Uracil Glycol Deoxynucleoside Triphosphate Is a Better Substrate for DNA Polymerase I Klenow Fragment Than Thymine Glycol Deoxynucleoside Triphosphate†

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Received August 28, 1997; Revised Manuscript Received October 30, 1997⊗

ABSTRACT: A major stable oxidation product of DNA cytosine is 5,6-dihydroxy-5,6-dihydrouracil (Ug). Ug can be formed directly in DNA or in the cellular nucleotide pools by deamination of the unstable primary product, cytosine glycol. Here, we synthesized dUgTP and showed that dUgTP was incorporated in place of dTTP and was a much better substrate for the model enzyme DNA polymerase I Klenow fragment lacking proofreading activity, Kf (exo$^-$), than deoxythymidine glycol triphosphate (dTgTP). The relative efficiency for dUgTP insertion opposite A was 10 times higher than for dTgTP; however, the extension of a primer with 3′ dUg was about 100 times more efficient than the extension of a primer with 3′ dTg. At the insertion step, the differences in V_{max} appeared to be responsible since the apparent K_ms for dUgTP and dTgTP were about the same. In contrast, both the apparent K_m and V_{max} for elongation of dUg were markedly different from those of dTg. Molecular modeling was performed with both Tg and Ug and provides a rational structural explanation for these observations.

Free radical-induced oxidative DNA damage has been implicated in mutagenesis, carcinogenesis, and aging (*1*−*3*). In the cell, oxidative DNA damage is usually caused by hydroxyl radicals formed in close vicinity to DNA as a result of ionizing radiation or cellular metabolism. In addition to strand breaks and damage to the deoxyribose, hydroxyl radicals also attack the heterocyclic purine and pyrimidine bases resulting in a large number of unstable and stable products (*4, 5*). Free radicals can also damage cellular dNTPs, producing oxidatively modified deoxynucleoside triphosphates (*6*). Hydroxyl radicals interact with cytosine residues primarily by addition to the 5−6 double bond; accordingly, a major product of DNA cytosine is cytosine glycol which is unstable in solution and deaminates easily to form uracil glycol (*7*). Cytosine glycol can also dehydrate to form 5-hydroxycytosine (5-OHC)[1] (*7*−*10*), while 5-hydroxyuracil (5-OHU) arises from sequential deamination and dehydration of cytosine glycol (*7*). Depending on the oxidizing conditions, uracil glycol and 5-hydroxycytosine are formed at comparable levels in DNA and are more abundant than 5-hydroxyuracil; furthermore, the background levels of these products are high in untreated DNA (*7*).

When present in template DNA, 5-OHU and 5-OHC are readily bypassed by DNA polymerase I Klenow fragment with 5-OHU most often pairing with A while 5-OHC most often pairs with G but also pairs with A (*11*). Because 5-OHU pairs with A and is derived from C, it would be expected to be premutagenic. Since 5-OHC mispairs at a significant rate, it would also be predicted to be premutagenic. It has been shown that 5-OHC leads to C → T transitions at a frequency of 2.5% (*12*). In fact, C → T transitions, predicted for 5-OHC and 5-OHU are commonly produced by chemical oxidants (*13, 14*) and ionizing radiation (*15*−*17*).

Like dihydrothymidine triphosphate (dDHTTP) (*18*), 8-oxo-7,8-dihydrodeoxyguanosine triphosphate (8-oxodGTP) (*6, 19*), 8-oxo-7,8-dihydrodeoxyadenosine triphosphate (8-oxodATP) (*20*), 2-hydroxydeoxyadenosine triphosphate (*21*), 5-formyldeoxyuridine triphosphate (fdUTP) (*22*), 5-OHdCTP (*12, 20*), and 5-OHdUTP (*20*) are good substrates for DNA polymerases. 8-OxodGTP, 5-OHdCTP, and fdUTP all mispair during *in vitro* incorporation (*6, 19, 20, 22, 23*), which could potentially result in mutation. In fact, 8-oxodGTP is removed from the metabolic pool in *Escherichia coli* by MutT, a nucleoside triphosphatase that hydrolyzes

† This work was supported by National Institutes of Health Grant R37 CA33657 awarded by the National Cancer Institute and by DE-FG02-88ER60742 awarded by the U.S. Department of Energy.

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⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1997.

[1] Abbreviations: Ug, 5,6-dihydroxy-5,6-dihydrouracil; dUg, 5,6-dihydroxy-5,6-dihydrodeoxyuridine; Tg, 5,6-dihydroxy-5,6-dihydrothymine; dTg, 5,6-dihydroxy-5,6-dihydrodeoxythymidine; dUgTP, 5,6-dihydroxy-5,6-dihydrodeoxyuridine 5′-triphosphate; dTgTP, 5,6-dihydroxy-5,6-dihydrodeoxythymidine 5′-triphosphate; dDHTTP, 5,6-dihydrodeoxythymidine 5′-triphosphate; dDHUTP, 5,6-dihydrodeoxyuridine 5′-triphosphate; 5-OHC, 5-hydroxycytosine; 5-OHU, 5-hydroxyuracil; 8-oxodGTP, 8-oxo-7,8-dihydrodeoxyguanosine 5′-triphosphate; Kf (exo$^-$), DNA polymerase I Klenow fragment lacking proofreading activity; DEAE, diethylaminoethyl; TEAB, triethylammonium bicarbonate; NMR, nuclear magnetic resonance, NOE, nuclear Overhauser effect; TPPI, time proportional phase incrementation, a method of quadrature phase detection; COSY, correlation spectroscopy.

S0006-2960(97)02153-3 CCC: $15.00
Published on Web 01/06/1998

8-oxodGTP to 8-oxodGMP (*6*). Cells lacking MutT exhibit a very high spontaneous mutation frequency (*24*). Even if oxidized deoxynucleoside triphosphates do not exhibit mispairing during incorporation, they provide a source of oxidative DNA lesions, and if they subsequently are able to mistemplate, they are potential contributors to the spontaneous mutational burden.

In this paper, we report the synthesis of dUgTP and its ability to serve as a substrate for DNA polymerase. We were interested in Ug because it is an abundant, stable, oxidative product of cytosine and because it is structurally very similar to Tg, a well-studied oxidative lesion (for a review, see ref 25). Our results show that dUgTP can be incorporated into DNA as dT by the model enzyme, *E. coli* DNA polymerase I Klenow fragment Kf (exo$^-$). Insertion of dUgTP was about 10-fold better than dTgTP while dUg on the 3′ end of a primer was extended by Kf (exo$^-$) almost 100 times more efficiently than dTg.

MATERIALS AND METHODS

Chemicals, Enzymes, and DNA. dTTP, dUTP, and DEAE Sephadex A-25 were obtained from Sigma; 2′-deoxynucleoside triphosphates, used in DNA polymerase reactions, and the Mono Q HR 5/5 column were purchased from Pharmacia; 2′,3′-dideoxynucleoside triphosphates, Klenow fragment (Kf), Sequenase version 2.0, T4 DNA ligase, shrimp alkaline phosphatase, and M13 mp18 DNA were obtained from U.S. Biochemicals; terminal transferase and T4 polynucleotide kinase were purchased from Boehringer Mannheim; Partisphere SAX 0.4 × 12.5 cm was obtained from Whatman; [γ-^{32}P]ATP (>5000 Ci/mmol, 10 mCi/ml) was purchased from DuPont.

Oligodeoxyribonucleotides. All oligonucleotides were synthesized by the standard phosphoramidite method on a ABI 380A DNA synthesizer (Department of Microbiology and Molecular Genetics, University of Vermont). The oligonucleotides were purified by Mono Q anion-exchange chromatography on a Milton Roy HPLC system and by electrophoresis in a 13% denaturing polyacrylamide gel containing 8 M urea. After purification, the oligonucleotides were desalted by gel-filtration on a NAP-5 column (Pharmacia) using water as an eluent.

The oligonucleotides were 5′-^{32}P-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase following the manufacturer's instructions. Labeled oligonucleotides were further purified using a NENSORB 20 Nucleic Acids Purification Cartridge (DuPont). To obtain the desired final specific radioactivity, labeled oligonucleotides were combined with the appropriate cold oligonucleotides.

Synthesis of 5,6-Dihydroxy-5,6-dihydrodeoxythymidine 5′-Triphosphate (dTgTP) and 5,6-Dihydroxy-5,6-dihydrodeoxyuridine 5′-Triphosphate (dUgTP). dTgTP was prepared as described by Ide *et al.* (*18*). To remove trace amounts of unreacted dTTP during dTgTP synthesis, instead of two rounds of purification on an anion-exchange SOTA AX 300 column, we used two rounds of purification on a Mono Q 5/5 column. Since dTgTP has no UV absorption maximum at 260 nm due to the loss of its aromaticity, elution products were monitored by UV absorption at 210 nm to detect dTgTP and at 260 nm to control the removal of dTTP. In the crude reaction mixture, dTgTP appeared as a single major product with a retention time of 22.5 min (data are not shown). The amount of dTTP remaining before the first round of purification was estimated to be less than 5% (retention time 21 min). After the second round of purification, dTTP was not detected at all.

dUgTP was obtained similarly by bromination of dUTP in water followed by silver oxide treatment using conditions essentially the same as described for the preparation of deoxythymidine glycol 5′-monophosphate by Rajagopalan *et al.* (*26*) or deoxythymidine glycol 5′-triphosphate by Ide *et al.* (*18*). The reaction mixture was separated on a Mono Q 5/5 column using a NaCl gradient (from 0.005 to 0.45 M over 54 min) in 20 mM Tris-HCl (pH 7.5) with UV detection at 210 nm or 260 nm. The major product (with a retention time of about 21 min) was collected, diluted about 5 times with water and loaded on a DEAE-Sephadex A-25 column in the HCO_3^- form. The column was washed with 0.1 M triethylammonium bicarbonate (TEAB), pH 7.4, and then the deoxynucleoside triphosphate fraction was eluted with 0.5 M TEAB. TEAB was removed by repeated evaporations at 50 °C with 50% ethanol. The crude reaction mixture contained just trace amounts of unreacted dUTP (detected at 260 and 210 nm; retention time on Mono Q column, 21 min) and a single major peak of dUgTP (detected at 210 nm; retention time 22.5 min). Two rounds of purification on a Mono Q 5/5 column provided dUgTP free of dUTP but containing 2−3% of dUgDP, a minor breakdown product of the triphosphate group which occurred during desalting on the DEAE Sephadex column (data are not shown). The extinction coefficient [$\epsilon(\lambda = 210\ \text{nm}) = 8500\ (\text{M}^{-1}\ \text{cm}^{-1})$] was used for both dTgTP and dUgTP to determine their concentration in solution.

NMR Spectroscopy of 5,6-Dihydroxy-5,6-dihydrodeoxyuridine 5′-Triphosphate (dUgTP). Samples for NMR spectroscopy were prepared in nonbuffered solutions of 99.96% D_2O. Final concentrations were 6 mM in a total volume of 0.6 mL and with a pD of 6.79. NMR spectra were obtained with a Varian INOVA 500 spectrometer system at a temperature of 25 ± 0.1 °C. Two-dimensional NOE spectroscopy (NOESY) (*27*) and two-dimensional correlation spectroscopy (COSY) (*28*) were recorded. NOESY data were recorded with a 300 ms mixing time, 512 indirect dimension increments, and TPPI phase detection. Processing was done with a shifted gaussian apodization function in both dimensions. For measuring coupling constants in the one dimensional spectrum, no apodization function was used. Assignments of all protons were made through a combination of the expected coupling patterns in the COSY spectrum and integration values from the one-dimensional spectrum.

Synthesis of 5,6-Dihydrodeoxythymidine 5′-Triphosphate (dDHTTP) and 5,6-Dihydrodeoxyuridine 5′-Triphosphate (dDHUTP). dDHTTP was prepared as described by Ide *et al.* (*18*). dTTP was hydrogenated by purging hydrogen gas through an aqueous solution containing rhodium as a catalyst. The same procedure was used to synthesize dDHUTP. Both dDHTTP and dDHUTP were purified on a Mono Q 5/5 column using the same chromatography conditions as described above for dTgTP and dUgTP. The extinction coefficient [$\epsilon(\lambda = 210\ \text{nm}) = 8500\ \text{M}^{-1}\ \text{cm}^{-1}$] was used for both dDHTTP and dDHUTP to determine their concentration in solution.

Synthesis of Oligonucleotides Containing a 3′ Terminal dUg or dTg. Oligonucleotides containing a 3′ terminal dTg or dUg were prepared by a modification of a method previously described (*11*, *29*). About 1−2.5 nmol of GCAGCCAAAACGTCC was incubated for 30 min at 30 °C in 65 μL of buffer containing 100 mM sodium cacodylate, pH 7.0, 1 mM $CoCl_2$, 0.1 mM EDTA, 50 μg/ml of BSA, 0.1 mM DTT, 0.1−0.12 mM dUgTP (or 0.18−0.2 mM dTgTP), and 100 units of terminal deoxynucleotidyl transferase. The oligonucleotides, extended from the 3′-end with a single dTg or dUg, were then HPLC purified on a Partisphere SAX column (0.4 × 12.5 cm, Whatman) using a linear gradient of sodium phosphate buffer, pH 6.3 (from 5 mM to 0.5 M over 60 min), containing 25% acetonitrile. The purified extended oligonucleotides, GCAGCCAAAACGTCCX (X = dTg or dUg), were desalted using NAP-5 columns (Pharmacia).

DNA Polymerase Reactions. For the "three dNTP" assay, the primer ^{32}pGTAAAACGACGGCCAGT was annealed to M13 mp18 DNA and extended using 0.2 unit of Klenow fragment lacking proofreading activity Kf (exo$^-$). The reaction mixture (6 μL) contained 15 mM Tris-HCl, pH 7.5, 7.5 mM $MgCl_2$, 30 mM NaCl, 4 mM DTT (buffer "P"), 20 nM of primed DNA, 0.1 unit of Kf (exo$^-$), and all four combinations (dA,dT,dC; dG,dT,dC; dG,dA,dC; and dG,dA,dT; 50 μM each) of three normal dNTPs. DNA polymerase reactions were incubated at 37 °C for 15 min in the absence or presence of 50 μM dTgTP or dUgTP. Similar reaction conditions were used for the extension of primers ^{32}pGCAGCCAAAACGTCCX (X = dTg, dUg, or dT) annealed to GACAGACCATCCAGGTTTTGGCTGC template.

Kinetic Experiments. To determine the kinetic parameters of incorporation of dTgMP or dUgMP, a steady state kinetic assay (*30*) was used. The reaction mixture was prepared by adding 3.5 μL of a solution containing primer ^{32}pGCAGCCAAAACGTCCC annealed to GTATGATTGATTGGGACGTTTTGGCTGC template, Kf (exo$^-$), and the buffer, to 2.5 μL of water containing dATP and dTgTP or dUgTP or dDHTTP or dDHUTP or dTTP. The final mixture (6 μL), contained 0.02 unit of Kf (exo$^-$), buffer "P", 50 nM of primer−template complex, 50 μM dATP and various concentrations of modified dNTP. For dTgTP and dUgTP, a concentration range of 10−300 μM was used; for dDHTTP and dDHUTP, a concentration range of 0.05−50 μM was used; and a concentration range of 0.025−5 μM was used for dTTP. Reactions were incubated at 4 °C. Reaction times were between 0.5 and 2 min.

To determine the kinetic constants for the extension of primers containing either a 3′-terminal dTg or dUg, the reaction mixture was prepared by adding 3.5 μL of a solution containing primer ^{32}pGCAGCCAAAACGTCCX (X = dTg, dUg, or dT), annealed to GACAGACCATCCAGGACGTTTTGGCTGC template, Kf (exo$^-$), and the buffer, to 2.5 μL of water solution containing dGTP. The final mixture (6 μL) contained 0.02 unit of Kf (exo$^-$), buffer "P", 50 nM of primer−template complex, and 0.025−10 μM of dGTP. Reactions were incubated at 4 °C. Reaction times were between 0.5 and 2 min.

Electrophoresis. All DNA polymerase reactions were terminated by the addition of an equal volume of loading buffer (95% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol, and 20 mM EDTA). Reaction products were analyzed by electrophoresis on 0.4 mm thick 13% denaturing polyacrylamide gels containing 8 M urea. The gels were electrophoresed in 50 mM Tris-borate, 2 mM EDTA buffer, pH 8.3, for 1.5−3 h at 2000 V, dried under vacuum, and exposed to X-ray film. The radioactivity in the bands corresponding to the products of enzymatic reactions was analyzed using a Model GS-250 Molecular Imager System (Bio-Rad).

Computational Analysis. We studied two conformers each of Tg and Ug, in which the hydroxyl groups at the C^5 and C^6 positions are in the pseudoequatorial and pseudoaxial conformations, respectively [5-eq-6-ax; Figure 1e and h, of Miaskiewicz *et al.* (*31*) show the two corresponding conformers of Tg]. Following Miller *et al.* (*32*), we refer to the *cis*-5*R*,6*S*/5-eq-6-ax conformer as the A conformer and the *cis*-5*S*,6*R*/5-eq-6-ax conformer as the B conformer. Miaskiewicz *et al.* (*31*) concluded that the 5-eq-6-ax structures of Tg were the most stable conformers based on *ab initio* quantum chemical calculations. Similarly, we find the 5-eq-6-ax structures of Ug to be the most stable conformers based on *ab initio* calculations (Derecski-Kovac, Wallace, and Bond, unpublished observation), consistent with the conclusion of Miaskiewicz *et al.* (*31*) that the stability of the 5-eq-6-ax structures is due to favorable dipolar interactions (which are not directly affected by the presence of the methyl group at C^5). The geometry and partial charges of Tg and Ug were obtained using Gaussian 94 (Gaussian, Inc.). Tg and Ug were modeled into the X-ray structure of the complex of Taq polymerase with DNA [(*33*) PDB entry 1tau) and the ternary complex of DNA polymerase β with DNA and ddCTP [(*34*), PDB entry 2bpf] by minimum RMS superposition of the N^1, C^2, N^3, and C^4 atoms on those of the appropriate pyrimidine (pq 51 and ddCTP, respectively). Superposition and graphical display were accomplished using Insight II (Biosym/MSI). Limited molecular mechanics calculations on a system consisting of a DNA template (CCAGG) (see primers 2−4 and template 2 in Figure 5), a lesion-containing primer (CCXG, X = Ug or Tg; cf. Figure 5), explicit solvent and counterions were performed using AMBER 4.1 (*35*, *36*) for the purpose of illustrating the structure of Tg and Ug in the context of DNA; structure predictions are not to be inferred.

RESULTS

Synthesis of dTgTP and dUgTP. In order to examine the efficiency of incorporation of dUgTP and compare it to dTgTP in DNA, it was necessary to synthesize both modified deoxynucleoside triphosphates. dTgTP and dUgTP were synthesized as previously described for dTgTP (*18*) by bromination in aqueous medium, followed by the treatment with silver oxide. dUgTP was prepared starting from dUTP using the same synthetic and purification procedures used for dTgTP.

The NMR spectra of the dUgTP were doubled in pattern, indicating the presence of two species in a ratio of 3:1 based upon integration of the proton spectrum (Figure 1). Upon the basis of measured coupling constant (*J*) of the uracil glycol H5 and H6 protons (∼3.6−3.8 Hz), it was determined that the glycol group must be in the *cis*-configuration, with both hydroxyl moieties on the same side of the heterocycle

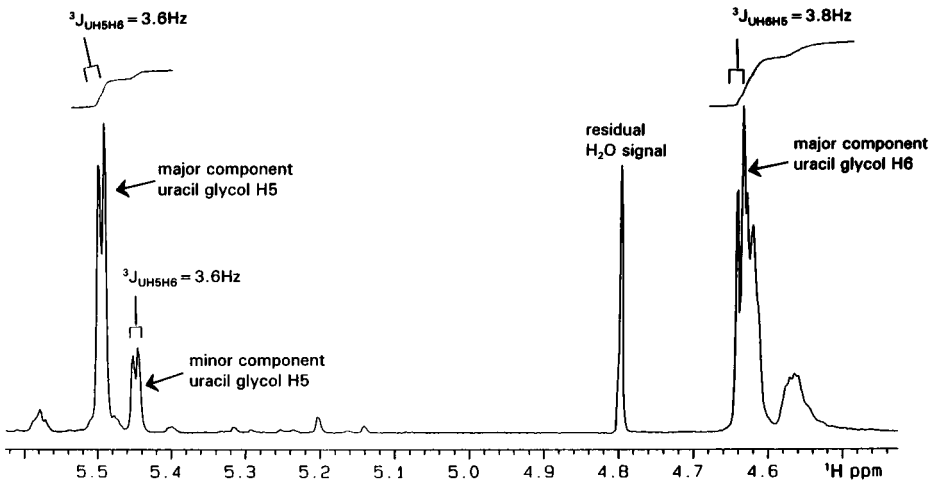

FIGURE 1: MHZ proton NMR spectrum of dUgTP in 99.96% D_2O. The expanded region displaying the uracil glycol H5 and H6 protons is shown.

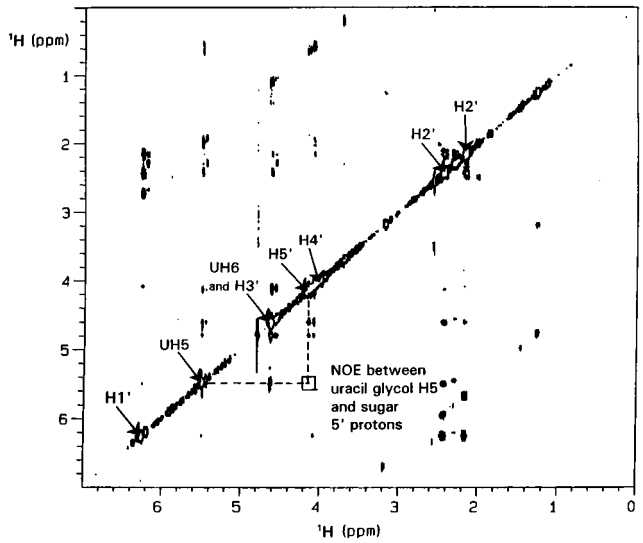

FIGURE 2: NOESY spectrum of dUgTP at 300 ms. Relevant assignments and NOE interactions are shown.

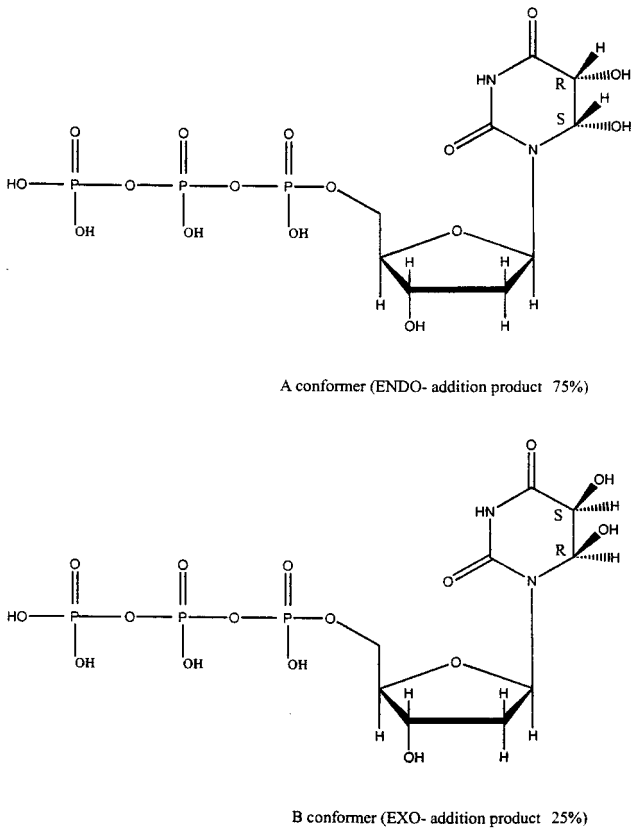

FIGURE 3: Schematic of the two major products resulting from bromination and treatment of dUTP with silveroxide, as determined by NMR analysis.

ring. The expected coupling in the comparable *anti*-addition product would range between 10 and 16 Hz (*37*). NOE interactions seen between the C5′ methylene protons and the uracil glycol H5 and H6 protons argue strongly that the major component of the mixture consists of the *endo*-addition product, *cis*-5*R*, 65/5 eq-6ax (A conformer) with the minor component being the *exo*-addition product, *cis*-55,6*R*/5eq-6-ax (B conformer) (Figures 2 and 3). This is not surprising in that preparation of glycols by the method described results in a higher ratio of *endo*- to *exo*-addition products in general (*38*).

The Specificity of Incorporation of dUgTP during DNA Synthesis. Both the efficiency and specificity of incorporation of dUgTP into DNA by Kf (exo⁻) was determined using the traditional "three dNTPs" assay (*39*). In this experiment, the ability of the modified dNTP to be incorporated into DNA in place of a normal dNTP is judged by the elongation of the primer in the primer−template complex incubated with DNA polymerase in the presence of only three of the four dNTPs ("minus" reaction) or in the presence of the same three dNTPs plus the modified dNTP ("plus" reaction). In the "minus" reaction, DNA polymerase-catalyzed primer elongation pauses or stops opposite each template position complementary to the "missing" normal dNTP. If, in the "plus" reaction, the added modified dNTP is incorporated in place of the missing dNTP, stimulation of the primer elongation reaction will be observed. The effectiveness of incorporation of the modified dNTP can be qualitatively estimated by comparing the lanes on the gel corresponding to primer elongation in the minus reaction in the presence of the modified dNTP with elongation in the presence of all four normal dNTPs. The specificity and effectiveness of dUgTP incorporation by Kf (exo⁻) was compared with dTgTP, which was previously shown to be very poor substrate for DNA polymerase (*18*). Figure 4 shows the results of the three nucleotide assay. Primer ^{32}pGTTTC-

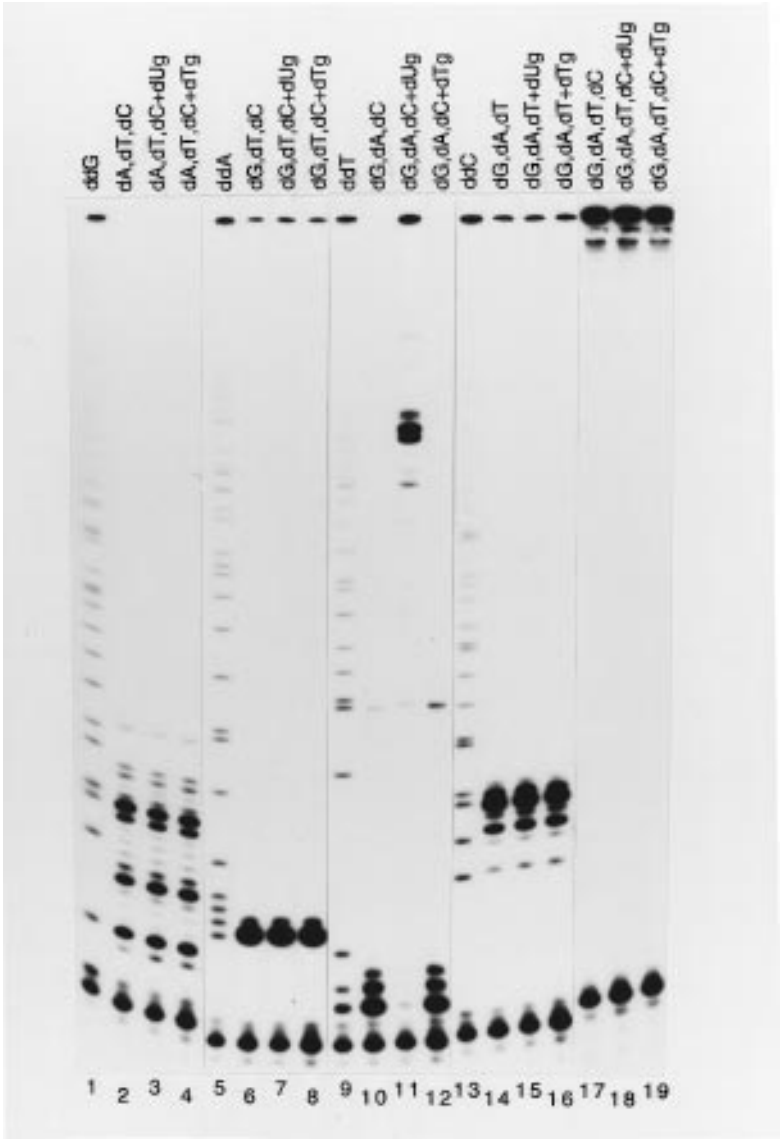

FIGURE 4: Specificity of incorporation of dUgMP into DNA catalyzed by *E. coli* DNA polymerase I Kf (exo$^-$) compared with incorporation of dTgMP. The extension of ^{32}pGTTTCCCAGTCACGAC annealed to M13 mp18 DNA using *E. coli* Kf (exo$^-$) was measured. The final concentration of Kf (exo$^-$ in the reaction mixture was 0.05 unit/μL). Lanes 2, 3, and 4 represent the extension in the absence of dGTP ("-dG" reaction) without (lane 2) and with addition of 50 μM dUgTP (lane 3) or 50 μM dTgTP (lane 4). Lanes 6, 7, and 8 represent "-dA" reaction without (lane 6) and with addition of 50 μM dUgTP (lane 7) or 50 μM dTgTP (lane 8). Lanes 10, 11, and 12 represent "-dT" reaction without (lane 10) and with addition of 50 μM dUgTP (lane 11) or 50 μM dTgTP (lane 12). Lanes 14, 15, and 16 represent "-dC" reaction without (lane 14) and with addition of 50 μM dUgTP (lane 15) or 50 mM dTgTP (lane 16). Lane 17, extension of the primer in the presence of all four dNTPs (50 μM each); lane 18 and 19 are same as 17, but containing in addition 50 μM dUgTP (lane 18) or 50 μM dTgTP (lane 19). Lanes 1, 5, 9, and 13 are standard dideoxy sequencing reactions using the same primer and Sequenase.

CCAGTCACGAC was annealed to M13 mp18 DNA and was extended using Kf (exo$^-$). Reaction mixtures contained all four combinations of three normal dNTPs, and DNA polymerase reactions were conducted in the absence or presence of dUgTP or dTgTP. The extension products were analyzed by sequencing gels. A comparison of these products confirmed the previous observation (*18*) that dTgTP is very poor substrate for Kf (exo$^-$). The efficiency of dTgTP incorporation was extremely low, just slightly higher than the background (compare lanes 10 and 12) and dTgTP only replaced dTTP. dUgTP also only replaced dTTP (compare lanes 10 and 11); however, the efficiency of incorporation of dUgTP in place of dTTP was much greater than for dTgTP in place of dTTP (compare lanes 11 and 12). The incorporation efficiency of dUgTP was comparable to the efficiency of incorporation of 8-oxodGTP opposite dC previously examined under the same reaction conditions (*20*). Neither dUgTP nor dTgTP inhibited the polymerase activity of Kf (exo$^-$) (compare lanes 17−19).

Steady State Kinetic Analysis of dUgTP Insertion. To quantify the insertion of dUg into DNA, a steady state kinetic assay was used. To compare kinetic parameters for incorporation of dUgTP and dTgTP, a 16 member primer (primer 1, Figure 5) and a 28 member template (template 1, Figure 5) were prepared. The target site, A in the template, was placed at the third position downstream from the primer. This provided a way to determine the kinetic parameters for the incorporation of dUgTP from a "running start" as described by Boosalis *et al.* (*30*). The primer contained ^{32}P-label to detect the extension products on the autoradiograph of the polyacrylamide gel and allowed for their quantification. The apparent K_m and V_{max} values for incorporation of dUgTP and

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    1                16

5' GCAGCCAAAACGTCCC              3'    primer 1
3' CGTCGGTTTTGCAGGGTTAGTTAGTATG 5'    template 1
                    1 2 3

5' GCAGCCAAAACGTCCX              3'    primers 2-4
3' CGTCGGTTTTGCAGGACCTACCAGACAG 5'    template 2
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X - dT (primer 2), dUg (primer 3) or dTg (primer 4)

FIGURE 5: Primers and templates used for kinetic analysis.

Table 1. Kinetic Parameters of Kf (exo$^-$) Catalyzed Insertion of Ring-Saturated Pyrimidine Deoxynucleoside Monophosphates Opposite A in the Template

substrate	K_m (μM)	V_{max} (μM) (% min^{-1})	V_{max}/K_m (% min$^{-1}/\mu$M)	relative efficiency[a]
dTTP	0.25	0.22	0.88	1
dUgTP	34	0.34	0.01	0.011
dTgTP	41	0.05	0.0012	0.0014
dDHUTP	5.1	0.48	0.094	0.11
dDHTTP	10	0.58	0.058	0.07

[a] V_{max}/K_m for incorporation of dTTP was normalized to 1 and the V_{max}/K_m values are given relative to this value.

dTgTP (Table 1) were determined based on the relative velocity of primer extension with the modified dNTP (with dTTP as a control) measured as I_3/I_2 at $t = 1$ min. where I_3 and I_2 correspond to the radioactivity of the extension product at sites 3 and 2, correspondingly, expressed as percentage of total primer (*30*). Both dUgTP and dTgTP have a similar apparent K_m, which was about 150-fold higher than the apparent K_m for dTTP. The major difference between dUgTP and dTgTP lies in the apparent V_{max} with that for dUgTP being 6.8-fold greater than the apparent V_{max} for dTgTP and about the same as that for dTTP. Taken together, the data show that, at the insertion stage, dUgTP was about a 7.9-fold better substrate for Kf (exo$^-$) than dTgTP and about 91-fold less efficient than dTTP.

Elongation of a 3′ Terminal dUg or dTg during DNA Synthesis. A 7.9-fold difference between the relative efficiencies of incorporation of dUgTP and dTgTP cannot explain their dramatically different behaviors during DNA polymerization as seen in Figure 4. Because primer elongation has been shown to be rate limiting for many base mismatches and mismatches containing DNA lesions (for a review, see ref 40), the abilities of oligonucleotides containing either a dUg or dTg on the 3′ end to serve as a primers for Kf (exo$^-$) were compared. To prepare oligonucleotides with a single dUg or dTg on the 3′ end, we used the terminal deoxynucleotidyl transferase reaction described previously (*29*). Pentadecamer GCAGCCAAAACGTCC was 3′ extended with dUg or dTg under the conditions optimized for one nucleotide extension (*20*), and hexadecamers containing a single 3′ dUg or dTg were purified by anion-exchange HPLC on a Partisphere SAX column. As was observed for Kf (exo$^-$), dUgTP was a better substrate for terminal transferase than dTgTP. To obtain the same yield and size distribution of the extension products of dTgTP as dUgTP, usually twice the concentration of dTgTP was required in the reaction mixture (data not shown).

Oligonucleotides containing a 5′ ^{32}P-label and a 3′ terminal dUg, dTg, or dT (primers 2−4, Figure 5) were annealed to a 28 mer template (template 2, Figure 5). Primers 2−4 were extended in the presence of Kf (exo$^-$) and four normal dNTPs. Analysis of the extension products by gel electrophoresis (Figure 6) showed that the extension efficiency of the primer containing 3′ dUg (Figure 6, lane 2) was just slightly lower than in the control experiment with the oligo containing 3′ dT (Figure 6, line 6). In contrast, the extension of the oligo containing 3′ dTg was not detected at all under the reaction conditions used (Figure 6, lane 4).

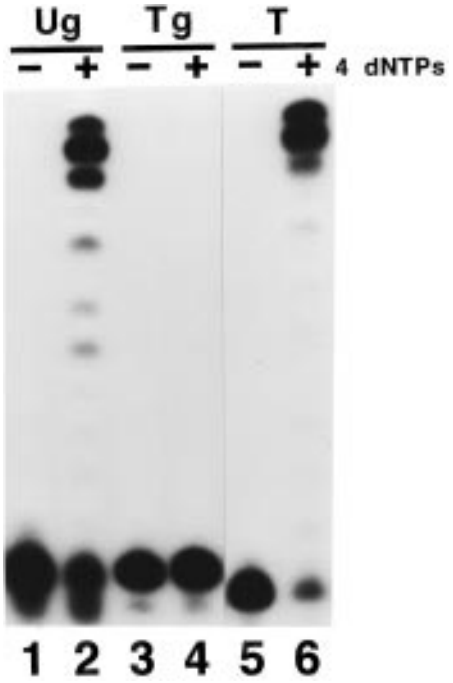

FIGURE 6: DNA polymerse Kf (exo$^-$) catalyzed extension of primers 2−4 annealed to template 2 in the presence of 50 μM dNTPs. Primers contained 3′ dUg (lane 2), dTg (lane 4) or dT (lane 6). Lanes 1, 3, and 5 are the same reactions in the absence of dNTPs.

Table 2. Kinetic Parameters of Kf (exo$^-$) Catalyzed Elongation of Primers 2−4 Containing 3′ dUg, dTg, or dT with dGTP

substrate	K_m (μM)	V_{max} (% min^{-1})	V_{max}/K_m (% min$^{-1}/\mu$M)	relative efficiency[a]
3′ dUg	0.18	5.6	31	0.34
3′ dTg	2.4	0.7	0.3	0.003
3′ dT	0.39	35	90	1

[a] V_{max}/K_m value for extension of the primer containing 3′ dT was normalized to 1 and the V_{max}/K_m values are given relative to this value.

In order to quantify Kf (exo$^-$) catalyzed primer extension, the steady state kinetic parameters for elongation of primers 2−4 annealed to template 2 in the presence of dGTP were determined. Under these conditions, primers 2−4 were elongated with only one or two dG members (see Figure 5). To determine the apparent K_m and V_{max} values, the total radioactivity of the bands in the gel corresponding to primers 2−4 extended with one and two dG residues was measured and expressed as percentage of total primer. As can be seen in Table 2, the primer with 3′ terminal dUg was a significantly better substrate for Kf (exo$^-$) than the primer with 3′ dTg. The relative efficiency of elongation of 3′ dUg was only about three times lower than that of 3′ dT, while the elongation of the 3′ dTg primer was about 2 orders of magnitude less than the extension of 3′ dT. In contrast to the insertion step, here the difference between dUg and dTg occurred at both the K_m and V_{max} levels with the K_m for

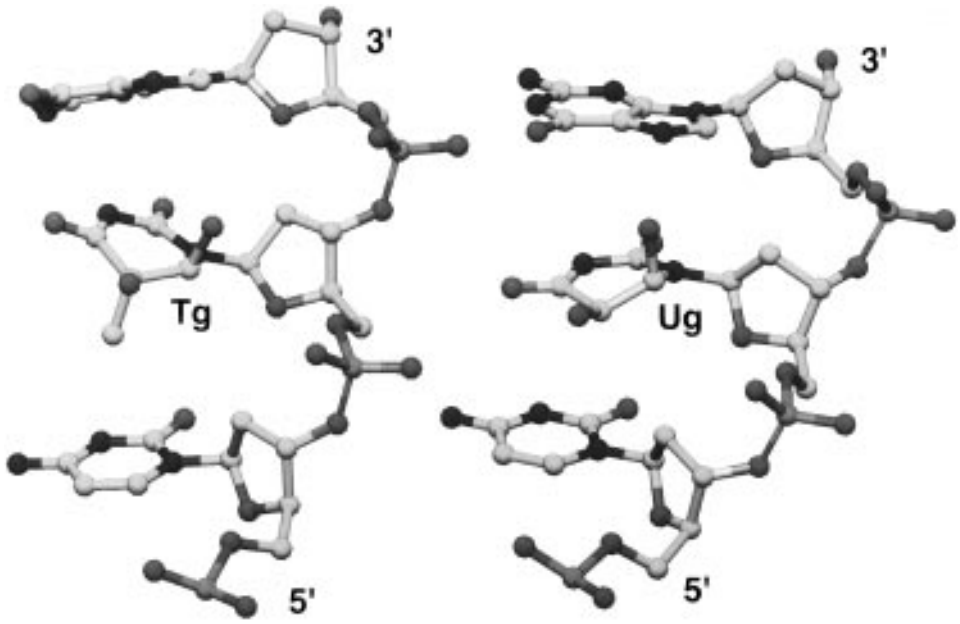

FIGURE 7: Illustration of Tg and Ug in the context of DNA. Three 3′ terminal nucleotides from a model primer strand containing Tg (left) or Ug (right) as the penultimate nucleotide (see Materials and Methods).

elongation of Ug being about 13-fold lower than that of Tg and even lower than for the normal primer containing dT while there was 2 orders of magnitude difference in apparent V_{max} between elongation of dUg compared to dTg.

DISCUSSION

Uracil glycol, a major stable oxidative product of cytosine, has been identified in cells that have been treated with chemical oxidants or ionizing radiation (*7*−*10*). Formed as a result of rapid deamination of cytosine glycol, uracil glycol is a close structural relative of thymine glycol, a major oxidative product of DNA thymine. Thymine glycol in DNA has been shown to constitute a strong block to DNA synthesis by DNA polymerases *in vitro* (*41*−*44*) and in the cell (*45*−*48*). Furthermore, dTgTP is a very poor substrate for DNA polymerase I. Surprisingly, dUgTP was a much better substrate for DNA polymerase I Kf (exo$^-$) than dTgTP which was incorporated into DNA at a minimal level (Figure 4).

Kinetic parameters of Kf (exo$^-$)-catalyzed DNA synthesis (Table 1) indicated that the lower efficiency of dUgTP and dTgTP insertion compared to dTTP was probably caused by the lower affinity of dUgTP and dTgTP to the binding site of DNA polymerase since the apparent K_m values for both dUgTP and dTgTP were about 150 times higher than for dTTP. In the case of dUgTP, the saturated pyrimidine ring did not appear to affect the catalytic stage of the DNA polymerase reaction since the apparent V_{max} determined for dUgTP was even slightly higher than the V_{max} for dTTP. The apparent V_{max} for dTgTP, however, was about 7 times lower than that of dUgTP, indicating that the C^5 methyl group of dTgTP reduced the efficiency of the catalytic stage of the DNA polymerase reaction. Taken together, dUgTP was about 100-fold and dTgTP about 1000-fold less efficiently inserted opposite A than dTTP. Once incorporated, however, dUg was relatively efficiently elongated (Table 2). In fact, the affinity of dUg at the primer terminus for Kf (exo$^-$) appeared to be slightly better than that of dT as indicated by the apparent K_m while the apparent V_{max} for dUg elongation was only about 6-fold lower than for dT giving dUg a relative efficiency of elongation compared to dT of about 34%. In contrast, dTg was about 300-fold less efficiently elongated than dT. The overall primer extension, as seen, for example, in Figure 4, reflects the combination of both the incorporation and elongation steps. Here, we would expect dUgTP to be about 400-fold and dTgTP about 4×10^6-fold less effective as substrates for Kf (exo$^-$) than dTTP. It should be pointed out that the kinetic parameters measured here are from diasteromeric mixtures of the substrates and thus the above interpretation may be subject to some modification.

Because we had shown earlier that deoxydihydrothymidine triphosphate (dDHTTP) was a good substrate for Klenow fragment polymerization, while dTgTP was not (*18*), we concluded that loss of aromaticity was not the sole determinant in the lack of ability of dTgTP to serve as a substrate but that the bulk of the hydroxyl groups must play an important role. Because of this, we were initially surprised that dUgTP was a reasonably good substrate. In an attempt to develop plausible hypotheses about the atomic interactions that result in the kinetic differences between the two substrates, we utilized a molecular modeling approach. To frame this discussion, two conformers each of Tg and Ug (A and B, see Materials and Methods) were used to consider steric interactions on the 5′ side (Figure 7) and on the 3′ side of a (Watson−Crick paired) base at or adjacent to the 3′ terminus of the primer. On the basis of energy minimization (*49*) and molecular dynamics (*32*) calculations, significant perturbations of DNA structure associated with replacement of T by Tg have been inferred. In particular, the pseudoaxial methyl group at the C^5 position in the A conformer of Tg should collide with the base on the 5′ side (Figure 7). A pseudoaxial hydroxyl group at C^6 that is directed toward the 5′ side, as in the B conformation of Tg or Ug, would also be expected to result in steric collisions that would decrease the efficiency of incorporation.

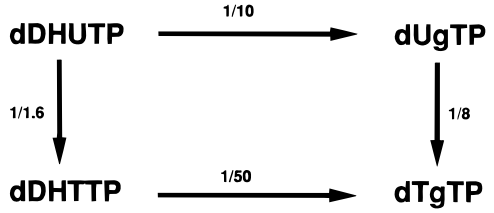

FIGURE 8: Relative efficiencies of incorporation of modified nucleotide triphosphates by Kf (exo⁻). Each number shown is the ratio of the relative efficiency of incorporation of the nucleotide at the tip of the arrow to that of the nucleotide at the base of the arrow.

Pseudoaxial methyl and hydroxyl substituents on the 3′ side of the Watson−Crick paired modified base, such as occur in conformation A of either Tg or Ug and in conformation B of Tg, may result in unfavorable steric collisions with the polymerase. In the structure of Taq DNA polymerase with DNA bound in the polymerase active site (*33*), the base on the 3′ end of the primer in a blunt-ended duplex DNA abuts phenylalanine 667 in the fingers domain of the DNA polymerase. The Taq polymerase is homologous to Kf, and the polymerase domain was found by Kim *et al.* (*50*) to be "nearly identical" in structure to that of Kf. In the ternary complex of polymerase β (which is not homologous to Kf) with its substrates (*34*), the incoming nucleotide abuts aspartate 276 in a similarly positioned helix as is found in the Taq polymerase. For both the Taq polymerase and polymerase β, replacing the relevant base with Tg (A or B) or Ug (B) resulted in some steric overlap with the polymerase (data not shown).

With these considerations in mind, steric collisions would likely occur during incorporation on *both* the 5′ and 3′ side of Tg and on *either* the 5′ or 3′ side of Ug, but not both. Because steric collisions should result in a reduction in the efficiency of incorporation of modified bases, the presence of steric collisions is consistent with the data shown in Table 1, i.e., the efficiency of insertion of dUgTP is reduced relative to that of dTTP, but not to the extent that the efficiency of insertion of dTgTP is reduced. In contrast, the efficiency for insertion of dDHTTP and dDHUTP (which have substituent hydrogens rather than hydroxyl groups) should be increased relative to the efficiency of insertion of dTgTP and dUgTP. This prediction is borne out by the data Table 1 which show that the apparent K_m for dDHUTP is almost 7-fold lower than that of dUgTP while that for dDHTTP is 4-fold lower than that of dTgTP. It is also possible that the differences in K_m arise in whole or in part from differences in the energy required to partially desolvate the bases upon formation of the enzyme−substrate complex. The reduction in the efficiency of insertion associated with the simultaneous addition to dDHUTP of a methyl group at C^5, and hydroxyl groups at C^5 and C^6 are greater than the product of the reductions associated with independent additions (Figure 8) (see ref 51 for an early example of the application of such "double mutant" cycles to enzymes kinetics). Such "anti-cooperative" interactions are consistent with the idea [suggested by the quantum chemical calculations of Miaskiewicz *et al.* (*31*)] that in progressing from dDHTTP to dTgTP (as in Figure 8) the methyl group at C^5 changes from a pseudoequatorial position to a pseudoaxial position, resulting in greater steric overlap on the 5′ side of the modified base. However, it is also reasonable to expect that the reduction in the efficiency of insertion associated with the addition of substituents that participate in steric collisions will be greater for damaged bases that already have substituents that collide with atoms of the DNA or polymerase.

While pseudoaxial substituents on the 3′ side of modified bases are proposed to decrease the efficiency of insertion, their effect on elongation may not be as severe because the helical twist places these substituents in the major groove. The results reported in Table 2 show that the efficiency of elongation of 3′ dUg was not dramatically reduced below that of 3′ dT. On the basis of the preceding discussion, this result is consistent with the possibility that the A conformer is the predominant conformer of dUgTP.

Because dUgTP is always incorporated in place of dTTP, at least at our level of detection, mispairing and thus a mutagenic outcome would be unlikely during incorporation of dUgTP resulting from oxidation of dCTP in the nucleotide pool. However, it is possible that if template Ug, incorporated in place of T, does not block polymerase and mispairs, subsequent rounds of replication past Ug could lead to mutation. In any case, Ug formed from oxidized C in DNA, is likely to be a potent premutagenic lesion if not repaired, since, if bypassed, it would most likely pair with its cognate A rather than G. These topics are currently under investigation.

ACKNOWLEDGMENT

The authors are grateful to Dr. Zafer Hatahet for useful discussions. The molecular modeling was accomplished using the resources of the Molecular Modeling Facility of the Vermont Cancer Center.

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BI972153D