Effects of a Guanine-derived Formamidopyrimidine Lesion on DNA Replication

TRANSLESION DNA SYNTHESIS, NUCLEOTIDE INSERTION, AND EXTENSION KINETICS*

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2,6-Diamino-4-hydroxy-5-formamidopyrimidine derived from guanine (FapyG) is a major DNA lesion formed by reactive oxygen species. In this study, a defined oligonucleotide template containing a 5-N-methylated analog of FapyG (mFapyG) was prepared, and its effect on DNA replication was quantitatively assessed in vitro. The results were further compared with those obtained for 7,8-dihydro-8-oxoguanine and an apurinic/apyrimidinic site embedded in the same sequence context. mFapyG constituted a fairly strong but not absolute block to DNA synthesis catalyzed by Escherichia coli DNA polymerase I Klenow fragment with and without an associated 3′-5′ exonuclease activity, thereby permitting translesion synthesis with a limited efficiency. The efficiency of translesion synthesis was G > 7,8-dihydro-8-oxoguanine > mFapyG > apurinic/apyrimidinic site. Analysis of the nucleotide insertion ($f_{ins} = V_{max}/K_m$ for insertion) and extension ($f_{ext} = V_{max}/K_n$ for extension) efficiencies for mFapyG revealed that the extension step constituted a major kinetic barrier to DNA synthesis. When mFapyG was bypassed, dCMP, a cognate nucleotide, was preferentially inserted opposite the lesion (dCMP (relative $f_{ins} = 1$) > dTMP (2.4 × 10^{-4}) > dAMP (8.1 × 10^{-5}) > dGMP (4.5 × 10^{-5})), and the primer terminus containing a mFapyG:C pair was most efficiently extended (mFapyG:C (relative $f_{ext} = 1$) > mFapyG:T (4.6 × 10^{-4}) > mFapyG:A and mFapyG:G (extension not observed)). Thus, mFapyG is a potentially lethal but not premutagenic lesion.

Reactive oxygen species formed by aerobic metabolism, phagocytic blood cells upon inflammation, ionizing radiation, and photosensitized reactions generate structurally diverse oxidative damage to DNA that stores vital genetic information of cells (1–3). Base lesions thus formed are generally restored by the base excision repair pathway involving multiple enzymes such as N-glycosylase/AP1 lyase, AP endonuclease, DNA polymerase I Klenow fragment with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: AP, apurinic/apyrimidinic; 8-oxoG, 7,8-dihydro-8-oxoguanine; FapyG, 2,6-diamino-4-hydroxy-5-formamidopyrimidine derived from guanine; mFapyG, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine derived from guanine; FapyA, 4,6-diamino-5-formamidopyrimidine derived from adenine; DMS, dimethyl sulfate; 7,8-dihydro-8-oxoguanine; FapyG, 2,6-diamino-4-hydroxy-5-formamidopyrimidine derived from guanine; mFapyG, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine derived from guanine; FapyA, 4,6-diamino-5-formamidopyrimidine derived from adenine; DMS, dimethyl sulfate; 7-MeG, 7-methylguanine; pol I Kf, E. coli DNA polymerase I lymerase, and DNA ligase (4, 5). However, if left unrepaired, they result in mutations and/or cell death. It has also been implied that oxidative DNA damage is involved in carcinogenesis and various degenerative diseases (6, 7).

7,8-Dihydro-8-oxoguanine (8-oxoG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine derived from guanine (FapyG) have been identified as major products in the reaction of DNA with reactive oxygen species (Fig. 1A) (1, 2). When formed in DNA, 8-oxoG in a template directs incorporation of non-cognate dAMP as well as cognate dCMP during translesion synthesis by DNA polymerases, thereby inducing GC-to-AT transversions (8, 9). Similarly, when formed in a cellular nucleotide pool, a 2′-deoxyribonucleotide form of 8-oxoG can be incorporated opposite adenine as well as cytosine in a DNA template, inducing AT-to-CG transversions (10–12). Mispair formation between 8-oxoG and adenine leading to mutation involves an unusual syn-conformer of 8-oxoG that can form two hydrogen bonds between O-6 and N-7–H in 8-oxoG and N-6–H and N-1 in adenine without introducing significant distortions in DNA (13, 14). Accordingly, the molecular basis of the mechanism of 8-oxoG-induced mutagenesis has been well established.

The amount of FapyG in DNA exposed to oxidizing agents is comparable to that of 8-oxoG (15–17). Despite this fact, the genotoxic effect of FapyG lesions has been less clarified than that of 8-oxoG. The effects of FapyG on DNA synthesis have been previously assessed by in vitro DNA polymerase reactions and transfection studies using DNA containing a 5-N-methylated analog of FapyG (2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (mFapyG)) (Fig. 1B) (18–20). In these experiments, single-stranded M13mp18 DNA was methylated by dimethyl sulfate (DMS) and then treated with 0.2 M NaOH to rupture the imidazole ring of 7-methylguanine (7-MeG), thereby introducing mFapyG as a major lesion (59%) together with 1-methyladenine (21%), 3-methyladenine (6%), and others (14%) as minor lesions. When the DMS/NaOH-treated DNA template was replicated by DNA polymerase I Klenow fragment (pol I Kf) or T4 DNA polymerase in vitro, DNA synthesis stalled 1 base prior to putative mFapyG sites as well as other adenine and cytosine lesions. Consistent with this, the transfection efficiency of DMS/NaOH-treated M13mp18 DNA decreased significantly relative to that of DMS-treated DNA.

Interestingly, the most frequent mutation in progeny phage was observed at adenine sites (A-to-G transitions) rather than guanine sites (putative mFapyG sites). Aside from the contribution of coexisting base lesions, these results strongly suggest
that mFapyG is a potentially lethal lesion due to its capacity to block DNA synthesis, but not a premutagenic lesion, although the mechanism involved is not clear. Possible mechanisms may involve (i) preferential insertion of cognate dCMP opposite mFapyG during translesion synthesis; (ii) very inefficient extension of primer termini containing mFapyG, mFapyGG, and mFapyG:T pairs formed by insertion of non-cognate nucleotides; and (iii) complete arrest of DNA synthesis by mFapyG (no translesion synthesis).

Recently, we developed a novel method to introduce mFapyG into DNA as a unique lesion without using DMS treatment (21). In this method, 7-MeG, a precursor of mFapyG, is site-specifically incorporated into oligonucleotides by a DNA polymerase reaction using the 2'-deoxyribonucleoside triphosphate of 7-MeG as a substrate, and then 7-MeG is quantitatively converted to mFapyG by mild alkaline treatment at pH 11.4. The oligonucleotides containing site-specific mFapyG have been successfully used to quantitatively characterize the activities of Escherichia coli formamidopyrimidine glycosylase (Fpg) and its human functional homolog (hOgg1) (21). More recently, we also demonstrated that endonuclease (Endo) III and Endo VIII from E. coli and a mammalian Endo III homolog (NTH1), which have been thought to be pyrimidine-specific enzymes, recognize mFapyG in a paired base-dependent manner (22).

Availability of the defined DNA containing mFapyG prompted us to further investigate the mechanistic and kinetic aspects of the effect of mFapyG on DNA synthesis. We report here that mFapyG is a fairly strong but not absolute block to DNA synthesis catalyzed by pol I Kf and that dCMP is preferentially incorporated opposite mFapyG when bypassed. Moreover, the reaction parameters reveal that extension of a mFapyG:C primer terminus rather than preceding insertion of dCMP opposite mFapyG constitutes a dominant kinetic barrier to DNA synthesis. The kinetic data and the thermal stability of the duplexes containing mFapyG are further compared with those of 8-oxoG and an AP site embedded in the same site of the templates.

**EXPERIMENTAL PROCEDURES**

**Materials**—T4 polynucleotide kinase and 7-methyl-2'-dGTP were purchased from Toyobo and Sigma, respectively. [γ-32P]ATP (110 TBq/mmol) and ultrapure dNTPs were obtained from Amersham Pharmacia Biotech. Pol I Kf and pol I Kf deficient in 3'-5' exonuclease (pol I Kfexo-1) were obtained from New England Biolabs Inc.

**Oligonucleotides**—The oligonucleotides used in this study are listed in Table I. The templates containing G (34G), 8-oxoG (34OG), and a tetrahydrofuran-type AP site (34AP) at the same position were synthesized by the standard phosphoramidite method and purified by 16% denaturing PAGE. Other oligonucleotides (except 34FP) were also synthesized and purified in a similar manner. The template containing mFapyG (34FP) was prepared by alkaline hydrolysis of a 7-MeG residue that was site-specifically introduced by a DNA polymerase reaction (21). Briefly, the primer 11OLG was 5'-end-labeled with [γ-32P]ATP and T4 polynucleotide kinase, purified, and mixed with a 9-fold excess of 11OLGp bearing a nonradioactive 5'-phosphate group. 11OLGp was annealed to the template 44OLG (2-fold molar excess) in buffer A (10 mM Tris-HCl (pH 7.5) and 25 mM NaCl). The template-primer (44OLG-11OLG, 125 nM) was incubated with pol I Kf (25 units) in the presence of 200 μM 7-methyl-2'-dGTP and 20 μM each dATP, dCTP, and dTTP in buffer B (66 mM Tris-HCl (pH 7.5), 5 mM mercaptoethanol, 50 μg/ml bovine serum albumin, and 6.6 mM MgCl2; total of 200 μl) at 25 °C for 40 min. A single 7-MeG residue was incorporated immediately after the original primer terminus (i.e. opposite cytosine in the template) under these conditions. After incubation, the reaction mixture was dialyzed against alkaline buffer (10 mM sodium phosphate (pH 11.4) and 2 mM EDTA) at 25 °C for 20 h to rupture the imidazole ring, followed by 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA at 4 °C for 6 h (twice). The strand containing mFapyG (34FP) from the template (44OLG) by 16% denaturing PAGE, extracted from the gel, and purified on a Waters Sep-Pak cartridge (23). Our previous study has shown that oligonucleotides prepared in this manner contain mFapyG exclusively (96%) at the lesion site (21).

**Analysis of Translesion DNA Synthesis**—The reactions to assay translesion synthesis were performed under running-start conditions using templates (34FP, 34OG, 34AP, and 34G) and a 32P-5'-end-labeled primer (11PRM) whose terminus was three nucleotides shorter than the lesion site. The template and primer were annealed in buffer A by briefly heating at 70 °C and cooling to room temperature. The template-primer (15 nM) was incubated with pol I Kf or pol I Kfexo-1 (both at 0.1 unit) and 4 dNTPs (50 μM each) in buffer B (5 μl) at 25 °C for 0.5–60 min. The reaction was terminated by adding gel loading buffer (0.05% xylene cyanol, 0.05% bromphenol blue, 20 mM EDTA, and 98% formamide). Products were separated by 16% denaturing PAGE, and the radioactivity in the gel was analyzed on a Fuji BAS 2000 phosphorimager analyzer. Alternatively, the gel was autoradiographed at −80 °C.

**Analysis of Nucleotides Inserted Opposite Lesions**—The nucleotides inserted opposite the lesions were analyzed by two methods. In the first method, a template (34FP, 34OG, or 34G) was primed by 11PRM and replicated with pol I Kf for 60 min as described above. The products were subjected to single-strand conformation polymorphism (SSCP) analysis. The SSCP analysis was performed following the reported method (24) with slight modifications. The upper phase (5 cm) of a 20% polyacrylamide gel containing 8% urea, and the lower phase (35 cm) contained no urea (native gel). The sample was electrophoresed at 4 °C for 30 h. The applied voltage was 600 V for the initial 24 h and was then shifted to 1200 V for the remaining 6 h. 5'-End-labeled oligonucleotides (28COM-N, where N is A, G, C, or T) were electrophoresed side by side as standard markers. In the second method, a single nucleotide incorporation opposite the lesion was analyzed by a primer extension assay under standing-start conditions. A template (34FP, 34OG, 34AP, or 34G) was primed by 5'-end-labeled 14PRM (15 μM as template-primer) and incubated with pol I Kf or pol I Kfexo-1 (both at 0.1 unit) and a single dNTP (100 μM) in buffer B (5 μl) at 25 °C for 5 min. The reaction was terminated by adding gel loading buffer, and products were analyzed by 16% denaturing PAGE.

**Kinetic Parameters for Nucleotide Insertion and Extension**—The parameters for nucleotide insertion and extension were determined by the gel fidelity assay (25). For the insertion parameter, a primer (5'-end-
Effects of mFapyG on DNA Synthesis

### Table I

| Name          | Sequence          |
|---------------|-------------------|
| 11OLG         | 3'-TCATCACAAG     |
| 11OLGp        | 3'-TCATCACAAGp    |
| 44OLG         | 5'-AGGATGTTGGATGAGGTTGACAGTATGTTTCAGCTAGAGCA |
| 11PRM         | 5'-GGTATGGAGG     |
| 14PRM         | 5'-GGTATGGAGGTTGAG |
| 15PRM-A       | 5'-GGTATGGAGGTTGAG |
| 15PRM-G       | 5'-GGTATGGAGGTTGAG |
| 15PRM-C       | 5'-GGTATGGAGGTTGAG |
| 15PRM-T       | 5'-GGTATGGAGGTTGAG |
| 34G           | 3'-TCATACCACATCCCCACCTTCATCATCAAGAG |
| 34FP          | 3'-TCATACCACATCCCCACCTTCATCATCAAGAG |
| 34OG          | 3'-TCATACCACATCCCCACCTTCATCATCAAGAG |
| 34AP          | 3'-TCATACCACATCCCCACCTTCATCATCAAGAG |
| 34COM-A       | 5'-AGGATGTTGGATGAGGTTGAGAGTAGTTTC |
| 34COM-G       | 5'-AGGATGTTGGATGAGGTTGAGAGTAGTTTC |
| 34COM-C       | 5'-AGGATGTTGGATGAGGTTGAGAGTAGTTTC |
| 34COM-T       | 5'-AGGATGTTGGATGAGGTTGAGAGTAGTTTC |
| 26COM-A       | 5'-GGTATGGAGGTTGAGAGTAGTTTC |
| 26COM-G       | 5'-GGTATGGAGGTTGAGAGTAGTTTC |
| 26COM-C       | 5'-GGTATGGAGGTTGAGAGTAGTTTC |
| 28COM-T       | 5'-GGTATGGAGGTTGAGAGTAGTTTC |

* F, mFapyG; O, 8-oxoG; X, AP site (tetrahydrofuran). Underscoring shows the sequence differences in individual oligonucleotide groups.

The temperature axis using the Science Lab 99 Image Gauge Version 3.4 was extended by pol I Kf (0.01–1.5 units) in the presence of a single dNTP (0.01–500 μM) for 5–30 min as described above (see “Analysis of Nucleotides Inserted Opposite Lesions”). Based on the results of preliminary experiments, the amount of pol I Kf, the concentration range of dNTP, and the reaction time were appropriately adjusted so that the extent of primer elongation was <30% in the actual measurement of the initial velocity. For the parameter of primer extension past the lesion, a primer (5'-end-labeled 15PRM-N, where N is A, G, C, or T) annealed to a template (34FP, 34OG, 34AP, or 34G) was extended by pol I Kf in an essentially similar manner, except that the amount of pol I Kf was 0.01–0.3 units and only dATP (complementary to template T next to the lesion) was added to the reaction mixture. The amounts of the original and extended primers were quantified by measuring the radioactivity of the corresponding bands on the Fuji BAS 2000 analyzer. The parameters ($V_{max}$ and $K_m$) were evaluated from the plot of the initial velocity versus the dNTP concentration using a hyperbolic curve-fitting program. Data are the average of two independent experiments, and all $V_{max}$ values were standardized as nm extended primer/min/unit of pol I Kf.

### Analysis of Melting Temperatures

The melting temperature ($T_m$) of duplexes containing a lesion was measured by temperature gradient gel electrophoresis (TGGE) (26–30). Duplexes (5 nM) comprising a 5'-end-labeled strand (34FP, 34OG, 34AP, or 34G) and a complementary strand (34COM-N, where N is A, G, C, or T) in TGGE loading buffer (0.25% xylene cyanol, 0.25% bromophenol blue, 50% glycerol, 5 mM Tris-HCl (pH 7.0), and 0.5 mM EDTA; total of 52.5 mM Tris-Borate (pH 8.0)) were loaded along the top of a 20% polyacrylamide gel (12 x 12 cm) containing 5 mM formamide and TBE buffer (90 mM Tris, 90 mM sodium borate, and 2 mM EDTA). Formamide was included to adjust the $T_m$ in a measurable temperature range of the present TGGE setup. Using a standard vertical gel electrophoresis apparatus, the sample was briefly electroforesed at 250 V for ~5 min at 4 °C, allowing the sample to run onto the gel. The gel was transferred to a TGGE apparatus Thermogradiant TG (TAE/TEC), and electrophoresis was carried out at 250 V for 3.5 h in a perpendicular TGGE mode (i.e., a temperature gradient was perpendicular to the electric field) using a linear temperature gradient between 20 and 60 °C. After electrophoresis, a digital image of the migrated radioactive DNA was acquired on the Fuji BAS 2000 analyzer. The amounts of the duplex and dissociated DNA were quantified every 0.15-mm slice (corresponding to a 0.05 °C temperature difference) along the temperature axis using the Science Lab 99 Image Gauge Version 3.4 software of the Fuji BAS 2000 analyzer. The $T_m$ value was determined from the melting curve (a plot of the fraction of duplex DNA ($\theta$) versus temperature) as a midpoint where the amounts of the duplex and dissociated DNA were equal.

### RESULTS

#### Translesion DNA Synthesis on Templates Containing mFapyG and Other Lesions

To elucidate the effect of mFapyG on DNA synthesis, a template (34FP) containing this lesion at the defined site was primed by 11PRM and replicated by pol I Kf or pol I Kf(exo) for up to 60 min. For comparison, similar experiments were performed using the templates containing G (34G), 8-oxoG (34OG), and an AP site (34AP) at the same site. Primer extension catalyzed by pol I Kf (Fig. 2A) and pol I Kf(exo) (Fig. 2B) was strongly arrested by mFapyG (lanes 7–11), 8-oxoG (lanes 13–17), and the AP site (lanes 19–23). The primary termination site of DNA synthesis was at the mFapyG and 8-oxoG sites for both pol I Kf and pol I Kf(exo). With the AP site, the primary site was one nucleotide prior to the lesion for pol I Kf, whereas it was one nucleotide prior to and at the lesion for pol I Kf(exo). The arrested bands were observed after 60 min of incubation for all lesions. In contrast, primer elongation on the intact template containing G (lanes 3–5) was rather rapid, although DNA synthesis was considerably distributive. Thus, almost fully extended products were observed after 3–10 min of incubation. With the templates containing mFapyG and 8-oxoG, products resulting from translesion synthesis (bands at the top of the gel) gradually accumulated with incubation time. This was also the case for the AP site, but the products accumulated very slowly. Note that the fully extended product on the AP template (lane 23) was one nucleotide shorter than the 34AP template (34-mer). Probably, a single nucleotide deletion occurred at the AP site by the misinsertion strand slippage mechanism (31–35). In the present case, incorporation of dAMP opposite the AP site (see below) was followed by misalignment of the primer terminus to 5'-T in the template (3'-TXT-5', where X is the AP site) and extension of the misaligned region. To compare the efficiency of translesion synthesis, the amount of bypassed products was quantified and plotted against incubation time (Fig. 3). The order of the efficiency of translesion synthesis was G > 8-oxoG > mFapyG > AP site, and the efficiencies were slightly higher for pol I Kf exo) (Fig. 3B) than for pol I Kf (Fig. 3A) with all lesions.

#### Nucleotides Incorporated Opposite mFapyG and Other Lesions

To analyze the nucleotide incorporated opposite mFapyG during translesion synthesis, the template 34FP was primed by 11PRM and replicated by pol I Kf for 60 min. The products, together with those obtained for 8-oxoG (34OG) and G (34G), were subjected to SSCP analysis, which can resolve a single nucleotide difference at the same site (34). Fig. 4A shows the results of SSCP analysis of the bypassed products. Comparison of the gel mobility of the product formed on the mFapyG template (lane 6) with those of standard markers

#### Table I

| Name          | Sequence          |
|---------------|-------------------|
| 11OLG         | 3'-TCATCACAAG     |
| 11OLGp        | 3'-TCATCACAAGp    |
| 44OLG         | 5'-AGGATGTTGGATGAGGTTGACAGTATGTTTCAGCTAGAGCA |
| 11PRM         | 5'-GGTATGGAGG     |
| 14PRM         | 5'-GGTATGGAGGTTGAG |
| 15PRM-A       | 5'-GGTATGGAGGTTGAG |
| 15PRM-G       | 5'-GGTATGGAGGTTGAG |
| 15PRM-C       | 5'-GGTATGGAGGTTGAG |
| 15PRM-T       | 5'-GGTATGGAGGTTGAG |
| 34G           | 3'-TCATACCACATCCCCACCTTCATCATCAAGAG |
| 34FP          | 3'-TCATACCACATCCCCACCTTCATCATCAAGAG |
| 34OG          | 3'-TCATACCACATCCCCACCTTCATCATCAAGAG |
| 34AP          | 3'-TCATACCACATCCCCACCTTCATCATCAAGAG |
| 34COM-A       | 5'-AGGATGTTGGATGAGGTTGAGAGTAGTTTC |
| 34COM-G       | 5'-AGGATGTTGGATGAGGTTGAGAGTAGTTTC |
| 34COM-C       | 5'-AGGATGTTGGATGAGGTTGAGAGTAGTTTC |
| 34COM-T       | 5'-AGGATGTTGGATGAGGTTGAGAGTAGTTTC |
| 26COM-A       | 5'-GGTATGGAGGTTGAGAGTAGTTTC |
| 26COM-G       | 5'-GGTATGGAGGTTGAGAGTAGTTTC |
| 26COM-C       | 5'-GGTATGGAGGTTGAGAGTAGTTTC |
| 28COM-T       | 5'-GGTATGGAGGTTGAGAGTAGTTTC |

* F, mFapyG; O, 8-oxoG; X, AP site (tetrahydrofuran). Underscoring shows the sequence differences in individual oligonucleotide groups.
lanes 1–4) revealed that dCMP was exclusively incorporated opposite mFapyG during translesion synthesis. Incorporation of other nucleotides was below the detection limit under these conditions. Consistent with previous reports (8, 9), both dCMP and dAMP were incorporated opposite 8-oxoG, with a preference for dCMP (lane 5). To confirm the SSCP data, the nucleotide incorporated opposite the lesion was also analyzed by a primer extension assay in the presence of a single dNTP using 14PRM as the primer. The results obtained for pol I Kf and pol I Kf(exo) are shown in Fig. 4 (B and C, respectively). Essentially similar results were obtained for both enzymes. Stepping of the primer band (i.e., incorporation of the added nucleotide) was observed with dCTP for mFapyG (lane 9) and with dCTP and dATP (incorporation of two consecutive As due to T on the 3′-side of 8-oxoG) for 8-oxoG (lanes 14 and 12, respectively). These results agree well with those obtained by SSCP analysis. For the AP template, dAMP was most efficiently incorporated (lane 17), consistent with previous studies (36–39).

Parameters for Nucleotide Insertion and Extension—To elucidate the quantitative aspect of nucleotide insertion opposite mFapyG and to compare the data with those for other lesions, parameters for nucleotide insertion ($V_{\text{max}}$ and $K_m$) were measured using pol I Kf. Using these data, the insertion efficiency of individual nucleotides ($f_{\text{ins}} = V_{\text{max}}/K_m$) was calculated (Table II). To better understand the data in Table II, log $f_{\text{ins}}$ was plotted against the nucleotide inserted opposite the lesion (Fig. 5A). As shown in Fig. 5A, dCTP was the much favored nucleotide for insertion opposite mFapyG (dCTP $>/=\$ dTTP $>/=\$ dATP $>/=\$ dGTP). The -fold decrease in $f_{\text{ins}}$ relative to that of dCTP was $4.2 \times 10^3$ for dTTP, $1.2 \times 10^3$ for dATP, and $2.2 \times 10^6$ for dGTP. The discrimination between dCTP and other nucleotides originated from both a reduction in $V_{\text{max}}$ (33–790-fold) and an increase in $K_m$ (47–2900-fold) (Table II). When the $f_{\text{ins}}$ values of the cognate nucleotide (dCTP) for template G and mFapyG were compared, insertion opposite mFapyG was 65-fold less efficient than that opposite G ($3.1 \times 10^2$ versus $2.0 \times 10^4$), showing that mFapyG constituted a moderate barrier to DNA synthesis in the insertion step. Also note that $f_{\text{ins}}$ of dCTP for mFapyG ($3.1 \times 10^2$) was comparable to that for 8-oxoG ($2.5 \times 10^2$), but was roughly an order of magnitude greater than that...
of dATP for 8-oxoG (1.9 × 10). The \( f_{in} \) value of dATP for the AP site was much lower (280-fold) than that of dCTP for mFapyG. Thus, the order of \( f_{in} \) of dNTPs preferentially inserted opposite the lesions and G was G:dCTP > mFapyG:dCTP ≈ 8-oxoG: dCTP > 8-oxoG:dATP > AP site:dATP (Fig. 5A).

The parameters of primer extension past the lesion (\( V_{max} \) and \( k_{cat} \)) were calculated for each lesion using the fraction of bypassed products formed by pol I Kf(exo-). The fraction of bypassed products formed by pol I Kf(exo-) was determined as described for A using the data from Fig. 2B. G; ▲, 8-oxoG; ■, mFapyG; ♦, AP.
and $K_m$ were also measured using pol I Kf, and the extension efficiency of individual termini ($V_{\text{max}} = V_{\text{max}}/K_m$) was calculated (Table II). The data were plotted in a manner similar to those for the insertion reaction (Fig. 5B). As shown in Fig. 5B, a mFapyG:C terminal was extended most efficiently in four possible primer termini (mFapyG:N, where N is A, G, C, or T). The second preferred terminus was a mFapyG:T pair, but the $V_{\text{max}}$ value was 220-fold lower than that of a mFapyG:C pair. The discrimination between the two termini (mFapyG:C and mFapyG:T) originated mostly from a reduction in $V_{\text{max}}$ (Table II). Extension of mFapyG:N and mFapyG:C termini was below the detection limit under the present conditions. Incorporation of G and T opposite 8-oxoG and that of C and T opposite the AP site were below the detection limit under the present conditions.

**TABLE II**

Reduction factors of insertion and extension efficiencies relative to those for a G (template):C (dNTP) pair

| Template:dNTP | Insertion | Extension | Insertion/Extension |
|---------------|-----------|-----------|---------------------|
|               | $V_{\text{max}}^{a,b}$ | $K_m^{a}$ | $f_{\text{ins}}^{c}$ |
| G:A           | 0.071     | 35        | $2.0 \times 10^{-3}$ |
| G:G           | 8.9       | 21        | $4.2 \times 10^{-1}$ |
| G:C           | 200       | $-0.01$   | $2.0 \times 10^{4}$  |
| G:T           | 4.9       | 53        | $9.2 \times 10^{-2}$ |
| mFapyG:A      | 0.081     | 3.3       | $2.5 \times 10^{-2}$ |
| mFapyG:C      | 0.028     | 200       | $1.4 \times 10^{-1}$ |
| mFapyG:T      | 22        | 0.070     | $3.1 \times 10^{2}$  |
| 8-oxoG:A      | 0.66      | 9.1       | $7.3 \times 10^{-2}$ |
| 8-oxoG:C      | 170       | 8.8       | $1.9 \times 10^{2}$  |
| AP:A          | 55        | 0.22      | $2.5 \times 10^{2}$  |
| AP:G          | 14        | 13        | 1.1                  |
|               | 0.057     | 12        | $4.8 \times 10^{-3}$ |

$^{a}$ Average values of two independent experiments.
$^{b}$ Data are expressed as $V_{\text{max}}$ extended terminus/min/unit of pol I Kf.
$^{c}$ $f_{\text{ins}} = V_{\text{max}}/K_m$ for nucleotide insertion opposite the lesion. $f_{\text{ext}} = V_{\text{max}}/K_m$ for extension past the lesion.

**DISCUSSION**

In this study, it has been shown that mFapyG site-specifically incorporated into template DNA constitutes a block to DNA synthesis catalyzed by pol I Kf and pol I Kf(exo -). The block was fairly strong but not absolute, thus permitting translesion DNA synthesis with a limited efficiency (Fig. 2). This was not clear in previous studies (18–20). Quantitation of the bypass product indicated that the efficiency of translesion synthesis for mFapyG was interposed between those of 8-oxoG and an AP site (Fig. 3). More detailed analysis of the insertion ($f_{\text{ins}}$) and extension ($f_{\text{ext}}$) parameters for individual dNTPs and primer termini revealed that the overall bypass efficiency ($f_{\text{ins}} \times f_{\text{ext}}$) was G:C > 8-oxoG:C > mFapyG:C > 8-oxoG:A > AP.
site:A (Fig. 5 and Table II), where the underlined C and A were nucleotides preferentially inserted during translesion synthesis. The primary kinetic barrier to DNA synthesis by mFapyG originated from the extension step after dCMP was inserted opposite the lesion, as was demonstrated by the reduction factors of $f_{\text{ins}}$ (65-fold) and $f_{\text{ext}}$ (9200-fold) relative to those of the G:C pair (Table III). This is consistent with the observation that the major termination band of DNA synthesis appeared at the mFapyG site (Fig. 2, A and B), but is in contrast to the previous observation that pol I Kf and T4 DNA polymerase stalled 1 base prior to the putative mFapyG sites when DMS/NaOH-treated M13 DNA was replicated in vitro (18). The dominant kinetic barrier to DNA synthesis by 8-oxoG also arose from the extension step, but the contribution of this step was less significant compared with mFapyG, particularly for the 8-oxoG:A pair (Table III).

When mFapyG was bypassed, dCMP was preferentially inserted opposite the lesion, which was also first demonstrated in this study. The second and third preferred nucleotides were dTMP and dAMP, respectively; but the $f_{\text{ins}}$ values for these

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**FIG. 5.** Comparison of nucleotide insertion, extension, and bypass efficiencies for the templates containing mFapyG, 8-oxoG, an AP site, and G:A, comparison of the nucleotide insertion efficiencies. The log $f_{\text{ins}}$ values were calculated from the data $f_{\text{ins}}$ in Table II and plotted against the inserted dNTP. B, comparison of the extension efficiencies. The log $f_{\text{ext}}$ values were calculated from the data $f_{\text{ext}}$ in Table II and plotted against the primer terminus nucleotide (N). C, comparison of the bypass efficiencies. The values of log($f_{\text{ins}} \times f_{\text{ext}}$) were calculated from the data ($f_{\text{ins}} \times f_{\text{ext}}$) in Table II and plotted against the inserted dNTP during translesion synthesis.

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**FIG. 6.** Typical TGGE data for $T_m$ measurement. A, a temperature gradient gel of a duplex containing a mFapyG:C pair (5'-end-labeled 34FP/34COM-C). Electrophoresis was carried out on a 20% polyacrylamide gel containing 5 M formamide and TBE buffer at 250 V for 3.5 h in a perpendicular mode (i.e., a temperature gradient was perpendicular to the electric field). The directions of the temperature gradient (20–60 °C) and DNA migration (shown as mobility) are indicated by arrows. B, a melting curve of 34FP/34COM-C. The fraction of 34FP/34COM-C in a duplex state ($f_{\text{duplex}}$) was determined every 0.05 °C slice along the temperature axis using the TGGE profile (A) and plotted against temperature.
TABLE IV
Melting temperatures of duplexes containing G, mFapyG, and 8-oxoG base pairs

| Base pair | \( T_m \) | \( \Delta T_m \) |
|-----------|-----------|-----------|
| G:A       | 35.6      | -6.5      |
| G:G       | 34.7      | -7.0      |
| G:C       | 41.7      | -7.0      |
| G:T       | 35.8      | -5.9      |
| mFapyG:A  | 37.2      | -0.6      |
| mFapyG:G  | 36.7      | -1.1      |
| mFapyG:C  | 37.8      | 0         |
| mFapyG:T  | 35.8      | -2.0      |
| 8-oxoG:A  | 36.4      | -1.5      |
| 8-oxoG:G  | 36.6      | -1.6      |
| 8-oxoG:C  | 38.2      | 0         |
| 8-oxoG:T  | 33.7      | -4.5      |

* Average values from two independent experiments.

\( \Delta T_m \) differences in \( T_m \) relative to the base pair containing C (G:C, mFapyG:C, and 8-oxoG:C).

nucleotides were 4200- and 12,000-fold lower than for dCMP, respectively (Table II). In addition, the \( f_{\text{ext}} \) of a mFapyG:T terminus was 220-fold lower than that of a mFapyG:C terminus, and that of a mFapyG:A terminus was below the detection limit (i.e. practically not extendable) (Table II). Thus, replication errors at mFapyG sites emerging in fully replicated DNA will be suppressed to a very low level of \(<1\text{E}^{-10}\) replicated mFapyG lesions based on the ratio of \( f_{\text{ins}} \times f_{\text{ext}} \) for insertion of C (3.7 × 10\(^{-5}\)) versus T (4.0 × 10\(^{-4}\)). This provides a reasonable model and explanation for the lack of (or very low) mutagenicity of mFapyG lesions found in the transfection assays of DMS/NaOH-treated M13 DNA (19, 20), although the DNA polymerase involved in \( \text{in vivo} \) is different from the present study (polymerase III holoenzyme in uninduced \( E. \text{coli} \) cells and possibly polymerase II, IV, or V in SOS-induced cells) (40, 41). Conversely, the replication error frequency at 8-oxoG sites emerging in fully replicated DNA is high (\(<1\text{E}^{-10}\) replicated 8-oxoG lesions according to the ratio of \( f_{\text{ins}} \times f_{\text{ext}} \) for insertion of C (9.5 × 10\(^{-4}\)) versus A (8.9 × 10\(^{-2}\)), which has been substantiated by a number of \( \text{in vivo} \) studies (9, 11, 42–44). Combining the present and previous data (18–20), the biological consequence of mFapyG is quite different from that of 8-oxoG and rather resembles thymine glycol, a major thymine lesion formed by reactive oxygen species. Like mFapyG, thymine glycol constitutes a fairly strong replication block in \( \text{in vitro} \) and is lethal in \( \text{in vitro} \) when introduced into transfecting M13 DNA (45–48). Moreover, DNA polymerase stalls at a thymine glycol lesion after inserting a cognate nucleotide dAMP (49). Thus, thymine glycol does not elicit mutations when bypassed in SOS-induced \( E. \text{coli} \) cells (48).

In this study, we attempted to delineate the physicochemical mechanism underlying selection of incoming dNTP at the mFapyG site and differential extension of the subsequently formed primer terminus. For this purpose, the \( T_m \) of the duplexes containing four possible mFapyG base pairs was measured by a TGGE method (Table IV), assuming that \( T_m \) changes in the duplexes reflect the local stability of mFapyG base pairs. The mFapyG:C pair showing the highest thermal stability was preferentially formed and extended by Pol I Kf (Fig. 5). This was also the case for G:C and 8-oxoG:C pairs, suggesting a common base pairing scheme involving three canonical hydrogen bonds for mFapyG:C, G:C, and 8-oxoG:C base pairs (Fig. 7). However, as inferred from the moderate decrease in \( T_m \) relative to G:C, a mFapyG:C pair seems less stable than a G:C pair. Plausible causes are weakened stacking interactions and increased conformational flexibility due to rupture of the imidazole ring. Thus, it is likely that a similar destabilization effect on incoming dCTP slows down the formation of a new phosphodiester bond at the mFapyG site. Judging from the very low value of \( f_{\text{ext}} \) (1.2) relative to \( f_{\text{ins}} \) (3.1 × 10\(^{-2}\)), the destabilized mFapyG:C pair exerts a more pronounced effect in the subsequent extension reaction. Conversely, in the mFapyG base pairs containing A, G, and T that showed lower stabilities compared with a mFapyG:C pair, there was no obvious correlation between their thermal stability and the insertion/extension efficiencies \( f_{\text{ins}} \) and \( f_{\text{ext}} \) (Fig. 5 and Table IV), although the difference in stability \( (\Delta T_m) \) was considerably small. This was also the case for G and 8-oxoG base pairs containing A, G, and T (Fig. 5 and Table IV). It is possible that steric exclusion (or geometric recognition) in the polymerase active site rather than the thermal stability of base pairs plays a crucial role in the insertion and extension steps (reviewed in Refs. 50 and 51) particularly when canonical hydrogen bonding and/or stacking interactions are absent. Such a mechanism has been implied for AP sites and thymine glycol on the basis of the lack of apparent correlations between the preference of insertion or proofreading excision of nucleotides and the \( T_m \) of oligonucleotides containing these lesions (52, 53). The mechanism involving steric exclusion also seems consistent with the observation that Endo III recognizes mFapyG paired with purines more...
efficiently than that paired with pyrimidines (activity with respect to the paired base: A ~ G > T > C) (22). Similar to other base lesions (54), steric clash between mFapyG and bulky purines in a helix promotes base flipping into the active-site pocket of Endo III. Thus, the order of $f_{max}(C > T > A > G)$ and $f_{ext}(C > T > A > G)$ for mFapyG can be interpreted as the combined outcome of hydrogen bonding specific for incoming dCTP and steric exclusion in the polymerase active site.

Toward the end of this study, the chemical synthesis of oligonucleotides containing FapyG (a form of mFapyG without the 5-N-methyl group) and 4,6-diamino-5-formamidopyrimidine derived from adenine (FapyA) was reported (55, 56). The reports have shown that the N-glycosidic bond of FapyG and FapyA in monomeric and single-stranded DNA substrates is stable, with half-lives of 108 days (FapyG, calculated from the deglycosylation rate at 55 °C) and 4.3 days (FapyA) at 37 °C. More interestingly, the nucleotide forms of FapyG and FapyA epimerize in solution, giving rise to a mixture of β- and α-anomers with respect to the orientation of the N-glycosidic bond around sugar C-1’. We have previously shown that the α-anomer of 2'-deoxyadenosine (αDA) site-specifically introduced into oligonucleotide templates or M13 vectors constitutes moderate insertion efficiency was limited. The order of the insertion efficiency was 2.34. Breen, A. P., and Murphy, J. A. (1995) J. Biol. Chem. 270, 8456–8464.

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