Primer Unblocking and Rescue of DNA Synthesis by Azidothymidine (AZT)-resistant HIV-1 Reverse Transcriptase

COMPARISON BETWEEN INITIATION AND ELONGATION OF REVERSE TRANSCRIPTION AND BETWEEN (-) AND (+) STRAND DNA SYNTHESIS*

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Azidothymidine (AZT) is a widely used inhibitor of type 1 human immunodeficiency virus reverse transcriptase (RT) that acts as chain terminator. Upon treatment, mutations conferring AZT resistance to RT are gradually selected. It has been shown that resistant RT is able to unblock the AZT-terminated primer by an ATP-dependent mechanism. However, this resistance mechanism has only been demonstrated for DNA-dependent DNA elongation. Here, we compared the AZT resistance of mutant RT during DNA elongation on DNA and RNA templates. We showed that, during DNA elongation, primer unblocking and rescue of DNA synthesis take place with similar rate constants on DNA and RNA templates. However, the fraction of a primer eventually repaired during RNA-dependent DNA synthesis is 2× lower compared with that of DNA-dependent synthesis, leading to reduced resistance. We also compared the initiation of reverse transcription, which uses tRNA\text{\textsuperscript{Lys}} as a primer and displays characteristic kinetic features, and the subsequent RNA-dependent elongation. Unlike during elongation, resistant RT was unable to unblock the AZT-terminated primer during initiation of (−) DNA strand synthesis. Our results demonstrate that the efficiency of primer unblocking conferred by the AZT resistance mutations greatly vary during the different steps of the provirus synthesis. These results also suggest that inhibitors specifically targeting the initiation of reverse transcription might prove to be advantageous, as compared with elongation inhibitors.

The introduction of highly active antiretroviral treatments against the type 1 human immunodeficiency virus (HIV-1) dramatically decreased the mortality linked to AIDS in developed countries (1). Highly active antiretroviral treatments usually combine inhibitors of the viral protease, which is required for maturation of the viral particles, with inhibitors of reverse transcriptase (RT), which converts the single stranded RNA genome into double-stranded DNA (2). The reverse transcriptase inhibitors are either deoxyribonucleoside analogs (NRTIs) that act as chain terminators or non-nucleotide inhibitors (NNRTIs) that bind to a hydrophobic pocket next to the RT catalytic site (for a review, see Ref. 3). In addition to the high cost that makes them unavailable in developing countries, the efficiency of highly active antiretroviral treatments is limited by long term adverse side effects and the rapid emergence of multiresistant HIV-1 strains (2).

AZT (3′-azido-3′-deoxythymidine), a nucleoside reverse transcriptase inhibitor, was the first drug approved by the Food and Drug Administration for the treatment of AIDS and is still widely used in combination with other antiretroviral drugs (2). The prolonged clinical use of AZT gives rise to resistant viruses that usually contain the mutations M41L, D67N, K70R, T215Y/F, and K219Q in their RT (4–6). In vivo, these mutations provide a 100–200-fold resistance toward AZT (4–7). However, the mechanism of resistance to AZTTP, the active form of AZT (8), remained unclear until recently. Indeed, AZT-resistant RT incorporates AZT as efficiently as the wild type (wt) polymerase (9–12). This situation contrasts with the enzymes that bear the mutations K65R (13, 14), L74V (15), and M184V (16–18) and that confer resistance toward ddC, ddI, and 3TC, respectively.

Recently, several lines of evidence indicated that resistance toward AZTTP is due to the increased unblocking of the AZT-terminated primer, rendering chain termination reversible (Fig. 1A). First, resistant RT was shown to bind AZT-terminated primers more tightly than wt RT does (19). Secondly, it was reported that resistant RT has an increased pyrophosphorolysis rate (20, 21), but this observation was not confirmed by other groups (9, 22). Finally, it was shown that the AZT-resistant enzyme, unlike wt RT, could efficiently unblock AZT-terminated primers using ATP instead of PP\text{\textsc{i}}, in a reaction analogous to pyrophosphorolysis (21–23) (Fig. 1A). However, all ATP lysis experiments performed so far used DNA/DNA primer-template complexes.

The high overall resistance provided by the M41L, D67N, K70R, T215Y/F, and K219Q mutations suggests that primer unblocking also works efficiently on DNA/RNA primer-template complexes. However, the relative resistance toward AZT during synthesis of the (−) and (+) proviral DNA strands is presently unknown. Several properties of the HIV-1 RT, such
as the polymerization rate (24), processivity (24), and fidelity (25), significantly differ on DNA and RNA templates. Thus, the efficiency of primer unblocking by the resistant RT might also considerably differ during synthesis of the (−) and (+) proviral DNA strands.

In addition, our studies of the initiation of HIV-1 reverse transcription, which corresponds to the addition of the first six nucleotides to the tRNA\textsuperscript{Lys}\textsuperscript{59} molecule annealed to the viral primer binding site (PBS) (26), led us to suspect that this step might remain sensitive to AZT despite the presence of the resistance mutations in RT. The initiation of reverse transcription requires intricate and specific interactions between the viral RNA, tRNA\textsuperscript{Lys}\textsuperscript{59}, and RT (27–29) and differs from the subsequent elongation by its slow polymerization rate and fast RT dissociation (26, 30). More important, we observed striking differences between the pyrophosphorolysis of AZT-terminated primers by wt RT during the initiation and elongation of (−) strand DNA synthesis. Indeed, during initiation the removal of AZT was hardly detectable at all (31). Given the strong similarity between PP\textsubscript{i}− and ATP-mediated primer unblocking reactions (22, 23, 32), it is conceivable that resistant RT is unable to unblock an AZT-terminated primer during the initiation of reverse transcription.

To address these points, we first compared ATP lysis of a primer terminal AZT during (+) strand and (−) strand DNA synthesis by AZT-resistant RT. Then, we compared the ATP lysis efficiency during the initiation and elongation of (−) strand DNA synthesis. We found that the excision of AZT was less efficient during (−) strand DNA synthesis as compared with (+) strand DNA synthesis because of an incomplete reaction. In addition, “AZT-resistant RT” was not resistant toward AZT during the initiation of reverse transcription. Hence, our results demonstrate that the efficiency of primer unblocking conferred by the AZT resistance mutations greatly vary during the different steps of the provirus synthesis. The results also suggest that inhibitors specifically targeting the initiation of reverse transcription might be advantageous as compared with elongation inhibitors.

**EXPERIMENTAL PROCEDURES**

**Template, Primers, and RT**—Natural tRNA\textsuperscript{Lys}\textsuperscript{59} was purified from beef liver as described (33). tRNA\textsuperscript{Lys}\textsuperscript{59} and ODN, an 18-mer oligodeoxyribonucleotide complementary to the PBS, were radioactively labeled at their 5′-ends as described (28). The chimeric primers ODN-dC-AZT and tRNA\textsuperscript{Lys}\textsuperscript{59}-dC-AZT (Fig. 1B) were obtained by an extension of ODN and tRNA\textsuperscript{Lys}\textsuperscript{59} hybridized at a 1:2 ratio to HIV-1 RNA-(1–311) with 15 μM HIV-1 RT, 50 μM dCTP, and 50 μM AZTTP for 1 h at 37 °C. After proteinase K treatment and phenol/chloroform extraction, chimeric primers were ethanol precipitated and purified by denaturing polyacrylamide gel electrophoresis as described (31).

The template strand was either an RNA encompassing nucleotides 1–311 (RNA-(1–311)) or 159–196 (RNA-(159–196)) of the HIV-1 Mal genomic RNA or a DNA corresponding to nucleotides 159–196 (DNA-(159–196)) of the same isolate. All templates contained the PBS (nucleotides 179–196). RNA-(1–311) was obtained by in vitro transcription as described (34), whereas RNA-(159–196) and DNA-(159–196) were chemically synthesized. Wild type RT and the resistant RT-bearing mutations D67N, K70R, T215F, and K219K were purified as previously described (20).

**Primer Unblocking**—2–5 nM labeled tRNA\textsuperscript{Lys}\textsuperscript{59}-dC-AZT or ODN-dC-AZT were hybridized to 20 nM template as described (35). The annealing efficiency was verified by electrophoresis on non-denaturing polyacrylamide gels. In all cases, >95% of the labeled primer (tRNA\textsuperscript{Lys}\textsuperscript{59}-dC-AZT or ODN-dC-AZT) was found to be hybridized to the template (RNA-(1–311), RNA-(159–196), or DNA-(159–196)) (Fig. 2E and data not shown). 20 nM wt or resistant RT were added to the primer-template complex for a 4-min pre-incubation at 37 °C in 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 6 mM MgCl\textsubscript{2}, and 1 mM dithiothreitol. Reactions were initiated by adding 3.5 mM ATP. Reactions were stopped, and the products were separated by denaturing gel electrophoresis and quantified as described (31).

**Rescue of DNA Synthesis**—Kinetics were performed as above, except that the reaction was initiated by the addition of 50 μM dTTP, 50 μM ddGTP, 3.5 mM ATP, and 0.005 units/μl inorganic pyrophosphatase (Sigma).

**RESULTS**

All ATP lysis experiments using AZT-resistant RT published to date were performed using DNA/DNA primer-template complexes (21–23,36). However, the efficiency of this reaction, like other properties of HIV-1 RT, might vary considerably during the different steps of the provirus synthesis. Because provirus synthesis requires both RNA- and DNA-dependent DNA elongation, we first compared the efficiency of the excision reaction during synthesis of the (+) and (−) DNA strands. To this aim, we used as a primer ODN-dC-AZT, an oligodeoxyribonucleotide complementary to the PBS and extended by dC and AZT, and either a DNA or an RNA template corresponding to nucleotides 159–196 of HIV-1 Mal (DNA-(159–196) and RNA-(159–196), respectively) (Fig. 1B). In addition, the excision of AZT from the primer terminus during (−) DNA strand synthesis was also studied with an RNA template encompassing nucleotides 1–311 of the same viral isolate (RNA-(1–311)). Using this template, we then compared the AZT excision from ODN-dC-AZT and tRNA\textsuperscript{Lys}\textsuperscript{59}-dC-AZT, thus allowing a comparison of the elongation and initiation steps of (−) DNA strand synthesis.

Because ATP lysis is sensitive to the presence of the next incoming nucleotide (22, 36), two series of experiments were performed. In the first one, the excision reaction was performed in the absence of any nucleoside triphosphate, and we monitored the appearance of a product one nucleotide shorter than the initial primer. In the second, the reaction was performed in the presence of dTTP and ddGTP. After removal of the terminal AZT, these two nucleotides could be incorporated (Fig. 1B), and we followed the rescue of DNA synthesis, which generated products extended by one nucleotide, as compared with the primer. In all experiments, the AZT-resistant RT was compared with the wt enzyme, and reactions were conducted in the absence and in the presence of ATP (3.5 mM).

**Comparison of AZT Excision and the Rescue of DNA Synthesis during DNA- and RNA-dependent DNA Elongation**

**Excision of the Terminal AZT**—When ODN-dC-AZT was annealed to the DNA-(159–196) template, excision of the terminal AZT in the presence of 3.5 mM ATP could be observed with both the wt and the resistant RT (Fig. 2A). However, the reaction was much more efficient with resistant RT, as confirmed by quantitative analysis of the data. Both reactions followed a first order kinetics (Fig. 2B), excision being 4-fold faster with resistant RT (k\textsubscript{exc} = 1.38 × 10^{-3} and 3.7 × 10^{-4} s\textsuperscript{-1} for resistant and wt RT, respectively). When no ATP was included in the reaction, no AZT excision was observed with wt RT, whereas a very limited excision could be detected when using resistant RT (data not shown).

When we used RNA-(1159–196) as a template, fast AZT excision was observed in the presence of 3.5 mM ATP with the AZT-resistant RT (Fig. 2C). Indeed, the excision rate deduced from the fit of the experimental data to a single exponential (2.1 × 10^{-3} s\textsuperscript{-1}) is slightly higher than that determined using the DNA template (Fig. 2D). However, the reaction rapidly reached a plateau, and only 40% of the primer was eventually repaired even after prolonged incubation (up to 3 h, Fig. 2D and data not shown). This situation contrasts with the DNA template, which allowed excision of more than 70% of terminal AZT (Fig. 2B). In addition, using wt RT, the amount of the repaired primer in the presence of ATP was less than 5% with RNA-(159–196) as the template as compared with 50% with DNA-
(159–196) (Fig. 2, B and D). Furthermore, no excision was observed with the former template in the absence of ATP, whichever RT we used (data not shown).

Incomplete annealing of the primer to the RNA template could be a trivial explanation for the different amplitudes of the AZT excision from ODN-dC-AZT annealed either to DNA-(159–196) or RNA-(159–196). To test this possibility, the primer-template complexes were analyzed by electrophoresis through a non-denaturing polyacrylamide gel (Fig. 2E). The autoradiography of the gel showed complete annealing of ODN-dC-AZT to RNA-(159–196) (Fig. 2E, lane 2), whereas >95% of the primer was bound to DNA-(159–196). In the latter case, the slightly retarded migration of the unbound primer as compared with free ODN-dC-AZT (compare lanes 1 and 3) and the smear between the two bands suggested that annealing was essentially complete and that a small fraction of the complex dissociated during electrophoresis. These controls clearly showed that the different amplitudes of the excision reactions were not due to incomplete annealing of the primer to RNA-(159–196).

Rescue of DNA Synthesis—With the DNA-(159–196) template, an efficient rescue of DNA synthesis in the presence of dTTP and ddGTP was observed with resistant RT when ATP was included in the reaction (Fig. 3A). A quantitative analysis of the rescue kinetics showed that the amplitude of this reaction (75%) is in keeping with that of the excision reaction (compare Figs. 2B and 3D). However, the rate of DNA synthesis rescue ($k_{\text{rec}} = 4.2 \times 10^{-4} \text{ s}^{-1}$) (Fig. 3D) is 3-fold lower than the rate of ATP lysis (Fig. 2B). Because ATP lysis was much slower than nucleotide addition to the 3'-end of an unblocked primer (30), our results suggest that ATP lysis was partially inhibited by the next incoming nucleotide (22, 36). The rescue of DNA synthesis was observed with the wt RT in the presence of ATP and with resistant RT in its absence, but these reactions were too weak to allow quantitative analysis (Fig. 3 and data not shown). No rescue was observed with wt RT in the absence of ATP.

Similar results were obtained when using RNA-(159–196) as a template (Fig. 3, B and D). An efficient rescue of DNA synthesis by resistant RT was observed in the presence of 3.5 mM ATP. Its rate, $6.4 \times 10^{-4} \text{ s}^{-1}$, is close to the one measured for rescue on the DNA template and 3-fold slower than the excision reaction on the RNA-(159–196) template, again suggesting that the excision reaction was somewhat inhibited by the presence of the next incoming nucleotide. As observed for the excision reaction, the amplitude of the rescue was 2-fold lower with the RNA-(159–196) template as compared with the equivalent DNA template (Fig. 3D). No significant rescue of DNA synthesis could be observed with the wt RT in the presence of ATP or with resistant RT in its absence (Fig. 3B and data not shown).

AZT Excision and Rescue of DNA Synthesis during the Initiation of (−) Strand DNA Synthesis

We showed previously that terminal dT and AZT could be removed with similar efficiencies from the primer by wt RT via pyrophosphorolysis during the elongation of (−) DNA strand synthesis (Fig. 1A), whereas only dT could be efficiently excised during initiation (31). As could be expected from the relative polymerization rates (26, 30), the pyrophosphorolysis of dT was slower during initiation as compared with elongation (31). However, it could be easily followed in 1-h time course experiments.

The similarity between pyrophosphorolysis and ATP lysis (22, 23, 32) prompted us to test whether AZT could be excised by resistant RT via ATP lysis during the initiation of reverse transcription. Because optimal initiation of (−) DNA strand synthesis requires complex interactions between the primer tRNA$^{3\text{tRNA}}$ and the viral RNA (28, 29), it cannot be studied with RNA-(159–196), which was replaced by RNA-(1–311) as the template.

As a first control, we compared the rescue of DNA synthesis using ODN-dC-AZT as the primer and RNA-(1–311) as the template with the one obtained under the same conditions with RNA-(159–196) as the template (Fig. 3, C and D). The amplitude of the reaction and its rate ($6.9 \times 10^{-4} \text{ s}^{-1}$) were identical (within experimental errors) to those determined with the
shorter RNA template, indicating that the rescue of DNA synthesis was essentially independent of the template length. Again, the low amplitude of the excision reaction could not be explained by incomplete annealing of the primer, because non-denaturing electrophoresis showed that $>98\%$ of ODN-dC-AZT was hybridized to RNA-(1–311) (Fig. 2E, lane 4).

We next studied the rescue of DNA synthesis from tRNA$^{\text{3Lys}}$-dC-AZT using either wt or resistant RTs. No extension of the

FIG. 2. Unblocking of an AZT-terminated primer during elongation of (+) and (−) strand DNA synthesis by wt RT and resistant RTs. A, unblocking of the ODN-dC-AZT-DNA-(159–196) complex in the presence of 3.5 mM ATP. Lanes 1–22 correspond to increasing reaction times as follows: 0, 6, 12, 18, 24, 30, 36, 42, 48, and 54 s, 1, 2, 3, 4, 5, 7.5, 10, 15, 30, 40, 50, and 60 min, respectively. B, the gels presented in A were quantified, and the experimental data were fitted to the equation $\%\text{ODN-dC-AZT} = A(e^{-k_{\text{exc}}t}) + C$, where $A$ is the amplitude of the burst, $k_{\text{exc}}$ is the apparent first order rate constant of AZT excision, and $C$ is a constant. The curves for the wild type (open circles) and resistant (filled circles) RTs correspond to $k_{\text{exc}} = 3.7 \times 10^{-4} \pm 5 \times 10^{-5}$ s$^{-1}$, $1.38 \times 10^{-3} \pm 7 \times 10^{-4}$ s$^{-1}$, respectively. C, unblocking of the ODN-dC-AZT-RNA-(159–196) complex in the presence of 3.5 mM ATP. Lanes 1–17 correspond to the following reaction times: 0, 1, 2.5, 5, 7.5, 10, 20, 30, 40, 50, 60, 75, 90, 105, 120, 150, and 180 min, respectively. D, experimental data obtained by quantifying the gels presented in C were fitted as in B. The curve for resistant RT (filled circles) corresponds to $k_{\text{exc}} = 2.1 \times 10^{-3} \pm 3 \times 10^{-4}$ s$^{-1}$. E, analysis of the primer-template complexes on a non-denaturing 6% polyacrylamide gel. Labeled ODN-dC-AZT incubated alone (lane 1), and annealed to RNA-(159–196) (lane 2), DNA-(159–196) (lane 3), and RNA-(1–311) (lane 4) were compared.
primer could be observed in a 2-h reaction with either wt or resistant RT irrespective of the presence of ATP in the reaction (Fig. 4A). To ensure that the lack of rescue of DNA synthesis was not due to inhibition by the next incoming nucleotide, we tested the excision of terminal AZT from tRNA\textsubscript{3Lys-dC-AZT} in the absence of nucleotides. Similarly, no excision could be detected under any condition within the 3-h time course of the reaction (Fig. 4B). To exclude the possibility that excision by ATP lysis could take place, although at a slower rate than pyrophosphorolysis (31), we extended the time course of the reaction up to 24 h and added fresh aliquots of RT after 4 and 8 h of incubation. Remarkably, we still failed to detect any AZT excision (data not shown). Parallel analysis of the tRNA\textsubscript{3Lys-dC-AZT-RNA-(1-311)} complex by electrophoresis through a non-denaturing gel showed almost complete primer annealing (> 95%, data not shown).

Two additional controls were performed. In the first one, tRNA\textsubscript{3Lys} was replaced by an 18-mer RNA complementary to the PBS (ORN) to test whether the absence of excision was due to the presence of the bulky tRNA\textsubscript{3Lys-dC-AZT} primer. However, we detected no AZT excision from ORN-dC-AZT (data not shown). In the second, we tested the excision of terminal dT from tRNA\textsubscript{3Lys-dC-dT} by ATP lysis using wt or resistant RT. No dT excision from the tRNA\textsubscript{3Lys-dC-dT} could be observed, even after a 24 h incubation in the presence of ATP (Fig. 4C).

**DISCUSSION**

Even though the selection in the HIV-1 RT gene of mutations conferring resistance toward AZT was first described more than 20 years ago (4–6), the mechanism of action has been unraveled quite recently (21–23), and ATP-dependent excision of AZT by the resistant RT has only been documented for DNA templates. Although the resistance mutations confer a strong resistance toward AZT (4–7), the virus is not insensitive to the drug. Thus, the resistance level might vary considerably, depending on the nature of the primer and template strands.

In this study, we first compared the repair of AZT-terminated primers with the rescue of DNA synthesis during elongation of the (−) and (+) DNA strands. We then compared the initiation and elongation steps of (−) strand DNA synthesis. The results we obtained using a DNA template and a DNA primer are consistent with previous studies (21–23, 36). They show that the mutant RT harboring mutations D67N, K70R, T215F, and K219K can efficiently repair an AZT-terminated DNA primer in the presence of 3.5 mM ATP. That a weak AZT excision could be observed with wt RT in the presence of ATP
and with resistant RT in its absence is also consistent with previous data (22). Because we worked at saturating RT concentrations, the excision of AZT and the repair of DNA synthesis followed apparent first order kinetics. Our results showed that the rescue of DNA synthesis is about 3-fold slower than the repair of the primer, suggesting that the removal of terminal AZT is significantly inhibited by the next incoming nucleotide. However, this inhibition is significantly less pronounced than that observed for the removal of terminal ddA or d4T (22, 36). The in vivo dNTP concentration varies considerably with the cell type and its activation state; it ranges from 29 to 150 μM in human H9 T cells (37), from 3 to 26 μM in activated peripheral blood mononuclear cells, and from 0.3 to 6 μM in quiescent ones (38). Thus, the inhibition of AZT excision by the next incoming nucleotide might be significant in the cell types with the highest dNTP pool. Noticeably, the most efficient rescue of DNA synthesis should be observed in the cells with the lowest dNTP pool, i.e. those cells in which inhibition by AZT is the most efficient (39).

This work is the first to study primer repair and the rescue of DNA synthesis by AZT-resistant RT on an RNA template. We found that these processes take place essentially at the same rate on the DNA and RNA templates. However, we observed an important difference between the two templates; whereas 80% of a primer was eventually unblocked on a DNA template, only half this amount of primer was repaired when annealed to RNA templates. The origin of this difference is unclear but was not due to incomplete annealing of the primer to the RNA templates. Because the reaction was much slower than the rate of RT dissociation from and re-association to the primer-template complex (30), our results suggest that the AZT-terminated primer/RNA template adopts two non-interconverting (or slowly converting) conformations, only one being repaired.

Whatever the explanation for the observed difference, its
consequence is that the overall AZT excision by resistant RT is significantly lower on RNA than on DNA templates. On the other hand, the AZT incorporation efficiency at a given site by the resistant enzyme is only slightly reduced during synthesis of the (−) strand, as compared with the (+) strand (10), and the number of possible incorporation sites are almost equal. Thus, the difference in AZT excision that we reported in this study indicates that the inhibition of a resistant virus is more likely to take place during elongation of the (−) strand.

When we used tRNA[^15]dC-AZT as a primer and RNA-(1–311) as a template, neither the rescue of DNA synthesis nor the excision of the terminal AZT could be observed, even after a 24 h incubation. Because both processes were inhibited, inhibition of the former cannot be attributed to the formation of a dead-end complex (22, 36). Our results indicate that the mutant polymerase harboring mutations D67N, K70R, T215F, and K219K is either totally unable to unblock the AZT-terminated primer during the initiation of reverse transcription or at least this process is too slow to be significant during provirus synthesis in vivo (41, 42). Thus, if a resistant virus incorporates AZT during this step, its replication is definitively blocked. This conclusion might seem contradictory in view of the fairly efficient replication of the resistant virus in the presence of AZT. However, because the probability of incorporating AZT during initiation is very low as compared with elongation, the lack of resistance during initiation has a limited effect on the overall reverse transcription. Indeed, the AZT incorporation efficiencies at a particular site are similar during initiation and elongation (31), but there are only two possible incorporation sites during initiation as compared with about 2500 during elongation.

The inability of resistant RT to unblock the AZT-terminated primer and to rescue DNA synthesis during the initiation of reverse transcription contrasts with the effect of the resistance mutations during the elongation phase. This lack of primer unblocking was not specific for the natural tRNA[^15]dC primer, because no AZT excision was observed when we used an 18-mer RNA complementary to the PBS as the primer. These observations suggest that the catalytic site of RT is distorted when bound to a RNA/RNA primer-template complex as compared with DNA/RNA and DNA/DNA complexes. This hypothesis is in keeping with the different effects of manganese ions on elongation and initiation (28), and with the reduced polymerization rate during initiation (26, 30).

The inability of resistant RT to remove terminal AZT by ATP lysis during initiation is reminiscent of the inability of wt RT to unblock the AZT-terminated primer by “classical” pyrophosphorolysis (31). However, this study reveals a significant difference between pyrophosphorolysis and ATP lysis. Whereas only AZT was resistant to pyrophosphorolysis by the wt enzyme during the initiation of reverse transcription (31), neither AZT nor dT could be removed via ATP lysis by AZT-resistant RT (this study). Thus, our data suggest that AZT-resistant RT is unable to accommodate ATP in a catalytically competent conformation when bound to the initiation complex.

Our results demonstrate the different effects of resistance mutations during the initiation and elongation of reverse transcription and during RNA-dependent and DNA-dependent DNA synthesis. These results highlight the need for detailed studies of these mutations at each step of the reverse transcription cycle (initiation of (−) and (+) strand synthesis, RNA- and DNA-dependent elongation, strand transfers, and termination at the central termination sequence). Such studies would improve our fundamental understanding of the resistance mechanisms. They could also point toward particular steps of the reverse transcription process that would constitute attractive targets for the development of new antiviral agents against which the emergence of resistance mutations would be limited.

Our present results obviously indicate that the initiation of reverse transcription could constitute one such target. Initiation is intrinsically less efficient than elongation (26, 30, 31), and optimal initiation requires intricate interactions between viral RNA, RT, and a cellular component, tRNA[^15][^3]H[^11]02[^2]Lys[^13]d[^14]C[^15]A[^18]ZT. Thus, if a resistant virus is only slightly reduced during synthesis of the provirus, RT, and a cellular component, tRNA[^15][^3]H[^11]02[^2]Lys[^13]d[^14]C[^15]A[^18]ZT. Thus, if a resistant virus...
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