Reverse transcriptase backbone can alter the polymerization and RNase activities of non-nucleoside reverse transcriptase mutants K101E+G190S

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Previous work by our group showed that human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) containing non-nucleoside RT inhibitor (NNRTI) drug resistance mutations has defects in RNase H activity as well as reduced amounts of RT protein in virions. These deficits correlate with replication fitness in the absence of NNRTIs. Viruses with the mutant combination K101E+G190S replicated better in the presence of NNRTIs than in the absence of drug. Stimulation of virus growth by NNRTIs occurred during the early steps of the virus life cycle and was modulated by the RT backbone sequence in which the resistance mutations arose. We wanted to determine what effects RT backbone sequence would have on RT content and polymerization and RNase H activities in the absence of NNRTIs. We compared a NL4-3 RT with K101E+G190S to a patient-isolate RT sequence D10 with K101E+G190S. We show here that, unlike the NL4-3 backbone, the D10 backbone sequence decreased the RNA-dependent DNA polymerization activity of purified recombinant RT compared to WT. In contrast, RTs with the D10 backbone had increased RNase H activity compared to WT and K101E+G190S in the NL4-3 backbone. D10 virions also had increased amounts of RT compared to K101E+G190S in the NL4-3 backbone. We conclude that the backbone sequence of RT can alter the activities of the NNRTI drug-resistant mutant K101E+G190S, and that identification of the amino acids responsible will aid in understanding the mechanism by which NNRTI drug-resistant mutants alter fitness and NNRTIs stimulate HIV-1 virus replication.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is a heterodimer comprised of a p51-kDa and a p66-kDa subunit, and has multiple enzymic functions: RNA-dependent DNA polymerase and DNA-dependent DNA polymerase activities as well as polymerase-dependent and polymerase-independent RNase H activities (Coffin & Cold Spring Harbor Laboratory Press, 2002). With these activities, RT converts the viral ssRNA genome into dsDNA that is subsequently integrated into the host cell chromosome. RT is an essential enzyme for HIV-1 virus replication and therefore an important target of antiretroviral therapy (Workowski & Berman, 2011).

There are two classes of RT inhibitors: nucleoside/nucleotide RT inhibitors (nRTIs) and non-nucleoside RT inhibitors (NNRTIs) (Vivet-Boudou et al., 2006). The NNRTI class is composed of four US Food and Drug Administration-approved drugs that are used commonly by clinicians: nevirapine (NVP), efavirenz (EFV), etravirine (ETR) and rilpivirine (RPV) (O’Neal, 2011). NNRTIs bind to a hydrophobic pocket adjacent to the polymerase active site (Spence et al., 1995). Mutations that arise in response to therapy are located in this pocket and reduce the NNRTI binding affinity. We have shown that NNRTI drug-resistant mutants have defects in RNase H activity and RT content which correlates with their replication fitness in cell culture (Archer et al., 2000; Domaoal et al., 2006; Gerondelis et al., 1999; Wang et al., 2006, 2010a). Due the high mutation rate of HIV-1 RT, HIV-1 exists in patients as a quasispecies, which is a related but diverse population of HIV-1 isolates (Eberle & Gürtler, 2012; Muñoz et al., 1993). Polymorphisms in the RT sequence have been shown to influence replication fitness and drug resistance of known drug resistance mutations (Betancor et al., 2010, 2012; Ceccherini-Silberstein et al., 2007; Dykes et al., 2000;
Garriga et al., 2009; Hachiya et al., 2012; Kemp et al., 1998; Marcelin et al., 2006; Puertas et al., 2009; Stürmer et al., 2003; Vergne et al., 2006).

We have previously reported that the replication of the NNRTI drug-resistant virus K101E+G190S can be stimulated by NVP and EFV (Wang et al., 2010b). Despite the finding that K101E+G190S is similar to other NNRTI drug-resistant mutants and has reduced RT content, we showed that NNRTIs did not stimulate virus replication by increasing RT content (Wang et al., 2011). Instead, stimulation occurred during early steps of virus replication, most likely during reverse transcription. In addition, D10, a virus with a clinical RT backbone containing M41L+T215Y and K101E+G190S, was also stimulated in the presence of NVP and EFV. We also showed that the nRTI drug-resistant mutants L74V and M41L are stimulated by NVP and EFV (Wang et al., 2010b). Despite the finding that K101E+G190S reduced the stimulatory potential of NVP and EFV, indicating that the backbone sequence in which K101E+G190S occurs can dramatically alter the RT content. There was no difference in the RT content between D10M41L+T215Y+K101E+G190S and D10K101E+G190S (P>0.05), indicating that for the D10 mutant, nRTI resistance mutations do not alter RT content.

We also determined whether the amount of gag processing and/or gag–pol incorporation could explain the reduced amounts of RT in the virions. Fig. 2(a) shows a Western blot of the gag precursor normalized by p24. As we have previously published, NL4-3_G190S and NL4-3_K101E+G190S have increased amounts of the p41 precursor, indicating a defect in gag processing compared to WT. The D10 viruses did not show this increase in precursor, indicating that the backbone sequence improves the gag processing of (K101E+G190S). We also performed Western blots for IN content to assess whether the RT backbone could alter the amount of gag-pol incorporated. The results show that the D10 viruses have significantly higher amounts of IN compared to the NL4-3 viruses (Fig. 2b, c). D10M41L+T215Y+K101E+G190S versus NL4-3 M41L+T215Y+K101E+G190S (P=0.0005), D10K101E+G190S versus NL4-3 K101E+G190S (P=0.0008). However, only L74V significantly increased the IN content of NL4-3_K101E+G190S (P=0.028). M41L+T215Y did not significantly increase the IN content of viruses with the NL4-3 or D10 backbones (P>0.05).

**RT backbone sequence can alter the polymerization activity of RT with the NNRTI drug resistance mutations, K101E+G190S**

Since we have shown that most NNRTI resistance mutations have normal polymerization activity, we wanted to determine if the NL4-3 and D10 RTs differed in polymerization activity. We measured the RNA-dependent DNA polymerization activity of recombinant RTs using an RNA corresponding to the first 200 nt of the HIV-1 genome and a radiolabelled DNA primer that annealed to the primer binding site (Fig. 3a). We compared the amounts of full-length products over time (Fig. 3b). The results show that NL4-3_G190S, NL4-3_K101E+G190S and NL4-3 L74V +K101E+G190S have polymerization activities that are not significantly different than WT (P>0.05; Fig. 3b). NL4-3_M41L+T215Y+K101E+G190S was similar to NL4-3_K101E+G190S (data not shown). However, D10M41L+T215Y+K101E+G190S and D10K101E+G190S are significantly slower than WT at the earlier time points (D10M41L+T215Y+K101E+G190S versus WT P<0.001; D10K101E+G190S versus WT P<0.001 at 15 s). D10M41L+T215Y+K101E+G190S was significantly slower than D10K101E+G190S, indicating that M41L and T215Y reduced the polymerization activity of the D10 RT (P=0.014 at 30 s). There were three substantial pauses during DNA synthesis for the D10 RTs. The first one

**RESULTS**

**RT backbone sequence can alter the RT content of viruses with NNRTI drug resistance mutations, K101E+G190S**

In order to directly compare the virion-associated RT content in the absence of EFV, we used Western blotting, probing for RT, integrase (IN) and capsid protein (p24) (Fig. 1). Consistent with our previous work, the mutant virions had reduced amounts of RT relative to WT, with NL4-3_K101E+G190S having a lower amount than NL4-3_G190S (P=0.01), which was increased significantly when L74V or M41L+T215Y was present (Fig. 1a, b; P>0.05 compared to G190S alone). However, the D10 mutant viruses had significantly higher RT content than the NL4-3 backbone mutants (D10M41L+T215Y+K101E+G190S versus NL4-3 M41L+T215Y+K101E+G190S P<0.001, D10K101E+G190S versus NL4-3_K101E+G190S P=0.007), indicating that the backbone sequence in which K101E+G190S occurs can dramatically alter the RT content. There was no difference in the RT content between D10M41L+T215Y+K101E+G190S and D10K101E+G190S (P>0.05), indicating that for the D10 mutant, nRTI resistance mutations do not alter RT content.

Since we have previously observed that NNRTI drug-resistant mutants can decrease RNase H activity and RT content we wanted to explore further the effects of the D10 polymorphisms on the virion-associated RT content, and the polymerase and RNase H activities of recombinant RT in the absence of NNRTIs. We found that the RT backbone sequence can decrease polymerization activity, while increasing RT content and RNase H activity.
occurred around base pair 30 of the RNA template, the second one at around base pairs 35–40, and the third at base pair 150, which was also a pause site for all the NL4-3 RTs. Relative differences for each mutant were confirmed with a second protein preparation (data not shown).

M41L + T215Y can compensate for the reduced polymerase-dependent RNase H activity of D10K101E + G190S

During reverse transcription, RT must degrade the RNA genome after minus-strand synthesis in order for plus-strand synthesis to proceed efficiently (Beilhartz & Götte, 2010). The RNase H activity of the enzyme is responsible for degrading the RNA still annealed to DNA and completes this function using two modes of activity: polymerase-dependent and polymerase-independent RNase H activities. During synthesis of the minus strand, the RT makes cuts periodically in the RNA genome using its polymerase-dependent RNase H activity. After minus-strand synthesis is completed, RNA fragments still annealed to DNA are further degraded by the polymerase-independent RNase H activity to sizes small enough to melt from the DNA.

We have previously shown that G190S, K101E + G190S and L74V + K101E + G190S RTs have reduced RNase H activities. Therefore, we next measured the polymerase-dependent RNase H activity of D10M41L + T215Y + K101E + G190S or D10K101E + G190S RTs compared to the NL4-3 RTs (Fig. 4). We found that substrate degradation for D10M41L + T215Y + K101E + G190S was faster than WT RT (WT...
**Fig. 2.** Western blot showing the gag processing and relative amounts of IN in WT and mutant virions. Virus stocks were prepared by transfecting 293 cells with either WT or mutant plasmid DNA. An equal amount of capsid protein (200 ng) was loaded per lane. All virus stocks were run on the same gel. Each gel was stripped and reprobed for IN and p24. The molecular masses in kilodaltons of the markers are shown on the left side. (a) Representative Western blot of the relative amounts of gag processing intermediates. (b) Representative Western blot probed with antibodies that recognize HIV-1 IN. (c) Relative amounts of IN for each mutant compared to WT in percent on the y-axis. The bars represent the means ± SD of triplicate Western blots. P-values were calculated using a Student t-test.
versus D10\textsubscript{M41L+T215Y+K101E+G190S} at 10 min $P \leq 0.001$; Fig. 4a, b). However, D10\textsubscript{K101E+G190S} was slower than WT and similar to the NL4-3 RTs (WT versus D10\textsubscript{K101E+G190S} at 10 min $P=0.002$). NL4-3\textsubscript{M41L+T215Y+K101E+G190S} was similar to NL4-3\textsubscript{K101E+G190S} suggesting that M41L+T215Y do not inherently improve RNase H activity, but when combined with other RT polymorphisms may have an advantage (data not shown). We also quantified the rate of primary and secondary product formation (Fig. 4c, d, respectively). More primary product was produced
over time for D10M41L+T215Y+K101E+G190S than for the other RTs (WT versus D10 backbone at 15 min \(P<0.001\)), while the same amounts of secondary product were produced. D10M41L+T215Y+K101E+G190S also had a significant increase in substrate degradation compared to D10K101E+G190S \((P<0.001)\). These data indicate that D10M41L+T215Y+K101E+G190S may have a faster rate of primary cleavage and that the RT backbone sequence can influence the rate of polymerase-dependent RNase H activity. Relative differences for each mutant were confirmed with a second protein preparation (data not shown).

**RT sequence backbone can alter polymerase-independent RNase H activity of recombinant RTs**

The polymerase-independent RNase H activity was also measured to determine if the RT backbone sequence could alter the rates for this mode of cleavage (Fig. 5). Similar to polymerase-dependent activity, polymerase-independent substrate degradation for D10M41L+T215Y+K101E+G190S was faster than WT and the other mutant RTs (WT versus D10M41L+T215Y+K101E+G190S at 15 min \(P=0.01\); Fig. 5b). However, there was no significant difference in the rate of primary product formation (data not shown). Instead, the...
rate of secondary cleavage was faster (WT versus D10M41L+T215Y+K101E+G190S at 10 min $P=0.02$; Fig. 5c). The rate of substrate degradation and secondary product formation for D10K101E+G190S RT was significantly slower than D10M41L+T215Y+K101E+G190S (at 10 min $P=0.01$), but not significantly different than NL4-3 K101E+G190S. These data indicate that M41L+T215Y may improve RNase H activity in the presence of certain polymorphisms and that differences in the RT backbone can also alter RNase H activity. Relative differences for each mutant were confirmed with a second protein preparation (data not shown).

**DISCUSSION**

The fitness of NNRTI drug-resistant HIV-1 correlates with biochemical characterizations of recombinant RT and virus particles. Drug-resistant mutants which are similar to WT in fitness have higher amounts of RT protein in virions, and normal polymerization and RNase H activities, while mutants which are low in fitness have low amounts of RT in virions and reduced RNase H activity (Archer et al., 2000, 2001; Gerondelis et al., 1999; Koval et al., 2006; Wang et al., 2006, 2010a, b, 2011). In addition, our group has reported that the backbone sequence of NNRTI drug-resistant mutant viruses can alter their replication capacity.
(Dykes et al., 2001). Here we wanted to determine whether the backbone sequence of the double mutant K101E+G190S could alter the RT content of virions and polymerization and RNase H activities of recombinant RT. We determined that the backbone sequence from a patient who failed EFV-containing therapy with the mutations K101E+G190S can significantly increase the amount of RT in virions and RNase H activity, and decrease polymerization activity.

Reduced polymerization activity should negatively impact virus replication, while improved RT content and RNase H activity should improve replication. Other groups have shown that decreases in polymerization activity have a larger impact on virus infectivity than decreases in RNase H, therefore it is possible that polymerization would play the dominant role (Julias et al., 2001). However, it is difficult to determine here what overall effect these three results would have on virus replication, but we postulate that the increased RT content would give the D10 virus an advantage in replication, despite the modest reduction in polymerization activity. It is also possible that the mechanisms by which the backbone affects stimulation by NNRTIs or fitness are different and that polymerization and RNase H would play different roles in each situation.

It is important to measure the effects of mutations and drugs on both polymerization-dependent (3‘-directed) and polymerization-independent (5‘-directed) RNase H activities because these activities are applied sequentially by the virus to clear away the plus-strand RNA genome to make way for plus-strand DNA synthesis. The 3‘-cleavage activity applied during synthesis makes cuts in the RNA template, reducing it to oligoribonucleotides. RTs then return to bind the 5‘-ends of these oligomers, cleaving them to sizes so short that they spontaneously dissociate from the newly made minus-strand DNA. The overall process of RNA removal may limit the rate of reverse transcription and viral reproduction. The presence of a drug that accelerates RNase H may then stimulate virus growth. Since the RNase H functions are sequential, more frequent cuts during polymerization will make it easier for the returning RTs to remove the shorter oligomers. If cuts during synthesis occur at a normal rate, more active 5‘-directed cuts will remove the oligomers more quickly. If both modes of RNase H are accelerated, short oligomers will be made during synthesis and rapidly removed by returning RTs. Acceleration of either mode of cleavage would then hasten complete RNA removal, but acceleration of both would be most effective.

We tested two preparations of each mutant, but acknowledge that the differences we see are modest even though they are significant and that the results may have been different if we tested additional preparations. However, in our experience, different preparations can have different specific activities due to differences in the quality of the preparation, but relative differences compared to WT and other mutants does not change. We acknowledge that the differences in polymerization between the D10 and NL4-3 backbones are significantly different only at the early time points. Given enough time, the D10 backbone will produce comparable amounts of product, but we feel these early differences may be significant enough to slow down virus replication kinetics and alter viral fitness. It is not clear from these experiments if the reduced rate of polymerization is due to differences in processivity, substrate binding or some other step of polymerization. The mechanistic difference between the backbones is beyond the scope of this work and will be addressed in future studies. The differences in RNase H activity were also tested with two preparations, but we feel that the magnitude of the differences detected are not due to differences in specific activity since we normalize the amount of protein input based on specific activity.

D10M41L+T215Y+K101E+G190S and D10K101E+G190S had significantly higher amounts of RT and IN in virions. The data suggested that the polymorphisms in the D10 significantly augmented the p51/p66 RT level in the virion by increasing incorporation of gag-pol. D10M41L+T215Y+K101E+G190S can be stimulated by NNRTIs 20-fold higher than NL4-3M41L+T215Y+K101E+G190S and NL4-3K101E+G190S (Wang et al., 2010b). Therefore, it is possible that the reason D10 can be stimulated more is because there are more copies of RT present in the virions to be stimulated.

Many RT polymorphisms have been reported to be involved in drug resistance and the polymorphisms in the D10 backbone are no exception. The D10 backbone sequence has the following polymorphic mutations compared to NL4-3: T275, K43N, K102Q, I142V, C162S, Q174K, Q207N, R211K, Q258L, R277K, T286A, A288S, V293I, E298K, K358R, A371T, A376T, T386I, E399D, A400T, T403S, I435V, D460N, R461K, V467I, P468S, Q480E and L491S. I424V, Q174K, Q207N, R211K, R277K, T286K, V293I, E399D and A400T are all involved in the drug resistance or fitness profile of HIV-1 (Betancor et al., 2010; Chung et al., 2012; Curr et al., 2006; Delviks-Frankenberg et al., 2009, 2013; Doualla-Bell et al., 2004; Garriga et al., 2009; Gupta et al., 2011; Handema et al., 2002; Kawamoto et al., 2008; Lengrubier et al., 2011; Michels et al., 2010; Ojesina et al., 2007; Pérez-Alvarez et al., 2003; Poveda et al., 2008; Romano et al., 2002; Stürmer et al., 2003; Tanuma et al., 2010; Vergne et al., 2000; Yu et al., 2009). Some resistance mutations against NNRTIs arise in the RNase H domain, such as R461K (Ordonez et al., 2012).

Crystallography studies have shown that residues in the fingers, palm, connection, thumb and RNase H domains are located at the interface between p66 and p51 (Srivastava et al., 2006). Mutations in these domains can alter dimerization efficiency and reduce RT content. In the patient backbone used in this study, mutations at amino acids 27, 162, 258, 286, 288, 358, 376, 386, 400, 403, 435 and 460 could be involved in heterodimer formation due
to their proximity to the heterodimer interface. We do not currently know which mutations are responsible for the differences seen between D10K101E+G190S and NL4-3K101E+G190S, but future work will attempt to identify which play a role in NNRTI stimulation or fitness of K101E+G190S.

Our findings indicate that drug resistance mutations have unpredictable effects depending on the backbone they arise in. The effect of polymorphisms on drug resistance has been documented for other drugs and genes (Armstrong et al., 2009; Cornelissen et al., 1997; Delviks-Frankenberg et al., 2009; Dykes et al., 2001; García-Lerma et al., 2000; Kantor & Katzenstein, 2003; Maiga et al., 2010; Montes et al., 2004; Rose et al., 1996; Van Marck et al., 2009; Velazquez-Campoy et al., 2002). Our work reinforces the finding that in order to understand the effect that drug resistance mutations have on virus replication they should be evaluated in a variety of backgrounds and subtypes. Our work will help solidify which mutants are primary NNRTI mutants and which are secondary.

Interestingly, we have previously published that K101E+G190S, in both the NL4-3 and D10 backbones, has very high levels of resistance and is stimulated in the presence of lower concentrations of NNRTIs (Wang et al., 2010b). However, the magnitude of the stimulation is higher for the D10 backbone than the NL4-3 background. This may be due to the higher levels of RT present in D10 virions. The IC50 for K101E+G190S is higher than the concentration measured in patient plasma for both backbones and therefore the resistance of this mutant likely plays a role in its selection in patients. Here we show that in the absence of drug, replication fitness may also play a role in selection. K101E and G190S are already recognized NNRTI drug resistance mutations (Johnson et al., 2011). Since we do not yet know which polymorphisms are responsible for the differences between the D10 and NL4-3 backgrounds, we do not know if our findings are relevant to screening naïve patients for resistance.

**METHODS**

**Cell culture and antibiotics.** The 293 cell line (ATCC) was grown in Dulbecco’s modified Eagle’s medium (Cellgro) with 10 % FBS, penicillin (100 U ml⁻¹) and streptomycin (100 U ml⁻¹). The following antibiotics were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: the HIV-1 RT mAb 8C4 was obtained from Dr D. Helland and Dr A. M. Szilvay, and recognizes both subunits of RT (Szilvay et al., 1992); p24 mAb 183-H12-5C was obtained from Dr B. Chesebro and K. Wehrly (Chesebro et al., 1992; Toohey et al., 1995); polyclonal HIV-1 IN antiserum recognizing epitopes mapping to amino acids 1–16 was obtained from Dr D. P. Grandgenett (Bukrinsky et al., 1993).

**Site-directed mutagenesis.** Each of the drug-resistant mutants (K103N, L74V, double mutations L100I+K103N and triple mutations L74V+L100I+K103N) was introduced into pRHA1 using the QuikChange II site-directed mutagenesis kit (Stratagene). A molecular clone of HIV-1, pNL4-3, was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from M. Martin. The drug-resistant mutations were subcloned into pNL4-3 using Apal and AgeI restriction sites, as previously described (Koval et al., 2006).

**Generation of NNRTI-resistant virus stock.** A human cell line, 293, was transiently transfected with WT NL4-3 or the drug-resistant mutants of NL4-3 using Superfect (Qiagen). After 72 h, clarified supernatants were harvested and the p24 capsid protein of each stock was measured using ELISA (Perkin Elmer). WT and mutant stocks in the pNL4-3 background were used for Western blot analysis to measure the relative RT content.

**Quantification of RT content in virions using Western blotting.** Supernatant solutions from transfected 293 cells with WT or drug-resistant mutants were pelleted by centrifugation at 50 000 g for 1 h at 4 °C. The RT content of virus pellets containing 200 ng of p24 antigen for WT and mutants was detected with Western blotting as previously described (Wang et al., 2010a). Briefly, the virus pellets were resuspended with 15 µl NuPAGE 2 x sample buffer (Invitrogen) and fractionated by electrophoresis according to the NuPAGE manufacturer’s instructions. The proteins were separated using a 4–12 % BisTris gel with 1 x MOPS running buffer (Invitrogen), transferred to nitrocellulose membranes, blocked by 5 % milk in TBS with 0.05 % Tween-TBST (0.1 % Tween 20) overnight and bound with primary antibody (anti–RT–horseradish peroxidase; Bio-Rad). Viral proteins were visualized using SuperSignal West Femto chemiluminescence substrate (Thermo Scientific) and quantified by 1D image analysis software (Kodak Digital Science). p51 and p66 subunits of RT were recognized by the RT mAb pool (8C4 and 5B2B2). Each blot was stripped, and the level of p24 and gag processing was also visualized by Western blot using the method described above except using an antibody specific to p24 (183-H12-5C). Stripping and reprobing of each blot was also done to determine the levels of IN using a polyclonal antibody that recognized amino acids 1–16 of IN.

**Expression and purification of recombinant RTs.** The full-length 6 x His-tagged p51, p66 subunits of WT and mutant RT sequences were expressed with pET21a (+) vector (Novagen), and were purified with a Q-Sepharose column, Talon column, and SesoSource S column used the AKTAPrim plus system (Amersham/GE), as previously described (Hou et al., 2004; Wang et al., 2010a). The fractions with equal relative amounts of p51 and p66 were determined using SDS-PAGE and chosen to measure DNA polymerization and RNase H activities. Two preparations were made for each mutant.

**RNase H assays and RNA-dependent DNA polymerization of recombinant RTs.** Specific DNA polymerization activity of each RT preparation was measured by using a poly(rA)/oligo(dT) template/primer and an [α-32P]dTTP substrate (Archer et al., 2000). A unit was defined as the amount of enzyme required to incorporate 1 nmol of dTTP into nucleic acid product in 10 min at 37 °C. Specific activity is a measure of the inherent polymerization activity of the mutant as well as the quality of the protein preparation. In order to prevent protein preparation quality from influencing the results, an equivalent number of units of specific polymerization activity were added to both the polymerization rate and RNase H assays. Using this method we means are comparing the ratio of polymerization activity on a complex template to a simple template [poly(rA)/oligo(dT)] and the ratio of polymerization to RNase H rather than the inherent RNase H activity per mass of protein. DNA 3'-end-directed and RNA 5'-end-directed RNase H activities were measured as previously described (Wang et al., 2006). Briefly, the substrate used to measure DNA 3'-end-directed RNase H cleavage was a 41-nt 3'-end 32P-labelled RNA annealed to a short DNA (26 nt) such that the 3'-end of the DNA was recessed. The substrate of RNA 5'-end-directed RNase H cleavage was the same 41-nt 5'-end 32P-labelled RNA annealed to a longer DNA.
(77 nt) such that the 5’-end of the RNA was recessed. Amounts of RT added to each reaction were normalized based on the relative activity of DNA polymerization. Reaction conditions were the same as for the RT specific activity assay, except that dNTPs were omitted. Products were resolved by 10% denaturing polyacrylamide–urea gel electrophoresis followed by autoradiography and were quantified using phosphorimaging.

To determine the rate of RNA-dependent DNA polymerization activity of each RT the polymerization activities were measured using a 5’-radiolabelled PBS oligomer DNA primer annealed to the RNA template D199, +1 to +199 of the pNL4-3 genomic RNA, which included the PBS binding site. The primer/template (15 nM/25 nM) was pre-bound with purified RT normalized by relative specific activities of polymerization, 0.5 units per reaction, in reaction buffer containing 25 mM Tris/HCl pH 8.0, 25 mM NaCl, 0.5 mM EDTA and DTT at 37 °C for 2 min. The reactions were initiated by adding 6 mM MgCl₂ and 500 μM dNTPs, and quenched at various times by an equal volume of 2 x DNA loading buffer (Ambion). Extension products were analysed by SDS-PAGE and quantified by phosphorimaging.

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