Repair of Propanodeoxyguanosine by Nucleotide Excision Repair in Vivo and in Vitro*

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Repair of the exocyclic DNA adduct propanodeoxyguanosine (PdG) was assessed in both in vivo and in vitro assays. PdG was site-specifically incorporated at position 6256 of M13MB102 DNA, and the adducted viral genome was electroporated into repair-proficient and repair-deficient Escherichia coli strains. Comparable frequencies of PdG → T and PdG → A mutations at position 6256 were detected following replication of the adducted genomes in wild-type E. coli strains. A 4-fold increase in the frequencies of transversions and transitions was observed in E. coli strains deficient in Uvr(A)BC-dependent nucleotide excision repair. A similar increase in the replication of the adduct containing strand was observed in the repair-deficient strains. No change in the frequency of targeted mutations was observed in strains deficient in one or both of the genes coding for 3-methyladenine glycosylase. Incubation of purified E. coli Uvr(A)BC proteins with a duplex 156-mer containing a single PdG adduct resulted in removal of a 12-base oligonucleotide containing the adduct. Incubation of the same adducted duplex with Chinese hamster ovary cell-free extracts also resulted in removal of the adduct. PdG was a better substrate for repair by the mammalian nucleotide excision repair complex than the bacterial repair complex and was approximately equal to a thymine-thymine dimer as a substrate for the former. The results of these in vivo and in vitro experiments indicate that PdG, a homolog of several endogenously produced DNA adducts, is repaired by the nucleotide excision repair pathway.

Exocyclic adducts that block the Watson-Crick base pairing region of DNA bases are produced from a range of exogenous and endogenous chemicals (1). Numerous exocyclic adducts have been identified including etheno and substituted etheno adducts to deoxyadenosine, deoxyguanosine, and deoxycytidine, propeno and substituted propano adducts to deoxyguanosine, and complex bicyclic adducts to deoxyguanosine (1, 2). A particularly important class of cyclic adducts include propeno and substituted propano adducts to deoxyguanosine (1, 2). These include the pyrimidinopurinone adduct, M1G,1 formed from malondialdehyde and the hydroxy-propanodeoxyguanosine adducts formed from crotonaldehyde and acrolein (Fig. 1).

(2) These adducts have been detected at levels from 1500–5000/ cell in liver DNA from healthy human beings (3, 4) and appear to be highly mutagenic in site-specific mutagenesis assays (5–11).

The high levels of propano adducts detected in human tissues suggests that their removal by DNA repair enzymes may be an important protective mechanism against genetic diseases that might be induced by these adducts. At present, no information is available on the enzymes involved in the repair of these adducts. Certain cyclic adducts such as N2-3-etheno-dodeoxyguanosine and 1,N2-etheno(deoxy)adenosine are substrates for the bacterial and mammalian 3-methyladenine glycosylases (12–15), and Singer and colleagues have characterized other human proteins that remove cyclic bases from duplex oligonucleotides in vitro (16–18). Propanodeoxyguanosine (PdG), which is a model for the chemically unstable M1G and hydroxy-PdG adducts, is not a substrate for these glycosylases (18).

We have undertaken an investigation of the repair of the prototypical exocyclic deoxyguanosine adduct, PdG, in vivo and in vitro. PdG was used as a model for the M1G and hydroxy-PdG adducts because of its stability during oligonucleotide synthesis and during the construction of large duplex oligonucleotides, which are necessary for in vitro repair assays. The removal of PdG was assessed in vivo by determining the mutation frequency and strand bias observed upon replication of M13 genomes containing this adduct in wild-type and repair-deficient strains of Escherichia coli. An increase in mutation frequency and adducted template replication in a repair-deficient strain was taken as an indication of the involvement of the deleted gene in repair. These experiments indicated that PdG residues are repaired by nucleotide excision repair. The in vivo results were confirmed by using an in vitro assay for the excision of the PdG adduct by both the E. coli and hamster excinucleases. Thus, PdG, a surrogate for endogenous adducts present in healthy humans, is a substrate for both bacterial and mammalian nucleotide excision repair proteins.

EXPERIMENTAL PROCEDURES

Materials—BsaHI and KspI restriction endonucleases and DNA ligase were purchased from Boehringer Mannheim. T4 polynucleotide kinase was purchased from U. S. Biochemical Corp. Calf thymus DNA and lauryl sulfate were purchased from Sigma. 5-Bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-gal) and isopropyl β-D-thiogalactoside were obtained from Gold Biotechnology (St. Louis, MO). Nitrocellulose transfer membranes (BA85 82.5-mm diameter) were from Schleicher & Schuell. [γ-32P]ATP (3000 Ci/mmol for hybridization experiments and 7000 Ci/mmol for excision assays) was from DuPont NEN. Unadducted and PdG-adducted 8-mer oligonucleotides for mutagenesis experiments, 5′-GGTTCTCCG-3′ (where X is G or PdG), and 12-mer oligonucleotide for the in vitro excision assay, 5′-GAAXTACGACG-3′ (where X is PdG), were synthesized by Midland Certified Reagent Co. (Midland, TX). The oligonucleotides were purified by reversed phase HPLC and run as single bands on denaturing polyacrylamide gels (6). Oligonucleotides used as hybridization probes were prepared using an Applied Biosystems automated DNA synthesizer in the Vanderbilt University Molecular Toxicology Molecular Genetics Core.

Bacterial Strains—The E. coli strain JM105 (supE, tri, rpsL, endA,
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nese hamster ovary cell line, K1, was used to assay excision in a mammalian system. Cells were grown to exponential phase as a suspension culture in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum. Cells were harvested and cell-free extract (CFE) was prepared as described (24) and stored at −80 °C in buffer that was 25 mM Hepes, pH 7.9, 10 mM KCl, 12 mM MgCl₂, 0.5 mM EDTA, 2 mM dithiothreitol, and 12.5% glycerol (v/v). The CFE concentration was 12.1 mg/mL, and the extract was stable for two cycles of thawing and refreezing.

Excision Assay with CFE—Using an internally labeled DNA substrate (at the fifth phosphodiester bond 5′ to the PdG residue or at the eleventh bond 5′ to T′), this assay detects the excited fragment resulting from both 5′ and 3′ incisions. The reaction mixtures contained 25 fmol of radiolabeled DNA, 121 µg of CFE, and 70 ng of PB322 in 50 µL of buffer that was 35 mM Hepes, pH 7.9, 10 mM Tris, pH 7.5, 60 mM KCl, 40 mM NaCl, 5.6 mM MgCl₂, 0.4 mM EDTA, 0.8 mM dithiothreitol, 2 mM ATP, and 3.2% glycerol with 20 µL each of dATP, dCTP, dGTP, and TTP, and bovine serum albumin at 0.2 mg/mL. The reaction was carried out at 30 °C for 90 min with 10 µL of the mixture (~5 fmol DNA) being removed at 15, 30, 60, and 90 min. DNA was deproteinized with protease K (0.2 mg/mL for 15 min at 37 °C) followed by phenol, phenol:chloroform, and ether extractions then precipitated with ethanol in the presence of 20 µg of oyster glycogen. Recovered DNA was resuspended in formamide/dye mixture and resolved on a 10% denaturing polyacrylamide gel followed autoradiography to visualize the repair products. The level of excision (oligomers in the 23–30-nt range) was quantitated by scanning the dried gel with an AMBIS systems scanner.

RESULTS

Recombinant M13 genomes containing a site-specifically positioned PdG adduct were constructed by ligation of the PdG 8-mer (5′-GTTT/PdG/TTTCCG-3′) into a gapped duplex DNA molecule containing uracil in the (−) strand and an 8-base gap in the (+) strand (7). Control genomes were constructed by ligation of the unmodified 8-mer. The oligonucleotides used for genome construction were purified by reversed phase HPLC and were judged greater than 99.3% pure by HPLC and denaturing polyacrylamide gel electrophoresis of 32P-labeled material. The structures of the oligonucleotides were verified by high field NMR analysis and by HPLC analysis of the deoxynucleosides released by nuclease digestion. The fully ligated genomes were isolated from the remaining starting material and incompletely ligated duplexes by agarose gel electrophoresis in the presence of 0.5 µg/mL ethidium bromide (6). Purified, fully ligated recombinant M13MB102 DNA was electrophoretized into the SOS-induced competent E. coli strains. Following overnight growth and elution of the entire plaque population from primary transformations, the eluate was diluted and replated on X-gal/isonicotinyl-β-ethiogalactoside indicator plates to give approximately 1,500 plaques/plate (6). These secondary plates were used in the hybridization assay (7). Elution of the primary plates enabled us to standardize the number of plaques/plate for the hybridization assay, as well as allowed screening of a large number of the transformants that were produced during electrophoresis. The mutation frequency was not significantly different when the DNA was screened for mutations after either the primary or secondary plating (data not shown). The base pair substitution mutation frequency was determined by screening for transitions and transversions at position 6256 by colony PCR (7). Recombinant single-stranded M13MB102 DNAs containing each of the four possible bases at the adduct site were hybridized in parallel as assay standards (7).

In Vivo Repair—Table I lists the mutation frequencies determined following electroporation of PdG-containing M13 genomes into strains deficient in nucleotide excision repair or one or both of the 3-methyladenine glycosylase genes. The base pair substitution mutation frequencies induced by the PdG adduct in M13MB102 replicated in LM102 and LM101 were similar to the mutation frequency previously reported in JM105 (7). LM102 and LM101 are wild-type for DNA repair. When PdG-

Excision Assay with UvrA/B/C—UvrA, UvrB, and UvrC were purified as described (25) and kindly provided by Dr. M.-S. Tang. The reaction mixtures contained 1.5 fmol of radiolabeled DNA, 15 µg UvrA, 15 µg UvrB, 15 µg UvrC, and 100 ng of dX74 DNA in 25 µL of buffer that was 50 mM Tris, pH 7.5, 100 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, and 1 mM ATP. Incubation was at 37 °C with the reaction being stopped by the addition of 2 µL of a 1:1 mixture of 0.25 mM EDTA and 5 mg/mL oyster glycogen, followed by ethanol precipitation. Recovered DNA was resuspended in formamide/dye mixture and resolved on an 8% denaturing polyacrylamide gel. Following autoradiography to visualize the repair products, the level of excision was quantitated by PhosphorImager analysis. For unmodified DNA, 5-µL aliquots were withdrawn from the 25-µL reaction mixture such that each time point was represented by ~0.3 fmol of DNA.

Preparation of Cell-free Extract—A repair-proficient (wild-type) Chi-
Values are the average of three independent DNA constructions, transformations, and hybridizations. LM102 and LM103 are the F- derivatives of AB1157 and AB1887, respectively. LM101, LM108, LM109, and LM110 are the F- derivatives of MV1161, MV1174, GC4800, and MV2157, respectively (see “Experimental Procedures”).

| Strain               | G → A | G → T |
|----------------------|-------|-------|
| LM102 (wild type)    | 1.5 ± 0.4 | 1.5 ± 0.3 |
| LM103 (uvrA-)        | 5.0 ± 1.0 | 6.0 ± 0.4 |
| LM101 (wild type)    | 1.7 ± 0.6 | 1.7 ± 0.4 |
| LM108 (alkA-)        | 1.5 ± 0.6 | 1.8 ± 0.4 |
| LM109 (tagA-)        | 1.8 ± 0.8 | 1.9 ± 0.9 |
| LM110 (alkA-tagA-)   | 1.6 ± 0.7 | 1.3 ± 0.3 |

The values for the replication of the (+)-strand contain a small contribution from mutagenic replication (PdG → A) of the (+)-strand. This value is assumed to be equivalent to the frequency of PdG → T induced by replication of the (-)-strand.

LM104 is the F- derivative of AB2463 (see “Experimental Procedures”).

In these initial in vitro experiments, uracil residues were randomly incorporated into the (+)-strand of the recombinant M13 genomes to minimize replication of the nonadducted strand. However, we have recently found that a significant amount of replication of the adducted genomes occurs in which the (+)-strand is used as the template (20). This accounts for the high percentage of progeny phage that contain G at position 6256 because the (+)-strand contained a C residue at this position. It is possible to estimate the percentages of replication that occur from the (+)-strand by placing T residues opposite the adduct in the recombinant genomes. Utilization of the (+)-strand as template leads to the incorporation of A residues at position 6256, whereas utilization of the (-)-strand as template leads to incorporation of whatever bases are inserted opposite PdG. For the experiments described below, recombinant M13 genomes were constructed that did not contain uracil residues in the (+)-strand. This reduces the apparent mutation frequency at position 6256 by approximately 50% (20).

M13MB102 genomes that contained G:T or PdG:T at position 6256 were electroporated into a wild-type strain (JM105), a recA- strain (LM104), and two nucleotide excision repair-deficient strains (LM103 and NR10148), and the identities of the bases incorporated were probed by hybridization analysis (Table II). For nonadducted genomes, the (-)-strand was used as template approximately 90% of the time, and no G → T transitions were detected within the limit of sensitivity of the assay (0.1%). When PdG was present in the (-)-strand, most of the replication occurred from the nonadducted (+)-strand. The total amount of replication from the (-)-strand was estimated by summing the frequencies of incorporation of G, T, and C and assuming that A was incorporated at the same frequency as T. The assumption of equal frequencies of PdG → A and PdG → T mutations was based on the results obtained with M13MB102 containing PdG:C at position 6256 (Table I). The remainder of the incorporation of A during replication of M13MB102 containing PdG:T at position 6256 was from utilization of the (+)-strand as template, and this clearly represented the majority of the replication events.

The PdG-containing (-)-strand was used as template approximately 6% of the time in JM105 and 4% of the time in LM104. The concordance of these results indicates that the shift in template utilization induced by PdG was not the result of recA-mediated strand switching. Rather, it arose from complete replication of the (+)-strand or synthesis using the (+)-strand as template following repair of PdG. The significant reduction in mutation frequency observed in LM104 supports prior findings that mutagenesis by PdG is enhanced by induction of the SOS response (7, 20).

The contribution of nucleotide excision repair to replication of the (+)-strand was estimated by comparing JM105 with LM103 and NR10148. The PdG-adducted strand was used as template 21% of the time in the uvrA- strain LM103. The relative frequency of (-)-strand utilization in JM105 and LM103 (3.5-fold) was comparable with the ratio of mutation frequencies measured with LM102 and LM103 (3.7-fold) (Table I). Because JM105 is a wild-type strain with regard to nucleotide excision repair, whereas LM103 is uvrA-, these results are consistent with a role for nucleotide excision repair in removing PdG residues. Removal of PdG in JM105 and LM102 by nucleotide excision repair would lead to increased incorporation of A residues during repair synthesis because of the presence of T at position 6256 on the (+)-strand.

Although the frequency of replication of PdG was higher in the uvrA- cells, the mutation frequency was similar to that observed in wild-type cells when corrected for template utilization. 26% of the (-)-strand replication events led to mutations in JM105, and 38% led to mutations in LM103. Electroporation of PdG-containing M13MB102 into NR10148, which is uvrB-, produced frequencies of template utilization (18%) and mutations (35%) comparable with those observed in LM103. Thus, mutations in two different genes of nucleotide excision repair led to similar effects on replication of PdG-containing M13 genomes in vivo.2

In Vivo Repair—Results from the in vivo mutagenesis studies suggested that the Uvr(A)BC excinuclease is involved in the repair of the PdG adduct in E. coli because the absence of the uvrA or uvrB genes increased the frequency of mutations induced by PdG. Repair of the PdG adduct by Uvr(A)BC excinuclease was further investigated by use of an in vivo assay in

2 Comparable results also were obtained in a second uvrA- strain that carries a Tn10 insertion in the uvrA gene. (S. P. Fink, unpublished results).
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which duplex DNA containing the PdG adduct was incubated with purified Uvr(A)BC repair enzymes to determine if excision occurred. The substrate for these in vitro experiments was a 156-mer duplex DNA bearing PdG near the center of the duplex. It was constructed by ligating a 5’-labeled 12-mer containing the PdG adduct at position 4, with seven other oligomers to obtain a 156-mer duplex (21). The 12-mers were purified by HPLC as described above for the 8-mers used for recombinant genome construction. The fully ligated DNA was purified from the partially ligated product by running the ligation mixture on a polyacrylamide gel and excising the band corresponding to the 156-mer. The Uvr(A)BC excinuclease repairs damaged DNA by excision of a 12-nucleotide fragment of DNA by hydrolysis of the eighth phosphodiester bond 3’- and the fourth phosphodiester bond 3’- to the adducted nucleotide (25, 26). By phosphorylating the oligonucleotide containing the adduct with 32P prior to ligation, we were able to determine the extent of excision by the appearance of a 12-nucleotide band on an autoradiogram of a polyacrylamide gel following incubation with the repair enzymes.

A 12-nucleotide fragment of DNA was excised when the 156-mer substrate adducted with PdG was incubated with the Uvr(A)BC excinuclease. The extent of excision increased linearly up to 40 min, at which point it plateaued (Fig. 2). In addition to the 12-mer product, a band corresponding to a 74-mer was detected, which represents uncoupled excision at only the 3’ end of the duplex. There was no excision in the samples incubated with boiled enzyme or the samples terminated immediately after addition of the enzyme. DNA that did not contain an adduct was also not excised by these enzymes (27). Approximately 1% of the PdG-containing 156-mer substrate was cut by the excinuclease. A 45-mer substrate containing a psoralen monoadduct was used as a positive control for these experiments, because psoralen is excised very efficiently by the Uvr(A)BC excinuclease (27). Approximately 50% of the psoralen-adducted DNA was cut. Thus, by comparison with psoralen, PdG appears to be a relatively poor substrate for the E. coli Uvr(A)BC excinuclease.

Three separate lines of evidence suggest that PdG is a substrate for nucleotide excision repair. First, the frequency of targeted base pair substitution mutations induced by PdG is higher in nucleotide excision repair-deficient strains than in wild-type strains in vivo. Second, the extent of PdG-containing template utilization is higher in nucleotide excision repair-deficient strains than in wild-type strains in vivo. Third, oligonucleotides of the expected size are removed from a PdG-containing 156-mer following incubation with reconstituted E. coli or hamster excinuclease complexes in vitro.

The studies of template utilization summarized in Table II are particularly informative in that they reveal a dramatic switch in template utilization when PdG is incorporated into

FIG. 2. Repair of PdG by Uvr(A)BC. A linear DNA molecule (1.5 fmol, 156-mer) containing a centrally located PdG adduct was incubated with Uvr(A)BC (15 nt each subunit) in a 25-μl reaction mixture. The reaction was stopped at 10, 20, 40, 60, and 90 min, and the recovered DNA was analyzed on an 8% sequencing gel (lanes 3–7). The 74-nt fragment arising from 3’-coupled incision is indicated as are the 13-mer from excision of psoralen and 12-mer from excision of PdG. Lane 2 is DNA alone. As a control, a 45-mer containing a psoralen monoadduct was tested in parallel (lane 1); 57% of the damage was excised.

FIG. 3. Repair of PdG by mammalian cell-free extract. Chinese hamster ovary K1 CFE was tested for excision activity by incubating CFE (2.4 mg/ml) in a 50-μl reaction mixture with linear DNA (12 fmol) containing either a centrally located PdG adduct (156-mer) or a T<->T lesion (140-mer). Aliquots were removed after 30 (lanes 2 and 6), 60 (lanes 3 and 7), and 90 min (lanes 4 and 8). Substrate DNA alone (0 min) is shown in lanes 1 and 5. End-labeled 24- and 30-mers were used as size markers (not shown). The primary excision products were 23–28 nt in length for PdG and 23–30 nt for T<->T.
M13 genomes. The low percentage of (+)-strand replication indicates that PdG is either a strong block to replication or is very efficiently repaired. Moriya et al. have shown that PdG is an effective block to replication when incorporated into single-stranded vectors (8). Incorporation of adducts that are strong blocks to replication into the (+)-strand of M13MB102 could lead to an apparent shift in template utilization because of the consecutive rather than bidirectional mechanism of strand replication employed by M13 genomes (29). Once the lesion is bypassed during the initial stage of DNA replication, the (+)-strand released can be rapidly copied to a duplex. The (+)-strand of this newly synthesized duplex will dominate viral DNA synthesis because the duplex produced in the initial phase of genome replication still contains the adduct in the (+)-strand.

The data in Table II indicate that a portion of the utilization of the (+)-strand as template occurs during DNA synthesis subsequent to nucleotide excision repair because replication of the adducted (+)-strand increases in cells that are deficient in either the uvrA or uvrB genes. This shift in template utilization masks the mutagenic potency displayed by PdG in duplex vectors. For example, the percentage of mutations induced by PdG in M13MB102 increases from approximately 1.5 to 28% when correction is made for the strand that is actually used as the template. The magnitude of the increase in the use of the adduct-containing strand as template is approximately the same as the magnitude of the increase in the mutation frequency. However, even in nucleotide excision repair-deficient cells, the nonadducted strand is still the preferred template for M13MB102 replication. This is likely due to replication blockade by the PdG residue on the (+)-strand, but we cannot rule out the possibility that another repair pathway such as base excision repair contributes to the utilization of the (+)-strand as template. Interestingly, deletion of the 3-methyladenine glycosylase gene (alkA), which has been reported to participate in the repair of etheno adducts to deoxyguanosine and deoxyadenosine residues (12–15), does not increase the mutation frequency measured following in vitro replication of PdG-adducted M13MB102.

Repair of PdG was further studied in an in vitro excision assay in which a 156-base pair substrate containing PdG was incubated with either the purified E. coli enzymes or a mammalian CFE. Removal of the adduct was detected by the appearance of the excised oligonucleotide fragments when reaction mixtures were analyzed by polyacrylamide gel electrophoresis. The E. coli enzymes excised approximately 1% of the adducted substrate. This low level of in vitro excision is similar to the amounts detected with other small adducts such as O6-methylguanine and DNA mismatches (27). This is considerably less than the extent of excision of larger adducts such as psoralen- or aflatoxin-derived adducts or thymine dimers (27, 30, 31). However, in contrast to excision by the E. coli excinuclease, PdG is a good substrate for removal by a mammalian excinuclease complex. The extent of excision of PdG-containing oligonucleotides by a hamster CFE is approximately the same as the extent of excision of thymine dimer-containing oligonucleotides, which are classic substrates for mammalian nucleotide excision repair.

The present findings establish that PdG is repaired by bacterial and mammalian nucleotide excision repair complexes. Although PdG is a relatively poor substrate for the bacterial excinuclease in vitro, the higher frequency of PdG-induced mutations in excision repair-deficient strains of E. coli indicates that repair by UvrABC is important in vivo. PdG appears to be a good substrate for repair by the mammalian excinuclease. This is interesting because several exocyclic deoxyguanosine adducts, including some hydroxy-PdGs, have been detected in DNA from healthy human beings (3, 4, 32). The levels of adducts detected in DNA reflect the steady-state balance between adduct generation and adduct removal, so if these exocyclic adducts are removed as efficiently as PdG by nucleotide excision repair, their contribution to endogenous DNA damage is significantly higher than one would estimate by determination of absolute adduct levels.

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