Site-specific Footprinting Reveals Differences in the Translocation Status of HIV-1 Reverse Transcriptase

IMPLICATIONS FOR POLYMERASE TRANSLLOCATION AND DRUG RESISTANCE*

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Resistance to nucleoside analogue inhibitors of the reverse transcriptase of the HIV-1 often involves phosphorolytic excision of the incorporated chain terminator. Previous crystallographic and modeling studies suggested that this reaction could only occur when the enzyme resides in a pre-translocational stage. Here we studied mechanisms of polymerase translocation using novel site-specific footprinting techniques. Classical footprinting approaches, based on the detection of protected nucleic acid residues, are not sensitive enough to visualize subtle structural differences at single nucleotide resolution. Thus, we developed chemical footprinting techniques that give rise to hyperreactive cleavage on the template strand mediated through specific contacts with the enzyme. Two specific cuts served as markers that defined the position of the polymerase and RNase H domain, respectively. We show that the presence of the next correct dNTP, following the incorporated chain terminator, caused a shift in the position of the two cuts a single nucleotide further downstream. The footprints point to monotonic sliding motions and provide compelling evidence for the existence of an equilibrium between pre- and post-translocational stages. Our data show that enzyme translocation is reversible and uncoupled from nucleotide incorporation and the release of pyrophosphate. This translocational equilibrium ensures access to the pre-translocational stage after incorporation of the chain terminator. The efficiency of excision correlates with an increase in the population of complexes that exist in the pre-translocational stage, and we show that the latter configuration is preferred with an enzyme that contains mutations associated with resistance to nucleoside analogue inhibitors.

Nucleoside analogue inhibitors of the retroviral reverse transcriptase (NRTIs) represent important components in currently used drug regimens to treat infection with the human immunodeficiency virus type 1 (HIV-1). 3′-Azido-3′-deoxythymidine (zidovudine or AZT), 2′,3′-dideoxyinosine (didanosine), and (−)-β-D-2′,3′-dideoxy-3′-thacytidine (lamivudine) are prominent members of this class of compounds. NRTIs are intracellularly phosphorylated and act as chain terminators. Previous biochemical studies have shown that the incorporated chain terminator can be removed from the primer terminus through phosphorolytic cleavage in the presence of either pyrophosphate (PPi) (1) or in the presence of ribonucleotide triphosphates (NTPs) (2), which were shown to act as pyrophosphate donors. Increasing evidence suggests that the latter reaction pathway provides an important mechanism for HIV resistance to AZT and, to a certain degree, also to other NRTIs (3–11).

Recombinant RT enzymes that contain mutations associated with resistance to AZT (M41L, D67N, K70R, T215Y/T215F, and K219Q) do not prevent or diminish the incorporation of AZT-MP (12). Based on findings showing enhanced binding of the 3′ end of AZT-terminated primer strands to HIV-1 RT, it has later been suggested that pyrophosphorylation might provide a possible reaction pathway involved in resistance to AZT (13). Excision of AZT-MP occurs with considerable efficiency with wild-type HIV-1 RT, and the rate of pyrophosphorylation appeared to be increased with resistant RT enzymes (1). The difference in regard to efficiency of the excision reaction between both enzymes is dramatically increased when NTPs act as a pyrophosphate donor (3). In the light of these findings it is now widely accepted that ATP can act as a physiologically relevant pyrophosphate donor. It appears that excision of AZT-MP is particularly efficient as compared with other NRTIs, even in the context of wild-type HIV-1 RT (14). The rate with which each individual inhibitor is removed from the primer terminus depends critically on the concentration of the dNTP that is complementary to the next template position (3, 10, 14, 15). Phosphorolytic cleavage of incorporated 2′,3′-dideoxynucleotides, including ddAMP and ddTMP, is compromised at nucleotide concentrations as low as 1 μM, whereas significantly higher concentrations are required to inhibit the excision of AZT-MP. The formation of a stable ternary dead end complex is diminished with AZT-terminated primer strands, which provides an explanation for the efficient removal of AZT-MP (16).

Based on crystallographic and modeling studies (7, 17, 18), it has been suggested that the excision reaction could only occur when the 3′ end of the primer terminus remains in the same position at the polymerase active site, referred to as nucleotide

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Figure 1. A, experimental strategy for the treatment of RT-DNA-DNA complexes with KOONO. The template was radioactively labeled at the 3' end (asterisks) to monitor the reaction. DNA synthesis was chain-terminated with HIV-1 RT in the presence of AZT-TP as described under "Experimental Procedures." Boldface C and Z point to newly incorporated dCMP and AZT-MP. The dNTP that is complementary to next template position (dGTP, offset) was added to reaction mixture at high concentrations of 1 mM to stabilize the complex. B, the footprint experiment was conducted by the addition of a stable alkaline solution of KOONO. Lane 1 shows the control in the absence of HIV-1 RT. Lanes 2–5 show reactions with HIV-1 RT containing a cysteine at position 280 in both subunits. Reactions were conducted in the presence of low concentrations of glycerol (0.5%, lanes 2 and 3) and at higher concentrations (5%, lanes 4 and 5). Lanes 6 and 7 show reactions with an enzyme that contains a serine at position 280. The arrow points to a highly specific KOONO-mediated cut with RT (Cys-280) at low concentrations of glycerol.

If this model is correct, there must be a mechanism that controls the formation of the pre-translocational stage after the incorporation of the chain terminator. This mechanism is unknown. Earlier studies suggested that translocation may take place during the release of PPi, immediately after incorporation of the last nucleotide (19). Thus, the 3' end of the primer may ultimately move into the P-site, which is a prerequisite for dNTP binding and continuation of DNA synthesis. It has indeed never been demonstrated that the primer terminus has free access to the N-site. Rather, recent studies (17, 18) suggested that the N-site complex is a short-lived, unstable polymerization intermediate that needs to be covalently trapped. The available crystal structure models without an incoming dNTP show the primer terminus exclusively in the P-site (20–22), unless the enzyme is cross-linked to its nucleic acid substrate immediately after catalysis and before translocation takes place (17). Here we employed site-specific footprinting experiments to characterize the relationship between the precise position of HIV-1 RT on its primer/template substrate and the efficiency with which chain-terminating nucleotides are removed from the primer terminus. Our data provide compelling evidence for the existence of a translocational equilibrium between pre- and post-translocational stages that ensures access of the 3' end of the primer to the N-site after nucleotide incorporation and the release of pyrophosphate.
Experimental Procedures

Enzymes and Nucleic Acids—Wild-type HIV-1 RT (p66/p51) was expressed in *Escherichia coli* and purified essentially as described previously (23). Mutant enzymes were generated through site-directed mutagenesis using the Stratagene Quick-change kit according to the manufacturer’s protocol. Substrates used in this study were derived from the HIV-1 polypurine tract (PPT) as described in our previous studies (24, 25): 5′-TTAAAAGAAAAGGGGGGA (PPT-1/18D); 5′-TTAAAAGAAAAGGGGGGA (PPT-1/17D); and 5′-TTAAAAGAAAAGGGGGGA (PPT-1/16D). The model template used in this study provides complementarity to these primers: 5′-CGTTGTCAGTGAATCAGCC-TCA (PPT-2/18D). The model template used in this study provides the template (2.7 pmol) with the complementary primer (8.1 pmol) and finally resolved on 8% polyacrylamide, 7 M urea gels.

Rescue of Chain-terminated DNA Synthesis—Rescue of DNA synthesis was studied at a single template position using a similar assay as we described earlier (11). The pre-hybridized duplex (8.5 pmol), composed of the aforementioned template and primer strands, was incubated with 25.5 pmol of HIV-1 RT in a buffer containing 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, and 6 mM MgCl₂ followed by the addition 10 μM dCTP and 10 μM AZT-TP (TriLink BioTechnologies) to generate primer strands terminated with AZT-TP. Chain termination with dUTP and AZA-TP (TriLink BioTechnologies) was performed analogously. Reactions were allowed to proceed for 20 min to ensure complete extension of the primer and its quantitative chain termination with the nucleotide analogue. The excision of the analogue was initiated with ATP (3.5 mM) or, as indicated in some cases, with PPi (150 mM). Excision and the ensuing rescue of chain-terminated DNA synthesis was monitored in time course experiments. Samples were heat-denatured for 5 min at 95 °C and finally resolved on 8% polyacrylamide, 7 M urea gels.

Site-specific Footprinting—Site-specific footprints with KOONO and Fe²⁺ were monitored on 3% gel-purified DNA templates. Hybridization of the template (2.7 pmol) with the complementary primer (8.1 pmol) was conducted in a buffer containing 20 mM sodium cacodylate, pH 7, and 20 mM NaCl. The duplex was incubated with HIV-1 RT (16.2 pmol)
in a buffer containing 120 mM sodium cacodylate, pH 7, 20 mM NaCl, 6 mM MgCl₂, and a mixture of nucleotide triphosphates that allow quantitative chain termination as described above. Prior to the treatment with KOONO or Fe²⁺ complexes were pre-incubated for 20 min with increasing concentrations of the next nucleotide at different temperatures as indicated in the figures. Treatment with Fe²⁺ and KOONO, respectively, was performed essentially as described recently (26, 27).

RESULTS

Site-specific Footprinting—It was our initial aim to develop novel footprinting techniques that would allow us to identify and to distinguish between pre- and post-translocational stages. We have previously applied high resolution footprinting techniques to study the interaction between HIV-1 RT and its DNA/DNA primer/template substrate (27, 28). Two chemically distinct sources of hydroxyl radicals were utilized: the classical method with [Fe(EDTA)]²⁻ that yields hydroxyl radicals via Fenton-like chemistry; and potassium peroxynitrite (KOONO), which is a metal-free source of hydroxyl radicals that are generated during decomposition of the conjugate acid HOONO. Despite a relatively high resolution of the hydroxyl radical footprints, the boundaries of the protected regions are not as clearly defined as required for the detection of structural differences associated with the translocation of RT. However, the same reactions give rise to hyperreactive cleavage at two distinct template positions. Treatment of RT-DNA-DNA complexes with divalent Fe²⁺ ions in the absence of EDTA yields oxidative cleavage at template position −17 and a minor cut at position −18. We showed that Fe²⁺ ions can bind in the vicinity of the RNase H active center, and oxidation of the bound metal ions yields a local source of hydroxyl radicals that cleave the template in site-specific fashion. Treatment with KOONO yielded hyperreactive cleavage at template position −7. Specific binding of the enzyme is a prerequisite for hyperreactivity, and therefore, both of these signals may serve as specific markers to identify the precise position of the enzyme in the presence and absence of an incoming nucleotide.

The KOONO-induced cut is of particular interest in this regard, because the observed hyperreactivity involves a single nucleotide in highly specific fashion. The model of the ternary complex shows Cys-280 of the large subunit of heterodimeric (p66/p51) HIV-1 RT in close proximity to the DNA template. Previous reports (29) have shown that peroxynitrite mediates oxidation of the thiol group of cysteines, which may provide a link to the mechanism involved in the reaction. To test a possible involvement of Cys-280 in the reaction with KOONO, we generated complexes composed of an AZT-terminated primer strand and RT enzymes that contained either cysteine or serine at position 280 (Fig. 1A). KOONO-induced cleavage is specific for the Cys-280 enzyme, whereas the reaction is undetectable with the Ser-280 mutant (Fig. 1B, lanes 3 and 7). The efficiency of the reaction is severely compromised in the presence of the radical scavenger glycerol, suggesting the involvement of either hydroxyl radicals and/or thyl radicals (Fig. 1B, lanes 3 and 5). Regardless of the precise chemical mechanism, the specific signal on the template strand is unambiguously assigned to the interaction between the cleaved nucleotide and Cys-280. We therefore refer to “site-specific footprinting” as opposed to classical footprinting techniques that do not yield information regarding the contribution of individual amino acid residues.

Pre- and Post-translocational Stages—Both techniques were utilized to study possible differences in regard to the position of the enzyme in the presence of the next dNTP (Fig. 2, A and B). RT-DNA-DNA complexes, with an AZT-terminated primer strand, were pre-incubated in the absence or in the presence of the next complementary dNTP. In the absence of dNTPs, we observed Fe²⁺-mediated cuts at position −18 and less pronounced at position −19 (Fig. 2A, lane 3). These cuts are seen a single position further downstream (−17/−18) when the next dNTP was present at high concentrations of 1 mM (Fig. 2A, lane 4). A similar shift is evident when comparing the positions of the KOONO-induced cut. In the absence of the next nucleotide, the cut is seen at position −8, whereas in the presence of the template dNTP, the cut is seen at position −7 (Fig. 2A, lanes 7 and 8). Thus, it appears that the formation of a ternary dead end complex is associated with enzyme translocation. Our data suggest that the cut at position −8 is indicative for the pre-translocational stage in which the primer terminus occupies the N-site, whereas the cut at position −7 is indicative for the post-translocational stage in which the primer terminus occupies the P-site.

A comparison of cleavage patterns with KOONO and Fe²⁺, respectively, shows that the ratio of cuts representing pre- and post-translocational stages is literally identical, for the two methods employed here, at any given dNTP concentration tested (Fig. 2C). Both methods suggest that dNTP concentrations of −22 μM are required to obtain 50% of complexes post-translocation (Fig. 2D). We note that the total amount of radioactivity increased selectively in the KOONO experiment at relatively high concentrations of dNTPs (≥100 μM), when 80% of the complexes exist already in the post-translocational stage. An increase in band intensities is not evident in the Fe²⁺ assay. It remains to be elucidated whether these data point to a local increase in the stability of the ternary complex, in close proximity to the polymerase active site, and/or to differences with respect to the efficiency of the KOONO-mediated reaction as the ternary complex is formed.

Impact of the Temperature—Fig. 2 shows unambiguously that low dNTP concentrations facilitate the pre-translocational N-configuration. This result appears to be in conflict with the crystal structure models that show the primer terminus exclusively in the P-site (21, 22). In fact, the structures of the binary and the ternary complex show Cys-280 in close proximity to the template position −7. Several factors may account for the apparent discrepancy between our biochemical data in solution and the crystal structure models. We initially evaluated the impact of the temperature. AZT-terminated complexes were pre-incubated in the presence of different dNTP concentrations and were kept at low temperatures on ice, i.e. conditions referred to as 0 °C, or at 37 °C, followed by treatment with

![Fig. 3. Temperature dependence of P-site and N-site occupations.](image-url)
Fig. 4. Efficiency of excision of 3'-azido-containing chain terminators and their 2,3'-dideoxy counterparts. A and B, schematic description of assays used to monitor the rescue of DNA synthesis through limited extensions of 5' end-labeled primers at a single template position as described under "Experimental Procedures." C, time course of the reaction with different chain terminators: AZT-MP and ddTMP (top), and AZA-MP and ddAMP (bottom). Lane 1 shows the unextended primer. Lane 2 the chain-terminated primer. Lanes 3–12 show different time points after the addition of ATP and the "rescue mixture" (1, 3, 6, 10, 15, 22, 30, 45, 60, and 90 min). D and E, effect of the different chain terminators on the translocational equilibrium. KOONO-footprinting of complexes containing AZT-MP and ddTMP (D) was performed and analyzed as described in Fig. 2. The footprints with AZA-MP and ddAMP (E) were performed in a different range of concentrations of the next nucleotide (dGTP). Lanes 1–11, 0.01, 0.025, 0.1, 0.25, 0.5, 1, 3, 12.5, 6.25, 1.25, and 25 μM, and lane 12 shows the binary complex in the absence of dGTP. F, effect of different temperatures on P/N-site occupations with AZA-MP and ddAMP-terminated primer strands. Reactions were performed at 37 °C (left) and on ice (right). Lane 1 shows a control in the absence of treatment with KOONO. Lane 2 is a control with KOONO treatment in the absence of RT. Lanes 3 and 5 show reactions in the absence of the next dNTP, and lanes 4 and 6 show reactions in the presence of 1 mM of the next dNTP.
KOONO at the same temperatures (Fig. 3). Reactions at 37°C show a gradual shift of the KOONO-induced cut from position H11002 towards position H11002H11002 as the concentration of the templated dNTP was increased (Fig. 3, left panel); however, the non-translocated complex with the primer terminus in the N-site is still detectable in the presence of 100 μM dNTP (Fig. 3, left panel, lane 8). In contrast, at 0°C the binary complex is only seen as a faint band in the pre-translocational stage (Fig. 3, lane 1, right panel), and the dNTP concentrations required to form the post-translocated stage is much lower as compared with the reaction at 37°C (Fig. 3, compare lanes 2–5, left and right panels). The temperature-dependent differences in regard to the relative N- and P-site occupations are completely reversible. Reactions conducted at 37°C, with a pre-incubation period of 30 min at low temperatures, gave essentially the same result as seen at 37°C in the absence of pre-incubation on ice (Fig. 3, middle). These data show that the 3' end of the primer does not ultimately translocate into the P-site to allow binding of the next dNTP. The primer terminus can occlude the N-site, and does this preferentially at physiologically relevant temperatures, i.e. at 37°C.

Impact of the Chemical Nature of the Chain Terminator—The aforementioned data form the basis to study the relationship between the precise position of HIV-1 RT on its nucleic acid substrate and the ability of the enzyme to excise the incorporated chain terminator via phosphorolytic cleavage. Excision is here monitored in assays that detect the removal of the chain terminator and the ensuing rescue of DNA synthesis at a single template position as described previously (11) (Fig. 4A). Our data are in good agreement with published results by others, showing efficient rescue of AZT-terminated DNA synthesis, whereas the excision of ddTMP is almost completely blocked under these conditions (3) (Fig. 4C, top). To analyze further whether the 3'-azido group may be an important structural feature that facilitates the excision reaction as suggested previously, we tested and compared the efficiency of excision with ddAMP and AZA-MP (Fig. 4B). Indeed, Fig. 4C (bottom) shows that rescue of AZA-terminated DNA synthesis is significantly increased, as compared with the reaction that involves ddAMP-terminated primers. The high efficiency with which AZT-MP and AZA-MP are removed from the primer terminus correlates with an increased population of complexes that exist in the N-conformation (Fig. 4, D and E). The site-specific footprints with KOONO revealed that pre-translocational stages with ddTMP or ddAMP-terminated primer strands are only seen at submicromolar concentrations of the templated dNTP or in the absence of dNTP when the translocational status is not influenced by the formation of a ternary complex. At 37°C, binary complexes with AZA-MP-terminated primer strands are found predominantly in the pre-translocational stage, whereas binary complexes with ddATP-terminated primer strands are characterized by a mixture of complexes in pre- and post-translocational stages (Fig. 4F, lanes 3 and 5, left panel). Importantly, at 0°C, binary complexes with ddAMP-terminated primer strands are found exclusively in the post-translocational stage. The fact...
FIG. 6. Effects of resistance conferring mutations on relative N-site and P-site occupations. A, efficiency of excision of AZT-MP with wild-type HIV-1 RT and a mutant enzyme containing AZT resistance conferring mutations M41L, T215Y, and T69S-SS. Rescue of DNA synthesis was monitored in time course experiments as described in Fig. 4. B, efficiency of translocation with AZT-terminated primers. The graphs show the nucleotide-dependent changes of the translocation status with wild-type RT and the mutant enzyme. Experiments were conducted as described in Fig. 2C. C, efficiency of excision of ddTMP with wild-type HIV-1 RT and the mutant enzyme. D, efficiency of translocation with ddTMP-terminated primers. The graphs show the nucleotide-dependent changes of the translocation status with wild-type RT and the mutant enzyme.
that the N-configuration is literally undetectable at lower temperatures helps to explain why the crystallographic data show exclusively the P-configuration. This result does not only confirm the temperature dependence of relative occupations of pre- and post-translocational stages, it also demonstrates the existence of an independent equilibrium between both configurations.

Impact of the Sequence—There are also clear differences between the relative occupations of N- and P-sites when comparing AZA-MP and AZT-MP or ddAMP and dTMP. In order to evaluate a possible impact of the sequence of the template on efficiency of the excision, we compared reactions with two different primers, termed PPT-1 and PPT-2, that were both terminated with AZT-MP (Fig. 5A). PPT-1 represents the primer that has been used throughout this study. PPT-2 binds at another location on the same template. In contrast to the high efficiency of rescue of AZT-terminated DNA synthesis with PPT-1, the reaction with PPT-2 is almost completely blocked (Fig. 5B). The analysis of populations of pre- and post-translocational stages shows that the 3′ end of the PPT-2 has no access to the N-site, not even in the absence of the templated dNTP (Fig. 5C). The formation of the post-translocational stage shows a similar dNTP-dependent appearance as seen with PPT-1, suggesting that the formation of the pre-translocational stage is selectively diminished with PPT-2.

Impact of Drug Resistance Conferring Mutations—Previous biochemical data have shown that RT enzymes with classical AZT resistance conferring mutations 41L and 215Y together with the insertion mutation (41L/215Y/69S-(SS)) are highly efficient with respect to the ATP-dependent unblocking of AZT-terminated primers (4–6, 8, 15). This mutational pattern is associated with high level resistance to AZT and medium levels of resistance to other NRTIs. Fig. 6A shows a comparison of rescue of AZT-terminated synthesis with wild-type RT and the mutant enzyme. The results are in good agreement with published reports, showing increased rates of the reaction with the mutant RT. Fig. 6B shows the analysis with respect to the relative populations of complexes in pre- and post-translocational stages. The insertion-containing mutant enzyme clearly facilitates access of the 3′ end of the primer to the N-site. The amount of radioactivity is almost identical in lanes 4–7 (top versus bottom); however, the ratio of cleavage at positions 7/8 differs significantly. The concentration of the templated dNTP required to obtain 50% of complexes in the post-translocational stages is ~25 times higher as seen with wild-type HIV-1 RT. This value is relatively high as compared with measurements on the basis of functional assays that monitor efficiency of excision. Thus, it is conceivable that not all of the complexes that exist in the pre-translocational stage are competent, and in some cases, the enzyme may dissociate from its nucleic acid substrate before excision can occur. The specific mutational background is likely to be an important factor in this regard (5).

Considering that resistance conferring mutations may also affect the affinity to ATP, we next tested whether the presence of ATP or PP_i may further facilitate the formation of the pre-translocational stage. However, the presence of physiologically relevant concentrations of 3 mM ATP and 150 μM PP_i did not affect the distribution of the two different configurations, neither in the context of wild-type HIV-1 RT nor in the context of
the mutant enzyme (data not shown). Although the same mutant enzyme can also increase rates of rescue of ddTMP-terminated DNA synthesis, this effect is by far not as pronounced as seen with AZT-terminated primers (Fig. 6C). Similar conclusions can be drawn from the analysis of the translocational stages (Fig. 6D). The N-site occupation is favored with the mutant enzyme; however, the concentration required to obtain 50% of complexes in the post-translocational stage is still significantly lower as compared with the situation with AZT-terminated primers (2–3 μM versus 570 μM). Taken together, our data show consistently a clear correlation between an increase in rates of excision of a chain-terminated nucleotide and an increase in the population of complexes in the pre-translocational stage.

**DISCUSSION**

**Characterization of Pre- and Post-translocational Stages**—Although both the polymerase and RNase H activities of HIV-1 RT have been studied in great detail, our current understanding in regard to mechanisms involved in translocation of this enzyme remains elusive. The study of mechanisms involved in polymerase translocation requires highly sensitive techniques that allow the detection of subtle structural differences at a single nucleotide resolution. We have recently reported that the precise position of the RNase H-mediated Fe²⁺ cut can differ, depending on the structure of the bound nucleic acid. All of these previous experiments were conducted in the presence of high concentrations of dNTPs that facilitate the formation of ternary complexes. Here we show that treatment of binary complexes yielded specific oxidative cleavage at template position −8 (KOONO-mediated) and −18/−19 (Fe²⁺-mediated), respectively. The specific cuts moved toward position −7 and positions −17/−18 as the concentration of the next correct dNTP was gradually increased. The single nucleotide shift defines the pre- and post-translocational stages (Fig. 7).

Our data suggest that the gross conformation of the enzyme and the bound nucleic acid substrate remains unchanged in these two configurations. We measured a constant number of 10 bp between Cys-280 and Glu-478, at any given dNTP concentration. Putative translocational intermediates with 11 or more base pairs between the two markers were not detected, suggesting that both the polymerase and the RNase H domain can translocate simultaneously. Such mode of translocation is not universal. For instance, a recent study with T7 RNA polymerase suggested that the presence of the next NTP could promote a discontinuous (30), inchworm-like mode of translocation, as described earlier for *E. coli* RNA polymerase (31).

**The Translocational Equilibrium Model**—Translocation is either coupled with or uncoupled from the catalytic step. Current mechanisms of a single cycle of nucleotide incorporation, which have been developed on the basis of kinetic measurements (19, 32, 33) and crystallographic snapshots (34–36), do not include the translocation as a distinct step. A widely accepted model involves nucleotide binding to an open RT-prim/template complex, followed by a rate-limiting conformational change that traps the incoming nucleotide in a closed conformation. The ensuing chemical step is followed by a rapid release of PPi. Enzyme translocation is kinetically invisible (19) and may occur in concert with PPi release. The difficulties to crystallize complexes in the pre-translocational stage may support such type of mechanism. The recent study by Sarafianos et al. (17) suggests that HIV-1 RT must be covalently cross-linked to the duplex in order to trap the 3′ end of the primer in the N-site before translocation takes place. The authors noted that the N-site configuration is conformationally strained, which may store part of the energy released after bond formation. However, our footprinting revealed considerable amounts of N-site complexes at 37 °C. Thus, higher temperatures may provide sufficient energy to overcome putative conformational strain. The temperature dependence of the relative populations of N- and P-site complexes makes clear that enzyme translocation is reversible, and the 3′ end of the terminus can regain access to the N-site after the incorporation of the chain terminator. The total amount of the N configuration depends also on the chemical nature of the chain terminator (Fig. 4F).
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differences in relative populations of pre- and post-translocation stages, seen even in the absence of the next dNTP, define the translocation as a distinct step during a single cycle of polymerization (Fig. 8).

Our data are consistent with the “translocational equilibrium model,” proposed by Sousa and colleagues (37, 38), that is based on a passive mode of polymerase translocation. These authors suggested that a given polymerase could rapidly slide between pre- and post-translocational stages. The pre-translocational stage in which the primer terminus occupies the N-site is favored in the absence of nucleotides, whereas the post-translocational P-site configuration is favored in the presence of nucleotide substrates. This hypothesis is based on the assumption that favored interactions between the polymerase and its substrates are maximized under these circumstances.

The model in its general form, as demonstrated by our data, does not make any specific distinctions between processive polymerases and other enzymes, such as HIV-1 RT that dissociate more frequently from their nucleic acid substrates. The site-specific footprints can be seen as snapshots taken within a few seconds that compose the short lifetime of HOONO of ~4 s at neutral pH (39). The relative distribution of the two bands at tional stage or in the post-translocational stage. These conditions, and the appearance of two bands points to a mixture of complexes that exist either in the pre-translocational stage or in the post-translocational stage.

Effects of Changes of the Relative Distribution of Pre- and Post-translocational Stages—We demonstrate that the relative distribution of both bands depends on several parameters that include the temperature, the sequence of the template, the overall equilibrium (Fig. 8, steps 5, 1 and 2) completely to the ternary complex in the post-translocational stage. However, the translocation equilibrium might play a role in the control of pausing or termination (40) or the extension of misaligned primers.

Here we demonstrate that this equilibrium plays a critical role in regard to the efficiency of excision of AZT-MP. Our data show consistently that increased rates of primer unblocking correlate with an increased population of complexes that exist in the pre-translocational stage. It appears that the drug-resistant enzyme can amplify this effect. This might be the result of a diminished stability of the ternary complex (6). Alternatively, resistance conferring mutation may exert a direct effect on the translocational equilibrium, and binding of the incoming nucleotide might be prevented by the occlusion of the N-site; however, in either case, the increase in the population of complexes in the pre-translocational stage correlates with increased rates of excision. We have also shown that the sequence of the primer/template substrate can affect relative populations of complexes in pre- and post-translocational stages. During the process of reverse transcription, the enzyme is probably in contact with a broad spectrum of different sequences that influence both the excision reaction and the translocational equilbrium to various degrees. The presence of sites that diminish the ATP-dependent unblocking reactions may well correlate with increased susceptibility to AZT; however, future studies should address whether the nucleotide analogues is also effective incorporated at these positions.

Conclusions—Taken together, the results of this study demonstrate that translocation of HIV-1 RT is a distinct step during a single cycle of DNA polymerization, which provides the basis for the determination of thermodynamic parameters of this process. The translocational equilibrium model provides a mechanism to ensure free access of the primer terminus to the N-site and also to allow binding of the pyrophosphate donor ATP, after the incorporation of the chain terminator and the release of pyrophosphate. These data validate the translocational equilibrium as a target for the development of novel antiviral agents that may act more effectively against various HIV-1 isolates including viruses that acquired resistance to NRTIs. The site-specific footprinting approach provides a valuable tool in this regard. Previous studies suggested that peroxynitrous acid participates in oxidation of the thiol group of cysteines, giving rise to thyl radicals at neutral pH (29). Thus, the thyl radical is presumably the active species that initiates cleavage through abstraction of hydrogens from the sugar moiety of the nearest nucleotide, as described for hydroxyl radical-mediated reaction. It is conceivable that substitutions of cysteine residues at other locations implicated in substrate binding may produce similar signals as described in this study with Cys-280. The distance between the thiol group and the nearest nucleic acid residue is certainly an important parameter in this regard. Further studies that address this problem are required to assess the potential and limitations of KOONO-mediated site-specific footprinting and its application to other systems.

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