

Mutagenic and Cytotoxic Properties of 6-Thioguanine, S⁶-Methylthioguanine, and Guanine-S⁶-sulfonic Acid^{*[S]}

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Thiopurine drugs, including 6-thioguanine (S^G), 6-mercaptopurine, and azathioprine, are widely employed anticancer agents and immunosuppressants. The formation of S^G nucleotides from the thiopurine prodrugs and their subsequent incorporation into nucleic acids are important for the drugs to exert their cytotoxic effects. S^G in DNA can be methylated by S-adenosyl-L-methionine to give S⁶-methylthioguanine (S⁶mG) and oxidized by UVA light to render guanine-S⁶-sulfonic acid (S⁶OHG). Here, we constructed single-stranded M13 shuttle vectors carrying a S^G, S⁶mG, or S⁶OHG at a unique site and allowed the vectors to propagate in wild-type and bypass polymerase-deficient *Escherichia coli* cells. Analysis of the replication products by using the competitive replication and adduct bypass and a slightly modified restriction enzyme digestion and post-labeling assays revealed that, although none of the three thionucleosides considerably blocked DNA replication in all transfected *E. coli* cells, both S⁶mG and S⁶OHG were highly mutagenic, which resulted in G→A mutation at frequencies of 94 and 77%, respectively, in wild-type *E. coli* cells. Deficiency in bypass polymerases does not result in alteration of mutation frequencies of these two lesions. In contrast to what was found from previous steady-state kinetic analysis, our data demonstrated that 6-thioguanine is mutagenic, with G→A transition occurring at a frequency of ~10%. The mutagenic properties of 6-thioguanine and its derivatives revealed in the present study offered important knowledge about the biological implications of these thionucleosides.

6-Mercaptopurine is a widely prescribed anticancer drug for treating acute lymphoblastic leukemia (1–3). Azathioprine is commonly used as an immunosuppressive agent for organ transplant patients and people suffering from chronic inflammatory diseases including ulcerative colitis and Crohn syndrome (4, 5). After oral administration and absorption, ~90% of the prodrug azathioprine is converted to 6-mercaptopurine from attack by sulfhydryl-containing compounds such as glutathione and cysteine (6, 7). Although it is established that the

conversion of these prodrugs to 6-thioguanine (S^G)² nucleotides and the subsequent incorporation of S^G into nucleic acids are essential for the drugs to be effective (1, 8), the exact mechanisms for the cytotoxic effects of these thiopurines remain poorly understood.

In light of the similarities between S^G and *N*-methyl-*N*-nitrosourea in exerting their cytotoxic effects, Swann *et al.* (9) proposed that, after metabolic activation, S^G in DNA may kill cells by triggering the post-replicative mismatch repair (MMR) pathway. In this context both S^G and *N*-methyl-*N*-nitrosourea exhibit delayed cytotoxic effect, and both introduce sister chromatid exchange, which is a type of chromosomal damage associated with post-replicative MMR. In addition, cells that are deficient in the MMR pathway are resistant to both S^G and *N*-methyl-*N*-nitrosourea (10, 11). Furthermore, *N*-methyl-*N*-nitrosourea can methylate guanine to give O⁶-methylguanine (12), and S^G in DNA can be methylated by S-adenosyl-L-methionine to afford S⁶-methylthioguanine (S⁶mG, see Fig. 1) (9). Both O⁶-methylguanine (O⁶mG) and S⁶mG can miscode during DNA replication and give rise to O⁶mG:T and S⁶mG:T mispairs, which can both be recognized by the post-replicative MMR pathway (9, 12, 13). Therefore, it was proposed that 6-thioguanine exerts its cytotoxic effect via its incorporation into DNA, its subsequent methylation by S-adenosyl-L-methionine, the misincorporation of dTMP opposite S⁶mG during DNA replication, and the provoking of the post-replicative MMR by the S⁶mG:T mispair (9, 14). In support of this hypothesis, it was found that the removal of methyl group from S⁶mG by O⁶-methylguanine DNA alkyltransferase is 10⁶ times slower than the corresponding transfer of a methyl group from O⁶-methylguanine (9). Although a S^G:T mispair in duplex DNA can be recognized by mammalian mismatch repair proteins as efficiently as the G:T mispair (15), the triggering of MMR by S^G in DNA was discounted because *in vitro* replication studies demonstrated that S^G in template DNA directs mostly the incorporation of the correct nucleotide, dCMP (9, 16, 17). It remains unclear whether the observations made in these *in vitro* replication studies can be extended to cells.

Unlike natural nucleobases, 6-thioguanine exhibits a UV absorption maximum at 342 nm. Thus, UVA exposure of S^G and oligodeoxynucleotides (ODNs) containing a S^G can result in the oxidation of the S^G to give guanine-S⁶-sulfonic acid

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² The abbreviations used are: S^G, 6-thioguanine; MMR, mismatch repair; CRAB, competitive replication and adduct bypass; ODN, oligodeoxynucleotide; REAP, restriction endonuclease and post-labeling; S⁶mG, S⁶-methylthioguanine; S⁶OHG, guanine-S⁶-sulfonic acid; LC, liquid chromatography; HPLC, high performance liquid chromatography; MS, mass spectroscopy; pol, polymerase.

Mutagenic Properties of 6-Thioguanine and Its Metabolites

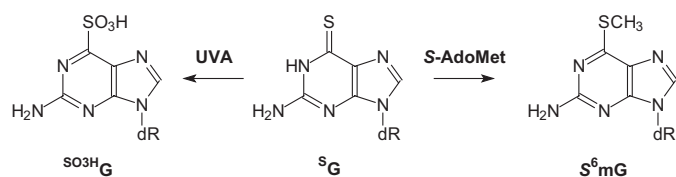


FIGURE 1. The formation of S^6mG and $S^{3H}G$.

($S^{3H}G$, Fig. 1) (18, 19). *In vitro* replication studies with purified DNA polymerases further showed that $S^{3H}G$ is a strong block to replicative DNA polymerase, and it exhibits ambiguous coding properties; significant misinsertion of dTMP and dAMP opposite the lesion was observed (18–20). The high mutagenic potential of $S^{3H}G$ and the UVA-induced formation of reactive oxygen species in S^6G -treated cells are thought to be associated with the increased skin cancer occurrence in those patients who have undergone azathioprine therapy (18). It remains elusive, however, to what extent $S^{3H}G$ can block DNA replication and cause mutations *in vivo*. In the present study we constructed single-stranded shuttle vectors containing a S^6G , S^6mG , or $S^{3H}G$ at a defined site and assessed how these thionucleosides perturb the efficiency and fidelity of DNA replication in *E. coli* cells.

EXPERIMENTAL PROCEDURES

Materials—The phosphoramidite building block of 6-thio-2'-deoxyguanosine was obtained from Glen Research (Sterling, VA). Unmodified ODNs used in this study were purchased from Integrated DNA Technologies (Coralville, IA). [γ - ^{32}P]ATP was obtained from PerkinElmer Life Sciences. Chemicals unless otherwise noted were obtained from Sigma-Aldrich. M13mp7(L2) and wild-type *Escherichia coli* strains were kindly provided by Prof. John M. Essigmann, and polymerase-deficient AB1157 strains ($\Delta pol B1::spec$ (pol II-deficient), $\Delta dinB$ (pol IV-deficient), $\Delta umuC::kan$ (pol V-deficient), $\Delta umuC::kan \Delta dinB$ (pol IV, pol V-double knock-out), and $\Delta umuC::kan \Delta dinB \Delta pol B1::spec$ (pol II, pol IV, pol V-triple knock-out)) were generously provided by Prof. Graham C. Walker (21).

Preparation of ODN Substrates Containing a 6-Thioguanine or Its Modified Derivatives—ODN substrates containing a S^6G , S^6mG , or $S^{3H}G$ were prepared following previously published procedures (19, 20, 22), and the identities of the modified ODNs were confirmed by electrospray ionization-MS and tandem MS (MS/MS) analyses.

Construction of ss-M13 Genomes Harboring a Site-specifically Inserted S^6G , S^6mG , or $S^{3H}G$ —The M13mp7(L2) viral genomes, either lesion-free or carrying a site-specifically inserted S^6G , S^6mG , or $S^{3H}G$, were prepared following the previously described procedures (23). Briefly, 20 pmol of ss-M13mp7(L2) was digested with 40 units of EcoRI at 23 °C for 8 h to linearize the vector. Two scaffolds, 5'-GCCCATG-GCACTGAATCATGGTCATAGC-3' and 5'-AAACGACG-GCCAGTGAATTAATTCGG-3' (25 pmol), each spanning one end of the cleaved vector and the modified ODN insert, were annealed with the linearized vector. The 16-mer insert (d(CCATGGGXCXCCGAATT), where X represents a guanine, S^6G , S^6mG , or $S^{3H}G$, 30 pmol) was 5'-phosphorylated with T4

polynucleotide kinase. The 5'-phosphorylated 16-mer inserts were ligated to the above vector by using T4 DNA ligase in the presence of the two scaffolds at 16 °C for 8 h. T4 DNA polymerase (22.5 units) was subsequently added, and the resulting mixture was incubated at 37 °C for 4 h to degrade the scaffolds and residual unligated vector. The solution was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v), and the aqueous phase was passed through a ProbeQuant-G50 micro column (GE Healthcare) to remove traces of phenol and salt. The constructed genomes were normalized against a lesion-free competitor genome, which was prepared by inserting a 19-mer unmodified ODN to the EcoRI-linearized genome, following the method described by Delaney *et al.* (23).

Transfection of *E. coli* Cells with ss-M13 Vectors Containing a Guanine, S^6G , S^6mG , or $S^{3H}G$ —Desalted genomes containing a lesion or unmodified guanine (150 fmol) were mixed with the competitor genome at a molar ratio of 6:1 (lesion/competitor) and transfected into the electrocompetent AB1157 *E. coli* cells. The M13 genome-carrying *E. coli* cells were grown in 3 ml of LB culture at 37 °C for 6 h after which the phage was recovered from the supernatant by centrifugation at 13,000 rpm for 5 min. The resulting phage was further amplified in SCS110 *E. coli* cells to increase the progeny/lesion-genome ratio (23). The phage recovered from the supernatant (700 μ l) was passed through a QIAprep Spin M13 kit (Qiagen, Valencia, CA) to isolate the ss-M13 DNA.

Determination of the Bypass Efficiency and Mutation Frequency Using Competitive Replication and Adduct Bypass (CRAB) and Restriction Endonuclease and Post-labeling (REAP) Assays—CRAB and REAP assays were carried out according to previously described procedures with some modifications (23). PCR amplification of the region of interest in the resulting progeny genome was performed by using Phusion high fidelity DNA polymerase. The primers were 5'-YCAGCTATGAC-CATGATTCACTGCCATG-3' and 5'-YTCGGTGCGGGC-CTCTTCGCTATTAC-3' (Y is an amino group), and the amplification cycle was 26, each consisting of 10 s at 98 °C, 30 s at 62 °C, 15 s at 72 °C, with a final extension at 72 °C for 5 min. The PCR products were purified by using QIAquick PCR purification kit (Qiagen).

For the bypass efficiency assay, a portion of the above PCR fragments was treated with 10 units of NcoI and 1 unit of shrimp alkaline phosphatase (USB Corp., Cleveland, OH) in 10- μ l of NEB buffer 2 (New England Biolabs, Ipswich, MA) at 37 °C for 2 h followed by heating at 65 °C for 20 min to deactivate the phosphatase. The above mixture was then treated in a 15- μ l of NEB buffer 2 with 5 mM dithiothreitol, ATP (50 pmol cold premixed with 1.66 pmol of [γ - ^{32}P] ATP) and 10 units of polynucleotide kinase. The reaction was continued at 37 °C for 1 h followed by heating at 65 °C for 20 min to deactivate the polynucleotide kinase. To the reaction mixture was subsequently added 10 units of Tsp509I, and the solution was incubated at 65 °C for 1 h followed by quenching with 15 μ l of formamide gel loading buffer containing xylene cyanol FF and bromophenol blue dyes. The mixture was loaded onto a 30% native polyacrylamide gel (acrylamide:bisacrylamide = 19:1), and products were quantified by phosphorimaging analysis. After the restriction cleavages, the DNA fragment of interest

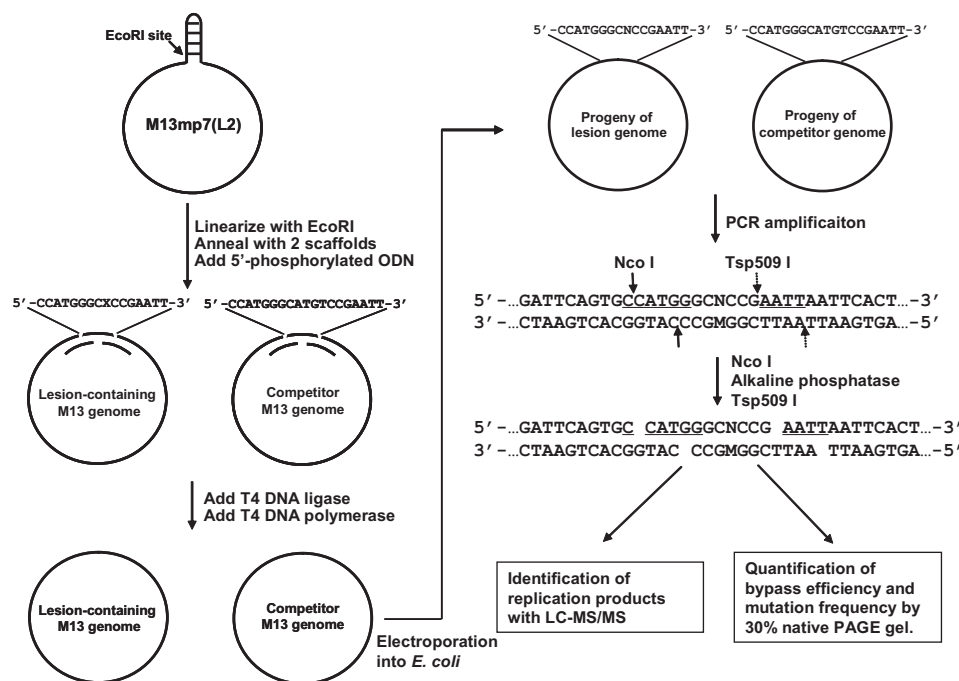


FIGURE 2. Method for the determination of the cytotoxicity and mutagenicity of ^5G , ^5mG , and $^{\text{SO}_3\text{H}}\text{G}$ in *E. coli* cells. X in the 16-mer ODN represents ^5G , ^5mG , $^{\text{SO}_3\text{H}}\text{G}$ or an unmodified guanine. N in the progeny of the lesion genome represents the nucleoside inserted at the initial lesion site. NcoI and Tsp509I restriction endonuclease recognition sites are underlined, and the cleavage sites induced by the two enzymes are designated by solid and broken arrows, respectively. Only partial sequence of PCR products for the lesion genome is shown, and the PCR products of the competitor genome are not shown.

from the full-length replication product was liberated as an 11-mer ODN, d(p*CATGGGCNCCG), where N designates the nucleobase present at the original lesion site after *in vivo* DNA replication, and p* represents the 5'-radiolabeled phosphate. The 11-mers with a single nucleotide difference could be resolved by 30% native polyacrylamide gel. On the other hand, the corresponding DNA fragment released from the competitor genome was a 14-mer ODN, d(p*CATGGGCATGTCCG). The mutation frequencies were determined from the relative amounts of different 11-mer products from the gel band intensities. The bypass efficiency was calculated using the formula % bypass = (lesion signal/competitor signal)/(non-lesion control signal/its competitor signal) (23).

Identification of Replication Products by Using LC-MS/MS—To identify the replication products using LC-MS, PCR products were treated with 50 units of NcoI and 20 units shrimp alkaline phosphatase in 250 μl of NEB buffer 2 at 37 $^\circ\text{C}$ for 2 h followed by heating at 65 $^\circ\text{C}$ for 20 min. To the resulting solution was then added 50 units of Tsp509I, and the reaction mixture was incubated at 65 $^\circ\text{C}$ for 1 h followed by extraction once with phenol/chloroform/isoamyl alcohol (25:24:1, v/v), and the aqueous portion was dried with SpeedVac and dissolved in 12 μl of water. The ODN mixture was subjected to LC-MS/MS analysis. A 0.5 \times 150-mm Zorbax SB-C18 column (5 μm in particle size, Agilent Technologies) was used for the separation, and the flow rate was 8.0 $\mu\text{l}/\text{min}$, which was delivered by using an Agilent 1100 capillary HPLC pump. A 5-min gradient of 0–20% methanol followed by a 35 min of 20–50% methanol in 400 mM 1,1,1,3,3,3-hexafluoro-2-propanol (pH was adjusted to 7.0 by the addition of triethylamine) was employed for the sep-

aration (24). The effluent from the LC column was coupled directly to an LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA), which was set up for monitoring the fragmentation of the $[\text{M}-3\text{H}]^{3-}$ ions of the 11-mer (d(CATGGGCNCCG), where N designates A, T, C, or G), and 14-mer (*i.e.* d(CATGGGCATGTCCG)) ODNs.

RESULTS

Preparation of ODNs Containing a 6-Thioguanine or Its Modification Products—We employed traditional phosphoramidite chemistry and synthesized a ^5G -containing ODN, d(CCATTGGGC $^5\text{GCCG}$ ATT). As reported previously (22), ^5G in ODNs can be selectively methylated to ^5mG by treatment with methyl iodide (CH_3I) in a phosphate buffer (pH 8.5). In addition, ^5G in ODNs can be oxidized selectively to $^{\text{SO}_3\text{H}}\text{G}$ upon treatment with magnesium monoperoxyphthalate (19, 20, 22). We employed similar procedures and isolated the desired

^5mG - and $^{\text{SO}_3\text{H}}\text{G}$ -containing ODNs from the reaction mixtures by HPLC. The identities of these thionucleoside-bearing ODNs were confirmed by electrospray ionization MS and MS/MS analyses (supplemental Figs. S1–S3).

Replication of 6-Thioguanine or Its Modification Products in *E. coli* Cells—We next asked how the presence of ^5G , ^5mG , and $^{\text{SO}_3\text{H}}\text{G}$ compromises DNA replication and which translesion synthesis-induced DNA polymerase is involved in bypassing the lesion in *E. coli* cells. To this end we inserted the aforementioned thionucleoside-bearing ODNs into single-stranded M13 genome and assessed the bypass efficiencies and mutation frequencies of these modified nucleosides by using the CRAB and REAP assays introduced by Essigmann and Delaney (Fig. 2) (23, 25, 26).

In the absence of deletion mutation, restriction digestion of the PCR products of the progeny M13 genome emanating from *in vivo* replication affords an 11-mer fragment harboring the site where the ^5G , ^5mG , or $^{\text{SO}_3\text{H}}\text{G}$ was initially incorporated. The corresponding digestion of PCR products of the progeny of the competitor genome gives a 14-mer fragment (Fig. 2). The failure to detect radiolabeled fragments with lengths shorter than 11-mer supports that none of the thiopurine derivatives gives rise to deletion mutations (Fig. 3). In this context, we employed 30% (19:1, acrylamide:bisacrylamide) native polyacrylamide gels to resolve the ^{32}P -labeled fragments, and it turned out that the 11-mers with a single nucleotide difference can be readily resolved from each other (Fig. 3).

The bypass efficiencies were calculated from the ratio of the combined intensities of bands observed for the 11-mer prod-

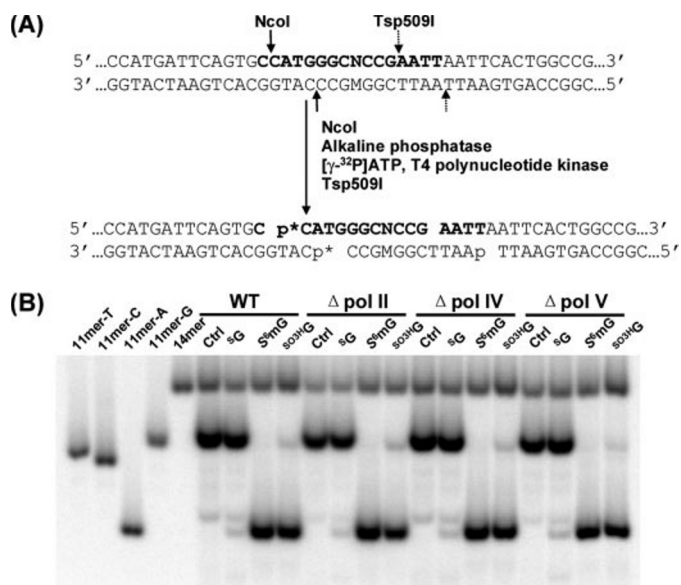


FIGURE 3. Measurement of the bypass efficiencies and mutation frequencies of ^5G , ^5mG , and ^5OHG in vivo by CRAB assay. A, sample processing (p^* represents ^{32}P -labeled phosphate group). B, gel image showing the 14- and 11-mer released from the PCR products of the progeny resulting from the replication of the competitor genome and the control (Ctrl) or lesion-carrying genome in wild-type (WT) and the isogenic AB1157 cells deficient in pol II, pol IV, or pol V. The restriction fragment arising from the competitor genome, i.e. d(CATGGGCATGTCGG), is designated with 14-mer; 11-mer-G, 11-mer-A, 11-mer-C, and 11-mer-T represent standard ODNs d(CATGGGCNCCG), where N is a G, A, C, or T.

ucts over the intensity of the 14-mer band with the consideration of the ratio of the lesion over competitor genome employed in the initial transfection. The bypass efficiencies for the lesion-carrying genomes were then normalized against that for the control lesion-free genome. It turned out that none of the thiopurine derivatives is a strong block to DNA replication in wild-type AB1157 cells, and the bypass efficiencies for ^5G , ^5mG , and ^5OHG are ~98, 91, and 87%, respectively. In addition, deficiency in pol II, pol IV, or pol V in the isogenic AB1157 background does not considerably affect the bypass efficiencies for these thionucleosides (Fig. 4A). Only a slight drop in bypass efficiency was found for ^5mG in AB1157 cells deficient in pol IV (7% drop relative to wild-type cells) or pol V (5% decrease) and for ^5OHG in pol V-deficient cells (9% decrease compared with wild-type cells; Fig. 4A). Depletion of both pol IV and pol V results in synergistic decrease in bypass efficiencies; relative to the results obtained for the wild-type cells, a decrease from 91 to 63% was found for ^5mG , and a drop from 87% to 48% was observed for ^5OHG (supplemental Fig. S4 and Fig. 4A). These data support that both pol IV and pol V are involved to some extent in the bypass of ^5mG and ^5OHG in *E. coli* cells. On the other hand, further deletion of pol II did not lead to an increased drop in bypass efficiency, underscoring the lack of involvement of this polymerase in bypassing ^5mG or ^5OHG .

The results from native PAGE analysis also allowed us to measure the mutation frequencies of ^5G , ^5mG , and ^5OHG in wild-type and bypass polymerase-deficient *E. coli* strains with the REAP assay (23, 26). The quantification data showed that both ^5mG and ^5OHG are highly mutagenic in wild-type AB1157 cells, with the G→A transition occurring at frequencies of 94 and 77%, respectively. Somewhat to our surprise, the

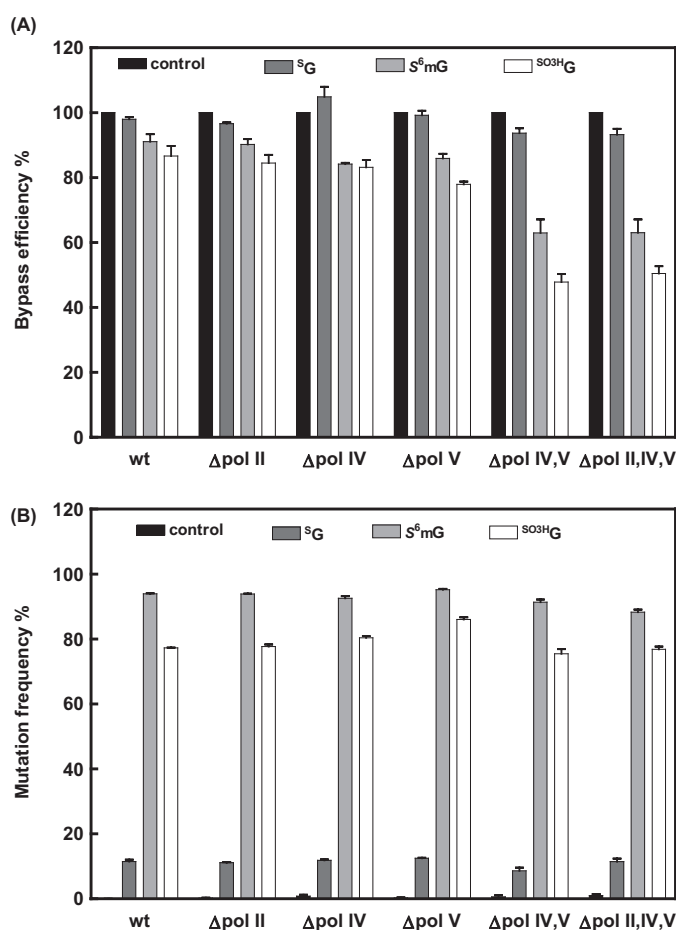


FIGURE 4. Bypass efficiencies (A) and mutation frequencies (B) of guanine, ^5G , ^5mG , and ^5OHG in wild-type (wt) and polymerase-deficient AB1157 *E. coli* cells. Black, dark gray, light gray, and white columns represent the results for substrates carrying dG, ^5G , ^5mG , and ^5OHG , respectively. The data represent the means and S.D. of results from three independent experiments.

presence of ^5G also results in G→A transition mutation at a frequency of 11%. The deficiency in translesion synthesis-induced polymerases did not confer significant alteration in the mutation frequencies for the replication of the three thionucleosides (Fig. 4B).

We also employed LC-MS/MS for interrogating the restriction fragments (Fig. 2) (27, 28). In this respect the restriction digestion mixture was analyzed by LC-MS/MS, and we monitored the fragmentation of the $[\text{M}-3\text{H}]^{3-}$ ions of d(CATGGGCNCCG), where N is an A, T, C, or G. It turned out that only d(CATGGGCGCCG) and d(CATGGGCACCG) could be detected in the digestion mixtures for samples arising from the *in vivo* replication of ^5G -, ^5mG -, and ^5OHG -containing substrates, which is in line with what we found from native PAGE analysis (LC-MS/MS results for monitoring the formation of d(CATGGGCGCCG) and d(CATGGGCACCG) are shown in supplemental Fig. S5).

DISCUSSION

The cytotoxicity of the thiopurine drugs involved the formation of ^5G nucleotide upon metabolic activation and its subsequent incorporation into DNA (29). In DNA, ^5G can be methylated by S-adenosyl-L-methionine to form ^5mG (9) and

converted to $^{32}\text{S}^3\text{H}_3\text{G}$ upon UVA irradiation (18). By using shuttle vector technology, we showed that although $^3\text{S}^3\text{G}$, $^3\text{S}^6\text{mG}$, and $^{32}\text{S}^3\text{H}_3\text{G}$ in a single-stranded M13 genome do not block considerably DNA replication, the three thiopurine derivatives are mutagenic in wild-type AB1157 *E. coli* cells, and the mutation frequencies for $^3\text{S}^3\text{G}$, $^3\text{S}^6\text{mG}$ and $^{32}\text{S}^3\text{H}_3\text{G}$ are 11, 94, and 77%, respectively.

Previous steady-state kinetic measurements showed that the nucleotide insertion by the Klenow fragment of *E. coli* DNA polymerase I opposite $^3\text{S}^3\text{G}$ is not mutagenic; the polymerase inserts predominantly the correct nucleotide opposite the lesion, with the incorporation of dTMP at a frequency of 0.3% of that for the insertion of dCMP (17). This is in stark contrast with an $\sim 11\%$ G \rightarrow A mutation observed in *E. coli* cells. In addition, the exonuclease-deficient Klenow fragment was found to insert dTMP opposite $^3\text{S}^6\text{mG}$ seven times more efficiently than dCMP (13); consistently, the insertion of dTMP accounts for 94% of the nucleotide incorporation opposite the lesion in *E. coli* cells. Several factors may contribute to the observed differences for nucleotide incorporation opposite $^3\text{S}^3\text{G}$ with Klenow fragment and in *E. coli* cells. First of all, DNA replication in *E. coli* cells may require both pol I and pol III (30). Second, the steady-state kinetic measurements were carried out in the presence of one kind of nucleotide at a time, which is different from *in vivo* polymerase synthesis conditions where all four nucleotides are mutually present. Indeed our recent LC-MS/MS results showed that when all four natural nucleotides are present, 72, 8, and 15% of the products arising from Klenow fragment-mediated primer extension are full-length replication products carry, respectively, dCMP, dTMP, and dAMP opposite $^3\text{S}^3\text{G}$ (20). Third, *in vivo* DNA replication is often carried out in the presence of auxiliary protein factors, which can alter both the efficiency and accuracy of nucleotide insertion by DNA polymerases (31).

The observation that $^3\text{S}^3\text{G}$ can introduce high frequency of G \rightarrow A mutation supports that the presence of 6-thioguanine in DNA can introduce $^3\text{S}^3\text{G}:\text{T}$ base pair, which may possibly stimulate post-replicative mismatch repair pathway (32). Along this line, it was observed that the $^3\text{S}^3\text{G}:\text{T}$ base pair can be recognized by mammalian mismatch repair factors to a similar extent as a G:T mispair (15). Previously it was estimated that ~ 1.6 of 10^4 $^3\text{S}^3\text{G}$ in DNA are methylated by *S*-adenosyl-L-methionine to $^3\text{S}^6\text{mG}$ (9). These results together with the observations that the $^3\text{S}^6\text{mG}:\text{T}$ mispair can be recognized less efficiently than the $^3\text{S}^3\text{G}:\text{T}$ mispair by MMR factor (14, 15) suggest the possibility that $^3\text{S}^3\text{G}$ may exert its cytotoxic effect by triggering the post-replicative mismatch repair pathway without being converted to $^3\text{S}^6\text{mG}$. In this context it is worth emphasizing that the present replication studies are carried out by using *E. coli* as host; it is important to examine whether the findings made for *E. coli* can be extended to human cells. In addition, single-stranded genome was employed for the current replication studies, which does not allow for the assessment of the implications of mismatch repair in the cytotoxic effects of $^3\text{S}^3\text{G}$.

Replication studies using bypass polymerase-deficient *E. coli* cells revealed that both pol IV and pol V are partially involved in the bypass of $^3\text{S}^6\text{mG}$, and pol V is involved to some degree in the translesion synthesis of $^{32}\text{S}^3\text{H}_3\text{G}$. Overall, all the three thionucleosides are not strong blocks to DNA replication. Thus, the

biological consequences of these thionucleosides may arise mostly from their mutagenic properties.

Since the approval of the thiopurine drugs by Food and Drug Administration in the 1960s, azathioprine, 6-mercaptopurine, and 6-thioguanine have been widely used as therapeutic agents in the treatment of a variety of human diseases (1). However, there is a high occurrence of certain cancers in long-term survivors of these patients (33–35). For example, 20 years after transplant, about 60–90% of the patients who have taken azathioprine as an immunosuppressant develop squamous cell carcinoma (36). The oxidation of $^3\text{S}^3\text{G}$ to $^{32}\text{S}^3\text{H}_3\text{G}$ and the high mutagenic potential of the latter as revealed by the present *in vivo* mutagenesis study may account for the development of cancers in those patients.

Different from the traditional REAP assay, which requires the elution of the post-labeled restriction fragments from denaturing PAGE, digestion of the eluted ODN fragments with 5' \rightarrow 3' exonuclease, and analysis of the resulting ^{32}P -labeled 5' terminal nucleotide by thin-layer chromatography, here we showed that 30% native PAGE analysis can allow for the direct determination of the identities of the restriction fragments. Nevertheless, it is important to note that it is often necessary, as illustrated in the present study, to confirm the identities of the restriction fragments by an alternative method, *i.e.* mass spectrometry, particularly when the *in vivo* replication gives a complicated mixture of products.

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