Replication of Template-Primers Containing Propanodeoxyguanosine by DNA Polymerase β

INDUCTION OF BASE PAIR SUBSTITUTION AND FRAMESHIFT MUTATIONS BY TEMPLATE SLIPPAGE AND DEOXYNUCLEOSIDE TRIPHOSPHATE STABILIZATION*

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Propanodeoxyguanosine (PdG) is a model for several unstable exocyclic adducts formed by reaction of DNA with bifunctional carbonyl compounds generated by lipid peroxidation. The effect of PdG on DNA synthesis by human DNA polymerase β was evaluated using template-primers containing PdG at defined sites. DNA synthesis was conducted in vitro and the products were analyzed by polyacrylamide gel electrophoresis and DNA sequencing. The extent of PdG bypass was low and the products comprised a mixture of base pair substitutions and deletions. Sequence analysis of all of the products indicated that the deoxynucleoside monophosphate incorporated “opposite” PdG was complementary to the base 5’ to PdG in the template strand. These findings are very similar to recent results of Efrati et al. (Efrati, E., Tocco, G., Eritja, R., Wilson, S. H., and Goodman, M. F. (1997) J. Biol. Chem. 272, 2559–2569) obtained in DNA replication of template-primers containing abasic sites and suggest that PdG is a non-informational lesion when acted upon by polymerase (pol) β. In addition to base pair substitutions and one- or two-base deletions, a four-base deletion was observed and the mechanism of its formation was probed by site-specific mutagenesis. The results indicated that this deletion occurred by one-base insertion followed by slippage to form a four-base loop followed by extension. All of the observations on pol β replication of PdG-containing template-primers are consistent with a mechanism of lesion bypass that involves template slippage and dNTP stabilization followed by deoxynucleoside monophosphate incorporation and extension. This mechanism of PdG bypass is completely different than that previously determined for the Klenow fragment of DNA polymerase I and is consistent with recent structural models for DNA synthesis by pol β.

Mutations are the cause of genetic disease and arise during the copying of normal or damaged DNA templates. The factors that determine the frequency and types of mutations include the identity of the DNA polymerase, the composition of the deoxynucleotide pool, the local sequence context of the template, and, in the case of damaged templates, the structure of the DNA lesion (1–16). Our laboratory and others have investigated the effect of propanodeoxyguanosine (PdG) on the fidelity of DNA replication in vivo and in vitro (17–22). PdG has been used as a model with which to assess the biological effects of several unstable DNA adducts derived from the reaction of dG residues with bifunctional aldehydes (23, 24). PdG is a relatively small lesion that completely blocks Watson-Crick base pairing but induces little distortion in the local structure of DNA molecules to which it is introduced (25, 26). Transformation of recombinant viral genomes or shuttle vectors containing site-specifically positioned PdG residues into bacterial and mammalian cells demonstrates that PdG is highly mutagenic and capable of inducing a range of base pair substitutions and frameshift mutations in vivo (17–19, 22). Likewise, PdG induces frameshift and base pair substitution mutations during in vitro replication of adducted template-primers (20, 21).

The outcome of replication of DNA molecules containing PdG is highly sequence context dependent (20). Using the Klenow fragment (exo− or exo−) of DNA polymerase I, we have demonstrated that the identity of the base pair 3’ to PdG in the template strand influences the choice of dNMP incorporated opposite PdG (20). More important, is the identity of the base 5’ to PdG in the template strand, which determines the outcome of synthesis beyond PdG (20). When the 5’ base can hydrogen bond to the base opposite PdG at the primer terminus, slippage and extension occur to generate one-base deletions at the site of the PdG residue (20, 21). When the 5’ base is T and the primer terminus contains A, the Klenow fragment extends the primer to a full-length product (20). The inability of the Klenow fragment to extend template-primers that contain C residues 5’ to PdG to full-length products correlates to the results of in vivo mutagenesis experiments in which PdG induces transitions to A and transversions to T when the base 5’ to it is T but not when it is C (18, 27).

To explore the generality of these observations on the replication of PdG by other DNA polymerases, we investigated the replication of PdG-containing template-primers by DNA polymerase β (pol β). Pol β is a small protein (39 kDa) that lacks a proofreading exonuclease and is highly error prone (28, 29). It plays a role in gap-filling during mammalian DNA repair that is analogous to the role of DNA polymerase I in Escherichia coli (30–34). However, recent results suggest that pol β also may play a role in mutagenic bypass of lesions that block replication (e.g. cis-Pt adducts and abasic sites) (35–37). Furthermore, a high resolution crystal structure of pol β is available which provides a structural framework with which to interpret the results of in vitro replication experiments (38, 39). The preliminary results of our studies on the replication by pol β of

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1 PdG, propanodeoxyguanosine; pol β, polymerase β; PAGE, polyacrylamide gel electrophoresis.
template-primers containing PdG residues appeared qualitatively similar to the results obtained with the Klenow fragment. However, more detailed analysis revealed that the factors that control deoxynucleoside-monophosphate insertion opposite PdG and extension to full-length are completely different. The structure of pol β suggests a possible explanation for these events that lead to PdG bypass and mutation.

MATERIALS AND METHODS

Chemicals and Enzymes—Deoxynucleoside 5’-triphosphates (ultrapure) were purchased from Pharmacia Biotech Inc. (Piscataway, NJ). [γ-32P]ATP (3000 Ci/mmol) and the Maxam-Gilbert Sequencing System were from NEB Life Sciences Products (Boston, MA). Osmium tetroxide (4 weight % solution in water) was purchased from Aldrich Chemical Co. 1,2-NAP-(1,3-Propano)-5’-O-DMT-3’-O-[2- (2-yanoethoxy-N,N-diisopropyl phosphinol)-2-deoxyguanosine] was obtained from Chem-Master International Inc. (Stony Brook, NY). T4 polynucleotide kinase and DNA polymerase I Klenow fragment (exo−) were obtained from Boehringer Mannheim. Human DNA polymerase β was purchased from Chimerx (Madison, WI).

Oligonucleotides—The 5’-dimethoxytrityl-protected phosphoramidites were incorporated into oligonucleotides by Midland Certified Reagent Co. (Midland, TX). The following 19-mer sequences (X = PdG) were synthesized: 19-mer 5’TATCGCGATCCGGCTAGAC-3’ (X = PdG); 19-mer 5’C-PdG-C-3’ sequence, 5’TATCGCGATCCGGCTAGAC-3’; 19-mer 5’G-PdG-C-3’ sequence, 5’TATCGCGATCCGGCTAGAC-3’; 19-mer 5’T-PdG-C-3’ sequence, 5’TATCGCGATCCGGCTAGAC-3’. The primer was sequenced as follows: 5’-GCTCATGCG-3’. The primer and all the unmodified 19-mer templates were obtained from the Molecular Genetics Core Laboratory of the Vanderbilt Center in Molecular Toxicology. Oligonucleotide sizing markers (8–32 bases) were obtained from Pharmacia Laboratory of the Vanderbilt Center in Molecular Toxicology. The following 19-mer sequences (X = PdG) were synthesized: 19-mer 5’-A-PdG-C-3’ sequence, 5’TATCGCGATCCGGCTAGAC-3’ (X = PdG); 19-mer 5’C-PdG-C-3’ sequence, 5’TATCGCGATCCGGCTAGAC-3’; 19-mer 5’G-PdG-C-3’ sequence, 5’TATCGCGATCCGGCTAGAC-3’; 19-mer 5’T-PdG-C-3’ sequence, 5’TATCGCGATCCGGCTAGAC-3’. The primer was sequenced as follows: 5’-GCTCATGCG-3’. The primer and all the unmodified 19-mer templates were obtained from the Molecular Genetics Core Laboratory of the Vanderbilt Center in Molecular Toxicology. Oligonucleotide sizing markers (8–32 bases) were obtained from Pharmacia Biotech Inc.

The template oligonucleotides were purified by the vendor by anion exchange high performance liquid chromatography. The 10-mer primer and the 19-mer unmodified templates were obtained in the 5’-dimethoxytrityl-protected form and were purified by nucleic acid purification columns (NEB Life Sciences Products). All the adducted and unadducted templates, and the primer were subjected to routine PAGE purification before use. The final purities of all the oligonucleotides were estimated by PAGE to be in excess of 98%.

General Procedure for in Vitro Replication—5’-End-labeling of the primers (2 μM) was carried out using T4 polynucleotide kinase and the labeled primers were purified by bio-spin columns (Bio-Rad). Annealing of template-primers was carried out by mixing template and 32P-labeled primer in a molar ratio of 5:1 and incubating for 5 min at 90 °C followed by slow cooling to room temperature in an annealing buffer comprising 50 mM Tris-HCl (pH 7.4), 50 μg/ml bovine serum albumin, and 5 mM MgCl2. The template-primer mixture (100 mM primer) was incubated in 10 μl of buffer containing 50 mM Tris-HCl (pH 6.5), 4 mM β-mercaptoethanol, 8 mM MgCl2, 4 mM dithiothreitol, and 2 mg/ml bovine serum albumin with 0.1 unit of Klenow fragment (exo−) or 4 units of pol β in the presence of normal dNTPs (100 μM). Incubations were carried out for 30 min at 25 °C and quenched by adding 10 μl of 10 mM EDTA in 90% formamide. The reaction products were analyzed by electrophoresis (20% PAGE) using the ultrapure Sequagel sequencing system (National Diagnostics, Atlanta GA). The positions of the bands were established by autoradiography and PhosphorImager analysis (Molecular Dynamics, Sunnyvale CA). To determine the sequences of the primer extension products, reactions were carried out on a 10–20-fold higher scale and the products were separated by PAGE. The bands were cut out of the gel and extracted by shaking overnight in distilled water. The extracted products were purified by ethanol precipitation and ultracentrifugation then subjected to Maxam-Gilbert chemical sequencing and a T-specific reaction (40, 41).

RESULTS

DNA Synthesis on a PdG-adducted Template—The effect of PdG on DNA replication catalyzed by pol β was determined with site-specifically modified template-primers (19-mer:10-mer) containing PdG at the eleventh position from the 3’ end of the template. A set of four PdG-adducted templates were utilized in which the base 5’ to the lesion was A, C, G, or T. The adducted templates were annealed to 32P-labeled 10-mer primers and the template-primers were used in primer extension assays carried out in the presence of all four dNTPs. The products of extension were analyzed by separation on a 20% denaturing polyacrylamide gel and detected with a PhosphorImager. When DNA synthesis was conducted on non-adducted template-primers, each of the primers was extended completely to a full-length 19-mer product (data not shown). However, when PdG was present in the template, a significant block to replication was observed in each template-primer at the position of the adduct (Fig. 1A). Bypass of the adduct was observed to the extent of 2–3% of that observed with unmodified template-primers. The extended primers comigrated with 19-mer standards when the base 5’ to PdG in the template was A, G, or T and with 18-mer standards in all sequence contexts. In addition, a 17-mer product was observed when C was 5’ to PdG and a 15-mer product was observed when G was 5’ to PdG. Parallel experiments were conducted with the Klenow fragment (exo−) of DNA polymerase I for comparison (Fig. 1B). The overall product pattern resembled that generated by pol β except that the 15-mer product detected in the 5’-[γ-32P]-G-PdG-C sequence context was absent in reactions catalyzed by the Klenow fragment.

Deoxynucleotide Incorporation Opposite PdG—Previous work from our laboratory has shown that the Klenow fragment incorporates dGMP and dAMP residues opposite PdG (dGMP > dAMP) but preferentially extends the template-primer containing PdG:dAMP at the primer terminus (20). The similarity in the profile of extension products generated by the Klenow fragment and pol β (Fig. 1) implied that a similar mechanism
might describe the course of adduct bypass by pol β. To test this, we performed single deoxynucleotide incorporation experiments with each of the template-primers. Contrary to our expectations, a constant ratio of incorporation of dGMP:dAMP was not observed but the identity of the dNMP incorporated opposite PdG changed with each sequence context. The principal dNMP incorporated in each case was the one complementary to the base 5' to PdG on the template strand (Fig. 2).

**Sequence Analysis of the Bypass Products**—The dependence of the identity of the dNMP incorporated opposite PdG on the nature of the base 5' to PdG prompted us to determine the sequence of all the extension products formed from each template-primer. The full-length (19-mer) and base-deleted (15-, 17-, and 18-mer) products were eluted from the polyacrylamide gels and subjected to Maxam-Gilbert chemical sequencing with T-specific reactions where required. In cases where multiple bands were observed opposite the adduct site in a sequence analysis gel, the major products was established by the intensity of the bands at the site of the adduct. Furthermore, deletion of CpG was observed on analysis of the band running parallel to a 17-mer that was synthesized from the 5'-C-PdG-C-3' sequence context (Fig. 6).

**Mechanism of Formation of the Four-base Deleted Product by Pol β**—Extension of the 5'-G-PdG-C-3' template-primer by pol β produced a 15-mer product that was not formed by the Klenow fragment (Fig. 1). Sequence analysis of the eluted product indicated that it arose by the deletion of the four-base sequence PdG-CGG (Fig. 7). This deletion could have occurred by two routes, one involving four-base slippage prior to incorporation of dNMP into the primer (Fig. 8, Model I) and the other involving polymerase-mediated incorporation of dCMP followed by slippage to form a four-base loop (Fig. 8, Model II). To elucidate the pathway of the four-base deletion product, primer extension was conducted with a PdG-adducted template in which the G residue four bases 5' to PdG was changed to A (5-G → 5-A template). If slippage occurred prior to incorporation of a dNMP, changing the identity of the fourth base 5' to PdG should significantly inhibit deletion because of the loss of the terminal base pair in the subsequent slippage intermediate. On the other hand, if dCMP was incorporated opposite PdG before slippage, changing the identity of the base four residues 5' to PdG should have no effect because the altered residue is not part of the slippage intermediate. On the other hand, if dCMP was incorporated opposite PdG after slippage, changing the identity of the base four residues 5' to PdG should significantly inhibit deletion because of the loss of the terminal base pair in the subsequent slippage intermediate (Fig. 9). In fact, replica- tion of the 5-G→5-A-substituted template-primer by pol β did not yield any band with an electrophoretic mobility of a 15-mer (Fig. 10).

**DISCUSSION**

Our initial comparative studies of replication of PdG-containing template-primers by pol β and the Klenow fragment suggested that the two enzymes act in a qualitatively similar fashion. Both enzymes are capable of bypassing the PdG residue albeit with low efficiency and both enzymes extend the primer to full-length when the base 5' to PdG is G, A, or T. However, analysis of the dNMP inserted opposite PdG revealed
dramatic differences between pol β and the Klenow fragment. Whereas the Klenow fragment incorporates G and A residues opposite PdG in a roughly constant ratio regardless of the sequence context (20), pol β incorporated residues complementary to the base 5’ to PdG in the template strand. Changing the identity of the 5’ base changes the identity of the inserted base. These findings indicate that the Klenow fragment uses PdG as a template base whereas pol β uses the base 5’ to PdG as the template (Fig. 11).

The ability of pol β to use the 5’ base as a template suggests that it can stabilize a template-primer structure that is formally equivalent to a one-base slippage intermediate of frameshift mutagenesis. This seems curious because there is no apparent hydrogen bonding in the template-primer that would stabilize positioning of the 5’ base at the template site. One possibility is that the PdG residue loops out of the template position and interacts with a residue on the enzyme. This interaction might stabilize the loop in the template-primer structure and position the 5’ base at the template site. However, another possibility is that the PdG residue does not loop out but the binding of the template strand to pol β positions the 5’ base close enough to the template site to hydrogen bond with the incoming dNTP. Hydrogen bonding to the incoming dNTP would help stabilize the slippage structure. This is essentially the mechanism recently proposed by Efrati et al. (37) to explain the pattern of dNMP incorporation opposite abasic sites by pol β. These investigators found that pol β utilizes the base 5’ to the abasic site as the template for dNMP incorporation in a manner analogous to that which we found for PdG. They term this bypass by dNTP stabilization (37).

The structural basis for the dNTP stabilization mechanism is provided by recent studies of the crystal structure of pol β (39). Pelletier et al. (39) proposed a model for the interaction of pol β with template-primers during gap filling synthesis which posits that the single-stranded template is bent 90° to span the distance between the catalytic domain and an 8-kDa domain that binds to the phosphate group at the 5’ end of the gap (39, 42). Model building suggests that as the template strand is
bent, the base 5' to the normal template base moves closer to the template site and rotates toward the incoming base. Although the present study did not employ gapped DNA substrates, the 8-kDa domain of pol β also has been shown to bind to the single-stranded template overhang (43). Bending of the template strand by pol β would move the base 5' to PdG into a position where it could be used as template preferentially to PdG, which contains relatively poor hydrogen bonding capacity.

Once a dNMP is inserted opposite the base 5' to PdG, continued synthesis to the end of the template would produce one-base-deleted products. In fact, these deletion products are seen with all template-primers extended by pol β regardless of 

FIG. 5. Sequence analysis of one-base deletion products synthesized on PdG-adducted 19-mer templates by pol β. Reaction products running parallel to 18-mers from the indicated templates were purified as described under “Materials and Methods.” The sequences were determined by the standard Maxam-Gilbert method and a T-specific reaction. The product sequences and their complementary template sequences are aligned on the right side of each gel to illustrate base deletion at the site of PdG.

FIG. 6. Sequence analysis of the two-base deletion product synthesized by pol β on a PdG-adducted 19-mer template. Sequence analysis of the primer extension product exhibiting the electrophoretic mobility of a 17-mer synthesized from a 5'-C-PdG-C-3' template. The primer extension product was purified as described under “Materials and Methods.” The sequence was determined by the standard Maxam-Gilbert method. The product sequence and its complementary template sequences are aligned on the right side of the gel to illustrate PdG induced two-base deletion.

FIG. 7. Sequence analysis of the four-base deletion product synthesized by pol β on a PdG-adducted 19-mer template. The primer extension product synthesized from a 5'-G-PdG-C-3' template was purified as described under “Materials and Methods.” The sequence was determined by the standard Maxam-Gilbert method and a T-specific reaction. The product sequence and its complementary template sequences are aligned on the right side of the gel to illustrate the PdG induced four-base deletion. The sequence of the primer is highlighted in the box.
sequence context. To generate full-length extension products, pol β must permit slippage of the template-primer so the newly inserted base is opposite PdG. Extension to the end of the template then produces a full-length product. Slippage may occur on the enzyme or following dissociation of the enzyme from the template-primer. Pol β is not a highly processive polymerase even when it is acting on undamaged template-primers so dissociation and reassociation with PdG-containing template-primers should occur readily (33). A similar observation also has been reported for an undamaged template in which a TCG3G transversion occurred at the 5' most T in the sequence 5'-CGTTTTAC (44). This base pair substitution was explained by a dislocation mutagenesis model (44).

In our studies, full-length extension was seen with template-primers containing A, G, or T residues 5' to PdG but not with a template-primer containing a C residue 5' to PdG. Since the relative yield of deletion and full-length extension products requires partitioning of the slipped intermediate produced by base insertion opposite the 5' base, the rate of slippage of the intermediate containing a base pair between a newly inserted G and the C 5' to PdG may be sufficiently slow to preclude slippage to an intermediate that can be extended to full-length. This would result in extension of only the slipped intermediate to produce a one-base-deleted product.

Pol β has the ability to bind to and extend slippage intermediates with loops containing more than one base (45). For example, a four-base deletion product was detected in our studies when the base 5' to PdG was G. Mechanistic studies suggested that this product arose by 1) insertion of dCMP opposite the 5' G; 2) slippage to form a four-base loop; and 3) extension. To our knowledge, this is the first report where a four-base-deleted product has been characterized by DNA sequence analysis and site-specific mutation. A similar four-base deletion product was not detected in reactions catalyzed by the Klenow fragment. This difference in the ability to catalyze deletions in vitro may result from differences in the ability of the two enzymes to accommodate extrahelical loops or bends in their binding sites. The structure of a duplex oligonucleotide containing PdG in a four-base loop has not been determined but Stone and co-workers (46, 47) have determined the structure of a
duplex containing a PdG-C loop. In fact, the DNA molecule does not exist as a duplex with an extrahelical loop but as two duplex segments bent at approximately a 25–35° angle with respect to each other and connected by the PdG-C strand (47). Similar structures may bind to the active sites of the Klenow fragment and pol β because both enzymes catalyze two-base deletions in vitro. However, the ability of pol β to generate four-base deletions may reflect its ability to bend the DNA molecules by up to 90° which would permit binding of orthogonally oriented duplex segments connected by single strands longer than two bases.

The results of our experiments with PdG do not appear to be general for exocyclic adducts with blocked Watson-Crick base pairing regions. Shibutani et al. (48) recently reported on the miscoding properties of another exocyclic adduct, 3,4′-etheno-2′-deoxyctydine (edC), in DNA synthesis catalyzed by pol β and other mammalian polymerases. They found that pol β incorporated dNMP's opposite edC in the order dCMP > dAMP > dTMP. Although the effect of sequence context was not explored, the base 5′ to edC in the template strand was C so one would have predicted preferential incorporation of dGMP opposite edC if template-slipping occurred analogous to what we observed for PdG. The contrast between the results with edC and PdG may relate to the ease with which the two adducts are bypassed by pol β. Template-primers containing edC are completely extended to full-length products on incubation at 25°C for 60 min with 0.5–1 units of enzyme; base-deleted products comprise less than 1% of the extension products (48). In contrast, PdG-containing template-primers incubated at 25°C for 30 min with 4 units of enzyme were extended by only 2–3% and base-deleted products comprised the majority of the products in most sequence contexts. This suggests that PdG is a much more powerful block to replication than edG and that template slippage as indicated in Fig. 11 is required for PdG bypass. The study by Shibutani et al. (48) establishes that edC provides hydrogen bonding information to direct deoxyxeno- side monophosphate incorporation and that edC:N base pairs can be formed and extended without the necessity for template slippage. PdG appears to have no ability to direct the incorporation of dNMP's when it is acted upon by pol β so slippage is required for bypass. Pol β may be especially sensitive to replication blockade by exocyclic adducts like PdG because it does not act efficiently on template-primers containing single-stranded templates even when they contain adducts, such as O6-methylguanine, that are readily bypassed by other polymerases (49, 50).

The similarity of the results of our experiments with PdG to those of Efrati et al. (37) with abasic sites suggest that PdG is functionally a non-informational lesion when it is being acted upon by pol β. However, this classification is not general for PdG replication by all DNA polymerases. For example, the Klenow fragment preferentially incorporates purines opposite PdG when it is replicating PdG-containing template-primers (20, 21). The ratio of dGMP to dAMP inserted depends upon the identity of the base 3′ to PdG in the template strand. No dependence of the deoxynucleoside monophosphate incorporated on the identity of the 5′ base was observed. Interestingly, although both dGMP and dAMP were incorporated opposite PdG, only template-primers containing dAMP opposite PdG were extended to full-length (20). Thus, PdG provides “information” for both base incorporation and extension when it is being acted upon by Klenow fragment but not in DNA replication by pol β.

Finally, it is important to note that the present observations provide support for a mechanism of adduct-induced frameshift mutagenesis in which template slippage precedes deoxynucleo- side monophosphate incorporation. Previous studies by Groll-
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