Acrolein is produced extensively in the environment by incomplete combustion of organic materials, and it arises endogenously in humans as a metabolic by-product. Acrolein reacts with DNA at guanine residues to form the exocyclic adduct, 8-hydroxypropanodeoxyguanosine (HOPdG). Acrolein is mutagenic, and a correlation exists between HOPdG levels in Salmonella typhimurium treated with acrolein and a resultant increase in mutation frequency. Site-specifically modified oligonucleotides were used to explore the mutagenic potential of HOPdG in *Escherichia coli* strains that were either wild-type for repair or deficient in nucleotide excision repair or base excision repair. Oligonucleotides modified with HOPdG were inserted into double-stranded bacteriophage vectors using the gapped-duplex method or into single-stranded bacteriophage vectors and transformed into SOS-induced *E. coli* strains. Progeny phage were analyzed by oligonucleotide hybridization to establish the mutation frequency and the spectrum of mutations produced by HOPdG. The correct base, dCMP, was incorporated opposite HOPdG in all circumstances tested. In contrast, in vitro lesion bypass studies showed that HOPdG causes misincorporation opposite the modified base and is a block to replication. The combination of these studies showed that HOPdG is not miscoding in vivo at the level of sensitivity of these site-specific mutagenesis assays.

The accumulation of DNA damage is believed to play a significant role in genetic diseases, including cancer. An important form of DNA damage results from the addition of electrophiles to form stable adducts to the bases. In the case of bifunctional electrophiles, cyclization can occur to form exocyclic adducts that obstruct Watson-Crick base pairing (1). These adducts are anticipated to be blocks to replication and, therefore, potential premutagenic lesions. This has been demonstrated to be the case with the exocyclic adducts, pyrimido[1,2-α]purin-10(3H)-one (M,G)³ and its structural analogue, 1,N²-propano-2'-deoxyguanosine (PdG) (2–4).

Acrolein, an α,β-unsaturated aldehyde, is an example of such a bifunctional electrophile. Acrolein is produced exogenously as a product of organic combustion and endogenously as a product of lipid peroxidation (5–7). Acrolein also arises during cyclophosphamide metabolism (8). It is highly mutagenic to bacterial and mammalian cells and exhibits tumor-initiating activity (9–11). The most likely mode of action for acrolein-induced mutagenicity is its ability to form adducts to DNA. The major adduct generated by the reaction of acrolein with deoxyguanosine residues in DNA is 8-hydroxypropanodeoxyguanosine (HOPdG), which is structurally related to MiG and PdG (Fig. 1) (12, 13). Acrolein has been shown to be mutagenic in *Salmonella typhimurium*, and a correlation has been observed between HOPdG levels in *S. typhimurium* treated with acrolein and the resultant increase in mutation frequency (14). The HOPdG adduct has been detected at relatively high levels in DNA from healthy human tissues using ³²P-postlabeling combined with high performance liquid chromatography (15, 16).

A major obstacle in attempts to evaluate the mutagenic potential of HOPdG has been the generation of oligonucleotides containing site-specifically incorporated HOPdG. The base liability of HOPdG prevents its direct incorporation into DNA by standard synthetic protocols. Recently, both Khullar et al. (17) and Nechev et al. (18) described strategies for the stable, site-specific incorporation of HOPdG into oligonucleotides using oxidative generation of the aldehyde function after the base deprotection step. This enables the construction of recombinant M13 genomes containing a single HOPdG adduct for use in mutagenesis assays in *Escherichia coli*. In the present study, we prepared double-stranded vectors containing HOPdG to evaluate its ability to block replication, to induce mutations, and to be repaired. In addition, single-stranded vectors were constructed to examine the ability of HOPdG to induce mutations in the absence of repair or of replication strand bias. The combination of these approaches revealed that HOPdG does not induce mutations at a level of sensitivity well below that needed to detect mutations caused by other structurally related exocyclic adducts.

**EXPERIMENTAL PROCEDURES**

*Materials—*T₄ polynucleotide kinase, T₄ DNA ligase, deoxynucleotide 5’-triphosphates, *Bst*HII, *Sac*II, EcoRI, and DNA polymerase I

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† To whom correspondence should be addressed: Dept. of Biochemistry, Vanderbilt University School of Medicine, 23rd Ave. at Pierce, Nashville, TN 37232. Tel.: 615-343-7329; Fax: 615-343-7534; E-mail: marnett@toxicology.mc.vanderbilt.edu.

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Construction of Adduct-modified M13MB102-1 Genomes—Preparation of gapped-duplex DNA and ligation of adducted or unadducted 8-mers followed an adaptation of the method described by Benamira and Marnett (19). Double-stranded M13MB102-1 was linearized with SacI and BsoHIII followed by dialysis with a 12-fold excess of single-stranded M13MB102-1 in decreasing concentrations of formamide. The resultant gapped-duplex was resolved by 0.8% low melting point agarose gel electrophoresis. The band corresponding to gapped-duplex DNA was excised, and the DNA was recovered by GE Lase treatment. HOPdG and unmodified 8-mers (300 pmol) were phosphorylated using T4 polynucleotide kinase and 1 mM ATP in the buffer supplied by New England BioLabs. For ligation, gapped-duplex DNA was added to each of the phosphorylated 8-mers, along with 400 units of T4 DNA ligase and 1 mM ATP. The reaction proceeded at 16 °C overnight in ligation buffer supplied by New England BioLabs. Reaction mixtures were brought up to 100 μl with H2O, and the DNA was purified by filtration through a modified polyvinylidene fluoride membrane from Millipore. Ligation products were purified on a 0.8% low melting point agarose gel and recovered using GE Lase enzyme.

Construction of M13mp7L2 Vectors Containing a Single HOPdG Adduct—Single-stranded M13mp7L2 genomes containing a unique site-specific HOPdG were constructed, following a modification of the procedure described by Banerjee et al. (20). Briefly, single-stranded M13mp7L2 DNA was linearized with EcoRI for 2 h at 25 °C, utilizing the unique EcoRI site within the hairpin linker region. The linearized DNA was recircularized with a 48-mer oligonucleotide creating a circular single-stranded DNA containing an 8-base gap at the EcoRI site. The ends of the 48-mer oligonucleotide are complementary to the EcoRI site, and its internal sequence of 8 bases is complementary to the dG-modified EcoRI. Oligonucleotides were synthesized using the method described by Nechev (18). After purification, the purity of the HOPdG 8-mer and 31-mer oligonucleotides was checked by a 20% denaturing polyacrylamide gel. Both HOPdG-modified oligonucleotides were determined to be >99.5% pure by capillary gel electrophoresis.

The primers and all unmodified template oligonucleotides for in vitro bypass assays were prepared using an Applied Biosystems automated DNA synthesizer in the Vanderbilt University Molecular Toxicology Molecular Genetics Core. A 12-mer oligonucleotide was used as a running start primer had the following sequence: 5′-CTGGTTCCCG-3′. The 13-mer standing start primer sequence was as follows: 5′-CTGGTTCCCGG-3′. After purification, the purity of the HOPdG 8-mer and 31-mer oligonucleotides was checked by a 20% denaturing polyacrylamide gel. Both HOPdG-modified oligonucleotides were determined to be >99.5% pure by capillary gel electrophoresis.

HOPdG

<a href="fig1.jpg" data-att-fig="1.png">Fig. 1. Structures of exocyclic adducts.</a>

Klenow fragment (exo- ) were purchased from New England BioLabs (Beverly, MA). MOPS and calf thymus DNA were purchased from Sigma Chemical Co. (St. Louis, MO). Tris-HCl (pH 8.0), EDTA, and sodium dodecyl sulfate solution were obtained from Life Technologies, Inc. (Grand Island, NY). Formamide was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). Ultrapure AquaPor LM agarose was from National Diagnostics (Atlanta, GA). Nylon membranes (0.45 μm, 82 mm) were from Schleicher & Schuell (Keene, NH). [γ-32P]ATP was purchased from PerkinElmer Life Sciences (Boston, MA). Bio-spin 6 columns were purchased from Bio-Rad (Hercules, CA). GELase enzyme was from Epicentre Technologies (Madison, WI). Isopropyl β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were purchased from Gold Biotechnology Inc. (St. Louis, MO).

Oligonucleotides—The unmodified 8-mer oligonucleotide for mutagenesis experiments, 5′-GGTTCCCGG-3′, was synthesized by Midland Reagent Co. (Midland, TX). HOPdG-modified 8-mer oligonucleotides, 5′-GGTTG/HOPdG/TCCGG-3′, and 31-mer oligonucleotides, 5′-GAGCATGTTCCGG/HOPdG/TGCACTGCACTG-3′, were synthesized using the method described by Nechev et al. (18). After purification, the purity of the HOPdG 8-mer and 31-mer oligonucleotides was checked by a 20% denaturing polyacrylamide gel. Both HOPdG-modified oligonucleotides were determined to be >99.5% pure by capillary gel electrophoresis.

Adduct—Single-stranded M13mp7L2 genomes containing a unique site-specific HOPdG were constructed, following a modification of the procedure described by Banerjee et al. (20). Briefly, single-stranded M13mp7L2 DNA was linearized with EcoRI for 2 h at 25 °C, utilizing the unique EcoRI site within the hairpin linker region. The linearized DNA was recircularized with a 48-mer oligonucleotide creating a circular single-stranded DNA containing an 8-base gap at the EcoRI site. The ends of the 48-mer oligonucleotide are complementary to the EcoRI site, and its internal sequence of 8 bases is complementary to the dG-modified EcoRI. Oligonucleotides were synthesized using the method described by Nechev (18). After purification, the purity of the HOPdG 8-mer and 31-mer oligonucleotides was checked by a 20% denaturing polyacrylamide gel. Both HOPdG-modified oligonucleotides were determined to be >99.5% pure by capillary gel electrophoresis.

The primers and all unmodified template oligonucleotides for in vitro bypass assays were prepared using an Applied Biosystems automated DNA synthesizer in the Vanderbilt University Molecular Toxicology Molecular Genetics Core. A 12-mer oligonucleotide was used as a running start primer had the following sequence: 5′-CTGGTTCCCGG-3′. The 13-mer standing start primer sequence was as follows: 5′-CTGGTTCCCGG-3′. After purification, the purity of the HOPdG 8-mer and 31-mer oligonucleotides was checked by a 20% denaturing polyacrylamide gel. Both HOPdG-modified oligonucleotides were determined to be >99.5% pure by capillary gel electrophoresis.
extension primer and were then quenched by the addition of 10 mM EDTA in 90% formamide. The products of the reaction were resolved on a 20% polyacrylamide gel using the Ultrapure Sequagel sequencing system (National Diagnostics, Atlanta, GA). The positions of the bands were visualized by autoradiography and imaged with PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

### RESULTS

**Mutagenicity of HOPdG-modified Double-stranded Vectors**—Construction of vectors containing HOPdG followed the basic procedure described by Benamira and Marnett (19). The M13MB102-1 vector was used during this preparation. M13MB102-1 contains a single deletion at position 6284 in the lacZa gene of the M13MB102 genome. Frameshift mutations that restore the reading frame of lacZ can be detected by phenotypic screening in the presence of IPTG and X-gal. Double-stranded M13MB102-1 DNA was linearized with BssHII and SacII, then dialyzed with an excess of (+)-single-stranded phage DNA to create a duplex with an 8-nucleotide gap in the (−) strand. Phosphorylated 8-mer oligonucleotides containing HOPdG or dG were ligated into the gap at a position corresponding to position 6256 in the M13 genome. The resultant closed circular duplexes were separated from incompletely ligated duplexes in ethidium bromide-containing agarose gels. The modified genomes were then transformed into *E. coli* strains that were either wild-type or deficient in DNA repair. The transformed cells were plated to produce a lawn of progeny phage. The plaques were eluted, and an aliquot of the stock was replated to yield ~500 plaques per plate. Plaque DNA from the secondary plating was lifted onto nylon membranes and probed by differential hybridization with radiolabeled probes specific for each type of base pair substitution. Frameshift mutations were detected by phenotypic screening using IPTG/X-gal during the secondary plating. HOPdG did not increase the frequency of frameshift mutations compared with unadducted genomes in any of the strains tested.

Table I lists the outcomes of *in vivo* replication when HOPdG-containing genomes were replicated in three separate *E. coli* strains. All strains were UV-irradiated before transformation to increase sensitivity to mutation by inducing the SOS response. No base pair substitutions were detected following replication of HOPdG-containing genomes in any of the strains with or without SOS induction. LM102 is wild-type for repair, whereas LM103 contains a uvrAS mutation that eliminates nucleotide excision repair. No increase in mutation frequency was detected when the HOPdG-modified vectors were replicated in the LM103 strain. LM119 contains deletions in two major apurinic/apyrimidinic endonucleases, exonuclease III and endonuclease IV. Apurinic/apyrimidinic endonucleases function to cleave the phosphodiester backbone at abasic sites, and their action is associated with the base excision repair pathway. If base excision repair is involved in the removal of...
Plaques were lifted onto nylon membranes and assayed for cells were plated to produce a lawn of progeny plaques. The presence of 100 μM dNTPs (indicated as dNTP), polymerase (molar ratio of enzyme to substrate greater than 1:1), a second product was observed, corresponding to an arrest of replication opposite HOPdG. By-pass of HOPdG to produce full-length extension products was observed at all enzyme concentrations tested for Klenow fragment. Increase in the overall mutation frequency compared with replication, using an in vitro lesion bypass assay. Extension of a 12-mer running-start primer annealed with a 31-mer template containing HOPdG at the sixteenth position from the 3′-end was compared with an unmodified template. The template: primer substrate was extended by the Klenow fragment of DNA polymerase I (exo-). The newly synthesized products of this reaction were separated on a 20% polyacrylamide gel and imaged using PhosphorImager analysis.

In Vitro Lesion Bypass with Purified Polymerase—The results of the in vivo experiments suggest that HOPdG is not highly mutagenic and is not a strong block to replication. Therefore, we tested the ability of HOPdG to block DNA replication, replication with Klenow fragment opposite an unadducted strand by Klenow fragment in the presence of all four dNTPs completely extended to the full-length product. However, full-length extension of template: primers containing HOPdG was substantially inhibited. Blockage of extension by Klenow fragment occurred opposite to the thymidine immediately preceding HOPdG (Fig. 2). At increased concentrations of polymerase (molar ratio of enzyme to substrate greater than 1:1), a second product was observed, corresponding to an arrest of replication opposite HOPdG. By-pass of HOPdG to produce full-length extension products was observed at all enzyme concentrations tested for Klenow fragment (exo-), although the extent of bypass was modest.

Primer extension studies were performed in the presence of individual dNTPs using a 13-mer standing-start primer. Klenow fragment (exo-) preferentially incorporated dAMP and dGMP residues opposite HOPdG (dGMP > dAMP), although some incorporation of dCTP was also observed (Fig. 3). Thus, in contrast to in vivo replication, in vitro replication with Klenow fragment reveals that HOPdG is able to strongly block replication and to miscode for insertion of purines opposite it.

**DISCUSSION**

In this report, we investigated both the mutagenic potential and repair of an HOPdG adduct site-specifically positioned in double-stranded and single-stranded M13 genomes and replicated in *E. coli*. Our results show that replication of double-stranded vectors containing HOPdG does not induce base pair substitutions or frameshift mutations at the level of sensitivity of the assay (10^-3). The same results were obtained in bacterial strains that are either wild-type for repair or deficient in nucleotide excision repair or base excision repair. This observation does not result from a masking effect due to the double-stranded nature of the vector, because HOPdG also was not mutagenic when incorporated into single-stranded vectors. Thus, even though the entire base pairing region of guanine is

**FIG. 3. Extension of 13-mer primer by Klenow fragment (exo-) in the presence of 100 μM individual dNTPs (as indicated) or a mixture of all four dNTPs (indicated as dNTP).** Replication of the template was performed in a 10-μl volume for 30 min at 37 °C. The final concentration of primertemplate was 5 nM, and the concentration of Klenow fragment (exo-) was 5 nM.

**HO-etanodG**

HOPdG, a deficiency in this repair pathway should result in an increase in the overall mutation frequency compared with repair-proficient cells. Replication of HOPdG-modified vectors in LM119 did not result in the occurrence of base pair substitutions.

**Mutagenicity of Modified Single-stranded Vectors**—The use of duplex vectors may mask the mutagenicity of lesions because of DNA repair or strand bias during replication. Therefore, we constructed single-stranded vectors containing HOPdG at a defined site to eliminate these possibilities. Vector molecules were constructed by the method described by Banerjee et al. (20). Single-stranded M13mp17L2 was digested with EcoRI, utilizing a unique site within a short hairpin region formed in the single-stranded DNA. The vector was then recircularized by electroporation into SOS-induced *E. coli*, and the transformed cells were plated to produce a lawn of progeny plaques. The plaques were lifted onto nylon membranes and assayed for mutations by differential hybridization with oligonucleotide probes specific for base pair substitutions. No mutations were observed in LM102 cells, complementing the results observed in the duplex vector studies (Table II).

**FIG. 4. Interconversion of the exocyclic adducts HOPdG, M1G, and HO-etanodG.**

**Fig. 3.** Extension of 13-mer primer by Klenow fragment (exo-) in the presence of 100 μM individual dNTPs (as indicated) or a mixture of all four dNTPs (indicated as dNTP). Replication of the template was performed in a 10-μl volume for 30 min at 37 °C. The final concentration of primertemplate was 5 nM, and the concentration of Klenow fragment (exo-) was 5 nM.
Principal DNA Adduct of Acrolein

The low mutagenicity of HO-PdG is quite different from that of other six-membered exocyclic guanine derivatives when compared in an identical sequence context. PdG and M1G induce base pair substitutions and frameshift mutations in vitro and in vivo (2, 4). HO-PdG, M1G, and PdG do not induce frameshift mutations in the sequence used in the present study, but PdG and M1G do induce frameshifts in a sequence containing reiterated CGs. The structural difference between PdG and HO-PdG is the presence or absence of a hydroxyl group, whereas M1G and HO-PdG also differ by the pi character of the exocyclic ring of M1G (Fig. 1). An analogy may be drawn to the study of Langouet and coworkers (25), which demonstrated that ethano-dG and etheno-dG, the five-membered homologues of PdG and M1G, are mutagenic in *E. coli*, whereas HO-ethano-dG, the homologue of HO-PdG, is only weakly mutagenic.

The lack of mutations induced by HO-PdG is not the result of it being a strong block to *in vivo* replication or to its removal by repair systems, as demonstrated by the use of single-stranded vectors in mutagenesis assays. One possible explanation for HO-PdG’s nonmutagenic nature is that the structure of HO-PdG may exist in multiple forms, where one form is miscoding but the other not (Fig. 4). A related interconversion has been observed for M1G, which exists in a hydrolytically ring-opened form (N2-(3-oxopropanyl)-dG) when it is opposite dC residues but in the ring-closed form when it is opposite dT residues (Fig. 4). The ring-opened form appears to be substantially less mutagenic than the ring-closed form. One can postulate the existence of an analogous ring-opened form for HO-PdG in which the ring-opened form, N2-(3-oxopropanyl)-dG, would be expected to be substantially less mutagenic. In fact, a very recent study by de los Santos et al. (26) indicates that HO-PdG exists entirely in the ring-opened form in duplex DNA and that the ring-opened form hydrogen bonds effectively to dC residues. This type of interconversion of opened and closed forms of exocyclic adducts may account for the low mutagenicity of HO-ethano-dG as speculated by Langouet et al. (25).

The relevance of our results to the mutagenicity and tumor-initiating activity of acrolein is supported by recent studies by Yang and coworkers (27), who reported that HO-PdG was not mutagenic in *E. coli*. It seems unlikely that HO-PdG is the principal adduct responsible for acrolein mutagenicity. This suggests that the mutagenic effects are mediated by other acrolein-DNA adducts. Isomeric 6-HOPdG isomers are formed on reaction of acrolein with DNA *in vitro* and have been detected in human tissue (12, 13, 15). Even if 6-HOPdG ring-opens in duplex DNA, it will still contain a 3-oxopropanyl group on N1 of dG, which should alter translesion synthesis. In addition, there are other adducts that may contribute to the genotoxicity of acrolein. Recently, Wang et al. (28) reported that the hydrolysis product of crotonaldehyde-β-hydroxybutyraldehyde adds to the exocyclic amino group of deoxyguanosine to form unstable imine adducts. The imine adducts are present in concentrations 50-fold higher than those of the 6-methyl-8-hydroxypropanodeoxyguanosine adduct generated by reaction of deoxyguanosine with crotonaldehyde. It is likely that related N2- imino adducts are formed by reaction of deoxyguanosine residues with hydrated acrolein (i.e. 3-hydroxypropionaldehyde). It will be interesting to evaluate the mutagenicity of this adducts using site-specific procedures analogous to those described in the present work. However, the chemical instability of these novel exocyclic and N2 adducts may make this a formidable experimental challenge.

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