Analysis of Efficiency and Fidelity of HIV-1 (+)-Strand DNA Synthesis Reveals a Novel Rate-limiting Step during Retroviral Reverse Transcription*

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We have analyzed the efficiency and accuracy of polymerization at several different stages during the initiation of human immunodeficiency virus type 1 (HIV-1) (+)-strand DNA synthesis. This reaction is of particular interest, as it involves the recruitment by reverse transcriptase of an RNA primer that serves as substrate for both the polymerase and RNase H activities of the enzyme. We found that the correct incorporation of the first two nucleotides was severely compromised and that formation of mismatches was completely absent at this stage of initiation. Although the fidelity of incorporations decreased concomitantly with ensuing polymerization, the elongation of mispaired primers was literally blocked. Instead, mispaired primer strands initiated a switch from active synthesis of DNA to premature RNase H-mediated primer removal. These findings suggest the existence of a fragile equilibrium between these two enzymatic activities that is shifted toward RNase H cleavage once the polymerization process is aggravated. Our data show that the initiation of HIV-1 (+)-strand DNA synthesis differs significantly from reactions involving other primer/template combinations, including tRNA-primed (+)-strand DNA synthesis.

Alterations in the availability of deoxynucleoside triphosphates (dNTPs) can profoundly affect retroviral reverse transcription in several ways (1, 2). Low concentrations of dNTPs have been shown to cause arrest of reverse transcription of human immunodeficiency virus type 1 (HIV-1) RNA in non-dividing macrophages and in quiescent T lymphocytes (3). This arrest resulted in incomplete reverse transcripts that were identified in the early G1 phase of the cell cycle. DNA synthesis was eventually re-initiated at later stages, as concentrations of dNTPs increased (4, 5). Endogenous reverse transcriptase (RT) reactions revealed that diminished or biased dNTP pools might also force the insertion of incorrect nucleotides (6, 7). The high error rate of HIV-1 RT results in about one mistake per round of replication (8). Together with the lack of proofreading, this is one of the major reasons for the enormous variability of HIV. This genetic diversity is also linked to the problem of drug resistance since the prolonged use of available RT inhibitors, including both nucleoside and non-nucleoside analogs, gives rise to mutant enzymes that confer resistance to the administered antiviral drug (9).

It is poorly understood whether fluctuations in dNTP concentrations affect reverse transcription indiscriminately or whether specific events in the reaction are particularly sensitive to such variations. Reverse transcription is complex and involves a variety of different stages in which the RT enzyme has to accommodate different primer/template substrates (10, 11). Reverse transcription of HIV-1 is primed by human tRNAgly⁹⁰, which binds via its 3′ terminus to the primer-binding site of the RNA genome. Synthesis of the first DNA strand, (+)-strand DNA, is initiated from the binary tRNA/primer-binding site complex and proceeds toward the 5′-end of the template, to yield so-called (+)-strand strong-stop DNA (12–14). The transcribed RNA of the newly formed RNA/DNA hybrid is degraded by the RT-associated RNase H activity. RNase H degradation can be seen at a fixed distance from the primer terminus, in temporal coordination with DNA synthesis, and, as well, in the absence of DNA synthesis, independent of the precise location of the 3′-end of the primer (15–19). This facilitates the release of the strong-stop DNA, which is transferred to the 3′-end of the RNA template to allow continuation of (+)-strand DNA synthesis (20). During this process, a purine-rich fragment near the 3′-end of the genomic RNA, i.e. polyuridine tract (PPT), remains resistant to RNase H degradation and serves as a primer for (+)-strand DNA synthesis (21–24). Following its selection, the PPT primer is initially extended, and specific RNase H cleavage at the RNA-DNA junction removes the RNA fragment from newly synthesized DNA. (+)-Strand DNA synthesis is a discontinuous process that may require initiation from a central polyuridine tract as well (25, 26). At a later stage of (+)-strand DNA synthesis, specific RNase H cleavage must also remove the tRNA from (+)-strand DNA to facilitate a second strand transfer, which is necessary for completion of synthesis of proviral DNA. Precise removal of both primers is important in regard to subsequent steps in the retroviral life cycle, as these cuts define the ends of the double-stranded DNA that is integrated into the genome.

Each of these various stages during reverse transcription may be affected by fluctuations of available dNTP pools in a different fashion. However, the initiation of retroviral (+)-strand DNA synthesis is unique in that both the polymerase...
and RNase H activities recruit the same strand as substrate. Hence, at this stage of the reaction, the RT-associated RNase H activity may directly influence the polymerization process.

We have recently studied the interplay between the two active sites under saturating dNTP concentrations and demonstrated that a specific pausing site at position +12 correlated precisely with the emergence of RNase H cleavage at the RNA-DNA junction that removes the RNA primer from newly synthesized DNA (23). In the present study, we have investigated the impact of diminished and biased dNTP pools on efficiency and accuracy of the polymerization process and the consequences regarding the interdependence between the two active sites of RT. Steady-state kinetics revealed severely diminished rates of nucleotide incorporation and unusually high fidelity during the initial steps of the reaction. Limited concentrations of dNTPs promoted a switch from active DNA synthesis to RNase H activity and caused premature removal of the primer before the enzyme reached the pause site at position +12. As a result, the lower limits of dNTP concentrations required to effectively and accurately initiate (+)-strand DNA synthesis are significantly higher compared with those identified in reactions that involve DNA/DNA primer/template substrates.

**EXPERIMENTAL PROCEDURES**

**Nucleic Acids and HIV-1 RT**—The oligonucleotides used in this study were chemically synthesized using the phosphoramidite method. We utilized a PPT-derived RNA primer (17R), its DNA counterpart (17D), and chimeric DNA-RNA primers (1D-17R and 3D-17R) that correspond to different stages during synthesis of (+)-strand DNA; “nD” and “nR” refer to numbers of DNA and RNA residues, respectively. The sequences of all oligonucleotides used are as follows (RNA residues are in italics, and the region of the template (57D) that provides complementarity to the RNA primer is in boldface): 17R, 5′-UUAAAA-GAAGGAGGACCC-3′; 1D-17R, 5′-UUAAAGAAAAACGAGGACCC-3′; 3D-17R, 5′-UUAAAGAAAAACGAGGACCC-3′; 1D-3′-UUAAAAAGAAAAAGGGGACT-3′; and 5D7, 5′-CTGGGGATGTAATGCCCTCCGACCTCCCCCCCTTTTTAAAAGGTGCCATAG-3′.

The crude oligonucleotides were purified on 7 M urea and 12% polyacrylamide gels containing 50 mM Tris borate (pH 8.0) and 1 mM EDTA. The presence of PPi solely facilitated the excision of the RNA-DNA or DNA/DNA substrates (100 nM) were preincubated for 5 min at 37 °C with the RNase H-deficient mutant enzyme (30 nM) in a reaction containing 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 5 mM MgCl₂, 200 nM enzyme. DNA synthesis and RNase H degradation were then initiated by the addition of MgCl₂ at a final concentration of 40 mM. Reactions were performed at 37 °C and stopped at different times by adding 1-µl aliquots of the reaction mixture to 9 µl of 95% formamide containing 40 µM EDTA.

**RESULTS**

**Experimental Design**—We recently devised an in vitro system that allows the study of structure-function relationships of HIV-1 RT at various stages of initiation of (+)-strand DNA synthesis through use of select primer/template combinations (23). This system has now been used to determine kinetic parameters regarding the efficiency of correct and incorrect nucleotide additions. The PPT-derived RNA primer (17R) has allowed us to study steady-state kinetics at early stages of the reaction, whereas the chimeric DNA-RNA primer (3D-17R), which corresponds to the addition of the first three nucleotides to the PPT primer, was employed to study nucleotide insertions as the enzyme accommodates increased numbers of DNA residues (Fig. 1A). Utilizing gel-based assays, we determined $K_{cat}$ and $k_{cat}/K_m$ values with respect to nucleotide incorporations at positions +1, +2, +3, and +6. Reactions were monitored with 5′-end-labeled primer strands; and unless otherwise indicated, the primer/template substrate was used at a 10-fold excess over RT to ensure that reaction conditions fulfilled steady-state criteria. To minimize the complexity of the assay, we initially used an RNase H-deficient mutant RT, i.e. RT-E478Q (29), to avoid cleavage of the extended RNA primer. These experiments were also performed with homologous DNA primers for purposes of comparison.

**Steady-state Kinetics of Correct and Incorrect Nucleotide Incorporations**—The results of our measurements are summarized in Table I, and important examples are shown in Fig. 1. We found that the efficiency of incorporation ($k_{cat}/K_m$) of the correct nucleotide at position +1 was diminished by ~50-fold when the RNA primer was used (Table I). The influence of the RNA primer was not restricted to the terminal RNA moiety since the diminution in rate of nucleotide incorporation was also seen at position +2. Following these initial studies, the efficiency of correct nucleotide incorporation increased significantly at position +3, and both the RNA- and DNA-primed reactions had similar $k_{cat}/K_m$ ratios once the enzyme had reached position +6 (Table I). A diminution in usage of the RNA primer was also noted when monitoring pyrophosphorylation, i.e. the reverse reaction of nucleotide incorporation (Fig. 1B). The presence of PPi solely facilitated the excision of the
terminal DNA residue of the chimeric primer 3D-17R. Shorter products were not even seen at a high concentration of PP_i (1 mM) that transferred ~40% of the 3D-17R primer into the 2D-17R fragment (Fig. 1B, left panel, lane 5). In contrast, relatively lower concentrations of PP_i (100–500 μM) were sufficient to convert 40–60% of the homologous 20D DNA primer.

**FIG. 1.** Reverse transcriptase kinetics at early stages during initiation of (+)-strand DNA synthesis. A, use of RNA/DNA primer/template combinations and experimental design. The pure RNA (17R) and the chimeric DNA-RNA primer (3D-17R) were employed to determine kinetic parameters at positions +1, +2, +3, and +6 as described under “Experimental Procedures.” All experiments were conducted with the RNase H-deficient RT-E478Q mutant enzyme. B, efficiency of pyrophosphorolysis monitored with primers 3D-17R (left panel) and 20D (right panel). The excision of terminal residues was analyzed at concentrations of 10, 50, 100, 500, and 1000 μM PP_i (lanes 1–5, respectively). Lanes C represent the control in the absence of PP_i. C, incorporation of incorrect nucleotides at position +1 using varying concentrations of dGTP. Reactions with the DNA primer 17D are shown in the left panel, and the corresponding reactions with the RNA primer 17R are shown in the right panel. Lanes C indicate controls in the absence of dNTPs. Reactions were allowed to proceed for 20 min.
Fidelity during Synthesis of Full-length DNA—The above findings suggest that the initiation of (+)-strand DNA synthesis may be sensitive to decreased or biased dNTP pools. Although our data show that formation of mismatches may occur at later stage of DNA synthesis, it is not clear whether such mispaired replication intermediates can be effectively extended. Therefore, we determined minimum concentrations of dNTPs needed to allow DNA synthesis to continue beyond these critical template positions by incrementally increasing the concentrations of one of the four nucleotides while using the other three dNTPs at saturating concentrations. For this purpose, we employed a chimeric primer that represents the PPT-derived RNA primer extended by a single nucleotide (1D-17R), which enabled us to monitor DNA synthesis at both initial and later stages. To determine the lower limits of dNTP concentrations required for full-length DNA synthesis, we used conditions that gave rise to high yields of the product under saturating nucleotide concentrations. Thus, reactions were allowed to proceed for 1 h using an excess of the RT-E478Q mutant enzyme over primer/template (Fig. 2A).

Consistent with steady-state kinetics, we detected a striking difference in regard to the yield of full-length DNA in reactions that compared nucleotide limitations and positions +2 and +3. First, a relatively high concentration of dCTP, i.e. >1 μM, was required to bypass position +2, which is the first incorporation event in this experiment (Fig. 2B and Table II). In contrast, 0.1 μM dTTP was sufficient to bypass position +3 and to yield 50% of the full-length product compared with reactions conducted under saturating dNTP concentrations of 10 μM (Fig. 2B and Table II). In either case, the majority of abortive reaction products were found at these initial steps of DNA synthesis. Later positions were relatively easy bypassed, unless the limiting nucleotide affected three or more consecutive bases. For instance, dGTP was required at a relatively high concentration of 0.8 μM since low levels of dGTP had the potential to aggravate DNA synthesis at multiple positions, i.e. positions 4, 5, and 8–10. In contrast, dATP, which is required at positions +6 and +7, was needed only at a relatively low concentration of 0.1 μM to achieve 50% full-length DNA synthesis. The full-length product was even detected in the absence of either dATP or dTTP, indicating that mispaired primers can, in principle, be extended, although the yield was relatively poor.

**Primer Removal Is Faster than Mismatch Formation and/or Extension**—Next, we increased the complexity of these assays and compared results obtained with the RT-E478Q mutant enzyme and with wild-type RT containing an intact RNase H domain. To analyze the influence of diminished dNTP pools on the interplay between the polymerase and RNase H activities of RT, we generated a 3′-end-labeled chimeric primer corresponding to the addition of a single radiolabeled DNA residue to the PPT primer, as in the experiments shown in Fig. 2. This type of labeling facilitates the analysis of RNase H-mediated processing of the newly synthesized DNA strand since the size of the processed reaction product directly points to the stage of DNA synthesis at which the RNA primer is removed (23). The temporal relationship between the polymerase and RNase H active sites of RT was monitored in a time course reaction in which one of the four nucleotides was omitted (Fig. 3, A and B). DNA synthesis was completely blocked in the absence of dCTP, i.e. the correct nucleotide at the second template position (Fig. 3B, panel 1). Thus, misincorporations did not take place at this stage of DNA synthesis. However, a diminution in the chemical step and decreased binding of the incoming incorrect dNTP are not the only possible factors that block DNA synthesis under these conditions. The time course shows that the RT-associated RNase H activity prematurely cleaved at the RNA-DNA junction, resulting in release of a single nucleotide
containing the radiolabel. The same result was obtained with reaction mixtures lacking dTTP or dGTP (Fig. 3B, panels 2 and 3). Here, effective DNA synthesis required incorporation of incorrect nucleotides at positions 13 and 14, respectively. DNA synthesis was again blocked even before the enzyme had reached these critical positions, and major RNase H cleavage products were found to correspond to short DNA fragments containing two and three residues, respectively. Thus, in these cases, polymerization stopped before an incorrect nucleotide was added, which finally resulted in premature removal of the primer.

A similar sequence of events was also seen when dATP was omitted from the reaction mixture (Fig. 3B, panel 4). However, under these conditions, we did observe incorrect insertions at position 16, although the misaligned primer was not further extended. In this case, the short cleavage fragment corresponded to a sequence of six DNA residues, indicating that primer removal was initiated after mismatch formation at position 16. Collectively, these data demonstrate that the RT-associated RNase H activity can seriously affect the initiation of (+)-strand DNA synthesis as the availability of dNTP pools is limited. The requirement for dATP incorporation at position 17 may be considered as an additional obstacle that compromises elongation of the mismatched intermediate. However, the homologous DNA primer was efficiently extended under the same reaction conditions (Fig. 3C). The RT enzyme efficiently bypassed critical, consecutive template positions at which mismatches were formed, which is consistent with previously published data (30–34) and demonstrates that high rates of misinsertions and extensions occur when DNA/DNA serves as substrate.

Switching from Active DNA Synthesis to RNase H Activity—To further assess the biological significance of these different dNTPs were kept constant at saturating levels of 100 μM, whereas the fourth dNTP was used at varying concentrations between 0 and 10 μM. Lanes 1–11 represent reactions with the following concentrations of the limiting nucleotide: 10, 5, 2.5, 1.3, 0.63, 0.31, 0.16, 0.08, 0.04, 0.02, and 0 μM, respectively. Reactions proceeded for 1 h using the enzyme in excess over primer/template. DNA synthesis was monitored using the 3'-end-labeled chimeric primer 1D-17R, which allowed us to study a broader spectrum of positions after the initially slower steps. The majority of abortive reaction products are seen at the level of initiation between positions 2 and 12. Nucleotide concentrations required to bypass these positions to yield 50% of the full-length DNA product are summarized in Table II. These data were obtained from the graphs shown in B. For each of the four dNTPs, concentrations of 5–10 μM were saturating. The graphs in C point to differences in product formation seen in the absence of one of the four dNTPs at low concentrations between 0 and 2 μM.

| Variable dNTP | Positions affected | Variable dNTP critical conc \( \mu M \) | DNA synthesis in absence of variable dNTP |
|---------------|-------------------|--------------------------------------|------------------------------------------|
| dCTP          | 2, 11             | 1.4–1.6                              | −                                        |
| dTTP          | 3, 12             | 0.1–0.2                              | +                                        |
| dGTP          | 4, 5, 8–10        | 0.8–1.0                              | −                                        |
| dATP \( ^b \) | 6, 7, 13, 14      | 0.1–0.2                              | +                                        |

^a Shown are the concentrations of the variable dNTP required to obtain 50% of the full-length product compared with reactions performed at a saturating concentration (10 μM) of this nucleotide. Critical concentrations were determined from data shown in Fig. 2B.

^b Note that dATP affected position +1 when using the pure RNA primer.
servations, we next determined the effects of fluctuations of dNTP concentrations on initiation of (+)-strand DNA synthesis. The time course in Fig. 4 (left panel) shows formation of the processed full-length product when dATP was used at a saturating concentration of 10 μM. In good agreement with our previous data (23), the first product that appeared in the time course corresponds to 12 DNA residues and therefore identifies the primer removal reaction after the enzyme paused at position 112. (The paused product was not seen under these conditions since the enzyme was used in excess over primer/template.) The short fragment disappeared with time as DNA synthesis resumed and the processed full-length product emerged. In contrast, 100 nM dATP, which was sufficiently high to produce the full-length product with the RT-E478Q mutant (Fig. 2 and Table II), blocked DNA synthesis entirely when using the wild-type enzyme (Fig. 4, right panel). Instead, two major abortive DNA species were identified. One of these corresponded to newly synthesized DNA that was cleaved from the RNA primer after RT paused at position 112. The shorter product, which contains six DNA residues, indicates premature removal of the primer before the enzyme has successfully passed position 16 (compare with Fig. 3). Another short product contains five DNA residues and thus indicates RNase H cleavage before the mispair was formed. Hence, both the natural pausing site at position 112 and template positions that require incorporation of the limited nucleotide represent obstacles that can block DNA synthesis and induce removal of the primer prematurely.

Secondary Initiation Events Can Rescue DNA Synthesis—The observation that the RNA primer is prematurely cleaved from newly synthesized DNA does not necessarily mean that DNA synthesis is blocked in irreversible fashion. We have recently demonstrated that chain-terminated DNA synthesis can be rescued since RT is capable of recognizing the 3'-end of the cleaved RNA and eventually initiates a second round of DNA synthesis (23). Therefore, we analyzed whether biased dNTP pools may likewise initiate such secondary priming events. For this purpose, we used the 5'-end-labeled chimeric DNA-RNA primer (3D-17R) and initiated DNA synthesis in the presence of only dATP and dGTP (Fig. 5A). The time course shows that DNA synthesis specifically stopped at position +10.
nucleotides, i.e. dCTP and dTTP, were included in the reaction mixture. Extension of the primary product is most likely more efficient since re-initiation from the RNA primer eventually involves displacement of the first DNA strand. However, these secondary reactions may be important, as the efficiency of extension of the primary product is diminished through incorrect nucleotide incorporations, e.g. as shown for position +6.

It is difficult to assess the extent to which such secondary initiation reactions may contribute to rescue of DNA synthesis, as RNase H cleavage continues into the RNA fragment (Fig. 5A). These cleavage events give rise to 3’-truncated PPT fragments that initiate DNA synthesis with diminished efficiency (35). A second round of DNA synthesis may be relevant when DNA synthesis from longer primary products is aggravated by a mispaired primer terminus, as shown for position +6. It remains to be seen whether such mechanisms might contribute to maintenance of the highly conserved sequence downstream from the PPT that is recognized by the viral integrase.

**DISCUSSION**

Structural differences among double-stranded DNA, double-stranded RNA, and DNA/RNA primer/template substrates can modulate reverse transcription in multiple ways, which include both overall efficiency of polymerization as well as the accuracy of this process. In this report, we investigated the efficiency and fidelity of the initiation of HIV-1 (+)-strand DNA synthesis, a stage of reverse transcription at which RT accommodates an RNA/DNA primer/template. Our data show that the efficiency of initial incorporation events was severely compromised compared with subsequent events when the active site interacts with the newly synthesized DNA duplex. This finding is reminiscent of recent biochemical studies showing slow rates of RNA-primed initiation of (−)-strand DNA synthesis and a sharp transition to a faster and more processive mode of polymerization once the sixth nucleotide has been incorporated (36, 37). Both RNA-primed reactions may therefore be considered as important rate-limiting steps during reverse transcription that respond to low concentrations of available dNTP pools in a particular sensitive fashion. Despite this analogy, our study also highlights significant functional differences between the two initiation events and reactions that involve double-stranded DNA or DNA/RNA primer/templates.

**Insertions of Correct Nucleotides**—Several lines of evidence suggest a functional distinction between the first two nucleotide additions and the ensuing polymerization events. Differences regarding the relative efficiencies of DNA- versus RNA-primed reactions, i.e. $(k_{cat}/K_m)_RNA/(k_{cat}/K_m)_DNA$, were significantly less pronounced beyond position +2 (Table I). The low efficiency of nucleotide incorporation at position +2 with the RNA primer is consistent with our recent observation of an RNA-specific pause site after a single nucleotide extension (23). The relatively sharp transition from initially low catalytic efficiencies to a more efficient mode of DNA synthesis at position +3 is seen with limited concentrations of enzyme as well as with an excess of RT over primer/template.

These data suggest a modulation of interactions between RT and its nucleic acid substrate that appears to specifically involve the first two steps of DNA synthesis. Our recent measurements of the numbers of base pairs that fit between the polymerase and RNase H active sites have indeed identified structural differences between complexes containing RNA/DNA and DNA/DNA (23). These variations most likely reflect differences in helical parameters of the bound nucleic acid substrates. However, it seems unlikely that such global structural alterations can explain the observed high $K_m$ values for initial nucleotide incorporations. In fact, despite a retained difference in the number of base pairs (23), the efficiency of
nucleotide addition at position +6 was literally identical with both RNA and DNA primers (Table I).

The sharp functional transition between positions +2 and +3 rather suggests that structural differences between both substrates may be critical, as they affect interactions in the vicinity of the active site. This interpretation is consistent with mutational analysis of the RT “primer grip,” which is implicated in interactions with the first one to approximately three nucleotides of the primer strand (38, 39). Some amino acid substitutions were shown to selectively impair RNA-primed DNA synthesis, whereas the same mutant enzymes were capable of efficiently recruiting the homologous DNA primer. These results, along with the kinetic data presented in this work, strongly suggest that the primer grip of RT plays a dominant role in substrate discrimination between RNA/DNA and DNA/DNA.

The so-called “minor groove binding track” (MGBT), which is located in the thumb subdomain of HIV-1 RT (40), is presumably less important in this regard, although particular mutations in this motif were shown to alter efficiency and specificity of the primer removal reaction (41). The crystal structure of HIV-1 RT cross-linked to a DNA duplex via residues of the MGBT shows contacts with the minor groove at a distance of ~5–7 base pairs upstream from the polymerase active site (42). Thus, the MGBT still interacts with the RNA/DNA segment as DNA synthesis proceeds relatively efficiently beyond position +2. In contrast, the location of this motif correlates well with the increased rates of polymerization seen after incorporation of the sixth nucleotide during RNA-primed (−)-strand DNA synthesis, suggesting that the MGBT might play a role in discriminating between RNA/RNA and DNA/RNA substrates (36, 37).

Specific structural features of the distinct chimeric replication intermediates that are generated during (−) and (+)-strand DNA synthesis may provoke altered interactions with RT and may, in turn, influence formation of a catalytically competent ternary complex. The NMR structure of a model substrate that mimics the chimeric duplex formed during initiation of (−)-strand DNA synthesis suggests an unusual narrow minor groove around the tRNA-DNA junction (43). The narrow minor groove and also the rigidity of the RNA/RNA segment are likely to be considered as structural parameters that alter optimal interactions with the MGBT (14, 43). In analogy, a compression of the minor groove correlates with termination of (+)-strand DNA synthesis after the second strand transfer (44). The unusual narrow minor groove of the chimeric replication intermediate generated during tRNA-primed (−)-strand DNA synthesis may also help to explain frequent pausing of RT, seen at positions +3 and +5 (45). In contrast, the NMR structure of the PPT-derived substrate that is accommodated during (+)-strand initiation points to relatively wide minor groove dimensions (46); and in fact, pausing during initiation of HIV-1 (+)-strand DNA synthesis is restricted to position +1 (23). This isolated initial pausing site suggests again a dominant role of the RT primer grip in discriminating between RNA/DNA and DNA/DNA substrates. However, specific pausing at position +12 shows that the structure of the PPT-derived hybrid remains important at a later stage of DNA synthesis, presumably through altered interactions with the RNase H domain.

Fidelity—The efficiencies with which incorrect nucleotides are incorporated during the initiation of (+)-strand DNA synthesis follow a pattern similar to that seen in regard to correct nucleotides. The result is more extreme, as misinsertions were never seen at positions +1 and +2. Fidelity of DNA synthesis at position +6 was similar to that seen with the homologous DNA primer, but the extension of misaligned primers was still diminished compared with DNA-primed reactions. When the two initial steps were bypassed, DNA synthesis could still be detected, even in the absence of one of the three nucleotides. Considering the high error rates reported for other types of substrates, the initiation of (+)-strand DNA synthesis, particularly the first two nucleotide incorporation events, is unusually accurate. These data, together with previous biochemical studies (30–34), suggest that fidelity increased, in order of dependence on the primer/template substrate, as follows: DNA/ DNA < DNA/RNA < RNA/RNA ≤ RNA/DNA. The availability of data regarding the initiation complex that contains the tRNA/RNA duplex is relatively limited. However, experiments with only two or three of the four dNTPs revealed that misincorporations were rarely observed, similar to early steps during initiation of (+)-strand DNA synthesis (47, 48). The high fidelity of both RNA-primed reactions may be advantageous for viral replication, considering that the initiation of (−)- and (+)-strand DNA synthesis and the corresponding primer removal reactions define the ends of the pre-integrative DNA that serves as substrate of the viral integrase (11).

The fidelity of the initiation of (+)-strand DNA synthesis is further increased, as reactions were conducted with the wild-type enzyme, which contains an intact RNase H domain. Nucleotide concentrations of ~100 nM, which allowed 50% full-length DNA synthesis with the RNase H-deficient RT-E478Q mutant, were not sufficient to bypass critical template positions when using the wild-type enzyme. Instead, RNase H cleavage at the newly formed RNA-DNA junction was seen, as these positions become involved in DNA synthesis. These data point to a fragile equilibrium between the polymerase- and RNase H-competent modes that is shifted toward RNase H degradation as the polymerase process is diminished. As for the natural pausing site at position +12, diminished or biased dNTP levels decrease the efficiency of DNA synthesis by an extent sufficient for initiation of RNase H activity. These data strongly support the existence of two competing binding modes of the RT enzyme on its PPT-derivatized substrate (23, 49), i.e. polymerase-competent binding mode, which involves specific interactions between the polymerase active site and the 3′-end of the primer terminus, and an RNase H-competent binding mode, in which the RNase H active site is positioned over the RNA-DNA junction in the same orientation as seen during synthesis of (−)-strand DNA. Thus, the concentration of available dNTP pools may also be considered as an important parameter that influences specificity for PPT primer usage. It is conceivable that intracellular dNTP concentrations are not high enough to favor a polymerase-competent binding mode with an RNA/DNA substrate that involves non-PPT primers (26, 49).

Taken together, our data reveal several unique features of the initiation of HIV-1 (+)-strand DNA synthesis that distinguish this stage of reverse transcription from other RNA- and DNA-primed reactions. The results point to an important rate-limiting step in reverse transcription since relatively high dNTP concentrations were required to overcome three distinct aspects of (+)-strand initiation. These are as follows: 1) the high fidelity, yet inefficient incorporation of the first two nucleotides into the (+)-strand; 2) the fragile equilibrium between the polymerase- and RNase H-competent binding modes; and 3) the specific pausing site at position +12 that aggravates DNA synthesis even after primer removal (Fig. 4). Further studies should now analyze the efficiency of (+)-strand DNA synthesis using mutant enzymes that display diminished dNTP-binding capacities, e.g. as described for amino acid substitutions at Tyr115 (50, 51). In this context, it is important to
note that the majority of amino acid substitutions in nucleoside analog-resistant RT molecules affect either directly or indirectly the nucleotide-binding pocket (42, 52). It is thus conceivable that this type of amino acid substitution may cause significant reductions in the efficiency of RNA-primed reactions that require per se relatively high concentrations of dNTPs. This may help to explain the attenuated replication of some drug-resistant viruses that can also be correlated, in some cases, with the diminished processivity displayed by the corresponding mutant enzymes (53–56).

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