Incorporation of the Guanosine Triphosphate Analogs 8-Oxo-dGTP and 8-NH$_2$-dGTP by Reverse Transcriptases and Mammalian DNA Polymerases*

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We have measured the efficiencies of utilization of 8-oxo-dGTP and 8-NH$_2$-dGTP by human immunodeficiency virus type 1 and murine leukemia virus reverse transcriptases and compared them to those of DNA polymerases α and β. Initially, we carried out primer extension reactions in the presence of dGTP or a dGTP analog and the remaining three dNTPs using synthetic DNA and RNA templates. These assays revealed that, in general, 8-NH$_2$-dGTP is incorporated and extended more efficiently than 8-oxo-dGTP by all enzymes tested. Second, we determined rate constants for the incorporation of each analog opposite a template cytidine residue using steady state single nucleotide extension kinetics. Our results demonstrated the following. 1) Both reverse transcriptases incorporate the nucleotide analogs; discrimination against their incorporation is a function primarily of $K_m$ or $V_{max}$ depending on the analog and the enzyme. 2) Discrimination against the analogs is more stringent with the DNA template than with a homologous RNA template. 3) Polymerase α exhibits a mixed kinetic phenotype, with a large discrimination against 8-oxo-dGTP but a comparatively higher preference for 8-NH$_2$-dGTP. 4) Polymerase β incorporates both analogs efficiently; there is no discrimination with respect to $K_m$ and a significantly lower discrimination with respect to $V_{max}$ when compared with the other polymerases.

Cellular DNA is subject to modifications by endogenous processes as well as from exposure to exogenous agents. If unrepaired, alterations of the nucleoside residues in DNA can result in misincorporations during DNA replication. Base damage can also occur at the level of the deoxynucleoside and/or deoxynucleotide (1, 2). For these to become mutagenic they first need to be incorporated into DNA at the time of DNA synthesis. In this paper, we have investigated the in vitro utilization of two potentially mutagenic analogs of dGTP, 8-oxo-dGTP and 8-NH$_2$-dGTP, by viral and mammalian DNA polymerases that lack 3’ $\rightarrow$ 5’ exonucleolytic activity. 8,8-Dihydro-8-oxodeoxyguanosine, also referred to as 8-hydroxodeoxyguanosine (8-oxo-dG),$^1$ in cellular DNA is a byproduct of oxygen damage. Reactive oxygen species such as singlet O$_2$, hydrogen peroxide (H$_2$O$_2$), hydroxyl (OH$^-$), and superoxide (O$_2^-$) radicals, generated from the actions of ionizing radiation, chemical mutagens, and endogenous processes, are believed to be responsible for this damage. In particular, hydroxyl radicals are implicated in reactions at the C-8 position of 2’-deoxyguanosine in DNA to produce the lesion 8-oxo-dG (3, 4). Alternatively, the lesion is generated at the level of the nucleoside triphosphate, where dGTP is converted to 8-oxo-dGTP by reactive oxygen species and subsequently incorporated into DNA. Irrespective of how it is generated, the lesion in DNA is mutagenic. DNA polymerases can insert nucleotides opposite the lesion and synthesize past it (5–7). Although 8-oxo-dG can theoretically form base pairs with each of the four deoxynucleosides, most polymerases studied incorporate either dATP or dCTP opposite 8-oxo-dG, the ratio of dA/dC inserted being dependent on the type of DNA polymerase (6). Cells have evolved multiple mechanisms to remove 8-oxo-dG from DNA as well as from the nucleoside triphosphate pool (8). However, if unrepaird in DNA, mispairing with dA results in G $\rightarrow$ T transversions.

8-NH$_2$-dG is a less extensively characterized analog. It is produced in rat liver nucleic acids on administration of the hepatocarcinogen, 2-nitropropane (9). 2-Nitropropane has been hypothesized to be metabolized to hydroxylamine-o-sulfonate or hydroxylamine-o-acetate, which generate reactive nitrogen ions that aminate DNA to produce 8-NH$_2$-guanine. 8-NH$_2$-dG has been shown to terminate human leukemia cell proliferation through induction of terminal differentiation (10). However, neither the mutagenic potential of 8-NH$_2$-dGTP nor repair of the analog in DNA have been characterized. Additionally, little is known about the structure of DNA containing this adduct. 8-NH$_2$-dG in DNA is predicted to be mutagenic, mispairing with dA and dT if it is present in the syn conformation and with dA if in the imino tautomeric form.$^2$

Extensive studies have been carried out with DNA templates containing 8-oxo-dG to examine miscoding by different DNA polymerases opposite the lesion and to determine whether synthesis proceeds beyond the adduct (5–7). We report here the kinetics of incorporation of the corresponding nucleoside triphosphate analog opposite a dC residue at a defined position in a DNA and an RNA template by HIV-1 and MLV reverse transcriptases and DNA polymerases α and β. For comparison, we also examined the insertion of 8-NH$_2$-dGTP that differs in the nature of the substituent at the same position.

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§ The abbreviations used are: 8-oxo-dG, 7,8-dihydro-8-oxodeoxyguanosine; HIV-1 RT, human immunodeficiency virus type-1 reverse transcriptase; MLV RT, murine leukemia virus reverse transcriptase; pol α, DNA polymerase α; pol β, DNA polymerase β; DTT, dithiothreitol; nt, nucleotide.

1 J. Essigmann, personal communication.
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EXPERIMENTAL PROCEDURES

Materials—DNA primer and template were synthesized and high performance liquid chromatography purified by Operon Biotechnologies Inc. (Alameda, CA), whereas the RNA template was synthesized and purified (purity >95%) by National Biosciences Inc. (Plymouth, MN). [γ-32P]ATP (specific activity, 3000 Ci/mol) used for 5′-end labeling of the primer was purchased from DuPont NEN. Ultrapure deoxynucleosides and deoxynucleotides (dNTPs) were obtained from Pharmacia Biotech Inc. 8-Oxo-dGTP was synthesized by the method of Kasaai et al. (4), and 8-NH₂-dGTP was synthesized by Darwin Molecular Corp.; both analogs were high performance liquid chromatography purified and >99% homogeneous. DNA polymerase α-prime complex from calf thymus (0.05 units/μl) was kindly provided by F. W. Perrino (Wake Forest University), and DNA polymerase β from rat liver (3 mg/ml) was a generous gift of S. H. Hughes (University of Texas, Galveston). Purified recombinant HIV-1 RT (20 units/μl) was purchased from U.S. Biochemical Corp., and homogeneous, recombinant HIV-1 RT (0.5 mg/ml and composed of p51-p66 heterodimers) was a kind gift of S. H. Hughes (National Cancer Institute, Frederick, MD). T4 polynucleotide kinase was obtained from New England BioLabs Inc., and RNasin was from Promega.

Oligonucleotides—DNA primer: 46-mer, 5′ GCTAGCGGGAATTCGGCGCG 3′; DNA template: 46-mer, 5′ GCGCGGAAAGTTGCGAATATT- GCTACGGGAATTTCGCCGC 3′; RNA template: 46-mer, 5′ GCGGCGGAAGUUGGCUGCAGAAUAUUGCUAGCGGGAAUUCGGCGCG 3′. Preparation of Primer-Template for Primer Extension—The 14-mer DNA primer was phosphorylated at the 5′ end by T4 polynucleotide kinase by using standard assay conditions (11). Briefly, 25 pmol of DNA primer was phosphorylated at the 5′ end with 20 units of T4 polynucleotide kinase at 37°C for 30 min. The reaction was carried out in a final volume of 20 μl in buffer containing 70 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 5 μM DTT. Following incubation, the kinase was inactivated by heating the reaction at 95°C for 10 min to prevent 5′-end labeling of the template strand.

The labeled primer (25 pmol in 20 μl) was mixed with a 2–3-fold molar excess of either the complementary DNA or RNA template in 50 μl NuPAGE (pH 7.5, 1 mM EDTA, and 150 mM NaCl). The oligonucleotides were denatured by first heating at 95°C for 10 min, then at 75°C for 10 min, and subsequently allowed to anneal by gradual cooling to room temperature (11). RNasin (40 units), a general inhibitor of RNase activity, was added to annealed RNA-DNA hybrids to minimize degradation of the RNA template.

Primer Extension—Each primer extension reaction contained 0.25 pmol of annealed primer-template. The 14-nt primer was extended by either HIV-1 RT (0.5 μg), MLV RT (100 units), polymerase α (0.05 units), or polymerase β (0.06 μg) in the presence of 50 μM dGTP, 8-oxo-dGTP, or 8-NH₂-dGTP and 50 μM each of dATP, dCTP, and dTTP. The reaction (typically 10 μl volume) was carried out in two steps in buffer optimal for each polymerase. In the first step, only dGTP or a dGTP analog was present so that it would be preferentially incorporated into the primer. Following incubation, the reaction was terminated by the addition of an equal volume of a solution containing 20 mM EDTA, 0.05% xylene cyanole, and 0.05% bromphenol blue, and the samples were stored at −80°C until electrophoresis.

Gel Electrophoresis—To visualize extension products, aliquots of each reaction mixture were electrophoresed through 14% denaturing acrylamide gels (11). Six microliters of each sample (3 μl of the extension solution) were denatured by incubation at 75°C for 10 min prior to electrophoresis. The gels were electrophoresed at a current of ~50 mA and at a temperature of 45–55°C until leading dye front was at the bottom of the gel. Gels were dried and exposed for autoradiography at room temperature.

In single nucleotide insertion assays, extension of the 14-mer primer was measured by PhosphorImager analysis of the dried gels. Quantitation was with the PhosphorImager model 400S (Molecular Dynamics, Sunnyvale, CA) at the PhosphorImager Analysis facility (Markey Molecular Medicine Center, University of Washington). Percent extension per min was calculated from the ratio of the amount of extended products to the total amount of oligonucleotides in each lane. The rates of incorporation of analogs were normalized to reflect the concentration of extended products measured by dGTP. The apparent Kₘ and Vₘₐₓ values for the incorporation of dGTP, 8-oxo-dGTP, and 8-NH₂-dGTP were calculated from Hanes-Woolf plots. The enzyme efficiency, designated f, was calculated as Vₘₐₓ (app)/Kₘ (app) and the discrimination factor (DF) was calculated from the ratio of fₐₘₐₓ for dGTP analog.

RESULTS

We analyzed the incorporation and extension of two dGTP analogs by viral and cellular DNA polymerases. 8-Oxo-dGTP can be generated by exposure of dGTP to oxygen free radicals in vitro and in cells; 8-NH₂-dGTP, a less well characterized analog, contains an amino instead of a hydroxyl group at the same position (C-8 on guanine).

Primer Extension Analysis—Primer extension experiments were carried out with dGTP or its analogs together with the other three dNTPs. Two reverse transcriptases, HIV-1 RT and MLV RT, and two mammalian DNA polymerases, pol α and pol β, were studied. Reaction mixtures contained sufficient enzyme to elongate 90% of the primers in reactions with the four normal dNTPs, as determined in preliminary titration experiments. Fig. 1A illustrates the extension of an end-labeled DNA primer (14-mer) hybridized to a DNA template by different polymerases.

HIV-1 RT, reported to be the most error-prone reverse transcriptase (13, 14), elongated the primer extensively in the presence of only three of the four complementary dNTPs (lane 1, -dGTP or analogs). The observed pause sites occurred either before or across from template cytidine residues. Inclusion of dGTP in the reaction generated a full-length product with <10% of the primer left unextended. Substitution of 8-oxo-dGTP for dGTP dramatically reduced both the amount and the length of the elongated products. The pattern of extended products appeared identical to that observed in the (−) dGTP control suggesting that 8-oxo-dGTP is not incorporated in place of dGTP under the assay conditions. On the other hand, 8-NH₂-dGTP substituted partially for dGTP resulting in extension of approximately 10% of the primers, the extended product being nearly equal in length to that achieved with all four dNTPs. That 8-NH₂-dGTP is fact incorporated is based on the following observations. (i) The patterns of extended products are different from that obtained either in the absence of dGTP, and (ii) the mobilities of the bands on the denaturing gel are different than those of bands containing the normal dNTPs. These extension experiments suggest that whereas HIV-1 RT is unable to incorporate 8-oxo-dGTP, it is able to utilize the closely related analog, 8-NH₂-dGTP.

Extension of the primer by MLV RT is different from that by
HIV-1 RT. Consistent with the fact that MLV RT has a higher fidelity (15), there was no detectable elongation of the primer when only dCTP, dATP, and dTTP were present. Addition of dGTP in the reaction resulted in near complete extension of the primer to the full-length, 46-nt product. In fact, longer extension products (up to 49 nt) were generated presumably by the terminal addition of a limited number of nucleotide residues as has been reported by others (16). However, replacement of dGTP with either 8-oxo-dGTP or 8-NH2-dGTP completely eliminated extension of the primer indicating that under these reaction conditions, MLV RT does not significantly incorporate either of the two analogs during DNA synthesis.

The pattern of extended products obtained using the cellular DNA polymerases was similar in many respects to that obtained with the reverse transcriptases, including preferential utilization of 8-NH2-dGTP over 8-oxo-dGTP. Both pol α and pol β incorporated non-complementary nucleotides and 8-NH2-dGTP to a greater extent than the viral RTs.

Polymerase α extended the 14-mer DNA primer to predominantly 15- and 19-mers in the presence of the three dNTPs, dCTP, dATP, and dTTP. The predominance of early termination sites, particularly those opposite dC residues, is in accord with poor extension of mismatched primer termini by pol α (17). The fact that this pattern does not change upon the addition of 8-oxo-dGTP suggests that this analog is not efficiently incorporated by DNA polymerase α. In contrast, a much greater degree of extension was observed with 8-NH2-dGTP. Although no full-length product was observed with 8-NH2-dGTP, >70% of the primer was extended, and distinct bands corresponding to sizes of 15, 18, 19, 27, 29, 30, 32, and 33 nt were apparent. The altered mobility of each of these bands suggests that the extended product contains 8-NH2-dG.

Primer extension by pol β with all four dNTPs was tested in buffer containing either MgCl2 or MnCl2. Full-length extension product was observed only in reactions with MnCl2, even a 10-fold higher concentration of pol β failed to generate greater than 10% full-length product in the presence of MgCl2. Thus Mn2+ was selected as the divalent cation in all of the assays carried out with pol β. Primer extension with pol β differed from that observed with pol α. The amount of full-length product in the reaction with all four dNTPs was less than that seen with pol α; in particular, there were strong pause sites between nt 34 and 44 that were absent in the reaction with pol α. Although the indications are relatively subtle, there appeared to be insertion and extension of 8-oxo-dG, as noted by the doublet at nt 15, the high intensity band at nt 18 (greater in amount than the corresponding band in the (-) dGTP control), and faint bands around 26–30 nt long. On the other hand, as seen with pol α, extension in the presence of 8-NH2-dGTP was considerable. Despite the absence of full-length product, greater than 80% of the primer was extended, and prominent bands were observed at around residues 18, 31, and 34.

Since RNA templates are efficiently utilized by reverse transcriptases and since RNA is the initial template copied during viral replication (18), we determined whether reverse transcriptases can utilize the dGTP analogs during DNA synthesis on an RNA template (Fig. 1B). We used an RNA template with a sequence that corresponds to the DNA template to minimize the contribution of sequence context to differences in extension of the two templates.

With HIV-1 RT, extension of the labeled DNA primer on the RNA template with all four dNTPs generated a ladder of products containing 15–46 nucleotides. This series of addition products is likely due to the low processivity of reverse transcriptases on this template, although we cannot rule out the presence or generation of incomplete extension products in the reaction. Incubation of a radiolabeled RNA template with the reverse transcriptase did not result in degradation of the oligonucleotide indicating the absence of contaminating nucleases in the enzyme preparation (not shown). The products of extension by HIV-1 RT on the RNA template were generally similar to those observed with the DNA template; there was (α) misincorporation in the absence of dGTP and its analogs, (b) no extension with 8-oxo-dGTP beyond that observed without dGTP, and (c) poor overall utilization of 8-NH2-dGTP, although it appears that, once incorporated, 8-NH2-dG can be extended relatively
FIG. 2. Insertion of dGTP, 8-NH₂-dGTP, or 8-oxo-dGTP as single nucleotides by MLV RT on the RNA template. A, the 14-mer DNA primer was end-labeled and annealed to the 46-mer RNA template. The annealed primer-template was extended at 37 °C in the presence of the indicated concentrations of dGTP and 0.4 units of enzyme for 4 min, or with 8-NH₂-dGTP and 20 units of enzyme for 10 min, or with 8-oxo-dGTP and 200 units of enzyme for 20 min. After termination by the addition of stop solution, aliquots were electrophoresed on a 14% denaturing gel and exposed for autoradiography as above. B, radioactivity on the dried gel was quantitated by phosphorimage analysis. The rates of incorporation of analogs were normalized to reflect the enzyme concentration used for the incorporation of dGTP. The Hanes-Woolf plots with each dNTP are presented. The $K_m$ and $V_{max}$ values were derived from the negative x axis intercept and the inverse slopes, respectively.

The $K_m$ for incorporating a single dGTP by HIV-1 RT on the DNA template was 0.3 μM (Table I). In contrast, the $K_m$ values for incorporation of the two analogs were 75–200-fold larger. Furthermore, the $V_{max}$ for 8-oxo-dG incorporation was approximately 100-fold lower than that of dGTP. The combination of a higher $K_m$ and lower $V_{max}$ resulted in a 20,000-fold discrimination against incorporation of 8-oxo-dG relative to dG opposite the template dC residue. The discrimination against 8-NH₂-dGTP was not as large (~350-fold) primarily because the $V_{max}$ for 8-NH₂-dGTP incorporation was only about 4-fold lower than that of dGTP. Interestingly, both analogs were incorporated more efficiently by HIV-1 RT on the RNA template. The discrimination factors (relative to dGTP) were 1200 and 75 for 8-oxo-dG and 8-NH₂-dG, respectively (Table I). The 16-fold reduction in discrimination against 8-oxo-dG on the DNA versus DNA template stemmed principally from a higher $V_{max}$. On the other hand, the reduced discrimination against 8-NH₂-dG arose from a 6-fold lower $K_m$ on the RNA template. No significant differences in the $K_m$ or $V_{max}$ values for dGTP were observed on the RNA template compared with those on the DNA template.

The higher fidelity of MLV RT was also manifested in the single nucleotide addition experiments; there was greater discrimination against both of the nucleoside analogs than displayed by HIV-1 RT (Table II). The $K_m$ of 0.5 μM for inserting dG on the DNA template with MLV RT was similar to that obtained with HIV-1 RT. However, the $V_{max}$ for incorporating dGTP by MLV RT was 30-fold higher than that by HIV-1 RT. The $K_m$ values for incorporating the analogs were similar to each other (10–30 μM) and not different from those observed with HIV-1 RT. A striking feature was the marked reduction in the $V_{max}$ for both modified nucleotides relative to the $V_{max}$ for dGTP, the reduction being greatest for 8-NH₂-dGTP. The maximum rate of incorporation of 8-oxo-dG was 40,000 and that for inserting 8-NH₂-dG was 1700-fold diminished relative to dG. The rates of incorporation of the two analogs were 75–200-fold larger. Furthermore, the $V_{max}$ for 8-oxo-dG incorporation was approximately 100-fold lower than that of dGTP. The combination of a higher $K_m$ and lower $V_{max}$ resulted in a 20,000-fold discrimination against incorporation of 8-oxo-dG relative to dG opposite the template dC residue. The discrimination against 8-NH₂-dGTP was not as large (~350-fold) primarily because the $V_{max}$ for 8-NH₂-dGTP incorporation was only about 4-fold lower than that of dGTP. Interestingly, both analogs were incorporated more efficiently by HIV-1 RT on the RNA template. The discrimination factors (relative to dGTP) were 1200 and 75 for 8-oxo-dG and 8-NH₂-dG, respectively (Table I). The 16-fold reduction in discrimination against 8-oxo-dG on the RNA versus DNA template stemmed principally from a higher $V_{max}$. On the other hand, the reduced discrimination against 8-NH₂-dG arose from a 6-fold lower $K_m$ on the RNA template. No significant differences in the $K_m$ or $V_{max}$ values for dGTP were observed on the RNA template compared with those on the DNA template.

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The two analogs differ from dGTP by a single modification, either an —OH or —NH₂ substituent, at the C-8 position on guanine. Both modifications are likely to render the nucleotides mutagenic (5). Two approaches were used to analyze the incorporation of these nucleotide analogs using DNA and RNA templates. First, we examined the pattern of extension products obtained with the modified nucleoside triphosphates in the presence of the other three normal nucleotides, dATP, dCTP, and dTTP. Subsequently, we measured the kinetics of their insertion as a single nucleotide (i.e. in the absence of other dNTPs).

The kinetics of single nucleotide additions have been used extensively by Goodman and colleagues (12, 23–25) to provide a facile method for quantitating the fidelity of nucleotide insertion by DNA polymerases. We have modified this approach to analyze the insertion of nucleoside triphosphate analogs. As defined by Boosalis et al. (12) this approach requires that the enzyme and template-primer are present in saturating concentrations, with the template-primer serving to limit the extent of misincorporation. Reduced incorporation of the modified nucleotides is observed in synthetic reactions with either DNA or RNA templates and is manifest as both higher 

\[ K_m \] and lower \[ V_{max} \] values, relative to dG. Of the two analogs, HIV-1 RT exhibits a greater preference for inserting 8-NH₂-dG than 8-oxo-dG, suggesting that the catalytic center accommodates an 8-NH₂-dG:dC base pair better than an 8-oxo-dG:dC base pair.

The extremely inefficient incorporation of 8-oxo-dGTP by HIV-1 RT was not anticipated. Evidence from CD, NMR, and crystallographic studies indicates that there are no global structural changes in DNA containing an 8-oxo-dG:dC base pair (27–30). However, the conformation assumed by 8-oxo-

### Table I

**Insertion kinetics of 8-oxo-dGTP and 8-NH₂-dGTP by HIV-1 RT**

| DNA template | RNA template |
|--------------|--------------|
| dGTP | dGTP |
| 0.3 | 0.25 |
| 60 | 35 |
| 9x10⁻² | 1.5x10⁻³ |
| 2x10⁴ | 1.6 |
| 8-Oxo-dGTP | 56 |
| 10 | 8-NH₂-dGTP |
| 3 | 75 |
| 7x10⁻³ | 8.8x10² |
| 3.4x10² | 5.6x10⁻³ |
| 8-NH₂-dGTP | 4 |

### Table II

**Insertion kinetics of 8-oxo-dGTP and 8-NH₂-dGTP by MLV RT**

| DNA template | RNA template |
|--------------|--------------|
| dGTP | dGTP |
| 0.5 | 0.8 |
| 10 | 19 |
| 2.8x10² | 1.1x10³ |
| 7x10⁻³ | 5.5x10⁻⁵ |
| 9.3x10⁴ | 2.5x10⁵ |
| 8-Oxo-dGTP | 31 |
| 27 | 8-NH₂-dGTP |
| 0.16 | 1.6 |
| 6x10⁻³ | 8.8x10² |
| 9.3x10⁴ | 2.5x10⁵ |

10⁴. The preferential utilization of 8-NH₂-dGTP compared with 8-oxo-dGTP was also observed with an RNA template. Although the \[ K_m \] values were not significantly different than for the DNA template, the \[ V_{max} \] values increased by a factor of 4, 14, and 200 with dGTP, 8-oxo-dGTP, and 8-NH₂-dGTP, respectively.

Since the cellular polymerases lack the ability to synthesize DNA on RNA templates, the insertion kinetics of the dGTP analogs by pol α and pol β were measured only with the DNA template (Table III). Polymerase α displayed a striking difference in its discrimination against 8-oxo-dGTP compared with that against 8-NH₂-dGTP. The diminished utilization of 8-oxo-dGTP occurred as a result of enhanced discrimination at both the \[ K_m \] and \[ V_{max} \] levels. Pol α, like the RTs, exhibited a higher \[ K_m \] (90-fold) and a much lower \[ V_{max} \] (>1000-fold) for 8-oxo-dGTP than for dGTP resulting in a 130,000-fold bias against this nucleotide. However, unlike the RTs, pol α did not discriminate against 8-NH₂-dG through a higher \[ K_m \]; the \[ K_m \] of 2 μM was only 5-fold higher than for dGTP. Additionally, pol α exhibited only about a 10-fold lower \[ V_{max} \] value for 8-NH₂-dG, discriminating against this nucleotide by only a 60-fold factor over dGTP.

Polymerase β is reported to exhibit the lowest fidelity of all eukaryotic DNA polymerases (19, 20). In the present studies as well, pol β exhibited the lowest discrimination against both dGTP analogs when compared with DNA polymerase α or even HIV-1 RT. Pol β exhibited a \[ K_m \] of 11 μM for dGTP which is ~30-fold higher than that of pol α and in the range reported by other investigators for dGTP and other dNTPs (21, 22). The \[ K_m \] values for 8-oxo-dGTP and 8-NH₂-dGTP were, within experimental error, similar to that for dGTP suggesting that the modifications at the C-8 position of dGTP do not interfere with their interaction with pol β. The \[ V_{max} \] values, however, were decreased resulting in 400- and 50-fold differences in the efficiencies of utilization of 8-oxo-dGTP and 8-NH₂-dGTP, respectively, relative to that of dGTP. These discrimination factors are significantly lower than the corresponding values obtained with both RTs using the DNA template.

**DISCUSSION**

We examined the utilization of two dGTP analogs, 8-oxo-dGTP and 8-NH₂-dGTP, by viral reverse transcriptases and cellular DNA polymerases that lack 3’ → 5’ exonuclease activity. The two analogs differ from dGTP by a single modification, either an —OH or —NH₂ substituent, at the C-8 position on guanine. Both modifications are likely to render the nucleotides mutagenic (5). Two approaches were used to analyze the incorporation of these nucleotide analogs using DNA and RNA templates. First, we examined the pattern of extension products obtained with the modified nucleoside triphosphates in the presence of the other three normal nucleotides, dATP, dCTP, and dTTP. Subsequently, we measured the kinetics of their insertion as a single nucleotide (i.e. in the absence of other dNTPs).

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We find that HIV-1 RT, despite being highly error-prone (13, 14) and promiscuous in its substrate interactions (26), is selective in that it distinguishes between dGTP and two modified dGTP analogs, 8-oxo-dGTP and 8-NH₂-dGTP. It also discriminates between modified deoxyguanosine triphosphates that differ by a single substituent at the identical position. This position (C-8) is neither implicated in phosphodiester bond formation nor in the formation of hydrogen bonds with the complementary nucleoside, dC. Reduced incorporation of the modified nucleotides is observed in synthetic reactions with either DNA or RNA templates and is manifest as both higher \[ K_m \] and lower \[ V_{max} \] values relative to dG. Of the two analogs, HIV-1 RT exhibits a greater preference for inserting 8-NH₂-dG than 8-oxo-dG, suggesting that the catalytic center accommodates an 8-NH₂-dG:dC base pair better than an 8-oxo-dG:dC base pair.

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The assay was carried out with DNA polymerases α and β as described under “Experimental Procedures.” The nomenclatures are as described previously in Table I.

| Polymerase α | K_m (µM) | V_max (min⁻¹) | f | DF | Polymerase β | K_m (µM) | V_max (min⁻¹) | f | DF |
|--------------|---------|---------------|---|----|--------------|---------|---------------|---|----|
| dGTP         | 0.4     | 3.3 x 10⁻⁵    | 6.3 x 10⁻⁵ | 1.3 x 10⁵ | dGTP         | 11      | 1.5 x 10⁴    | 1.4 x 10⁴ | 36  |
| 8-Oxo-dGTP   | 35      | 0.23          | 6.6 x 10⁻³ | 1.3 x 10⁵ | 8-Oxo-dGTP   | 7       | 2.5 x 10⁵    | 36  | 3.9 x 10² |
| 8-NH₂-dGTP   | 2       | 27            | 13.5     | 62  | 8-NH₂-dGTP   | 6       | 1.6 x 10⁵    | 2.7 x 10⁵ | 51  |

8-Oxo-dGTP in solution can be a major factor in governing its accommodation within the active sites of DNA synthesizing enzymes. 8-Oxo-dGTP exists in an anti or syn conformation. If the energetically favorable syn conformation is accommodated and fixed within the active site of HIV-1 RT, the relaxed discrimination against 8-oxo-dGTP was meditated through higher rates of incorporation on the RNA template. Of particular significance is the rate of insertion of 8-NH₂-dGTP, which is 200-fold higher on the RNA versus DNA template.

Like the reverse transcriptases, pol α exhibits a strong bias against the incorporation of 8-oxo-dG with respect to both the K_m and V_max. The 126,000-fold discrimination is consistent with the high fidelity of pol α relative to that of HIV-1 RT and with the published reports that pol α favors both the formation and extension of 8-oxo-dG:dA rather than 8-oxo-dG:dC base pairs (32). The generally higher fidelity of pol α, presumably a manifestation of enhanced discrimination at the nucleotide binding site, does not apply to the insertion of 8-NH₂-dGTP. Pol α exhibits only a 60-fold discrimination against this analog relative to dG. In this respect, it is even more efficient than the reverse transcriptases in incorporating this analog over 8-oxo-dGTP. The facile utilization of 8-NH₂-dGTP by pol α is apparent in the primer extension assay; pol α is able to insert the analog and to extend it efficiently with the other dNTPs when it is present as an 8-NH₂-dGdC base pair at the 3′ primer terminus. Clearly, the local DNA structure is not sufficiently distorted to prevent phosphodiester bond formation with the incoming nucleotide.

DNA polymerase β is involved in DNA repair, in particular the filling of small gaps arising in base excision repair (33). It has a different primary sequence and structure than that of other DNA polymerases (34) and reverse transcriptases (35). Pol β has been shown to exhibit the lowest fidelity of all eukaryotic DNA polymerases (20, 36); this is perhaps due to a decreased ability to discriminate nucleotides at the level of binding. In accord with its low fidelity, pol β exhibits the lowest discrimination against both analogs. In our studies, it is even less accurate than HIV-1 RT is during synthesis on the DNA template. A direct comparison is not possible since Mn²⁺ was used as the metal activator in the studies with pol β; however, the error rate of pol β is similar with Mn²⁺ or Mg²⁺ as the metal activator (37).

The lack of discrimination in binding nucleotide substrates is reflected in the near identical K_m values of pol β for dGTP and its analogs. Introduction of substituent groups at C-8 on the guanine base does not perturb the interaction of pol β with either 8-oxo-dGTP or 8-NH₂-dGTP. This feature is unique to pol β. Discrimination against these nucleotides thus occurs only at the level of the V_max (like pol α, pol β shows a large preference for inserting 8-NH₂-dG during primer extension, both as a single nucleotide and in the presence of the other three dNTPs. Also, it appears to extend a terminal 8-NH₂-dGdC base pair with the same efficiency as pol α. Unlike pol α and the reverse transcriptases, however, and in accord with its lack of discrimination in binding nucleoside triphosphate substrates, pol β shows orders of magnitude higher efficiency in incorporating 8-oxo-dG on the DNA template. Repair poly-
merases like pol β have been shown to preferentially form 8-oxo-dG:dC over 8-oxo-dG:dA base pairs implying that the favorable conformation of 8-oxo-dG in the active site of pol β is the anti form (6). Since the other DNA polymerases exhibited large discriminations against inserting 8-oxo-dG in the same sequence context as pol β, it is tempting to speculate that the analog assumes the syn conformation in their catalytic centers. This would make base pairing with a pyrimidine nucleoside, like dC, a low efficiency process, as we observed in the single nucleotide insertion assays. The conformation assumed by 8-oxo-dG in its interactions with different DNA polymerases may thus contribute to enzyme fidelity. Since the syn conformation of 8-oxo-dG favors purine:purine base pairs, it would appear that reverse transcriptases preferentially form 8-oxo-dG:dA base pairs like pol α.

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