ERROR PRONE TRANSLESION SYNTHESIS PAST

γ-HYDROXYPROPAANO DEOXYGUANOSINE, THE PRIMARY ACROLEIN
DERIVED ADDUCT IN MAMMALIAN CELLS

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SUMMARY

8-Hydroxy-5,6,7,8-tetrahydropyrimido[1,2-α]purin-10(3H)-one, 3-(2'-deoxyriboside) (1,N2-γ-hydroxypropanodeoxyguanosine, γ-HOPdG) is a major DNA adduct that forms as a result of exposure to acrolein, an environmental pollutant and a product of endogenous lipid peroxidation. γ-HOPdG has been shown previously not to be a miscoding lesion when replicated in *E. coli*. In contrast to those prokaryotic studies, *in vivo* replication and mutagenesis assays in COS-7 cells using single stranded DNA containing a specific γ-HOPdG adduct, revealed that the γ-HOPdG adduct was significantly mutagenic. Analyses revealed both transversion and transition types of mutations at an overall mutagenic frequency of 7.4 x 10^{-2}/translesion synthesis. *In vitro* γ-HOPdG strongly blocks DNA synthesis by two major polymerases, pol δ and pol ε. Replicative blockage of pol δ by γ-HOPdG could be diminished by the addition of proliferating cell nuclear antigen, leading to highly mutagenic translesion bypass across this adduct. The differential functioning and processing capacities of the mammalian polymerases may be responsible for the higher mutation frequencies observed in this study when compared with the accurate and efficient nonmutagenic bypass observed in the bacterial system.
INTRODUCTION

Xenobiotics from the environment can cause damage to DNA that may lead to mutations following replication past these lesions. DNA damage can arise from both endogenous metabolic end-products of cellular respiration, as well as from hazardous industrial chemicals. Numerous compounds have been investigated for their ability to form DNA adducts and their mutagenic potential during replication in prokaryotic and eukaryotic systems (1-4). One such compound that has recently become the target for evaluation is acrolein (CH$_2$=CHCHO), an $\alpha,\beta$-unsaturated aldehyde that is released into the environment (140 tons/yr) via a number of sources (5-7). Acrolein is also formed endogenously during metabolic oxidation of polyamines, lipid peroxidation and cyclophosphamide metabolism (8-10).

Acrolein has been shown to be mutagenic in *Salmonella typhimurium* (11) as well as in DNA repair-deficient xeroderma pigmentosum (XP) human cells (12). It also has been demonstrated to have tumor-initiating activity (13). Molecular analysis of mutations induced by acrolein in human fibroblasts and XP cells has been performed using a supF shuttle vector. These studies revealed that in both types of cells, the majority of the mutations were single and tandem base substitutions that predominantly occurred in G:C base pairs (14).

Being a bifunctional electrophile, acrolein reacts with DNA bases *in vitro* to form several exocyclic DNA adducts (15), among which 8-Hydroxy-5,6,7,8-tetrahydropyrimido[1,2-$\alpha$]purin-10(3$H$)-one,1,N$^2$-(\(\gamma\)-hydroxypropano) deoxyguanosine (\(\gamma\)-HOPdG) (Fig. 1), was identified as a major product of reaction with
deoxyguanosine (16). This adduct is formed by conjugate addition of the acrolein to N\textsuperscript{2} of dG followed by ring closure of the resulting N\textsuperscript{2}-(3-oxopropyl)dG at N1 to form the cyclic adduct. The acyclic and cyclic adducts are in dynamic equilibrium. At physiological pH, the acyclic species cannot be detected spectrophotometrically. However, it can be trapped as the N\textsuperscript{2}-(3-hydroxypropyl) adduct by treatment with NaBH\textsubscript{4}. Using \textsuperscript{32}P-postlabelling combined with high performance liquid chromatography, evidence has been obtained for the presence of the \(\gamma\)-HOPdG adduct in liver cells of humans and rodents (17,18) and also in the lymphocyte DNA of cancer patients under treatment with cyclophosphamide (19).

The discovery of a variety of guanine-derived exocyclic adducts in healthy mammalian tissues and cells of cancer patients (17-20) has generated interest in evaluating its mutagenicity by site-specific approaches. 1,N\textsuperscript{2}-Propanoguanine (PdG) and pyrimido[1,2-\(\alpha\)]purin-10(3\(H\))-one guanine (M\textsubscript{1}G) (Fig. 1) share structural similarities with the \(\gamma\)-HOPdG, and have been utilized as model compounds for biological studies (21-25). Experiments designed to replicate past DNA containing a PdG adduct reported a major block to DNA synthesis. However, the relative mutagenicity of this lesion when replicated in \textit{E. coli} has varied greatly, with Burcham and Marnett (23) reporting a 2\% mutagenesis of G to T and A, while Moriya et al (22) found 100\% G to T transversions. Further, Moriya et al (22) reported that PdG was mutagenic in COS-7 cells, with 7.5\% G to T transversions, and 1\% G to A transitions. It is probable that these mutagenic variations could be accounted for by differences in the vectors used, with one study (22) employing single-stranded DNA while the other (23) used double stranded DNA.

Recent progress in the synthesis of oligodeoxynucleotides containing \(\gamma\)-HOPdG (26, 27) has greatly facilitated structural studies (28) and made it possible to investigate
the biological fate of this adduct using site-specific approaches (29, 30). An oligodeoxynucleotide containing a single \( \gamma \)-HOPdG adduct was incorporated into heteroduplex DNA (29) and its cellular processing as well as mutagenic potential were examined in *E. coli* using wild type and repair-deficient strains. Analyses of progeny plasmids revealed that \( \gamma \)-HOPdG was not mutagenic *in vivo* and that accurate translesion synthesis, nucleotide excision repair and recombination repair protect *E. coli* from the genotoxicity of the adduct. Inactivation of *polB*, *dinB*, and *umuD,C* genes did not affect significantly the efficiency or fidelity of translesion synthesis suggesting that “SOS” polymerases were not essential for replication across \( \gamma \)-HOPdG. Another study (30) using the same adduct in single stranded as well as double stranded shuttle vectors, also showed that \( \gamma \)-HOPdG was not a strong block to DNA replication and was not a miscoding lesion in *E. coli*. *In vitro*, the Klenow exo\(^{-}\) fragment of *E. coli* polymerase I exhibited a limited, mostly error-prone DNA synthesis across \( \gamma \)-HOPdG (29,30).

Therefore, it has been concluded that DNA polymerase III, the major replicative DNA polymerase in *E. coli*, catalyzes error-free translesion synthesis across \( \gamma \)-HOPdG (29).

NMR spectroscopy of a DNA duplex containing the \( \gamma \)-HOPdG adduct (28) established that there is a fundamental difference between \( \gamma \)-HOPdG and PdG within duplex DNA, in that the former predominantly exists as an open ring form (Fig. 1), enabling the modified base to participate in a standard Watson-Crick base pairing. The chemical conversion of the \( \gamma \)-HOPdG to \( N^2 \)-(3-oxopropyl)dG during replication has been proposed to account for the lack of mutagenicity of the \( \gamma \)-HOPdG adduct as observed in biological studies (29, 30).
In the present study, accuracy of translesion DNA synthesis across the γ−HOPdG adduct has been evaluated in vivo in both mammalian (COS-7) and bacterial (repair deficient E. coli AB2480 (uvrA recA)) cells using a site-specifically modified single stranded pMS2 shuttle vector (31). To further explore the mechanism of replication bypass and to identify the polymerases involved in both error free and error prone synthesis, in vitro replication assays were carried out with two major mammalian DNA polymerases, pol δ and pol ε (32).
EXPERIMENTAL PROCEDURES

Materials—T4 DNA ligase, T4 polynucleotide kinase, and EcoRV were obtained from New England BioLabs (Beverly, MA). S1 nuclease and proteinase K were purchased from Life Technologies, Inc. (Rockville, MD). Calf thymus DNA polymerase δ and proliferating cell nuclear antigen (PCNA) were generous gifts from Dr. K. M. Downey (University of Miami, FL) and were isolated according to the published procedures (33, 34). DNA polymerase ε from HeLa cells was kindly provided by Dr. S. Linn (University of California, Berkeley, CA) and was purified as described (35). [γ³²P] ribo-ATP was purchased from NEN Life Science Products Inc. (Boston, MA). Bio-Spin columns were purchased from Bio-Rad (Hercules, CA). Centricon 100 concentrators were obtained from Amicon Inc. (Beverly, MA). Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum, Opti-MEM (reduced serum medium), L-glutamine, antibiotic-antimycotic and lipofectin reagent for tissue culture were obtained from Life Technologies, Inc (Rockville, MD). Trypsin-EDTA and HEPES buffer were purchased from Cellgro Mediatech, VA. Phosphate buffered saline was from Sigma Chemical Company, (St. Louis, MO).

Oligodeoxynucleotides—γ–HOPdG (Fig. 1) was inserted into a 12-mer oligonucleotide sequence [5’-GCTAGC (γ–HOPdG) AGTCC-3’] and purity determined by a 20% denaturing polyacrylamide gel, by previously described procedures (27). Nondamaged 12-mer with a dG in place of γ–HOPdG was purchased from Midland Certified Reagent
Co. (Midland, TX). All other oligodeoxynucleotides were synthesized by the Molecular Biology Core Laboratory of the NIEHS Toxicology Center at the University of Texas Medical Branch, Galveston, Texas, and purified by electrophoresis through a 15% denaturing PAGE (in the presence of 7M urea).

*Bacterial strains and vectors*—Single stranded (ss) pMS2 DNA was a generous gift from Dr. M. Moriya (State University of New York, Stony Brook, NY). COS-7 cells were purchased from American Type Culture Collection, (Rockville, MD). *E. coli* DH10B cells that were used for transformation of DNA isolated from COS-7 cells after transfection with modified and control DNA, and helper phage M13KO7 were purchased from Life Technologies Inc. (Rockville, MD). Repair-deficient *E. coli* AB2480 (uvrA<sup>−</sup> recA<sup>−</sup>) was used for propagating the pMS2 shuttle vector during isolation of ss DNA by helper phage rescue using M13KO7 (10<sup>11</sup> pfu/ml). Purity of ss pMS2 was assayed by electrophoresis through a 1.4% agarose gel and compared with standard ss pMS2 DNA as supplied by Dr. M. Moriya.

*Construction of circular ss pMS2 DNA modified with γ-HOPdG*—ss pMS2 (29 pmols, 50 µg) was annealed to a 58–mer scaffold (145 pmols/2.34 µg) in the presence of a buffer (NEB # 3) containing 100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM di-thiothreitol (DTT) and 100 µg/ml bovine serum albumin (BSA), in 200 µl of reaction mix, and incubated at 90°C overnight. The hairpin loop in the ss pMS2 DNA that contains a single *EcoRV* restriction site (Fig. 2), was digested with 40 units of *EcoRV* in the presence of NEB#3 and BSA in a 600 µl reaction mixture, by incubation at 37°C for 3 hr. Non-annealed 58–mer scaffold and the excised hairpin loop were removed from the reaction by centrifugation in a Centricon-100 tube. The 58–mer scaffold was
complementary to the two termini of the digested vector (after removal of the hairpin loop), and the central sequence was complementary to the 12-mer oligonucleotide bearing the γ−HOPdG adduct, except there was complete substitution of uracil for thymine (Fig. 2). The gap created by annealing of ss pMS2 DNA to the scaffold was filled by ligation with a 10-fold excess of the γ−HOPdG containing oligonucleotide, or the unmodified 12-mer as control. The 12-mer oligodeoxynucleotides were phosphorylated at the 5′ end with ribo-ATP and ligated in a buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 10 mM ATP, 25 μg/ml BSA, using T4 polynucleotide kinase (5 U/pmol) and T4 Ligase (325 U/pmol), in the reaction mixture of 200 μl, at 4 °C for 48 hr. The two ligated samples were designated ss pMS2(dG) and ss pMS2(γ−HOPdG).

Ligation products were visualized by ethidium bromide staining, following electrophoresis through a 1.4% agarose gel and compared with standard circular and linearized ss pMS2 DNA. Modified samples were purified to remove non-ligated 12-mer in Centricon-100 tubes after dilution to 600 μl. After repeating this step three times, DNA was recovered by ethanol precipitation and the DNA concentration determined by measuring absorbance at 260 nm. In order to avoid the 58−mer scaffold being used as a primer for (-) strand replication, ligated DNAs were incubated with uracil-DNA glycosylase for 1 hr to remove uracil bases and generate abasic sites.

Transfection of ss pMS2(dG) and ss pMS2(γ−HOPdG) into COS-7 cells—Modified DNAs were allowed to replicate in COS-7 cells and DNA was isolated for transforming *E. coli* DH10B cells to assess mutations which might have arisen during replication in COS-7 cells. For transfection of ligated DNAs, COS-7 cells (5 X 10⁵ cells per 6 cm petri
dish), were first maintained in (DMEM) supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml), incubated for 24 hr at 5% CO₂. Individually, 30 µg of lipofectin and 200 ng of ligation mixture were dissolved in 1.5 ml of Opti-MEM, combined and incubated for 15 min at room temperature. A total of 3 ml of this solution was added to each 6 cm petri plate containing the attached COS-7 cells and incubated for 18 hr at 37°C. Transfection medium was replaced with 5 ml of fresh DMEM and cultured for 48 hr. Cells were then washed twice with 5 ml of phosphate buffered saline, and progeny phagemid DNA was recovered by the method described by Hirt (36).

Site-specific mutagenesis assay—DNA extracted from COS-7 cells was successively treated with S1 nuclease (0.2 U/60 µl reaction mix) and EcoRV (20 U/60 µl reaction mix) in order to remove any input ssDNA and progeny associated with the original ss pMS2 vector and then replicated in E. coli DH10B. A total of 50 µl of electrocompetent DH10B cells was transformed with 5 µg of DNA by electroporation in chilled 2-mm gap cuvettes using ECM 600 (BTX, San Diego), at 25µF, 2.5kV, R5 (Resistance timing) 129 OHMS. 2X yeast- tryptone (YT) (0.95 ml) was immediately added and incubated with shaking at 37°C for 1 hr to fully express the ampicillin resistant gene. Aliquots of the transformation mixture were plated out onto 1X YT agar plates containing 100 µg/ml ampicillin. After incubation overnight at 37°C, the number of transformants per plate was counted to determine replication efficiency.

In addition, 5 µg of undamaged and damaged, modified ss pMS2 DNA were added to 40 µl of competent cells of E. coli AB2480 (uvrA⁻ recA⁻) by electroporation
under similar conditions described above and allowed to replicate on LB-ampicillin plates overnight, for comparison of mutagenicity in mammalian and bacterial cells.

A total of 480 colonies from each plate of transformed cells were individually picked with sterile toothpicks and grown in 2X YT containing ampicillin in 96 well plates overnight. The cultures from each 96 well plate were transferred using 48 pin replicators, onto Whatman 541 filter papers placed on ampicillin containing 1X YT agar plates, in four replicates (one for each probe: [5′-GATGCTAGCNAAGTCCATC-3′] where N refers to A, T, G, or C), and incubated overnight at 37°C to form well defined colonies. Alternately, colony lifts were performed on four replicate plates of transformants in order to determine the actual mutation frequency of the entire population. Filter papers were removed from the agar plates, cell lysis achieved by soaking in 0.5 N NaOH for 5 min followed by neutralization in 0.5 M Tris-HCl, (pH 7.4) and washed in 1X SSC (0.15 M NaCl; 0.15 M Sodium Citrate-C₆H₅O₇Na₃, pH 7.0). DNA was crosslinked onto the paper in a UV Stratalinker for 1.5 min.

Hybridization was carried out with γ²³P ATP labeled probes, designed to determine the nature of the base at the site of the adducted guanine in progeny plasmid DNA. Hybridization temperature was calculated using the formula where the oligonucleotide melting temperature equals [4(G+C) +2(A+T)] – 14°C. Filters were incubated in prehybridization solution containing 25 ml of 20X SSPE (3 M NaCl; 2 mM Na₂HPO₄; 2 mM EDTA), 20 ml of formamide, 5 ml of Denhardt’s solution (1% Ficoll; 1% polyvinylpyrrolidone; 1% BSA), 1 ml of 10 mg/ml fish milt DNA and 1 ml of 10% SDS, for 20 min to restrict non-specific binding of the probe. Four filters were separated and probed with 100 pmol each of four 18–mer oligodeoxynucleotides containing one of the four canonical bases at the adducted site within the centrally located sequence of the
damaged and non-damaged 12-mer oligonucleotide. After overnight hybridization with probes in hybridization solution, filters were washed with 2X SSPE at 2°C above hybridization temperature to remove nonspecifically bound probes. Dried filters were exposed to XR-OMAT film overnight and autoradiographs were developed to identify mutation frequency and types of mutations. Representative colonies were subjected to dideoxy sequencing (37) to confirm the sequence of the mutated DNAs. A 20-mer primer (5′-CCATCTTGTTCATCAGCG-3′) was used for sequencing progeny plasmid DNA.

Construction of linear template for in vitro replication assays—The γ−HOPdG 12-mer oligodeoxynucleotide utilized in the construction of the pMS2 vector, as well as its nondamaged analog, were used to construct 135-mer linear templates. Nondamaged or γ−HOPdG containing 12-mer oligodeoxynucleotide, the 62-mer 5′-flanking DNA, and the 61-mer 3′-flanking DNA (500 pmols of each) were annealed to an equimolar amount of the 42-mer scaffold DNA. Prior to annealing, the 12-mer and 61-mer oligodeoxynucleotides were 5′-phosphorylated using ribo-ATP (non-labeled) and T4 polynucleotide kinase. To visualize the ligation products, 1/100 of the 12-mer oligodeoxynucleotides were phosphorylated with [γ32P] riboATP. Ligations were performed with 400 U of the T4 DNA ligase in the reaction volume of 100 µl at 13°C for 20 hr. Ligation products were separated by electrophoresis on a 10 % denaturing PAGE. The 135-mer bands were excised, DNAs were eluted with buffer consisting of 500 mM ammonium acetate, 10 mM magnesium acetate, and 1 mM EDTA and precipitated with ethanol. The sequence of resulting oligodeoxynucleotides were identical: 5′-

GGGACCTGAACACGTACGGGAGCAGCATCGAGCCAGATCTGCGCCAGCTGGCCACCCCTGCTAGCGAGTCCGCGCCAAGCTGGGCTGCAGCAGGTCGACT

12
CTAGAGGATCCGGCGAGCTCGAATTCGCC-3’, where the underlined G is either γ–HOPdG or non-adducted.

*In vitro replication of γ–HOPdG-containing linear template*—21-mer oligodeoxynucleotides were used as primers for *in vitro* polymerase reactions, using the following sequences: 5’-CCTGCTGCAGCCCAAGCTTGG-3’, which is complementary to the template DNA from positions –9 to –29 relative to the site of lesion (-9 primer), 5’-AGCCCAAGCTTGGCGCGGACT-3’, (–1 primer), and 5’-TGGCGCGGACTCGCTAGCAGG-3’, which is complementary to the template DNA from positions +9 to –11 overlapping the lesion site (+9 primer). Primer oligodeoxynucleotides were phosphorylated with T4 polynucleotide kinase using [γ³²P] riboATP. The [γ³²P]-labeled primers were mixed with the oligodeoxynucleotide substrates at a molar ratio of 1:2 in the presence of 50 mM KCl, heated at 90°C for 2 min, and cooled to room temperature overnight. DNA samples were purified using P-6 Bio-Spin columns supplied with 10 mM Tris-HCl buffer (pH 7.4). To confirm the completion of primer annealing, aliquots were tested on a 5% native PAGE, which was also used for verification of DNA concentrations.

Assays with polymerase δ and polymerase ε were carried out as described (33, 38). The polymerase δ reaction mixture contained 2 nM primer annealed to a template in 40 mM HEPES-KOH (pH 6.8), 10% glycerol, 200 µg/ml of BSA, 6 mM MgCl₂, 1 mM DTT, 70 ng calf thymus PCNA where indicated, polymerase δ and dNTPs (each of the four dNTPs or individually) at the concentrations as given in figure legends. Reactions were incubated for 10 min at 37°C in a final volume of 5 µl. The pol ε reaction mixture contained 2 nM primer/template DNA, 50 mM HEPES-KOH (7.5), 100 mM potassium
glutamate, 20% glycerol, 200 µg/ml of BSA, 15 mM MgCl₂, 10 mM DDT, 0.03% Triton X-100, 100 µM each of the dNTP’s and 2.5 mU of polymerase ε (where 1 unit of polymerase is defined as the amount that catalyzes the incorporation of 1 nmol of dTTP into an acid-insoluble form per hr using dA₃₀₀₀ and oligo(dT)₁₂₋₁₈). Reactions were incubated for 20 min at 37°C in a final volume of 12.5 µl. Incubation of the primer/template DNA substrates under the same reaction conditions without polymerase was used as a negative control reaction. Reactions were terminated by the addition of 2X of stop solution consisting of 95% (v/v) formamide, 20 mM EDTA, 0.02 % (w/v) xylene cyanol, and 0.02 % (w/v) bromphenol blue. Reaction products were resolved on a 15% denaturing PAGE and visualized by autoradiography. Quantitative analyses was performed using a PhosphorImager screen and Image-Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA).
RESULTS

*Mutagenicity of γ−HOPdG modified single stranded vectors in simian kidney cells*—

Single stranded pMS2 DNA which was purified as described in Materials and Methods, was annealed with a partially complementary scaffold DNA and digested with *Eco*RV (Fig. 2, step 1). The *Eco*RV restriction site spontaneously forms within a hairpin in the ss pMS2 and completion of linearization was tested by differential electrophoretic mobility through a 1.4% agarose gel. Into the resulting gap (Fig. 2, step 2), control unmodified and γ−HOPdG modified 12-mer oligodeoxynucleotides were ligated (Fig. 2, step 3). Efficiencies of ligation were assessed by electrophoretic analyses of the conversion of linear to circular DNA molecules. The scaffolding DNA was severely damaged by digestion with uracil DNA glycosylase (Fig. 2, step 4). COS-7 cells were transfected with 200 ng of modified ss pMS2(dG) and ss pMS2(γ−HOPdG), and following a 72 hr replication, progeny plasmid DNA was isolated. Following treatment of this DNA with S1 nuclease and *Eco*RV to remove any contaminating non-ligated ss pMS2 vector, *E. coli* DH10B cells were transformed and selected for ampicillin resistance.

Table 1 shows the results of transformation of ss pMS2(dG) and ss pMS2 (γ−HOPdG). The modified construct containing the acrolein adduct yielded slightly more transformants than control ss pMS2(dG). To assess the mutagenic potential of the adduct, 480 colonies were picked and grown in 96 well plates for analysis using a differential hybridization strategy to reveal the kind of mutations that may have occurred at the site of the adducted guanine. Of the 480 colonies that were randomly picked, 417
hybridized with one of the probes, while 63 colonies did not hybridize with any of the four probes. Of those transformants that did not hybridize with any sequence-specific probe, none of those hybridized to sequences immediately upstream of the oligodeoxynucleotide ligation site, suggesting that this deletion was not caused by the adduct. Further, approximately the same number of nonhybridized colonies were observed with the control ss pMS2(dG). Although most colonies (~93%) revealed no targeted mutations (Fig. 3), 2.6% (11/417) of the transformants had targeted G-C transversions as evidenced by hybridization with the probe containing C at the site of the adducted base (Fig. 4). Sequencing of plasmid DNA prepared from these colonies confirmed the presence of G opposite the site. Further, 1% of the transformants (4/417) hybridized with the probe containing A at the site of the adduct indicating G -A transition, and 3.8% of the transformants (16/417) hybridized with the probe containing T at the site of the adduct (Fig. 4). A total of 7.4% of the transformants was found to have point mutations. None of the transformants carried the sequence of the 58-mer scaffold. Differential hybridization of control, nonadducted ss pMS2(dG) revealed no mutations (Figs. 4 and 5). Transfection and transformation in COS-7 cells and E. coli DH10B cells, as well as differential hybridization with the four probes were repeated three times.

In order to increase the total number of E. coli transformants that could readily be assayed, direct lifts were also performed on transformants, and probed with site-specific A, T, G or C probes. Since all of the transformed colonies were lifted for mutational analysis by the differential hybridization strategy, sequencing for these DNAs was not possible, but mutation frequencies were confirmed with Fig. 5 showing the pattern of differential hybridization. In excellent agreement with the previous data, the transformants contained 1.8% G -C transversions, 1.2% G -A transitions and 3.4% G -T
transversions. Thus, a total of 6.4% mutations were observed by the direct lift method while 7.4% mutations were obtained using randomly chosen samples (Fig.3). Sequencing data confirmed base substitution at the adducted site for all the mutant colonies tested.

*Replication and mutagenicity of γ−HOPdG in E. coli AB 2480*—Since the γ−HOPdG lesion had been previously shown to be very weakly mutagenic in *E. coli* (29, 30), it was considered to be important to test these vector constructs in *E. coli* mutagenesis assays. For direct comparison of results in the bacterial system, modified ss pMS2(dG) and ss pMS2(γ−HOPdG) were introduced into repair-deficient AB2480 cells (*uvrA− recA−*) by electroporation. The percentage of mutations obtained in *E. coli* cells was <1% (Fig. 3). This essentially error-free replication past the γ−HOPdG adduct in *E. coli* suggests that the fidelity of the bacterial polymerases is substantially different from the mammalian polymerases, that appear to have bypassed the lesion efficiently, but misincorporated and extended a substantial number of bases opposite the lesion.

*In Vitro Lesion Bypass with Polymerases δ and ε* — The data within this and other reports (29, 30) indicate that in *E. coli*, γ−HOPdG is not a strong block for DNA synthesis, nor a miscoding lesion. *In vitro*, Klenow exo− fragment of *E. coli* pol I is capable of limited, error-prone DNA synthesis across γ−HOPdG (29). The “SOS” polymerases do not seem to be essential for replication across this lesion, as evidenced by the observation that inactivation of *polB*, *dinB*, and *umuD,C* genes did not affect significantly the efficiency or fidelity of translesion synthesis (29). It has been concluded therefore that DNA polymerase III, the major replicative DNA polymerase in *E. coli*, catalyses error-free translesion synthesis across γ−HOPdG. Our *in vivo* replication studies
using the pMS2 vector and COS-7 cells suggest that in the eukaryotic system the γ-HPdG lesion also does not pose a significant block for DNA replication machinery; however, it was pro-mutagenic in this system. Taking these data into consideration, it was assumed that at least one of the major eukaryotic DNA polymerases, pol δ or pol ε (32), may be capable of translesion synthesis, presumably error-prone, across γ-HPdG. To test this assumption, *in vitro* replication studies were performed using both calf thymus pol δ and human pol ε.

In order to determine whether calf thymus pol δ was able to replicate across a γ-HPdG adduct, primer extension experiments were carried out in which running start (-9 primer annealed to template DNA) and standing start (-1 primer annealed to template DNA) conditions were simulated (Fig. 6). To control for the integrity of the template preparations, a +9 primer was also utilized in this study. Under the conditions used, all three primers were efficiently extended by pol δ when they were primed with nondamaged DNA template (Fig. 6, lane 3, 6, and 9). Due to the distributive mechanism of pol δ replication (39), extension of each primer led to the formation of a multitude of products that were shorter than full-length product. On the γ-HPdG-adducted template, -9 primer was efficiently extended to the position one nucleotide before the lesion (Fig. 6, lane 12), but no bands were detectable that would reflect nucleotide incorporation opposite the adducted site or beyond it. Accordingly, no extension was observed when γ-HPdG-modified template was primed with –1 primer (Fig. 6, lane 15). Thus, γ-HPdG presents a major replication block to pol δ.

It has been shown earlier that proliferating cell nuclear antigen (PCNA), the known pol δ processivity factor (32, 34), facilitates pol δ translesion bypass through a
number of DNA lesions, including abasic sites, 8-oxo-deoxyguanosine, aminofluorene-deoxyguanosine (40), thymine dimers (41), and 1,N\(^6\)-ethenodeoxyadenosine (42). In our experiments, addition of PCNA to reactions resulted in formation of full-length products (approximately 64% of the primers) when non-damaged DNA template was examined (Fig. 6, lanes 4, 7, and 10). On the γ-HOPdG-modified template, full-length products were also formed, but only at ~7% under running start conditions (Fig. 6, lane 13) and ~5% under standing start conditions (Fig. 6, lane 16). In addition, an accumulation of products was observed that could reflect nucleotide incorporation opposite the lesion (13% of the product under running start and 12% under standing start conditions). Thus, replicative blockage of pol δ caused by γ-HOPdG was diminished in the presence of PCNA. Confirming the integrity and purity of the γ-HOPdG-adducted DNA template, extensions of the + 9 primer, without or with PCNA (Fig. 7, lanes 18 and 19), were as efficient as that observed on non-damaged substrate.

The specificity of nucleotide incorporation by pol δ opposite the γ-HOPdG was also examined. Single nucleotide incorporation experiments were carried out under the same conditions as the primer extension experiments. In agreement with primer extension experiments, no incorporation opposite γ-HOPdG was detected when reactions were incubated without PCNA (Fig. 7, lanes 11-14). On the non-damaged template, 69% of primers were extended with a C (Fig. 7, lane 3) and about 1% with a T (Fig. 7, lane 5). In the presence of PCNA, an A (Fig. 7, lane 15), a G (Fig. 7, lane 17), and a T (Fig. 7, lane 18) were inserted opposite γ-HOPdG, forming 3%, 2%, and 3% of the products, respectively. Surprisingly, no detectable nucleotide incorporation was found when the correct nucleotide was added to the reaction (Fig. 7, lane 16). On nondamaged substrate,
pol δ displayed an unexpectedly high propensity for misincorporation in the presence of PCNA (Fig. 7, lanes 6-9). In this case, correct nucleotide C was preferentially incorporated opposite a G (80% of the extension), but the primers were also readily extended by a T and an A (45% and 16% of the products, respectively). Reduction of the fidelity of DNA synthesis by pol δ in the presence of PCNA has been reported previously (43, 44). Particularly, interaction of PCNA with polymerase led to a decrease in $K_m$ of dNTPs, and consequently to an increase of nucleotide incorporation efficiency. This is true both for correct and incorrect nucleotide incorporation. Under conditions of single nucleotide incorporation experiments in which there is no competition between correct versus incorrect dNTP, this increased efficiency of incorporation is reflected in effective misincorporation. After the conditions of the reactions in the presence of PCNA were adjusted so that misincorporation by pol δ opposite G was effectively eliminated (5 µM dNTP and 10 mU of the enzyme) (Fig. 7, lanes 24-27), no nucleotide incorporation was observed opposite $\gamma$-HOPdG (Fig. 7, lanes 33-36). Thus *in vitro*, pol δ has limited ability to replicate through the $\gamma$-HOPdG. This replication is highly mutagenic and occurs only at high concentrations of dNTPs and in the presence of PCNA.

Primer extension experiments were also performed with human pol ε using the same set of primers as described in pol δ experiments. Under the conditions used, approximately 97% of the primers were extended by pol ε replicating through unmodified G (Fig. 8, lanes 2 and 6). Using the $\gamma$-HOPdG-modified DNA template, primer extension by pol ε was strongly blocked one nucleotide before the lesion and DNA synthesis was completely aborted after extremely weak incorporation opposite the lesion (less than 2% of the products) both under running start and standing start conditions (Fig. 8, lanes 4 and
8). In the presence of human PCNA, replication by pol ε through the γ−HOPdG was as inefficient as in the absence of PCNA (data not shown).

Therefore, these data indicate that major eukaryotic polymerases, pol δ and pol ε, are unlikely to be involved in translesion bypass across the γ−HOPdG in vivo, and that neither of them contributes to error-free replication across this lesion.
DISCUSSION

The results of transformation of mammalian cells with control ss pMS2(G) and modified ss pMS2(γ−HOPdG) reported in this paper indicate that at least a subset of mammalian polymerases are capable of efficiently bypassing the adduct, but with extensive misincorporation opposite the lesion. Use of the single stranded shuttle vector in this study allowed for a sensitive evaluation of *in vivo* translesional synthesis and also eliminated the possibility of repair and preferential replication of the unadducted complementary strand that could conceivably occur when double stranded vectors are used. It is evident that in a subset of vector progeny (7.4%), all nucleotides are being incorporated opposite the lesion with a subsequent extension. This work represents the first report of error-prone replication for γ−HOPdG adducts.

Comparison of these data with that reported by Moriya *et al* (22) for the mutagenic bypass of PdG, also using the same pMS2 vector in COS-7 cells, reveals significant similarities. Both of these studies report that neither the γ−HOPdG nor PdG were severe blocks to *in vivo* DNA replication, suggesting that one or a combination of DNA polymerases can efficiently bypass this lesion. Further the PdG adduct yielded ~ 7.5% G to T and ~ 1% G to C, while in this study, γ−HOPdG conferred 3.8% G to T, 2.6% G to C and 1% G to A. Considering that there is differential statistical power to these reported values based on the total number of colonies screened, these mutagenic spectra are remarkably similar. In agreement with the *E. coli* study of Burcham and Marnett (23) for PdG and both studies on γ−HOPdG in *E. coli* (29, 30), we also find very
low mutagenic frequencies in *E. coli*, indicating different functioning and processing abilities of the mammalian polymerases, when compared with bacterial polymerases. A reasonable hypothesis is that the γ−HOPdG base is presented to the bacterial polymerase as the acyclic \( N^2 -(3\text{-oxopropyl})dG \), while in the mammalian system it remains in the cyclic γ−HOPdG form, at least to some extent.

In order to identify the polymerase associated with *in vivo* DNA synthesis past γ−HOPdG and to understand how the adduct gives rise to mutations, *in vitro* replication studies were performed using two major replicative mammalian DNA polymerases, pol δ and pol ε. Surprisingly, DNA synthesis by both pol δ and pol ε was almost completely terminated by the γ−HOPdG one nucleotide prior to the lesion, but in the presence of PCNA, pol δ could perform highly error-prone replication across the adduct.

Although the present study was not aimed at uncovering the basis of replicative blockage and mutagenicity caused by γ−HOPdG, some assumptions concerning this matter can be made. Weak nucleotide incorporation opposite the lesion by pol ε was observed and, in the case of pol δ, incorporation was achieved by addition of PCNA. Based on this, formation of the competent ternary complex containing γ−HOPdG within the polymerase active site is evident. The current model for error-free DNA synthesis past is γ−HOPdG based on a recently established (28) structure of the adducted DNA duplex, in which γ−HOPdG was paired with C, as well as on the observation that in *E. coli*, the replication across γ−HOPdG is very accurate. Within the duplex, all base pairs including γ−HOPdG:C displayed a Watson-Crick alignment, and the adduct predominantly existed as an open form with the \( N^2 \)-propyl chain pointed away from the minor groove (28). In contrast, γ−HOPdG predominantly exists as a ring-closed form in...
solution (28) and may have the same conformation in the single-stranded DNA region within a polymerase active site. Such a ring closed form would be structurally similar to that of the PdG lesion. It has been proposed therefore that at the replicative fork, an incoming dCTP would trigger a structural rearrangement of the adduct from the ring-closed form to the ring-open form, promoting the formation of the correct Watson-Crick hydrogen bonds, stabilizing the structure, and facilitating a consequent extension of DNA synthesis (28). The data presented here demonstrate that pol δ can incorporate A, G, and T opposite γ-HOPdG, but that incorporation of the correct nucleotide, C, was not detected. This observation that incorrect nucleotides are preferentially incorporated opposite the adduct is similar to the error-prone nucleotide insertion by *E. coli* pol I Klenow fragment (exo⁻) opposite the same adduct (29,30), where insertions of A and G were much more efficient than insertion of C. Thus these data may suggest that the active sites of such polymerases either restrict the ability of the exocyclic ring to open and thus force infrequent mutagenic bypass of the ring-closed form of the lesion or possess unfavorable geometric interactions between dCTP and the adducted base, whether ring-opened or closed. It is not clear whether nucleotides are incorporated opposite the adduct that remains in the ring-closed conformation or whether interactions between an incoming nucleotide and the adduct promotes the ring-opening. Also, it cannot be excluded that nucleotides are preferentially incorporated opposite the ring-open form, which may be present as a minor subpopulation in the single-stranded DNA. Independent of how non-canonical base pairs are formed, the resulting structure does not facilitate the subsequent steps of extension efficiently.

Both polymerases investigated in the present study possess a 3' to 5' exonucleolytic activity. Therefore, it is likely that upon encountering the lesion, the
polymerase is stalled performing nonproductive cycling of polymerization/nucleotide removal. Interestingly, the impact of a polymerase exonucleolytic activity to bypass \(\gamma\)-HOPdG was observed in the case of \textit{E. coli} Klenow pol I fragment: the exonucleolytically proficient protein was completely blocked by \(\gamma\)-HOPdG (unpublished data), whereas the protein lacking an exonucleolytic activity exhibited a capacity for a limited error-prone DNA synthesis past the lesion (29,30). Thus, none of the three polymerases tested so far utilizes the above mentioned mechanism for error-free replication across \(\gamma\)-HOPdG. Since the adduct at the replicative fork is presumed to exist in ring-closed form, it is suggested that for some polymerases the mechanism of \(\gamma\)-HOPdG translesional synthesis would resemble the mechanism of synthesis past the model PdG adduct.

\textit{In vivo} replication studies revealed that DNA synthesis past \(\gamma\)-HOPdG was not significantly inhibited in a mammalian system and that correct nucleotide was incorporated in approximately 93\% of the translesional events. In contrast, \(\gamma\)-HOPdG was a severe block for \textit{in vitro} replication by both pol \(\delta\) and pol \(\varepsilon\). In addition, incorporation of correct nucleotide opposite the lesion was not detected. The data indicate therefore, that in mammalian cells the major replicative polymerases are unlikely to be responsible for replication across \(\gamma\)-HOPdG. We hypothesize that a polymerase switching mechanism occurs at replication forks that are blocked by encountering a \(\gamma\)-HOPdG lesion. The identity of this polymerase is currently unknown but reasonable candidates are the recently identified lesion bypass polymerases (45). The role of such a polymerase would be to carry out short bypass synthesis, and then be subsequently displaced by the major replicative enzyme complex. It is not evident whether one
polymerase is solely responsible for the bypass and mutagenic spectrum in COS-7 cells or whether multiple polymerases are involved, in which one polymerase carries out error-free bypass and another is primarily error-prone.

The present study clearly indicates the $\gamma$-HOPdG adduct can contribute to the mutagenicity of acrolein in a mammalian system, being responsible for at least some of the acrolein-induced base substitutions. Moreover, the potential for $\gamma$-HOPdG to form secondary DNA adducts such as DNA-DNA (46) and DNA-polypeptide cross-links (A. J. Kurtz, I. G. Minko, unpublished data) may extend further the spectrum of mutagenicity of this adduct.
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TABLE 1
TRANSFORMATION EFFICIENCY AND BASE PAIR SUBSTITUTIONS IN
COS-7 CELLS AND E. coli (AB 2480) TRANSFORMED WITH
ss pMS2 (dG) AND ss pMS2 (γ-HOPdG)

| SPECIES TRANSFORMED | Total Transformants | Non-Hybridized Colonies | Base Pair Substitutions | TOTAL MUTATION % |
|----------------------|---------------------|-------------------------|-------------------------|-----------------|
| COS-7                |                     |                         |                         |                 |
| ss pMS2 (dG) Colony pick | 288 | 42 | 246 | 0 | 0 | 0 | 0 |
| ss pMS2 (dG) Colony lift | 744 | 250 | 494 | 0 | 0 | 0 | 0 |
| ss pMS2 (γ-HOPdG) Colony pick | 480 | 63 | 387 | 4 | 16 | 11 | 7.4 |
| ss pMS2 (γ-HOPdG) Colony lift | 1064 | 563 | 468 | 6 | 17 | 9 | 6.3 |
| E. coli (AB 2480)    |                     |                         |                         |                 |
| ss pMS2 (dG) Colony lift | 2488 | 598 | 1890 | 0 | 0 | 0 | 0 |
| ss pMS2 (γ-HOPdG) Colony lift | 3200 | 530 | 2643 | 12 | 7 | 8 | 0.96 |
FIGURE LEGENDS

Fig. 1. Structures of exocyclic adducts.

Fig. 2. Schematic representation of construction of modified ss pMS2 DNA. Single stranded shuttle vector pMS2 was sequentially annealed to a 58-mer scaffolding DNA stabilizing the EcoRV site hairpin, restricted with EcoRV, hybridized to a 12-mer control or γ−HOPdG containing single-stranded oligodeoxynucleotide and ligated to recircularize the ss DNA.

Fig. 3. Percentage mutations observed following replication of DNAs containing a site-specific γ−HOPdG adduct in COS-7 cells and E. coli (AB2480). Percentage of mutations formed in COS-7 cells as assayed in E. coli by: Panel A Individual colonies picked and grown in 96 well plates or Panel B Colonies lifted out on Whatman 541 filter paper. Panel C Percentage of mutations observed following replication of modified DNA in E. coli (AB2480).

Fig. 4. Differential hybridization of colonies grown in 96 well plates. Panel A Non-damaged control plates (No mutations detected). Panel B γ−HOPdG plates. Base substitutions detected at the site of the adducted guanine in progeny plasmid DNA of COS-7 cells. G, A, C and T denote the base present at the site of the adducted guanine

Fig. 5. Differential hybridization of colony lifts. Panel A Non-damaged control (No mutations detected). Panel B Base pair substitutions at the site of the adducted guanine in progeny plasmid DNA derived from COS-7 cells. Panel C Base pair substitutions at the site of the adducted guanine in E. coli (AB 2480). G, C, A and T denote the base present at the site of the adducted guanine.
Fig. 6. Primer extension reactions catalyzed by calf thymus DNA polymerase δ on the γ−HOPdG-adducted template. Nondamaged (ND) or γ−HOPdG-adducted 135-mer DNA templates were annealed to one of three primers. The DNA substrates (2 nM) were incubated for 10 min at 37°C in the presence of all four dNTPs (100 µM) without (-) or with (+) 50 mU of polymerase δ and in the presence of 70 ng of calf thymus PCNA where indicated. The positions of the 21-nt primers and the 97-nt, the 89-nt and the 79-nt full-length products are indicated. G* indicates the position of the modified G on the template.

Fig. 7. Single-nucleotide incorporation by calf thymus DNA polymerase δ on the γ−HOPdG-adducted template. Nondamaged (dG) or γ−HOPdG-adducted 135-mer DNA templates were annealed to the –1 primer. The DNA substrates (2 nM) were incubated for 10 min at 37°C with 50 mU (A) or 10 mU (B) of polymerase δ in the presence of 100 µM (A) or 5 µM (B) of each of the four dNTPs (A = dATP, C = dCTP, G = dGTP, T = dTTP) and 70 ng of calf thymus PCNA where indicated. The positions of the 21-nt primer and 22-nt products are indicated.

Fig. 8. Primer extension reactions catalyzed by human DNA polymerase ε on the γ−HOPdG-adducted template. Nondamaged (ND) or γ−HOPdG-adducted 135-mer DNA templates were annealed to one of three primers. The DNA substrates (2 nM) were incubated for 20 min at 37°C in the presence of all four dNTPs (100 µM of each) without (-) or with (+) 2.5 mU of polymerase ε. The positions of the 21-nt primers and the 97-nt, the 89-nt and the 79-nt full-length products are indicated. G* indicates the position of the modified G on the template.
3-(2'-Deoxyribosyl) -pyrimido[1, 2-a]purin-10(3H)-one (M1G)

1,N^2-Propano-2'-deoxyguanosine (PdG)

γ-Hydroxy-1,N^2-propano-2'-deoxyguanosine (γ-HOPdG)

N^2-(3-Oxopropyl)-2'-deoxyguanosine
FIG. 2

STEP 1: Annealing to scaffold

```
    GC
   GC   
  C   G
 A  T
 G  C
 A  T
 C  G
 T  A
 C  G
 T  A
 G  C
 C  G
 T  A
 A  T
```

**EcoRV**

pMS2 DNA - AGATCGAATTGCTAGGTACC TGCAGGGGCCCTCGAGATCTGAT
3' - CUUAAGCUCGAGCCAUGG - 5' (SCAFFOLD)

STEP 2: EcoRV digestion

pMS2 DNA - TTCGAGCTCAGGAT TGCAGGGGCCCTCG -
3' - CUUAAGCUCGAGCCAUGGUCGCUA CGAUCGCUCAGG UAGCGAACGUCCCCGGGAGCUC - 5'

STEP 3: Ligation of DNA adduct

```
GCTAGCXAGTCC
```

pMS2 DNA - TTCGAGCTCAGGAT TGCAGGGGCCCTCG -
3' - CUUAAGCUCGAGCCAUGGUCGCUA CGAUCGCUCAGG UAGCGAACGUCCCCGGGAGCUC - 5'

STEP 4: Digestion of scaffold with UDG

pMS2 DNA 3' - TTCGAGCTCAGGAT TGCAGGGGCCCTCG -
(MODIFIED ss pMS2 DNA)
A

92.6 (G)

1.0 (A)

2.6 (C)

3.8 (T)

B

93.6 (G)

1.2 (A)

1.7 (C)

3.5 (T)

C

99 (G)

0.4 (A)

0.3 (C)

0.3 (T)
FIG. 4
5'----gctggccacccgtagcGagtccgcgcaccagcttggtgcacgcagcgg----3'  
gttcgacccgacgtcgc-5' (- 9 primer)  
tcaggcgggtcgaaccga-5' (-1 primer)  
ggacgatcgcagcggctggt-5' (+ 9 primer)

| DNA substrate: | Pol δ: | PCNA: |
|----------------|--------|-------|
|                | ND     | γ-HOPdG|
| Pol δ:         | - + + - + + - + + | - + + - + + - + + |
| PCNA:          | - - + - - + - - + | - - + - - + - - + |
| 1 2 3 4 5 6 7 8 9 10 | 11 12 13 14 15 16 17 18 19 |

Figure 6
5'----gctggccaccctgctagcG\*Gagtccgcgccaagcttggtgcagcagg----3' 
tcaggcgcgggtcgaaccgga-5' (-1 primer)

| DNA substrate: | dG | \(\gamma\)-HOPdG |
|----------------|----|-----------------|
| Pol \(\delta\): | – + + + + + + + + + | – + + + + + + + + + |
| PCNA: | – – – – – – + + + + + | – – – – – – + + + + + |
| dNTP: | – A C G T A C G T | – A C G T A C G T |
| 1 2 3 4 5 6 7 8 9 | 10 11 12 13 14 15 16 17 18 |

A

B

21

22
5'----gctggccaccctgctagcGagtcgcgcggcaagctgggtgcagcagg----3'  
gttcgaaccctgacgtcgtcc-5' (- 9 primer)  
tcaggccgggtcgaaccgga-5' (- 1 primer)  
ggacgatcgctcaggcgcgggt-5' (+ 9 primer)  

DNA substrate:  
| Pol ε: | - | + | - | + | - | + | - | + | - | + |
|--------|---|---|---|---|---|---|---|---|---|---|
| 1      | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10| 11| 12| 13 |

-9 primer  -1 primer  +9 primer  

- G*  
- G*  
- 21  
- 97  
- 89  
- 79  

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Error prone translesion synthesis past γ-hydroxypropano deoxyguanosine, the primary acrolein derived adduct in mammalian cells
Manorama Kanuri, Irina G. Minko, Lubomir V. Nechev, Thomas M. Harris, Constance M. Harris and R. Stephen Lloyd

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