Replication across O\(^6\)-Methylguanine by Human DNA Polymerase \(\beta\) in Vitro

INSIGHTS INTO THE FUTILE CYTOTOXIC REPAIR AND MUTAGENESIS OF O\(^6\)-METHYLGUANINE*\(^a\)

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Jatinder Singh, Lin Su, and Elizabeth T. Snow‡

From the Nelson Institute of Environmental Medicine, New York University Medical Center, Tuxedo, New York 10987

Replication in vivo across unrepaird O\(^6\)-methylguanine (m\(^6\)dG) lesions by mammalian DNA polymerase \(\beta\) (pol \(\beta\)) during short patch repair may contribute to the cytotoxicity and mutagenesis of m\(^6\)dG. We have employed in vitro steady state kinetic analysis to investigate the replication of oligonucleotide templates containing site-specific m\(^6\)dG by human pol \(\beta\). Our results show that m\(^6\)dG is a strong but not absolute block to replication by pol \(\beta\). pol \(\beta\) exhibits mixed kinetic discrimination during overall replication across dG and m\(^6\)dG. pol \(\beta\) preferentially inserts dTMP rather than dCMP opposite m\(^6\)dG. However, pol \(\beta\) extends from the dC-m\(^6\)dG base pair more efficiently than from the dT-m\(^6\)dG base pair. This is in strong contrast to other polymerases such as the exonuclease-deficient Klenow fragment of Escherichia coli DNA polymerase I (exo \(^{-}\)KF) that preferentially extends dT-m\(^6\)dG by a factor of 10 over dC-m\(^6\)dG. When both insertion and extension are considered, pol \(\beta\) has a 15-fold overall preference for incorporation of the mutagenic substrate dTTP rather than the nonmutagenic substrate dCTP during replication across m\(^6\)dG. This suggests that pol \(\beta\) in concert with the T:G-specific thymine DNA glycosylase, may be intrinsically involved in the futile cytotoxic repair induced by m\(^6\)dG. Our results also suggest that replication across m\(^6\)dG by pol \(\beta\) may contribute to m\(^6\)dG-induced G \(\rightarrow\) A transition mutations.

Eukaryotic DNA polymerase \(\beta\) (pol \(\beta\))\(^1\) is a nuclear DNA repair polymerase extensively characterized by Wilson (1, 2). It mainly carries out gap filling during short patch base excision repair (3–7). pol \(\beta\) fills short (up to six nucleotides long) DNA gaps (4–8) and has been found to act in concert with uracil glycosylase (3) and the human G:T-specific thymine glycosylase (9). pol \(\beta\) is also implicated in the repair of DNA damage induced by anticancer agents such as bleomycin, \(\gamma\)-irradiation (10), cisplatin (11), and alkylating agents (12). Resistance to cisplatin may be partially due to overexpression of pol \(\beta\) (11). Tumors may also develop resistance to radiation therapy and chemotherapy (e.g. cisplatin and alkylating agents) by increased efficiency of DNA repair, pol \(\beta\) has been found to be altered in some human cancers (13, 14), suggesting its importance in maintaining genomic stability. However, pol \(\beta\) is the most error prone of all known polymerases tested in vitro (15).

Mechanistic studies of pol \(\beta\) are important for understanding its diverse roles in DNA replication and repair under circumstances leading to mutagenesis, cytotoxicity, tumor progression, and the resistance of tumors to anticancer therapy. Furthermore, the small size, single subunit composition, availability of crystal structure, and absence of confounding associated activities (such as 3’-5’ exonuclease) make pol \(\beta\) a good model for the study of DNA polymerase structure and function.

O\(^6\)-Methylguanine (m\(^6\)dG) is a mutagenic and cytotoxic DNA adduct that can be formed in vivo by such diverse agents as tobacco smoke, methyl/nnitrosourea, and other \(S\),\(1\) methylating agents (16). In vitro studies (17–19), as well as in vivo mutagenesis assays (20, 21) have shown that m\(^6\)dG preferentially base pairs with dTMP instead of dCMP, thus giving rise to G to A transition mutations. The importance of these lesions has been repeatedly demonstrated. A strong correlation was found between the persistence of m\(^6\)dG lesions and tumors in rodents (22). More recently, the persistence of m\(^6\)dG after treatment with 4-(methyl/nnitrosamoino)-1-(3-pyridyl)-1-butane (a component of tobacco smoke) has been found to correlate with the activation of the Ki-ras oncogene in lung tumors in mice (23). Introduction of m\(^6\)dG into codon 12 of a synthetic c-Ha-ras gene and transfection into normal NIH-3T3 cells resulted in focus formation and the production of G to A mutations at the position of the m\(^6\)dG (24).

The m\(^6\)dG lesion can be readily repaired in a saturable manner by the suicide enzyme O\(^6\)-methylguanine-DNA methyltransferase (MGMT) (25). This type of repair is error free and noncytotoxic. However, in many human solid tumor cell lines and in some non-tumor tissues the ability to repair m\(^6\)dG is lacking due to the inactivation of MGMT (25–29), thereby favoring the persistence of unrepaird m\(^6\)dG lesions. Persistence of these lesions is associated with methylation-dependent cytotoxicity. Methylating agents that form m\(^6\)dG in vivo also induce sister chromatid exchanges (29), which are thought to result from persistent gaps (30). The cytotoxicity of unrepaird m\(^6\)dG in eukaryotic cells may be related to its replication blockade and a type of mismatch repair that is independent of MGMT (9, 31, 32). The initial event in m\(^6\)dG-induced mismatch repair may be the recognition (“tagging”) of dT-m\(^6\)dG (or dT-dG after removal of methyl group by MGMT) by the hMSH2-p160 heterodimer (33). Functional deficiency of these mismatch recognition/tagging proteins imparts resistance to alklylation-in-
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We have investigated the kinetics of replication across m^6dG by pol β in order to dissect the dual roles of this DNA polymerase in the cytotoxic repair and mutagenesis induced by m^6dG. In this paper we report the kinetic parameters of steady state replication (reviewed in Ref. 38) across dG and m^6dG by pol β. We find that m^6dG acts as a strong, but not complete, block to replication by pol β and that pol β, like other polymerases preferentially inserts dT opposite the lesion. Surprisingly, in striking contrast to other polymerases, we also find that the “correct” base pair, dC:m^6dG is preferentially extended relative to the “incorrect” base pair, dT:m^6dG.

EXPERIMENTAL PROCEDURES

Materials— Purified human pol β was very generously supplied by Dr. Samuel Wilson (Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, TX). Kleen fragment of Escherichia coli DNA polymerase I (KF) was purchased from Boehringer Mannheim Biochemicals. The exonuclease deficient Kleen fragment of E. coli DNA polymerase I (exo KF) was purchased from U. S. Biochemical Corp. T7 DNA polymerase and T4 polynucleotide kinase were purchased from New England Biolabs. Primer-Template Annealing— The 32P-labeled primers were annealed to the normal and m^6dG containing templates (100–200 pmol/reaction) in a hybridization buffer containing 20 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 50 mM NaCl, and 1 mM dithiothreitol (DTT). The primer to template ratio was 2:1 (2:1 in some starting point extension studies). The reaction mixture was incubated for 5 min at 90 °C and then cooled slowly to room temperature over a period of about 2 h.

FIG. 1. DNA primers and templates for the in vitro DNA replication system. Sequences of the 44-mer templates are written 3′ to 5′ to show complementation with the primers. X equals either m^6dG or G.

The in vitro DNA replication reaction (Fig. 1) consists of short oligonucleotide primers and complementary templates containing either m^6dG (abbreviated as X) or dG at a defined site in the template strand. The 44-mer template is a biologically relevant M13 DNA sequence (nucleotides 118 to 152 of the minus strand).

Primer End Labeling—The primers were 5′ end-labeled by T4 polynucleotide kinase using [γ-32P]ATP as described by Sambrook et al. (1989).

Primer-Template Annealing—The 32P-labeled primers were annealed to the normal and m^6dG containing templates (100–200 pmol/reaction) in a hybridization buffer containing 20 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 50 mM NaCl, and 1 mM dithiothreitol (DTT). The primer to template ratio was 2:1 (2:1 in some starting point extension studies). The reaction mixture was incubated for 5 min at 90 °C and then cooled slowly to room temperature over a period of about 2 h.

Primer Extension Studies—10–100 pmol of 32P-labeled primer-template were replicated with different DNA polymerases for different times at 37 °C in 10-μl reactions containing variable concentrations of dNTPs. Primer extension with pol β was carried out in the presence of 50 mM Tris-HCl (pH 7.5), 2 mM DTT, and 10 mM MgCl2. Primer extension with KF or exo KF was carried out in the presence of 10 mM Tris-HCl (pH 7.8), 7 mM MgCl2, and 12 mM DTT. Replication with T7 DNA polymerase was carried out in the presence of 40 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM DTT, and 0.1 mg/ml bovine serum albumin.

Primer extension reactions were stopped by the addition of stop solution containing 20 mM EDTA in 90% formamide and kept on ice. Before loading onto the gel, the reactions were denatured by heating at 100 °C for 5 min. The replication products were resolved on 8% polyacrylamide gels and the bands were visualized by autoradiography. Autoradiograms were scanned to quantitate the bands using the BioImage Application system (Millipore Corp., Ann Arbor, MI). Film exposures were such that the intensities of the bands to be quantitated were well within the linear range of the apparatus. Relative velocity was determined by the ratio of integrated optical density of pol β to pol α (37). Time course studies (data not shown) indicated that under our standard conditions using pol β, the reaction velocity is linear (i.e. obeys steady state kinetics) for up to 5 min. Sequencing—Primed templates were sequenced using Sanger’s dideoxynucleotide sequencing method (41). In short, 3 units of Sequenase 2.0, 40 μM of all four dNTP’s, 40 μM each dideoxynucleotide triphosphate, 50 mM NaCl, 3.6 mM MnCl2, and 16.7 mM DTT were mixed and primer extension was carried out for 5 min before termination of the reaction by the addition of stop solution. The sequencing lanes were run side-by-side with the primer extension reactions on denaturing polyacrylamide gels, as described above.

RESULTS

Replication of Normal and m^6G-modified Templates by pol β—We have performed in vitro DNA replication studies of primed normal and m^6dG modified templates by pol β and three other DNA polymerases in the presence of all 4 dNTPs. The polymerases encounter m^6dG after a four-base running start. Fig. 2 shows the replication of normal (TG) and m^6G-modified (TXG) 44-mer templates by pol β, KF, exo KF, and T7 DNA polymerases. During replication of the TXG template, each of the polymerases exhibited pausing immediately before and at m^6dG, as well as bypass replication. pol β was blocked by m^6dG to a greater extent than KF and exo KF, while T7 polymerase was blocked the most. In this experiment, since the
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FIG. 2. Replication of the 20-mer-44TXG primer-template by different DNA polymerases. Lane 1 shows the replication of the control template 44TGG by pol β (0.3 unit). Note that a small amount of fully extended primer can be seen. Lane 2 shows replication of the 44TXG template by pol β showing very limited bypass of the m6dG lesion. Lanes 3–5 show replication of the 44TXG template by exo-κF (4.6 units), κF (4.3 units), and T7 polymerase (4.4 units), respectively. Enzyme concentrations were varied in order to keep the total amount of primer extension approximately equal. In the experiments shown here the amount of extended primer ranged from 41% (exo-κF) to 74% (T7 polymerase) with κF and pol β at 53 and 56%, respectively. dNTP concentrations were kept at 20 μM each and the reaction time was 5 min at 37°C. The ability to bypass the m6dG lesion varied in the order exo-κF (63% ± 2%) > κF (41% ± 6%) > pol β (18 ± 7%) > T7 (7 ± 2%). X denotes m6dG. The sequences shown are those of the template DNA.

FIG. 3. Running start nucleotide insertion by pol β. Representative gel autoradiograms showing (A) insertion of dCMP opposite template dG and (B) insertion of dTMP opposite m6dG by pol β (0.3 unit). For A the primer template was 20-mer-44TGG (10 pmol) and dATP and dTTP were present at a concentration of 50 μM each. Incubation time was 0.5 min at 37°C. For B the primer-template was 20-mer-44TXG (10 pmol) and each of the other three normal dNTPs were present at a concentration of 50 μM. Incubation time was 5 min at 37°C. X denotes m6dG. The sequences shown are those of the template DNA.

concentration of pol β is limiting, intermediate pause sites as indicated by weak intensity bands in the control (TGG) lane (lane 1) indicate that pol β is functioning in a slightly processive mode. Each of the polymerases is able to bypass (replicate beyond) m6dG, although with limited efficiency. The relative extension past the lesion varied with each polymerase in the order: exo-κF (63%), followed by κF (41%), then pol β (18%), and most poorly by T7 polymerase (7%).

Steate State Kinetics of Nucleotide Insertion Opposite dG and m6dG—The kinetic parameters of nucleotide insertion opposite dG and m6dG by pol β in a running start mode were determined by individually varying concentrations of dCTP and dTTP. Typical autoradiograms of gels showing dose-dependent insertion of dCMP opposite template dG and dTMP opposite m6dG are shown in Fig. 3. Fig. 3A shows efficient insertion and extension opposite a normal base pair, while Fig. 3B shows reduced insertion of dTMP opposite the modified base in the presence of each of the other three dNTPs. None of the other nucleotides competed significantly with dTTP for insertion opposite m6dG (i.e. no band at m6dG in was seen absence of dTTP). Fig. 3B also illustrates the preleision block at dT, the 3’ neighboring base to m6dG. Fig. 4, A and B, show, respectively, the Hanes-Woolf plots of insertion of dTMP and dCMP opposite m6dG by pol β in a running a start mode. The kinetic parameters of nucleotide insertion opposite dG and m6dG are presented in Table I.

Insertion of dCMP opposite dG was chosen as the standard determination with a relative efficiency value of 1.0 to allow for comparison between insertion of dCMP and dTMP opposite both template dG and m6dG. For the correct (dCMP) versus incorrect (dTMP) insertion opposite the normal base, dG, in the running start mode the Vmax(rel) discrimination is only 5-fold (39.4 rIOM min⁻¹ for dCMP versus 8.5 rIOM min⁻¹ for dTMP) while there is a very strong, 8000-fold, Km(app) bias (1.6 μM for dCMP versus 12.9 μM for dTMP) (Table I). The predominantly Km(app)-mediated discrimination suggests that binding of the incoming dTTP to pol β-primer-template complex and formation of the T-G base pair is highly inefficient because T-G is an unstable, non-Watson-Crick (wobble) type of base pair (42).

The Vmax(rel) for nucleotide insertion opposite m6dG by pol β is drastically reduced compared to insertion opposite template dG (i.e. Vmax(rel) discrimination) (Table I). There is an almost 1000-fold decrease in the Vmax(rel) for insertion of dCMP opposite m6dG (0.04 rIOM min⁻¹ versus dG (39.4 rIOM min⁻¹), and a 71-fold decrease in the Vmax(rel) for insertion of dTMP opposite m6dG (0.12 rIOM min⁻¹ versus dG (8.5 rIOM min⁻¹) (Table I). This indicates that the new phosphodiester bond 5’ to dC or dT paired with m6dG forms with difficulty, probably because of sterochemical hindrances due to the base pairing of the incoming nucleotide with m6dG (42) or because of interference with hydrogen bonding between arginine 283 of the polymerase and the template base (43). Meanwhile, the Km(app) for insertion of dCMP opposite m6dG (83.5 μM) is 52-fold greater than the Km(app) for insertion of dCMP opposite dG (1.6 μM) (Table I). This indicates that pol β employs a mixed (Km(app) and Vmax(rel) based) discrimination (with Vmax(rel) bias playing a predominant role) to reduce the efficiency (an index of the ease of the given step in DNA replication) of insertion of dCMP opposite m6dG by 10^-2-fold relative to its insertion opposite normal dG. It is striking that when the template base is m6dG instead of dG, the discrimination between insertion of dCMP versus dTMP is mixed, but milder and reversed (i.e. both Vmax(rel) and Km(app) differences are seen during the insertion of dCMP opposite dG and during the insertion of dTMP opposite m6dG) (Table I). However, there is a drastic 1700-fold increase in the Km(app) for insertion of dTMP opposite dG (12.9 μM) relative to the insertion of dTMP opposite m6dG (7.6 μM) (Table I). This is consistent with the formation of a relatively unstable T-G wobble base pair compared to the formation of a relatively stable Watson-Crick alignment for the incoming dTTP base pair with m6dG. This inference from kinetic replication studies is supported by structural NMR studies (42). It is notable that the overall efficiency of insertion of dTMP opposite m6dG is 24-fold greater than the insertion of dTMP opposite dG (Table I).

In summary, pol β has a 10-fold lower Km(app) and a 3-fold higher Vmax(rel) for insertion of the mutagenic nucleotide dTMP opposite m6dG as compared to insertion the nonmutagenic nucleotide dCMP. With this mixed Km(app) and Vmax(rel) bias, pol β has greater than a 33-fold greater efficiency for the insertion of dTMP relative to the insertion of dCMP opposite
m<sup>6</sup>dG (Table I). Thus, pol β suffers a decrease in both efficiency and fidelity during nucleotide insertion opposite m<sup>6</sup>dG relative to insertion opposite dG.

**Standing Start Insertion Opposite m<sup>6</sup>dG**—Although running start insertion is the more physiological mode of replication, we also investigated the effect of m<sup>6</sup>dG on the kinetics of standing start nucleotide insertion by pol β using 24 nucleotide primers ending immediately prior (5′) to the m<sup>6</sup>dG in the template strand (Table II). Using these primer-templates, we again found that discrimination between dCTP and dTTP for insertion opposite the normal dG is mediated by differences in kinetic parameters of base pair extension are tabulated in Table III. In the standing start mode, there is no significant difference in the K<sub>m(app)</sub> and only a 2-fold difference in the V<sub>max</sub> for extension from dC-dG and dC-m<sup>6</sup>dG base pairs by pol β (Table II). Thus the efficiency varies only by 2-fold. However, the K<sub>m(app)</sub> for extension from dT-m<sup>6</sup>dG (21.6 μM) is 7-fold less than that from dT-dG (142 μM) (Table II). Concomitantly, the V<sub>max</sub> for extension from dT-m<sup>6</sup>dG (0.11 rIOD min<sup>-1</sup>) is 3-fold higher than that from dT-dG (0.03 rIOD min<sup>-1</sup>) (Table II). This mixed discrimination leads to a 24-fold preference for extension from dT-m<sup>6</sup>dG rather than dT-dG. The K<sub>m(app)</sub> values for extension from dC-m<sup>6</sup>dG versus dT-m<sup>6</sup>dG are similar. However, the V<sub>max</sub> for extension from dC-m<sup>6</sup>dG (0.21 rIOD min<sup>-1</sup>) is 2-fold higher than that from dT-m<sup>6</sup>dG (0.11 rIOD min<sup>-1</sup>) (Table II). This leads to an overall 2-fold preference for standing start extension from dC-m<sup>6</sup>dG versus dT-m<sup>6</sup>dG. The relative efficiency of extension from the four terminal base pairs varies in the order: dC-dG > dC-m<sup>6</sup>dG > dT-m<sup>6</sup>dG > dT-dG.

**Running Start Extension**—Running start extensions from dC-dG, dC-m<sup>6</sup>dG, and dT-m<sup>6</sup>dG base pairs with dCTP as the next nucleotide substrate were also evaluated (results not shown). A comparison of the kinetics of running start extension from dC-dG and dC-m<sup>6</sup>dG base pairs also shows 2-fold overall preference for extension from dC-dG versus dC-m<sup>6</sup>dG. Running start extension from dC-m<sup>6</sup>dG (K<sub>m(app) = 1.1 μM) and dT-m<sup>6</sup>dG (K<sub>m(app) = 13.5 μM) shows an exclusively K<sub>m(app)</sub> based 13-fold preference of extension from dC-m<sup>6</sup>dG versus dT-m<sup>6</sup>dG. This bias is much more pronounced than, but in agreement with, the 2-fold preference for standing start extension from dC-m<sup>6</sup>dG versus dT-m<sup>6</sup>dG. For all three base pairs: dC-dG, dC-m<sup>6</sup>dG, and dT-m<sup>6</sup>dG.
Experiments were performed as described under “Experimental Procedures” using 10 pmol of 24-mer primer hybridized to the 44TXG template with limiting enzyme concentration (0.7 unit/reaction) and varying the appropriate dNTP.

| Template base | dNTP | $K_{m(app)}$ | $V_{max(rel)}$ | $f$ | Relative $f$ |
|---------------|------|-------------|---------------|-----|-------------|
| dG           | dCTP | 1.52 ± 0.15 | 9.2 ± 0.2     | 6.1 | 1           |
| dG           | dTTP | 950 ± 390   | 10.1 ± 2.5    | $3.1 	imes 10^{-2}$ | $2.1 	imes 10^{-3}$ |
| mG           | dCTP | 5.04 ± 0.91 | 0.022 ± 0.001 | $4.4 	imes 10^{-3}$ | $7.2 	imes 10^{-4}$ |
| mG           | dTTP | 8.30 ± 4.18 | 0.16 ± 0.03   | $1.9 	imes 10^{-2}$ | $3.1 	imes 10^{-3}$ |

**Fig. 5. Time course of extension from dC-m6dG and dT-m6dG by exo-KF and pol β.** Extension from dC-m6dG and dT-m6dG terminal base pairs by A, exo KF (2.6 units), and B, pol β (0.2 unit) was determined as a function of time. The primer-templates (10 pmol each) were 5'-end-labeled 25-mer (C) hybridized to the 44TXG template (25-mer(C)-44TXG) and the 5'-labeled 25-mer(T)-44TXG, respectively. dATP, dCTP, and dTTP were present at a concentration of 10 μM each.

**DISCUSSION**

m6dG Lesions Inhibit Repair Replication by pol β—pol β is likely to replicate across persistent m6dG lesions in vivo when MGMT-mediated repair is saturated or lacking. We have performed in vitro kinetic studies to characterize the mechanisms of replication across site-specific m6dG adducts by pol β. Our results suggest that pol β, in concert with the G:T-specific thymine glycosylase, may contribute to the cytotoxic repair induced by m6dG. Our results also indicate that the infrequent bypass replication across m6dG by pol β may lead to insertion of dTMP opposite m6dG and thus contribute toward G → A mutagenesis by methylating agents in vivo.

pol β was previously reported to be unable to bypass m6dG (46, 47). We have demonstrated that pol β is able to bypass m6dG, but at a much reduced efficiency. In the study conducted by Aebkes et al. (47), m6dG was located in a run of dTs, and the activating metal ion was manganese. In our experiments m6dG is located in a heterogeneous sequence context (Fig. 1) and the activating metal ion is magnesium. It is interesting to note that...
Preferential Insertion of dTMP opposite m6dG and Extension from dC-m6dG—pol β has a 33-fold preference for insertion of the mutagenic nucleotide substrate dTMP, rather than the nonmutagenic dCMP, opposite m6dG. In this respect pol β is similar to KF, T4 and T5 polymerases which also preferentially insert dTMP (17–19). On the other hand, pol β has an opposing 2-fold (13-fold from a running start) preference for extension from dC-m6dG rather than dT-m6dG. This contrasts with the 10-fold preferential extension from dT-m6dG by exo-KF, whereas previously published experiments using KF in difference sequence contexts have observed up to a 3-fold preference for extension from dT-m6dG (45). The efficiency of incorporation of the next correct nucleotide to extend a terminal base pair depends on the degree of base pairing stability of the terminal base pair and the ensuing local helical distortion. Phosphodiester bonds 3’ and 5’ to dC paired with m6dG have been shown to be distorted (42). The observation that dC-m6dG is extended almost as well as dC-dG suggests that, in contrast to KF and exo-KF, pol β is able to extend from poorly bonded terminal base pairs and is relatively insensitive to local helical perturbations.

The finding that pol β extends from dC-m6dG better than from dT-m6dG is difficult to rationalize in view of NMR data which shows that the dT-m6dG base pair more stable, has Watson-Crick alignment, and the alkyl group of m6dG has an anti orientation, causing less distortion of local DNA structure, while dC-m6dG is a less stable wobble base pair, and the alkyl group of m6dG has an syn orientation which causes more distortion of local DNA structure (42, 52). The lack of 3’-5’ exonuclease function, lower processivity, fidelity, and other less understood features of pol β may be involved in imparting such reduced and reversed discrimination during extension from this unusual base pair. It has been previously reported that pol β efficiently extends from primer templates with one or more terminal mismatches (53).

Preferential Incorporation of dTMP during Overall Replication across m6dG—With regard to the overall replication across m6dG, pol β has a 16-fold preference for incorporating dTTP rather than dCTP. This mutagenic bypass replication of m6dG...
is 10⁴-fold less efficient than replication across dG. This indicates that pol β is strongly, but not completely, blocked at m⁶dG and that bypass replication is predominantly mutagenic and would contribute to production of G to A mutations in vivo.

pol β exhibits mixed kinetic discrimination (i.e., both $K_{\text{m(app)}}$ and $V_{\text{max(rel)}}$ differences) and decreased fidelity during replication across template m⁶dG. In agreement with our observations, Boosalis *et al.* (54) have also reported a mixed discrimination by pol β. pol β employs a more rigorous kinetic discrimination (i.e., severalfold differences in $K_{\text{m(app)}}$ and $V_{\text{max(rel)}}$) during the initial nucleotide insertion step that contrasts with the much decreased discrimination during subsequent base pair extension. Our results show that enhanced kinetic discrimination may not always be concordant with fidelity (in terms of mutagenesis). pol β exhibits reduced fidelity but greater kinetic discrimination during nucleotide insertion opposite m⁶dG, but has greater fidelity and reduced kinetic discrimination during subsequent base pair extension from m⁶dG.

### Table V

| Terminal base pair | Primer-template | $K_{\text{m(app)}}$ (μM) | $V_{\text{max(rel)}}$ | $f$ | Relative $f$ |
|-------------------|-----------------|--------------------------|----------------------|----|-------------|
| dC-dC             | 25C-TGG         | 0.47 ± 0.35              | 8.3 × 10⁻¹          | 1.0| 1.0         |
| dT-dT             | 25T-TGG         | 146 ± 24                 | 3.4 × 10⁻³          | 4.1 × 10⁻³ | 4.1 × 10⁻³ |
| dC-m⁶dG           | 25C-TXG         | 29.9 ± 1.6               | 2.1 × 10⁻¹          | 2.5 × 10⁻² | 2.5 × 10⁻² |
| dT-m⁶dG           | 25T-TXG         | 2.83 ± 0.39              | 1.8 × 10⁻¹          | 2.2 × 10⁻¹ | 2.2 × 10⁻¹ |

### Table VI

| Base pair | $f_1$ (insertion) | $f_2$ (extension) | $f_1 \times f_2$ | Relative $f_1 \times f_2$ |
|-----------|-------------------|-------------------|------------------|--------------------------|
| dCMP → dG | 1.3               | 8.3 × 10⁻¹        | 1.08             | 1.0                      |
| dTMP → dG | 3.0 × 10⁻³        | 3.4 × 10⁻³        | 1.0 × 10⁻⁵       | 9.4 × 10⁻⁶               |
| dCMP → m⁶dG| 1.2 × 10⁻³        | 2.1 × 10⁻²        | 2.5 × 10⁻⁵       | 2.3 × 10⁻⁵               |
| dTMP → m⁶dG| 3.1 × 10⁻¹        | 1.8 × 10⁻¹        | 5.6 × 10⁻²       | 5.2 × 10⁻²               |

### Table VII

| Favored versus unfavorable event | $K_{\text{m(app)}}$ (μM) | $V_{\text{max(rel)}}$ | $f$ |
|---------------------------------|--------------------------|----------------------|----|
| dCMP versus dTMP opposite dG    | ++                        | ++                   | + ++ ++ ++ |
| dTMP versus dCMP opposite m⁶dG  | +                         | ++                   | ++ ++ ++ ++ |
| dCMP opposite dG versus m⁶dG    | ++                        | +                    | ++ ++ ++ ++ |
| dTMP opposite m⁶dG versus dG    | ++                      | +                    | ++ ++ ++ ++ |

The role of *pol β* in the futile repair of m⁶dG—During genomic replication by replicative polymerases, dTMP may preferentially incorporate opposite persisting m⁶dG lesions. The dTMP/m⁶dG base pair is recognized by a G:T-specific thymine glycosylase which preferentially excises the thymine (31). The subsequent action of AP endonucleases creates a short gap. When filling the short gap containing m⁶dG, pol β preferentially incorporates dTMP opposite m⁶dG with a low efficiency. The dT-m⁶dG base pair is not efficiently extended, thus leaving a long-lived gap or nick. Once the nick is sealed, the G:T-specific thymine glycosylase may again excise the thymine opposite the m⁶dG, followed by another round of action of AP endonucleases (and/or pol β/deoxyribonuclease V), gap filling by pol β, etc. This futile cycling of the repair process (32) may sequester the repair machinery at m⁶dG lesions. The resulting long lived gaps may also induce sister chromatid exchanges and other types of cytotoxic responses. Incision by thymine glycosylase has also been shown in different DNA environments to be either sensitive or insensitive to the nature of the 5’ neighboring base to dG or m⁶dG base paired with T (55, 56). Thus the local DNA sequence may modulate the cytotoxicity of m⁶dG during the futile cyclic repair mechanism.

**Summary**—This is the first report to dissect the kinetic parameters of replication across m⁶dG by pol β in *vitro*. Our results are physiologically significant since they provide mechanistic insight into the involvement of pol β in the futile repair of m⁶dG that may lead to cytotoxicity. This study also provides a basis to explain how pol β may be partially blocked by m⁶dG and contribute to the mutagenesis of m⁶dG in *vitro*. Moreover, this study provides important functional and mechanistic information regarding the kinetic discrimination, fidelity, and processivity of pol β during *in vitro* replication across normal and miscoding template bases.
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