Reduced dNTP Interaction of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Promotes Strand Transfer*

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We have recently demonstrated that HIV-1 RT mutants characterized by low dNTP binding affinity display significantly reduced dNTP incorporation kinetics in comparison to wild-type RT. This defect is particularly emphasized at low dNTP concentrations where WT RT remains capable of efficient synthesis. Kinetic interference in DNA synthesis can induce RT pausing and slow down the synthesis rate. RT stalling and slow synthesis rate can enhance RNA template cleavage by RT–RNase H, facilitating transfer of the primer to a homologous template. We therefore hypothesized that reduced dNTP binding RT mutants can promote template switching during minus strand synthesis more efficiently than WT HIV-1 RT at low dNTP concentrations. To test this hypothesis, we employed two dNTP binding HIV-1 RT mutants, Q151N and V148I. Indeed, as the dNTP concentration was decreased, the template switching frequency progressively increased for both WT and mutant RTs. However, as predicted, the RT mutants promoted more transfers compared with WT RT. The WT and mutant RTs were similar in their intrinsic RNase H activity, supporting that the elevated template switching efficiency of the mutants was not the result of the mutations enhancing RNase H activity. Rather, kinetic interference leading to stalled DNA synthesis likely enhanced transfers. These results suggest that the RT-dNTP substrate interaction mechanistically influences strand transfer and recombination of HIV-1 RT.

Mechanisms involved in the interaction of DNA polymerases with dNTP substrates have been extensively studied. In general, DNA polymerases contain dNTP binding pockets that consist of residues that form direct or indirect contacts with different moieties on the dNTP substrate during initial dNTP binding, subsequent conformational change, and chemical reaction (1–4). Human immunodeficiency virus type 1 (HIV-1)3 reverse transcriptase (RT) uniquely interacts with the sugar moiety of the incoming dNTP (5). The side chain of the Gln151 (Q151) residue of HIV-1 RT forms an H-bond with the 3’-OH of the incoming dNTP (6, 7). In the Q151N mutant, this interaction is disrupted because of the shorter side chain of Asn compared with Gln. Accordingly, the Q151N mutant displays greatly reduced binding affinity for dNTP substrates, albeit without affecting chemical catalysis ($k_{cat}$) (6, 8).

One major consequence of the reduced dNTP binding affinity is poor DNA synthesis at low dNTP concentrations (9). Whereas WT HIV-1 RT efficiently synthesizes DNA even at low dNTP concentrations, mutants with reduced dNTP binding, like Q151N RT, are only fully active at high dNTP concentrations (9). The dNTP binding step ($K_{d}$) of HIV-1 RT likely becomes rate-limiting in the polymerization reaction with the Q151N mutant at low dNTP concentrations (8). We identified a second HIV-1 RT mutant with low dNTP binding, V148I (10). Structural modeling suggested that the V148I mutation, which lies near the Gln151 residue, likely positions the Gln151 residue away from the active site, preventing the Gln151 residue from forming an H-bond with the 3’-OH of the incoming dNTP, consequently reducing the dNTP binding affinity (6). Both these mutant viruses display restricted DNA synthesis at low dNTP concentrations, but show WT-like high DNA synthesis activity under saturating dNTP conditions (9).

HIV-1 is well known to infect both activated CD4+ T cells and terminally differentiated macrophage, which contain high and low cellular dNTP concentrations, respectively (9). We recently demonstrated that HIV-1 variants harboring reduced dNTP binding mutant RTs fail to infect macrophage (~0.05 μM dNTP) even though these mutant viruses are capable of infecting T cells (~5 μM dNTP) (9). Another retrovirus, MuLV, infects only actively dividing cells containing high dNTP concentrations (11, 12). Our recent studies demonstrate that MuLV RT has between a 6- and 121-fold lower dNTP binding affinity than WT HIV-1 RT, depending on the dNTP (13). These studies suggest that the tight dNTP binding affinity of WT HIV-1 RT contributes to the efficient replication of HIV-1 in macrophage containing low dNTP concentrations as well as dividing CD4+ T cells. Consistent with these biochemical observations, HIV-1-based vectors containing the Q151N mutant RT preferentially transduce cells containing very high dNTP concentrations such as tumor and transformed cell lines, but fail to transduce normal human cells such as human lung fibroblasts and keratinocytes containing relatively lower dNTP concentrations than tumor cell lines (14).
dNTP Interaction Mechanism of HIV-1 RT and Strand Transfer

All retroviral virions, including HIV-1, contain two copies of a highly structured RNA. It has been long studied that the sequence and secondary structure of an RNA template can interrupt processive DNA synthesis of RTs, which can lead to stalling of reverse transcription (15–17). Stalling of synthesis promotes local degradation of the RNA template by the RT–RNase H activity (17, 18). Degradation of the original (donor) template allows DNA synthesis to continue only if the nascent DNA anneals to a second homologous RNA template (acceptor). It has been demonstrated that template-switching between two non-identical RNA genomes contained within the virion contributes to viral recombination, promoting viral genomic diversity and viral (19–22). Studies have shown that retroviral recombination events can contribute to progeny virus with high and multidrug resistance (21, 22).

Indeed, RT pause-induced template switch is one of the best studied mechanisms of the viral recombination process (18, 23–25). In addition to RNA template sequence and secondary structure, restricting dNTP incorporation kinetics of RTs can also interrupt reverse transcription and promote RT pausing. Previous reports have shown that RT pausing induced by restricting dNTP substrate availability elevates template switch efficiency of HIV-1 RT (18, 26).

In this study, we address the role of RT-dNTP interactions in HIV-1 reverse transcription and recombination. Since the two reduced dNTP binding HIV-1 RT mutants, Q151N and V148I, display kinetic interruption in reverse transcription at low dNTP concentrations compared with WT HIV-1 RT, we postulated that these mutant RTs would have elevated strand transfer efficiency at physiological dNTP concentrations, compared with WT HIV-1 RT. Biochemical data presented in this study support that the dNTP interaction of HIV-1 RT can mechanistically influence strand transfer efficiency of HIV-1 RT, and in turn, HIV-1 recombination.

EXPERIMENTAL PROCEDURES

Cells, Plasmids, and Chemicals—Escherichia coli XL-1 Blue (Stratagene) was used during the construction of NL4-3 WT and mutant RT expression plasmids; E. coli Rosetta II (DE3) pLysS (Novagen, WI) was used for the overexpression of all RT proteins. The pD3-GFP series of plasmids used as the templates for strain NL4-3 RT genes has been previously described (9). Plasmid pET-28a (Novagen) was used in the construction of NL4-3 RT expression plasmids. DNA oligomers used in this study were obtained from Integrated DNA Technologies (IDT). [γ-32P]ATP was obtained from PerkinElmer Life Sciences. The 72 amino acid nucleocapsid (NC) protein was chemically synthesized as described by de Rocquigny (27) and was a kind gift from Dr. Bernard P. Roques.

Preparation of HIV-1 RT Expression Plasmids—To create expression plasmids encoding wild-type and mutant RTs, the regions containing the RT gene of plasmids pD3-GFP, pD3-GFP-VI, and pD3-GFP-QN (9) were each amplified with primers NL4RT NdF 1F’-AAAAAATAATGC-CCATTTAGTCCTATGGAGAC-3’ and NL4RT C-T HIIIR 5’-AAAAAAGCTTATATGGACTTCTGACAG-3’ (sequences encoding the restriction sites for Ndel and HindIII underlined). Amplified RT genes were inserted into the backbone of pET-28a using Ndel and HindIII. Confirmation of correct entire RT inserts was achieved through sequencing with T7 promoter and T7 terminator primers (Macrogen).

RT Purification—Purification of hexahistidine-tagged RT proteins was performed using protocols, reagents, and buffers provided by the manufacturer (Novagen) as previously described (28) with some modification. Briefly, expression plasmids were transformed to Rosetta II (DE3) pLysS via heat shock. Transformed bacteria were grown in shaking culture at 37 °C to an A600 = 0.2 and then induced with isopropyl-1-thio–β-d-galactopyranoside (1 mM final concentration). Cultures were allowed to shake at 37 °C for an additional 3 h to allow protein expression. Bacteria were then harvested and cell pellets were resuspended in 1× binding buffer and stored overnight at −70 °C. Frozen pellets were thawed on ice for 2 h, and then centrifuged 25 min (12,000 × g at 4 °C). Supernatants were applied to Ni2+ charged resin for Ni2+ chelation chromatography as previously described (28, 29). Purified protein was then dialyzed against 1× dialysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 200 mM NaCl, 10% glycerol) overnight at 4 °C. Purified proteins were then dialyzed for an additional 3 h against 1× dialysis buffer with 1 mM dithiothreitol. Dialyzed protein was stored at −70 °C prior to usage in assays.

Preparation of Nucleic Acid Substrates—Primer MB22 as well as pol donor and acceptor templates used in this study were produced as previously described (30, 31). RNAs were generated using an in vitro run-off transcription reaction using T7 RNA polymerase with BamHI-linearized plasmids as templates. These templates were then gel purified on 6% polyacrylamide-urea denaturing gel. 38-mer RNA templates were obtained from Dharmaco (Dharmaco Research, CO). [32P]5′-end labeling was performed using [γ-32P]ATP and T4 polynucleotide kinase (New England Biolabs). RNAs labeled in this manner were gel-purified on either 6% (for donor and acceptor RNAs) or 12% (for 38-mer RNA, previously described, (28)) polyacrylamide-urea denaturing gel.

DNA Polymerization and Strand Transfer Assays—The dNTP-dependent DNA polymerization and strand transfer assays were performed as previously described (31) but with slight modifications. Briefly, 32P 5′-end labeled 19-nt primer (MB22) was mixed with pol donor in a 2:1 ratio in 0.1 mM NaCl, incubated at 95 °C for 2 min and slow cooled to room temperature. Template primer mixtures were preincubated with RT protein for 2 min at 37 °C, and reactions were initiated by the addition of dNTP and MgCl2. Final reactions contained 6 nM primer, 4 nM donor template (strand transfer assays contained in addition, 16 nM acceptor), RT protein (666 homodimer, concentrations described in figure legends), 16 μM oligo(dT), 50 mM Tris-HCl (pH 8.0), 0 mM MgCl2, 50 mM KCl, 10 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 50, 5, or 2 μM dNTP in a final reaction volume of 12.5 μL. For reactions with NC, sufficient NC was added to achieve 200% coating (2 NC molecules per 7 nucleotides of template) of donor and acceptor RNAs. The reaction mixture was incubated with NC for 5 min at room temperature before addition of RT. Reactions were terminated by adding an equal volume of 2× stop buffer (10 mM EDTA (pH 8.0), 90% formamide, 0.1% bromphenol blue, 0.1% xylene cyanol) and products were resolved on 6% polyacrylamide-urea.
denaturing gel. Reaction products were visualized using the Storm Phosphorimager (GE Healthcare) and quantitated using ImageQuant software (GE Healthcare). Transfer efficiencies were calculated as 100/transfer product/(transfer product/fully extended donor product), as described previously (30, 31). Statistical differences between efficiency values were calculated using a Student’s t test.

## Donor Template Degradation Assay
Donor template degradation assays were performed as above, except that the pol donor RNA was 32P-labeled and mixed with unlabeled MB22 primer mixed in a 1:2 (50:100 fmol) donor:primer ratio. Donor RNA degradation assays were performed in the presence of 5 μM dNTP. Products were analyzed on 6% polyacrylamide-urea denaturing gel and visualized and quantitated as above.

## Intrinsic RNase H Assay
Intrinsic RNase H assays were performed using identical reaction conditions as above, but using a 32-mer DNA primer (RNase H assay 32-mer DNA; 5'-CCGGCCGAATTCGGCTAGGAATATGC-3') annealed to a 32P-labeled 38-mer RNA template (5'-AAGGTCTGCTGGAATATTCGGCCGAGGAAATTCGGCCG-3') at a 1:4 (25:100 fmol) template:primer ratio. The intrinsic RNase H assays were performed in the absence of dNTP. Products were analyzed on 14% polyacrylamide-urea denaturing gel and visualized and quantitated as above.

## RESULTS

### [dNTP]-dependent DNA Polymerase Activity and Pausing Profile of HIV-1 RT Proteins
To determine the amount of protein giving equivalent RNA-dependent DNA polymerase activity for the WT, V148I, and Q151N HIV-1 RT proteins, we performed single nucleotide incorporation assays on a short RNA template:DNA primer substrate at 50 μM dNTP. Products were analyzed using 6% polyacrylamide-urea denaturing gel and visualized and quantitated as above.

In previous studies, we demonstrated that because of their reduced dNTP binding affinity, the V148I and Q151N HIV-1 RT mutants exhibit significantly reduced DNA synthesis at low dNTP concentrations of 5–0.5 μM (9). This is in contrast to WT HIV-1 RT, which efficiently polymerizes DNA at these same dNTP concentrations. We verified this using a 184-nt RNA template comprising a portion of the HIV-1 pol coding region, which has been used previously (30, 31). The 32P-5’-end-labeled primer annealed to the 3’-end of the RNA template was extended using the equivalent polymerase activities of the WT, V148I, and Q151N RT proteins described above. We specifically chose this template sequence for the present study because of the relatively fewer and weaker RT pause sites

![Figure 1. dNTP concentration-dependent reverse transcription activity of HIV-1 RT proteins. A 32P-labeled 19-mer primer annealed to the 184-nt long RNA template was extended using 10 nm WT, V148I, or Q151N HIV-1 RT proteins, which showed similar RT activity at 50 μM dNTP. Synthesis to the end of the template yields a 177-nt cDNA product. Primer extension reactions were performed at 50 μM (A) 5 μM (B), or 2 μM (C) dNTP. Reactions were sampled at 1, 2, 4, 8, 15, and 30 min of reaction time. Lanes marked C show control reactions in the absence of RT, F, 177-nt fully extended product; P, 19-nt unextended primer; M, 10-nt DNA ladder.](image-url)
observed on this template. All of the three RT proteins exhibited similar synthesis kinetics for formation of the 177 nt long fully extended product at 50 μM (Fig. 1A). However, as the dNTP concentration was lowered to 5 or 2 μM dNTP, the mutant RTs became increasingly inefficient at generating full-length cDNA product (Fig. 1, panels B and C). This was particularly evident at the early time points of the reactions. The amount of full-length extension products at 30 min was also significantly reduced for the V148I and Q151N mutant RTs compared with WT HIV-1 RT. Quantitation of extension products showed that as the dNTP concentration was reduced from 50 μM to 5 μM and 2 μM dNTP, the amount of fully extended products for V148I decreased from ~39% to ~22 and ~10%, respectively. Similarly, fully extended products for Q151N decreased from ~44% at 50 μM dNTP to ~29% at 5 μM and 10% at 2 μM. In comparison, fully extended products made by WT RT decreased from ~47% at 50 μM to ~29%, at 2 μM. In addition to the decreased synthesis efficiency all three enzymes displayed increased pausing at the lower dNTP concentrations. Increased pausing at low dNTP levels has been previously described for WT HIV-1 RT (26). Overall, reaction characteristics observed with the 184-nt pol template were similar and consistent with our previous study using a shorter RNA template (9). Basically, unlike with WT HIV-1 RT, dNTP binding becomes a rate-limiting step during reverse transcription at low dNTP concentrations for the two mutant RTs. This results in significant kinetic interference in DNA synthesis at limiting dNTP levels, where WT HIV-1 RT still efficiently polymerizes DNA.

Strand Transfer Activity of HIV-1 RT Proteins—Since pausing during DNA synthesis has been shown to promote RT template switching, we hypothesized that the V148I and Q151N RT mutants would exhibit increased strand transfer compared with WT HIV-1 RT, especially at low dNTP concentrations. To test this prediction we employed a previously established template switch assay system, which is illustrated in Fig. 2A (30). The donor template-primer in this system was the same as that used in the primer extension reactions described in Fig. 1. To detect template switching, a second 227-nt long (acceptor) RNA template, was included in the reaction. The donor and acceptor templates share 140-nt of sequence identity, allowing for interstrand template switching events within this region (see thick dotted lines in Fig. 2A). The acceptor RNA is longer at its 5′-end (upstream region with reference to the primer) by 70 nt. All reactions were terminated at 30 min. T, strand transfer products; F, fully extended product on donor template; P, 19-mer unextended primer; M, 10-nt DNA ladder.

FIGURE 2. Strand transfer activity of HIV-1 RT proteins. A, schematic of the strand transfer system. 32P-labeled 19-mer primer (thick arrow) annealed to 184-nt donor RNA was extended by WT, V148I, or Q151N RT proteins (80 nM) in the presence of a 227-nt acceptor RNA template. The donor and acceptor RNAs (solid lines) share 140 nt of sequence homology within which template switching can occur (thick dashed arrow). The donor RNA also possesses a 16-nt nonviral sequence at its 5′-end (slashed box), which disrupts homology with the acceptor template preventing transfers from the 5′-end of the donor template. The acceptor has an additional 70-nt upstream of the sequence identity and lacks the primer binding site at its 3′-end. B, strand transfer reactions with WT, V148I, or Q151N RT proteins were performed at dNTP concentrations of 50, 5, or 2 μM in the presence (+ NC) or absence (− NC) of viral NC protein. Reactions with NC were performed at 200% coating (2 NC molecules per 7 nt). All reactions were terminated at 30 min. T, strand transfer products; F, fully extended product on donor template; P, 19-mer unextended primer; M, 10-nt DNA ladder.

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The pol template system generates low transfer efficiency with WT HIV-1 RT, making it an ideal system for detecting increases in strand transfer efficiency. We performed strand transfer assays at 50, 5, and 2 μM dNTP, using equivalent activities of each of the three RT proteins (Fig. 2B). Reaction conditions were as described in Fig. 1, but with eight times the total RT activity. Control primer extension assays were also performed on the acceptor template alone to ensure that the extension capabilities of the three enzymes were similar on this template (data not shown). As observed in Fig. 2B, decreasing dNTP concentration promoted increased transfer with all three RT proteins (T in Fig. 2B). Transfer efficiencies of the three RT proteins at 50, 5, and 2 μM dNTP are summarized in Fig. 3. With each of the RTs, transfer efficiency doubled when the dNTP concentration was reduced from 50 to 2 μM, increasing from 10.7 to 20.6% for WT, 13.2 to 27.8% for V148I and 12.8 to 25.5% for Q151N. Indeed, at 5 and 2 μM dNTP, V148I RT exhibited 39 and 35% higher transfer efficiencies than WT RT (p values <0.001). Unexpectedly, although low dNTP concentrations had a greater inhibitory effect on DNA synthesis by the Q151N mutant than the V148I mutant, the effects on transfer were not statistically as significant. As shown in Fig. 3A, the Q151N RT mutant showed a transfer efficiency of 25.5% at 2 μM dNTP (24% difference from transfer efficiency compared with WT RT; p values <0.025 at 5 and 2 μM dNTP).

The viral nucleocapsid (NC) protein has been shown to stimulate transfers by HIV-1 RT by improving template degradation, promoting strand exchange and annealing of complimentary strands (32–34). We therefore examined the effects of NC on the transfer kinetics of WT and V148I RT proteins. The donor and acceptor RNAs were premixed with sufficient NC protein to achieve 100% coating of the templates (two NC molecules per 7 nt) prior to initiating the transfer reaction. Transfer reactions with NC protein are shown in the right-hand panel of Fig. 2B. As shown, in addition to the 247-nt transfer product, transfer products of larger size are observed. These products are the result of fold-back synthesis on the acceptor template and have been observed in other transfer systems (23, 32). These products are much more significant in reactions with
NC, and therefore have been included in analysis of transfer efficiency. NC enhanced transfer efficiency by ~100% for all three RTs at 50 μM dNTP. However as with reactions with no NC, the V148I RT mutant exhibited higher overall transfer efficiencies than WT RT at lower dNTP concentrations. At 5 and 2 μM dNTP, the transfer efficiencies of V148I were 11 and 47% higher than the respective efficiencies of WT RT (p < 0.01 at 5 μM and <0.005 at 2 μM). Interestingly, unlike with the V148I mutant, transfer efficiency of Q151N was not significantly increased over that of WT RT as dNTP concentrations were decreased. At 2 μM dNTP, the transfer efficiency of Q151N was only 17% difference compared with WT RT.

Similar to the primer extension assays in Fig. 1, a larger proportion of the primer extension products in the low dNTP (5 and 2 μM) transfer reactions with the V148I and Q151N mutant RTs were the result of partial extensions compared with reactions with WT RT. However, of the primer extensions that went to completion, a larger proportion had switched to the acceptor template in reactions with the mutant RTs compared with WT RT. We hypothesize that the reduced ability to polymerize DNA efficiently is associated with an elevation in transfer efficiency for the two dNTP binding mutant RTs.

Donor Template Degradation during Reverse Transcription—Pausing during reverse transcription promotes degradation of the donor RNA template by the RT-RNase H activity. Increased donor degradation is likely to increase the opportunity for RT template switching. As shown above, at 5 μM dNTP both the V148I and Q151N mutants displayed reduced processive synthesis compared with WT RT. This altered DNA synthesis was coupled to an increase in transfer efficiency. To verify this, primer extension reactions were performed using unlabeled 19mer primer annealed to 5′-end 32P-labeled 184-nt RNA donor template. Reactions were performed at 5 μM dNTP using the same template primer and RT concentrations as used in the transfer assays. As seen in Fig. 4, relative to the V148I and Q151N mutant RTs, WT RT displayed increased RNA cleavage products at the end of the template, consistent with WT RT synthesizing DNA more efficiently at 5 μM dNTP compared with the mutant RTs. Quantitation of the cleavage products at 30 min of reaction showed that 65% of the products resulted from cleavages at the 5′-end of the template in the case of WT RT. In the case of the mutants this class of products comprised only 40–45% of the cleavage products. Instead, cleavage products in the 100–150-nt size range were more prevalent with the mutant RTs than with the WT RT. This is consistent with WT RT synthesizing donor RNA more efficiently at 5 μM dNTP compared with the mutant RTs. These data support the idea that kinetic interference in DNA synthesis can promote frequent stalling of RT, thereby increasing donor RNA degradation, for the V148I and Q151N RT mutants. The slow synthesis rate combined with the increased template degradation very likely contributes to the elevated transfer efficiencies of the mutant RTs, compared with WT RT.

Intrinsic RNase H Activity of HIV-1 RT Proteins—The ability to effectively cleave the donor template is important in promoting transfer. An increase in intrinsic RNase H activity of the RT mutants can lead to increased overall degradation of the donor template, but in a manner independent of RT pausing and consequently promote increased transfer efficiency. We therefore compared the intrinsic RNase H activities of the Q151N and V148I RT mutants to that of WT RT. For this assay, a 38-nt RNA template was 32P-labeled at its 5′-end and annealed to an
unlabeled 32-nt DNA primer such that there is a 6-nt overhang at the 5’-end of the RNA (Fig. 5A). Equivalent amounts of RT activity were added to the template primer and incubated in the absence of dNTP. The RT activities used in this assay were 20% of that used in the transfer assays. RT initially binds the substrate at the primer terminus and cleaves the RNA template ~18-nt downstream from the 3’-end of the primer to generate the 24-nt cleavage product (P1 in Fig. 5A). In the absence of synthesis, RT subsequently repositions 8–9-nt downstream from the primer 3’ terminus to make a second cut, generating the shorter 13-nt radiolabeled product (P2 in Fig. 5A). To compare the RNase H cleavage efficiencies of the WT and mutant RTs, the percentage of cleaved RNA was plotted over time. As observed in Fig. 5B, both WT and mutant RTs displayed overall similar rates of template cleavage, indicating that the mutant and WT RTs have similar intrinsic RNase H activities. These data suggest that the ability of the two dNTP binding mutant RTs to promote more transfers is not because of an increase in their intrinsic RNase H activity.

DISCUSSION

The role of RT pausing in promoting strand transfer has been the focus of several studies. Stable secondary structures within the RNA template can stall RT synthesis and subsequently elevate RNA template degradation and promote transfer (23, 26, 32, 34, 35). Strong pause sites however are not always the primary determinant, nor a prerequisite for transfers as inferred from studies on templates with minimal RT pausing (18, 26, 30, 31). Derebel and DeStefano (18) reported a higher efficiency of transfers with an RNA template which induced weak RT pauses, in comparison to a highly structured RNA template that induced stronger RT pausing. These data indicate that a combination of factors is likely involved in facilitating the transfer process on any given template system.

In contrast to previous studies that have examined template features that promote pausing, in this study we address how restricted DNA polymerization kinetics, resulting from disruption of RT–dNTP interactions, can lead to stalling of synthesis and consequently increased strand transfer. DNA polymerization kinetics can be modulated by altering dNTP substrate availability. Lowering dNTP concentration slows the DNA synthesis rate by RT, causing the enzyme to stall more frequently, thereby increasing transfer efficiency (26). Our study suggests another mechanistic element that can contribute to the strand transfer efficiency of HIV-1 RT, namely the RT–dNTP binding interaction. Our data show that reducing RT affinity for dNTP substrates can further amplify the effect of lowering dNTP concentration on transfer efficiency. The higher transfer efficiency of the reduced dNTP binding mutant RTs very likely results from the kinetic interference in DNA synthesis at low dNTP concentrations. Increased transfers with the mutant RTs could also have resulted from their increased intrinsic RNase H activity. However, this possibility was excluded by the data that both V148I and Q151N RTs show similar levels of intrinsic RNase H activity as WT HIV-1 RT. The binding affinity of HIV-1 RT to template-primer also could affect pausing and template degradation. However, our previous pre-steady state kinetic studies demonstrated that the template-primer binding affinities of the two mutant RTs are similar to that of WT RT (K_D, Ref. 8). Because the post-pausing events in the strand transfer process such as pause-induced RNase H cleavage and strand invasion are unlikely to be affected by changing the dNTP substrate availability, the enhanced transfer efficiency of the V148I and Q151N HIV-1 RT proteins over WT HIV RT at the low dNTP concentrations are very likely induced by the slower DNA synthesis kinetics of RT during reverse transcription.

Unexpectedly, although the Q151N mutant showed similar to larger defects in DNA synthesis compared with V148I, it did not show significant increases in transfer at the low dNTP concentration unlike V148I. One possibility with the Q151N mutant is that more than one template switch occurs during the course of completion of synthesis on this template. The 240-nt transfer product results when a template switch results in completion of synthesis on the acceptor. However, if two (or any even number of) crossovers occur during DNA polymerization, with the primer returning back and completing synthesis on the donor template, the resultant transfer products will be indistinguishable from donor extension products and therefore not scorable. In such a scenario, the transfer efficiency of the mutant would be underestimated, as measured by this system. Because the Q151N mutant is more defective than V148I in DNA synthesis at low dNTP concentrations, an underestimation of transfer efficiency due to multiple template switching is a distinct possibility for transfer reactions involving the Q151N mutant in comparison to the V148I mutant. A more extensive analysis, involving sequence analysis of the donor extension and acceptor extension products will be required to accurately measure template-switching frequency of the dNTP binding mutants. Such analyses, however, are beyond the scope of the present study. A second possibility is that the restricted synthesis compromises accurate quantitation of the donor extension and transfer products. Therefore, it is plausible that the transfer efficiency of the Q151N RT proteins, particularly at 2 μM dNTP, might be underestimated due to the severely reduced DNA synthesis rate. This could explain the modest elevation in transfer efficiency of the Q151N RT, compared with WT HIV-1 RT, at 2 μM dNTP.

Secondary structures within an RNA template induce RT pausing at specific sites within RNA template. In contrast, pausing induced by kinetic interference of synthesis, such as by lowering dNTP concentration or disruption of RT–dNTP interaction, should occur throughout the RNA template sequences regardless of the nature of RNA template. The absence of prominent secondary structure-induced pause sites within the pol template allowed us to examine the effects of RT stalling induced by kinetic interference of synthesis. However, similar transfer efficiency differences between the WT and dNTP-binding mutant RTs are also likely to occur on highly structured RNA templates. To address this we also measured transfers on a highly structured RNA template that was previously shown to promote frequent and strong pausing by WT HIV-1 RT (36). Similar to observations made with the pol template, we observed that both Q151N and V148I RT proteins exhibited higher transfer efficiency than WT HIV-1 RT at low dNTP
concentrations with this highly structured template (data not shown).

A recent study by the Pathak group (37) reported that HIV-1 variants containing various drug resistant RT mutations showed altered viral recombination frequencies. Among the RT mutations tested, HIV-1 harboring the Q151N RT showed the highest recombination frequency, or ~80%. These data imply that as much as 80% of the functional proviral DNAs synthesized from Q151N virus infection were a product of recombination. This also indicates that the interstrand viral recombination process could function as a survival mechanism for HIV-1, particularly when proviral DNA synthesis is disrupted by defective replication machinery (Q151N RT). The viral infections in the Pathak group study were performed using a transformed cell line. Established transformed or tumor cell lines such as HeLa cells have ~5 μM cellular dNTP (38).

In terms of the relationship between reverse transcription and cellular dNTP concentration, a factor which can alter proviral DNA synthesis kinetics and HIV-1 recombination, studies have yielded contradictory results. Chen et al. (39) reported no significant difference in HIV-1 recombination frequency during replication in macrophage and T cells, which contain low and high dNTP concentrations, respectively. In contrast to these findings, Levy et al. (40) reported higher viral recombination frequency in macrophage than T cells, although treatment of macrophage with DN did not alter HIV-1 recombination frequency. The dN concentration used in the study by Levy et al. was only 20–40 μM. Our dNTP quantitation assays have demonstrated that at least 1 mM dN is required for sustaining elevated dNTP levels in macrophage, and no significant increase in cellular dNTP content was measurable with 20–40 μM dN.4 The discrepancy between these two studies for HIV-1 recombination frequency in macrophage and T cells may be due to technical differences in terms of assay sensitivity and genetic markers employed in these two studies. Irrespective of the reasons for these differences, it is clear that there is a need for additional studies that will elucidate and resolve these conflicting findings.

Structural modeling proposed that the oxygen amide on the side chain of the Gln151 residue forms an H-bond with the 3'-OH of the incoming dNTP (6, 7). The shorter side chain resulting from the Q151N mutation, compared with WT Gln151, prevents formation of this H-bond, leading to reduced dNTP binding affinity. In contrast, the V148I mutation, which has a bulkier side chain than the WT valine residue may move the neighboring Gln151 residue away from the active site, making it difficult for the WT Gln151 side chain to form an H-bond with the 3’-OH of the incoming dNTP substrate. However, as demonstrated in the pre-steady state kinetic analysis, these structural changes appear not to influence chemical catalysis for polymerization (kpol) (6, 8). Despite these unique mechanistic and structural alterations, both V148I and Q151N mutant RTs remain highly active at high dNTP concentrations, like WT HIV-1 RT.

The biochemical data presented in this study suggest that the RT interactions with the incoming dNTP substrate mechanistically influence the efficiency of DNA synthesis which in turn can influence RT strand transfer. These data therefore offer a biochemical interpretation for the observation that virus harboring the Q151N mutation show highly elevated viral recombination frequency (37). Our studies indicate that RT-dNTP interaction is an important contributing factor in driving HIV-1 recombination, genomic mutagenesis, as well as cell type specific replication.

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