LETTER

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6-Thioguanine and S^6-Methylthioguanine Are Mutagenic in Human Cells

Bifeng Yuan[†], Timothy R. O'Connor[‡], and Yinsheng Wang[†,*]

[†]Department of Chemistry, University of California, Riverside, California 92521 and [‡]Biology Division, Beckman Research Institute, City of Hope National Medical Center, Duarte, California 91010

ABSTRACT Thiopurines are effective immunosuppressants and anticancer agents. However, the long-term use of thiopurines was found to be associated with a significantly increased risk of various types of cancer. To date, the specific mechanism(s) underlying the carcinogenicity associated with thiopurine treatment remain(s) unclear. Herein, we constructed duplex pTGFP-Hha10 shuttle vectors carrying a 6-thioguanine (^{S}G) or S^6-methylthioguanine (S^6mG) at a unique site and allowed the vectors to propagate in three different human cell lines. Analysis of the replication products revealed that although neither thionucleoside blocked considerably DNA replication in any of the human cell lines, both ^{S}G and S^6mG were mutagenic, resulting in G→A mutation at frequencies of ~8% and ~39%, respectively. Consistent with what was found from our previous study in *E. coli* cells, our data demonstrated that the mutagenic properties of ^{S}G and S^6mG provided significant evidence for mutation induction as a potential carcinogenic mechanism associated with chronic thiopurine intervention.

Since the approval of the thiopurine drugs by FDA in the 1950s, azathioprine, 6-mercaptopurine, and 6-thioguanine (^{S}G) have been extensively used as anticancer and immunosuppressive agents for over half a century (*1*). Azathioprine is widely used as an immunosuppressive agent in organ transplantation and for treating autoimmune disease (*2, 3*). As a prodrug, azathioprine is converted efficiently (~90%) to 6-mercaptopurine from attack by sulfhydryl-containing compounds such as glutathione and cysteine after oral administration and absorption (*1*). 6-Mercaptopurine, a prescribed anticancer drug, is commonly used for acute lymphoblastic leukemia treatment (*4, 5*). ^{S}G is the ultimate active metabolite of all thiopurine prodrugs and can be converted to ^{S}G nucleotides and incorporated into nucleic acids (*1, 6*). Some studies have been carried out toward understanding the molecular mechanisms of action of the thiopurine drugs. Along this line, it was found that azathioprine and its metabolites could induce apoptosis in human T cells (*7*). The induction of apoptosis requires costimulation with CD28 and is mediated by specific blockade of Rac1 activation through binding of 6-thioguanosine triphosphate (^{S}GTP) in lieu of GTP (*7*). Although the molecular mechanisms for the anticancer activity of the thiopurine drugs remain unclear, it was observed that DNA ^{S}G could be spontaneously methylated by *S*-adenosyl-L-methionine (*S*-AdoMet) to afford S^6mG (Figure 1), which may trigger the mismatch repair pathway and induce cell death through futile cycles of repair synthesis (*8*). A major concern for thiopurine therapy is a high occurrence of certain cancers in long-term survivors of these patients following the drug treatment (*9–15*). Azathioprine has been shown to be genotoxic and classified as a human carcinogen because of its associated cancer risk (*16*). To date, the specific mechanism(s) underlying the *in vivo* carcinogenicity of thiopurines remain(s) unclear. Our recent study showed that both ^{S}G and S^6mG are mutagenic in *E. coli* cells and could lead to G→A mutation at frequencies of 10% and 94%, respectively (*17*). We also carried out the measurement of the level of 6-thio-2′-deoxyguanosine (^{S}dG) and S^6-methylthio-2′-deoxyguanosine (S^6mdG) in genomic DNA in four ^{S}G-treated human leukemia cell lines by employing a sensitive HPLC coupled with tandem mass spectrometry (LC-MS/MS) method (*18*). Our results revealed that, upon treatment with 3 μM ^{S}G for 24 h, approximately 10%, 7.4%, 7%, and 3% of guanine in genomic DNA was replaced with ^{S}G in Jurkat T, HL-60, CCRF-CEM, and K-562 cells, respectively (*18*). In addition, a small percentage of the DNA ^{S}G was observed to be methylated to S^6mG (*18*). Therefore, the mutation induced by the ^{S}G and S^6mG in DNA may contribute to the carcinogenic effects of thiopurines. However, it remains undefined whether the mutagenic properties of the two mercaptopurine derivatives found in *E. coli* cells can be observed in human

***Corresponding author, yinsheng.wang@ucr.edu.**

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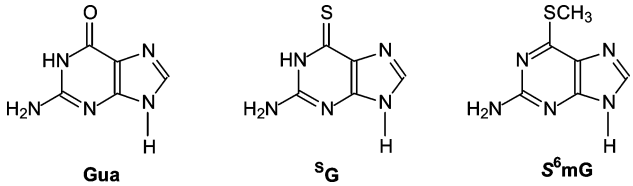

Figure 1. Structures of guanine (Gua), 6-thioguanine (^{S}G), and *S*^{6}-methylthioguanine (*S*^{6}mG).

cells because human cells are equipped with a more intricate DNA replication machinery than prokaryotic cells. In addition, it would certainly be more significant to investigate the cytotoxic and mutagenic properties of these thiopurine nucleosides in human cells because of the increased occurrence of cancers in humans receiving the thiopurine drugs.

In the present study, we investigated the mutagenic and cytotoxic properties of the two thiopurine derivatives in human cells by developing a novel shuttle vector-based method with the use of double-stranded plasmids containing a ^{S}G or *S*^{6}mG at a defined site. We first synthesized a 17-mer ^{S}G-containing oligodeoxyribonucleotide (ODN) 5′-GCGCAAA^{S}GCTAGAGCTC-3′. ^{S}G in ODNs can be selectively methylated to *S*^{6}mG by treatment with methyl iodide (CH_3I) in a phosphate buffer (pH 8.5) (*19*). We employed similar procedures and isolated the desired *S*^{6}mG-containing ODN from the reaction mixtures by HPLC. The identities of these thionucleoside-bearing ODNs were confirmed by electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) analyses (Supplementary Figures S1 and S2).

We next constructed double-stranded pTGFP-Hha10 vectors housing a ^{S}G or *S*^{6}mG (Figure 2, panel A). The pTGFP-Hha10 plasmid harbors two unique N.BstNB I recognition sites, and this restriction enzyme nicks only one strand of duplex DNA at the fourth nucleotide 3′ to the GAGTC restriction recognition site (Figure 2, panel B) (*20*). The resulting 33mer single-stranded ODN can be removed from the nicked plasmid by annealing the cleavage mixture with its complementary ODN in 50-fold molar excess. The gapped plasmid was isolated and ligated with a 5′-phosphorylated 17-mer lesion-carrying insert and a 16-mer unmodified ODN, and the resulting lesion-carrying double-stranded plasmid was isolated from the mixture by using agarose gel electrophoresis (Figure 2, panel C).

Constructing a lesion-bearing plasmid is among the most challenging steps when a double-stranded shuttle vector is employed for *in vivo* replication experiments. However, the use of the pTGFP-Hha10 vector allows for the facile preparation of the lesion-containing plasmid because the ligation only necessitates the insertion of a single-stranded damage-containing ODN into a gapped double-stranded vector. As depicted in Figure 2, panel C, the above-described strategy facilitated the efficient generation of lesion-containing double-stranded vector, and agarose gel electrophoresis afforded convenient purification of the resulting double-stranded vector from the ligation mixture (Figure 2, panel C); we could routinely produce the ^{S}G- and *S*^{6}mG-containing pTGFP-Hha10 vectors at an overall yield of ~30%.

We further confirmed the incorporation of the lesion-containing insert by employing a restriction digestion/postlabeling assay. In this experiment, the aforementioned double-stranded genomes were digested with EcoR I (the unique EcoR I site is shown in Figure 2, panel B). The nascent terminal phosphate groups were removed by using alkaline phosphatase, and the 5′-termini were subsequently rephosphorylated with [γ-^{32}P]ATP. The linearized vector was further cleaved with Nhe I (the unique Nhe I site is shown in Figure 2, panel B), which affords a 52-mer lesion-containing ODN if the ligation is successful (Figure 2, panel B). Indeed denaturing PAGE analysis revealed a distinct 52-mer ^{32}P-labeled fragment, and no shorter fragments could be detected (Figure 2, panel D), supporting the successful incorporation of the 17-mer lesion-containing insert and the 16-mer unmodified ODN into the gapped construct.

When the lesion-containing double-stranded shuttle vector is replicated in mammalian cells, the undamaged strand may be preferentially replicated over the lesion-carrying strand (*21, 22*), rendering it difficult to determine accurately the mutation frequencies. To overcome this difficulty, we employed a similar strategy as reported by Moriya *et al*. (*21, 22*) and incorporated an A:A mismatch three nucleotides away from the lesion site (Figure 2, panel B and Figure 3, panel A). This method facilitated the independent assessment of the products arising from the replication of the lesion-containing strand and its opposing unmodified strand (Figure 3, panel A).

We next asked how the presence of ^{S}G and *S*^{6}mG compromises DNA replication. The lesion-containing and the control lesion-free vectors were transfected separately into 293T human kidney epithelial cells. To assess whether nucleotide excision repair is involved with the repair of ^{S}G and *S*^{6}mG, we also evaluated the replication of the lesion-carrying and control lesion-free vectors in SV40-transformed XPA-deficient (GM04429) and repair-proficient (GM00637) human fibroblast cells. The pTGFP-Hha10 vector contains an SV40 origin, which allows the vector to be replicated in the SV40-transformed cells. The isolated progeny vectors from the host cells were digested with Dpn I to remove the residual unreplicated vectors prior to PCR amplification; thus, only the progeny vectors can be amplified by PCR. The bypass efficiencies and mutation frequencies of these modified nucleosides were assessed by using the modified REAP assay (Figure 3) (*17, 23*).

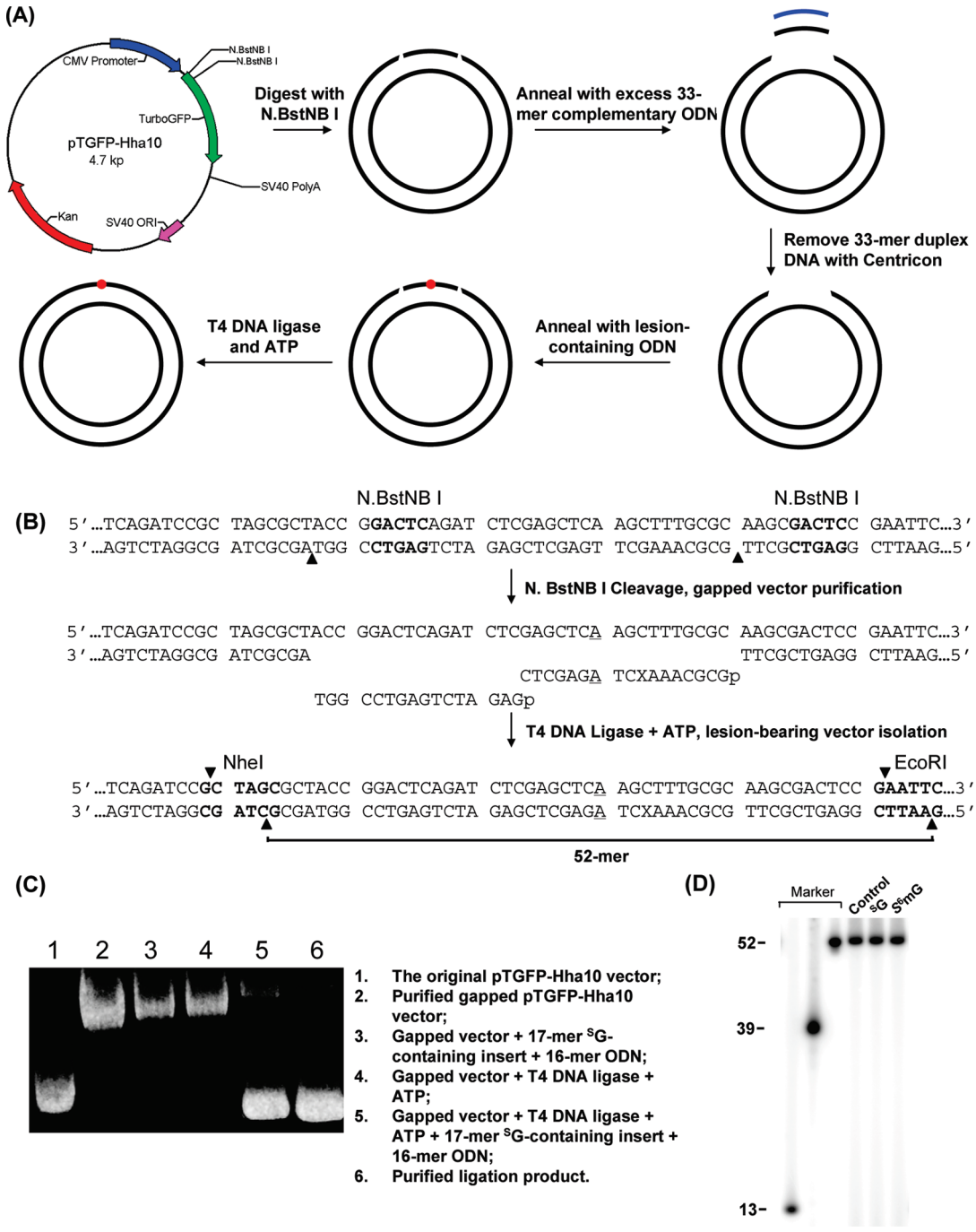

Figure 2. Method for the construction of lesion-containing duplex vector. A) Schematic diagram showing the procedures for the preparation of the lesion-containing plasmid. See Methods for details. B) Enzymatic digestion and ligation for the insertion of ^{S}G- or *S*^{6}mG-carrying ODN into gapped pTGFP-Hha10 vector. "X" represents guanine, ^{S}G, or *S*^{6}mG, and the A:A mismatch site is underlined. The restriction recognition sites are highlighted in bold, and cleavage sites are indicated by solid triangles. C) Agarose (1%) gel electrophoresis for monitoring the processes for the construction of the ^{S}G-bearing pTGFP-Hha10 vector. D) Restriction digestion and postlabeling for assessing the integrity of the control and lesion-containing vectors (see text).

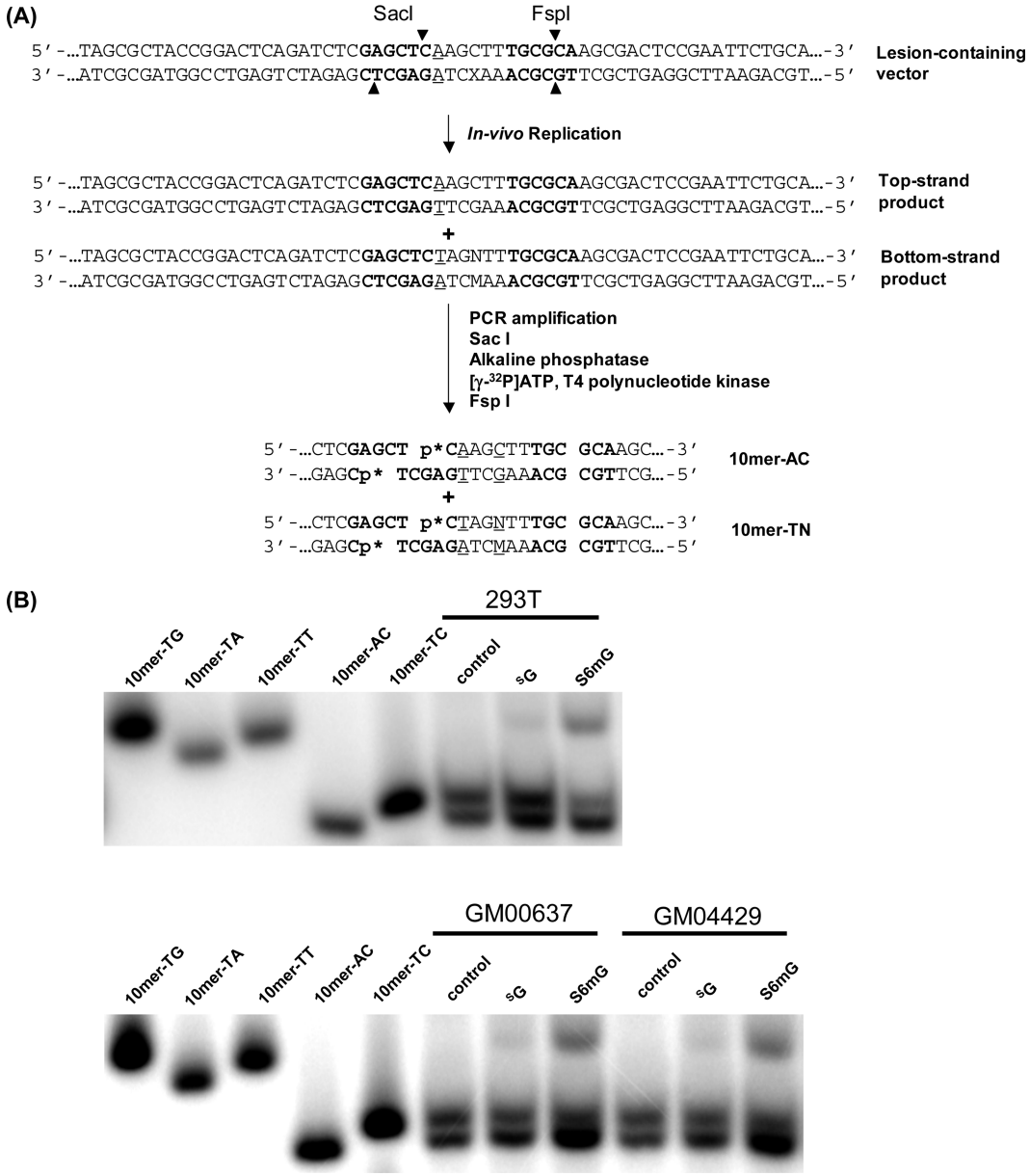

Figure 3. Determination of the bypass efficiencies and mutation frequencies of ^{S}G or *S*^{6}mG. A) Restriction digestion of PCR products of progeny genome arising from *in vivo* replication of ^{S}G- and *S*^{6}mG-bearing genomes in mammalian cells. The PCR fragments were digested with SacI, and the nascent terminal phosphate groups in the resulting DNA fragments were removed by shrimp alkaline phosphatase. The 5′ termini were subsequently labeled with [γ-^{32}P]ATP, and the resulting DNA fragments were further digested with FspI and resolved on a 30% denaturing PAGE gel. B) Denaturing PAGE for assessing the bypass efficiencies and mutation frequencies of ^{S}G and *S*^{6}mG in 293T, GM00637, and GM04429 cells.

In the absence of deletion mutation, restriction digestion of the PCR products of the progeny genome emanating from *in vivo* replication affords a 10-mer fragment harboring the site where the ^{S}G or *S*^{6}mG was initially incorporated (Figure 3). The failure to detect radio-labeled fragments with lengths shorter than 10-mer supports that neither thiopurine derivative gives rise to deletion

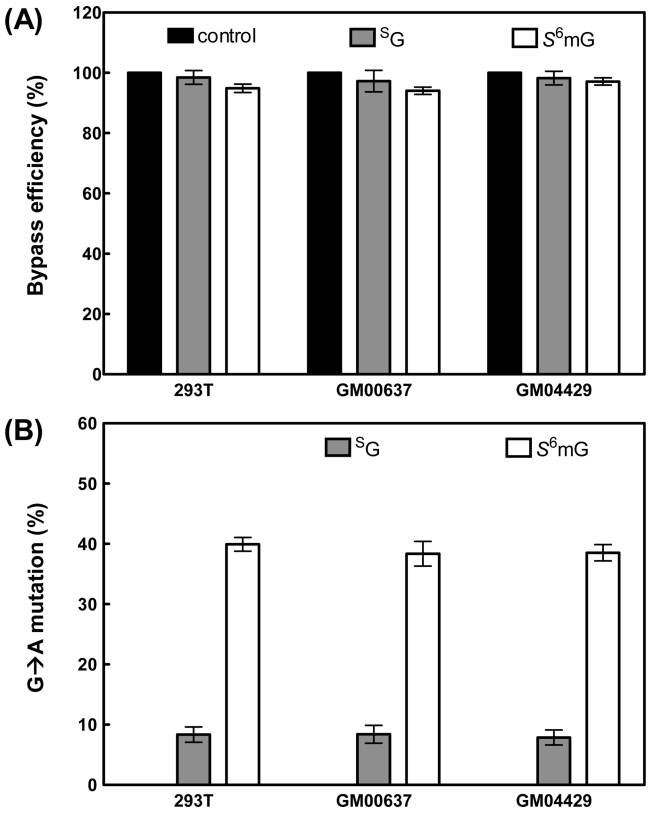

Figure 4. Bypass efficiencies (A) and mutation frequencies (B) of guanine, ^{S}G, and S^{6}mG in 293T, GM00637, and GM04429 cells. Black, gray, and white columns represent the results for substrates carrying guanine, ^{S}G, and S^{6}mG, respectively. The data represent the means and standard deviations of results from three independent transfection experiments.

mutations (Figure 3). In this context, we employed 30% (19:1, acrylamide:bisacrylamide) denaturing polyacrylamide gels containing 5 M urea to resolve the ^{32}P -labeled fragments and it turned out that the 10-mers with a single nucleotide difference can be resolved from each other (Figure 3, panel B).

The bypass efficiencies were calculated from the ratio of the combined intensities of bands for the 10-mer products from the replication of the lesion-bearing bottom strand over the intensity of the band for the 10-mer product from the replication of the lesion-free top strand. The bypass efficiencies for the lesion-carrying genomes were then normalized against that for the control lesion-free genome. Our results revealed that neither ^{S}G nor S^{6}mG is a strong block to DNA replication in human 293T cells, and the bypass efficiencies for ^{S}G and S^{6}mG are approximately 98% and 95%, respectively. In addition, deficiency in NER factor XPA does not affect appreciably the bypass efficiencies for these thionucleosides when we compared the bypass efficiencies of ^{S}G and S^{6}mG in GM00637 (repair-proficient) and GM04429 (XPA-deficient) cell lines (Figure 4, panel A). These data suggest that NER is not involved in the repair of ^{S}G or S^{6}mG in human cells. The results from denaturing PAGE analysis also allowed us to measure the mutation frequencies of ^{S}G and S^{6}mG in human cells with the restriction endonuclease and postlabeling (REAP) assay (*24, 25*). The mutation frequencies were calculated from the ratio of the intensity of the band for the 10-mer mutated product over the combined intensities of bands for the 10-mer products from the replication of the lesion-bearing bottom strand. The quantification data showed that S^{6}mG are highly mutagenic in human 293T cells and in human fibroblast cells that are XPA-deficient or repair-proficient, with G⟶A transition occurring at frequencies of 40%, 39%, and 38%, respectively. The presence of ^{S}G also results in G⟶A transition mutation at a frequency of ~8% in all three cell lines. Thus, deficiency in NER did not confer significant alteration in the mutation frequencies for the replication of the two thionucleosides (Figure 4, panel B).

We also employed LC-MS/MS for interrogating the restriction fragments (Figure 3) (*17, 23, 26, 27*). In this respect, the restriction digestion mixture was analyzed by LC-MS/MS, and we monitored the fragmentation of the $[M - 4H]^{4-}$ ions of d(GCAAAMCTAGAGCT), where "M" is an A, T, C, or G. It turned out that only d(GCAAAGCTAGAGCT) and d(GCAAAACTAGAGCT) could be detected in the digestion mixtures for samples arising from the *in vivo* replication of ^{S}G- and S^{6}mG-containing substrates, which is in line with what we found from PAGE analysis (LC-MS/MS results are shown in Supplementary Figure S3).

The observation that ^{S}G can induce a high frequency of G⟶A mutation supports that the presence of ^{S}G in DNA can introduce ^{S}G:T base pair, which may trigger the post-replicative mismatch repair (MMR) pathway. Along this line, it was observed that the ^{S}G:T base pair can be recognized by mammalian MMR factors to a similar extent as a G:T mispair (*28*). Recently we observed that, upon a 24-h treatment with 3 μM ^{S}G, ~3−10% of guanine was replaced with ^{S}G in 4 different leukemic cell lines; however, less than 0.02% of DNA ^{S}G was converted to S^{6}mG in the above cell lines (*18*). These results, in conjunction with the observation that the S^{6}mG:T mispair can be recognized less efficiently than the ^{S}G:T mispair by MMR factors (*28, 29*), suggest that ^{S}G can exert its cytotoxic effect by triggering the post-replicative MMR pathway without being converted to S^{6}mG. A direct ^{S}G-associated cytotoxicity could be a reason that thiopurine drugs have had success as antileukemic agents.

Long-term immunosuppression with azathioprine in organ transplant patients is as-

sociated with an increased risk of certain types of cancer (*9*–*15*), and azathioprine has been designated as a human carcinogen (*16*). Although other factors may also contribute to the carcinogenic effect, it is reasonable to believe that the mutagenic properties of ^{S}G and *S*^{6}mG in human cells, as observed in the current study, may play a significant role in the carcinogenicity of thiopurine drugs. Viewing the extremely low frequency of conversion of ^{S}G to *S*^{6}mG in genomic DNA (*18*), it is very likely that the mutagenic effect of the thiopurine drugs arises predominantly from the mutagenic bypass of ^{S}G in DNA. In this context, it is of note that in the current study we employed a shuttle vector to evaluate the mutagenic properties of ^{S}G and *S*^{6}mG, where the replication across ^{S}G and *S*^{6}mG occurs extrachromosomally; this may differ, to some extent, from the replication of these nucleobases when present in genomic DNA.

Lastly, it is important to note that the modified version of the REAP assay developed in the present study may be generally applicable to examine the cytotoxic and mutagenic properties of other DNA lesions in mammalian cells.

METHODS

Unmodified ODNs used in this study were purchased from Integrated DNA Technologies (Coralville, IA). [γ-^{32}P]ATP was obtained from Perkin-Elmer (Piscataway, NJ). The phosphoramidite building block of 6-thio-2′-deoxyguanosine was obtained from Glen Research (Sterling, VA). Shrimp alkaline phosphatase was from USB Corporation (Cleveland, OH), and all other enzymes were from New England Biolabs (Ipswich, MA). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was purchased from TCI America (Portland, OR). Chemicals unless otherwise noted were obtained from Sigma-Aldrich (St. Louis, MO). The 293T cells (HEK 293T/17) were purchased from ATCC (Manassas, VA). The XPA (xeroderma pigmentosum complementation group A)-deficient (GM04429) and repair-proficient human fibroblast cells (GM00637) were kindly provided by Prof. Gerd P. Pfeifer.

The detailed experimental procedures are described in the Supporting Information. Briefly, the lesion-containing genomes were constructed by inserting a 17-mer ^{S}G- or *S*^{6}mG-bearing ODN into a gapped pTGFP-Hha10 vector *via* enzymatic ligation. The lesion-carrying and the control lesion-free genomes were transfected separately into cultured human cells. After *in vivo* replication for 24 h, the progeny vectors were isolated, digested with DpnI to remove the residual unreplicated parent vector, and amplified by PCR, and the PCR products were digested with restriction enzymes. The restriction fragments were selectively labeled with [γ-^{32}P]ATP and resolved on polyacrylamide gels to determine the bypass efficiencies and mutation frequencies (Figure 3). The restriction fragments were also subjected to LC-MS/MS analysis to identify unambiguously the replication products.

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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