Effect of Ribonucleotides Embedded in a DNA Template on HIV-1 Reverse Transcription Kinetics and Fidelity*

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Background: Under limiting dNTP concentrations, HIV-1 RT incorporates rNTPs during DNA synthesis. HIV-1 RT utilizes dNTP less efficiently around rNMPs, and mismatch extension fidelity is significantly reduced.

Results: HIV-1 RT utilizes dNTP less efficiently around rNMPs, and mismatch extension fidelity is significantly reduced.

Conclusion: Presence of an rNMP in DNA template slows HIV-1 RT-mediated DNA synthesis and reduces fidelity.

Significance: This study provides insight into how rNMP incorporation during proviral DNA synthesis can affect HIV-1 replication kinetics and fidelity.

Human immunodeficiency virus type 1 (HIV-1) uniquely infects both activated CD4⁺ T cells and terminally differentiated/non-dividing macrophages (1, 2). These cells differ in the concentrations of their intracellular deoxyribonucleoside triphosphates (dNTPs). Activated CD4⁺ T cells, which are dividing cells, contain higher levels of dNTPs (1–16 μM) as compared with terminally differentiated macrophages (20–40 μM) (3, 4). Recently, we and others reported that the low dNTP pool in macrophages is partially due to active hydrolysis of cellular dNTPs by SAMHD1, a cellular dNTP triphosphohydrolase (5, 6). Although the dNTP pools differ considerably, both cell types have similarly high concentrations of ribonucleoside triphosphates (rNTPs)² (4). In addition, it has been shown that this disparity exists in yeast (7). Because macrophages contain much lower dNTP levels, the disparity between dNTPs and rNTPs is far greater in these cells as compared with activated CD4⁺ T cells. Given that the only difference between a dNTP and an rNTP is the absence of a 2’ OH on the dNTP, it is important that DNA polymerases possess a mechanism to minimize rNTP incorporation into genomic DNA during synthesis. Indeed, most DNA polymerases, including HIV-1 RT, have evolved to discriminate against rNTPs using a bulky residue located in their active site as a steric gate (8, 9). HIV-1 RT uses a tyrosine residue at position 115 (Tyr-115) as steric gate, and mutations of this residue have been shown to promote greater rNTP incorporation (10). Despite this discriminatory mechanism, it has been shown that cellular DNA polymerases do incorporate rNTPs during DNA synthesis (7, 11, 12). We have previously demonstrated that HIV-1 RT frequently incorporates rNTPs during DNA synthesis under macrophage dNTP/rNTP conditions at ratio of 1 rNTP every 146 bases (4, 13). HIV-1 RT synthesizes the first strand (negative) proviral DNA from the viral RNA genome and the second strand (positive) DNA from the newly synthesized first strand DNA. Thus, the incorporation of rNTPs during first strand synthesis generates an rNMP-containing DNA template for second strand DNA synthesis. However, until now the mechanistic and kinetic impacts of chimeric DNA templates containing rNMPs on HIV-1 RT have not been explored.

Structural studies with DNA duplexes have shown that rNMPs embedded in DNA induce a global structural change, shifting it from a B-form helix to an A-form helix, which is more typical of dsRNA (14–16). However, another study suggested a more localized structural change around an rNMP embedded in a DNA duplex (17). More recent studies by McElhinny et al. (7, 18) on cellular DNA polymerases have shown that an rNMP present in a DNA template causes pause of a yeast replicative DNA polymerase and is mutagenic if not repaired. This is in

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‡ This article contains supplemental Tables 1 and 2 and Figs. 1–3.

§ The abbreviations used are: rNTP, ribonucleoside triphosphate; dNTP, deoxyribonucleoside monophosphate; rNMP, ribonucleoside monophosphate; AVV, avian myeloblastosis virus; T/P, template/primer; SA, streptavidin-coated; SIV, simian immunodeficiency virus.
agreement with other studies that implicatedpause sites as 
mutation hot spots for DNA polymerases, including HIV-1 RT 
(19, 20). Furthermore, it has been shown that rNMPs in dsDNA 
are targeted by RNase H2-initiated repair mechanism and 
could be mutagenic if not removed (21). However, we have 
shown that RNase H2-mediated repair for rNMPs embedded in 
DNA is significantly delayed in macrophages as compared with 
dividing cells (13).

Collectively, our previous findings indicate that HIV-1 RT 
frequently incorporates rNTPs particularly in macrophages. 
Thus, in this study we biochemically tested whether rNTPs 
icorporated during first strand proviral DNA synthesis affect 
polymerization kinetics and enzyme fidelity of HIV-1 RT during 
second strand DNA synthesis. This study provides invaluable 
aspects as to how rNMP incorporation during proviral DNA 
synthesis, which is mechanistically promoted by extremely limited canonical dNTP levels in macrophages, 
affects HIV-1 RT kinetics and enzyme fidelity.

**EXPERIMENTAL PROCEDURES**

**Protein Expression**—SIVagm Sab-1 RT gene was previously 
cloned and purified (22), and avian myeloblastosis virus (AMV) 
protein was obtained from New England Biolabs. Hexahistidine-
tagged HXB2 HIV-1 RT gene (23) was introduced into 
PET28a (Novagen) and overexpressed in BL21 *Escherichia coli* 
(Novagen). The RT protein was purified using Ni²⁺ chelating 
chromatography as described previously (24, 25). The 
concentration and purity of the protein was analyzed by 10% SDS-
polyacrylamide gel using 1.5 μg of bovine serum albumin 
(Sigma) as a control.

**Primer Extension Assay**—An HIV-1, SIVagm, and AMV RT 
primer extension assay was performed as previously described 
but with minor modifications (4). Briefly, a 17-mer primer was 
5′ end ³²P-labeled and annealed to 48-mer rNMP-containing or 
rNMP-free DNA templates in the presence of 100 mM NaCl, 10 
mm Tris-HCl (pH 8.0), and 1 mm EDTA. Reactions with a final 
volume of 20 μl contained equal amounts of RT, 10 nm template/ 
primer (T/P), and macrophage or T cell dNTP concentrations. After incubation of reactions at 
37 °C for 10, 20, or 60 min, the reactions were terminated with 
0.3 m KOH without RT for 1 h. The products were resolved on 
a 14% urea-PAGE gels under denaturing conditions and visualized by Personal Molecular Imager.

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**Surface Plasmon Resonance**—To analyze HIV-1 RT T/P interaction, we utilized a surface plasmon resonance technol-

**Processivity Assay**—A trap assay was used to examine the 
productivity of HIV-1 RT as previously described (29). Briefly, a 
17-mer primer was 5′ end ³²P-radiolabeled and annealed to 
48-mer rNMP-containing or rNMP-free template (2:1 primer 
to template ratio). The trap was prepared by annealing poly-rA 
to oligo-dT at a 1:2 ratio. RT was incubated with the annealed 
template (8 nM)/primer (16 nM) complex for 3 min at 37 °C in 
RT reaction buffer. The reaction was initiated with the addition 
of MgCl₂ (6 mM), 200-fold excess trap, and macrophage or 
T cell dNTP concentrations. After incubation of reactions at 
37 °C for 10, 20, or 60 min, the reactions were terminated with 
EDTA. For a trap control, RT was added to a mixture of T/P and 
trap, and then extension was initiated after 3 min. In the 
positive control reaction, RT was incubated with T/P, and the 
reaction was initiated in the absence of trap. The products were 
resolved on a 14% urea-PAGE gel under denaturing conditions and visualized by Personal Molecular Imager.

**Steady-state Kinetic Analysis**—The reaction condition for 
the steady-state kinetic analysis is the same as the primer 
extension reaction with minor modifications (30, 31). Briefly, the 
17-, 18-, and 19-mer primers with matched 3′ ends were 5′ end 
³²P-radiolabeled and annealed to rNMP-containing or rNMP-
free templates. For each T/P pair and dNTP to be examined, RT 
and dNTP concentrations that extend 50% of 20 nM primer
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were established. Reactions to determine the $K_m$ and $V_{\text{max}}$ were performed with the predetermined protein amount and different dNTP concentrations. To assess a mismatch extension at rNMP site using a steady-state kinetics assay, a 19-mer primer was annealed to an rNMP-containing or rNMP-free template, and the incorporation of the correct dNTP after a mismatched 3’ end of the primer was investigated. The reactions were resolved on 14% urea-PAGE under denaturing conditions and visualized by Personal Molecular Imager. The product was then quantified to determine the product formation rate, which was plotted against dNTP concentrations. The $K_m$ and $V_{\text{max}}$ were determined by fitting data obtained with the Michaelis-Menten equation ($[\text{product}] = (V_{\text{max}}[S])/(K_m + [S])$). $K_{\text{cat}}$ was calculated using the obtained values and RT concentration used in the reaction. The incorporation efficiency was determined by the expression $K_{\text{cat}}/K_m$.

Mismatched Primer Extension Assay—The assay was performed as described before but with minor modifications (32). The amount of protein that gave equal activity on matched rNMP-containing and rNMP-free T/P complex was determined by primer extension assay described above. This RT concentration was set as $1 \times$. 19-mer primers with varying 3’ end nucleotide, either matched or mismatched, were 5’-32P-radiolabeled and annealed to a 38-mer DNA template containing rNMP or dNMP at position 19 from 3’ end. These primers were then extended at 37°C with varying concentrations of RT. The protein concentrations are $1 \times$, 0.5 $\times$, or 0.25 $\times$ for matched primers and 16 $\times$, 8 $\times$, and 4 $\times$ for mismatched primers. The reactions were terminated after 5 min with the addition of 40 mM EDTA. The products were analyzed as described above. To determine the amount of product formed, the band corresponding to the extended primer and the entire lane were quantified, and percent of primers extended was calculated. This was then multiplied by the total primer concentration in the reaction (20 nM) to obtain the amount of product formed.

RESULTS

An rNMP Embedded within a DNA Template Induces Pausing of HIV-1 RT—We recently reported that HIV-1 RT incorporates rNMPs frequently at macrophage dNTP/rNTP concentrations but not at activated CD4+ T cell concentrations. This is due to the higher discrepancy between dNTP and rNTP levels in macrophages (4). Also, previously published studies have shown that the presence of rNMPs in a DNA template causes DNA polymerases to pause during DNA synthesis (7, 23). Thus, we tested whether the presence of rNMPs in DNA templates also has the same pausing impact on HIV-1 RT-mediated DNA synthesis. For this test we performed a time course primer extension assay of HIV-1 RT on 48-nucleotide DNA templates containing a single rNMP (rAMP, a purine, or rUMP, a pyrimidine) at position 23 from 3’ end of the template (see N in Fig. 1A and B, and supplemental Table 1 showing sequences of all templates and primers used) under macrophage (20–40 nM) or T cell (2–5 $\mu$M) dNTP conditions. As shown in Fig. 1A, at the macrophage dNTP concentration, HIV-1 RT generated two paused products near the rNMP site (N site: arrows 1 and 2), whereas no evident RT pausing was observed in the reaction with the template containing normal dAMP at the correspond-
FIGURE 1. HIV-1 RT-mediated extension of DNA primers annealed to rNMP-containing templates. A 5' end 32P-labeled DNA primer (10 nM) annealed to a 48-mer DNA template containing normal dAMP or rAMP at the 23rd position from the 3' end (N: see Supplemental Table 1 for sequences) was extended with an equal activity of HIV-1 RT protein (green circle) for indicated time points. The reactions were conducted under macrophage dNTP pool (20 – 40 nM) (A) or activated T cell dNTP (2–5 μM) pools (B). C, primer extension on DNA templates containing rAMP at the 33rd position (lane 5–7) was conducted under macrophage dNTP pool for indicated duration. D, a 5' end 32P-labeled 48-mer rAMP or dATP-containing DNA template annealed to the 23-mer DNA primer was incubated with HIV-1 RT, KOH, or none (C) for 60 min. The products were resolved by 14% denaturing PAGE and analyzed. F indicates fully extended product, P indicates an unextended primer, and CP indicates cleaved product. The arrow shows the pausing sites. The letter N in the T/P diagram shows the location of embedded rAMP (red) and corresponding dAMP (blue). The template sequences from the 3' end of the annealed primer are marked at the sides of the gels. E, shown are circular dichroism results comparing a 22-mer primer annealed to 48-mer rNMP-free (black solid line) or rNMP-containing (red dashed line) template. The wavelength-showing discrepancy is marked as gray bars.
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plate, we utilized CD, which has been employed to monitor changes in the structure of DNA (16, 33). Because one of the pause sites was observed at the position corresponding to rNMP present in the DNA template, we analyzed the structure of the same DNA template while annealed to a 22-mer primer (see the diagram in Fig. 1E). In this configuration the rNMP will be at the position of RT active site and will be in the single-stranded region of the T/P duplex. Although rAMP is present in a single strand region of the T/P, the CD spectra did show an elevated ellipticity near wave length 280 nm (see box in Fig. 1E), that is indicative of a structural shift toward an A-form helix, and this is a similar effect of rNMP as previously observed when in duplex DNA (16). In addition, we observed a similar shift when rAMP is within the duplex region of our T/P (supplemental Figs. 2, A and B). Our CD data suggest that the observed two pause sites could occur due to DNA structural change by the embedded rNMP, which may affect the interaction of RT with template and dNTP incorporation kinetics. Collectively, the data shown in Fig. 1 support that rNMPs embedded in DNA template induces HIV-1 RT pausing more frequently in limited dNTP pools, possibly due to local structural changes in the T/P, which may slow down the overall HIV-1 RT-mediated DNA synthesis. Importantly, it also suggests that HIV-1 RT could bypass the rNMP-induced pausing when RT kinetics is optimal due to abundant dNTPs.

Effect of an rNMP Embedded within a DNA Template on T/P Binding of HIV-1 RT—Although previously it had been shown that HIV-1 RT binding affinity to DNA-primed DNA and RNA templates are similar (34), HIV-1 RT may interact with an rNMP-containing DNA template differently, particularly when the 3′ end of the primer lies near the rNMP site (N site). In addition, the structural change observed previously and, with our CD data combined with the pause site observed during primer extension, prompted us to test whether the embedded rNMP can alter the T/P binding kinetics of HIV-1 RT. The overall T/P binding affinity of polymerases is determined by two opposing events. Initial binding was measured by the association rate or on-rate (kₐ or kₐ off), and release from T/P, was measured by the dissociation rate or off-rate (kₐ or kₐ off). Together this results in the binding affinity (Kₐ), which is determined by kₐ/kₐ off (35, 36). To examine these parameters, we employed a surface plasmon resonance-based biosensor technology, which has been used previously for the same purpose (35). We determined the Kₐ values of HIV-1 RT with primers aligned at the two paused products, marked by arrows 1 and 2 in Fig. 1, which were generated by the delayed incorporation at the −1 and +1 sites relative to the N site (see diagram in Table 1 and supplemental Fig. 3). As shown in Table 1 and supplemental Fig. 3, when the 3′ end of the primer is aligned at the positions corresponding to the site of pause products, the experimentally determined kₐ, kₐ off, and Kₐ values of HIV-1 RT to T/P were not significantly altered by presence of rNMP. Therefore, the data presented in Table 1 and supplemental Fig. 3 together with the CD data indicate that although an rNMP embedded in DNA template alters the T/P structure, this alteration does not significantly affect the T/P interaction of HIV-1 RT. Furthermore, because the T/P binding kinetics of HIV-1 RT at the rNMP-mediated paused products were not altered by the rNMP, this supports that the T/P binding step of HIV-1 RT is not a part of the mechanistic cause for the rNMP-mediated pausing of HIV-1 RT.

### Table 1

The Kₐ, Kₐ off, and Kₐ values of HIV-1 RT on rAMP-containing and rAMP-free templates

| Primer and \( \text{rNMP} \) position | Nucleotide at (N) | \( Kₐ \) (M⁻¹S⁻¹) | \( Kₐ \) (M) | \( Kₐ/Kₐ \) (M⁻¹S⁻¹) |
|-------------------------------|-----------------|-----------------|--------------|---------------------|
| \( \text{dAMP} \) | \( \text{dAMP} \) | 480.1±27.6 | 0.033±0.01 | 1473.8± 2585 (4s) |
| \( \text{rAMP} \) | \( \text{rAMP} \) | 205.1±27.6 | 0.084±0.01 | 3503.6± 285 (4s) |
| \( \text{dTTP} \) | \( \text{dTTP} \) | 558.6±37.3 | 0.035±0.01 | 9973.9± 578 (1s) |
| \( \text{rAMP} \) | \( \text{dTTP} \) | 402.0±32.2 | 0.054±0.009 | 7534.0± 443 (1s) |
| \( \text{dCTP} \) | \( \text{dCTP} \) | 390.0±64.0 | 0.068±0.001 | 5429.7± 5002 (6s) |

* The dashed arrow indicates the position of rNMP relative to RT active site. The solid arrow indicates the position of RT active site.

** Kₐ values were obtained from data fitted with the Michaelis-Menten equation.

** Kₐ values were derived from equation \( Kₐ = V_{max}/E \), where \( V_{max} \) is maximum velocity (obtained from graph), \( E \) is enzyme concentration and \( t \) is time in minutes.

### Table 2

Steady-state kinetics values of HIV-1 RT for incorporation of correct dNTP on rNMP-containing and rNMP-free DNA templates

| T/P layout | Nucleotide at (N) | dNTP | \( Kₐ \) (M⁻¹S⁻¹) | \( Kₐ \) (M) | \( Kₐ/Kₐ \) (M⁻¹S⁻¹) |
|------------|-----------------|-----------------|--------------|---------------------|
| \( \text{dAMP} \) | \( \text{dAMP} \) | 480.1±27.6 | 0.033±0.01 | 1473.8± 2585 (4s) |
| \( \text{rAMP} \) | \( \text{rAMP} \) | 205.1±27.6 | 0.084±0.01 | 3503.6± 285 (4s) |
| \( \text{dTTP} \) | \( \text{dTTP} \) | 558.6±37.3 | 0.035±0.01 | 9973.9± 578 (1s) |
| \( \text{rAMP} \) | \( \text{dTTP} \) | 402.0±32.2 | 0.054±0.009 | 7534.0± 443 (1s) |
| \( \text{dCTP} \) | \( \text{dCTP} \) | 390.0±64.0 | 0.068±0.001 | 5429.7± 5002 (6s) |

* The dashed arrow indicates the position of rNMP relative to RT active site. The solid arrow indicates the position of RT active site.
and rAMP sites (9973.9 ± 578 and 7534.0 ± 443 μM⁻¹ min⁻¹, respectively), suggesting that the rAMP in the template does not affect the dNTP incorporation efficiency of HIV-1 RT at the actual site complementary to the rAMP. In contrast, when we measured the dNTP incorporation efficiency of HIV-1 RT when the rNMP is present at the +1 or −1 sites relative to the rAMP (N) site, where Fig. 1 showed the delayed kinetics and consequent generation of paused products, HIV-1 RT showed a 4- and 6-fold reduction in the dNTP incorporation efficiency (Table 2). In addition, HIV-1 RT displays a 3-fold reduction in dNTP incorporation efficiency at the −1 position with respect to rUMP (supplemental Table 2), a position corresponding to where delayed kinetics and consequent generation of paused product were observed (supplemental Fig. 2). Interestingly, although the $K_{cat}$ values of HIV-1 RT at the −1 and +1 sites were not affected by the rAMP, the $K_m$ values were increased at those sites on rAMP containing template as compared with rAMP-free template. Thus, this kinetic finding supports that the rNMP-mediated pausing of HIV-1 RT at the −1 and +1 positions was generated by inefficient dNTP incorporation, leading to a severe kinetic block at those sites when the dNTP availability become limited (below the $K_m$ values).

Next, we tested if the rAMP embedded in DNA template directly affects the DNA synthesis rate at low macrophage and high T cell dNTP concentrations during single round primer extension. To establish the single round primer extension, we employed a molar excess of unlabeled T/P trap, which captures the HIV-1 RT molecules released from the template after the initial primer extension and prevents the rebinding of RT to the 5′ end 32P-labeled T/P thus limiting the extension to a single round (37–39). As shown in Fig. 2, at both low macrophage (Fig. 2, A and C) and high T cell (Fig. 2, B and D) dNTP concentrations, without the trap (−T), a large amount of fully extended products was observed due to the multiple rounds of primer
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extension. However, when HIV-1 RT was preincubated with the trap before the primer extension (TC), no product was observed, showing that the trap was successfully able to capture and prevent HIV-1 RT from initiating the DNA synthesis. For the single round of the primer extension, the primer extension reactions were initiated by mixing the HIV-1 RT, which was prebound to the 5’ end 32P-labeled T/P, with a mixture of dNTPs (macrophage or T cell concentration), Mg2+, and the unlabeled T/P trap and terminated at three different time points (T1-T3). The reactions with the rAMP containing T/P produced strong pauses (see the arrows) but only at the macrophage/low dNTP concentrations (Fig. 2A), which is similar with what we observed during the multiple rounds of the primer extension. However, no pausing was observed with the dAMP-containing template regardless of the dNTP concentrations used. More importantly, as seen in the level of the fully extended products (F in Fig. 2A) and quantified product (Fig. 2C), the reactions with the rAMP-containing T/P at the macrophage dNTP concentrations generated reduced fully extended product compared with the reactions with the dAMP-containing T/P, supporting that the rAMP embedded in the DNA template restricts the processive DNA synthesis of HIV-1 RT at limiting dNTP concentrations. In contrast, under T cell dNTP concentrations no pause site was observed, and the fully extended product was similar on both rNMP-containing and rNMP-free templates (Fig. 2, B and D). Interestingly, the paused products in the rAMP containing T/P (Fig. 2A) gradually decreased during incubation under the single round primer extension condition. In addition, fully extended product on the rAMP-containing template continued to increase in a time-dependent manner (Fig. 2C), suggesting that some of the HIV-1 RT molecules, which paused at the −1 and +1 sites relative to rNMP, remained bound to the template and were able to continue extending the prematurely terminated primers at the pause sites. Basically, this kinetic study supports that the rNMPs in DNA template can further slow down the processive DNA polymerization kinetics of HIV-1 RT at low dNTP concentrations.

Effect of an rNMP in the Template DNA on Enzyme Fidelity of HIV-1 RT—It has been reported that the rNMPs contained in DNAs are mutagenic, and cells harbor a repair system with RNase H2 that specifically removes rNMPs molecules in DNA duplex incorporated by cellular DNA polymerases during DNA replication (18, 40, 41). Thus, we tested whether rNMPs embedded in DNA templates could become mutational hotspots by affecting the enzyme fidelity of HIV-1 RT at the rNMP site by using a steady-state single nucleotide fidelity assay. The complete synthesis of a single mutation during DNA polymerization consists of two sequential mechanistic steps; 1) misinsertion (incorporation of incorrect dNTPs) and 2) extension of the mismatches generated by the misinsertion (32). Therefore, first we measured the misinsertion fidelity of HIV-1 RT during dNTP incorporation opposite a dAMP or rAMP. For the dAMP site, the misinsertion fidelity for the incorrect dGTP was 6.6 × 104. When the misinsertion fidelity was measured with the rAMP-containing template, similar misinsertion fidelity was observed (Table 3). This suggests that an rNMP embedded in the DNA template does not alter the misinsertion fidelity of HIV-1 RT at the N site.

Next, we compared the capability of HIV-1 to extend matched and mismatched primers at the N site using the multiple nucleotide incorporation assay. In this assay the 5’ end-matched and -mismatched primers annealed to the rAMP- or dATP-containing DNA template at the N site were extended by HIV-1 RT. As shown in Fig. 3, A and B, HIV-1 RT showed similar matched primer extension capability, which was assessed by the amount of the full-length product (F), with both rAMP-containing and rAMP-free templates. However, when provided with mismatched primers on templates containing rAMP at the N site, HIV-1 RT showed enhanced primer extension on rAMP-containing template as compared with rAMP-free templates (Fig. 3, A and B). Thus the data presented in Tables 2 and 3 combined with Fig. 3 suggest that the rNMPs

| T/P layout | dNTP | $K_{cat}$ (min$^{-1}$) | $K_{d}$ (nM)$^a$ | $K_{cat}/K_{d}$ (min$^{-1}$ nM$^{-1}$) | Misinsertion fidelity |
|------------|------|---------------------|----------------|------------------------------------|---------------------|
| rAMP      | dATP | 25.69 ± 0.7         | 1999 ± 1.0     | 20.7 ± 0.7                          | 6.3 × 10$^4$       |
| rAMP      | dGTP | 26.69 ± 0.7         | 233 ± 1.0      | 22.7 ± 1.0                          | 6.3 × 10$^4$       |

$^a$ $K_{cat}$ values were obtained from data fitted with Michaelis-Menten equation.

| Primer 3’ end (N) | dNTP | $K_{cat}$ (min$^{-1}$) | $K_{d}$ (nM)$^a$ | $K_{cat}/K_{d}$ (min$^{-1}$ nM$^{-1}$) | Mismatch extension fidelity |
|-------------------|------|---------------------|----------------|------------------------------------|---------------------|
| dGTP              | 170 ± 0.7 | 0.37 ± 0.06 | 1.5 × 10$^2$ |
| dATP              | 203 ± 0.7 | 0.54 ± 0.06 | 1.6 × 10$^2$ |

$^a$ $K_{cat}$ values were derived from equation $K_{cat} = V_{max}/[E]_t$, where $V_{max}$ is maximum velocity (obtained from graph), [E] is enzyme concentration and t is time in minute.

$^b$ Mismatch extension fidelity = (Kcat/Kd)correct/(Kcat/Kd)incorrect.

$^c$ dGMP values were obtained from data fitted with Michaelis-Menten equation.

$^d$ dAMP values were derived from equation $K_{cat} = V_{max}/[E]_t$, where $V_{max}$ is maximum velocity (obtained from graph), [E] is enzyme concentration and t is time in minute.
embedded in the DNA template alter the enzyme fidelity of HIV-1 RT by decreasing the mismatch extension fidelity.

Effect of rNMPs in DNA Template on Pausing of Other Reverse Transcriptase—Next, we tested whether the rNMP-induced pausing, which was observed with HIV-1 RT, is also observed with other retroviral RTs. For this test we first employed RT of the lentiviral simian immunodeficiency virus (SIVagm Sab-1 (SIV)). As shown in Fig. 4A, SIV RT also generated the two paused products near the N site of the rNMP-containing template but only at macrophage dNTP concentrations, which is the same pattern as HIV-1 RT (Fig. 1). Finally we tested the rNMP-mediated pausing of AMV RT (oncoretrovirus). Importantly, unlike lentiviral RTs, which are functional in both high dividing cell and low non-dividing cell dNTP concentrations, oncoretroviral RTs are not functional at the low non-dividing cell dNTP concentration because oncoretroviruses do not replicate in non-dividing cells such as macrophages (42, 43).

In addition we have shown biochemically that oncoretrovirus RTs, including AMV RT, function poorly in low dNTP concentration (30). Previously, it has been shown that a yeast replicative DNA polymerase, which normally functions with abundant dNTPs, pauses under high dNTP concentrations (7). This prompted us to investigate whether AMV RT may pause on the rNMP-containing template under the dNTP conditions in which it normally functions. Thus we tested the impact of rNMP on AMV RT at only high dNTP concentration found in T cells. Indeed, as shown in Fig. 4B, AMV RT did not display any pausing near the N site. Thus, the data shown in Fig. 4 demonstrate that the rNMP-mediated pausing, which can induce kinetic delay of the DNA synthesis, is specific for the lentiviral RTs such as HIV-1 and SIV RT proteins.

**DISCUSSION**

DNA polymerases have evolved to maintain an effective “gate” mechanism that prevents the entry of cellular rNTPs, which tends to be at 100–1000 times higher concentrations than cellular canonical dNTPs, to the active site of DNA polymerases. This mechanism is engineered by the molecular clash between the conserved residues of DNA polymerases near the dNTP binding site and the 2’OH of the rNTP (8, 12). However, this mechanism appears not to be completely successful, leading to the incorporation of noncanonical cellular rNTPs during chromosomal DNA replication. This is apparent because almost all organisms maintain a specific cellular repair mechanism that removes single rNMPs embedded in dsDNA initiated by RNase H2. The ubiquitous nature of the RNase H2 repair system strongly supports that the rNMP molecules embedded in DNA significantly impacts cellular DNA metabolisms (41, 44). It has been reported that host DNA polymerases also pause near the sites of DNA-containing rNMPs (7). Two possible consequences that can be induced by rNMP-induced pausing are 1) the kinetic delay of chromosomal replication and 2) mutation synthesis.
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Our previous study reported that HIV-1 frequently incorporates rNTPs during proviral DNA synthesis with a rate of 1/146 in macrophages due to a greater discrepancy between cellular dNTP and rNTP concentration but not in dividing cells with much smaller rNTP/dNTP discrepancy (13). The incorporation of rNMPs during the first (+) strand synthesis produces DNA templates containing rNMPs, which will be used for the second (+) strand synthesis. Our followup study here clearly demonstrates that HIV-1 RT also pauses near the rNMP sites of the DNA template, which can delay viral replication kinetics, and the pauses occur due to the delayed dNTP incorporation kinetics at one nucleotide before and after the rNMP site instead of the interrupted T/P interaction of HIV-1 RT. Furthermore, HIV-1 RT becomes more error-prone specifically at the rNMP sites specifically by facilitating the mismatch extension capability. Our data also suggest that it is plausible that the rNMP-mediated kinetic delay and fidelity change may be mechanistically related with the local structural change of the T/P near the rNMP site. The rNMP-induced pausing was also observed for SIV RT at the limited dNTP pools, suggesting that consequences of the rNMP incorporation are common for lentiviral RTs. The oncoretroviral AMV RT, which is active only with high dNTP concentrations, failed to generate the rNMP-initiated pausing under these conditions.

We recently reported that the RNase H2-mediated repair function is significantly less in macrophages as compared with the activated CD4+ T cells and other dividing cells (13). Thus, we envision the postulated kinetic delay and potential mutagenic consequence of the rNMP incorporation could be mechanistic bottlenecks that lentiviruses encounter in macrophages. Our biochemical simulation in Fig. 1 clearly demonstrates that the rNMP-mediated pausing disappears when HIV-1 RT synthesizes DNA at a fast rate with elevated dNTP concentrations. In fact, we and others recently reported that the limited dNTP pool found in macrophages is due to the expression of host restriction factor, SAMHD1, which is a dNTP triphosphohydrolase (5, 45). Indeed, we also reported that HIV-2 and some SIVs encode an accessory protein, Vpx, that antagonizes the anti-viral function of SAMHD1 by proteasomal degradation. This led to the elevation of cellular dNTPs in macrophages (5, 46), dendritic cells (47), and resting T cells (48, 49), which ultimately rescued the delayed reverse transcription process. Previously, we have shown that dNTP levels in macrophage are lower than the $K_m$ values of RT (4, 50). However, Vpx-mediated degradation of SAMHD1 elevates dNTP levels above the $K_m$ values of RT (5, 50), accelerating proviral DNA synthesis (46). Therefore, we further envision that Vpx-induced elevation in the non-dividing target cell types enables RTs to effectively overcome the rNMP-induced pausing as shown in Fig. 1B and consequently alleviating the kinetic delay in proviral DNA synthesis.

In summary, we have demonstrated that DNA templates containing rNMPs slow down HIV-1 RT-mediated DNA synthesis under limiting dNTP concentrations, and this impact is reversed under elevated dNTP concentrations. In addition, we show that rNMPs embedded in DNA templates decreases RT fidelity. Overall, this study demonstrates the effect of chimeric DNAs, which are generated during the first strand of proviral DNA synthesis, on HIV-1 replication kinetics and mutagenesis, particularly in macrophages.

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