The Roles of DNA Polymerses κ and ι in the Error-free Bypass of N²-carboxyalkyl-2′-deoxyguanosine Lesions in Mammalian Cells*5

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To counteract the deleterious effects of DNA damage, cells are equipped with specialized polymerases to bypass DNA lesions. Previous biochemical studies revealed that DinB family DNA polymerases, including Escherichia coli DNA polymerase IV and human DNA polymerase κ, efficiently incorporate the correct nucleotide opposite some N²-modified 2′-deoxyguanosine derivatives. Herein, we used shuttle vector technology and demonstrated that deficiency in polk or poli in mouse embryonic fibroblast (MEF) cells resulted in elevated frequencies of G mutations. Kinetic measurements revealed that human DNA polymerase ι preferentially inserts the correct nucleotide, dCMP, opposite N²,2-carboxymethyl-2′-deoxyguanosine (N²-CMdG) sites. Steady-state analysis revealed five branches of Y-family polymerases, which include UmuC, DinB, polymerase η, polymerase κ, and REV1 (4, 5). All of these, except for UmuC, are found in eukaryotic cells (4, 5). Apart from the Y-family DNA polymerases, some B-family DNA polymerases also participate in TLS. These include DNA polymerase II in Escherichia coli and polymerase ζ, which is composed of the catalytic subunit REV3 and the regulatory subunit REV7, in eukaryotic cells (4, 5).

These specialized DNA polymerases are characterized by their relatively low fidelity in replicating undamaged DNA but have the capability to bypass those DNA lesions that normally block DNA synthesis by replicative DNA polymerases (4). It was advocated that specialized DNA polymerases are evolved by nature to copy DNA base damage accurately, but they no longer bear the ability to replicate unmodified DNA with high fidelity (6). The best known example of this is the efficient and accurate bypass of cis,syn-cyclobutane pyrimidine dimers by polymerase η (7, 8), which is encoded by the xeroderma pigmentosum-variant gene (i.e. POLH) in humans (9). Xeroderma pigmentosum-variant patients exhibit elevated susceptibility to developing skin cancer (10). Several recent studies also demonstrated that DinB DNA polymerase, a Y-family polymerase conserved in all three kingdoms of life (11), can insert the correct dCMP opposite several N²-substituted guanine lesions at an efficiency that is similar to or better than opposite an unmodified guanine (12–14). The X-ray crystal structure of the catalytic core of human DNA polymerase κ, along with primer/template DNA and an incoming nucleotide, reveals the lack of steric hindrance in the minor groove at the primer-template junction (15), which may account for the tolerance of polymerase κ toward the minor groove N²-DG lesions. By using shuttle vector technology, we also showed that DinB (i.e. polymerase IV) is the major polymerase involved in the accurate bypass of the two diastereomers of N²-(1-carboxyethyl)-2′-deoxyguanosine (N²-CMdG) in E. coli cells (14). However, it remains unclear whether this finding can be extended to mammalian cells and whether other mammalian TLS polymerase(s) are also required for the error-free bypass of N²-CMdG.
N²-CEdG is the major stable DNA adduct formed from methylglyoxal, a glycolytic metabolite (Fig. 1) (16). This modified nucleoside was found to be present in urine samples from healthy human subjects (17), and the lesion was observed more frequently in kidney and aortic cells of diabetic and uremic patients than in the corresponding cells of healthy human subjects (18). N²-CEdG can also be detected in cultured human cells, and the exposure of these cells to methylglyoxal or glucose further enhanced the formation of this lesion (14). Moreover, recent studies illustrated that the exposure of isolated DNA to the structurally related glyoxal can give rise to the formation of N²-carboxymethyl-2'-deoxyguanosine (N²-CmdG) (19, 20). The major objective of the present study is to define the roles of polymerases κ and other mammalian TLS DNA polymerases in bypassing the N²-CEdG and N²-CmdG lesions in cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unmodified ODNs used in this study were purchased from Integrated DNA Technologies (Coralville, IA). [γ-³²P]ATP was obtained from Perkin Elmer Life Sciences, and chemicals unless otherwise noted were from Sigma-Aldrich. Enzymes for these studies were purchased from New England Biolabs. Shrimp alkaline phosphatase was obtained from USB Corp. (Cleveland, OH), and all other enzymes unless otherwise specified were from New England Biolabs. The Polk, Rev3-, and Poli-deficient mouse embryonic fibroblast (MEF) cells were kindly provided by Professors Haruo Ohmori (21), Richard D. Wood (22), and Roger Woodgate (23), respectively. The C-Tag cells (POLH-deficient cells), which are SV40-immortalized cells derived from xeroderma pigmentosum-variant patients, were provided generously by Professor William K. Kaufmann (24).

**Preparation and LC-MS/MS Characterizations of ODN Substrates Containing an (S)-N²-CEdG, (R)-N²-CEdG, or N²-CmdG**—The 17-mer ODN 5’-GGCGAAAAXCTAGAGCT-C-3’ (X = N²-CEdG) was synthesized following previously reported procedures (25). The corresponding ODN bearing an N²-CmdG was prepared by using the same procedures except that glycine was used in lieu of alanine to displace the fluorine atom in 2-fluoro-2’-deoxyinosine after ODN assembly.

The identities of N²-CEdG- and N²-CmdG-containing 17-mer ODNs were confirmed by on-column capillary HPLC electrospray ionization-MS/MS (supplemental Figs. S1 and S2) using an Agilent 1100 capillary HPLC pump (Agilent Technologies) interfaced with an LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA), which was configured for monitoring the fragmentation of the [M – H]+ ions of the 17-mer ODNs. A 5-min linear gradient of 0–30% methanol followed by a 30 min of 30–50% methanol in 400 mM 1,1,1,3,3,3-hexafluoro-2-propanol buffer (pH adjusted to 7.0 by addition of triethylamine) was employed.

Construction of pTGFP-Hha10 and pmTEX4 Genomes Harboring a Site-specifically Inserted (S)-N²-CEdG, (R)-N²-CEdG, N²-CmdG, or dG—A previously described method (26) was used to insert a structurally defined lesion into a unique site in the pTGFP-Hha10 vector. Briefly, we first nicked the original vector with N.BstNI to produce a gapped vector and a 33-mer single-stranded DNA (see Fig. 2, A and B). In this respect, the pTGFP-Hha10 vector contains two N.BstNI recognition sites upstream of the coding region of the Turbo GFP (TGFP) gene, and N.BstNI nicks only one strand of duplex DNA at the fourth nucleotide 3’ to the GAGTC restriction recognition site. The 33-mer single-stranded ODN was removed subsequently from the nicked plasmid by annealing the cleavage mixture with the complementary 33-mer ODN in 50-fold molar excess. The gapped plasmid was isolated from the mixture by using 100-kDa cut-off ultracentrifugation filter units (Centricon 100 from Millipore). The purified gapped construct (see Fig. 2C, lane 2) was subsequently annealed with a 5’-phosphorylated 17-mer lesion-carrying insert and a 16-mer unmodified ODN and ligated with T4 DNA ligase (see Fig. 2B). In this regard, we used a 17-mer rather than 33-mer lesion-containing insert because of the relative ease in the synthesis and purification of the former substrate. The ligation mixture was incubated with ethidium bromide, and the resulting supercoiled, lesion-carrying double-stranded plasmid was isolated from the mixture by agarose gel electrophoresis (see Fig. 2C, lanes 4 and 5). The pTGFP-Hha10 vector was nicked with Nb.BbvCI to produce a gapped vector and a 41-mer single-stranded DNA (supplemental Fig. S3). The subsequent procedures were similar as those for the construction of lesion-containing pTGFP-Hha10 vector as described above (supplemental Fig. S3B).

**Cell Culture, Transfection, and in Vivo Replication**—The 293T, POLH-deficient human fibroblast cells and all MEF cells were cultured at 37 °C in a 5% CO₂ atmosphere and in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 µg/ml streptomycin (ATCC, Manassas, VA). Cells (5 × 10⁵) were seeded in six-well plates and cultured overnight, after which they were transfected with 300 ng of the lesion-containing or lesion-free pTGFP-Hha10 or pmTEX4 vector using Lipofectamine 2000 (Invitrogen). After in vivo replication for 24 h, DNase I was added to digest the residual DNA adsorbed to the exterior of cells (27). Cells were subsequently detached by treating with trypsin-EDTA (ATCC), the progenies of the plasmid were isolated by using an alkali lysis method (28), and residual unreplicated plasmid was further removed by DpnI digestion.

**Determination of Bypass Efficiency and Mutation Frequency**—The bypass efficiencies and mutation frequencies were deter-
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determined by a method adapted from the restriction endonuclease and post-labeling assay (29, 30), which was developed initially by Essigmann and co-workers (31–33) for assessing the cytotoxic and mutagenic properties of DNA lesions in E. coli cells using the single-stranded M13 genome. The progeny genomes were amplified subsequently by PCR using Phusion high fidelity DNA polymerase. The primers were 5'-GCAGAGCTGGTTT-AGTGAAACCGTCAG-3' and 5'-CTCTGCTGAGCCAGTTACCTCGG-3' for pTGFP-Hha10 vector, 5'-GCTATCGAATATACGACTTATTAGG-3' and 5'-CCTTCCGAAAAGAGTTGGTAGC-3' for pMTEX4 target. Target DNA was amplified for 35 cycles, each consisting of 10 s at 98 °C, 30 s at 62 °C, 2 min at 72 °C, with a final extension at 72 °C for 5 min. The resulting 3950-bp PCR products (from pTGFP-Hha10 progenies) and 3412-bp PCR products (from pMTEX4 progenies) were purified by using a QIAquick PCR purification kit (Qiagen, Valencia, CA). For the bypass efficiency assay, a portion of the above PCR fragments was treated with 10 units of SacI and 1 unit of shrimp alkaline phosphatase (USB Corp.) in 10-μl New England Biolabs buffer 4 at 37 °C for 20 min to deactivate the phosphatase. The above mixture was then treated in a 15-μl NEB buffer 4 with 5 mM DTT, ATP (50 pmol (cold), premixed with 1.66 pmol [γ-32P]ATP), and 10 units of polynucleotide kinase. The reaction was continued at 37 °C for 1 h, followed by heating at 65 °C for 20 min to deactivate the polynucleotide kinase. 10 units of FspI was added to the reaction mixture (see recognition sites highlighted in boldface in Fig. 3A), and the solution was incubated at 37 °C for 1 h, followed by quenching with 15 μl of formamide gel loading buffer containing xylene cyanol FF and bromphenol blue dyes. The mixture was loaded onto a 30% polyacrylamide gel (29) and run for 12–14 h at 200 V. The 14-mer products were visualized by autoradiography. The resulting 3950-bp PCR products (from pTGFP-Hha10) were analyzed by agarose gel electrophoresis, and products were quantified by a phosphorimaging analysis (Molecular Dynamics). The 238-bp PCR products were treated with 50 units of SacI, 1 unit of shrimp alkaline phosphatase (USB Corp.) in 10-μl New England Biolabs buffer 4 at 37 °C for 2 h, followed by heating at 65 °C for 20 min. The resulting solution was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v), and the aqueous portion was dried with Speed-vac and dissolved in 12 μl of water. The ODN mixture was subjected to LC-MS/MS analysis. A 0.5 × 150 mm Zorbax SB-C18 column (5 μm in particle size, Agilent Technologies) was used for the separation, and the flow rate was 8.0 μl/min, which was delivered by using an Agilent 1100 capillary HPLC pump. A 5-min gradient of 0–20% methanol followed by a 35-min of 20–50% methanol in 400 mM 1,1,1,3,3,3-hexafluoro-2-propanol (pH was adjusted to 7.0 by the addition of triethylamine) was employed for the separation. The effluent from the LC column was coupled directly to an LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA), which was configured for monitoring the fragmentation of the [M – 3H]− ions of the 14-mer ODNs (i.e. d(GCAAAMCTAGAGCT), where M designates A, T, C, or G).

siRNA Knockdown of POLK Gene in 293T Cells and Replication Studies in siRNA-treated Cells—POLK ON-TARGETplus SMARTpool (L-002108) and siGENOME Nontargeting siRNA (D-001210) were purchased from Dharmacon (Lafayette, CO). 293T cells were grown in DMEM medium (ATCC) containing 10% fetal bovine serum (Invitrogen) and penicillin/streptomycin (ATCC). Cells were seeded in six-well plates at 70% confluence and transfected with 1 μg of synthetic duplex siRNAs using Lipofectamine 2000 following the manufacturer’s instructions. After a 48-h incubation, cells were co-transfected with another aliquot of siRNA and pTGFP-Hha10 lesion-containing vector followed by isolation of progeny vectors after a 24-h incubation with the same methods used previously. The isolated progeny vectors were processed using a protocol identical to that described above.

Real-time Quantitative RT-PCR and Immunoblot Analysis—
Total RNA was extracted from the cells 48 and 72 h after transfection with siRNA using the RNasy Mini Kit (Qiagen). cDNA was synthesized by using iScript™ CDNA synthesis kit (Bio-Rad) according to the manufacturer’s recommended procedures. Briefly, 1 μg of total RNA was reverse-transcribed with 1 μl of iScript reverse transcriptase in a 20-μl volume reaction. The reaction was carried out at 25 °C for 5 min and at 42 °C for 30 min. The reverse transcriptase was then deactivated by heating at 85 °C for 5 min. Analyses of the transcripts for POLK were performed by real-time quantitative RT-PCR using iQ™ SYBR Green Supermix kit (Bio-Rad) according to the manufacturer’s recommendations on a Bio-Rad iCycler system (Bio-Rad). The following primers were used for the real-time PCR: 5'-TGCTGATTTTCCACATCCCTTGAG-3' and 5'-CTCCTTTGGTTGTTCTCTGTGCC-3' for POLK, 5'-TTTGTCAAGCTCATTTCCTGATAG-5' and 5'-TCTCTCCTCTCTTGGCTCTTTGCTG-3' for GAPDH. The comparative cycle threshold (Ct) method (ΔΔCt) was used for the relative quantification of gene expression (34). Immunoblot analysis was performed with a total of 40 μg of whole cell lysate. Antibody that specifically recognizes human DNA polymerase κ (Santa Cruz Biotechnology) was used at a 1:2000 dilution. Human β-actin antibody (Abcam, Cambridge, MA) was used at a 1:5000 dilution. Horseradish peroxidase-conjugated secondary goat anti-mouse antibody (Santa Cruz Biotechnology) and goat anti-rabbit antibody (Abcam) were used at a 1:10,000 dilution.
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Steady-state Kinetic Measurements—A 24-mer ODN, d(5′-GCGAAAXCTAGCTGAGCTCAGATCT-3′) (X = S(R)-N2-CEdG) and a 5′-32P-labeled 16-mer primer, d(AGATCTCGAGCTCAGT), were employed for steady-state kinetic measurements.

The 24-mer N2-CEdG-containing ODNs, 5′-GCGAAAXCTAGCTGAGCTCAGATCT-3′, were prepared and steady-state kinetic analyses were performed following procedures described previously (14). The 24-mer lesion-containing template or nondamaged template (20 nm) with dG in place of the N2-CEdG was annealed with a 5′-32P-labeled 16-mer primer d(AGATCTCGAGCTCAGT), to give the primer-template complex. The primer-template complex (10 nm) was incubated at 37 °C with human polymerase η (5 ng) for 10 min or polymerase τ (5 ng) for 30 min in the presence of various concentrations of an individual dNTP and in a buffer containing 20 mm Tris-HCl (pH 7.5), 2 mm dithioreitol, 100 μg/ml bovine serum albumin, 10% glycerol, and 5 mm (for polymerase η) or 2 mm (for polymerase τ) MgCl2. The individual dNTP concentration was optimized for different insertion reactions to allow for a <20% primer extension (35). The products were resolved by denaturing PAGE analysis, and 20% (29:1, acrylamide/bisacrylamide) polyacrylamide gels containing 8 mm urea were used. Gel band intensities for the substrates and products were quantified by using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences) and ImageQuant (version 5.2; Amersham Biosciences). The observed rate of dNTP incorporation (Vobs) was plotted as a function of dNTP concentration, and the apparent Km and Vmax steady-state kinetic parameters for the incorporation of both the correct and incorrect nucleotides were determined by fitting the rate data with the Michaelis-Menten equation.

$$V_{\text{obs}} = \frac{V_{\text{max}} \times [\text{dNTP}]}{K_m + [\text{dNTP}]} \quad (\text{Eq. 1})$$

The $k_{\text{cat}}$ values were then calculated by dividing the $V_{\text{max}}$ values with the concentration of the polymerase used. The efficiency of nucleotide incorporation was determined by the ratio of $k_{\text{cat}} / K_m$, and the fidelity of nucleotide incorporation was calculated by the frequency of misincorporation ($f_{\text{inc}}$) with Equation 2.

$$f_{\text{inc}} = \frac{(k_{\text{cat}} / K_m)_{\text{incorrect}}}{(k_{\text{cat}} / K_m)_{\text{correct}}} \quad (\text{Eq. 2})$$

RESULTS

Construction and Characterization of Plasmids Housing a Site-specifically Inserted N2-CEdG or N2-CMdG—Our previous studies revealed that DinB is the major DNA polymerase involved in the accurate and efficient bypass of N2-CEdG in E. coli cells. The goal of the present investigation is to understand how N2-CEdG and its structurally related N2-CMdG are replicated in mammalian cells. To this end, we first constructed double-stranded pTGFP-Hha10 and pMTEX4 vectors housing these lesions (Fig. 2 and supplemental Fig. S3).

Constructing the lesion-bearing plasmid is among the most challenging steps when a double-stranded shuttle vector is employed for in vivo replication experiments. However, the use of the pTGFP-Hha10 and pMTEX4 vectors allows for the relatively easy preparation of the lesion-containing plasmid because the ligation only necessitates the insertion of a single-stranded damage-containing ODN into a gapped double-stranded vector. As depicted in Fig. 2C (lane 4), the strategy facilitated the efficient generation of lesion-containing double-stranded vector, and agarose gel electrophoresis afforded convenient purification of the resulting double-stranded vector from the ligation mixture (Fig. 2C); we could routinely produce the N2-CMdG- and N2-CEdG-containing pTGFP-Hha10 vectors at an overall yield of ~30%.

We further confirmed the incorporation of the lesion-containing insert by employing a restriction digestion/post-labeling assay. In this experiment, the aforementioned double-stranded genomes were digested with EcoRI (the unique EcoRI site is shown in Fig. 2B). The nascent terminal phosphate groups were removed by using alkaline phosphatase, and the 5′-termini were subsequently phosphorolyated with [γ-32P]ATP. The linearized vector was further cleaved with NheI (unique NheI site shown in Fig. 2B), which affords a 52-mer lesion-containing ODN if the ligation is successful (Fig. 2B). Indeed, denaturing PAGE analysis revealed a distinct 52-mer 32P-labeled fragment, and no shorter fragments were detected (Fig. 2D), supporting the successful incorporation of the 17-mer lesion-containing insert and the 16-mer unmodified ODN into the gapped construct.

Effect of N2-CEdG and N2-CMdG on Efficiency and Fidelity of DNA Replication in Mammalian Cells—When the lesion-containing, double-stranded shuttle vector is replicated in mammalian cells, the undamaged strand may be replicated preferentially over the lesion-carrying strand (36, 37), rendering it difficult to determine accurately the mutation frequencies. To overcome this difficulty, we employed a similar strategy as reported previously (26, 36, 37) and incorporated an A:A mismatch three nucleotides away from the lesion site (Figs. 2B and 3A). This method facilitated the independent assessment of the products arising from the replication of the lesion-containing strand and its opposing unmodified strand (Fig. 3A).

The lesion-containing and the control lesion-free vectors were transfected separately into several mammalian cell lines, which include the 293T cells and wild-type MEF cells. To assess the roles of various TLS DNA polymerases in bypassing the above-mentioned N2,dG lesions, we also evaluated the replication of the lesion-carrying and control lesion-free vectors in immortalized MEF cells deficient in Polk (21), Rev3 (22), or Poli (23), as well as C-Tag cells, which are deficient in human POLH (24).

Our results revealed that none of the N2-dG lesions are mutagenic in human 293T cells or wild-type MEF cells. However, all of them are mutagenic in MEF cells that are deficient in Polk. As depicted in Fig. 3B, replication of A:A mismatch-containing, lesion-free genome (labeled as “control”) in wild-type MEF gives two 10-mer products, i.e. d(CAGCTTTTG) (10-mer-AC) and d(CAGCTTTTG) (10-mer-TC), from the respective replication of the top and bottom strands of the plasmid. The observation of similar amounts of products originating from the replication of the top and bottom strands indicates the lack of repair of the A:A mismatch in the plasmid. The replication of
the N²-CEdG- and N²-CMdG-harboring genomes in Polk-deficient background results in the appearance of two new bands in the gel (Fig. 3B), which are attributed to d(CTAGTTTTGC) (10-mer-TT) and d(CTAGATTTGC) (10-mer-TA) based on LC-MS/MS monitoring of the complementary 14-mer fragments from the same restriction digestion mixtures (supplemental Fig. S4 and Fig. 3A). In this context, the latter analysis revealed the formation of only the two mutated products as labeled to the right of Fig. 3B; other mutagenic products are below the detection limit of the LC-MS/MS method. Quantification revealed that the replication of (S)-N²-CEdG-, (R)-N²-CEdG-, and N²-CMdG-containing genomes in Polk-deficient background can give rise to G→A transition at frequencies of 23.3, 23.6, and 23.9%, respectively, and G→T transversion at frequencies of 15.1, 15.8, and 15.0%, respectively (Fig. 4B). The bypass efficiencies for the three lesions are similar in 293T cells and in wild-type MEF cells, and the deficiency in Polk did not compromise the bypass efficiencies. In this regard, the bypass efficiencies for (S)-N²-CEdG, (R)-N²-CEdG, and N²-CMdG in wild-type MEF are 99.4, 100.6, and 99.0%, respectively, whereas the corresponding bypass efficiencies in the Polk-deficient MEF are 97.8, 95.4, and 98.3% (Fig. 4A).

The above results support that the polymerase κ-mediated bypass of N²-substituted dG lesions in mammalian cells is accu-
This result is in keeping with our recently published results showing that polymerase IV, the *E. coli* ortholog of mammalian polymerase α, is the major polymerase involved in the accurate bypass of N2-CEdG in *E. coli* cells and that the human polymerase α-mediated in vitro nucleotide incorporation opposite N2-CEdG is both accurate and efficient (14).

We next assessed whether other TLS DNA polymerase(s) are also involved in the bypass of N2-CEdG and N2-CMdG in mammalian cells. Our results revealed that Pol α deficiency also led to elevated frequencies of G→A (18.0, 19.3, and 20.4% for (S)-N2-CEdG, (R)-N2-CEdG, and N2-CMdG, respectively) and G→T mutations (12.6, 13.1, and 13.6%, respectively, Fig. 4B). In addition, the bypass efficiencies for the three lesions in Pol α-deficient cells were 105.3, 101.2, and 99.5%, respectively (Fig. 4A), which were similar to those found in the wild-type MEF cells.

The observation of substantial frequencies of G→A and G→T mutations in Pol α-deficient MEF suggests that the polymerase ε-mediated nucleotide insertion opposite the above N2-dG lesions is also accurate. To examine whether this is the case, we performed steady-state kinetic measurements for human polymerase ε-induced nucleotide incorporation opposite the two diastereomers of N2-CEdG as well as an unmodified dG. The results indeed demonstrated that human polymerase ε preferentially inserts the correct dCMP across the N2-CEdG lesions (Table 1). Importantly, the efficiencies for dCMP incorporation opposite the two N2-CEdG derivatives are only slightly lower than that for dCMP insertion opposite an unmodified dG (Table 1). This is consistent with the previous observations showing that polymerase ε-mediated nucleotide insertion opposite other minor groove N2-dG adducts is both accurate and efficient (38, 39).

We next assessed the role of polymerases η, ε, and ζ on the bypass of N2-CEdG and N2-CMdG in mammalian cells. It turned out that the replication of N2-CEdG and N2-CMdG-
bearing plasmids in POLH- or Rev3-deficient cells did not give rise to detectable mutations. These data underscored the lack of involvement of polymerase η or ι in bypassing those lesions in a background that is proficient in Polk or Poli.

**DISCUSSION**

We developed a shuttle vector-based method for assessing quantitatively how \((S)\)-N\(^2\)-CEdG, \((R)\)-N\(^2\)-CEdG, and N\(^2\)-CMdG perturb the efficiency and fidelity of DNA replication in mammalian cells. With the use of this method and mammalian cells that are deficient in TLS DNA polymerases, we investigated the roles of various mammalian TLS DNA polymerases in bypassing these lesions. The most important finding made from the present study is that, in mammalian cells, the polymerase κ- and polymerase ε-mediated nucleotide incorporation opposite N\(^2\)-CEdG and N\(^2\)-CMdG is error-free. This result reinforced the notion that N\(^2\)-substituted dG lesions are cognate substrates for DinB DNA polymerases (12, 14), and it also ascertained that polymerase ι, a Ζ-family polymerase present only in higher eukaryotes (4), accurately bypasses these lesions.

Our current study also underscored that, in a genetic background lacking polymerase κ or ι, some other polymerase(s) can also bypass the above two lesions, though with compromised fidelity. In this context, our mutagenesis data revealed substantial frequencies of dAMP and dTMP misincorporation opposite the N\(^2\)-dG lesions when the replication was performed in Polk- or Poli-deficient cells. The *in vitro* steady-state kinetics experiments with purified human polymerase η illustrated that, aside from inserting the correct nucleotide (dCMP), the polymerase can incorporate dAMP and dTMP at efficiencies that are similar or slightly lower than for dCMP insertion (Table 1), suggesting that polymerase η might be responsible for the mutagenic bypass of the N\(^2\)-dG lesions in a Polk- or Poli-deficient background. This finding is reminiscent of what was recently observed for the bypass of cis,syn-cyclobutane thymine dimer in mammalian cells. In this regard, polymerase η can perform the accurate and efficient nucleotide insertion opposite the lesion (40); in the absence of polymerase η, the lesion also can be bypassed by other TLS polymerases, but with poor accuracy (40, 41). In addition, a previous report showed that another N\(^2\)-modified 2’-deoxyguanosine derivatives, N\(^2\)-benzo[a]pyrene-guanine, can be bypassed efficiently and accurately.

![Figure 4](image-url)

**FIGURE 4.** Bypass efficiencies (A) and mutation frequencies (B) of dG, (S)-N\(^2\)-CEdG, (R)-N\(^2\)-CEdG, and N\(^2\)-CMdG lesions in wild-type, POLH-, Rev3-, Polk-, and Poli-deficient mammalian cells. Black, dark gray, light gray, and white columns represent the results for substrates carrying dG, (S)-N\(^2\)-CEdG, (R)-N\(^2\)-CEdG and N\(^2\)-CMdG, respectively. The data represent the means and S.D. of results from three independent experiments.

### TABLE 1

Fidelity of nucleotide incorporation by human polymerases η and ι on N\(^2\)-CEdG-containing substrates and the undamaged substrate as determined by steady-state kinetic measurements. \(k_{cat}\) and \(K_m\) are average values based on three independent measurements.

| dNTP                | \(k_{cat}\) \(\text{min}^{-1}\) | \(K_m\) \(\text{nM}\) | \(k_{cat}/K_m\) \(\text{min}^{-1}\text{nM}^{-1}\) | \(I_{nu}\) |
|---------------------|-------------------------------|---------------------|-----------------------------------------------|---------|
| \((S)\)-N\(^2\)-CEdG-containing substrate |                               |                     |                                               |         |
| dATP                | \((5.06 \pm 0.01) \times 10^{-2}\) | \((3.53 \pm 0.29) \times 10^{3}\) | \((1.43 \pm 10^{-5})\) | 0.63    |
| dGTP                | \((5.73 \pm 0.37) \times 10^{-2}\) | \((2.05 \pm 0.42) \times 10^{4}\) | \((2.79 \pm 0.04) \times 10^{-6}\) | 0.12    |
| dCTP                | \((3.01 \pm 0.02) \times 10^{-2}\) | \((1.32 \pm 0.04) \times 10^{4}\) | \((2.28 \pm 0.01) \times 10^{-5}\) | 1       |
| dTTP                | \((1.50 \pm 0.02) \times 10^{-2}\) | \((6.90 \pm 0.64) \times 10^{2}\) | \((2.17 \pm 0.04) \times 10^{-5}\) | 0.95    |
| \((R)\)-N\(^2\)-CEdG-containing substrate |                               |                     |                                               |         |
| dATP                | \((3.53 \pm 0.01) \times 10^{-2}\) | \((2.85 \pm 0.25) \times 10^{3}\) | \((1.24 \pm 10^{-5})\) | 0.52    |
| dGTP                | \((4.86 \pm 0.01) \times 10^{-2}\) | \((5.60 \pm 0.62) \times 10^{4}\) | \((8.69 \pm 10^{-7})\) | 0.069   |
| dCTP                | \((2.61 \pm 0.02) \times 10^{-2}\) | \((1.10 \pm 0.09) \times 10^{5}\) | \((2.37 \pm 10^{-5})\) | 1       |
| dTTP                | \((1.57 \pm 0.02) \times 10^{-2}\) | \((5.62 \pm 0.36) \times 10^{4}\) | \((2.79 \pm 0.01) \times 10^{-5}\) | 1.2     |
| Control dG-containing substrate |                               |                     |                                               |         |
| dATP                | \((4.33 \pm 0.01) \times 10^{-2}\) | \((9.27 \pm 0.52) \times 10^{5}\) | \((4.67 \pm 10^{-6})\) | 0.00013 |
| dGTP                | \((1.53 \pm 0.003) \times 10^{-2}\) | \((2.52 \pm 0.44) \times 10^{5}\) | \((6.07 \pm 10^{-6})\) | 0.00017 |
| dCTP                | \((3.57 \pm 0.04) \times 10^{-2}\) | \((9.92 \pm 0.72) \times 10^{-1}\) | \((3.61 \pm 10^{-2})\) | 1       |
| dTTP                | \((3.22 \pm 0.001) \times 10^{-2}\) | \((2.56 \pm 0.21) \times 10^{5}\) | \((1.26 \pm 10^{-6})\) | 3.5 \times 10^{-5} |
| Human polymerase ι* |                               |                     |                                               |         |
| \((S)\)-N\(^2\)-CEdG-containing substrate |                               |                     |                                               |         |
| dCTP                | \((1.16 \pm 0.01) \times 10^{-2}\) | \((2.23 \pm 0.38) \times 10^{4}\) | \((5.24 \pm 10^{-7})\) | 1       |
| \((R)\)-N\(^2\)-CEdG-containing substrate |                               |                     |                                               |         |
| dCTP                | \((1.07 \pm 0.02) \times 10^{-2}\) | \((1.97 \pm 0.12) \times 10^{4}\) | \((5.43 \pm 10^{-7})\) | 1       |
| Control dG-containing substrate |                               |                     |                                               |         |
| dCTP                | \((1.32 \pm 0.04) \times 10^{-2}\) | \((1.06 \pm 0.08) \times 10^{4}\) | \((1.24 \pm 10^{-6})\) | 1       |

* No apparent incorporation of dAMP, TMP, or dGMP was observed even when high concentrations of dNTP (2 mM) were used. Thus, steady-state kinetic parameters were determined only for dCMP incorporation.
by polymerase η in wild-type MEF cells; however, the bypass of this adduct was error-prone in the absence of polymerase η (42). Along this line, polymerase η was found to be important to protect mammalian cells against the lethal and mutagenic effects of benzo[a]pyrene (43).

To further examine whether polymerase η was involved in mutagenic bypass of the N²-dG lesions in cells, we employed siRNA to knock down the POLK gene in POLH-deficient cells to determine whether the genetic suppression of POLK in POLH-deficient background could abrogate the G→A and G→T mutations found in Polk⁻/⁻ MEFS. As a control experiment, we also treated 293T cells with POLK siRNA while reasoning that siRNA-induced depletion of polymerase η in 293T cells should recapitulate the findings made for Polk-deficient MEFS. However, no mutagenic products could be found from replication of the N²-CEdG- or N²-CMdG-containing plasmids in 293T cells treated with POLK siRNA. This result suggests that, although siRNA treatment results in an ~85% decrease in POLK expression as demonstrated by real-time quantitative RT-PCR and Western analysis (supplemental Fig. S5), the remaining polymerase η might be sufficient for bypassing the N²-carboxyalkyl-dG lesion in transfected plasmids. We also attempted but failed to effectively knock down the expression of Polh in the Polk-deficient MEF cells using siRNA.

Based on the above findings, we put forward a plausible model for the bypass of the N²-dG lesions in mammalian cells. In wild-type cells, polymerase η and/or τ insert the correct dCMP opposite the lesion and the resulting N²-dG:dC base pair at the primer/template junction can be extended by polymerase ζ, a general mismatch extender (44), or by another TLS polymerase. In cells deficient in Polk or Poli, another polymerase (e.g. polymerase η) can insert a nucleotide opposite the N²-dG lesion, but the nucleotide incorporation is error-prone, with substantial frequencies of dAMP and dTMP misincorporation. The nascent base pair at the primer/template junction can again be extended by polymerase ζ or another TLS polymerase.

N²-dG adducts can arise from byproducts of an array of cellular processes, including glycolysis (14, 18) and lipid peroxidation (45). In addition, there is supporting evidence that these minor groove adducts may be resistant toward excision repair (46). In this vein, the N²-dG adduct is the least abundant dG lesion produced from acetylaminofluorine, but it persists in tissues of animals treated with this carcinogen (47). The ubiquitous formation of N²-dG adducts from byproducts of endogenous metabolism and the poor repair of these lesions may justify the need of multiple polymerases (polymerase η and ζ) for their accurate bypass in mammalian cells. In this context, little is known about how N²-CEdG and N²-CMdG lesions are repaired, and such studies are currently ongoing in our laboratory.

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