0026-895X/92/050802-06$03.00/0

MOLECULAR PHARMACOLOGY, 42:802-807

Consequences of 6-Thioguanine Incorporation into DNA on Polymerase, Ligase, and Endonuclease Reactions

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Received June 15, 1992; Accepted August 24, 1992

SUMMARY

The incorporation of 6-thioguanine (S^6G) in place of guanine proceeds readily in DNA synthesis reactions catalyzed by mammalian and bacterial polymerases. This report summarizes the consequences of such incorporation studied to date. S^6G was incorporated into one strand of a defined M13mp18 phage sequence in a (+) reaction catalyzed by the Klenow fragment of *Escherichia coli* DNA polymerase I. After denaturation of the newly synthesized strand (containing S^6G) and annealing with a reverse (−) ^{32}P-labeled primer, polymerization catalyzed by the Klenow enzyme as well as by human DNA polymerases α, γ, and δ was slowed considerably, compared with that across the corresponding guanine-containing template. To evaluate S^6G-containing DNA as a substrate for DNA ligases, two oligodeoxynucleotides (19- and 20-mers) antisense to a 40-mer were synthesized so that the 40-mer coded for guanine at the 3′ terminus of the 19-mer. After annealing of the synthetic oligonucleotides to form a duplex DNA containing a one-nucleotide gap (opposite cytosine in the 40-mer), the 19-mer was extended with 2′-deoxythioguanosine 5′-triphosphate using DNA polymerase, forming a nicked duplex DNA. The abilities of T_4 DNA ligase and HeLa and calf thymus DNA ligase I to join the 5′-phosphate with the 3′-S^6G-OH were severely inhibited, compared with the 3′-guanine-extended control. This finding suggests that incorporation of S^6G at the 3′ terminus of Okazaki fragments would inhibit lagging strand DNA synthesis. In other experiments, cleavage of S^6G-containing DNA by some but not all restriction endonucleases progressed poorly, compared with the control guanine-containing DNA, independently of the location of S^6G at recognition or cleavage sites, as previously observed by Iwaniec *et al.* [*Mol. Pharmacol.* **39**:299–306 (1991)] with a different spectrum of enzymes. These findings indicate altered DNA-protein interactions due to S^6G incorporation. The poor template function of S^6G-containing DNA is consistent with the known delayed cytotoxicity and DNA damage previously reported to occur in S^6G-treated cells.

S^6G has proven activity for the treatment of childhood and adult leukemias, particularly acute myelogenous leukemia, when used with arabinosyl cytosine and doxorubicin (1). A major mechanism proposed for the cytotoxic action of S^6G is its conversion via purine salvage pathways to S^6dGTP and subsequent incorporation into DNA (2). In previous reports, we demonstrated that S^6dGTP is a relatively good substitute for dGTP in DNA synthesis catalyzed by human (3) and *Escherichia coli* (4) DNA polymerases. Although several studies using different approaches have proposed that the incorporation is causally related to cytotoxicity (5), DNA damage (6–9), and chromosomal aberrations (10), the precise mechanism by which incorporation leads to cell death is presently unknown. Theoretically, S^6G residues in a Watson-Crick DNA double helix should generate a distortion in cytosine base pairs due to the bulkier sulfur atom, compared with the normal oxygen in guanine (11). The proposed deviation from normal G-C base pairs due to the presence of S^6G is minimal for a single residue; however, the presence of two or more residues in proximity could magnify the distortion (7). Because of these proposed and demonstrated features of S^6G-DNA, we have been examining the consequences of S^6G incorporation into DNA on the nucleic acid function (12, 13).

Here, we have enzymatically synthesized S^6G-DNA to evaluate it as a template for DNA synthesis and to determine its substrate activity toward type II restriction endonucleases. Additionally, we have evaluated the substrate activity toward DNA ligases using S^6G at the 3′ terminus of a synthesized oligonucleotide. The results demonstrate that S^6G substitution for guanine results in a poor template for DNA replication catalyzed by human and *E. coli* DNA polymerases, that substrate activity toward restriction endonuclease is variable, and that ligation reactions are dramatically decreased. Preliminary

This work was supported by National Institutes of Health Grants CA-28034 from the National Cancer Institute and GM-25530 from the National Institute of General Medical Sciences.

ABBREVIATIONS: S^6G, 6-thioguanine; S^6dGTP, 6-thiodeoxyguanosine triphosphate; G-DNA, guanine-containing DNA; S^6G-DNA, 6-thioguanine-containing DNA.

reports of this work have been presented in abstract form (14, 15).

Experimental Procedures

Materials. Unlabeled forms of ATP and deoxynucleoside triphosphates, ultrapure grade, were purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ); [γ-^{32}P]ATP (3000 Ci/mmol) from ICN Radiochemicals (Irvine, CA); universal primer (17-mer), M13mp18 bacteria phage single-strand DNA (+), *Bam*HI, *Sph*I, *Hind*III, and *Xba*I from New England Biolabs (Beverly, MA); ultrapure urea, T_4 polynucleotide kinase, T_4 ligase, and the Klenow fragment of *E. coli* DNA polymerase I (endonuclease free) from United States Biochemicals (Cleveland, OH); and *Sam*I, *Pst*I, and *Sal*I, from Boehringer Mannheim (Indianapolis, IN). Oligonucleotides for ligase analyses were synthesized by Genosys Biotechnologies, Inc. (Woodlands, TX). HeLa cell and calf thymus DNA ligases were purified by the method of Yang *et al.* (16).

Preparation of 5′-labeled primer. The universal primer (17-mer) or synthetic oligonucleotide (19-mer) was labeled at the 5′ end with ^{32}P in a 40-μl reaction mixture containing 50 mM Tris·HCl (pH 7.5), 7 mM $MgCl_2$, 10 mM dithiothreitol, 10 pmol of primer or oligomer, 20 μCi of [γ-^{32}P]ATP (3000 Ci/mmol), and 5 units of T_4 polynucleotide kinase. The mixture was incubated at 37° for 30 min, and the reaction was stopped by the addition of 50 mM Na_2EDTA (pH 8.0).

Primer-template annealing. Annealing of the ^{32}P-labeled primer to M13mp18 template or of the labeled 19-mer oligonucleotide to the 40-mer was performed in a buffer containing 50 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, 500 mM NaCl, 5 pmol of primer, and 10 pmol of template. The primer-template solution was incubated at 65° for 5 min and then allowed to cool slowly to room temperature.

Preparation of S⁶G-containing duplex DNA. The ^{32}P-labeled universal primer was annealed to the multiple cloning site region of M13mp18 DNA strand (+) as described above. Extension of the primer along the template was performed using reaction conditions described previously (4). Briefly, 100 μl of reaction mixture contained 20 μM dATP, dCTP, and dTTP, 20 μM dGTP or 50 μM S⁶dGTP as the fourth deoxynucleoside triphosphate, 8 mM $MgCl_2$, and 30 mM Tris·HCl (pH 7.4). The reaction was initiated by the addition of 1 unit of the Klenow fragment of *E. coli* DNA polymerase I and was incubated at 30° for 30 min. After addition of 20 mM Na_2EDTA, the reaction products were purified by cold ethanol precipitation. The size of the polymer was analyzed by electrophoresis on an 8% denaturing polyacrylamide gel.

Digestion by restriction enzymes. Restriction enzyme digests of the labeled M13mp18 duplex DNA were performed using the conditions recommended by the enzyme suppliers. Briefly, 0.1 pmol of duplex DNA sample was digested at 37° for 1 hr in a reaction volume of 100 μl, in a tightly capped polypropylene tube. The amounts of enzyme in the reaction mixture ranged from 0.5 to 10 units. After the reactions were terminated by heating at 100° for 5 min, the digested products were analyzed by electrophoresis using a 16% denaturing polyacrylamide gel.

Measurement of ligation reactions. ^{32}P-labeled 19-mer and 5′-phosphorylated 20-mer (10 pmol) oligonucleotides were annealed to the 40-mer (10 pmol) so that the guanine or S⁶G could be inserted into the site opposite a complementary cytosine in the 40-mer. Extension of the 19-mer to the resulting [5′-^{32}P]guanine- or S⁶G-containing 20-mer was performed using 1 unit of the Klenow fragment of *E. coli* DNA polymerase I in the presence of 10 μM dGTP or S⁶dGTP. Ligation of this product to the unlabeled 20-mer was performed as described by Maniatis *et al.* (17). The 50-μl reaction mixture consisted of 30 mM Tris·HCl (pH 7.5), 1 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, and 0.1 pmol of ^{32}P-labeled oligomer substrate. The ligation reaction was started by the addition of T_4 DNA ligase or mammalian cell DNA ligases. After incubation at room temperature for various times, aliquots of the reaction mixture were removed and denatured at 100° for 5 min. The ligation products were then subjected to electrophoresis on an 8 M urea-16% polyacrylamide gel.

Polyacrylamide gel electrophoresis and autoradiography. The 8 or 16% gels were prepared from a 40% (w/v) solution of acrylamide (38%) and bisacrylamide (2%) in TBE buffer (100 mM Tris, pH 8.3, 100 mM borate, 2 mM EDTA) and 8 M urea. Reaction samples (5–10 μl) were denatured at 100° for 5 min in the presence of a formamide loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 50 mM Tris-borate, 1 mM EDTA), chilled in an ice bath, and electrophoresed under standard conditions (18). The products from restriction enzyme and ligase reactions were visualized by autoradiography after overnight exposure to Kodak X-OMAT film at −80°.

Densitometer scanning. The digestion and ligation reactions were quantitated by scanning densitometry (E-C Apparatus Corporation, St. Petersburg, FL). Densitometer curves were analyzed by manually integrating the area under the bands above a base line drawn from points of minimum absorbance.

Results

Template function of S⁶G-DNA. Nonradioactive primer was annealed with single-stranded M13mp9 DNA as illustrated in Fig. 1. Primer extension was accomplished using dATP, dCTP, dTTP, and dGTP or S⁶dGTP as shown. After denaturation and reannealing with ^{32}P-labeled reverse primer, as described in Experimental Procedures, the ability of S⁶G-DNA to serve as a template was evaluated by monitoring of the rate

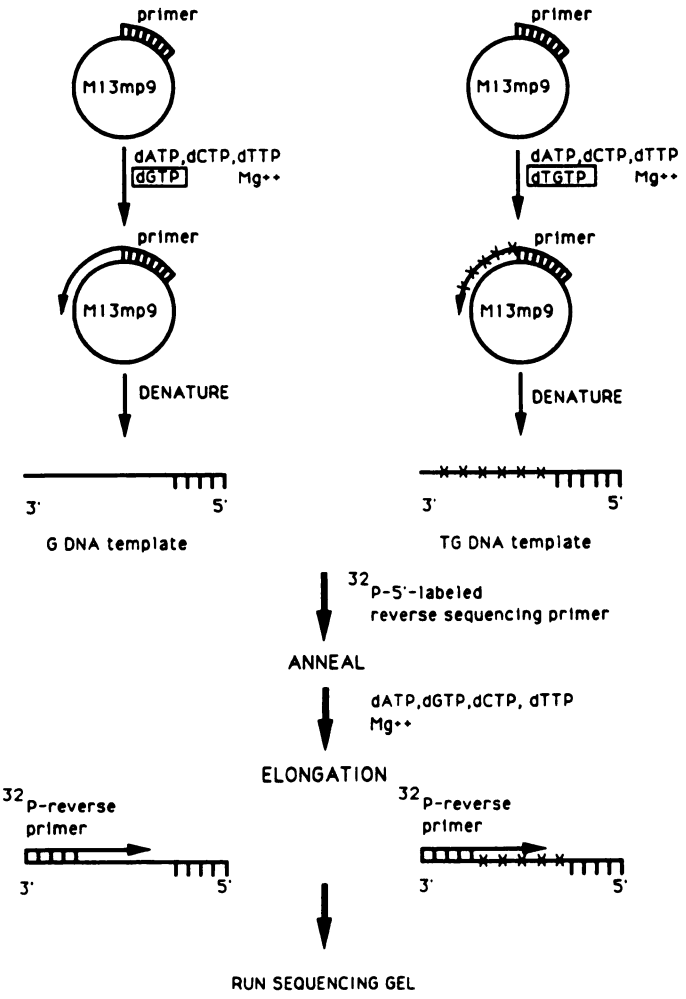


Fig. 1. Scheme for determining template activity of S⁶G-substituted DNA. *TG*, S⁶G.

of elongation of the radioactive primer using gel electrophoresis. The rate of polymerization across the S^6G-DNA template was slowed, compared with that across the control, G-DNA (Fig. 2). Specifically, whereas the primer elongation reached completion in the controls by 0.5 min, the earliest time sampled, several "stop sites" were observed in the elongation of the primer across the S^6G-DNA. Elongation was impaired at S^6G sites and at other sites, particularly across adenine residues. Whether the presence of S^6G impedes elongation at sites other than those coding for cytosine is not known. Because the elongation was impaired even at the first S^6G site at 0.5 min (Fig. 2, *lane 12*), the other sites might have represented normal pause sites for elongation of the primer in this system had the reaction with control G-DNA been measured at earlier times or with smaller amounts of polymerase. Results similar to those shown in Fig. 2 were obtained using purified human α, γ, and δ DNA polymerases (data not shown).

Substrate activity of S^6G-DNA for DNA ligases. We designed a set of synthetic oligomers, a 19-mer, a 20-mer, and a 40-mer, to determine the effect of S^6G substitution for guanine on DNA ligase activity. The sequences of these oligomers are presented in Fig. 3. As outlined, the 19-mer was labeled at its 5′ end with [γ-^{32}P]ATP and the 20-mer was phosphorylated at the 5′ end with nonradioactive ATP by T_4 polynucleotide kinase. Hybridization of the two oligomers with a complementary 40-mer, synthesized to leave a cytosine gap site opposite the missing base between the 19- and 20-mers, was then performed, allowing insertion of guanine or S^6G at the 3′ terminus of the 19-mer. Using the site-specific insertion reaction described by Boosalis *et al.* (19), guanine or S^6G was incorporated at the 3′ terminus of the 19-mer with the Klenow fragment of DNA polymerase I. DNA ligation activity was then determined by monitoring the generation of ^{32}P-labeled 40-mer catalyzed by DNA ligase in the presence of ATP, as illustrated in Fig. 3. The ligation products were analyzed by 16% denaturing polyacrylamide gels, and the relative rate of ligation was measured by densitometry of the bands corresponding to substrate (20-mer) and product (40-mer). As shown in Fig. 4, T_4 ligase (0.001 unit) rapidly joined the normal substrate (guanine-extended 20-mer) to yield the radiolabeled 40-mer. Within 0.5 min of incubation at 30° the relative rate of ligation was approximately 24%, and after 5 min it reached the plateau level of about 48%. Under the same reaction conditions, S^6G-containing 20-mer as substrate resulted in a dramatic reduction of ligation rate. For example, within 0.5–1.0 min of incubation only small amounts of ligation product were detected and the relative rate was only about 1.5%. Nonetheless, the amount of ligation product increased linearly during continuous incubation, reaching about 16% after 10 min of incubation. We also determined whether S^6G altered the efficiency of ligation catalyzed by mammalian cell DNA ligases. Compared with the ligation rate observed with the normal guanine-extended 20-mer substrate, the rate of ligation with S^6G-containing substrate was approximately 70% lower using purified DNA ligase I from HeLa cells (Fig. 5A) or from calf thymus (Fig. 5B).

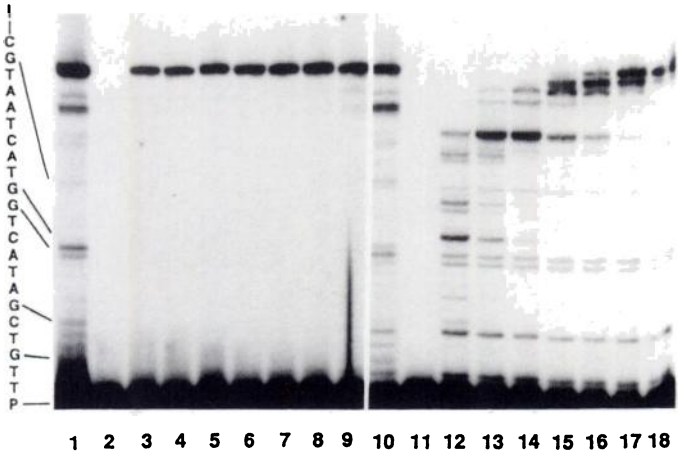


Fig. 2. Poor template activity in a DNA containing S^6G in place of guanine. A DNA template for DNA polymerase was prepared as described in Fig. 1, by elongation of a nonradiolabeled primer. After denaturation and reannealing with radiolabeled reverse primer, elongation by *E. coli* Klenow fragment DNA polymerase was monitored by gel electrophoresis. *Lanes 1* and *10*, guanine sequence determined by dideoxyguanosine incorporation; *lanes 2–9*, incubation for 0, 0.5, 1, 2.5, 5, 10, 20, and 30 min, respectively, with a G-DNA template; *lanes 11–18*, incubation for the same times with a S^6G-DNA template. *P*, primer, as shown in Fig. 1.

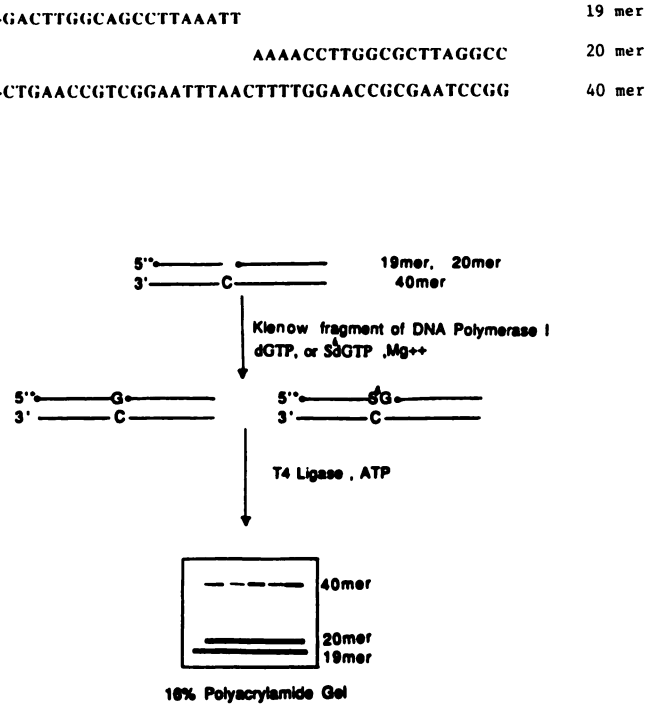
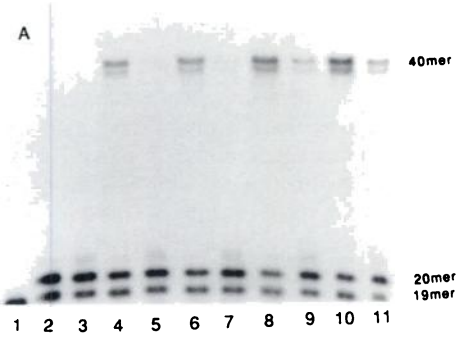


Fig. 3. Scheme for the preparation of guanine or S^6G substrates for assay of DNA ligase. 5′-^{32}P-labeled 19-mer and 5′-phosphorylated 20-mer were annealed with the 40-mer as shown. The guanine or S^6G was inserted opposite the cytosine site with DNA polymerase I to form a ^{32}P-labeled 20-mer with 3′-terminal guanine or S^6G. The ligation reaction was carried out as described in Experimental Procedures, and ligation products were analyzed on a 16% denaturing polyacrylamide gel.

Substrate activity toward type II restriction endonucleases. Table 1 and Fig. 6 summarize the results obtained in reactions of different restriction endonucleases with control (G-DNA) and S^6G-DNA. S^6G substitution for guanine completely blocked cleavage activities by some enzymes, such as *Bam*HI and *Sph*I. On the other hand, *Sal*I and *Sma*I recognized S^6G-DNA, i.e., the cleavage activity was only slightly reduced. *Pst*I, whose recognition and digestion site is CTGCA↓G, almost completely retained its ability to cut the A↓S^6G. *Hind*III and *Xba*I exhibited similar patterns of digestion with control and S^6G-containing DNA, suggesting that S^6G-modified nucleotides located outside the cleavage sites did not markedly affect the enzyme activity.



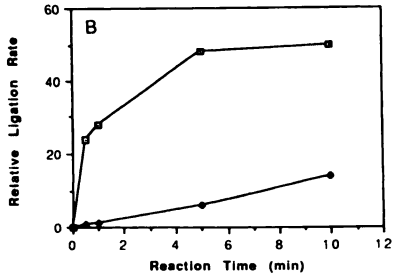
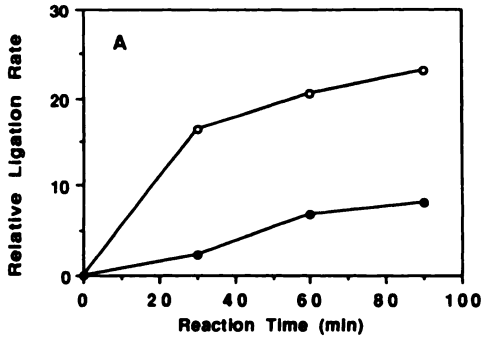


Fig. 4. Time course of T_4 ligase reaction with control or S⁶G-terminated substrate. The ligation reaction was carried out as described in Experimental Procedures and as illustrated in Fig. 3. A, Autoradiogram of joining products catalyzed by T_4 ligase (0.001 unit) at 30° for various times. *Lane 1*, 19-mer; *lanes 2* and *3*, guanine- and S⁶G-terminated 20-mer, respectively; *lanes 4*, *6*, *8*, and *10*, ligation reaction with guanine-terminated 20-mer for 0.5, 1, 5, and 10 min, respectively; *lanes 5*, *7*, *9*, and *11*, ligation reaction with S⁶G-terminated 20-mer for 0.5, 1, 5, and 10 min, respectively. B, Relative rate of ligation calculated as described in Experimental Procedures. The intensities of the bands for substrate and products were determined by scanning densitometry. Each *point* represents the average of two or three separate experiments.

Discussion

Incorporation into DNA is a significant mechanism for the cytotoxicity of S⁶G toward several tumor cell lines (2, 5, 20). The available data suggest that S⁶G, as its deoxyribonucleoside 5′ triphosphate, is readily utilized in place of dGTP (3, 4, 21) for the synthesis of a new DNA strand that is functional for a first round of cell division, i.e., cells package the DNA and divide in a normal fashion (22). However, chromosomal aberrations (10), DNA damage assessed by alkaline elution analysis (7–9, 22), progression of cells through G_2 (23, 24), and frank cytotoxicity (20) become manifest in daughter cells. Thus, the presence of S⁶G in daughter cells is responsible for the most important events implicated thus far in the drug-induced cytotoxicity. With regard to DNA replication, Pan and Nelson (22) reported that elongation of DNA was not impaired when S⁶G was being incorporated into DNA; however, elongation was slowed in a second round of cell division. This result suggested that S⁶G-DNA is a poor template for DNA replication. Evidence that S⁶G-DNA is a poor template for DNA replication is given by the direct *in vitro* assessment of template function reported herein (Fig. 2). This effect of S⁶G on DNA function appears, therefore, to be a plausible explanation for its cytotoxic mechanism. As described below, the presence of S⁶G in DNA has additional influences on proteins that interact with and modify DNA that might also relate to the mechanism for the antitumor action of this antimetabolite.



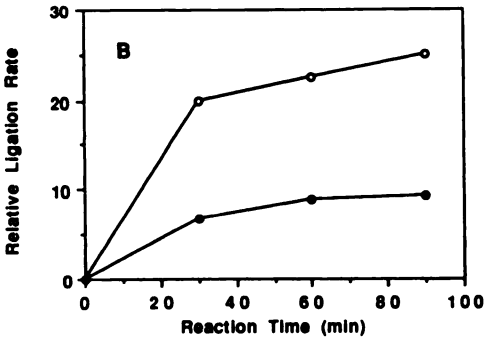


Fig. 5. S⁶G-terminated oligomer as a substrate for mammalian cell DNA ligases. Guanine- or S⁶G-containing oligomer substrate, as illustrated in Fig. 3, was incubated in the presence of HeLa cell ligase (A) or calf thymus ligase (B). After incubation at 37° for the indicated times, aliquots were analyzed by polyacrylamide gel electrophoresis. The band densities representing the substrate (20-mer) or product (40-mer) were determined by scanning densitometry.

TABLE 1
Restriction enzyme activities toward a S⁶G-substituted DNA

Enzyme	Recognition sequence	S⁶G-DNA cleavage*
*Bam*HI	5′...G↓GATCC...3′ 3′...CCTAG↑G...5′	−
*Hin*dIII	5′...A↓AGCTT...3′ 3′...TTCGA↑A...5′	+
*Pst*I	5′...CTGCA↓G...3′ 3′...G↑ACGTC...5′	+
*Sal*I	5′...G↓TCGAC...3′ 3′...CAGCT↑G...5′	±
*Sma*I	5′...CCC↓GGG...3′ 3′...GGG↑CCC...5′	±
*Sph*I	5′...G↓CATGC...3′ 3′...CGTAC↑G...5′	−
*Xba*I	5′...T↓CTAGA...3′ 3′...AGATC↑T...5′	+

* Evaluation of S⁶G-DNA cleavage relative to G-DNA control: −, almost no enzyme activity; ±, reduced substrate activity; +, good substrate activity.

Protein-DNA interactions play important roles in several cell functions, including DNA replication, recombination, repair, and transcription (25, 26). Some proteins, such as DNA polymerases, DNA helicase, and DNA ligase, have little or no sequence specificity, whereas others, such as transcriptional activators, repressors, and restriction endonucleases, have extremely high sequence specificity for their target sites (27–29). To determine the effect of S⁶G substitution for guanine in

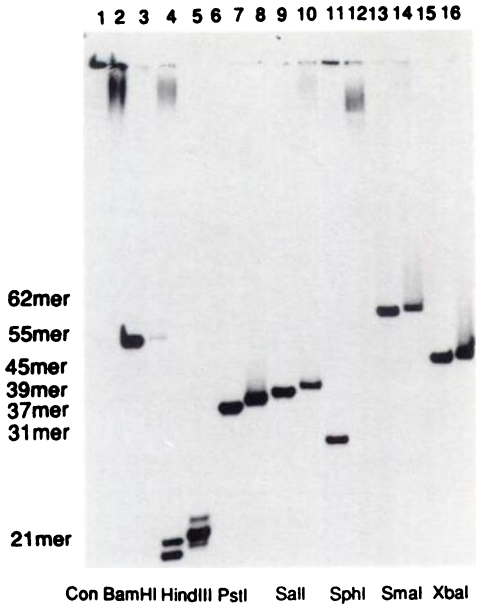


Fig. 6. Autoradiogram of cleavage products of control or S^6G-substituted M13mp18 duplex DNA with different restriction endonucleases. The reaction was carried out as described in Experimental Procedures, the products of the digestion reaction were analyzed on a 16% denaturing polyacrylamide gel, and the product bands were visualized by autoradiography. *odd-numbered lanes*, digestion products of control DNA; *even-numbered lanes*, digestion products of the S^6G-DNA.

duplex DNA on cleavage reactions catalyzed by restriction enzymes, we employed the multiple cloning site region of M13mp18 as a template to prepare a S^6G-containing duplex DNA. This region contains 10 unique hexanucleotide sites for 13 different restriction enzymes, and these enzymes cleave M13mp18 duplex DNA at only one site in this region (30). M13mp18 single-strand DNA (+) was annealed to 5′-^{32}P-labeled universal primer (17-mer), and the primer was extended along the template by the Klenow fragment of DNA polymerase I using S^6dGTP or dGTP to prepare S^6G-DNA or G-DNA. After a 30-min reaction, the primer extension was longer than 200 bases for both dGTP and S^6dGTP as the fourth nucleotide, consistent with our previous reports (3, 4). Because the sites for the restriction enzymes chosen in these experiments are located within 70 bases or less of the primer sequences, this duplex DNA could be used as a substrate to study the effects of S^6G substitution on restriction enzymes. Our results indicated that S^6G substitution for guanine resulted in a complete blockage or a dramatic reduction of digestion ability for some enzymes in which the DNA sequences of recognition and cleavage contained one or more S^6G bases. However, other enzymes, such as *Hind*III and *Xba*I, for which the DNA sequences of recognition and cutting did not contain or were beyond the S^6G bases, retained the ability to digest the S^6G-containing DNA. Thus, effects of the S^6G substitution on the restriction enzymes depend on the DNA sequences of recognition or cleavage. It is interesting that *Sma*I and *Pst*I retained their ability to recognize and digest the S^6G residues in the DNA (Fig. 4). Jerieny and Martin (31) reported that some restriction enzymes with relaxed recognition specificities could also cleave the DNA when a modified nucleotide was present within the recognition sequence. Apparently, these two enzymes belong to this group, i.e., they can recognize and cleave S^6G-DNA. These data are also consistent with the reports by Iwaniec *et al.* (32), who also found less efficient primer extension and inhibition of restriction endonuclease activity, albeit with a different spectrum of enzymes.

To determine the influence on restriction endonucleases of guanine modification at another site in the purine ring, we used 7-deazaguanine in place of guanine to make a 7-deazaguanine-containing M13mp18 (−) strand. Using this substituted duplex DNA as a substrate, the patterns of inhibition of enzyme activities were very similar to those observed in S^6G-containing DNA (data not shown). In addition, we prepared a corresponding arabinosylguanine-containing duplex DNA to test whether the alteration of the sugar moiety might change recognition and cleavage by restriction enzymes. The results indicated that arabinosylguanine-containing DNA was also a poor substrate for some restriction enzymes (data not shown). These data suggest that alteration either in base pairing by modified purines or in the sugar-phosphate backbone could lead to changes in enzyme activity. Several investigators have reported that the replacement of the natural nucleotides by analogs can distort hydrogen-bond formation between enzyme and recognition sequences, particularly when the analog bases are located on the major groove (33). Whether S^6G or other substitutions interrupt the enzyme binding, interfere with the recognition sequences, or arrest the scissor ability for these enzymes remains to be determined.

DNA ligases, enzymes necessary for the processes of DNA replication and repair, can join the single-strand breaks in double-stranded DNA by catalyzing the formation of phosphodiester bonds (34). Although details of the mechanism for phosphodiester bond formation catalyzed by ligase remain unknown, a number of reports suggest that a nucleophilic attack on an activated 5′-phosphorylated group by the 3′-hydroxyl terminus of the DNA fragment occurs in the reaction (35). In this work, we demonstrated that S^6G present at the 3′ terminus caused a dramatic decrease of the ligation efficiencies of the prokaryotic T_4 DNA ligase and eukaryotic DNA ligase I from HeLa cells and calf thymus (Figs. 4 and 5). Recently, the direct inhibition of DNA ligase I activity by another antileukemic agent (2-fluoroadenine arabinoside), as its triphosphate, has been reported (36). Further, the activity of the DNA ligase I was reduced, compared with the deoxyadenosine control, when the analog nucleotide was used to extend the 3′ terminus of the substrate, similar to results with S^6G as reported herein (Figs. 4 and 5).

In summary, this study demonstrates that guanine replacement by S^6G in DNA strongly alters the interactions of restriction enzymes or DNA ligases. Additionally, the poor template activity of S^6G-DNA for DNA replication by DNA polymerases is consistent with the known delayed effects of S^6G on DNA structure and cell viability.

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