The 3' → 5' Exonuclease of T4 DNA Polymerase Removes Premutagenic Alkyl Mispairs and Contributes to Futile Cycling at O6-Methylguanine Lesions*

Received for publication, December 6, 2000, and in revised form, March 29, 2001
Published, JBC Papers in Press, April 4, 2001, DOI 10.1074/jbc.M011025200

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We have studied the processing of O6-methylguanine (m6G)-containing oligonucleotides and N-methyl-N-nitrosoaurine (MNU)-treated DNA templates by the 3' → 5' exonuclease of T4 DNA polymerase. In vitro biochemical analyses demonstrate that the exonuclease can remove bases opposite a defined m6G lesion. The efficiency of excision of a terminal m6G-T is similar to that of m6G-C, and both were excised as efficiently as a G-T substrate. Partitioning assays between the polymerase and exonuclease activities, performed in the presence of dNTPs, resulted in repeated incorporation and excision events opposite the m6G lesion. This idling produces dramatically less full-length product, relative to natural substrates, indicating that the 3' → 5' exonuclease may contribute to DNA synthesis inhibition by alkylating agents. Genetic data obtained using an in vitro herpes simplex virus-thymidine kinase assay support the inefficiency of the exonuclease as a "proofreading" activity for m6G, since virtually all mutations produced by the native enzyme using MNU-treated templates were G → A transitions. Comparison of MNU dose-response curves for exonuclease-proficient and -deficient forms of T4 polymerase reveals that the exonuclease efficiently removes 50–98% of total premutagenic alkyl mispairs. We propose that idling of exonuclease-proficient polymerases at m6G lesions during repair DNA synthesis provides the biochemical explanation for cellular cytotoxicity of methylating agents.

O6-Methylguanine (m6G) is a prototype DNA adduct produced by alkylating agents that is both cytotoxic and mutagenic. Two mechanisms potentially govern the cytotoxic effects of the m6G lesion. O6-Methylguanine lesions decrease the efficiency of DNA synthesis by replicative polymerases in vitro (1, 2) and inhibit in vitro DNA replication in cell-free extracts (3). Alternatively, the persisting m6G can be cytotoxic through lethal interactions with the mismatch repair pathway in mammalian cells (reviewed in Ref. 4). Loss of O6-methylguanine-DNA methyltransferase activity results in the persistence of unrepaired m6G lesions, and such cells are sensitive to killing by alkylating agents. Additional loss of mismatch repair proteins renders O6-methylguanine-DNA methyltransferase-deficient cells tolerant of the cytotoxic effects of alkylating agents (5, 6). However, the precise biochemical mechanism mediating this toxicity has not been completely defined (4). In one model, mismatch repair activity causes persistent DNA strand breaks which signal cell cycle arrest and apoptosis (4, 7).

Replicative and repair-associated DNA polymerases contain 3' → 5' proofreading exonucleases, which act in coordination with the polymerase activity to enhance the efficiency of error discrimination. Despite the critical role of polymerases in mediating DNA damage mutagenesis, little is known regarding the biochemistry of this polymerase exonuclease activity at DNA lesions. Eukaryotic DNA polymerases δ and ε contain the polymerase domain and the exonuclease domain within the same polypeptide. Thus, during DNA synthesis, the nascent 3'-primer terminus must be shuttled between the two active sites in order for excision to occur. In studies using 2-aminopurine fluorescence, the rate-limiting step in excision by the T4 polymerase is translocation of the DNA from the polymerase to the exonuclease site (8, 9). Part of the molecular “switch” for this process is thought to be the structure of the DNA (10–12).

The preferred DNA substrate for the polymerase domain is base-paired duplex DNA, whereas that for the exonuclease is melted duplex DNA containing a few single-stranded bases. Any factor that destabilizes duplex DNA is expected to enhance the exonuclease catalytic rate relative to the polymerase catalytic rate. For example, the significant distortion of DNA within the DNA polymerase binding cleft may destabilize duplex DNA and enhance excision by affecting the equilibrium between single-stranded and double-stranded states (13). Alternatively, the melting capacity of the primer terminus determines the rate of editing by the Klengow polymerase (10). The physical structures of m6G-T and m6G-C base pairs have been determined using crystal (14, 15) and solution (16, 17) methods. Structurally, the m6G-T mispair simulates Watson-Crick alignments, and the m6G-T pair is indistinguishable in the minor groove from G-C (reviewed in Ref. 18). Thermodynamically, however, the m6G-T pair is less stable than both the m6G-C and G-T pairs in DNA and displays a lower melting temperature (15, 19). We report here the efficiency of exonuclease removal of m6G and other alkylation mispairs and the resulting effects on alkylation-induced errors by the T4 DNA polymerase.

EXPERIMENTAL PROCEDURES

Reagents—Wild type and D219A T4 DNA polymerases were gifts of Dr. Stephen Benkovic (Pennsylvania State University, University Park, PA). The D324A T4 polymerase was a gift of Dr. Linda Rehak-Krantz (University of Alberta, Edmonton, Canada). All enzymes for

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molecular biology procedures were supplied by Life Technologies, Inc. and used according to the manufacturer’s protocol. N-Methyl-N-nitrosourea (MNU) and 5-fluoro-2-deoxyuridine were purchased from Sigma.

DNA Substrates—DNA primer-templates were made by hybridizing combinations of synthetic oligonucleotides in which the 33-mer template oligonucleotide (HPLC-purified, Midland Certified Reagents, Midland, TX) had either a G or a site-specific m6G adduct, and the complementary 15-mer primer oligonucleotides (gel-purified; Pierce) contained either a terminal C or T residue: template, 3′-CGCGACGCCTGCGTCT/5′Gm6GCTCGAGCCAGAC-3′, mutagenic primer, 5′-CGCGACGCCTGCGTCTGAGCCAGAC-3′; mutagenic primer, 5′-GCGCGACGCCTGCGTCTGAGCCAGAC-3′ (boldface type indicates location of differences between substrates).

The presence of m6G in the final template preparation was verified by matrix-assisted laser desorption-ionization-time-of-flight mass spectrometry by the manufacturer. The 15-mer primers were labeled at the 5′-end using [γ-32P]ATP (5000 Ci/mmol) and T4 polynucleotide kinase according to the manufacturer’s protocol. The labeled primers were annealed with the template oligonucleotides by combining a 1:3 primer-template molar ratios of each DNA in the presence of SSC. The mixtures were heated at 75 °C for 5 min and gradually cooled to room temperature. The primer-template preparations were purified using G25 Sephadex Quickspin columns prior to use.

Enzymatic Assays—The exonuclease reaction mixture contained 50 mM Tris-OAc (pH 7.4), 10 mM dithiothreitol, 10 mM Mg(OAc)2, 150 mM KAc, 10 nM DNA primer-template, and 0.5 nM enzyme. Reactions were initiated by the addition of enzyme and were incubated at 37 °C. At the indicated times, aliquots were added to an equal volume of stop solution (containing 20 mM EDTA and 90% formamide) and quenched, and the products were separated on a 16% denaturing polyacrylamide gel. Band intensities were quantitated using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). The radioactivity at respective time points was normalized to the amount of radioactivity in the control (no polymerase) lane.

In Vitro Polymerase/Exonuclease Partitioning Assay—T4 polymerase partitioning assays were carried out in the presence of either 200 μM dGTP (next correct nucleotide) or 200 μM four dNTPs, 5 or 50 nM wild-type enzyme, and 10 nM primer-template using the reaction buffer described above. Aliquots were removed at the indicated times and quenched, and the products were separated on a 16% denaturing polyacrylamide gel. For reactions containing dGTP only, radioactivity was measured for full-length (33-mer) product, 1% extension, and 1% extension 16–32-mer, and extension products (n − 1, n − 2) relative to the total radioactivity in each sample. The experimental conditions used for the D324A extension assay were the same as above, except the protein concentration was 5 μM.

In Vitro Polymerase Reactions—Alkylated DNA substrates were created by random MNU modification (MeSO solvent) of primed, single-stranded oligonucleotide templates and purified prior to use in polymerase reactions, as previously described (20). The in vitro reactions for mutational analyses contained 2 pmol of ssDNA template at 40 nM concentration. Reaction conditions were 50 mM Tris-OAc (pH 7.4) or 25 mM Hepes-HCl (pH 7.4) 150 mM KAc, 10 mM Mg(OAc)2, 10 mM dithiothreitol, 200 μM dNTPs, and 40 pmol of T4 polymerase. Reactions were incubated at 37 °C for 60 min and terminated with 15 mM EDTA. The extent of DNA synthesis was determined by parallel reactions (0.2 pmol of DNA; same molar ratios of enzyme to substrate as above) supplemented with 5 μCi of [α-32P]dCTP (3000 Ci/mmol). A 64-mer oligonucleotide oligonucleotide was radiolabeled at the 5′-end using [γ-32P]ATP (5000 Ci/mmol) and T4 polynucleotide kinase, and 50 fmol were added to each reaction after termination for use as an internal loading standard. The DNA products were analyzed on an 8% denaturing polyacrylamide gel together with a DNA sequencing ladder generated from the same primer-template, followed by PhosphorImager analysis. The amount of synthesis in each reaction was normalized first to the internal standard and then to the solvent-treated control for each polymerase. No reaction products less than 210 nucleotides in length were observed for any polymerase with either solvent or MNU treatment.

HSV-tk Forward Mutational Assay—Mutational analyses of the reaction products were performed as reported previously (20). Briefly, the ssDNA synthesis products were digested with EcoRV and MluI restriction enzymes, and 203-base pair double-stranded products were purified. An equivalent yield of product (50–100 ng; 0.4–0.8 pmol, as estimated by gel electrophoresis, was obtained for all solvent and MNU-treated polymerase reactions. These fragments were hybridized to gapped duplex molecules (0.05 pmol) containing one chlorophenicol-resistant strain and one chlorophenicol-sensitive strain. To select for HSV-tk mutations, an aliquot of the final hybridization was used to transform recA13, upp, tdl E. coli strain P7334 by electroporation and plated on VBA-selective media. Progeny of the DNA strand produced during in vitro synthesis were isolated with 50 μg/ml chloramphenicol and HSV-tk mutant plasmids were selected with 40 μg 5-fluoro-2-deoxyuridine. The HSV-tk mutant frequency is defined as the number of 5-fluoro-2-deoxyuridine + chlorophenicol-resistant colonies divided by the total number of chlorophenicol-resistant (CmR) colonies. In the experiments presented here, a total of 0.14–3 × 10^6 CmR transformants (solvent treatment) and 0.06–6 × 10^5 CmR transformants (MNU treatment) were analyzed for mutants for each polymerase.

Mutational spectra were derived from independent mutants, as described (21). Differences in proportions of specific types of errors were analyzed statistically using Fisher’s exact test (two-tailed).

RESULTS

O6-Methylguanine-containing Templates Are Substrates for the 3′ → 5′ Exonuclease Activity of T4 Polymerase—The intrinsic exonuclease activity of T4 polymerase on various natural and m6G-containing templates was examined by incubating the enzyme and DNA in the absence of dNTP substrates. As expected for control templates, the T4 exonuclease activity exhibited “proofreading” activity by degrading terminal G/T mispair-containing substrates with a greater efficiency (t1/2 = 6 min) than correctly paired G-C-containing substrates (t1/2 = 60 min), under enzyme-limiting conditions (Fig. 1). In contrast, the exonuclease exhibited little or no discrimination between the two m6G-containing substrates. Both m6G-C and m6G-T substrates were degraded with similar efficiencies (t1/2 = 4.5 min and 4 min, respectively), near that observed for the G-T mispaired substrate (Fig. 1). More rapid excision was observed for all four substrates during the first minute of the reaction, compared with later times. This observation may reflect the high processivity of the T4 exonuclease, since excision of the 15-mer primers was complete under the substrate excess conditions utilized. These results show that although m6G-containing templates are substrates for the T4 exonuclease activity, this activity may not contribute a significant “proofreading” function to DNA polymerase errors, because both m6G-C and m6G-T substrates are excised with similar efficiencies.

T4 Polymerase Partitioning Between Polymerase and Exonuclease Active Sites at m6G Lesions—Partitioning of the T4 enzyme between the polymerase and exonuclease activities was assayed in the presence of the next correct nucleotide, dGTP (Fig. 2). The correctly paired natural substrate (G-C) was efficiently extended by the enzyme with minimal exonuclease degradation. In contrast, polymerase activity was highly inhibited using a mispaired G-T substrate (<1% extension), relative to the paired substrate (90% extension), and exonuclease degradation products were predominant over the time course analyzed. However, inclusion of m6G in the substrate eliminated this discrimination, and the exonuclease activity predominated in reactions containing either the m6G-C or the m6G-T substrate (Fig. 2, C and D).

T4 Polymerase Exhibits Idling on m6G Substrates—The 3′ → 5′ exonuclease assays (Figs. 1 and 2) demonstrate that this activity could excise both nonmutagenic (C) and mutagenic (T) bases incorporated opposite m6G lesions. We next examined whether the polymerase or the exonuclease activity predominates in the presence of all four dNTP substrates (Fig. 3). Experiments performed using natural G templates with or without a mispaired primer terminus resulted in the accumulation of full-length product (Fig. 3, A and B). As expected, the 3′ → 5′ exonuclease activity was not detectable in the presence
of the G-C substrate and 200 μM dNTPs. However, using the mispaired G/T substrate, excision products accumulated during the initial time points with little extension (Fig. 3B). With increased time of incubation, full-length product accumulated concomitant with disappearance of excision bands, suggesting that efficient extension occurred only after complete excision of the terminal mispaired thymine.

The enzyme polymerization activity was found to be highly inhibited using lesion-containing templates. For both m6G-C and m6G-T substrates, excision products persisted throughout the course of the reaction and predominated over extension products (Fig. 3, C and D). Increasing the enzyme concentration 10-fold (enzyme excess conditions) did not affect the polymerase/exonuclease balance, since the ratio of extension/excision products remained ~1:1 (Table I). Accumulation of the 15-mer products probably results from the repeated cycles of incorporation and excision of bases at the lesion-containing primer terminus. In order to examine the m6G substrate preference of the polymerase activity, extension of m6G-containing substrates was examined using the exonuclease-compromised D324A T4 polymerase (Fig. 3, E and F). The enzyme was found to extend the m6G-T substrate somewhat more efficiently than the m6G-C substrate (Table I). However, even under these large enzyme excess conditions, the m6G lesion is inhibitory to the T4 polymerase, since 20–50% of the substrates remained unextended. The appearance of excision products (Fig. 3, E and F) may represent either residual exonuclease activity or non-enzymatic cleavage in the presence of Tris buffer, as has been reported by others (22).

 Genetic Analyses Reveal That the T4 Exonuclease Activity Can Proofread Alkyl-directed DNA Mismatches—In the biochemical assays, we observed a similar yield of full-length reaction products for both the nonmutagenic (m6G-C) and mutagenic (m6G-T) substrates (Fig. 3). In order to quantitate the extent to which the exonuclease contributes to error avoidance, we performed the in vitro HSV-tk forward mutation assay (20). The wild-type, proofreading-proficient enzyme was compared with two proofreading-deficient forms: D324A, which exhibits a 104 to 105-fold reduction in exonuclease activity, and D219A, which exhibits a 104-fold reduction in exonuclease activity (22, 23). At 200 μM dNTPs, using unmodified DNA templates, the mutation frequency for wild-type T4 polymerase was 2.4 × 10−4, approximately 5-fold lower than either the D324A (21 × 10−4) or the D219A (10 ± 2.5 × 10−4) exonuclease-deficient enzymes and near the background for this assay (20).

In the HSV-tk forward mutation assay, chemically treated, oligonucleotide-primed ssDNA is used as a template for DNA polymerase reactions. The DNA reaction products are digested with restriction enzymes, and a 203-base pair DNA synthesis product is purified. To recover and analyze these DNA fragments for the presence of mutations, a gapped duplex molecule is used that is formed by hybridization of a linear chloramphenicol-resistant DNA fragment to a chloramphenicol-sensitive ssDNA. DNA synthesis fragments containing potential mutations within the thymidine kinase gene are rescued by hybridization to the gapped duplex, forming heteroduplex plasmid molecules that are used to transform E. coli. Incubation of the transformed bacteria in the presence of chloramphenicol selects progeny of the DNA strand synthesized in vitro (20). HSV-tk mutant plasmids are selected by plating the bacteria in the presence of 5-fluoro-2′-deoxyuridine. The resulting HSV-tk mutant frequency is a measure of the proportion of DNA fragments containing mutations.

Modification of oligonucleotide-primed single-stranded DNA templates with MNU resulted in a dose-dependent inhibition of DNA synthesis for the three polymerases. However, at low levels of modification, the inhibition of synthesis observed for the wild-type polymerase was greater than that observed for either exonuclease-deficient enzyme (Fig. 4A). Genetic analyses of the reaction products from these modification reactions are shown in Fig. 4B. For the wild-type polymerase, a linear mutation versus dose-response curve was observed, with a 30-fold increased frequency at 5 mM MNU (110 ± 30 × 10−4), relative to solvent control (3.6 ± 3.9 × 10−4). We repeated the wild-type dose-response curve in Hepes buffer to eliminate potential nonenzymatic exonuclease activity, and again observed a 37-fold increased mutation frequency at 5 mM MNU (270 × 10−4), relative to solvent-treated templates (7.3 × 10−4). The mutation rate (defined by the slope of the dose-response curves) of the D324A polymerase was 7-fold greater than that of wild-type T4, while that of the D219A was 2-fold greater than wild-type (Fig. 4B). At the highest dose tested (5
mM MNU), the absolute mutation frequencies measured for the D324A and D219A polymerases were $7.4 \times 10^{-2}$ and $2.5 \times 10^{-2}$, respectively.

Comparison of the wild-type and exonuclease-deficient dose-response curves demonstrates that the exonuclease of T4 polymerase is able to remove 50–86% of total alkylation mismatches. Random modification of ssDNA by MNU results in alkylation at template bases in the order: G $\rightarrow$ A $\rightarrow$ C (24).

DNA sequence analyses revealed that the wild-type enzyme produced exclusively G $\rightarrow$ A transition mutations using alkylated templates (Fig. 5). The frequency of G $\rightarrow$ A transition mutations at 5 mM MNU treatment was increased 2000-fold, relative to solvent treatment (Table II). At the same dose, the G $\rightarrow$ A mutation frequency for the D324A polymerase was 2.6-fold higher than that for the wild-type polymerase (Table II), indicating that the exonuclease activity removed only $\sim 38\%$ of m6G$\rightarrow$T mismatches. Inspection of the mutational spectra derived from all three forms of the T4 polymerase reveals that the majority of errors removed by the exonuclease involve methylated C and A lesions (Table II). In the D324A spectrum, eight mutational events (26%) induced by MNU were C $\rightarrow$ T transitions. This corresponds to a frequency of $2.3 \times 10^{-2}$, nearly equivalent to the G $\rightarrow$ A frequency and 190-fold greater than the solvent-treated control frequency. Other prevalent mutations in the D219A and D324A MNU-induced spectra include C $\rightarrow$ A transversions (18 and 10%, respectively), A $\rightarrow$ T transversions (15 and 10%, respectively), and one-base deletions at template A (7 and 22%, respectively).
and exonuclease enzymatic activities. As has been observed previously by others (2), exonuclease-deficient T4 polymerase displays a preference for extension of m6G-T over m6G-C substrates (Fig. 3), consistent with a polymerase activity that discriminates on the basis of structural configuration of bases (18). Physical determinations have shown that the m6G-C base pair is in altered wobble geometry, while the mutagenic m6G-T base pair simulates Watson-Crick geometry. In contrast, we observed that the T4 3’ → 5’ exonuclease activity displayed little discrimination between nonmutagenic and mutagenic base pairs opposite the m6G lesion, and both were excised as efficiently as the natural G-T mispair (Fig. 2). This result is consistent with a thermodynamic mechanism of substrate recognition by the exonuclease. Both m6G-C and m6G-T base pairs are less stable than correct G-C (∆ΔG = 46 and 46.8 kJ/mol, respectively) (15). Moreover, the m6G-T base pair is of equivalent stability as an m6G-C base pair (∆ΔG = 0.8 kJ/mol), whereas the natural G-T base pair is substantially less stable than a G-C base pair (∆ΔG = 30.9 kJ/mol). Thus, the significant thermodynamic difference for correctly paired and mispaired natural bases is eliminated by formation of the m6G lesion. Interestingly, we observed that the exonuclease activity was highly effective in removing alkylated mispairs at template A and C residues, resulting in a 7-fold reduction in the MNU-induced T4 polymerase mutation frequency (Fig. 5 and Table II). The premutagenic lesions for these mutational events have not been defined by site-specific lesion studies. However, the nearly 200- and 40-fold increases in the frequency of C → T transitions and C → A transversions, respectively, observed for the D324A enzyme upon MNU treatment are consistent with earlier studies demonstrating misincorporation of dAMP > TMP on methylated poly(dC) templates (25). We presume that the premutagenic lesion for these events is 3meC, which is formed in significant amounts by MNU modification of single-stranded DNA (24). The mutational events at methylated A residues may result from 1meA adducts (25) and/or apurinic sites resulting from spontaneous decay of 7meA lesions. Alkylation of purine and pyrimidine base pairing positions may destabilize the primer/terminus, allowing for efficient exonuclease removal.

DNA alkylation lesions inhibit forward DNA synthesis by 3’ → 5’ exonuclease-proficient DNA polymerases (Fig. 4A; Ref. 26). Our biochemical observations (Figs. 1–3) indicate that the exonuclease activity acts as a kinetic barrier to DNA synthesis on m6G lesion-containing templates by preventing accumulation of 3’-terminal m6G substrates. Furthermore, the m6G-C and m6G-T intermediates are extended inefficiently by T4 polymerase. However, the presence of m6G is not an absolute barrier to DNA synthesis, since we observed 3% full-length product with wild-type T4 polymerase in the presence of 200 μM dNTPs at approximately equimolar concentrations of enzyme and DNA (Table I). Moreover, the presence of accessory proteins may enhance the ability of T4 polymerase to synthesize past lesions, as has been observed for E. coli polIII (27) and pol δ (28) in the presence of their cognate processivity factors. Recent evidence in S. cerevisiae has led to the suggestion that specific translesion synthesis polymerases may assist in overcoming m6G inhibition (29). Together, these biochemical observations are consistent with in vivo data demonstrating that m6G-mediated cytotoxicity is not observed in the first S phase after alkylation treatment (30, 31). Nonetheless, the idling we observed at the site of m6G, which we interpret as repeated cycles of base incorporation and exonuclease excision, could lead to disruption of local dCTP and TTP pools during DNA replication, resulting in a biochemical signal for cell cycle arrest or apoptosis.
The most profound implication of the idling by proofreading-proficient polymerases at sites of m6G lesions is during repair DNA synthesis. In eukaryotic cells, m6G has been suggested to be cytotoxic when used as a substrate by mismatch repair enzymes, in what has been termed a “futile cycle.” In this model, DNA repair of the strand opposite the lesion will produce an m6G-containing gapped DNA substrate for either polymerase δ or polymerase ε (32). The presence of the lesion in this gap will severely inhibit the efficiency of DNA repair synthesis, since we have observed that less than 10% of m6G-containing templates give rise to full-length product by wild-type T4 polymerase. Our observations provide a biochemical explanation for how persistent gaps arise in genomic DNA from methylated substrates. Moreover, human cell lines have been reported that are O6-methylguanine-DNA methyltransferase-deficient and mismatch repair-proficient yet exhibit full resistance to the cytotoxic action of MNU (5). Extrapolating our data, these cell lines may harbor a mutation in the exonuclease domain of polymerase δ or polymerase ε that alleviates the idling and thus the cytotoxicity of MNU.

Our study demonstrates the biochemical significance of the polymerase-associated 3'→5' exonuclease activity in processing alkylation adducts, particularly the m6G lesion. The idling we observed opposite m6G could have implications for improving.

FIG. 5. Solvent and MNU-induced HSV-tk mutational spectra for wild-type and exonuclease-deficient T4 polymerases. The DNA template sequence shown (middle lines) is the 203-base pair HSV-tk mutational target. Letters above the middle line indicate base substitutions; symbols below the line represent deletion mutations. , one-base deletion; , two-base deletion; arrows, larger deletion end points. A, D324A polymerase and MeSO-treated templates; B, wild-type polymerase and MeSO-treated templates; C, D324A polymerase and 5 mM MNU-treated templates; D, D219A polymerase and 5 mM MNU-treated templates; E, wild-type polymerase in Tris (top) or Hepes (bottom) reaction buffer and 5 mM MNU-treated templates.

TABLE II

| T4 polymerase | DNA treatment | HSV-tk mutation frequency \( \times 10^{-4} \) (no. observed) | Base substitutions | Frameshifts |
|---------------|---------------|---------------------------------|-------------------|------------|
|               |               |                                 |                   |            |
| Wild type     | Solvent*      | 0.06 (1) 0.37 (6)               | <0.06 (0)         | <0.06 (0) |
|               | MNUb          | 120 (20) <6 (0)                  | <6 (0)            | <6 (0)    |
| D324A         | Solventc      | 3.6 (3) <1.2 (0)                | 2.4 (2)           | <1.2 (0)  |
|               | MNUd          | 320 (11) 230 (8)                | 86 (3)            | 86 (3)    |

* Other mutations were one A→G, two G→T base substitutions, two complex mutations, and three insertions (23 total mutational events; \( f = 1.4 \times 10^{-4} \)).

b 20 total mutational events; \( f = 120 \times 10^{-4} \).

c Other mutation: one T→A base substitution (10 total mutational events; \( f = 12 \times 10^{-4} \)).

d Other mutations were one A→C, one G→T base substitutions, one deletion (31 total mutational events; \( f = 890 \times 10^{-4} \)).
ing the therapeutic potential of chemotherapeutic agents. Nucleoside analogs or DNA adducts that thermodynamically destabilize the nascent DNA are expected to be substrates for the exonuclease activity; thus, the most effective cytotoxic agents will be those that produce substrates favored by the exonuclease domain over the polymerase domain, as has been observed for the prototype m6G lesion.

Acknowledgments—We thank Guang Yan for expert technical assistance and Dr. Linda Bloom for critical reading of the manuscript.

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