In Vitro Bypass of Malondialdehyde−Deoxyguanosine Adducts: Differential Base Selection during Extension by the Klenow Fragment of DNA Polymerase I Is the Critical Determinant of Replication Outcome†

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ABSTRACT: The major malondialdehyde-derived adduct in DNA is 3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-pyrimido[1,2-α]purin-10(3*H*)-one (M_1dG). M_1dG undergoes hydrolytic ring opening in duplex DNA to 9-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-N^2-(3-oxo-1-propenyl)guanine (N^2OPdG). Template-primers were constructed containing M_1dG or N^2OPdG in a $(CpG)_4$ repeat sequence and replicated with the Klenow fragment of DNA polymerase I (Kf). Incorporation opposite the lesion and replication beyond the adduct sites by Kf was reduced compared to unadducted controls. The amount of bypass to full-length products was significantly greater with the acyclic adduct, N^2OPdG, than with the cyclic adduct, M_1dG. Sequence analysis indicated that the fully extended primers contained dC opposite both adducts when replication was conducted with Kf exo$^+$. In contrast, with Kf exo$^-$, primers extended past M_1dG contained T opposite the adduct, but primers extended past N^2OPdG contained dC opposite the adduct. Single nucleotide incorporation experiments indicated that Kf exo$^-$ incorporates all four nucleotides opposite M_1dG or N^2-OPdG. Kf exo$^+$ removed dA, dG, and T opposite M_1dG and N^2OPdG but was much less active when dC was opposite the adduct. NMR studies on duplex DNA indicated that N^2OPdG hydrogen bonds with dC in the complementary strand. The fact that base pairing can occur for the acyclic adduct may explain why N^2OPdG is less blocking than M_1dG. These results support in vivo findings that the ring-closed adduct, M_1dG, is more mutagenic than the ring-opened adduct, N^2OPdG. They also provide a detailed picture of in vitro replication in which the outcome is determined primarily by the selectivity of template-primer extension beyond rather than insertion opposite the adducts.

Considerable interest exists in the identification of endogenous factors that contribute to the development of genetic disease (*1*, *2*). 3-(2′-Deoxy-β-D-*erythro*-pentofuranosyl)-pyrimido[1,2-α]purin-10(3*H*)-one (M_1dG)[1] is an endogenously occurring DNA adduct that has been found in human liver, breast, pancreas, and white cells (*3*−*9*). It arises by reaction of deoxyguanosine residues with the lipid oxidation product, malondialdehyde (MDA), or with the DNA oxidation products, base propenal (Figure 1) (*10*−*13*). Site-specific mutagenesis experiments in which M_1dG is incorporated at defined positions in viral genomes or shuttle vectors and then replicated indicate that M_1dG in *Escherichia coli* and mammalian cells is mutagenic (*14*, *15*). Mao et al. reported that M_1dG undergoes a rapid and quantitative hydrolytic ring opening to 9-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-N^2-(3-oxo-1-propenyl)guanine (N^2OPdG) when it is present in duplex DNA opposite dC (*16*). Ring opening was shown to be reversible upon duplex denaturation. M_1dG placed opposite T residues remains ring-closed in the duplex as does M_1dG placed in a two-base loop in a $(CpG)_4$ repeat sequence. The conversion of M_1dG to N^2OPdG does not occur in single-stranded DNA at physiological pH (*17*, *18*).

These discoveries suggest that M_1dG exists in two different chemical forms in DNA, which are likely to exhibit different genotoxic and mutagenic properties. In fact, considering that MDA or base propenal will react preferentially with G·C base pairs in DNA, it is possible that N^2OPdG is the predominant adduct in genomic DNA. This is supported by the observation that MDA and the chemically related base propenals preferentially react with DNA via the minor groove (*13*). Therefore, it is important to define the biochemical and biological effects of N^2OPdG as well as M_1dG.

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[1] Abbreviations: M_1dG, 3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-pyrimido[1,2-α]purin-10(3*H*)-one; N^2OPdG, 9-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-N^2-(3-oxo-1-propenyl)guanine; Kf exo$^+$, Klenow fragment of DNA polymerase I; Kf exo$^-$, Klenow fragment of DNA polymerase I with the 3′−5′ exonuclease activity deleted; dsTXT:ACA, 5′-GGTXTCCG-3′ annealed to its complement where X is M_1dG; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy.

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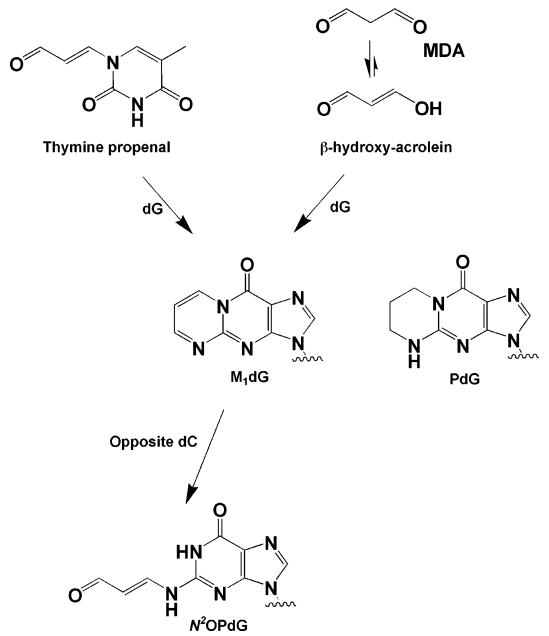

FIGURE 1: Structures of malondialdehyde, its tautomer β-hydroxy-acrolein, thymine propenal, N^2OPdG, M_1dG, and its structural analogue PdG.

We recently described a general method for the synthesis of oligonucleotides containing M_1dG that uses commercially available reagents (*19*). Using this method, we have constructed oligonucleotides containing M_1dG at defined positions (*19*). Model studies indicate that the conversion of M_1dG to N^2OPdG occurs rapidly at basic pH (>10.5) and that N^2OPdG is relatively stable at physiological pH (*17*, *18*). This observation provides an opportunity to directly compare the effects of M_1dG and N^2OPdG on DNA replication by studying the ability of appropriately modified template-primers to serve as substrates for purified DNA polymerases.

We describe here the results of a study performed with the Klenow fragment of DNA polymerase I with or without proofreading (Kf exo$^+$ or Kf exo$^-$). DNA polymerase I was chosen because it has been used for studies with a range of DNA adducts including propanodeoxyguanosine (PdG) (Figure 1), a close structural homologue of M_1dG (*20*−*23*). Template-primers containing M_1dG or N^2OPdG were incubated with polymerase, and the outcome of replication was evaluated by gel electrophoresis. The identities of the bases inserted opposite the adducts in full-length extension studies were determined either by Maxam−Gilbert sequencing or by long gel electrophoresis with comparison to authentic standards. NMR studies demonstrate that N^2OPdG in duplex oligonucleotides can base pair with dC in the complementary strand and may explain why only dC incorporation at the adduct site is observed in full-length products. The results of these experiments indicate that both adducts represent strong blocks to DNA replication. However, the cyclic adduct, M_1dG, is more blocking to polymerase bypass than the acyclic adduct, N^2OPdG. In the absence of proofreading, M_1dG is more mutagenic than N^2OPdG. The results of these in vitro experiments contrast sharply with similar experiments performed with PdG, which highlights the exquisite sensitivity of DNA polymerases toward subtle changes in substrate structure.

MATERIALS AND METHODS

Chemicals and Enzymes. Deoxyribonucleotides were obtained from Pharmacia Biotech Inc. (Piscataway, NJ). [γ-^{32}P]-ATP (6000 Ci/mmol) and Maxam−Gilbert sequencing reagents were from NEN Life Sciences Products (Boston, MA). Osmium tetroxide (4 wt % solution in water) was purchased from Aldrich Chemical Co. (Milwaukee, WI). MOPS was purchased from Sigma (St. Louis, MO). T_4 polynucleotide kinase and DNA polymerase I Klenow fragment (Kf exo$^+$, 9600 units/mg) from *E. coli* were purchased from Boehringer Mannheim Biochemica (Indianapolis, IN). Exonuclease-free DNA polymerase I Klenow fragment (Kf exo$^-$, 19608 units/mg) was from Amersham Life Sciences Inc. (Cleveland, OH). NMR experiments were performed on Bruker DRX 600 or DRX 800 spectrometers.

Oligonucleotides. Oligonucleotides (20-mers) bearing M_1-dG, 5′-TATCGCGCXCGGCATGAGCT-3′ (X = M_1dG), were prepared and purified as described (*19*). Chemically distinct forms of the adduct on the oligonucleotide, M_1dG or N^2OPdG, were obtained by dissolving adducted oligonucleotides in 10 mM MOPS buffer containing 0.1 mM EDTA at pH 5.5 or 9.5, respectively, and allowed to stand at room temperature for at least 1 h. The unmodified 20-mer template, various primers, and the synthetic standards were obtained from the Molecular Physiology Core Laboratory of Vanderbilt University. The sequences of the standards were complementary to the full-length products containing different bases at the 12th position, across from the site of the adduct in the template. The unmodified template, primers, and synthetic standards were purified by polyacrylamide gel electrophoresis. The sequences of the primers were as follows: 5′-AGCTCATGCCG-3′ (11-mer) and 5′-AGCT-CATGCCGN (N = A, C, G, or T).

General Procedure for in Vitro Assays. 5′-End labeling was carried out in a phosphorylation buffer (100 μL) of 50 mM MOPS (pH 7.3), 10 mM $MgCl_2$, 100 μM EDTA, and 5 mM dithiothreitol containing 3 μM primer, 0.25 mM [γ-^{32}P]-ATP, and 10 units of T_4 polynucleotide kinase. Labeled primers were purified using Bio-Spin columns (Bio-Rad, Hercules, CA). Template-primer complexes were obtained by annealing adducted/unadducted template with appropriate 5′-end-labeled primer in a molar ratio of 5:1 (template: primer) at 90°C for 3 min and then slowly cooling to room temperature in an annealing buffer containing 50 mM MOPS (pH 6.0 to give M_1dG in the template or pH 10.0 to give N^2OPdG in the template), 50 μg/mL BSA, and 5 mM $MgCl_2$. Replication assays contained the template-primer complex (15 nM primer) and 1.5 nM Klenow fragment (exo$^+$)/ (exo$^-$) in 10 μL of polymerase reaction buffer containing 50 mM MOPS (pH 7.5), 8 mM $MgCl_2$, 4 mM dithiothreitol, and 2 μg/mL BSA. Reactions were initiated by the addition of all four dNTPs (100 μM each) or a single dNTP (100 μM) and were incubated at 30 °C for 15 min. Reactions were quenched by adding 10 μL of 10 mM EDTA in 90% formamide. The reaction products were analyzed on a 20% polyacrylamide gel using the ultrapure sequagel sequencing system (National Diagnostics, Atlanta, GA). The positions of the bands were established by autoradiography and PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). Detailed analysis of the primer extension products were carried out with products formed and purified from a 10−20-fold scale-up reaction.

The identities of the purified full-length products were established by Maxam−Gilbert chemical sequencing (*24*, *25*) and reanalysis of the purified products on a 88 cm polyacrylamide gel with comparison to synthetic standards.

NMR Spectroscopy. The sequence 5′-GGTXTCCG-3′, X = M_1dG, was synthesized and purified by HPLC as described above. The oligonucleotide was annealed to its complement with dC opposite the adduct site to a final concentration of 2.0×10^{-4} M in 0.01 M sodium phosphate buffer (pH 6.5), 0.05 M NaCl, and 2.0×10^{-5} M EDTA. NMR experiments were performed at either 600.13 or 800.13 MHz. Experiments were performed at 12 °C in a 9:1 mix of H_2O and D_2O to observe exchangeable imino protons. NMR measurements were made using a watergate pulse sequence for solvent suppression (*26*). Phase-sensitive NOESY experiments were performed with a 250 ms mixing time. TOCSY experiments were recorded with either 42 or 120 ms mixing times.

RESULTS

DNA Synthesis on M_1dG- or N^2OPdG-Adducted Templates. The effect of M_1dG and N^2OPdG on DNA synthesis was investigated using 3′−5′ exonuclease-proficient (Kf exo$^+$) or deficient (Kf exo$^-$) forms of the Klenow fragment of DNA polymerase I from *E. coli*. M_1dG or N^2OPdG was site-specifically incorporated at the 12th position from the 3′-end of the 20-mer template containing a $(CpG)_4$ repeat. Control experiments indicated that N^2OPdG did not cyclize to M_1dG during the time course of the experiments (*27*). The adducted templates were annealed to ^{32}P-labeled 11-mer primers and used in primer extension assays. The extension reactions were carried out in the presence of all four dNTPs. Analysis of the extended products employed a 20% denaturing polyacrylamide gel with phosphorimaging. Bypass product identification was accomplished by comigration of standard markers and chemical sequencing of the extended products.

Figure 2 illustrates the extent of DNA synthesis from the adducted template-primers in the presence of all four dNTPs under enzyme-limiting conditions. Table 1 lists the quantitation of the extension products. DNA synthesis was blocked primarily opposite and at sites 5′ to the adducts to give intermediate extension products. Some bypass synthesis to fully extended products was evident from the slow-moving bands that comigrated with the full-length standard marker (20-mer). A maximum of 2% full-length extension was observed with N^2OPdG and 0.3% with M_1dG, indicating that both lesions are strong blocks to replication by the Klenow fragment (exo$^+$). Bypass synthesis did not increase significantly in the absence of the 3′→5′ exonuclease activity of the Klenow fragment (see Figure 2 and Table 1).

Analysis of the Bypass Products. Bypass synthesis products that comigrated with full-length 20-mer standards were eluted from the polyacrylamide gel. One aliquot was subjected to Maxam−Gilbert chemical sequencing and another reanalyzed on a long sequencing gel with comparison to authentic standards of 20-mers differing only in the identity of the base at the 12th position. Maxam−Gilbert chemical sequencing with a T-specific reaction was used to analyze the composition of the bypass synthesis products. Figures 3 and 4 illustrate the sequence analysis of the full-length products synthesized on M_1dG- and N^2OPdG-adducted templates by

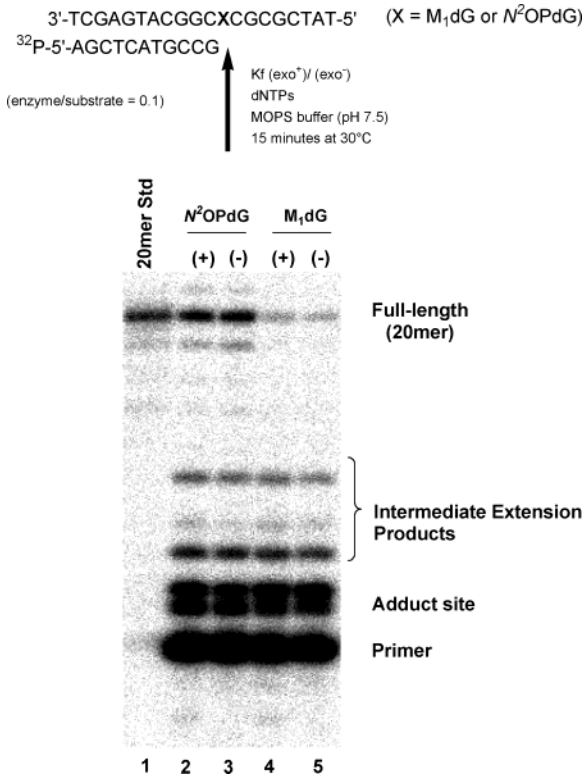

FIGURE 2: DNA synthesis on M_1dG- and N^2OPdG-adducted templates by Kf exo$^+$ and Kf exo$^-$. ^{32}P-Labeled 11-mer primers were annealed to templates containing M_1dG or N^2OPdG at position 12 from the 3′-end (15 nM) and were allowed to replicate in the presence of all four dNTPs (100 μM). Polymerization was carried out with each enzyme (1.5 nM) at 30 °C for 15 min. Lanes: 1, standard molecular size marker; 2, primer extension products synthesized by Kf exo$^+$ on the N^2OPdG-adducted 20-mer template; 3, primer extension products synthesized by Kf exo$^-$ on the N^2-OPdG-adducted 20-mer template; 4, primer extension products synthesized by Kf exo$^+$ on the M_1dG-adducted 20-mer template; 5, primer extension products synthesized by Kf exo$^-$ on the M_1-dG-adducted 20-mer template. Product length and block sites are indicated on the right side of the gel.

Table 1: Percent Composition of Extension Products Synthesized on M_1dG- and N^2OPdG-Adducted Templates

		premature termination		full extension	
	unextended primer (%)	adduct site (%)	5′ block sites (%)	−1 (19-mer) (%)	full length (20-mer) (%)
Kf (exo$^+$)					
M_1dG	80	15	3	0	0.4
N^2OPdG	81	14	3	0.2	2
Kf (exo$^-$)					
M_1dG	82	15	3	0	0.3
N^2OPdG	84	11	3	0.3	2

Kf exo$^+$ and Kf exo$^-$, respectively. Sequence alignment of the products with the templates verified that the products comigrating with full-length standard arose by complete extension from each template-primer. Full-length product sequence analysis revealed the presence of dC and T residues opposite the adduct. However, the proportion of dC and T incorporated depended on the identity of the adduct (M_1dG/N^2OPdG) and the exonuclease status of the Klenow fragment. Bands in the C lanes opposite both M_1dG and N^2OPdG were most prominent in the products synthesized by Kf exo$^+$, indicating that the major full-length product arose by the

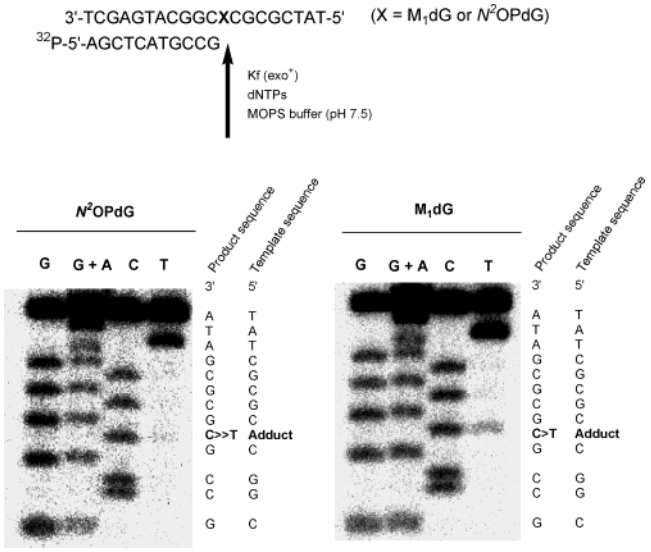

FIGURE 3: Sequence analysis of full-length (20-mer) bypass products synthesized on M_1dG- and N^2OPdG-adducted templates by Kf exo$^+$. Reaction products running parallel to 20-mers synthesized on the indicated templates were purified as described under Materials and Methods. The sequences were determined by the Maxam−Gilbert method utilizing a T-specific reaction. The product sequences and their complementary template sequences are aligned on the right side of each gel to illustrate base incorporation at the site of M_1dG or N^2OPdG.

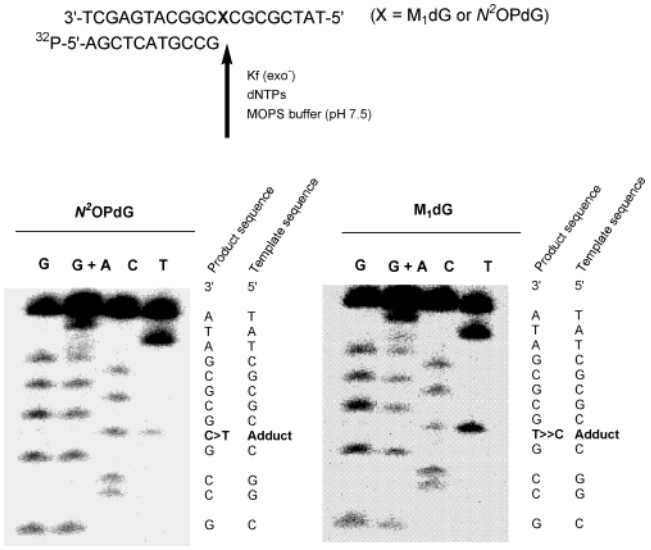

FIGURE 4: Sequence analysis of full-length (20-mer) bypass products synthesized on M_1dG- and N^2OPdG-adducted templates by Kf exo$^-$. Reaction products running parallel to 20-mers synthesized on the indicated templates were purified as described under Materials and Methods. The sequences were determined by the Maxam−Gilbert method and by a T-specific reaction. The product sequences and their complementary template sequences are aligned on the right side of each gel to illustrate base incorporation at the site of M_1dG or N^2OPdG.

incorporation of dC opposite M_1dG and N^2OPdG. In contrast, bands in the T lane opposite M_1dG and the C lane opposite N^2OPdG were the most intense in the full-length products synthesized by Kf exo$^-$.

The appearance of multiple bands and the differences in the band intensities at the site of the adduct in the Maxam−Gilbert chemical sequencing gel prompted us to determine the quantitative composition of the full-length products. The eluted full-length products were completely resolved on a long (88 cm) 20% denaturing polyacrylamide gel. The identities of the full-length products were established by comparison to 20-mer synthetic standards complementary to the template sequence and containing A, C, G, or T across from the adduct site. Figure 5 and Table 2 show the relative band intensities of the full-length products. Approximately 90% of full-length products synthesized by Kf exo$^+$ contained C across from M_1dG and N^2OPdG. However, a striking difference was observed in the specificity of base fixation opposite the two adducts when Kf exo$^-$ was used. Eighty percent of the full-length products synthesized on an M_1dG-adducted template contained T across from the adduct. In contrast, C was present in 75% of the full-length products inserted opposite N^2OPdG synthesized by Kf exo$^-$.

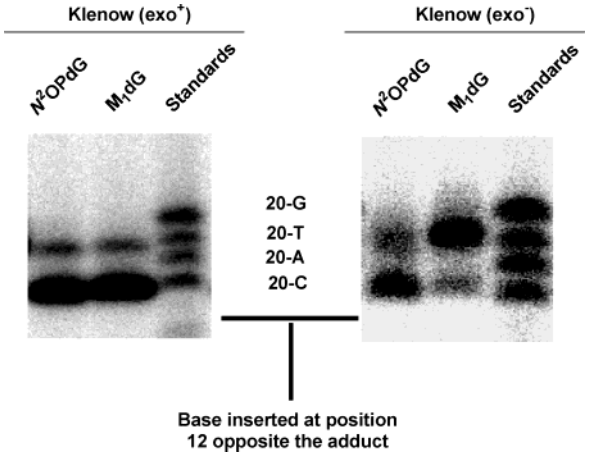

FIGURE 5: Separation of full-length (20-mer) products. Reaction products running parallel to 20-mers were purified as described under Materials and Methods. The purified products were resolved on a long (88 cm) 20% polyacrylamide gel. The identities of the full-length products were established by comigration of 20-mer synthetic standards complementary to the template sequence that contained dA, dC, dG, or T at position 12 from the 5′-end.

Table 2: Identity of Full-Length Products Synthesized on M_1dG- and N^2OPdG-Adducted Templates

	base incorporation across from adduct			
	A (%)	C (%)	G (%)	T (%)
Kf (exo$^+$)				
M_1dG		88		12
N^2OPdG		89		11
Kf (exo$^-$)				
M_1dG		18		82
N^2OPdG		75		25

Nucleotide Incorporation opposite M_1dG or N^2OPdG. To determine if the identities of the bases opposite the adduct in the extended primers reflected the specificities of nucleotide incorporation, we conducted single nucleotide incorporation experiments with the adducted template-primers. As shown in Figure 6, Kf exo$^-$ incorporated all four nucleotides opposite both M_1dG and N^2OPdG (dC > dG, dA > T). In contrast, Kf exo$^+$ produced a consistent pattern of preferential incorporation of C opposite the lesions. These observations indicate that the identities of the bases opposite the adducts in the primers extended to full length were not determined solely by the nucleotide incorporation step. They also indicate that the 3′−5′ exonuclease activity of Kf was less active when C and, to a lesser extent, G were present at the primer terminus opposite M_1dG and N^2OPdG. To test this hypoth-

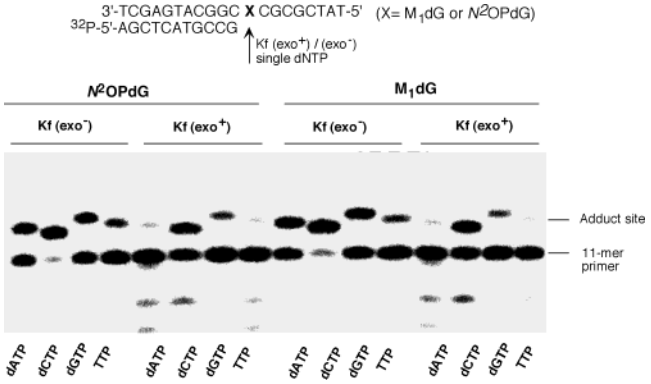

FIGURE 6: Nucleotide incorporation opposite M_1dG and N^2OPdG by Kf exo$^+$ and Kf exo$^-$. ^{32}P-Labeled 11-mer primers were extended on M_1dG- or N^2OPdG-adducted 20-mer templates (10 nM) in the presence of a single dNTP (100 μM). Reactions were catalyzed by exo$^+$/exo$^-$ Klenow fragment (1 nM) at 30 °C for 15 min.

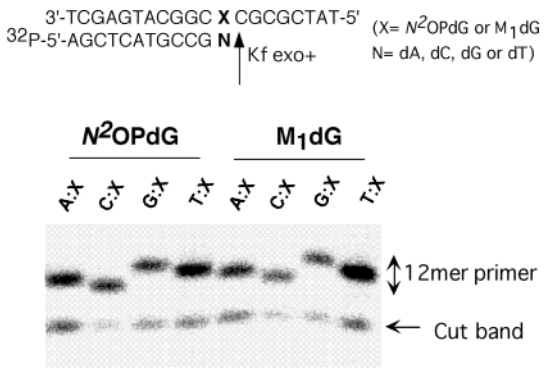

FIGURE 7: Exonucleolytic cleavage of the base opposite M_1dG and N^2OPdG by Kf exo$^+$. ^{32}P-Labeled 12-mer primers having dA, dC, dG, or T at the 3′-terminus were annealed to M_1dG- or N^2OPdG-modified 20-mer templates (10 nM). Exonucleolytic cleavage was carried out by Kf exo$^+$ (1 nM) by incubating at 30 °C for 15 min.

esis, we incubated the adducted template-primer (20-mer/12-mer) in the presence of Kf exo$^+$ at 30 °C for 15 min. The 12-mer primer had either dA, dC, dG, or T at the 3′-terminus positioned opposite M_1dG and N^2OPdG. The specificity of the removal of the base across from M_1dG and N^2OPdG is shown in Figure 7. As can be seen, dC positioned opposite M_1dG and N^2OPdG is cleaved to a lesser extent by the exonucleolytic activity of the Kf than the other primer termini. This observation may partially explain the exclusive incorporation of dC into the full-length products synthesized by exonuclease-proficient Kf.

Specificity of Extension from the Base opposite M_1dG or N^2OPdG. The previous experiments suggest that the specificity of extension is an important determinant of the bases present opposite M_1dG or N^2OPdG in the full-length products. To test this, separate template-primers were constructed containing each of the four bases at the primer terminus opposite M_1dG or N^2OPdG in the template strand. These template-primers were then incubated with Kf exo$^-$ in the presence of all four dNTPs. Gel electrophoretic analysis of the extension products is shown in Figure 8. Only template-primers containing pyrimidines opposite the adduct were extended to full length. Primers containing dC or T opposite N^2OPdG were extended to full length whereas the primer containing T opposite M_1dG was the primary substrate for full-length extension.

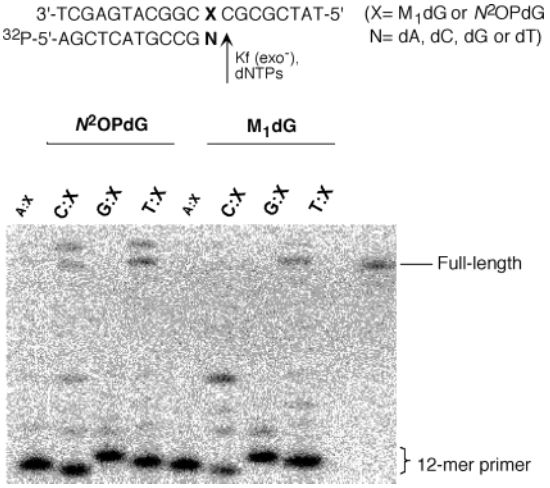

FIGURE 8: Dependence of extension on the identity of the base opposite M_1dG and N^2OPdG by Kf exo$^-$. ^{32}P-Labeled 12-mer primers having dA,dC, dG, or T at the 3′-terminus were extended on M_1dG- or N^2OPdG-modified 20-mer templates (10 nM). Polymerization was carried out by Kf exo$^-$ (1 nM) in the presence of all four dNTPs (100 μM). The reaction was conducted at 30 °C for 15 min.

NMR Spectroscopy. Mao et al. (*16*) reported that, in the presence of dC opposite M_1dG, quantitative ring opening to N^2OPdG occurred. This was reversible upon duplex melting. Subsequently, Mao et al. (*28*) reported more detailed structural information for the N^2OPdG adduct when placed opposite dC in a reiterated CpG sequence. The latter work revealed that the oxopropenyl group of N^2OPdG projects into the minor groove of the duplex. The rearrangement of M_1-dG to N^2OPdG when placed opposite dC in duplex DNA and the resulting orientation of N^2OPdG in the minor groove were anticipated to allow Watson−Crick hydrogen-bonding interactions to be maintained between N^2OPdG and dC. One of the spectroscopic "signatures" of the N^2OPdG adduct was a single exchangeable resonance at approximately 11.1 ppm in the ^{1}H NMR spectrum. This resonance was assigned as the N1 imino proton resonance of M_1dG. The anticipated chemical shift for the N1 imino proton resonance of dG when hydrogen bonded to dC is in the 12−13 ppm region of the ^{1}H spectrum; thus, it was concluded that Watson−Crick hydrogen bonding between N^2OPdG and dC was weak or absent (*16*, *28*). This conclusion was reexamined in the present work.

The oligonucleotide (GGTXTCCG) containing a single M_1dG adduct was synthesized and annealed with its complement. The resulting duplex was maintained in phosphate-buffered solution (pH 6.5) at temperatures below 15 °C to ensure that it was annealed completely. As anticipated, formation of N^2OPdG was observed in duplex DNA, with the characteristic exchangeable resonance at approximately 11.1 ppm in the ^{1}H NMR spectrum. A series of two-dimensional ^{1}H NMR experiments established that the previous assignment of this resonance in the (GGTXTCCG) duplex (*16*) as the N1 imino proton was incorrect and inferred that its assignment in the spectrum of the reiterated CpG sequence (*28*) was probably also incorrect. The critical experiment is summarized in a ^{1}H-TOCSY spectrum (Figure 9) that identifies the 11.1 ppm resonance as arising from the N^2OPdG N2 amino proton. The 11.1 ppm resonance shows the expected coupling to the H6 and H7 resonances

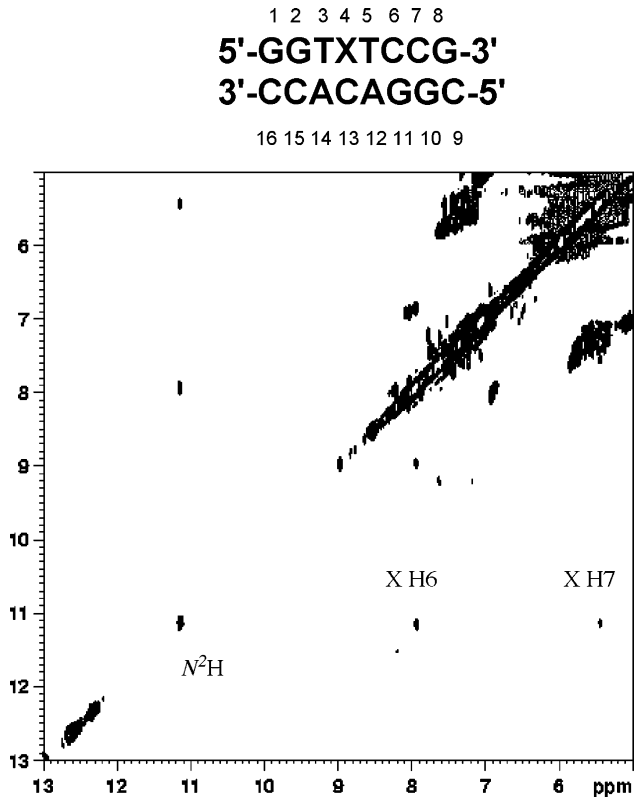

FIGURE 9: 2D-TOCSY spectrum of M_1dG opposite dC in duplex DNA. A sample of the double-stranded TXT:ACA 8-mer was prepared in H_2O at a final concentration of 2×10^{-4} M in 0.05 M sodium phosphate, pH 6.5, 0.1 M NaCl, and 2×10^{-5} M EDTA. The sample was cooled to 10 °C in an 800 MHz field NMR instrument. The sample was referenced to the chemical shift of water (4.7), and total correlation spectroscopy experiments were performed at a mixing time of 42 ms (Figure 9) or 120 ms (data not shown).

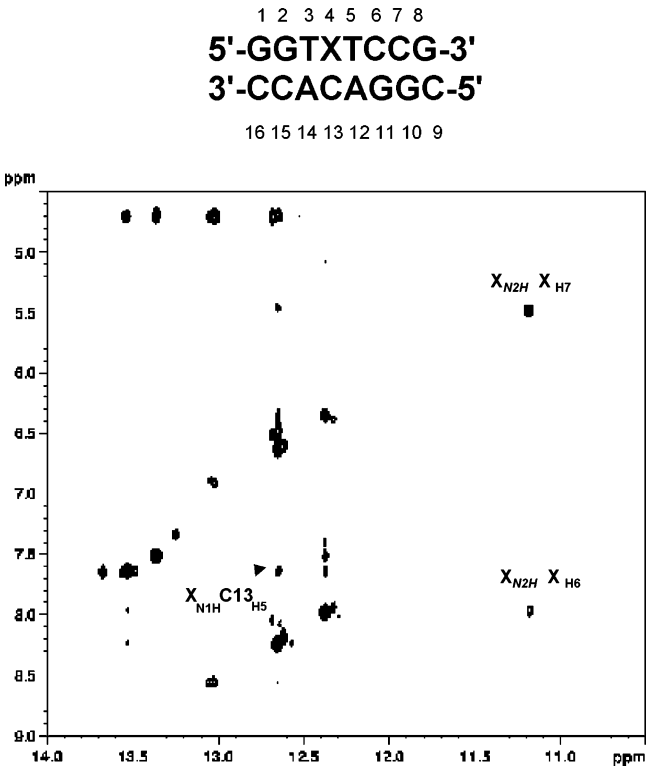

FIGURE 10: 2D-NOESY spectrum of M_1dG opposite dC in duplex DNA. A sample of the double-stranded TXT:ACA 8-mer (see Materials and Methods) was prepared in H_2O at a final concentration of 2×10^{-4} M in 0.05 M sodium phosphate, pH 6.5, 0.1 M NaCl, and 2×10^{-5} M EDTA. The sample was cooled to 10 °C in a 600 MHz NMR instrument equipped with a cryoprobe. The sample was referenced to the chemical shift of water (4.7), and nuclear Overhauser effect (NOE) experiments were performed at a mixing time of 250 ms.

of the N^2OPdG propenyl moiety in the ^{1}H TOCSY experiment, which would not be observed for the N^2OPdG N1 imino proton. Moreover, NOE data obtained at low temperature revealed that the 11.1 ppm resonance exhibited an NOE to a proton at 12.6 ppm in the ^{1}H spectrum that was assigned to the N1 imino proton of N^2OPdG (Figure 10). The chemical shift of the N^2OPdG N1 imino proton at 12.6 ppm is consistent with participation of the imino proton in a hydrogen bond with the imine nitrogen of dC in the complementary strand. This corrected assignment establishes unequivocally that N^2OPdG participates in Watson−Crick hydrogen bonding with dC in the complementary strand.

DISCUSSION

The ability of DNA adducts to exist in different forms during replication can be a function of several chemical factors arising from both DNA and DNA−protein interactions at the active site of the polymerase. Physical factors leading to different placement of an adduct in DNA can lead to varying mutagenic results. For example, eight stable conformations of the benzo[*a*]pyrene-7,8-dihydrodiol epoxide−dG adduct have been hypothesized to be the cause of its ability to induce different kinds of mutations (G → T, G → A, etc.) during DNA replication (*29*). Alternatively, chemical factors can cause different forms and effects of DNA adducts. Experimental evidence indicates that certain exocyclic DNA adducts exist in multiple forms in DNA (*16*, *28*, *30*). The possibility that lesions exist in multiple forms in DNA poses a challenge to understanding the molecular mechanisms by which they induce mutations. The results from the present study are the first to clearly demonstrate that M_1dG and N^2OPdG, the interchangeable forms of the major MDA−dG adduct in DNA, are significantly different both in their ability to block DNA replication and in their mutagenic potential.

M_1dG and N^2OPdG are strong blocks to in vitro replication by the Klenow fragment of DNA polymerase I; the exocyclic adduct, M_1dG, is approximately 6−7-fold more potent as a block than N^2OPdG. Blockage is observed opposite the adduct site in the template strand and one to three nucleotides beyond the position of the adduct (Figure 2). Interestingly, the intensities of the bands corresponding to premature termination of primer extension appear to be comparable in reactions with template-primers containing M_1dG and N^2-OPdG. These blockage sites may arise from either incomplete extension by the polymerase or slippage by the polymerase to yield shortened products. The major differential in blockage between the two adducts appears in the intensity of the full-length product band. Sequence analysis of the fully extended template-primers indicates that the products differ depending on the presence of 3′−5′ exonuclease activity in the DNA polymerase. Template-primers extended by Kf exo$^+$ contain primarily dC opposite either M_1dG or N^2OPdG, whereas template-primers extended by Kf exo$^-$ contain T opposite M_1dG or dC > T opposite N^2OPdG.

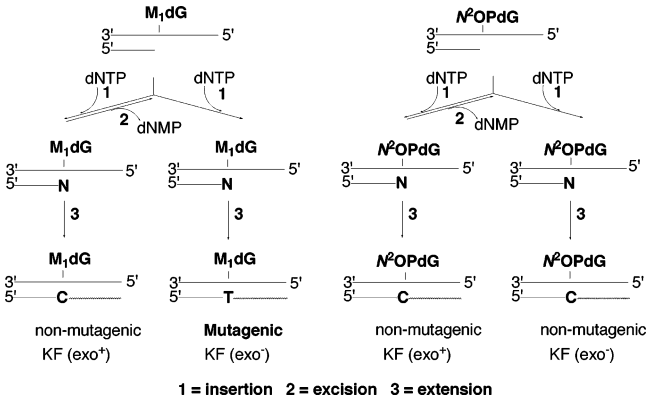

FIGURE 11: Model summarizing full-length extension of template-primers containing M_1dG or N^2OPdG.

Further evidence for the differential exonuclease activity on the removal of bases incorporated opposite M_1dG and N^2OPdG is apparent from the results of single nucleotide insertion experiments (Figure 6). Kf exo$^-$ inserts all four nucleotides opposite M_1dG or N^2OPdG with relative efficiencies of dC > dA ~ dG > T. Thus, both adducts appear ambiguous in their coding properties. In contrast, single nucleotide incorporation experiments performed with Kf exo$^+$ reveal that dC is preferentially incorporated followed by dG. Very little incorporation of dA or T is observed with either adduct. This differential between enzymes appears to be due to the preferential removal of dA and T and, to a certain extent, dG by the exonuclease activity of Kf.

Although dA and dG are efficiently incorporated opposite M_1dG or N^2OPdG by Kf exo$^-$, template-primers containing these residues opposite the adducts are not efficiently extended. By contrast, template-primers containing dC or T opposite N^2OPdG are extended to intermediate-length and full-length products. Template-primers containing T opposite M_1dG or dC or T opposite N^2OPdG are extended to full length. Thus, the identity of the products generated during in vitro replication of template-primers containing M_1dG or N^2OPdG is determined primarily by the relative efficiency of extension beyond the lesion rather than the relative efficiency of insertion opposite the lesion. This is similar to the replication of template-primers containing the model compound PdG except that purines are preferentially inserted opposite PdG but only template-primers containing dA opposite PdG are extended to full length (*23*, *31*).

The sequence of events that lead to full-length extension of template-primers containing M_1dG or N^2OPdG is summarized in Figure 11. With both polymerases (exo$^+$ or exo$^-$), all four nucleotides are incorporated opposite either adduct. The exo$^+$ enzyme removes dA and T and extends the template-primer containing dC. This gives rise to fully extended products containing primarily dC opposite either M_1dG or N^2OPdG; some full-length products with T opposite the adduct are observed with M_1dG. Although Kf exo$^-$ is incapable of removing any of the nucleotides inserted opposite M_1dG or N^2OPdG, it only extends template-primers containing pyrimidines to full length. Template-primers containing T opposite M_1dG are preferentially extended to full length whereas template-primers containing dC opposite N^2OPdG are preferentially extended to full length. As mentioned above, the observation that the specificity of extension rather than the specificity of incorporation determines the outcome of full-length extension was previously noted with the structurally related adduct, PdG. However, both the specificity of incorporation and the specificity of extension differed with PdG, indicating that significant differences in replication can be observed with structurally related adducts.

There are also sequence differences observed between PdG and M_1dG. Template-primers containing PdG and a dC residue 5′ to the adduct in the template strand are not extended to full length. Instead, one base pair deletions are observed in the final products that occur due to slippage of the 5′-dC residue. The base 5′ to the lesion site in the template strand forms a base pair with the dG that was originally inserted opposite PdG by the polymerase. In contrast to the results with PdG, template-primers containing either M_1dG or N^2OPdG and a 5′-dC residue in the template strand are extended to full length. Although products arising by one-base deletion are detected by gel electrophoresis, they are present in very low yield, which precluded efforts to identify their composition by sequence analysis.

This differential in "in vitro mutagenicity" corresponds to the differential observed in in vivo experiments (*14*). Site-specifically engineered vectors containing M_1dG opposite T were five times more mutagenic than vectors containing M_1dG opposite dC. M_1dG remains ring closed opposite T whereas it ring opens to N^2OPdG opposite dC. Since N^2-OPdG is capable of hydrogen bonding to an inserted dC residue, extension beyond the lesion should be error-free. This hypothesis assumes that the interconversion of M_1dG and N^2OPdG does not occur or is slow in the active site of a DNA polymerase. Recent measurements of the rates of ring opening and ring closing suggest that this is the case (*27*). The rate of ring closing of N^2OPdG in a single-stranded oligonucleotide is comparable to the rate measured in the mononucleoside. It is conceivable that these rates are altered significantly in the active site of a DNA polymerase. However, the differential outcome of replication of template-primers containing M_1dG or N^2OPdG (Tables 1 and 2) suggests that this is not the case for the Kf.

Although the relative mutagenicities of M_1dG and N^2OPdG correlate reasonably well between in vitro and in vivo experiments, the actual mutations induced do not. M_1dG induces mainly transversions to T and transitions to dA in either *E. coli* or monkey kidney cells but only transitions to dA in the present in vitro experiments (Figures 3 and 4). This discrepancy is not surprising because the replication machinery and the process involved in the bypass of M_1dG in vivo are more complex than simple bypass by the repair polymerase, Kf. In vivo mutagenesis by M_1dG in *E. coli* requires prior induction of the SOS system and is abolished in a umuC$^-$ background even following SOS induction. UmuC has been demonstrated to be the catalytic subunit that constitutes DNA polymerase V when in a complex with umuD′ (*32*). These in vivo results suggest the likely involvement of pol V in the mutagenic bypass of M_1dG and N^2OPdG in *E. coli*. The identity of the DNA polymerase responsible for M_1dG bypass in monkey kidney cells has not been established. It will be interesting to perform in vitro bypass experiments with the mammalian DNA polymerases that play a role in lesion bypass.

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