Sequence-dependent Induction of Base Pair Substitutions and Frameshifts by Propanodeoxyguanosine during in Vitro DNA Replication*

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Template primers containing propanodeoxyguanosine (PdG) in two different sequence contexts (C-PdG-C and T-PdG-T) were replicated by the Klenow fragment of DNA polymerase I. The presence of PdG in the template strand reduced the extent of in vitro DNA synthesis 10^2-10^3-fold compared with unmodified template primers. Partial blockade was observed 1 base 3' to the adduct and opposite the adduct. Purines were preferentially incorporated opposite the adduct; the $V_{max}/K_m$ values for incorporation of dGMP were similar in both sequence contexts, whereas the $V_{max}/K_m$ for dAMP incorporation increased 4.7-fold when the base pair 3' to PdG was changed from C:G to T:A. Oligonucleotides containing 1- and 2-base deletions were major products of replication in both sequence contexts. Full-length products were observed with templates containing T-PdG-T but not C-PdG-C. The major full-length product resulted from incorporation of dAMP residues opposite PdG. Kinetic analysis revealed that the major factor contributing to the selective incorporation of dAMP in full-length products was preferential extension of template primers containing PdG:da termini rather than preferential incorporation of dAMP opposite PdG. The observation of PdG → T mutations in the T-PdG-T context but not the C-PdG-C context during in vitro DNA replication parallels findings of in vivo experiments that base pair substitutions are induced by PdG in the former sequence context but not the latter.

Propanodeoxyguanosine (PdG) has been used as a model for several naturally occurring DNA adducts in in vivo mutagenesis and in vitro DNA replication experiments (1-5). PdG significantly reduces the transforming ability of recombinant viral genomes and induces base pair substitutions in bacterial and mammalian cells (1-3). The primary mutation observed in both cell types is PdG → T, but a significant frequency of PdG → A is also detected (2, 3, 6). PdG reduces the rate of incorporation of nucleoside monophosphates in the order dGMP > dAMP = dTMP > dCMP (4). Although detailed kinetics of insertion and extension have not been reported, it has been found that when the base 5' to PdG in the template strand is dC, 1-base deletions arise by slippage to form a dC:dG base pair followed by extension (4) (Structure 1).

The induction of base pair substitutions by PdG appears to be strongly sequence context-dependent. When viral genomes containing a single PdG residue in the context T-PdG-T are transformed into SOS-induced E. coli, the predominant mutations are PdG → T and PdG → A (3, 6). In contrast, no base pair substitutions are observed when the PdG residue is incorporated in the context C-PdG-C (7). These sequence context effects prompted us to investigate the replication of template primers containing PdG in the two different contexts by wild-type or exonuclease-deficient Klenow fragment. Standard steady-state kinetic techniques were used to evaluate the incorporation of single or multiple nucleoside monophosphates into the primer strand (8-11). Dramatic differences were observed in the insertion of bases opposite PdG and in the extension of the PdG:dn termini in the different sequence contexts. The results of the in vitro replication experiments mimic the results of the in vivo experiments and provide insight into the factors that control the induction of base pair substitution and frameshift mutations in these sequences.

MATERIALS AND METHODS

Chemicals and Enzymes—Ultrapuré solutions of dNTPs were purchased from Pharmacia Biotech Inc. [α-32P]ATP (3000 Ci/mmol) was from DuPont NEN. T4 polynucleotide kinase and DNA polymerase I Klenow fragment (exo-) were from New England Biolabs. The following sequences were synthesized: 19-mer T-PdG-T sequence, 5'-TATCGCGN-3' (X = PdG); 19-mer T-PdG-T sequence, 5'-CAGTGGTXXTCGATTG-3' (X = PdG); 19-mer C-PdG-N sequence, 5'-TATCGCGNXXCGGCTAG-3' (X = PdG). The templates were high pressure liquid chromatography purified by the vendor. 5'-Dimethoxytrityl-protected phosphoramidites of PdG was synthesized as described and incorporated into oligomers by Midland Certified Reagent Co (12). The following sequences were synthesized: 19-mer C-PdG-C sequence, 5'-TATCGCGXCGGCTAG-3' (X = PdG); T4 polynucleotide kinase (exo-) was purchased from U.S. Biochemical Corp.

Oligonucleotides—The 5'-dimethoxytrityl-protected phosphoramidite of PdG was synthesized as described and incorporated into oligomers by Midland Certified Reagent Co (12). The following sequences were synthesized: 19-mer C-PdG-C sequence, 5'-TATCGCGXCGGCTAG-3' (X = PdG); T4 polynucleotide kinase (exo-) was purchased from U.S. Biochemical Corp.

The templates were high pressure liquid chromatography purified by the vendor. 5'-Dimethoxytrityl-protected unmodified 19-mers, the various primers, and synthetic markers were obtained from the Molecular Genetics Core Laboratory of the Vanderbilt Center in Molecular Toxicology. Sequences of the synthetic markers were complementary to the templates and corresponded to the sequences that are full-length or 2 base-deleted at the site of the PdG (see text). The unmodified oligonucleotides were purified with nucleic acid purification columns (NEN Research Products). The purities of all the oligonucleotides were estimated by polyacrylamide gel electrophoresis to be in excess of 98%.

The primer sequences were as follows: C-PdG-C sequence, 5'-GCTCATGCC-3' (9-mer), 5'-GCTCATGCGG-3' (10-mer), 5'-GCTCATGCG-3' (11-mer, N = A, C, G, or T); T-PdG-T sequence, 5'-TCATTCGGA-3' (9-mer), 5'-TCAATTCGGA-3' (10-mer), 5'-TCAUTTCGGA-3' (11-mer, N = A, C, G, or T).

General Procedures—5' end labeling of the primers (2 μM) was carried out using T4 polynucleotide kinase (13). Labeled primers were...
purified by biospin columns (Bio-Rad). Annealing of the appropriate template with 5' end labeled primer in a molar ratio of 5:1 (template: primer) was carried out by incubating at 90°C for 10 min and slow cooling in an annealing buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM β-mercaptoethanol, 50 μg/ml bovine serum albumin, and 5 mM MgCl₂. Incubations with DNA polymerase were performed as follows. The template primers (100 nM primer) were incubated in 10 μl of 50 mM Tris-HCl (pH 6.5), 4 mM β-mercaptoethanol, 8 mM MgCl₂, 4 mM dithiothreitol, and 2 μg/ml bovine serum albumin with 0.1 unit of DNA polymerase I Klenow fragment (exo⁻ or exo⁺) in the presence of all four normal dNTPs (100 μM). Incubations were conducted at 25°C for 30 min. Reactions were quenched by adding 10 μl of 10 mM EDTA in 95% formamide. The reaction products were analyzed by 20% denaturing polyacrylamide gel electrophoresis using sequagel (National Diagnostics). The positions of the bands were established by autoradiography or PhosphorImager analysis (Molecular Dynamics). The sequences of the primer extension products were characterized by chemical sequencing utilizing base-specific cleavage reactions (14). The bands corresponding to primer extension products obtained with 100 pmol of labeled primer were cut out of the gel and extracted by shaking overnight in distilled water. The extracted products were purified by biospin columns (Bio-Rad) and sequenced.

RESULTS

DNA Synthesis on a PdG-adducted Template—The effect of PdG on DNA synthesis by the Klenow fragment of DNA polymerase I was evaluated using template primers containing PdG in C-PdG-C and T-PdG-T sequence contexts. PdG was incorporated at position 11 from the 3' end of a 19-mer template hybridized to a nine-nucleotide primer. Replication in the presence of all four dNTPs was carried out at pH 6.5 to minimize hybridization of the primer to the template primers containing PdG in DNAsynthesis by the Klenow fragment of DNA polymerase I was evaluated using template primers containing PdG in the presence of all four dNTPs. Polymerization was carried out with Klenow fragment (exo⁻ or exo⁺) at 25°C for 30 min. Electrophoretic mobilities of the extended products were compared with synthetic markers. The template sequence for A is 5'-TATCGCGCAATGATTGA-3', and the template sequence for B is 5'-CATGGAATGCTTGGCCATT-3' (X = PdG or dG).

Deoxyguanosine monophosphate and dAMP were preferentially incorporated opposite PdG in both sequence contexts. The incorporation of dGMP opposite the adduct site was higher than that of dAMP in the C-PdG-C context, whereas the reverse was true in the T-PdG-T sequence. Only trace amounts of dCMP or dTTP were incorporated in either sequence. The two bands of extended primer that appeared at position 11 corresponded to the electrophoretic mobility of bands produced by preferential incorporation of dGMP and dAMP.

Extension past the lesion was studied using 11-mer primers with different bases at the 3' termini (Fig. 4). Comparison of the intensity of the extended bands indicated that the primer with dG opposite PdG was the most favorable substrate for extension from the C-PdG-C template (Fig. 4A). A significant amount of extension was also observed with the primer containing dC at the 3' terminus. No significant extension was obtained from either the dT-PdG- or dA-PdG-paired termini. Identical results were obtained when 3' → 5' exonuclease-deficient Klenow fragment was used under similar conditions. Maxam-Gilbert sequencing of the bypass products arising from dG-PdG and dC-PdG primer termini in the C-PdG-C sequence context revealed that they were the result of a 1-base deletion (C) and a 2-base deletion (CG) at the adduct site, respectively (data not shown). Thus extension of template primers containing PdG in this context only occurred following slippage of the template thereby leading to frameshifts.

Investigation of the dependence of extension on the identity of the base opposite PdG in the T-PdG-T template revealed that the primer containing dA at the terminus was preferentially extended and yielded both 19- and 18-nucleotide products (Fig. 4B).
A small amount of extension was also observed when dC was at the primer terminus; the product comigrated with a 2 base-deleted standard (17-mer). Elongation past PdG from primers containing dT or dG at the 3' end was not observed under the experimental conditions. The data show that in the T-PdG-T sequence, 1 and 2 base-deleted products resulted from insertion of dAMP and dCMP followed by slippage-mediated extension of the bases complementary to neighboring 5' bases. Full-length bypass synthesis occurred by insertion of dAMP opposite PdG and extension without slippage.

The presence of PdG in the template was essential for production of frameshifts. Incubation of Klenow fragment or exonuclease-deficient Klenow fragment with a (19–11)-mer template primer containing dG at the primer terminus opposite a dG residue in the C-PdG-C template led to no extension (Fig. 5). Thus, slippage and extension to the 1 base-deleted product occurred from the PdG:dG mismatch but not the dG:dG mismatch.

Nucleoside monophosphate incorporation opposite PdG preceded slippage leading to frameshifts. Changing the identity of the base 5' to PdG in the template strand did not change the pattern of nucleoside monophosphate incorporation opposite PdG (Fig. 6). If slippage leading to frameshifts occurred before nucleoside insertion, a different pattern of nucleoside monophosphate incorporation would have been observed with each 5' base.

Kinetics of Insertion and Extension at PdG—The 3' → 5' exonuclease-deficient Klenow fragment was used to measure the kinetics of nucleotide insertion and extension past PdG using (19–10)-mer template primers and (19–11)-mer template primers, respectively. Steady-state conditions were used to measure kinetic parameters according to the polyacrylamide gel assay described by others (8, 9, 11). A Hanes-Woolf plot was used to determine the $K_m$ and $V_{max}$ values, and the efficiency of incorporation and extension was determined from the ratio $V_{max}/K_m$ (8, 9, 15–17). The relative efficiencies ($V_{max}/K_m$) of nucleotide insertion opposite PdG in both the sequence contexts are listed in Table I. The efficiency of dGMP incorporation across from PdG was 3-fold higher than for dAMP and 10-fold higher than both dCMP and dTMP in the C-PdG-C sequence context. The efficiency of dAMP insertion was approximately 1.4 times higher than dGMP in the T-PdG-T context.

The efficiency of extension was measured using kinetic parameters for the addition of the next nucleotide onto a primer containing dG or dA opposite PdG. The relative efficiencies of extension are presented in Table II. When dG was opposite PdG in the C-PdG-C sequence context, the efficiency of extension by the addition of dCMP was about 130 times higher than for the addition of dGMP. Elongation by the addition of dCMP onto the primer containing dA at the 3' terminus was not observed over the range of concentrations used. The addition of
dGMP onto the dA-PdG primer terminus was observed but at a rate 36-fold lower than for the addition of dCMP onto the dG-PdG primer terminus. In T-PdG-T sequence, the efficiency of extension by the addition of dAMP onto the primer with dA opposite PdG was 85-fold higher than for a primer containing dG at the 3′ terminus (Table II). Therefore, unlike the C-PdG-C sequence, kinetic parameters were favorable for direct extension from a primer terminus containing dA opposite PdG in the T-PdG-T sequence context. Slippage prone extension kinetic parameters for the addition of dCMP onto the primer containing dA at the 3′ terminus could not be determined due to three contiguous dGs in the template that complicated the measurement of band intensity.

**DISCUSSION**

The objective of the present experiments was to determine and compare the spectrum and specificity of mutations induced by in vitro replication past a single PdG adduct in two different sequence contexts that have been used for in vivo mutagenesis experiments. As reported by Shibutani and Grollman (4), PdG is a strong block to replication; at prolonged incubation times, approximately 2–3% of the primers in template primers containing PdG were extended beyond the position of the adduct. The \( \frac{V_{\text{max}}}{K_m} \) values for insertion of individual deoxynucleoside monophosphates opposite PdG ranged from 7 to 70 \( \times 10^4 \) slower than the \( \frac{V_{\text{max}}}{K_m} \) for insertion of dC opposite dG in the C-G-C sequence context (Table I). Likewise, the \( \frac{V_{\text{max}}}{K_m} \) values for extension of template primers beyond the PdG-nucleoside monophosphate mispair were significantly decreased relative to extension from G:C termini (Table II).

The principal bases incorporated opposite PdG were purines, and the relative incorporation of deoxyguanosine and deoxyadenosine residues depended to some extent on sequence context. Deoxyguanosine was incorporated more frequently than deoxyadenosine in the C-PdG-C context (dG/dA = 5), whereas the reverse was true in the T-PdG-T context (dA/dG = 1.5). The differences in relative efficiency of purine incorporation in the two sequences were due to the higher value of \( \frac{V_{\text{max}}}{K_m} \) for dAMP incorporation in the T-PdG-T context (4.7-fold). The \( \frac{V_{\text{max}}}{K_m} \) ratios for incorporation of dGMP in both contexts were the same. Similar patterns of incorporation were observed with normal and exonuclease-deficient Klenow fragment, so exonucleolytic removal of bases did not appear to contribute significantly to the relative extent of nucleoside incorporation.

The nature of the base pair 3′ to PdG in the template primer contributed to the increased rate of dAMP incorporation opposite PdG in the T-PdG-T context. This was mainly the result of a decrease in \( K_m \) for dATP. This may reflect favorable stacking interactions between the incoming dAMP and the dA residue 5′ to it in the primer strand. An effect of the base 5′ to the incoming nucleotide has been previously noted in experiments on the in vitro replication of unmodified or modified templates.

\[ \text{FIG. 5. Requirement for PdG for the induction of frameshifts.} \]

\[ \text{32P-Labeled 11-mer primers with dG at the 3′ end terminus were extended on unadducted (lane 1) and adducted (lane 2) 19-mer templates (sequence, CXC, X = PdG/dG). Polymerization was catalyzed by the Klenow fragment (exo−) in presence of all four dNTPs. Reactions were conducted for 30 min at 25 °C. Lane 3 shows extension of an 11-mer primer having dC at the 3′ end terminus on an unadducted template.} \]

\[ \text{FIG. 6. Dependence of nucleotide incorporation opposite PdG on the nature of the 5′-flanking base.} \]

\[ \text{32P-Labeled 10-mer primers were extended on PdG-adducted 19-mer templates having dA, dC, dG, or dT on the 5′ side of the PdG. Polymerization was catalyzed by the Klenow fragment (exo+) in the presence of a single nucleotide. Reactions were conducted for 30 min at 25 °C.} \]
Propanodeoxyguanosine Replication

TABLE I
Kinetic parameters for nucleotide incorporation opposite PdG

| pair X | $K_m$ (μM) | $V_{max}$ (%/min) | $V_{max}/K_m$ |
|--------|------------|------------------|---------------|
| C:G    | 0.025      | 15               | 600           |
| A:PdG  | 75         | 1                | $13 \times 10^{-3}$ |
| G:PdG  | 30         | 1.2              | $41 \times 10^{-3}$ |
| C:PdG  | 25         | 0.10             | $4 \times 10^{-3}$  |
| T:PdG  | 42         | 0.17             | $4 \times 10^{-3}$  |

| pair X | $K_m$ (μM) | $V_{max}$ (%/min) | $V_{max}/K_m$ |
|--------|------------|------------------|---------------|
| A:PdG  | 22         | 1.4              | $61 \times 10^{-3}$ |
| G:PdG  | 30         | 1.4              | $45 \times 10^{-3}$ |
| C:PdG  | ND         |                   |               |
| T:PdG  | ND         |                   |               |

*ND, not determined.

TABLE II
Kinetic parameters for extension from nucleotides located opposite PdG

| C-PdG-C | Event          | $K_m$ (μM) | $V_{max}$ (%/min) | $V_{max}/K_m$ |
|---------|----------------|------------|------------------|---------------|
| $5'\text{-}G \text{G} \downarrow$ | dGTP         | 14.2       | 0.4              | $30 \times 10^{-3}$ |
| $3'\text{-}X \text{C} \text{G} \uparrow$ | 5'--G | 4.7        | 0.5              | $106 \times 10^{-3}$ |
| $3'\text{-}X \text{C} \text{G} \uparrow$ | dCTP        | 0.18       | 0.7              | $3856 \times 10^{-3}$ |
| $3'\text{-}X \text{C} \text{G} \uparrow$ | 5'--A | ND         |                   |               |
| $3'\text{-}X \text{C} \text{G} \uparrow$ | T:PdG-T      | 49         | 0.22             | $4.5 \times 10^{-3}$ |
| $3'\text{-}X \text{T} \text{G} \uparrow$ | dATP         | 0.7        | 0.27             | $385 \times 10^{-3}$ |
| $3'\text{-}X \text{T} \text{G} \uparrow$ | ND         |                   |               |

*ND, could not be determined with accuracy due to contiguous run of G.

Frameshift mutations, the most common of which were 1-base deletions, were observed in both sequence contexts. Frameshifts required the presence of the PdG residue in the template primer, no frameshifts were detected following replication of template primers containing dG:dG mismatches at the termini (Fig. 5). The identity of the 1 base-deleted product was consistent with the previous report of Shibutani and Grollman (4) and supportive of the model proposed by Kunkel and Bebenek (29) and Fuchs et al. (30) in which nucleotide incorporation opposite the lesion precedes slippage and extension (Fig. 7). As stated above, dGMP and dAMP residues are preferentially incorporated opposite PdG in both sequence contexts studied in the present experiments. In the C-PdG-C context, the template primer containing dG at the 3' primer terminus slips to form a base pair with the dC residue 5' to PdG. Conversely, in the T-PdG-T context, the template primer containing dA at the 3' primer terminus slips to form a base pair with the dT residue 5' to PdG. Thus, even though dG and dA residues are inserted opposite PdG in both sequence contexts, slippage and formation of a base pair with the 5' residue determines which template primer is extended. Two base slippage and extension was also observed in both sequence contexts when the residue opposite PdG was dC because of the presence of a dG residue 2 bases 5' to PdG in both sequences. Equimolar amounts of the four deoxynucleoside triphosphates were used in most of the in vitro replication experiments, and because dCMP was poorly incorporated opposite PdG, the frequency of 2-base deletion was much lower than the frequency of 1-base deletion. However, the yield of the 2-base deletion product could be increased by increasing the content of dCTP in the deoxynucleoside triphosphate mixture. The 2-base deletion product was increased approximately 2-fold when the ratio of dCTP to dGTP was 20:1, which approximates the relative pool size in replicating mammalian cells (31). Thus, in vivo DNA synthesis conditions may favor incorporation of dC and the subsequent 2-base slippage and deletion.

The most dramatic effect of sequence context on replication of PdG-containing template primers was the difference in full-length extension. No full-length extension was observed when PdG was present in the C-PdG-C context; only 1- and 2-base deletion products were detected. However, full-length extension was the predominant process when PdG was in the T-PdG-T context. DNA sequence analysis indicated that dA was
A mutation is not mimicked by extension of dAMP opposite PdG in the T-PdG-T context. The preferential incorporation and extension of dAMP opposite the T-PdG-T context but not the C-PdG-C context correlates well with the presence of all four dNTPs with the T-PdG-T template further supporting the preferential incorporation and extension of dAMP opposite PdG in the template strand. The factors responsible for this selective extension and its generality for other exocyclic deoxyguanosine adducts such as pyrimidopurinone are under investigation.

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