Miscoding Properties of 3,N^4-Etheno-2′-deoxycytidine in Reactions Catalyzed by Mammalian DNA Polymerases†

Shinya Shibutani,*,‡ Naomi Suzuki,‡ Yoshihiro Matsumoto,§ and Arthur P. Grollman‡

Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794-8651, and Department of Radiation Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Received June 17, 1996; Revised Manuscript Received September 16, 1996⊗

ABSTRACT: We have investigated the miscoding properties of the exocyclic DNA adduct, 3,N^4-etheno-2′-deoxycytidine (ϵdC), using an experimental system designed to detect and quantify base substitutions and deletions generated by primer extension in reactions catalyzed by DNA polymerases α, β, and δ. Oligodeoxynucleotides modified site-specifically with ϵdC were used as DNA templates for this study. Pol α catalyzed incorporation of dTMP and dAMP opposite ϵdC, accompanied by lesser amounts of dCMP and dGMP and some two-base deletions. Pol β promoted incorporation of dCMP and dAMP, along with small amounts of one-base and two-base deletions. Pol δ catalyzed incorporation of dTMP and lesser amounts of dAMP and dGMP. The frequency of nucleotide insertion opposite ϵdC and of chain extension from the 3′-primer terminus in reactions catalyzed by pol α and pol β was established by steady-state kinetic analysis. Results of this study were consistent with those obtained in primer extension experiments. The miscoding properties of ϵdC determined *in vitro* are consistent with observations of ϵdC→A transversions and ϵdC→T transitions in site-specific mutagenesis experiments in mammalian cells (Moriya *et al.* (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11899). We conclude from this study that DNA polymerases may differ significantly in their miscoding potential and that *in vitro* analysis can be used to predict mutagenic specificity of exocyclic DNA adducts in mammalian cells.

Occupational exposure to vinyl chloride, a chemical used for the production of synthetic polymers, is associated with the induction of hepatic sarcomas (IARC Monographs, 1979; Merletti *et al.*, 1984). Ethyl carbamate (urethane), found in certain foods, wine, and distilled liquor (Ough, 1976), and used as a co-solvent for certain analgesic agents (Miller, 1991), is carcinogenic in rodents (Nettleship *et al.*, 1943; Mirvish, 1968). Both carcinogens are activated by liver microsomes to form α-chloroethylene oxide, chloroacetaldehyde, and epoxyethyl carbamate (Barbin *et al.*, 1985; Scherer *et al.*, 1986). Metabolites of vinyl chloride and urethane react with DNA to form the exocyclic nucleoside adducts ϵdC,[1] ϵdA, and ϵdG (Malaveille *et al*., 1975; Scherer *et al*., 1981; Barbin & Bartsch, 1986). These adducts, which also arise through the reaction of lipid peroxidation products with DNA, have recently been detected in genomic DNA (Chaudhary *et al.*, 1994; Nath & Chung, 1994; Scheller *et al.*, 1995). The ubiquitous nature and persistence of exocyclic DNA adducts suggest the possibility of their involvement as initiators of human cancer. Their physiological importance is underscored by reports that these lesions undergo cellular repair (Matijasevic *et al.*, 1992; Dosanjh *et al.*, 1994).

The mutagenic properties of ϵdC in bacteria have been established by transfecting plasmid vectors modified with ϵdC into *Escherichia coli* (Palejwala *et al*., 1991; Basu *et al*., 1993; Moriya *et al.*, 1994). The miscoding properties of this adduct *in vitro* have been studied, using the Klenow fragment of *E. coli* DNA polymerase I (Simha *et al*., 1991; Zhang *et al*., 1995a). In this paper, we explore the miscoding properties of ϵdC in mammalian cells by measuring translesional synthesis on a site-specifically modified DNA template in reactions catalyzed by pol α, pol β, and pol δ. Fully-extended products were analyzed quantitatively (Shibutani, 1993) with an improved experimental system. Kinetic parameters of nucleotide insertion and chain extension were determined under steady-state conditions (Mendelman *et al.*, 1989, 1990). We find that miscoding properties of ϵdC vary significantly, depending on the DNA polymerase used to catalyze the reaction. The significance of this observation with respect to translesional DNA synthesis, mutagenesis, and carcinogenesis is discussed.

EXPERIMENTAL PROCEDURES

Materials and Methods. Organic chemicals used for the synthesis of oligodeoxynucleotides were supplied by Aldrich Chemical Co., Inc. HPLC grade acetonitrile, triethylamine, and distilled water were purchased from Fisher Chemical. [γ-^{32}P]ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Corp. Calf thymus DNA pol α (30 000 units/mg of protein) and human pol β (100 000 units/mg of protein) were purchased from Molecular Biology Resources, Inc. T4 polynucleotide kinase was obtained from Stratagene.

† This research was supported by NIH Grants CA17395 and ES04068.

* Corresponding author. Telephone: (516) 444-8018; Telefax: (516) 444-7641.

‡ State University of New York at Stony Brook.

§ Fox Chase Cancer Center.

⊗ Abstract published in *Advance ACS Abstracts*, November 1, 1996.

[1] Abbreviations: ϵdC, 3,N^4-etheno-2′-deoxycytidine; K_m, Michaelis constant; V_{max}, maximum rate of reaction; F_{ins}, frequency of insertion; F_{ext}, frequency of extension; dNTP, 2′-deoxynucleoside triphosphate; pol α, DNA polymerase α; pol β, DNA polymerase β; pol δ, DNA polymerase δ; pol I, DNA polymerase I; PCNA, proliferating cell nuclear antigen; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; Δ, deletion.

S0006-2960(96)01446-8 CCC: $12.00

Table 1: Sequence of Templates, Primers and Standard Markers[a]

Number	Sequence 5′ → 3′
1	CATGCTGATGAATTCCTTCXCTACTTTCCTCTCCATTT
2	CCTTCXCTACTTTCCTCTCCATTT
3	AGAGGAAAGT
4	AGAGGAAAGTAG
5	AGAGGAAAGTAGN
6	AGAGGAAAGTAGNGAAGG
7	AGAGGAAAGTAGGAAGG
8	AGAGGAAAGTAGAAGG
9	AGAGGAAAGTAGNGAAGGAATTCATCAGCATG
10	AGAGGAAAGTAGGAAGGAATTCATCAGCATG
11	AGAGGAAAGTAGAAGGAATTCATCAGCATG
12	AGAGGAAAGTAGGAGG
13	AGAGGAAAGTAGTAGG
14	AGAGGAAAGTAGCAGG

[a] X = dC or ϵdC; N = C, A, G, or T.

*Eco*RI restriction endonuclease (100 units/μL) was purchased from New England BioLabs. A Waters 990 HPLC instrument, equipped with a photodiode array detector, was used for the separation and purification of oligodeoxynucleotides. UV spectra were measured using a Hewlett Packard 8452A diode array spectrophotometer.

Synthesis of Oligodeoxynucleotides. DNA templates, primers, and standard markers listed in Table 1 were prepared by solid-state synthesis, using an automated DNA synthesizer (Takeshita *et al.*, 1987). DNA templates containing a single ϵdC adduct were prepared, using a DMT-phosphoramidite derivative of ϵdC (Zhang *et al.*, 1995b). Modified and unmodified oligomers were purified on a reverse-phase μBondapak C_{18} column (0.39 × 30 cm, Waters), using a linear gradient of 0.05 M triethylammonium acetate, pH 7.0, containing 10−20% acetonitrile, with an elution time of 60 min and a flow rate of 1.0 mL/min (Shibutani *et al.*, 1991a). DNA templates and primers were further purified by electrophoresis on a 20% polyacrylamide gel in the presence of 7 M urea (35 × 42 × 0.04 cm) (Shibutani *et al.*, 1991a). Bands detected under ultraviolet light were extracted with 2.0 mL of distilled water overnight at 4 °C. Extracts were concentrated on a Centricon 3 filter (Amicon) by centrifugation at 5000 rpm for 2 h; urea was removed by HPLC (Shibutani *et al.*, 1991a). Oligonucleotides were labeled at the 5′-terminus by treatment with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (Maniatis *et al.*, 1982) and subjected to acrylamide gel electrophoresis. The position of bands in the gel and homogeneity was determined by autoradiography, using Kodak X-Omat XAR film.

Primer Extension Studies. A 38-mer (sequence 1 in Table 1) or 24-mer (sequence 2) template (0.75 pmol), annealed to a ^{32}P-labeled 10-mer primer (0.5 pmol; sequence 3), was used for primer extension experiments. Reaction mixtures containing pol α, pol β, or pol δ were incubated at 25 °C for the designated time in 10 μL of buffer containing the template and all four dNTPs (100 μM each) (Shibutani *et al.*, 1991b, 1993). For reactions with pol α and pol β, the buffer consisted of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and BSA (0.5 μg/μL). Pol δ and proliferating cell nuclear antigen (PCNA) were prepared from *Xenopus laevis* oocytes (Matsumoto *et al.*, 1994). For reactions with pol δ, the buffer contained 50 mM Tris-HCl (pH 6.5), 10 mM KCl, 6 mM $MgCl_2$, 2 mM DTT, BSA (0.04 μg/μL), and PCNA (6 ng/μL). Reactions were stopped by adding formamide dye and heating the sample to 95 °C for 3 min. Samples were subjected to electrophoresis on a 20% polyacrylamide gel containing 7 M urea (35 × 42 × 0.04 cm). Bands were located by autoradiography and excised from the gel; radioactivity was determined by liquid scintillation counting.

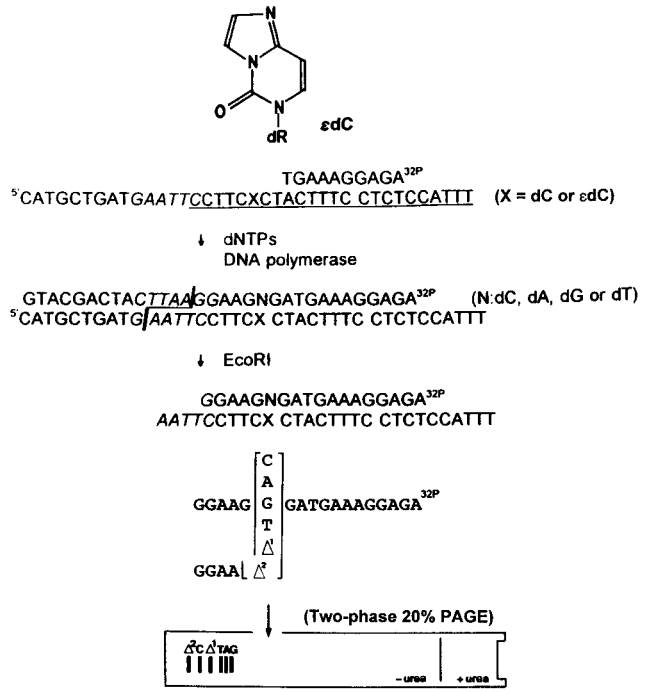

FIGURE 1: Diagram of method used to determine miscoding specificities.

Quantitation of Miscoding Specificity. Using a 38-mer template (0.75 pmol, sequence 1) and a ^{32}P-labeled 10-mer primer (0.5 pmol, sequence 3), reaction mixtures, catalyzed by one of several DNA polymerases, were incubated at 25 °C for 1 h in the presence of four dNTPs. Reactions were stopped by heating at 95 °C for 3 min and then subjected to electrophoresis on a 20% polyacrylamide gel containing 7 M urea (35 × 42 × 0.04 cm). Fully extended products were recovered from the gel and then cleaved with *Eco*RI after annealing with an unmodified 38-mer (sequence 1). Samples were incubated with *Eco*RI (100 units) for 1 h at 30 °C, and then 1 h at 15 °C to ensure complete digestion of the reaction products. Electrophoresis was carried out on two-phase 20% polyacrylamide gels (15 × 72 × 0.04 cm) containing 7 M urea in the upper phase (Shibutani, 1993). The modified method for determining miscoding specificity is shown in Figure 1.

Kinetic Studies of Nucleotide Insertion and Chain Extension. Kinetic parameters associated with nucleotide insertion opposite the lesion and chain extension from the 3′ primer terminus were determined in reactions containing a single dNTP (Mendelman *et al.*, 1989, 1990). Reaction mixtures containing 0.1−0.25 unit of pol α or 0.005−0.05 unit of pol β, 10 μL of Tris-HCl (pH 8.0), and 1.0 pmol of 38-mer template (sequence 1), primed with 0.5 pmol of ^{32}P-labeled 12-mer (sequence 4), were used to measure nucleotide insertion or, primed with 0.5 pmol of ^{32}P-labeled 13-mer (sequence 5), to measure chain extension.

In studies with pol α, nucleotide insertion was determined by incubating reaction mixtures containing 0.1 unit of enzyme for 90 s (G:C pair), 0.25 unit for 90s (A:ϵC, T:ϵC), and 0.25 unit for 5 min (C:ϵC, G:ϵC). Chain extension

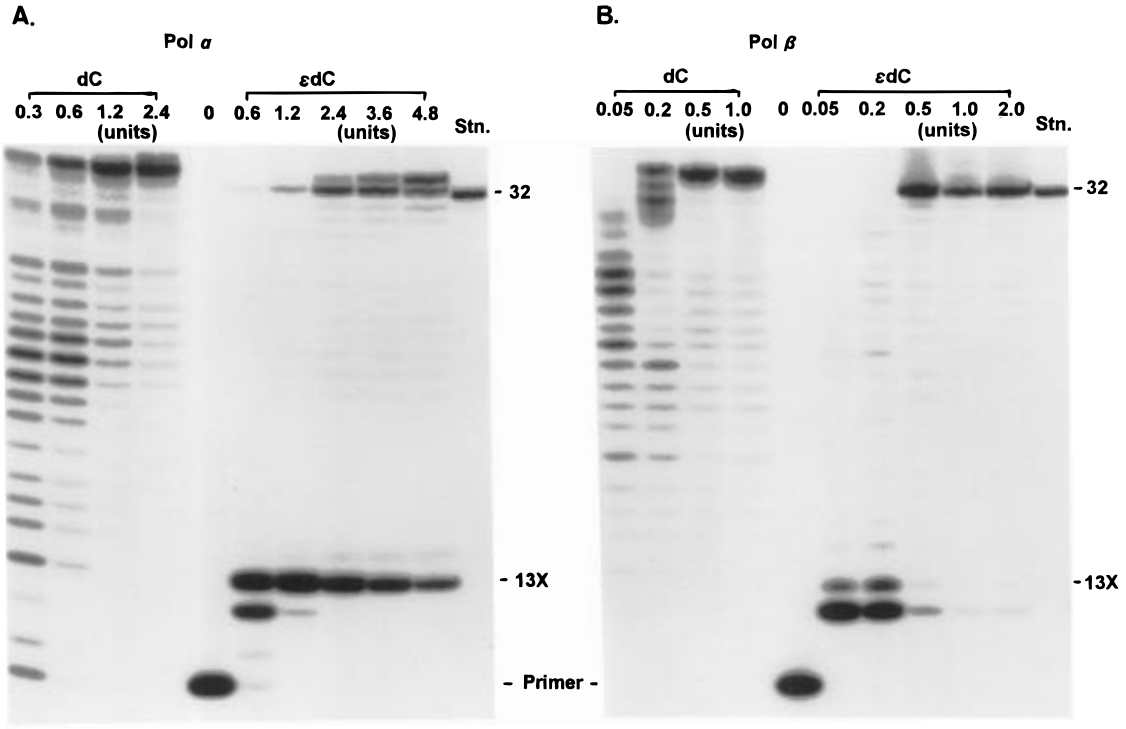

FIGURE 2: Primer extension on a 38-mer template catalyzed by pol α or *β*. Using an unmodified or ϵdC-modified 38-mer template (5′CATGCTGATGAATTCCTTCXCTACTTTCCTCTCCATTT, X = dC or ϵdC, sequence 1) primed with a ^{32}P labeled 10-mer (5′AGAGGAAAGT, sequence 3), primer extension reactions with DNA pol α (A) and pol *β* (B) were carried out for 1 h at 25 °C as described under Experimental Procedures. One-third of the reaction mixture was subjected to denaturing 20% polyacrylamide gel electrophoresis (35 × 42 × 0.04 cm). A 32-mer containing dA opposite the lesion (5′AGAGGAAAGTAGAGAAGGAATTCATCAGCATG, sequence 9) was used as a standard marker to determine the position of fully-extended products.

kinetics were measured by incubating reactions containing 0.1 unit of pol α for 90 s (G:C), 0.25 unit for 90 s (C:ϵC, T:ϵC), and 0.25 unit for 5 min (A:ϵC, G:ϵC). In studies with pol *β*, nucleotide insertion was determined in reactions containing 0.005 unit of pol *β* for 90 s (G:C), 0.05 unit of pol *β* for 90 s (C:ϵC, A:ϵC, T:ϵC), and 0.05 unit for 5 min (G:ϵC). Chain extension was measured in reactions using 0.005 unit for 90 s (G:C), 0.05 unit for 90 s (C:ϵC, A:ϵC, T:ϵC), and 0.05 unit for 5 min (G:ϵC). Reaction mixtures were subjected to electrophoresis on 20% polyacrylamide gels (35 × 42 × 0.04 cm) in the presence of 7 M urea. The Michaelis constant (K_m) and maximum rate of reaction (V_{max}) were obtained from Hanes−Woolf plots of the kinetic data. Frequencies of insertion (F_{ins}) and extension (F_{ext}) were determined relative to the dG:dC base pair according to the equation developed by Mendelman *et al*. (1989, 1990), where $F = (V_{max}/K_m)_{[wrong\ pair]}/(V_{max}/K_m)_{[correct\ pair=dG:dC]}$ is defined for any base pair containing a single modified nucleoside. All reactions were linear over the course of the experiment. Data reported represent the average of 2−4 separate experiments in which less than 20% of the primer was extended (Mendelman *et al.*, 1989).

RESULTS

Primer Extension Studies. Primer extension reactions catalyzed by pol α were conducted in the presence of four dNTPs using unmodified or ϵdC-modified 24-mer templates (5′CCTTCXCTACTTTCCTCTCCATTT, X = dC or ϵdC, sequence 2) (data not shown). Using small amounts of pol α, fully-extended products were generated on unmodified and ϵdC-modified templates. When the amount of enzyme was increased, blunt-end addition to the fully-extended reaction product (Clark *et al.*, 1987) interfered with the quantitative analysis of miscoding. To avoid this problem, we modified our previously-described (Shibutani, 1993) experimental system by creating a 38-mer template containing an *Eco*RI restriction site (Figure 1). Fully-extended products are first digested by *Eco*RI and then subjected to two-phase gel electrophoresis (Shibutani, 1993). When a template containing dC or ϵdC was annealed with a complementary strand containing dC, dA, dG, or dT opposite the lesion, or with a strand containing one- or two-base deletions, cleavage by *Eco*RI was >96% complete (data not shown).

In reactions containing pol α and pol *β*, primer extension on an unmodified template produced a fully-extended product (Figure 2). Primer extension on ϵdC-modified templates in reactions catalyzed by pol α was partially blocked opposite the lesion; some fully-extended products were formed (Figure 2A). In reactions catalyzed by pol *β*, primer extension was blocked one base before the lesion (Figure 2B); fully-extended products were formed only when higher concentrations of pol *β* were used.

Miscoding Specificity of ϵdC. Fully-extended products formed in reactions catalyzed by pol α or pol *β* were digested by *Eco*RI and analyzed by two-phase polyacrylamide gel electrophoresis. A mixture of ^{32}P-labeled oligodeoxynucleotides containing dC, dA, dG, dT, and one- or two-base deletions served as standard markers for these experiments (Figure 3A, lanes 2 and 8). Using pol α and pol *β*, primer extension on an unmodified template led to the expected product (Figure 3A, lane 1; Figure 3B, lane 1). Using pol α and an ϵdC-modified template, dTMP and dAMP were preferentially incorporated opposite the lesion (Figure 3A,

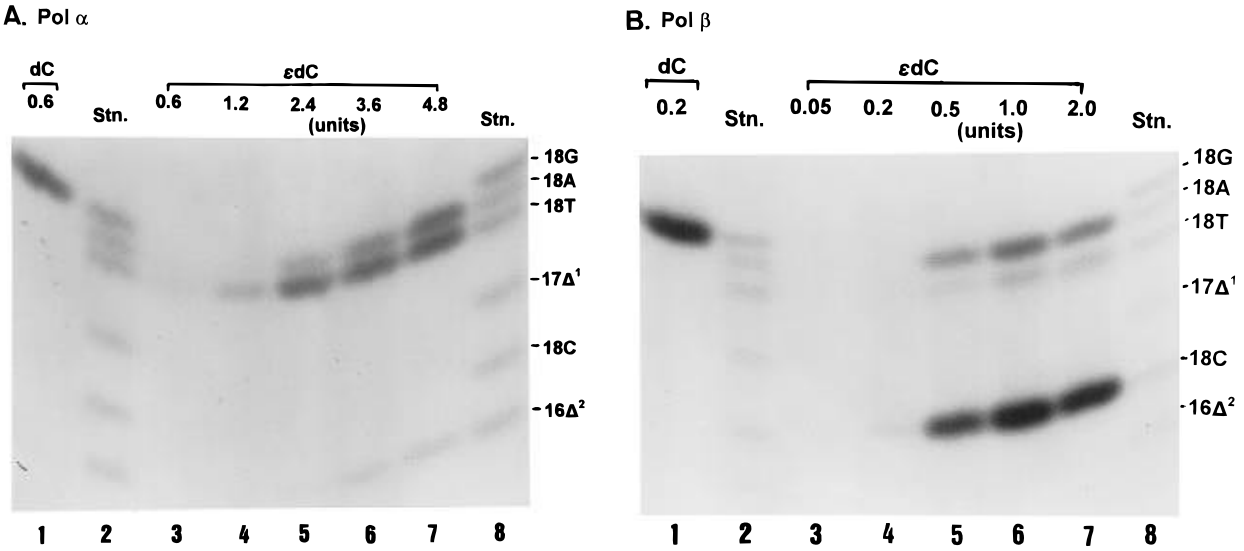

FIGURE 3: Miscoding specificities in reactions catalyzed by pol α or pol β. The fully-extended products formed by pol α (A) or pol β (B) in the experiment shown in Figures 2A and 2B were recovered from the gel and digested by treatment with 100 units of *Eco*RI for 1 h at 30 °C and, additionally, for 1 h at 15 °C, as described under Experimental Procedures. Aliquots removed from the reaction mixture were subjected to two-phase 20% polyacrylamide gel electrophoresis. Mobilities of reaction products were compared with those of 18-mer standards (Table 1, sequences 6−8) containing dC, dA, dG, or dT opposite the lesion and one-base (Δ^1) or two-base (Δ^2) deletions (lanes 2 and 8).

Table 2: Miscoding Properties of ϵdC in Reactions Catalyzed by Pol α and Pol β[a−c]

lesion	enzyme (units)	dC[b] (%)	dA (%)	dG (%)	dT (%)	Δ^1 (%)	Δ^2 (%)
				Polymerase α			
dC	0.3			9.9 ± 0.8[d]			
	0.6			14.4 ± 1.3			
	1.2			40.6 ± 4.6			
	2.4			57.0 ± 1.3			
ϵdC	0.6	ND	0.10 ± 0.01	ND	1.26 ± 0.07	ND	0.17 ± 0.02
	1.2	0.04 ± 0.01	2.01 ± 0.13	ND	7.29 ± 0.38	ND	0.38 ± 0.04
	2.4	0.23 ± 0.01	7.33 ± 0.35	0.18 ± 0.02	21.2 ± 1.7	ND	1.40 ± 0.11
	3.6	0.31 ± 0.04	13.4 ± 1.6	0.38 ± 0.08	21.4 ± 1.1	ND	3.11 ± 0.28
	4.8	0.38 ± 0.03	19.7 ± 1.1	0.43 ± 0.04	26.9 ± 0.8	ND	5.63 ± 0.25
				Polymerase β			
dC	0.05			2.0 ± 0.1			
	0.2			27.0 ± 1.1			
	0.5			69.6 ± 3.2			
	1.0			71.4 ± 2.8			
ϵdC	0.05	0.02 ± 0.01	ND	ND	0.02 ± 0.01	ND	ND
	0.2	0.38 ± 0.03	0.08 ± 0.01	ND	0.11 ± 0.01	ND	
	0.5	47.4 ± 0.8	9.97 ± 0.65	ND	3.72 ± 0.16	0.07 ± 0.02	0.08 ± 0.01
	1.0	45.8 ± 3.1	10.6 ± 0.5	ND	3.84 ± 0.09	0.09 ± 0.01	0.12 ± 0.02
	2.0	46.1 ± 5.0	17.2 ± 0.4	ND	9.14 ± 0.41	0.11 ± 0.01	0.14 ± 0.04

[a] Primer extension reactions were conducted for 1 h at 25 °C as described in the legend of Figure 3. [b] Expressed as fraction of starting primer converted to fully-extended product. [c] ND indicates not detectable. [d] Data were expressed as means ± SD.

lanes 3−7). Small amounts of dCMP and dGMP also were incorporated. In addition, two-base deletions were observed. The sequence of these products was confirmed by the Maxam−Gilbert technique (1980). The frequency of translesional synthesis in reactions catalyzed by pol α follows the order: dTMP > dAMP > $\Delta^2 \gg$ dCMP, dGMP.

When pol β was used to catalyze primer extension on the ϵdC-modified template, dCMP was incorporated preferentially opposite the lesion. Lesser amounts of dAMP and dTMP incorporation also were observed. dGMP was not incorporated (Figure 3B, lanes 3−7, and Table 2). With longer exposure, small amounts of fully-extended products containing one-base and two-base deletions were detected (Table 2). The frequency of translesional synthesis catalyzed by pol β follows the order: dCMP > dAMP > dTMP $\gg$ Δ^2, Δ^1. In reactions catalyzed by pol α and pol β, miscoding frequency increased proportional to the amount of enzyme used (Table 2) and the time of the reaction (data not shown).

Kinetic Studies of Nucleotide Insertion and Extension. Steady-state kinetic parameters were established for nucleotide insertion opposite ϵdC and for chain extension from 3′-termini containing this lesion. When pol α was used (Table 3), the frequency of dAMP insertion (F_{ins}) opposite ϵdC was 1.7 times higher than that of dTMP and 57 and 87 times higher than that for dCMP and dGMP, respectively. The frequency of chain extension (F_{ext}) from 3′-termini containing dA:ϵdC was 2.3 and 3.8 times lower than chain extension from dC:ϵdC and dT:ϵdC, respectively, and similar to extension from 3′-termini containing dG:ϵdC. The relative frequency of translesional synthesis ($F_{ins} \times F_{ext}$) past ϵdC in reactions involving dT is 2.3-fold higher than for dA and 55- and 240-fold higher than for dC and dG, respectively.

Table 3: Kinetic Parameters for Nucleotide Insertion and Chain Extension Reaction Catalyzed by Pol α[a]

	Insertion dNTP ↓GATGAAAGGAGA[32]P 5′CCTTCXCTACTTTCCTCTCCATTT			Extension dGTP ↓NGATGAAAGGAGA[32]P 5′CCTTCXCTACTTTCCTCTCCATTT			
N:X	K_m (μM)	V_{max} (% min^{-1})	F_{ins}	K_m (μM)	V_{max} (% min^{-1})	F_{ext}	$F_{ins} \times F_{ext}$
G:C	2.3 ± 0.3[b]	75.0 ± 11.0	1.0	2.6 ± 0.3	36.8 ± 0.2	1.0	1.0
C:ϵdC	813 ± 52	7.2 ± 1.6	2.71×10^{-4}	444 ± 67	7.1 ± 0.7	1.16×10^{-3}	3.14×10^{-7}
A:ϵdC	50 ± 6	24.8 ± 1.4	1.54×10^{-2}	243 ± 12	1.7 ± 0.1	4.95×10^{-4}	7.62×10^{-6}
G:ϵdC	919 ± 132	5.3 ± 0.4	1.77×10^{-4}	327 ± 14	1.9 ± 0.1	4.09×10^{-4}	7.24×10^{-8}
T:ϵdC	87 ± 12	26.0 ± 0.1	9.20×10^{-3}	199 ± 26	5.2 ± 0.1	1.87×10^{-3}	1.72×10^{-5}

[a] Kinetics of nucleotide insertion and chain extension reactions were determined as described under Experimental Procedures. Frequencies of nucleotide insertion (F_{ins}) and chain extension (F_{ext}) were estimated by the equation: $F = (V_{max}/K_m)_{[wrong\ pair]}/(V_{max}/K_m)_{[correct\ pair]}$. X = dC or ϵdC lesion. [b] Data were expressed as means ± SD.

Table 4: Kinetic Parameters for Nucleotide Insertion and Chain Extension Reactions Catalyzed by DNA Pol β[a]

	Insertion dNTP ↓GATGAAAGGAGA[32]P 5′CCTTCXCTACTTTCCTCTCCATTT			Extension dGTP ↓NGATGAAAGGAGA[32]P 5′CCTTCXCTACTTTCCTCTCCATTT			
N:X	K_m (μM)	V_{max} (% min^{-1})	F_{ins}	K_m (μM)	V_{max} (% min^{-1})	F_{ext}	$F_{ins} \times F_{ext}$
G:C	88 ± 3[b]	261 ± 29	1.0	63 ± 18	98.3 ± 14	1.0	1.0
C:ϵdC	181 ± 36	5.98 ± 0.04	1.13×10^{-2}	181 ± 17	10.6 ± 1.3	3.70×10^{-2}	4.18×10^{-4}
A:ϵdC	174 ± 28	2.47 ± 0.04	4.79×10^{-3}	278 ± 25	11.0 ± 0.9	2.49×10^{-2}	1.19×10^{-4}
G:ϵdC	174 ± 58	0.11 ± 0.02	2.40×10^{-4}	164 ± 6	0.9 ± 0.1	3.48×10^{-3}	8.35×10^{-7}
T:ϵdC	345 ± 5	0.94 ± 0.12	9.10×10^{-4}	235 ± 13	30.4 ± 3.3	8.09×10^{-2}	7.30×10^{-5}

[a] Kinetics of nucleotide insertion and chain extension reactions were determined as described under Experimental Procedures. Frequencies of nucleotide insertion (F_{ins}) and chain extension (F_{ext}) were estimated by the equation: $F = (V_{max}/K_m)_{[wrong\ pair]}/(V_{max}/K_m)_{[correct\ pair]}$. X = dC or ϵdC lesion. [b] Data were expressed as means ± SD.

Using pol β, F_{ins} for dCMP opposite ϵdC is 2.4 and 12.4 times higher than for dAMP and dTMP, respectively (Table 4). F_{ext} from 3′-termini containing dC:ϵdC is 1.5 times higher than from dA:ϵdC, but 2.2 times lower than from dT:ϵdC. F_{ins} for dGMP and F_{ext} from dG:ϵdC are much less than for other DNA bases. The relative frequency of translesional synthesis past ϵdC in reactions involving dC is 3.5- and 5.4-fold higher than for dA and dT, respectively, and 500-fold higher than for dG.

Primer Extension Reactions Catalyzed by Pol δ. In experiments with pol δ, 38% of the primer was extended on an unmodified template to form a single fully-extended product. Twenty-five percent of the starting primer was degraded by the 3′→5′ exonuclease of this enzyme (Figure 4A, lane 2). Primer extension on ϵdC-modified templates was blocked opposite and one base before the lesion. Small amounts (1.68%) of fully-extended products were formed (lane 3). Using two-phase polyacrylamide gel electrophoresis to separate components of the reaction, preferential incorporation of dTMP (1.45%) was detected (Figure 4B, lane 3). With longer exposure, small amounts of dAMP (0.12%) and dGMP (0.11%) were observed (data not shown).

Generation of Deletions. Two-base deletions in fully-extended products were detected when pol α was used to catalyze primer extension on an ϵdC-modified template (Figure 3A and Table 2). These experiments utilized a 13-mer primer containing dA, dG, dT, or dC at the 3′-terminus with this base positioned opposite ϵdC. When dT or dC was positioned opposite the lesion, 79% and 67% of the primer was converted to fully-extended products (data not shown). When dA or dG was at the 3′-primer terminus, the yield of fully-extended products was 17% and 18%, respectively.

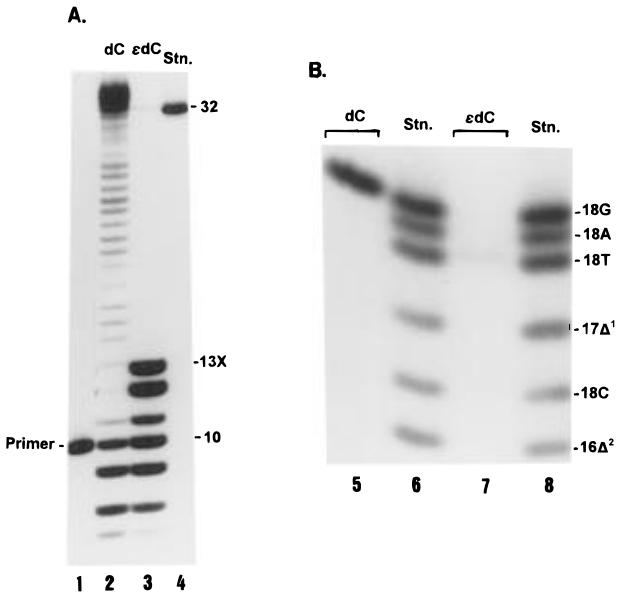

FIGURE 4: Primer extension catalyzed by pol δ. (A) Primer extension reactions on templates containing ϵdC or dC at the lesion site were conducted at 25 °C for 1 h using 0.03 unit of pol δ, as described in the legend of Figure 2. (B) Fully-extended products recovered from a mixture of five separate reactions obtained from the experiment shown in panel A were digested by *Eco*RI and then subjected to two-phase gel electrophoresis. Experimental conditions are as described in the legend to Figure 3.

Following cleavage of fully-extended products by *Eco*RI, [32]P-labeled fragments were analyzed by two-phase gel electrophoresis. In experiments in which the 13-mer contains dA at the 3′-primer terminus, 3.6% of the full length products contained two-base deletions; when dG was positioned opposite the lesion, 2.6% of the product contained one-base

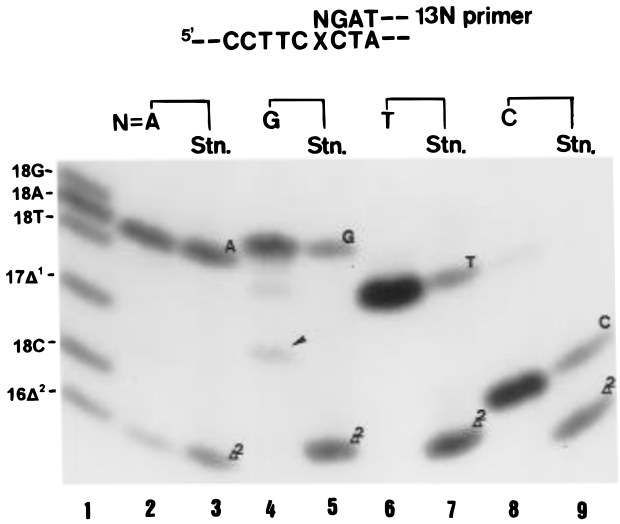

FIGURE 5: Chain extension from the 3′-primer terminus in reactions catalyzed by pol α. Using an ϵdC-modified 38-mer template (5′CATGCTGATGAATTCCTTCXCTACTTTCCTCTCCATTT, X = dC, ϵdC, sequence 1) primed with a 13-mer (5′AGAGGAAAG-TAGN, sequence 5) containing dA (lane 2), dG (lane 4), dT (lane 6), or dC (lane 8) positioned opposite the lesion, primer extension reactions were conducted for 1 h at 30 °C using 2.4 units of pol α. Fully-extended products were digested by *Eco*RI and then subjected to two-phase gel electrophoresis as described in the legend in Figure 3. Mobilities of reaction products were compared with 18-mers containing dA (lane 3), dG (lane 5), dT (lane 7), or dC (lane 9) and with comparable sequences containing a two-base deletion (sequences 7, 12−14).

Table 5: Summary of Miscoding Properties of DNA Polymerases

polymerase	miscoding specificity
pol α	T > A > $\Delta^2 \gg$ C, G
pol β	C > A > T $\gg$ Δ^1, Δ^2
pol δ	T $\gg$ A, G
pol I[a]	A, T > C

[a] Data for pol I reported by Zhang *et al.* (1995a).

deletions (see arrow in Figure 5, lane 4). When a 13-mer with dT or dC at the 3′-terminus was used as primer, deletions were not detected (lanes 6 and 8).

DISCUSSION

Miscoding Properties of ϵdC. The miscoding properties of ϵdC, observed during translesional synthesis in reactions catalyzed by mammalian DNA polymerases, are summarized in Table 5. The *in vitro* experimental system used for these experiments detects all base substitutions and deletions targeted to the site of the lesion. In reactions catalyzed by pol α, dTMP and dAMP were incorporated preferentially opposite ϵdC, accompanied by two-base deletions. dTMP was incorporated preferentially in reactions catalyzed by pol δ. In contrast, pol β preferentially catalyzed incorporation of dCMP.

Analysis of full length reaction products should reflect relative rates of nucleotide insertion opposite the lesion. This assumption was confirmed by steady-state kinetic analysis, in which the frequency of nucleotide insertion opposite ϵdC and chain extension from the 3′-primer terminus are measured in the presence of a single dNTP. With pol α and pol β, F_{ins} for dTMP was 1.7 and 5.3 times lower, respectively, than for dAMP, while F_{ext} from a dT:ϵdC terminus was 3.2 and 3.6 times higher than from dA:ϵdC. The frequency of translesional synthesis ($F_{ins} \times F_{ext}$) with pol α is 1.6-fold higher for dTMP than for dAMP and 2.3-fold lower with pol β. $F_{ins} \times F_{ext}$ for dCMP in reactions catalyzed by pol β is 1000-fold higher than for pol α.

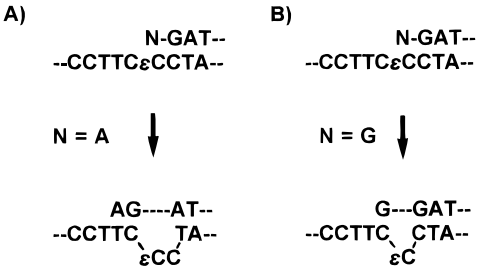

FIGURE 6: Proposed mechanism for one- and two-base deletions.

We conclude from these results that ϵdC promotes misincorporation of dTMP, dAMP, and dCMP, the relative predominance of a given nucleotide depending on the DNA polymerase used to catalyze the reaction. The miscoding potential of the mammalian replicative polymerases, pol α and pol δ, for ϵdC is similar and differs significantly from that observed with polymerase, pol β. Pol I, the repair polymerase of *E. coli*, shows similar frequencies for dAMP and dTMP on templates containing ϵdC (Zhang *et al.*, 1995a).

It is instructive to compare the miscoding properties of ϵdC with those of another endogenous-generated DNA lesion, 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG) (Shibutani *et al.*, 1991b). Pol α and pol δ preferentially catalyze incorporation of dAMP opposite 8-oxodG while pol β and the Klenow fragment of pol I preferentially incorporate dCMP, the nonmutagenic base, opposite the lesion. The difference in miscoding specificity contributes to the effective repair of 8-oxodG in *E. coli* (Tchou & Grollman, 1993). It appears that the miscoding specificity of DNA polymerases for any given adduct may vary, depending on the polymerase involved.

Translesional synthesis past ϵdC in simian kidney (COS) cells is accompanied by targeted ϵdC→A and ϵdC→T base substitutions (Moriya *et al.*, 1994). This result, established by site-specific techniques, is consistent with the miscoding specificity of pol α and δ observed in the present study.

Mechanism of Deletions. One- and two-base deletions generated during primer extension reactions *in vitro* may be explained by a general mechanism proposed for frameshift (deletion) mutations in which the propensity for template misalignment can be shown to depend on the (a) sequence context of the lesion, (b) nature of the base inserted opposite the lesion, and (c) frequency of translesional synthesis (Shibutani & Grollman, 1993; Kunkel, 1990). With pol α, F_{ins} opposite ϵdC follows the order: dAMP > dTMP $\gg$ dCMP > dGMP; F_{ext} from dA:ϵdC is relatively low. Thus, when dAMP is inserted opposite ϵdC, the newly incorporated base is predicted to pair preferentially with dT located two positions 5′ to the lesion. The template becomes misaligned, generating a bulge and promoting two-base deletions (Figure 6A). This prediction was confirmed by annealing an ϵdC-modified template to a primer containing dA, dG, dT, or dC at the 3′-terminus. With pol α, two-base deletions were detected only when dA was positioned opposite the lesion (Figure 6A). When dG is incorporated opposite ϵdC, the newly inserted base could pair with dC 5′ to the lesion to form a one-base deletion (Figure 6B). However, F_{ins} for dGMP was much lower than for other dNMP's; thus, one-

base deletions were not detected. In experiments with pol β, F_{ext} was much higher than F_{ins} for all bases tested. Since primer extension is not blocked as strongly as with pol α, formation of deletions is minimized. DNA templates used in COS cell experiments (TAϵdCGT) (Moriya *et al.*, 1994) should generate one-base deletions since dTMP inserted opposite ϵdC can pair with dA 5′ to the lesion. This reaction was not observed. Presumably, the proofreading exonuclease functions of pol δ (Brynes *et al.*, 1976) operates to minimize deletions during DNA synthesis *in vivo*. Alternatively, the replication complex in cells may stabilize the DNA template, minimizing the tendency to misalign.

Structural Basis for Miscoding by ϵdC. As shown in this paper, ϵdC promotes incorporation of dAMP, dTMP, or dCMP *in vitro*, depending on the DNA polymerase used to catalyze the reaction. In bacteria, dG is predominantly targeted to the lesion site (Moriya *et al.* 1994); thus, under appropriate experimental conditions, any of the four DNA bases may be inserted opposite the lesion.

ϵdC can adopt *anti* or *syn* conformations in DNA. In either conformation, only a single hydrogen bond can potentially form between ϵdC and certain DNA bases. Furthermore, steric considerations preclude structures in which dA or dG are accommodated in a coplanar arrangement with ϵdC in a right-handed undistorted helix. Nevertheless, the apparent fidelity of DNA replication in noninduced *E. coli* is relatively high (Moriya *et al.*, 1994).

2D-NMR studies of ϵdC have been performed in which the lesion is positioned opposite dA and dT in a DNA duplex and permitted to assume its preferred conformation (Cullinan *et al.*, 1996; Korobka *et al.*, 1996). These solution structures reveal that ϵdC:dA pair adopts a staggered conformation with each residue displaced toward the 5′-terminus and partially intercalated between bases on the opposite strand. Both ϵdC and dA are *anti*. Intercalation allows partial stacking of ϵdC with dA and with base pairs adjacent to the lesion. These hydrophobic interactions may stabilize ϵdC during translesional synthesis, permitting a high frequency of extension when ϵdC:dA is at the 3′-primer terminus.

When ϵdC is positioned opposite dT, a coplanar structure is formed, stabilized by a single hydrogen bond between the imino protein of dT and N4 of ϵdC. Thus, it appears that structures containing single hydrogen bonds or partial base-stacking participate in translesional synthesis past ϵdC. Structures of duplexes in which dC or dG are positioned opposite this lesion have not been reported.

ACKNOWLEDGMENT

We thank Mr. R. Rieger and Ms. M. C. Torres for synthesizing the modified oligodeoxynucleotides used in this study and Ms. S. Rigby for expert assistance in preparing the manuscript.

REFERENCES

Barbin, A., & Bartsch, H. (1986) In *The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis*, IARC Scientific Publications 70, pp 345−358, IARC, Lyon, France.

Barbin, A., Besson, F., & Perrard, M (1985) *Mutat. Res. 152*, 147−156.

Basu, A. K., Wood, M. L., Niedernhofer, L. J., Ramos, L. A., & Essigmann, J. M. (1993) *Biochemistry 32*, 12793−12801.

Brynes, J. J., & Downey, K. M. (1976) *Biochemistry 15*, 2817−2823.

Chaudhary, A. K., Nokubo, M., Reddy, G. R., Yeola, S. N., Morrow, J. D., Blair, I. A., & Marnett, L. J. (1994) *Science 265*, 1580−1582.

Clark, J. M., Joyce, C. M., & Beardsley, G. P. (1987) *J. Mol. Biol. 198*, 123−127.

Cullinan, D., Korobka, A., Grollman, A. P., Patel, D. J., Eisenberg, M., & de los Santos, C. (1996) *Biochemistry 35*, 13310−13318.

Dosanji, M. K., Chenna, A., Kim, E., Fraenkel-Conrat, H., Simson, L., & Singer, B. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1024−1028.

IARC Monograph Series (1979) in *Evaluation of the Carcinogenic Risk of Chemicals to Humans*, Vol. 19, pp 348−377, IARC, Lyon, France.

Korobka, A., Cullinan, D., Cosman, M., Grollman, A. P., Patel, D. J., Eisenberg, M., & de los Santos, C. (1996) *Biochemistry 35*, 13319−13327.

Kunkel, T. A. (1990) *Biochemistry 29*, 8003−8011.

Malaveille, C., Bartsch, H., Montesano, R., Barbin, A., & Camus, A. M. (1975) *Biochem. Biophys. Res. Commun. 63*, 363−370.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Matijasevic, Z., Sekiguchi, M., & Ludlum, D. B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9331−9334.

Matsumoto, Y., Kim, K., & Bogenhagen, D. F. (1994) *Mol. Cell. Biol. 14*, 6187−6197.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499−560.

Mendelman, L. V., Boosalis, M. S., Petruska, J., & Goodman, M. F. (1989) *J. Biol. Chem. 264*, 14415−14423.

Mendelman, L. V., Petruska, J., & Goodman, M. F. (1990) *J. Biol. Chem. 265*, 2338−2346.

Merletti, F., Heseltine, E., Saracci, R., Simonato, L., & Wilbourn, J. (1984) *Cancer Res. 44*, 2244−2250.

Miller, J. M. (1991) *Jpn. J. Cancer Res. 82*, 1323−1324.

Mirvish, S. S. (1968) *Adv. Cancer Res. 11*, 1−42.

Moriya, M., Zhang, W., Johnson, F., & Grollman, A. P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11899−11903.

Nath, R. G., & Chung, F.-L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7491−7495.

Nettleship, A., Henshaw, P., & Meyer, H. (1943) *J. Natl. Cancer Inst. 4*, 309−319.

Ough, C. S. (1976) *Agric. Food Chem. 24*, 323−328.

Palejwala, V. A., Simha, D., & Humayun, M. Z. (1991) *Biochemistry 30*, 8736−8743.

Scheller, N., Sangaiah, R., Ranasinghe, A., Amarnath, V., Gold, A., & Swenberg, J. A. (1995) *Chem. Res. Toxicol. 8*, 333−337.

Scherer, E., Van Der Laker, C. J., Gwinner, L. M., & Laib, R. J. (1981) *Carcinogenesis 2*, 671−677.

Scherer, E., Winterwerp, H., & Emmelot, P. (1986) *The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis*, IARC 70, pp 109−125, IARC, Lyon, France.

Shibutani, S. (1993) *Chem. Res. Toxicol. 6*, 625−629.

Shibutani, S., & Grollman, A. P. (1993) *J. Biol. Chem. 268*, 11703−11710.

Shibutani, S., Gentles, R., Johnson, F., & Grollman A. P. (1991a) *Carcinogenesis 12*, 813−818.

Shibutani, S., Takeshita, M., & Grollman, A. P. (1991b) *Nature 349*, 431−434.

Shibutani, S., Bodepudi, V., Johnson, F., & Grollman, A. P. (1993) *Biochemistry 32*, 4615−4621.

Shibutani, S., Takeshita, M., & Grollman, A. P. *J. Biol. Chem.* (in press).

Simha, D., Palejwala, V. A., & Humayun, M. Z. (1991) *Biochemistry 30*, 8727−8735.

Takeshita, M., Chang, C.-N., Johnson, F., Will, S., & Grollman, A. P. (1987) *J. Biol. Chem. 262*, 10171−10179.

Tchou, J., & Grollman, A. P. (1993) *Mutat. Res. 299*, 277−287.

Wang, T. S.-F. (1991) *Annu. Rev. Biochem. 60*, 513−552.

Zhang, W., Johnson, F., Grollman, A. P., & Shibutani, S. (1995a) *Chem. Res. Toxicol. 8*, 157−163.

Zhang, W., Rieger, R., & Johnson, F. (1995b) *Chem. Res. Toxicol. 8*, 148−156.

BI961446O