THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 9, Issue of February 28, pp. 5892–5898, 1997

Printed in U.S.A.

Incorporation of the Guanosine Triphosphate Analogs 8-Oxo-dGTP and 8-NH_2-dGTP by Reverse Transcriptases and Mammalian DNA Polymerases*

(Received for publication, June 19, 1996, and in revised form, November 22, 1996)

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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.

7,8-Dihydro-8-oxodeoxyguanosine, also referred to as 8-hydroxydeoxyguanosine (8-oxo-dG),[1] in cellular DNA is a byproduct of oxygen damage. Reactive oxygen species such as singlet O_2, hydrogen peroxide (H_2O_2), hydroxyl ($OH^{\cdot}$), and superoxide ($O_2^{\cdot-}$) 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 unrepaired in DNA, mispairing with dA results in G → T tranversions.

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 nitrenium 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.

* This work was supported by National Cancer Institute Grant CA39903 and NIAID Grant AI38180 (to L. A. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] 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.

[2] J. Essigmann, personal communication.

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-^{32}P]ATP (specific activity, 3000 Ci/mmol) used for 5′ end labeling of the primer was purchased from DuPont NEN. Ultrapure deoxynucleoside triphosphates (dNTPs) were obtained from Pharmacia Biotech Inc. 8-Oxo-dGTP was synthesized by the method of Kasai *et al.* (4), and 8-NH_2-dGTP was synthesized by Darwin Molecular Corp.; both analogs were high performance liquid chromatography purified and >99% homogeneous. DNA polymerase α-primase 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. Wilson (University of Texas, Galveston). Purified recombinant MLV RT (200 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: 14-mer, 5′ CGCGCCGAATTCCC 3′; DNA template: 46-mer, 5′ GCGCGGAAGCTTGGCTGCAGAATATTGCTAGCGGGAATTCGGCGCG 3′, RNA template: 46-mer, 5′ GCGCGGAAGCUUGGCUGCAGAAUAUUGCUAGCGGGAAUUCGGCGCG 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 primer were incubated with 1 μM (60 μCi) [γ-^{32}P]ATP in the presence of 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 $MgCl_2$, and 5 mM 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 of 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 150 mM NaCl. The oligomers 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-NH_2-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 opposite the complementary nucleotide (dC) at the +1 position of the extended product. Incubation was for 10 min at 37 °C; thereafter, a mixture of dATP, dCTP, and dTTP was added, and incubation was continued for an additional 30 min. Following incubation, the reactions were stored at −80 °C until analysis by denaturing gel electrophoresis. Additional RNasin (10 units) was included in extension reactions with the RNA template to minimize degradation during the incubation period.

The buffers used with each of the enzymes contained 0.1 mg/ml bovine serum albumin in addition to the components listed as follows. (i) HIV-1 RT, 25 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 40 mM KCl, 2 mM DTT. (ii) MLV RT, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT. (iii) DNA polymerase α, 50 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 1.5 mM DTT. (iv) DNA polymerase β, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM $MnCl_2$, 3 mM DTT.

Single Nucleotide Extension Kinetics—Assays measuring single nucleotide additions of either dGTP, 8-oxo-dGTP, or 8-NH_2-dGTP were carried out by using a protocol modified from that of Boosalis *et al.* (12). The labeled primer-template (indicated above) was mixed with unlabeled primer-template in a 1:10 ratio and used at a final concentration of 50 nM in each reaction. In preliminary experiments, reactions were carried out at saturating or near-saturating of dNTPs (50 μM dGTP or 100 μM either 8-oxo-dGTP or 8-NH_2-dGTP), and the enzyme concentration and reaction time were systematically varied. These experiments established the requisite conditions for subsequent kinetic analysis, *i.e.* linearity of incorporation with time during the fixed incubation period and utilization of less than or equal to 20% of the template-primer. Extension was monitored as a function of dNTP concentration, keeping the template and enzyme concentrations and reaction time fixed; the rate-limiting factor was the dNTP concentration. The reactions were incubated at 37 °C in a volume of 10 μl containing buffers specified above for each DNA polymerase. Following incubation, reactions were terminated by the addition of an equal volume of a solution containing 90% formamide, 20 mM EDTA, 0.05% xylene cyanol, 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 reaction + 3 μl of stop 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 the 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 enzyme in the reaction with dGTP. The apparent K_m and V_{max} values for the insertion of dGTP, 8-oxo-dGTP, and 8-NH_2-dGTP were calculated from Hanes-Woolf plots. The enzyme efficiency, designated f, was calculated as $V_{max\ (app)}/K_{m\ (app)}$, and the discrimination factor (DF) was calculated from the ratio of f_{dGTP} to $f_{dGTP\ analog}$.

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-NH_2-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. 1*A* illustrates the extension of an end-labeled DNA primer (14-mer) hybridized to a DNA template by the 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-NH_2-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-NH_2-dGTP is in fact incorporated is based on the following observations. (i) The profile of extended products is different from that obtained either in the absence or presence 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-NH_2-dGTP.

Extension of the primer by MLV RT is different from that by

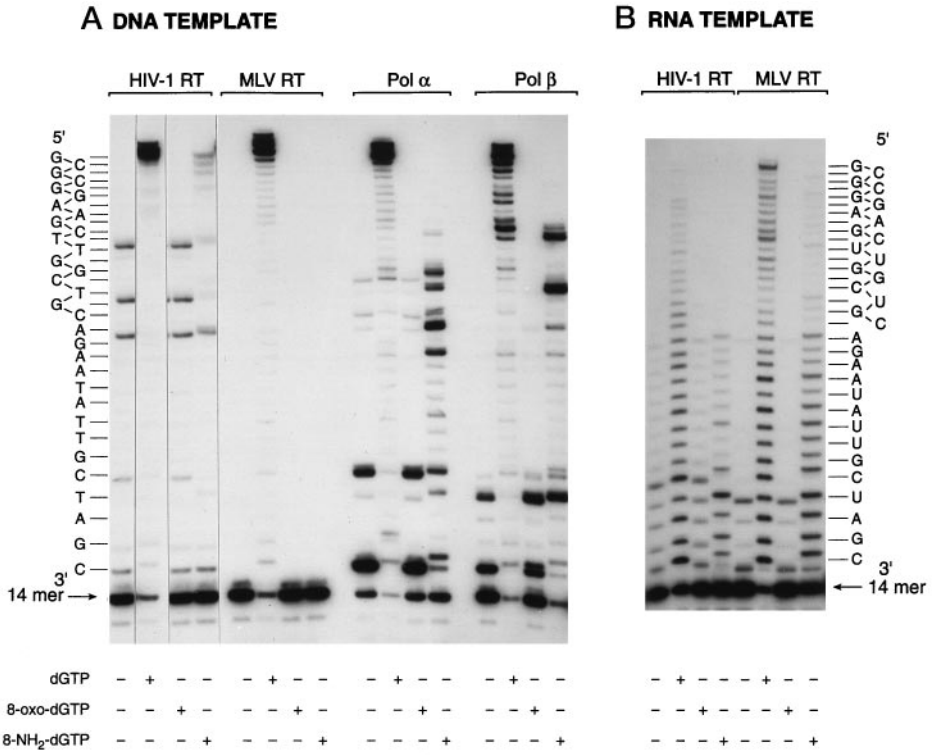

FIG. 1. **Primer extension by viral (HIV-1 and MLV RTs) and cellular (pol α and pol β) DNA polymerases.** The 5′-end-labeled 14-mer DNA primer, annealed to the unlabeled 46-mer DNA (*A*) or RNA (*B*) template, was incubated in the absence or presence of dGTP, 8-oxo-dGTP, or 8-NH_2-dGTP; subsequently, a mixture containing dCTP, dATP, and TTP was added as detailed under "Experimental Procedures." Following incubation with the indicated DNA polymerases, reaction aliquots were electrophoresed through a 14% urea polyacrylamide gel. The gel was dried and exposed to x-ray film to visualize the extension products. The resulting autoradiogram is presented. The positions of migration of the unextended primer (14 nt), the fully extended primer (46 nt), and the sequence of the template strand are indicated.

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-NH_2-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-NH_2-dGTP over 8-oxo-dGTP. Both pol α and pol β incorporated non-complementary nucleotides and 8-NH_2-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 template 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-NH_2-dGTP. Even though no full-length product was observed with 8-NH_2-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 mobilities of each of these bands suggests that the extended product contains 8-NH_2-dG.

Primer extension by pol β with all four dNTPs was tested in buffer containing either $MgCl_2$ or $MnCl_2$. Full-length extension product was observed only in reactions with $MnCl_2$; even a 10-fold higher concentration of pol β failed to generate greater than 10% full-length product in the presence of $MgCl_2$. Thus Mn^{2+} 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-NH_2-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. 1*B*). 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 (*a*) 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-NH_2-dGTP, although it appears that, once incorporated, 8-NH_2-dG can be extended relatively

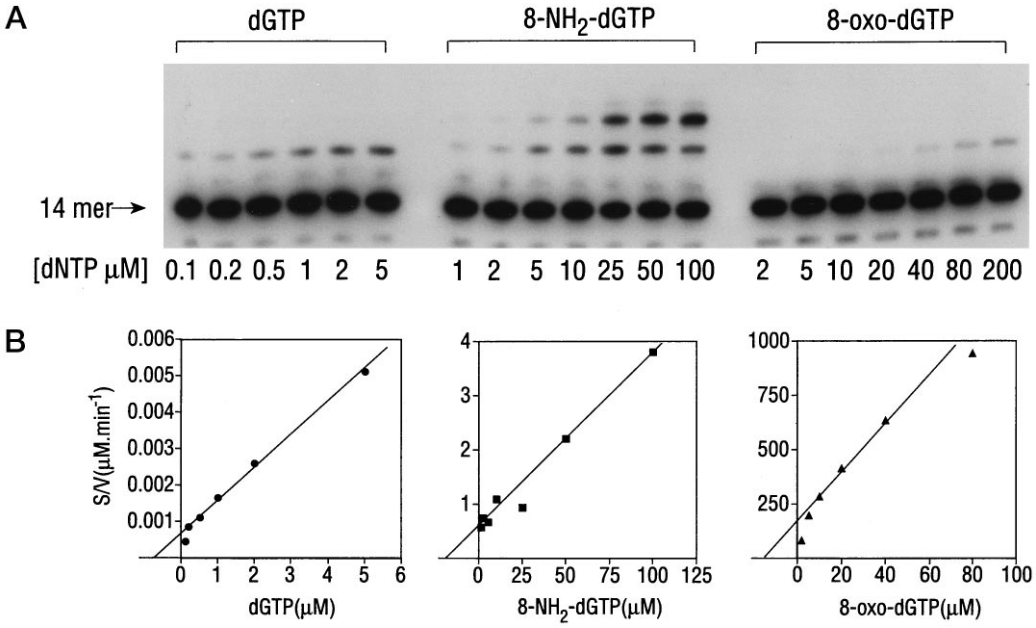

FIG. 2. **Insertion of dGTP, 8-NH_2-dGTP, or 8-oxo-dGTP as single nucleotides by MLV RT on the RNA template.** *A*, the 14-mer DNA primer was end-labeled and annealed to the 46-mer RNA template. The annealed primer-template was extended at 37 °C in the presence of the indicated concentrations of dGTP and 0.4 units of enzyme for 4 min, or with 8-NH_2-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.

efficiently (Fig. 1*B*). A similar ladder of extension products was observed during primer extension on the 16 S rRNA template. Additionally, the two analogs were utilized to the same extent (data not shown).

The results with MLV RT were also similar to those obtained with the DNA template, with one major exception. Although there was only a low extent of misincorporation in the absence of dGTP and no apparent insertion of 8-oxo-dGTP, there was a surprisingly efficient utilization of 8-NH_2-dGTP by MLV RT on the RNA template. About 40% of the primer was extended on the RNA template (Fig. 1*B*). This is in contrast to the extension profile on the DNA template where there was no trace of incorporation of this nucleotide. The utilization of the analogs was similar on the rRNA template (not shown).

Insertion of dGTP, 8-Oxo-dGTP, and 8-NH_2-dGTP as Single Nucleotides on DNA and RNA Templates—In order to determine the kinetics of incorporation of the nucleotide analogs, we used steady state conditions and measured single nucleotide additions (12). This approach avoids the complexities of analyzing multiple nucleotide addition steps and possible competition with the other dNTPs. The apparent K_m and V_{max} values for incorporating 8-oxo-dGTP or 8-NH_2-dGTP were calculated with each of the four enzymes and compared with those obtained for dGTP.

The 14-mer DNA primer was extended in the presence of increasing concentrations of either dGTP, 8-oxo-dGTP, or 8-NH_2-dGTP. The experimental conditions were such that the dNTP concentration was rate-limiting, incorporation was a linear function of time, and less than or equal to 20% of the template-primer was extended (see "Experimental Procedures"). Quantitation of the extended product by using the PhosphorImager made it feasible to calculate the kinetic constants that are summarized in Tables I–III. A representative autoradiogram showing extension with the analogs as single nucleotides and the corresponding Hanes-Woolf plots derived from phosphorimage analysis of the same gel are presented in Fig. 2, *A* and *B*, 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-NH_2-dGTP was not as large (~350-fold) primarily because the V_{max} for 8-NH_2-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-NH_2-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-NH_2-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-NH_2-dGTP. The maximum rate of incorporation of 8-oxo-dG was 40,000 and that for inserting 8-NH_2-dG was 1700-fold diminished relative to dG (Table II). As a result, the overall discrimination against 8-oxo-dGTP was 8×10^5 and that against 8-NH_2-dGTP was 9.3 $\times$

TABLE I
Insertion kinetics of 8-oxo-dGTP and 8-NH_2-dGTP by HIV-1 RT

The incorporation kinetics of dGTP and the dGTP analogs were measured from the extension of a labeled 14 mer DNA primer ("Experimental Procedures"). The rate constants were derived from Hanes-Woolf plots. K_m and V_{max} represent apparent values for the interaction and incorporation respectively, of each dNTP tested on the DNA and RNA template, as indicated. *f*, the enzyme efficiency, is the ratio of the V_{max} to K_m values, while DF, the discrimination factor, is the ratio of f_{dGTP} to f_{dGTP} analog.

	DNA template					RNA template			
	K_m	V_{max}	f	DF		K_m	V_{max}	f	DF
	μM	min^{-1}	$\mu M^{-1}min^{-1}$			μM	min^{-1}	$\mu M^{-1}min^{-1}$	
dGTP	0.3	9	30		dGTP	0.25	14	56	
8-Oxo-dGTP	60	9×10^{-2}	1.5×10^{-3}	2×10^{4}	8-Oxo-dGTP	35	1.6	4.6×10^{-2}	1.2×10^{3}
8-NH_2-dGTP	23	2	8.7×10^{-2}	3.4×10^{2}	8-NH_2-dGTP	4	3	0.75	75

TABLE II
Insertion kinetics of 8-oxo-dGTP and 8-NH_2-dGTP by MLV RT

The experimental procedure and nomenclature are as in Table I except that MLV RT was used instead of HIV-1 RT.

	DNA template					RNA template			
	K_m	V_{max}	f	DF		K_m	V_{max}	f	DF
	μM	min^{-1}	$\mu M^{-1}min^{-1}$			μM	min^{-1}	$\mu M^{-1}min^{-1}$	
dGTP	0.5	2.8×10^{2}	5.6×10^{2}		dGTP	0.8	1.1×10^{3}	1.4×10^{3}	
8-Oxo-dGTP	10	7×10^{-3}	7×10^{-4}	8×10^{5}	8-Oxo-dGTP	18	0.1	5.5×10^{-3}	2.5×10^{5}
8-NH_2-dGTP	27	0.16	6×10^{-3}	9.3×10^{4}	8-NH_2-dGTP	19	31	1.6	8.8×10^{2}

10^4. The preferential utilization of 8-NH_2-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-NH_2-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-NH_2-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-NH_2-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-NH_2-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-NH_2-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-NH_2-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-NH_2-dGTP, by viral reverse transcriptases and cellular DNA polymerases that lack $3' \rightarrow 5'$ exonuclease activity. The two analogs differ from dGTP by a single modification, either an —OH or —NH_2 substituent, at the C-8 position on guanine. Both modifications are likely to render the nucleotides mutagenic (5).[2] 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 amounts and that the rate of incorporation is limited by the nucleoside triphosphate concentration. Under steady state conditions, we compared the kinetics of incorporation of the nucleotide analogs with that of the corresponding complementary nucleoside triphosphate substrate.

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-NH_2-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-NH_2-dG than 8-oxo-dG, suggesting that the catalytic center accommodates an 8-NH_2-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 III

Insertion kinetics of 8-oxo-dGTP and 8-NH_2-dGTP by polα and polβ on the DNA template

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					Polymerase β			
	K_m	V_{max}	f	DF		K_m	V_{max}	f	DF
	μM	min^{-1}	$\mu M^{-1} min^{-1}$			μM	min^{-1}	$\mu M^{-1} min^{-1}$	
dGTP	0.4	3.3×10^2	8.3×10^2		dGTP	11	1.5×10^5	1.4×10^4	
8-Oxo-dGTP	35	0.23	6.6×10^{-3}	1.3×10^5	8-Oxo-dGTP	7	2.5×10^2	36	3.9×10^2
8-NH_2-dGTP	2	27	13.5	62	8-NH_2-dGTP	6	1.6×10^3	2.7×10^2	51

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, base pairing with dC would be reduced. The predicted interaction would be a Hoogsten type base pairing in which the bases are stabilized by two instead of three hydrogen bonds and in which there is potential for repulsion between the two carbonyl groups, making this an unfavorable interaction (27, 31). Alternatively, within the nucleotide binding site of HIV-1 RT, the addition of the carbonyl group on 8-oxo-dG may cause solvent reorganization in the vicinity of the incoming nucleotide analog and/or cause sequence-dependent changes in base stacking and base pairing strengths (30). These changes may be thermodynamically unfavorable for formation of an 8-oxo-dG:dC base pair. This is borne out in the results from the standard primer extension assays (in the presence of dCTP, dATP, and dTTP) where the large bias against 8-oxo-dGTP precludes its incorporation by HIV-1 RT during synthesis on DNA and RNA templates. The presence of the analog in the reaction, however, does not alter the extent of misincorporation of the other noncomplementary nucleoside triphosphates as reflected by the striking identity of pause sites in the absence and presence of 8-oxo-dGTP (Fig. 1, compare *lanes 1* and *3*).

MLV RT is the most accurate of the reverse transcriptases tested (15), and its fidelity even exceeds those of some cellular DNA polymerases (20). In accord, it exhibited greater selectivity for dGTP *versus* the modified dGTPs on both DNA and RNA templates than HIV-1 RT. With one exception, the discrimination against the analogs was between 50–200-fold higher with MLV RT. As opposed to HIV-1 RT, which exhibits a mixed discrimination through equivalent contributions from changes in K_m and V_{max}, MLV RT discriminates against these analogs predominantly on the basis of their maximal rates of incorporation. Like HIV-1 RT and the cellular polymerases (see discussion following), MLV RT preferentially inserts 8-NH_2-dGTP compared with 8-oxo-dGTP. Formation of the 8-NH_2-dG:dC base pair appears to be energetically more favorable than the corresponding pairing with 8-oxo-dG. These kinetic studies with single nucleotides are concordant with the extension profiles generated by MLV RT when dGTP is replaced with either of the two analogs in the standard primer extension assay (Fig. 1).

Both reverse transcriptases utilized the analogs more efficiently on the RNA template than on the DNA template, whereas utilization of dGTP was similar. Thus, discrimination against the analogs was relatively relaxed. In the case of HIV-1 RT, the 16-fold relaxation of discrimination against 8-oxo-dGTP was mediated primarily a heightened V_{max}. Apparently, formation of the phosphodiester bond involving the 8-oxo-dG:dC base pair is more favorable in the context of the RNA template, due in part to differences in the structures of RNA and DNA templates. In contrast, the 5-fold relaxation of discrimination against 8-NH_2-dGTP arose through a lower K_m, suggesting that HIV-1 RT may have a higher affinity for 8-NH_2-dGTP when bound to the RNA template. In the case of MLV RT, the relaxed discrimination against the analogs was mediated through higher rates of incorporation on the RNA template. Of particular significance is the rate of insertion of 8-NH_2-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-NH_2-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-NH_2-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-NH_2-dG:dC 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 Mn^{2+} was used as the metal activator in the studies with pol β; however, the error rate of pol β is similar with Mn^{2+} or Mg^{2+} 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-NH_2-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-NH_2-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-NH_2-dG:dC 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.

Acknowledgments—We are extremely grateful to the following investigators for their generous gifts of purified DNA polymerases and nucleotide analogs: S. H. Hughes (National Cancer Institute, Frederick, MD), F. W. Perrino (Bowling Green School of Medicine, Wake Forest University, NC), J. Tabone (Darwin Molecular Corporation, Bothell, WA), and S. H. Wilson (Sealy Center for Molecular Science, University of Texas, Galveston, TX). We thank members of the laboratory for their helpful comments and insightful discussions during the course of this work and to Ann Blank for critical reading of the manuscript.

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