0026-895X/91/040508-07$03.00/0

MOLECULAR PHARMACOLOGY, 40:508–514

2′-Deoxy-6-Thioguanosine 5′-Triphosphate as a Substrate for Purified Human DNA Polymerases and Calf Thymus Terminal Deoxynucleotidyltransferase *In Vitro*

YI-HE LING, J. ARLY NELSON, Y.-C. CHENG, ROSS S. ANDERSON, and KENNETH L. BEATTIE

Department of Experimental Pediatrics, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 (Y.-H.L., J.A.N.), Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510-8066 (Y.-C.C.), and Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030 (R.S.A., K.L.B.)

Received April 15, 1991; Accepted July 22, 1991

SUMMARY

2′-Deoxy-6-thioguanosine 5′-triphosphate (S^6dGTP), a metabolite of the antileukemia agent 6-thioguanine, was evaluated as a substrate for purified human DNA polymerases. Using bacteriophage M13 single-strand DNA as a template, S^6dGTP substituted efficiently for dGTP and stimulated DNA synthesis in reactions without dGTP, with DNA polymerases α, δ, and γ from the human leukemia cell line K562. The apparent K_m values for dGTP and S^6dGTP were very similar, i.e., 1.2 μM each for polymerase α, 2.8 and 3.6 μM, respectively, for polymerase δ, and 0.8 μM each for polymerase γ; however, the relative V_{max} values for the modified nucleotide were 25–50% lower than those of the corresponding natural substrate. Using a highly sensitive electrophoretic assay of chain elongation across M13mp9 (+)-strand DNA by the aforementioned human DNA polymerases, S^6dGTP was shown to be incorporated at the 3′ end of the nascent growing DNA chain, and the patterns of chain extension with S^6dGTP as substrate were identical to those obtained in the presence of dGTP. There were no major differences using S^6dGTP in place of dGTP with these DNA polymerases; however, at higher concentrations (1–10 μM) the analog stimulated primer elongation in reactions without dATP, indicating some misincorporation at sites of $S^6G \cdot T$ base pairs during DNA synthesis. Using $p(dA)_{12-18}$ as the initiator for calf thymus terminal deoxynucleotidyltransferase, S^6dGTP inhibited the incorporation of all four natural deoxyribonucleoside 5′-triphosphates into the primer, in a competitive manner. The apparent K_i values for the analog were 6–20 times lower than the K_m values for the four endogenous substrates. As a substrate, S^6dGTP was added to the 3′-hydroxyl termini of primer, although tailing efficiency with the analog was lower than that in the presence of the natural substrate. These findings indicate that S^6dGTP is a relatively good substrate for several mammalian DNA polymerases, including terminal deoxynucleotidyltransferase.

The guanine analog S^6G is an active antileukemia agent in humans (1–3). Although the exact mechanism for the antitumor activity of this compound is not completely understood, many findings are consistant with the hypothesis that the incorporation of S^6G into DNA is the major cause of its cytotoxicity (4–6). By alkaline elution analysis, Christie *et al.* (6) indicated that S^6G incorporation into the DNA of Chinese hamster ovary cells was associated with cell killing and single-strand DNA damage. Other investigators showed that double-strand DNA breaks, DNA-protein cross-links, and interstrand cross-links occur in TG-treated L1210 cells (7–9). Recently, Pan and Nelson (10) found that TG produced single-strand breaks in S^6G-containing and daughter DNA strands, presumably following its incorporation during DNA replication.

Yoshida *et al.* (11) reported that S^6dGTP was a substrate for DNA polymerase α from calf thymus. In previous work, we have applied a high resolution DNA-sequencing gel technique to demonstrate substrate activity of the analog with the Klenow fragment of *Escherichia coli* DNA polymerase I.[1] Herein, we have used this technique to determine whether mammalian cell DNA polymerases can utilize S^6dGTP as a substrate during DNA replication. We used two kinds of purified DNA replication enzymes, polymerase α and polymerase δ, one of mitochondrial DNA synthesis, polymerase γ, and TdT. The results indicate that these enzymes readily utilize this modified substrate in place of dGTP. A preliminary report of this work has appeared (12).

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

[1] Y. H. Ling, J. A. Nelson, D. Farquhar, and K. L. Beattie. Utilization of 2′-deoxy-6-thioguanosine 5′-triphosphate in DNA synthesis catalyzed by DNA polymerase: I. Klenow fragment of *Escherichia coli*. Submitted for publication.

ABBREVIATIONS: S^6G, 6-thioguanine; S^6dGTP, 2′-deoxy-6-thioguanosine triphosphate; dNTP, 2′-deoxynucleoside 5′-triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TdT, terminal deoxynucleotidyltransferase; BSA, bovine serum albumin.

Materials and Methods

Chemicals. High performance liquid chromatography-purified dNTPs, the corresponding 2′,3′-dideoxynucleotides, and $p(dA)_{12-18}$ were purchased from LKB Pharmacia Biotechnology, Inc. (Piscataway, NJ). S^6dGTP was synthesized and purified as previously described.[1] Seventeen-mer synthetic oligonucleotide primer was obtained from New England Biolabs (Beverly, MA). M13mp9 and M13mp18 single-stranded circular DNA were prepared as described by Messing (13). [γ-^{32}P]ATP (specific activity, >7000 Ci/mmol) and [α-^{32}P]dNTPs (specific activity, >600 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, CA). T4 polynucleotide kinase (10 units/μl) and Klenow fragment of *E. coli* DNA polymerase I (5 units/μl) were from New England Biolabs. TdT from calf thymus was obtained from United States Biochemicals (Cleveland, OH). DNA polymerases α, δ, and γ were prepared and purified from the human chronic myelogenous leukemia cell line K562, according to the method described by Parker *et al.* (14). Ultrapure acrylamide and urea were from United States Biochemicals. DE-81 ion exchange discs (24-mm diameter) were obtained from Whatman (Maidstone, UK). Other chemicals were purchased from sigma Chemical Co. (St. Louis, MO).

Assay of DNA polymerase activity. Activities of DNA polymerases were measured with M13mp9 or M13mp18 single-stranded DNA as templates. The reaction mixture contained 30 mM Tris·HCl (pH 7.5), 8 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 pmol of primer-M13mp9 DNA, 100 μg/ml BSA, 20 μM each of dATP and dTTP, 2 μM [α-^{32}P] dCTP (1000 cpm/pmol), various concentrations of dGTP or S^6dGTP, and 0.25 units of DNA polymerase, in a total reaction volume of 50 μl. After incubation at 37° for the indicated times, aliquots of the reaction mixture were spotted onto DE-81 discs, washed three times with 5% Na_2HPO_4 and twice with 95% ethanol, and dried. Radioactivity associated with the discs was then determined using a Beckman LS8100 liquid scintillation counter.

Primer 5′-end labeling. Universal 17-mer primer (3 pmol) was 5′-^{32}P-labeled in a 30-μl reaction buffer containing 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 50 μg/ml BSA, 0.25 mCi of [γ-^{32}P]ATP (>7000 Ci/mmol), and 10 units of T4 polynucleotide kinase. After incubation at 37° for 30 min, the reaction was terminated by addition of 5 μl of 0.25 M EDTA (pH 8.0) and heating at 85° for 5 min. The labeled primer was precipitated with 0.3 M sodium acetate and cold ethanol.

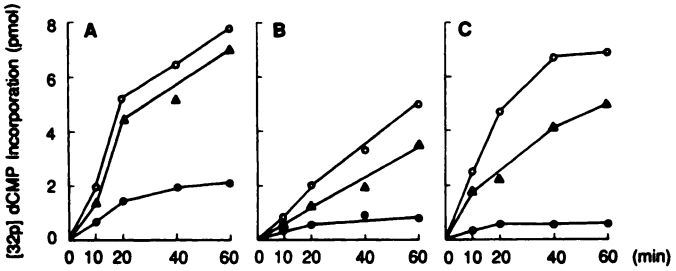

Fig. 1. A, DNA synthesis using M13mp9 single-strand DNA as template and human DNA polymerase α. The reaction mixture contained 20 μM dATP, 20 μM dTTP, and 2 μM [α-^{32}P]dCTP, in the absence (●) or presence of 20 μM dGTP (○) or with 20 μM S^6dGTP (▲). B, using human DNA polymerase δ. C, using human DNA polymerase γ. The average values for two separate experiments performed in duplicate are given.

TABLE 1

Kinetic analysis of S^6dGTP as a substrate for human DNA polymerases

Incubations were performed as described for the DE-81 assay in Materials and Methods. $K_{m_{app}}$ and V_{max} values were estimated by double-reciprocal plots of initial velocity data. The results shown are the average values for two separate experiments, each performed in duplicate.

Polymerase	Substrate	$K_{m_{app}}$	Relative V_{max}
		μM	
α	dGTP	1.2	1
α	S^6dGTP	1.2	0.75
δ	dGTP	2.8	1
δ	S^6dGTP	3.6	0.53
γ	dGTP	0.8	1
γ	S^6dGTP	0.8	0.68

Primer-template annealing. The procedure for annealing the primer-template was as previously described.[1] Briefly, 5′-end-labeled primer (0.5 ng, 0.1 pmol) was annealed to M13mp9 or M13mp18 (+)-single-strand DNA (0.5 μg, 0.2 pmol) in 20 μl of annealing buffer containing 0.5 M NaCl, 40 mM Tris·HCl (pH 7.5), and 1 mM EDTA (pH 8.0). After heating of the mixture at 65° for 5 min and slow cooling to room temperature, the primer-template complex was passed over a Sepharose 2B column (0.7 cm × 20 cm) and eluted with 10 mM Tris, 1 mM EDTA buffer. The first peak of radioactivity, containing the ^{32}P-labeled primer-template complex, was collected, precipitated with cold ethanol, and finally dissolved in Tris-EDTA buffer.

Elongation of ^{32}P-labeled primers by DNA polymerases. The mixture for measurement of primer elongation contained, in a total volume of 10 μl, 30 mM Tris·HCl (pH 7.5), 8 mM $MgCl_2$, 20 μM each of three dNTPs, 2 nM ^{32}P-primer/M13mp9 template, 100 μg/ml BSA, 0.15 units of human DNA polymerase α, δ, or γ, and the indicated concentrations of omitted corresponding natural substrates or S^6dGTP. The reaction was performed at 37° for 1 hr and terminated by addition of 5 μl of stopping solution containing 0.3% xylene cyanol FF, 0.3% bromphenol blue, and 0.37% Na_2EDTA. The terminated solutions were denatured at 100° for 5 min and quickly cooled in an ice bath, and aliquots (2–5 μl) were taken and subjected to electrophoresis in 7 M urea-8% polyacrylamide gels (23 cm × 36.5 cm × 0.4 mm). For each reaction minus dNTP, a corresponding dideoxy sequencing reaction was carried out, as described by Sanger *et al.* (15). Briefly, a reaction mixture volume of 8 μl contained 0.1 pmol of ^{32}P-primed M13mp9 or M13mp18 single-stranded DNA template, 100 μM dideoxynucleoside triphosphate, 1.25 μM corresponding dNTP, 100 μM each of the other three dNTPs, 40 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, and 1 unit of Klenow fragment of *E. coli* DNA polymerase I. After a 30-min incubation at 30°, the reaction was terminated by the addition of 4 μl of 95% (v/v) deionized formamide, 10 mM Na_2EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol FF.

Measurement of TdT activity. Calf thymus TdT activity was assayed with $p(dA)_{12-18}$ as the initiator and one of four [α-^{32}P]dNTPs (1000 cpm/pmol) as the nucleotide substrate. The reaction mixture contained, in a total volume of 10 μl, 50 mM HEPES-KOH (pH 7.5), 1 mM β-mercaptoethanol, 0.6 mM $ZnSO_4$, 0.25 mM $MnCl_2$, 10 μM $p(dA)_{12-18}$, 100 μM [^{32}P]dNTP, various concentrations of S^6dGTP, and 5 units of TdT. After a 30-min incubation at 37°, the reaction mixture was applied to DE-81 discs, washed twice with 5% Na_2HPO_4 and twice with 95% ethanol, and dried, and the radioactivity was determined by liquid scintillation counting.

Gel electrophoretic assay of primer tailing reaction by TdT. The preparation of 5′-end-labeled ^{32}P-primer was performed as described above. The reaction mixture contained, in a total volume of 10 μl, 50 mM HEPES (pH 7.5), 0.25 mM $MnCl_2$, 1 mM β-mercaptoethanol, 0.6 mM $ZnSO_4$, 0.1 pmol of 5′-^{32}P-17-mer primer, and 10 μM dGTP or S^6dGTP. The tailing reaction was initiated by addition of 5 units of calf thymus TdT. After incubation at 37° for the indicated times, the reaction was stopped by the addition of an equal volume of deionized formamide dye. The samples were denatured at 100° for 5 min and subjected to electrophoresis in 7 M urea-16% polyacrylamide denaturing gels.

Gel electrophoresis. The products from the chain elongation by DNA polymerases or from the tailing reaction by TdT were dissolved in formamide dye solution, denatured at 100° for 5 min, and quickly cooled in an ice bath. After loading of the samples (5 μl/well), the gels were electrophoresed at 1200–1600 V until the xylene cyanol marker had migrated 18 cm. The gels were then exposed to Kodak X-Omat XAR-5 film at −70° overnight.

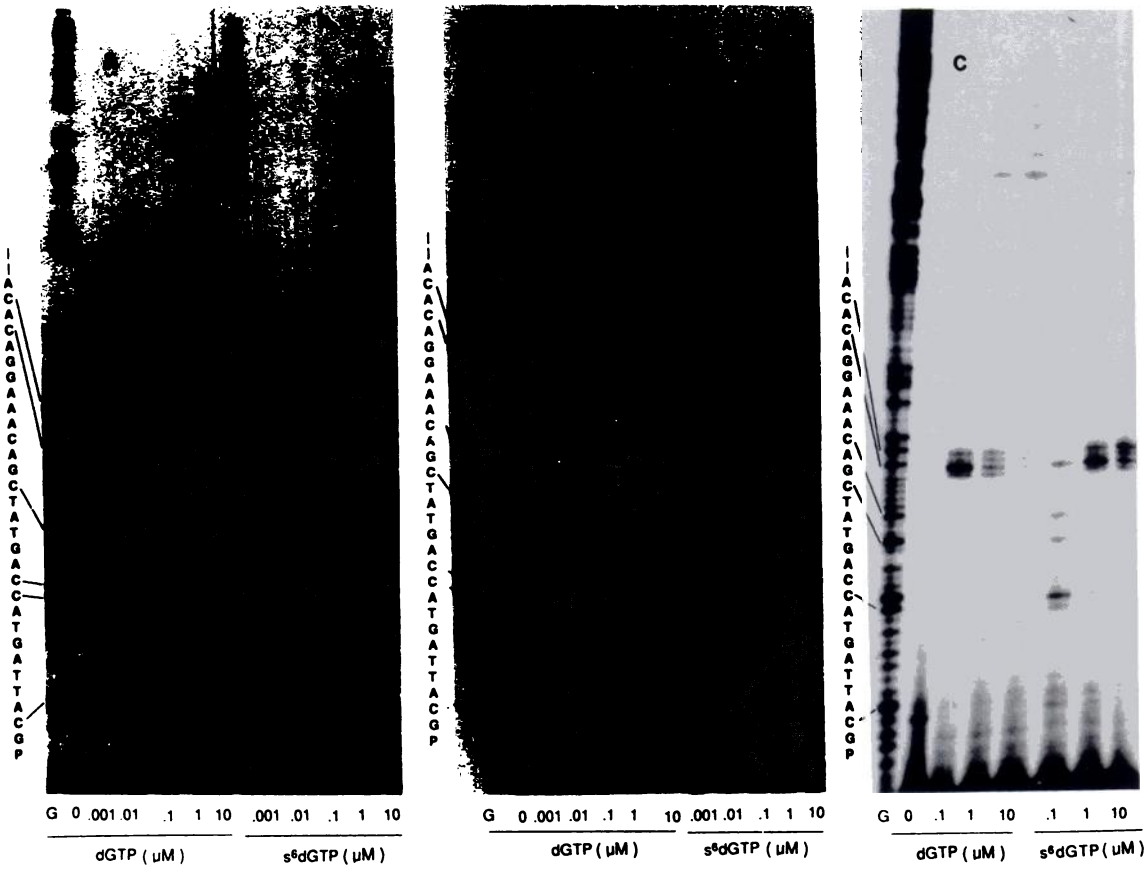

Fig. 2. Elongation of 5′-^{32}P-end-labeled primer from M13mp9 single-strand DNA (+)-template by human DNA polymerases. A, Reactions without dGTP, in the presence or absence of dGTP or S^{6}dGTP, with DNA polymerase α; B, DNA polymerase δ; C, DNA polymerase γ. The reaction conditions and guanine-sequencing were performed as described in Materials and Methods. This result is representative of three separate experiments.

Results

Utilization of S^{6}dGTP versus dGTP in the DE-81 assay of incorporation. Human DNA polymerases α, δ, and γ prepared from K562 cells were used to measure the incorporation of [α-^{32}P]dCMP into primed M13mp9 DNA template, as described in Materials and Methods. As shown in Fig. 1A, polymerization in the absence of dGTP (due to misincorporation) catalyzed by polymerase α was about 27–30% of that in the complete reaction during 10–60 min of incubation. When 10 μM S^{6}dGTP was added to the mixture without dGTP, the rate of DNA synthesis achieved was about 70% that of the complete reaction within 10 min of incubation and reached approximately 80–85% of the complete reaction rate during 20–60 min of incubation. These results indicate that S^{6}dGTP is a good substrate in place of dGTP in DNA synthesis catalyzed by human polymerase α. Qualitatively similar results were obtained with DNA polymerases δ and γ (Fig. 1, B and C).

The apparent K_m values for dGTP and S^{6}dGTP were 1.2 μM each for DNA polymerase α, 2.8 and 3.2 μM, respectively, for polymerase δ, and 0.8 μM each for polymerase γ, (Table 1). Thus, these DNA polymerases possess similar binding efficiency for dGTP and S^{6}dGTP. In comparison with the corresponding natural substrate, the relative V_{max} values for S^{6}dGTP were 0.76, 0.53, and 0.68 for polymerases α, δ, and γ, respectively.

Utilization of S^{6}dGTP by human DNA polymerases, analyzed by a gel electrophoretic assay. To gain further insight into the specificity of incorporation of the analog during polymerization catalyzed by mammalian DNA polymerases, we monitored 5′-^{32}P-labeled primer elongation along an M13mp9 single-stranded template DNA, using high resolution DNA sequencing gels.[1] As shown in Fig. 2A, in the reaction without dGTP, primer elongation catalyzed by polymerase α was mainly arrested one nucleotide before the guanine insertion site. After addition of 0.001 μM dGTP, the primer chain was extended past one to four cytosine residues in the template. Increasing dGTP concentrations led to increased accumulation of longer primer chains, until at 10 μM dGTP the primer bands appeared at the top of the gel. When various concentrations of S^{6}dGTP were added to the reaction without dGTP catalyzed by human polymerase α, stimulation of primer elongation was also seen, indicating that the analog was incorporated into nascent growing primer strands in place of guanine. Although the patterns of primer elongation with S^{6}dGTP as substrate were identical to those produced with dGTP, there was a reduction in the efficiency of primer extension in the case of S^{6}dGTP substitution for dGTP. For instance, with 0.1 μM S^{6}dGTP the extent of primer elongation was similar to that with 0.01 μM dGTP, and at 10 μM S^{6}dGTP the extent of chain elongation was similar to that seen at 1 μM dGTP. As shown in Fig. 2, B and C, DNA polymerase δ or γ also readily incorporated S^{6}dGTP into elongating primer chains, with a reduction in elongation when S^{6}dGTP replaced dGTP similar to that seen with polymerase α. However, the efficiency of primer extension by polym-

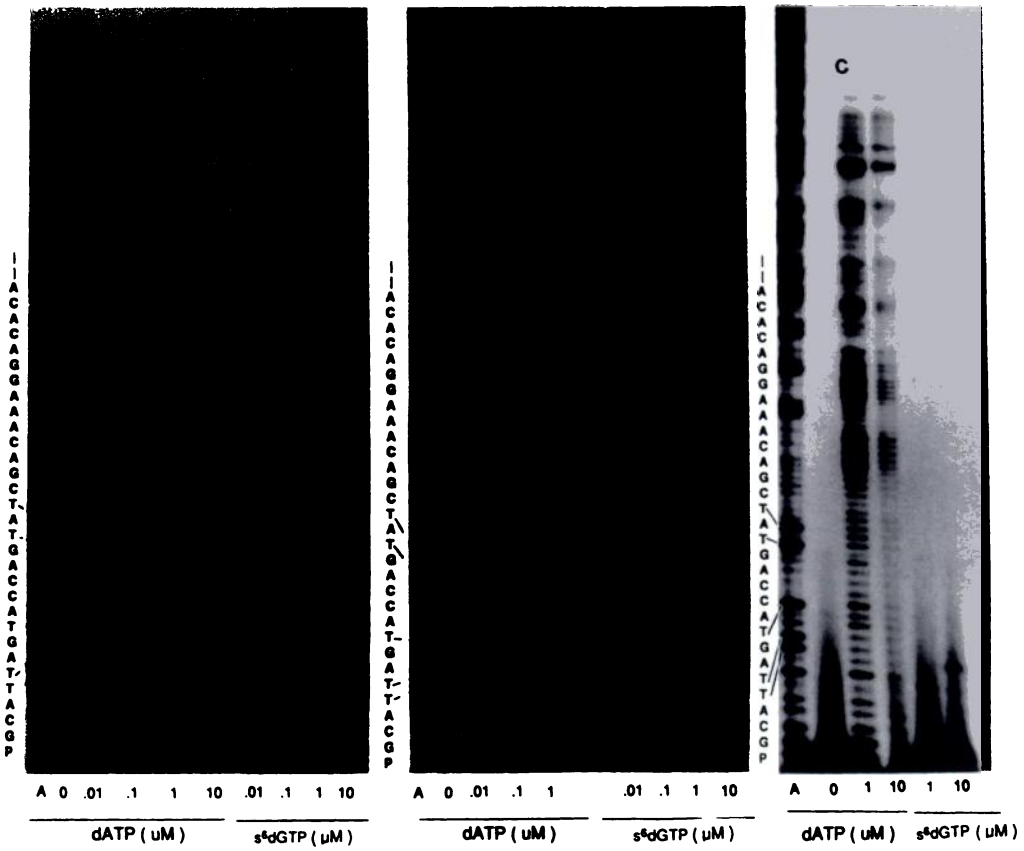

Fig. 3. Misincorporation of S⁶G in place of adenine by human DNA polymerases. Reactions without dATP were performed in the absence or presence of S⁶dGTP, analogous to the reactions without dGTP illustrated in Fig. 2. A, DNA polymerase α; B, DNA polymerase δ; C, DNA polymerase γ.

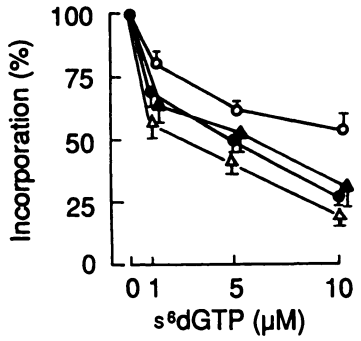

Fig. 4. Inhibitory effect of S⁶dGTP on calf thymus TdT. Experiments were performed using $p(dA)_{12-18}$ as initiator, under the assay conditions described in Materials and Methods. ●, [α-^{32}P]dGTP; ▲, [α-^{32}P]dATP; ○, [α-^{32}P]dCTP; △, [α-^{32}P]dTTP. Values are the average of three separate experiments; *bar*, ± standard deviation.

erase δ was lower than that with polymerases α and γ, possibly due to polymerase δ being more slowed by secondary structure in the template.

Additionally, we conducted experiments to ascertain whether human polymerase α, δ, or γ can misincorporate S⁶dGTP in place of dATP during *in vitro* primer elongation, as we had observed previously with the *E. coli* Klenow I enzyme.[1] Bands due to the formation of S⁶G·T mismatched base pairs were observed in the gel electrophoretic autoradiograms (Fig. 3) at higher concentrations (1–10 μM) of S⁶dGTP in place of dATP. These data suggest a greater propensity of polymerase α to misincorporate S⁶G in place of adenine than the other two mammalian polymerases. Because the reaction mixture contained 20 μM dGTP, these mammalian DNA polymerases did not misincorporate guanine for adenine under these conditions (i.e., absence of bands in the 0 μM S⁶dGTP lanes).

S⁶dGTP-induced inhibition of utilization of dNTPs by calf thymus TdT. Inhibitory effects of various concentrations of S⁶dGTP on TdT activity were assessed using $p(dA)_{12-18}$ as

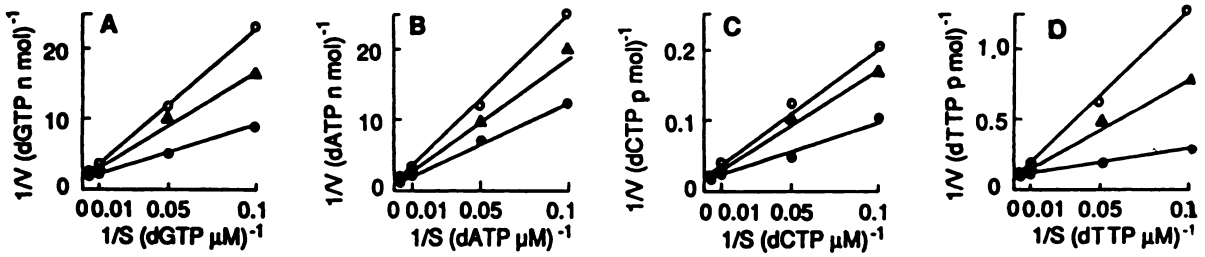

Fig. 5. Inhibition of calf thymus TdT by S⁶dGTP. The conditions described in Materials and Methods and in Fig. 4 were used to assay TdT activity. The concentrations of S⁶dGTP used were 0 (●), 5 μM (▲), or 10 μM (○). The substrates were [α-^{32}P]dGTP (A), [α-^{32}P]dATP (B), [α-^{32}P]dCTP (C), or [α-^{32}P]dTTP (D).

TABLE 2

Inhibitory effect of S^{6}dGTP on calf thymus TdT activity using p(dA)$_{12-18}$ as initiator

Assay conditions were described in Materials and Methods. $K_{m_{app}}$ and K_i values were calculated from replots of the slopes of Lineweaver-Burk regression lines. The results shown are the average values for three separate experiments, each performed in duplicate.

Substrate	Inhibitor	$K_{m_{app}}$	K_i
		μM	μM
dGTP	S^{6}dGTP	86	5.0
dATP	S^{6}dGTP	120	6.2
dCTP	S^{6}dGTP	65	10.8
dTTP	S^{6}dGTP	75	3.5

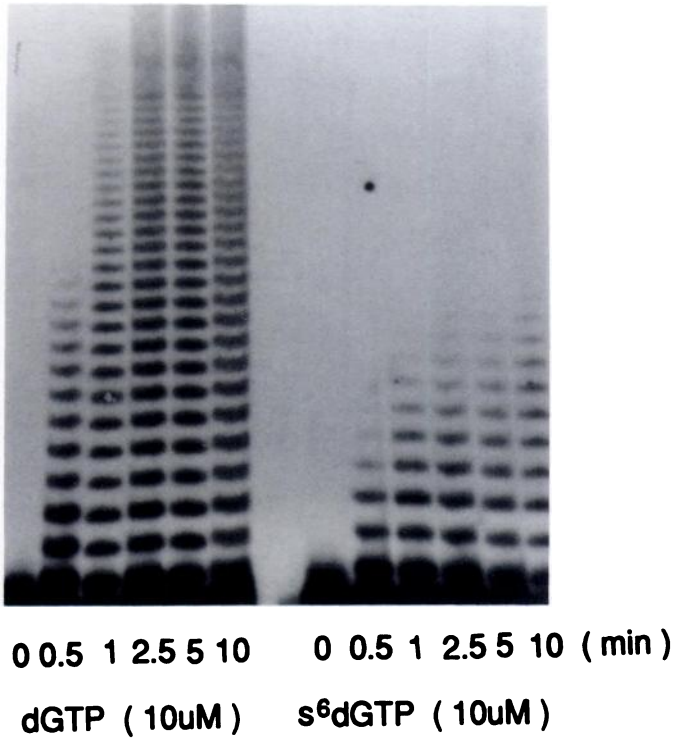

Fig. 6. Time-course for ^{32}P-primer tailing reaction catalyzed by calf thymus TdT in the presence of 10 μM dGTP or S^{6}dGTP. The measurements were performed as described in Materials and Methods. After incubation at 37° for the indicated times, aliquots of the reaction mixtures were subjected to electrophoresis in 7 M urea-16% polyacrylamide gels.

the initiator and all four dNTPs as substrates. As shown in Fig. 4, S^{6}dGTP inhibited the dNTP incorporation into initiator by TdT in a concentration-dependent manner. The utilization of dNTPs by TdT was decreased by approximately 50% in the presence of 5 μM S^{6}dGTP and by more than 75% at 10 μM S^{6}dGTP. Initial velocities of dNTP incorporation into initiator in the absence or presence of 5 or 10 μM S^{6}dGTP were determined at different concentrations of the four natural dNTPs. Double-reciprocal plots indicated that S^{6}dGTP was a typical competitive inhibitor with respect to the endogenous dNTPs (Fig. 5). The kinetic parameters are presented in Table 2. The K_m values for the normal dNTPs are similar to each other, in agreement with the report by Modak (16). The K_i values for S^{6}dGTP were lower than the K_m values for the natural dNTPs.

Gel electrophoretic assay of the TdT-catalyzed homopolymer tailing reaction in the presence of S^{6}dGTP. In order to determine whether TdT can use S^{6}dGTP as a substrate and catalyze its addition to the 3′-hydroxyl terminus of the primer, the tailing products generated by incorporation of substrate onto the 3′-hydroxyl of 5′-^{32}P-labeled-primer were subjected to gel electrophoresis and visualized by autoradiography. As shown in Fig. 6, S^{6}dGTP, like the natural substrate dGTP, was utilized by TdT and rapidly resulted in a number of extended primer bands during the 0.5–10-min incubation. However, the tailing efficiency for S^{6}dGTP was markedly lower than that for dGTP. For instance, within 0.5 min of incubation, only one to five S^{6}G residues were added to each primer, whereas more than 12 guanine residues were incorporated per primer. Moreover, during 10 min of incubation, the largest extent of S^{6}G tailing was about 11 residues, compared with at least 35 guanine residues. The extent of primer tailing by TdT at various concentrations of dGTP or S^{6}dGTP is shown in Fig. 7. The length of tailing products dramatically increased as a function of dGTP concentrations from 0.1 to 1 μM, reaching a plateau between 1 and 10 μM. Further, the bulk of the extended primers centered in the 9–13-base addition range, with some extending to about 35 residues. In the case of S^{6}dGTP, however, there was increased primer extension in the range of 0.1–10 μM (but less than with dGTP), with marked inhibition of primer tailing at 50–200 μM.

Discussion

DNA polymerases α and δ are considered the main candidate enzymes for DNA replication in eukaryotic cells (17–19). DNA polymerase γ is located in mitochondria, where it is involved in DNA synthesis in this organelle (20). In previous work, we demonstrated that prokaryotic DNA polymerase (Klenow fragment of *E. coli* DNA polymerase I) can use S^{6}dGTP, incorporating the analog into DNA in place of dGTP and also misincorporating the analog in place of dATP.[1] From the results presented herein, we conclude that purified human leukemia DNA polymerases α, δ, and γ are able to utilize S^{6}dGTP as a substrate in place of dGTP and incorporate it into the growing primer chain. Interestingly, kinetic analysis showed that the apparent K_m values for dGTP and S^{6}dGTP with these three human DNA polymerases are similar, suggesting that replacement of the O^6-position of guanine by sulfur does not markedly alter the binding affinity of the nucleotide for the DNA polymerase. The same K_m values for dGTP and S^{6}dGTP were observed for the Klenow fragment of *E. coli* DNA polymerase I[1] and for calf thymus DNA polymerase α (11). Although base pair recognition/discrimination generally occurs at the level of binding of substrate (21, 22), the discrimination against S^{6}dGTP utilization apparently occurs at a step of the polymerase reaction after nucleotide binding.

Using a highly sensitive electrophoretic assay to measure chain elongation of 5′-^{32}P-labeled primer on bacteriophage M13 single-stranded DNA template, we found that 0.001–10 μM dGTP stimulated primer extension by human DNA polymerases in a reaction without dGTP, in a concentration-dependent manner. At higher concentrations of dGTP (10 μM), polymerases α and γ extended the primer by about 250 bases, whereas polymerase δ extended the primer chain by only about 35 bases (Fig. 2). When S^{6}dGTP replaced dGTP, the extent of primer elongation also increased as a function of nucleotide concentration with polymerases α, δ, and γ. However, the efficiency of primer elongation with the analog was reduced approximately 1 order of magnitude. For instance, the pattern of primer elongation produced with 1 μM S^{6}dGTP was similar to that

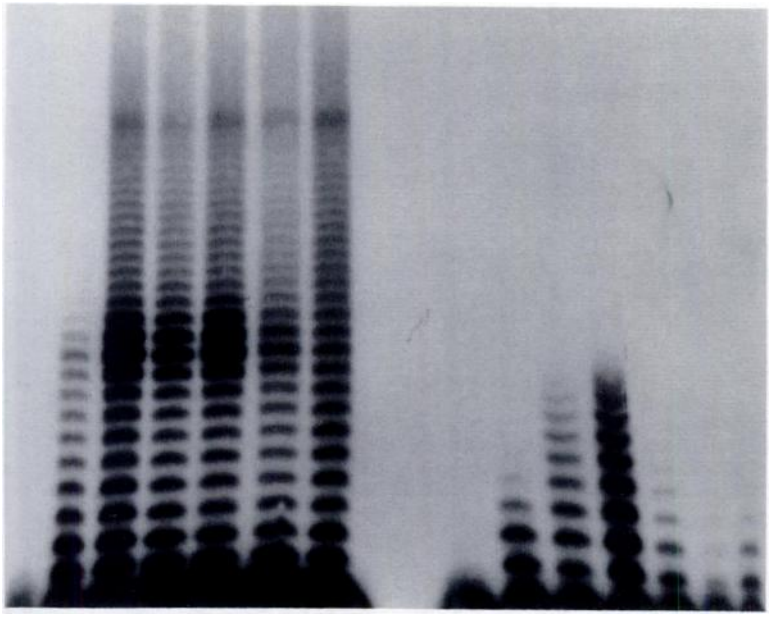

Fig. 7. Concentration effects of dGTP or S^{6}dGTP on the ^{32}P-labeled primer tailing reaction. The tailing reactions were as described in Materials and Methods. The tailing products were analyzed with 7 M urea-16% polyacrylamide gels, as shown in Fig. 6.

with 0.1 μM dGTP (Fig. 2). There are several possibilities to account for the reduced primer elongation when S^{6}dGTP replaces dGTP. The formation of S^{6}G·C base pairs may destabilize or distort the base-paired structure at the 3′-end of the primer-template, such that binding of the polymerase or addition of the next nucleotide is disrupted. Analysis of the structures of S^{6}G and guanine by X-ray diffraction demonstrated that replacement of the O^6 atom in guanine by sulfur increased the hydrogen bond lengths in the respective base pairs (23). Another possibility is that alteration in the conformation of the DNA polymerase-dNTP-primer-template complex after S^{6}G incorporation may slow down the chemical step (phosphodiester bond formation). The kinetic data suggest that the analog is able to bind normally in the polymerase active cleft but that a rate-limiting step for formation of phosphodiester bonds is affected.

The ability of S^{6}dGTP to be misincorporated in place of dATP, to form S^{6}G·T mismatched base pairs, was also demonstrated herein (Fig. 3). Such misincorporation during DNA synthesis could produce biological effects such as induction of mutations or distortion of normal gene structure and functions. The mechanism of S^{6}G misincorporation could involve tautomerization, ionization, or wobbling (24, 25).

TdT is a very unique DNA polymerase-like enzyme, which exists only in the thymus and bone marrow. This nuclear enzyme may contribute to both immunoglobulin and T cell receptor diversity, through the addition of nucleotides at certain functional regions during gene rearrangement (26–28). In addition, it has been reported that the expression of TdT is elevated in T lymphocytic leukemia cells. Certain nucleotide analogs that are active against neoplastic cell growth or virus proliferation, such as the triphosphates of arabinosyl cytosine, arabinosyl adenine, azidothymidine, and 2′3′-didehydro-2′-3′-dideoxythymidine, can competively inhibit TdT activity (29–31). In the present work, we tested the inhibitory effect of S^{6}dGTP on calf thymus TdT. The compound inhibited TdT activity with all four natural dNTPs as substrates. Kinetic analysis demonstrated that S^{6}dGTP is a typical competitive inhibitor (Fig. 5). Furthermore, the K_i values for S^{6}dGTP were lower than the K_m values for the endogenous dNTPs, suggesting that the analog has a higher affinity for TdT than do the normal dNTPs (Table 2). Similar results were reported regarding the inhibitory effects of Ara-CTP and Ara-ATP on Molt-4 human leukemia TdT (29). In the analysis of primer tailing by TdT, the enzyme was observed to use S^{6}dGTP as a substrate, adding it onto the 3′-hydroxyl of the primer, although the tailing efficiency with S^{6}dGTP as substrate was lower than with dGTP as substrate (Fig. 6). At higher concentrations (>10 μM), S^{6}dGTP inhibited the tailing reaction by TdT (Fig. 7). As observed with the DNA polymerases, incorporation of S^{6}G onto the 3′-end of the primer may inhibit subsequent nucleotide incorporation. Because some investigators have demonstrated that reduction or cessation of TdT activity in T lymphocytic leukemia cells may be associated with the blockage of cell growth or induction of maturation (32, 33), we also tested whether S^{6}dGTP could suppress the activity of TdT prepared from human T lymphocytic leukemia cells (NCLB-6). Preliminary results indicated that the analog competitively inhibited the utilization of dNTPs by the enzyme when p(dA)$_{12-18}$ was used as initiator (data not shown). However, the possible relationship between TdT inhibition and S^{6}G-induced cytotoxicity for T lymphocytic leukemia cells is not known.

In summary, the data presented herein clearly demonstrate that S^{6}dGTP is a substrate for mammalian cell DNA polymerases, including TdT, being incorporated into the nascent DNA strands in place of guanine. Several important questions remain, including those regarding the behavior of S^{6}G-containing DNA strands when used as templates by DNA polymerases and

the resultant changes in structure and function of the newly replicated DNA. Further studies may reveal how S^6G incorporation into DNA can ultimately lead to the death of target cells.

References

1. Elion, G. B., E. Burgi, and G. H. Hitchings. Studies on condensed pyrimidine systems. IX. The synthesis of some 6-substituted purines. *J. Am. Chem. Soc.* **74:**411–414 (1952).
2. Hitchings, G. H., and G. B. Elion. The chemistry and biochemistry of purine analogs. *Ann. N. Y. Acad. Sci.* **60:**195–199 (1954).
3. Elion, G. B. The purine path to chemotherapy. *Science (Washington D. C.)* **244:**41–47 (1989).
4. Lepage, G. A. Basic biochemical effects and mechanism of action of 6-thioguanine. *Cancer Res.* **23:**1202–1206 (1963).
5. Tidd, D. M., and A. R. Paterson. A biochemical mechanism for the delayed cytotoxic reaction of 6-mercaptopurine. *Cancer Res.* **34:**738–746 (1974).
6. Christie, N. T., S. Drake, R. E. Meyn, and J. A. Nelson. 6-Thioguanine-induced DNA damage as a determinant of cytotoxicity in cultured Chinese hamster ovary cells. *Cancer Res.* **44:**3665–3671 (1984).
7. Lee, S. H., and A. C. Sartorelli. The effects of inhibitors of DNA biosynthesis on the cytotoxicity of 6-thioguanine. *Cancer Biochem. Biophys.* **5:**189–194 (1981).
8. Covey, J. M., M. D'Incalci, and K. W. Kohn. Production of DNA-protein crosslinks by 6-thioguanine and 2′-deoxy-6-thioguanosine. *Proc. Am. Assoc. Cancer Res.* **27:**17 (1986).
9. Fairchild, C. R., J. Maybaum, and K. A. Kennedy. Concurrent unilateral chromatid damage and DNA strand breakage in response to 6-thioguanine treatment. *Biochem. Pharmacol.* **35:**3533–3541 (1986).
10. Pan, B. F., and J. A. Nelson. Characterization of the DNA damage in 6-thioguanine-treated cells. *Biochem. Pharmacol.* **40:**1063–1069 (1990).
11. Yoshida, S., M. Yomada, S. Masaki, and M. Saneyoshi. Utilization of 2′-deoxy-6-thioguanosine 5′-triphosphate in DNA synthesis *in vitro* by DNA polymerase α from calf thymus. *Cancer Res.* **39:**3955–3958 (1979).
12. Nelson, J. A., Y. H. Ling, D. Farquhar, and K. L. Beattie. 6-Thioguanine (TG) as a substrate for DNA polymerase: 7th International Symposium on Purine and Pyrimidine Metabolism in Man, Bournemouth, England (1991). *Int. J. Purine Pyrimidine Res.* **2 (Suppl. 1):**71 (1991).
13. Messing, J. New M13 vectors for cloning. *Methods Enzymol.* **101:**20–78 (1983).
14. Parker, W. B., A. R. Bapat, J. X. Shen, A. J. Townsend, and Y.-C. Cheng. Interaction of 2-halogenated dATP analogs (F, Cl, and Br) with human DNA polymerases, DNA primase, and ribonucleotide reductase. *Mol. Pharmacol.* **34:**485–491 (1988).
15. Sanger, F., S. Nicklen, and R. A. Coulson. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:**5463–5467 (1977).
16. Modak, M. J. Biochemistry of terminal deoxynucleotidyl transferase. *Biochemistry* **18:**2679–2684 (1979).
17. Fry, M., and L. A. Loeb. *Animal Cell Polymerases.* CRC Press, Boca Raton, FL. (1986).
18. Weissbach, A. Eukaryotic DNA polymerases. *Annu. Rev. Biochem.* **46:** 25–47 (1977).
19. Focher, F., M. Gassman, P. Hafkemezer, E. Ferrari, S. Spadari, and U. Hübscher. Calf thymus DNA polymerase δ independent of proliferating cell nuclear antigen (PCNA). *Nucleic Acids Res.* **17:**1805–1821 (1989).
20. Bolden, A., G. P. Noz, and A. Weissbach. DNA polymerase of mitochondria is a γ-polymerase. *J. Biol. Chem.* **252:**3351–3356 (1977).
21. Boosalis, M. S., J. Petraska, and M. D. Goodman. DNA polymerase insertion fidelity: gel assay for site-specific kinetics. *J. Biol. Chem.* **262:**14689–14696 (1987).
22. Perrino, F. W., and L. A. Loeb. Differential extension of 3′ mispairs is a major contribution to the high fidelity of calf thymus DNA polymerase-α. *J. Biol. Chem.* **264:**2898–2905 (1989).
23. Thewalt, U., and C. E. Bugg. Effects of sulfur substituents on base stacking and hydrogen bonding: the crystal structure of 6-thioguanosine monohydrate. *J. Am. Chem. Soc.* **94:**8892–8898 (1972).
24. Driggers, P. H., and K. L. Beattie. Effect of pH on the base-mispairing properties of 5-bromouracil during DNA synthesis. *Biochemistry* **27:**1729–1735 (1988).
25. Beattie, K. L., M.-D. Lai, and R. Maldonado-Rodriquez. Base pairing and mispairing during DNA synthesis, in *The Eukaryotic Nucleus, Molecular Biochemistry and Macromolecular Assemblies* (P. R. Strauss and S. H. Wilson, eds.). Telford Press, West Caldwell, NJ, Vol. I. 337–359 (1990).
26. Brasheim, C. J., J. H. Kersey, F. J. Bollum, and T. W. LeBien. Ontogenic studies of lymphoid progenitor cells in human bone marrow. *Exp. Hematol.* **10:**886–892 (1982).
27. Yancopoulos, G. D., T. K. Blackwell, H. Suh, L. Hood, and F. W. Alt. Introduced T cell receptor variable region gene segments recombine in pre-B cells: evidence that B and T cells use a common recombinase. *Cell* **44:**251–259 (1986).
28. Bollum, F. J. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. *Blood* **54:**1203–1215 (1979).
29. Dicioccio, R. A., and B. I. S. Srivastava. Kinetics of inhibition of deoxynucleotide-polymerizing enzyme activities from normal and leukemia human cells by 9-β-D-arabinofuranosyladenine 5′-triphosphate and 1-β-D-arabinofuranosylcytosine 5′-triphosphate. *Eur. J. Biochem.* **79:**411–418 (1977).
30. Müller, W. E. G., R. K. Zahn, and J. Arendess. Differential mode of inhibition of terminal deoxynucleotidyl transferase by 3′-dATP, ATP, β-araATP and α-araATP. *FEBS Lett.* **94:**47–50 (1978).
31. Ono, K., H. Nakane, P. Herdewijn, J. Balzarini, and E. Declercq. Differential inhibitory effects of several pyrimidine 2′,3-dideoxynucleoside 5′-triphosphates on the activities of reverse transcriptase and various cellular DNA polymerases. *Mol. Pharmacol.* **35:**578–583 (1989).
32. Wolf, S. C., P. G. Steinberg, N. R. Landau, and A. E. Silverstone. Measurement of terminal deoxynucleotidyl transferase mRNA in clinical samples: new parameter in analysis of leukemia cells. *Am. J. Hematol.* **25:**259–279 (1987).
33. Beland, J. L., M. A. R. Yuille, M. Hugunin, X. Zhang, and A. E. Silverstone. Phorbol ester regulation of terminal deoxynucleotidyl transferase, proliferation, and TcRα in a pre-T cell line. *J. Cell Physiol.* **142:**523–532 (1990).

Send reprint requests to: Dr. J. Arly Nelson, Department of Experimental Pediatrics, University of Texas M. D. Anderson Cancer Center, Box 88, 1515 Holcombe Boulevard, Houston, TX 77030.