prone TLS. Studies with XP variant cells, which lack pol η, could be used to determine the role of pol η in mutagenesis induced by this adduct.

With regard to miscoding frequency and specificity, results obtained in HeLa and HCT116 cells are quite different from those obtained using simian kidney cells (COS; 5). In our COS cell experiments, miscoding frequency reached 70% with ϵdA→dG mutations being strongly dominant. There are several differences in the design of these two studies, including ss *versus* ds vector, sequence context, location of the DNA adduct relative to the replication origin, and host cells. One or several of these factors could have contributed to the differences observed. Because the experiment using ssDNA in HeLa cells yielded a miscoding frequency similar to that using dsDNA, the

[5] I-Y. Yang, G. A. Pandya, and M. Moriya, unpublished results.

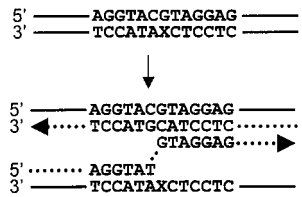

Fig. 6. Template strand switching model. DNA synthesis is blocked at the site of DNA adduct. The 3′ terminus of the blocked strand switches a template to the newly synthesized strand (*dotted line*) of the sister molecule, and then DNA synthesis resumes. *X*, ϵdA.

difference between ssDNA and dsDNA does not account for the discrepancy.

The analysis of tumors in humans and animals exposed to VC or vinyl carbamate showed A:T→T:A transversions in the *p53* and *ras* genes (13–18). The results reported here show that ϵdA efficiently induces this type of transversion in human cells. There are two possible mechanisms by which ϵdA might induce ϵdA→dT transversions. One possibility is the incorporation of dAMP opposite the adduct. The other is via the formation of an abasic site. ϵdA is known to be removed by human 3-methyladenine DNA glycosylase (34), which creates an abasic site that is then repaired by apurinic/apyrimidinic endonuclease (35). If TLS occurs at the abasic site, A→T transversions will be induced as the consequence of dAMP insertion opposite the lesion (36, 37). It is reported that an imbalance between the DNA glycosylase activity and the endonuclease activity increases the spontaneous mutation frequency (38). However, we believe this mechanism to be unlikely because ϵdA embedded in ssDNA shows mutational events similar to those in dsDNA (Table 3). The DNA glycosylase is not active on ϵdA located in ssDNA (39). Therefore, we conclude that the A:T→T:A transversions observed in human and animal tumors were induced by misreplication of ϵdA. A remaining question is why ϵdA→dG transitions were not observed equally in those tumors. This type of base substitution at the sites (described in the "Introduction") in the *p53* and *ras* genes are not silent as discussed in the "Introduction," and ϵdA induces this mutation at a frequency similar to that for ϵdA→dT. In one tumorigenicity study with VC (15), A→T, A→G, and A→C mutations were observed in the rat *p53* gene, consistent with the results of our study.

Our results show that ϵdA shows a similar miscoding frequency when located in either strand. This contrasts with the finding in *E. coli* that acetylaminofluorene-dG adduct showed a marked difference in its mutagenic activity when located in the leading or lagging strand (40).

ϵdA is produced endogenously, and its level in DNA is high in Wilson's disease and primary hemochromatosis (10). These metal storage disorders are associated with increased frequencies of liver tumors (41–43). The finding of its endogenous origin and increased formation following enhanced lipid peroxidation, coupled with the demonstrated mutagenic activity in human cells, implies that ϵdA is a significant threat to genomic integrity.

In addition to targeted single mutations, other sequence changes were observed in the adducted region (Tables 3 and 4). Whereas targeted single mutations are derived from TLS because they are flanked by 5′-C and 3′-A, the origin of the other sequence changes is not clear. These sequence changes can be explained by untargeted single-point mutations in either strand (Table 4). Because progeny plasmid was digested with *Sna*BI, any change in the recognition sequence in the complementary strand renders progeny resistant to the digestion. Mutants b and c could arise, respectively, from C→T and G→T mutations in 5′-CGT of the complementary strand (Table 4). Mutant d could be derived from C→A in 3′-AXC of the adducted strand. Among these sequence changes, mutant b accounts for the majority. In addition to the above possibility, this dominant sequence change can be generated by template strand switching at the adduct site (Fig. 6). If this mechanism operates, the blocked 3′ end of the nascent strand must be extended from a G:T mismatched terminus. It is reported that a G:T mismatch is the easiest to be extended among various mismatches (44). Additional studies are under way to distinguish these possibilities.

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