Efficient and accurate bypass of N^2-(1-carboxyethyl)-2′-deoxyguanosine by DinB DNA polymerase *in vitro* and *in vivo*

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DinB, a Y-family DNA polymerase, is conserved among all domains of life; however, its endogenous substrates have not been identified. DinB is known to synthesize accurately across a number of N^2-dG lesions. Methylglyoxal (MG) is a common byproduct of the ubiquitous glycolysis pathway and induces the formation of N^2-(1-carboxyethyl)-2′-deoxyguanosine (N^2-CEdG) as the major stable DNA adduct. Here, we found that N^2-CEdG could be detected at a frequency of one lesion per 10^7 nucleosides in WM-266-4 human melanoma cells, and treatment of these cells with MG or glucose led to a dose-responsive increase in N^2-CEdG formation. We further constructed single-stranded M13 shuttle vectors harboring individual diastereomers of N^2-CEdG at a specific site and assessed the cytotoxic and mutagenic properties of the lesion in wild-type and bypass polymerase-deficient *Escherichia coli* cells. Our results revealed that N^2-CEdG is weakly mutagenic, and DinB (i.e., polymerase IV) is the major DNA polymerase responsible for bypassing the lesion *in vivo*. Moreover, steady-state kinetic measurements showed that nucleotide insertion, catalyzed by *E. coli* pol IV or its human counterpart (i.e., polymerase κ), opposite the N^2-CEdG is both accurate and efficient. Taken together, our data support that N^2-CEdG, a minor-groove DNA adduct arising from MG, is an important endogenous substrate for DinB DNA polymerase.

glycolysis | mutagenesis | polymerase κ | translesion synthesis

Living cells are constantly exposed to environmental and endogenous agents, which can damage DNA (1). To counteract the deleterious effects of DNA lesions, cells have evolved an intricate DNA repair system (2). When DNA repair is not efficient enough, the presence of unrepaired DNA lesions in replicating DNA may lead to replication fork stalling, thereby inducing cell death. It was proposed that, when a high-fidelity replication fork is arrested at the lesion site, translesion synthesis polymerases can take over from replicative polymerases temporarily to bypass synthetically the lesion residing in the template (2, 3). Among the Y-family DNA polymerases, DinB [also known as polymerase (pol) IV in *Escherichia coli* and pol κ in mammalian cells] is conserved among all domains of life; however, the basis for this marked conservation remains unclear (4). In particular, the physiological substrates for this polymerase remain to be identified.

Similar to eukaryotic pol η, which is specialized in bypassing efficiently and accurately TT cyclobutane pyrimidine dimer (5–7), *E. coli* pol IV and human pol κ can insert preferentially the correct nucleotide, dCMP, opposite a number of N^2-dG lesions (8, 9). In addition, DinB polymerase is capable of bypassing the N^2-dG adduct induced by benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE) (10–14).

Aside from the difference in substrate specificity, *E. coli* pol IV distinguishes from pol V in expression levels (4). In uninduced cells, pol V is not detectable by Western analysis, whereas pol IV can be detected at a level of ≈250 copies per cell. Upon SOS induction, there are only ≈15 molecules of pol V per cell; however, the level of pol IV reaches ≈2,500 copies per cell (4). The high level of expression of pol IV suggests there is an important, basic, and yet-to-be-discovered function of pol IV in general metabolism (4).

In addition to reactive oxygen species, which constitutes a major endogenous source of DNA damage (1, 15), genomic DNA in living cells is susceptible to damage from exposure to reactive carbonyl species, and methylglyoxal (MG) is one of them. In this respect, MG can be produced endogenously in all cells and all organisms from the nonenzymatic fragmentation of triose phosphates, which include glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, and are produced as metabolites of the highly conserved glycolysis pathway (16–18). In this regard, treatment of human red blood cells with increasing concentrations of glucose *in vitro* can result in increases of intracellular MG concentration (19). The MG concentration was also found to be elevated in the kidney (cortex and medulla), lens, and blood of streptozotocin-induced diabetic rats (20) and in blood samples of diabetic patients (21).

N^2-(1-carboxyethyl)-2′-deoxyguanosine (N^2-CEdG) was the major stable adduct formed in calf thymus DNA upon exposure to MG at physiological concentration and temperature (Fig. 1) (22). A competitive enzyme-linked immunosorbant assay showed that the adduct can be detected in urine samples of 121 healthy human subjects at levels ranging from 1.2- to 117-ng N^2-CEdG equivalent per milligram of creatinine (23). Immunohistochemistry using a monoclonal antibody against N^2-CEdG revealed that the level of the lesion is enhanced in the kidney and aorta of patients with diabetic nephropathy and uremic atherosclerosis, respectively (24).

DNA adducts arising from MG could induce mutations in *E. coli* cells and G → C and G → T transversions in *supF* gene in mammalian cells (25, 26). However, the lesion-containing DNA substrates used in these previous mutagenesis experiments were prepared by the direct treatment of undamaged DNA with dihydroxyacetone or MG (25, 26); thus, the identity and homogeneity of the DNA adducts were not carefully assessed. In light of the previous findings that DinB is capable of bypassing, in an error-free fashion, a number of N^2-dG lesions (8–14), we reason that this polymerase may also be involved in the bypass of N^2-CEdG.

In the present study, we prepared single-stranded M13 genomes carrying individual diastereomers of N^2-CEdG and assessed the cytotoxic and mutagenic properties of the lesion in *E. coli* cells. Our results showed that pol IV was the major poly-

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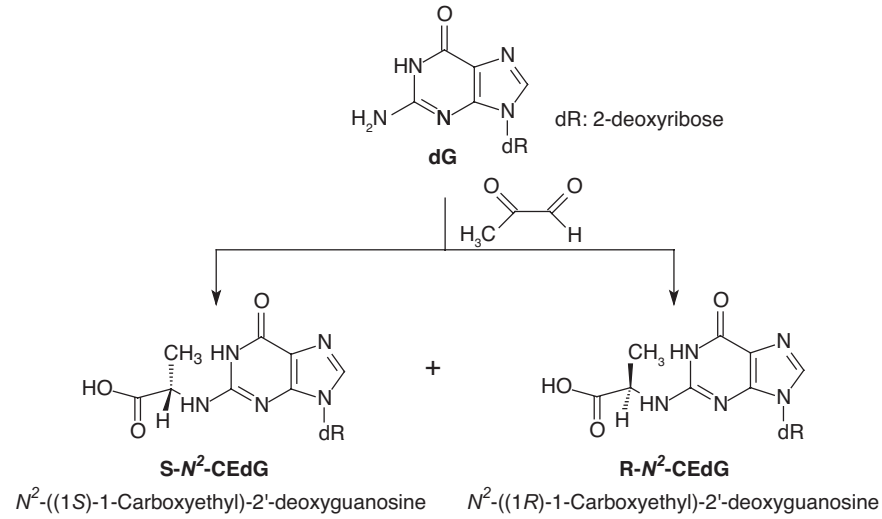

Fig. 1. Formation of N^2-CEdG.

merase responsible for the error-free bypass of N^2-CEdG *in vivo*. Steady-state kinetic measurements revealed that the nucleotide incorporation, catalyzed by *E. coli* pol IV or its human homolog (i.e., pol κ), is both accurate and efficient. In addition, N^2-CEdG could be detected in untreated human cells, and exposure of cells to glucose or MG enhanced the formation of N^2-CEdG. These observations support that N^2-CEdG is an important endogenous substrate for DinB DNA polymerase.

Results

To explore the possibility that N^2-CEdG might constitute an endogenous substrate for DinB DNA polymerase, we first assessed the formation of N^2-CEdG in WM-266-4 human melanoma cells that are either untreated or treated with glucose or MG. LC-MS/MS analysis using the accurate isotope-dilution method revealed that both diastereomers of N^2-CEdG could be detected in untreated cells at a level of one lesion per 10^7 nucleosides [Fig. 2; LC-MS/MS data and calibration curves are shown in supporting information (SI) Figs. S1 and S2]. In addition, incubation of the melanoma cells with MG led to a dose-dependent increase in the level of N^2-CEdG (Fig. 2).

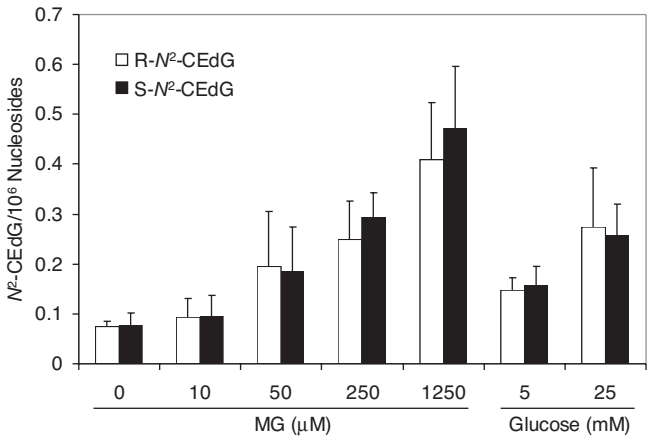

Fig. 2. The dose-dependent formation of N^2-CEdG in WM-266-4 cells, either untreated or treated with MG for 3 h or D-glucose for 5 days. The concentrations of MG and D-glucose are indicated. The data represent the mean and standard deviations of results from three independent cell culture and treatments.

Moreover, culturing of these cells in media containing 5 and 25 mM glucose for 5 days resulted in the increase of the levels of the lesions to approximately 2.5 and 4 lesions per 10^7 nucleosides (Fig. 2). In this context, it is worth noting that, in response to change in blood glucose level, glucose transporters are regulated in some types of cells (27). Therefore, the intracellular glucose concentration in WM-266-4 cells may not increase proportionally with the glucose concentration in the culture media, which may explain why the levels of N^2-CEdG lesions induced in glucose-treated cells are not proportional to the applied glucose dose. Taken together, both diastereomers of N^2-CEdG can be induced endogenously in WM-266-4 human melanoma cells, and the exposure of these cells to MG or glucose further enhances the formation of N^2-CEdG. In this context, it is worth noting that the two diastereomers of N^2-CEdG can also be detected readily in HeLa-S3 cells (data not shown).

We next asked how the presence of N^2-CEdG compromises DNA replication, and which SOS-induced DNA polymerase is involved in bypassing the lesion in *E. coli* cells. To this end, we synthesized oligodeoxyribonucleotides (ODNs) harboring a site-specifically incorporated S- or R-N^2-CEdG, following our procedures (28). We then ligated the ODNs into single-stranded M13 genome and assessed the bypass efficiencies and mutation frequencies of the two diastereomers by using the competitive replication and adduct bypass (CRAB) and restriction endonuclease and postlabeling (REAP) assays introduced by Essigmann and coworkers (Fig. 3) (29–31).

If there is no deletion mutation, restriction digestion of the PCR products of the progeny M13 genome arising from the replication of the lesion-carrying vector affords a 8-mer fragment harboring the site where the N^2-CEdG was initially incorporated. The corresponding digestion of PCR products of the progeny of the competitor genome gives an 11-mer fragment (Fig. 3). The failure to detect any radiolabeled fragments with lengths shorter than 8 mer supports that neither diastereomer gives rise to deletion mutations (Fig. S3). The bypass efficiency can be calculated from the ratio of the 8- over the 11-mer product with the consideration of the genome ratio used in the initial transfection experiment. The bypass efficiency for the lesion-carrying genome is then normalized against that for the control lesion-free genome (Fig. 4*A*).

It turned out that the bypass efficiencies for S- and R-N^2-CEdG in wild-type AB1157 cells are ≈75% and 39%, respectively; deficiency in pol II or V in the isogenic AB1157 background does not appreciably affect bypass efficiencies for the two

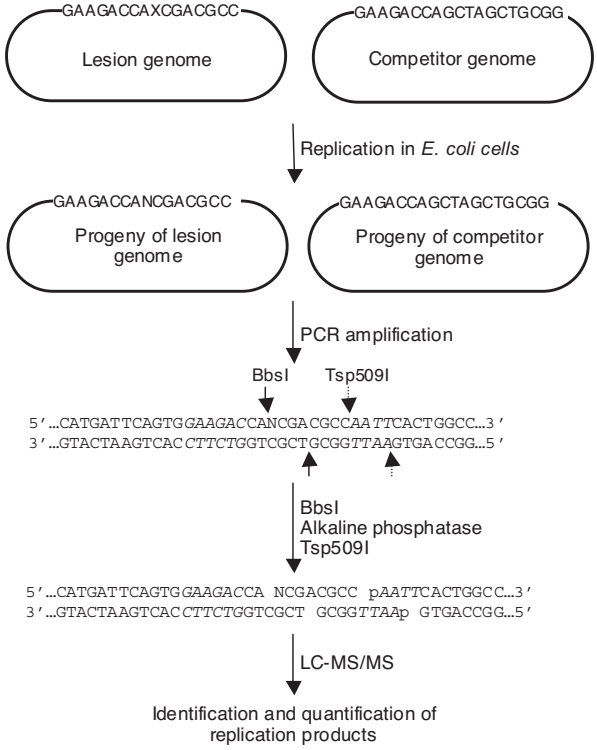

Fig. 3. Method for the determination of the cytotoxicity and mutagenicity of N^2-CEdG in *E. coli* cells. "X" in the 16-mer ODN represents S-N^2-CEdG, R-N^2-CEdG or unmodified dG. "N" in the progeny of lesion genome represents the nucleoside inserted at the initial lesion site. BbsI and Tsp509I restriction endonuclease recognition sites are indicated in italic, and the cleavage sites induced by the two enzymes are designated by solid and broken arrows. Only partial sequences of the PCR products for the lesion genome are shown, and the PCR products of competitor genome are not depicted.

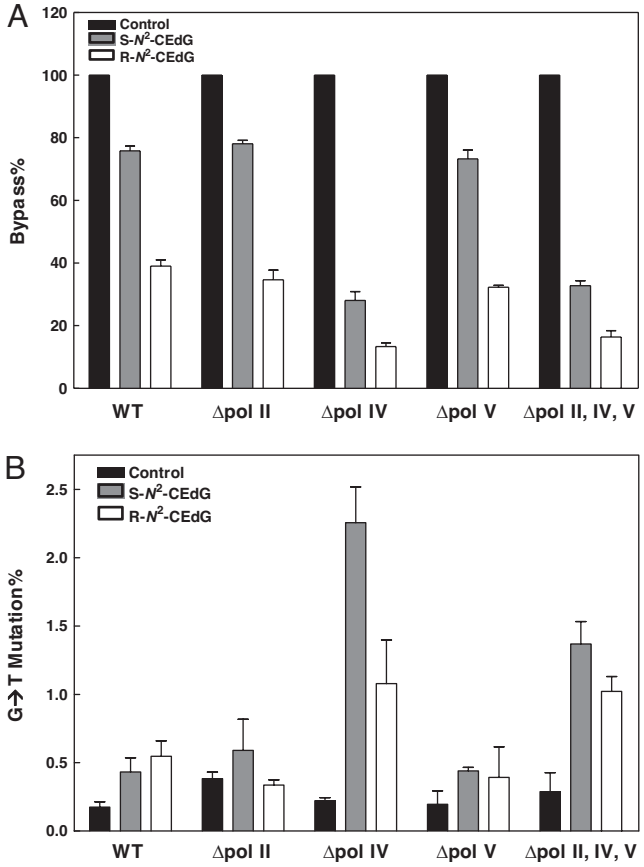

Fig. 4. Bypass efficiencies (*A*) and mutation frequencies (*B*) of dG, S-N^2-CEdG, and R-N^2-CEdG lesions in wild-type; pol II-, pol IV-, and pol V-deficient; and triple-knockout AB1157 *E. coli* cells. Black, gray, and white columns represent the results for substrates carrying dG, S-N^2-CEdG and R-N^2-CEdG, respectively. The data represent the mean and standard deviations of results from three independent experiments.

diastereomers of N^2-CEdG (Fig. 4*A*). In stark contrast, the bypass efficiencies dropped to ≈28% and 13%, respectively, in the corresponding pol IV-deficient cells (Fig. 4*A*). Therefore, pol IV is the major DNA polymerase involved in the bypass of N^2-CEdG in *E. coli* cells. In addition, regardless of the *E. coli* strains used, the bypass efficiency for R-N^2-CEdG is approximately one-half of that for S-N^2-CEdG (Fig. 4*A*), demonstrating that R-N^2-CEdG is a stronger block to DNA replication than S-N^2-CEdG. Moreover, the bypass efficiencies for N^2-CEdG were similar in pol IV-deficient and triple-knockout AB1157 cells (Fig. 4*A*), underscoring the lack of involvement of pol II and V in bypassing the N^2-CEdG *in vivo*. We also measured the bypass efficiencies by using the recently introduced LC-MS/MS method (32), and the results are in keeping with those measured by using the conventional CRAB assay (Fig. S4).

We then assessed the mutation frequencies of N^2-CEdG in wild-type and bypass polymerase-deficient *E. coli* strains with the REAP assay (29, 31), and we used LC-MS/MS for interrogating the replication fragments (Fig. 3) (32). In this respect, the restriction digestion mixture was analyzed by LC-MS/MS, and we monitored the fragmentation of the $[M–2H]^{2-}$ ions of d(NC-GACGCC), where "N" is an A, T, C, or G. It turned out that only d(GCGACGCC) and d(TCGACGCC) could be detected in the digestion mixture. We then quantified the relative amounts of different replication products with the consideration of the difference in ionization efficiencies for different ODNs [LC-MS/MS for monitoring the formation of d(GCGACGCC) and d(TCGACGCC) are shown in Fig. S5, and calibration curves are depicted in Fig. S6]. Our results revealed that the two diastereomers of N^2-CEdG are weakly mutagenic in wild-type AB1157 cells, and the deficiency in pol II or V does not confer apparent increase in mutation frequency (Fig. 4*B*). However, deficiency in pol IV causes considerable increase in G → T mutation. Moreover, the mutation frequency induced by R-N^2-CEdG is approximately one-half of that by S-N^2-CEdG in the pol IV-deficient background (1.1% and 2.3%, respectively; Fig. 4*B*). Along this line, triple-knockout cells again exhibited significant increase in G → T mutation relative to the wild-type strain (Fig. 4*B*). On the grounds that low mutation frequencies were found for N^2-CEdG, we may conclude that DinB plays an important role in avoiding the cytotoxic, rather than the mutagenic, effects of N^2-CEdG and its structurely related derivatives.

The above results unveiled that approximately two-thirds of the bypass of N^2-CEdG in AB1157 *E. coli* cells requires pol IV, and the deficiency in pol IV leads to a marked increase in mutation frequency. Therefore, the pol IV-mediated bypass of N^2-CEdG in *E. coli* cells is error-free. To further substantiate this conclusion, we assessed quantitatively the efficiency and fidelity of pol IV-mediated nucleotide insertion opposite both S- and R-N^2-CEdG by using the steady-state kinetic measurements (Figs. S7 and S8 and Table 1) (33, 34). It turned out that the nucleotide insertion opposite both diastereomers of N^2-CEdG is remarkably accurate, with misinsertion frequencies similar to those found for nucleotide incorporation opposite an unmodified dG. Furthermore, the replacements of dG with an S- or R-N^2-CEdG caused decreases in the efficiency (V_{max}/K_m,

Table 1. Steady-state kinetic parameters for nucleotide incorporation opposite the two diastereomers of N^2-CEdG and unmodified dG by *E. coli* DNA polymerase IV (K_m and V_{max} are average values based on three independent measurements)

dNTP	V_{max}, nM min^{-1}	K_m, nM	V_{max}/K_m, min^{-1}	f_{inc}
S-N^2-CEdG-containing substrate				
dATP	0.62 ± 0.07	(8.41 ± 0.74) × 10^5	7.37 × 10^{-7}	2.91 × 10^{-4}
dGTP	0.23 ± 0.02	(6.83 ± 0.63) × 10^5	3.36 × 10^{-7}	1.33 × 10^{-4}
dCTP	0.19 ± 0.02	75 ± 8.4	2.53 × 10^{-3}	1.00
dTTP	0.46 ± 0.06	(5.83 ± 0.68) × 10^5	7.89 × 10^{-7}	3.12 × 10^{-4}
R-N^2-CEdG-containing substrate				
dATP	0.16 ± 0.01	(7.90 ± 0.89) × 10^5	2.02 × 10^{-7}	1.44 × 10^{-4}
dGTP	0.49 ± 0.08	(1.49 ± 0.26) × 10^6	3.28 × 10^{-7}	2.34 × 10^{-4}
dCTP	0.16 ± 0.03	(1.14 ± 0.28) × 10^2	1.40 × 10^{-3}	1.00
dTTP	0.48 ± 0.05	(1.23 ± 0.05) × 10^6	3.89 × 10^{-7}	2.78 × 10^{-4}
dG-containing substrate*				
dCTP	0.34 ± 0.07	53 ± 8.4	6.42 × 10^{-3}	1.00
dTTP	0.073 ± 0.001	(4.61 ± 0.56) × 10^4	1.58 × 10^{-6}	2.46 × 10^{-4}

*The incorporation of dATP and dGTP opposite undamaged dG was barely detectable even at extraordinarily high dNTP concentrations (Fig. S8).

Table 1) for nucleotide insertion by 2.5- and 4.6-fold, respectively, which is consistent with the observation that R-N^2-CEdG is a stronger block to DNA replication than S-N^2-CEdG in *E. coli* cells (see above).

We also measured the steady-state kinetic parameters for nucleotide incorporation opposite the N^2-CEdG lesion by human pol κ (Figs. S9–S11). The nucleotide insertion by human pol κ is again highly accurate, and the polymerase inserts preferentially dCMP opposite the lesion (Table S1). More strikingly, the efficiencies for human pol κ to incorporate the correct nucleotide, dCMP, opposite S- and R-N^2-CEdG, were increased by ≈6- and 3.5-fold, respectively, relative to the unmodified substrate (Table S1). Thus, N^2-CEdG is a better substrate for human pol κ than an unmodified dG.

Discussion

MG is induced endogenously as a byproduct of glycolysis (16, 18), a metabolic process conserved in all organisms (17). The concentration of MG in human cells can be elevated under various pathological conditions (e.g., diabetes) (19–21). It was found recently that MG induces modifications in calf thymus DNA mainly on dG to give N^2-CEdG (22). N^2-CEdG could also be detected in urine samples of healthy human subjects (23) and in kidney and aorta cells of diabetic and uremic patients (24). LC-MS/MS with the isotope dilution method revealed that N^2-CEdG can be formed in untreated WM-266-4 cells at a level of approximately one lesion per 10^7 nucleosides; treatment of cells with MG or glucose can further enhance the formation of the lesion, supporting that N^2-CEdG is an endogenous DNA lesion, and the amount of the lesion can be increased by byproducts of glycolysis.

Replication studies using single-stranded M13 shuttle vectors harboring a site-specifically incorporated N^2-CEdG revealed that the two diastereomers of the lesion exhibited significantly different bypass efficiencies in *E. coli* cells. The R-N^2-CEdG is twice as effective as S-N^2-CEdG in blocking DNA replication in all strains of *E. coli* cells that we examined. Although the absence of pol II or V does not give rise to apparent alteration in bypass efficiency, deficiency in pol IV results in a significant drop in bypass efficiency by 63% and 66% for the S and R diastereomers, respectively (Fig. 4*A*).

Both diastereomers are weakly mutagenic in wild-type AB1157 cells and isogenic *E. coli* cells deficient in pol II or V. However, the frequency of G → T mutation in the pol IV-deficient background was increased significantly for both diastereomers, supporting that the pol IV-mediated lesion bypass is largely error-free. Along this line, it was found that G → T transversion accounts for 70% of benzo[*a*]pyrene-induced mutations in pol κ-defective cells, whereas G → T and G → A mutations occur at an equal frequency of ≈30% of total mutations in parental cells (35).

These results are also in accordance with *in vitro* replication data showing that nucleotide insertion opposite N^2-CEdG is both accurate and efficient. In this respect, the efficiencies (V_{max}/K_m) for *E. coli* pol IV to incorporate the correct nucleotide, dCMP, opposite dG, S-, and R-N^2-CEdG were 6.42 × 10^{-3}, 2.53 × 10^{-3}, and 1.40 × 10^{-3} min^{-1}, respectively (Table 1). In addition, the corresponding efficiencies for human pol κ to insert dCMP were 5.40 × 10^{-5}, 3.10 × 10^{-4}, and 1.91 × 10^{-4} min^{-1}, respectively (Table S1). In keeping with our observations, *E. coli* pol IV and human pol κ insert dCMP opposite N^2-furfuryl-dG with 10- to 15-fold greater catalytic efficiency than opposite an undamaged dG (8). It is of note that N^2-furfuryl-dG is a structure analog of the principal N^2-dG adduct induced by nitrofurazone (8), and there is no evidence showing that N^2-furfuryl-dG is an endogenously induced DNA adduct. Furthermore, *E. coli* pol IV and human pol κ can bypass accurately the bulky N^2-dG-BPDE adduct (11–14), and the M13 genome bearing an N^2-dG-BPDE gave 4-fold fewer plaques when transfected into SOS-induced pol IV-deficient *E. coli* cells relative to the isogenic wild-type cells (13). Different from what we found for the bypass of N^2-CEdG, the efficiency for human pol κ to insert dCMP across N^2-dG-BPDE was at least 70 times less than opposite an undamaged dG (11).

Recently, the x-ray crystal structure for the catalytic core of human pol κ in ternary complex with DNA and an incoming nucleotide has been solved (36). The structure reveals the absence of steric hindrance in the minor groove at the primer-template junction (36), which may explain the tolerance of the polymerase toward the minor-groove adduct, N^2-CEdG.

Taken together, the results from the present study offer solid evidence supporting that N^2-CEdG, a DNA adduct arising from MG, is an endogenous substrate for DinB DNA polymerase.

Materials and Methods

A full description of *Materials and Methods* can be found in the *SI Text*.

[2,2,2-D$_3$]-N^2-CEdG (D$_3$-N^2-CEdG) was synthesized from 2-fluoro-2′-deoxyinosine and D$_3$-DL-alanine by using reported procedures (28). The resulting two diastereomers of D$_3$-N^2-CEdG were separated by HPLC and used as internal standards for the LC-MS/MS quantification of the lesion formed in human melanoma cells. ODNs containing a site-specifically inserted and ste-

reochemically defined N^2-CEdG were synthesized according to recently described procedures (ESI-MS and MS/MS shown in Fig. S12) (28).

Quantification of N^2-CEdG Formed in WM-266-4 Human Melanoma Cells. WM-266-4 human melanoma cells [American Type Culture Collection (ATCC)] were cultured under conditions recommended by ATCC. At 80% confluence, cells were detached and harvested by centrifugation. The cell pellets were washed twice with PBS and resuspended in 20 ml of PBS (10^6 cells per ml) containing the desired concentrations of MG. The cells were incubated with MG at room temperature for 3.0 h with occasional shaking. For D-glucose treatment, the cells were cultured in the media containing 5 or 25 mM D-glucose. The media were discarded, and the cells were cultured in fresh media containing the same concentration of D-glucose on day 3. The cells were then harvested at the end of day 5.

Nuclear DNA was isolated from cell lysates with phenol extraction and desalted by ethanol precipitation. The resulting DNA was digested to mononucleosides with four enzymes (details shown in *SI Text*), and to the digestion mixture were added the two diastereomers of D_3-N^2-CEdG. Quantitative analysis of N^2-CEdG in the above DNA hydrolysates was performed by online HPLC-ESI-MS/MS on an LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific), which was set up for monitoring the fragmentation of protonated ions of N^2-CEdG and D_3-N^2-CEdG. To eliminate the isobaric impurities present in MS/MS, we quantified the N^2-CEdG by using MS^3, which monitored the further fragmentation of the ions of *m/z* 224 and 227, corresponding to the protonated nucleobase portions of N^2-CEdG and D_3-N^2-CEdG, respectively.

Construction of N^2-CEdG-Carrying Single-Stranded M13 Genome and *in Vivo* Replication Studies. The lesion-carrying genomes were constructed by inserting a 5′-phosphorylated d(GAAGACCAXCGACGCC), where ''X'' designates S- or R-N^2-CEdG, into the EcoRI-linearized M13mp7(L2) genome via enzymatic ligation (31, 32). To determine bypass efficiency, we also prepared a competitor genome by inserting a 19-mer unmodified ODN, d(GAAGACCAGCTAGCTGCGG), into the linearized M13 genome. The amount of the lesion-containing genome was normalized against that of the competitor genome (31). The lesion-containing genome was then mixed with the competitor genome at a molar ratio of 6/1, transformed into wild-type and DNA polymerase-deficient AB1157 *E. coli* cells [Δ*pol B1*::spec (pol II-deficient), Δ*dinB* (pol IV-deficient), Δ*umuC*::*kan* (pol V-deficient), and Δ*umuC*::*kan* Δ*dinB* Δ*pol B1*::spec (triple knockout)] (37) by electroporation, and allowed for replication in the host *E. coli* cells for 6 h. In this regard, the AB1157 strains are proficient in nucleotide excision repair. The AB1157 cells were then pelleted and the supernatant, which contained progeny phages, was collected. To minimize the effect of residual lesion-containing genomes in subsequent analysis, the progeny/lesion-genome ratio was increased by several orders of magnitude via infecting SCS110 *E. coli* with the viable progeny phage (31). After culturing the cells at 37°C for 5 h, single-stranded DNA was extracted from the progeny phage.

PCR amplification of the region of interest in the resulting progeny genome was performed by using primers 5′-YCAGGGTTTTCCCAGTCACGACGTTGTAA-3′ and 5′-YCAGCTATGACCATGATTCAGTGGAAGAC-3′ (Y is an amino group) (31). PCR products were treated sequentially with BbsI, shrimp alkaline phosphatase, and Tsp509I, which gave rise to d(NCGACGCC) (''N'' designates the nucleobase present at the original lesion site after DNA replication *in vivo*) and d(GCTAGCTGCGG) for the lesion-carrying and competitor genomes, respectively.

Restriction fragments were subjected to LC-MS/MS analysis, and a gradient of 5 min of 0−20% methanol followed by 35 min of 20–50% methanol in 400 mM hexafluoro-2-propanol buffer (pH was adjusted to 7.0 by the addition of triethylamine) was used for the separation (32, 38, 39). The mass spectrometer was set up for monitoring the fragmentation of the $[M-2H]^{2-}$ ions of the 8- and 11-mer ODNs.

For the CRAB assay, the ODN fragments were 5′ ^{32}P-labeled before digestion with Tsp509I, and the resulting ODN fragments were resolved by denaturing PAGE analysis and quantified by using a phosphorimager.

Primer Extension Assays and Steady-State Kinetic Measurements. A 20-mer ODN, d(ATGGCXCACTATGATCCTAG) (X = S/R-N^2-CEdG) and a 5′-[^{32}P]-labeled 15-mer primer, d(GCTAGGATCATAGTG), were used for primer extension assays and steady-state kinetic measurements.

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