Combining mutations in HIV-1 reverse transcriptase with mutations in the HIV-1 polypurine tract affects RNase H cleavages involved in PPT utilization

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Abstract

The RNase H cleavages that generate and remove the polypurine tract (PPT) primer during retroviral reverse transcription must be specific to generate linear viral DNAs that are suitable substrates for the viral integrase. To determine if specific contacts between reverse transcriptase (RT) and the PPT are a critical factor in determining the cleavage specificity of RNase H, we made HIV-1 viruses containing mutations in RT and the PPT at the locations of critical contacts between the protein and the nucleic acid. The effects on titer and RNase H cleavage suggest that combining mutations in RT with mutations in the PPT affect the structure of the protein of the RT/nucleic acid complex in ways that affect the specificity and the rate of PPT cleavage. In contrast, the mutations in the PPT (alone) and RT (alone) affect the specificity of PPT cleavage but have much less effect on the overall rate of cleavage.

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Introduction

Reverse transcription and integration, the copying of the single-stranded RNA genome found in virions into double-stranded linear DNA and the subsequent insertion of this DNA into the host genome, are the hallmarks of retroviral replication (Coffin et al., 1997; Whitcomb and Hughes, 1992). The conversion of the retroviral RNA genome into DNA is performed by the viral enzyme reverse transcriptase (RT). RT has two enzymatic activities that are essential for retroviral replication: a DNA polymerase that can copy either an RNA or a DNA template and an RNase H that cleaves RNA if, and only if, the RNA is part of an RNA–DNA duplex (Repaske et al., 1989; Schatz et al., 1990; Tisdale et al., 1991). In retroviral replication, the primer used to initiate the first, or minus, strand DNA is a host tRNA that is base-paired near the 5′ end of the RNA genome (Coffin et al., 1997; Whitcomb and Hughes, 1992). Synthesis of the second (plus) strand of viral DNA is initiated from a primer derived from the RNA genome by RNase H cleavage (Coffin et al., 1997; Pullen and Champoux, 1990; Rattray and Champoux, 1989). The plus-strand segment of the viral genome used as the second-strand primer is purine-rich and is called the polypurine tract (PPT). The PPT is relatively resistant to RNase H cleavage, which allows it to be used as the primer for second-strand DNA synthesis. The removal of the RNA primers used to initiate the minus and plus DNA strands defines the ends of the linear viral DNA. Most cleavages by RNase H do not need to be specific; however, the cleavages that generate the PPT and remove the PPT and tRNA primers must be specific in order to generate linear DNAs that are appropriate substrates for integrase. The PPT primer is removed at the RNA/DNA junction (Kulkosky et al., 1990;
The structure of RT in a complex with the RNA–DNA substrate, together with the structures of RT in complexes with DNA–DNA substrates, provides useful information about the contacts between RT and its nucleic acid substrates (Huang et al., 1998; Jacobo-Molina et al., 1993; Sarafianos et al., 2001). RT makes numerous contacts with its nucleic acid substrates through both the polymerase domain and the RNase H domain. The two domains cooperate in binding the nucleic acid, and mutations in either the RNase H domain or the polymerase domain can affect both enzymatic activities (Dudding and Mizrahi, 1993; Dudding et al., 1991; Gao et al., 1998; Hughes et al., 1998; Julias et al., 2002; Mizrahi et al., 1993; Dudding et al., 1991; Gao et al., 1998; Hughes et al., 1998; Julias et al., 2002; Mizrahi et al., 1993, 1994).

However, there are elements near the polymerase active site that have a particularly important role in positioning the nucleic acid for polymerization (for example, the primer grip) and there are elements near the RNase H active site (in particular the RNase H primer grip) that are especially important in positioning the nucleic acid for RNase H cleavage (Julias et al., 2002; Rausch et al., 2002). Additionally, sequence-dependent properties of the nucleic acid substrate such as the width of the minor groove and the trajectory of the RNA/DNA hybrid are important for the cleavage of substrates, or the resistance of substrates to cleavage, by RNase H. For example, A-tracts have a narrow minor groove width; the presence of the A-tracts in the 5' end of the PPT should (help) position the RNA strand away from the RNase H active site and contribute to the PPT's resistance to RNase H cleavage (Sarafianos et al., 2001).

We have identified elements in both the PPT and the RNase H primer grip that are important for defining RNase H cleavage specificity. Based on alanine scanning mutagenesis, we identified Y501 and Q475 as two amino acids that are particularly important for proper RNase H cleavages (Julias et al., 2002; Rausch et al., 2002). When HIV-1 RT is modeled with the RNase H active site at the PPT/U3 junction, the 6 nucleotides of the PPT that lie immediately upstream of the cleavage site are all Gs (see Fig. 1A). This segment of the HIV-1 PPT is called the G-tract. If a model is generated in which an RNA–DNA duplex containing the PPT is positioned with the RNase H active site at the PPT/U3 junction, Y501 would contact the phosphate backbone of the DNA strand between 5 and 6 nucleotides upstream (3') of the cleavage site (see Fig. 1B). Q475 contacts the DNA strand 4 nucleotides upstream of the cleavage site (through the deoxyribose ring) and, also through the deoxyribose ring, 5 nucleotides upstream of the cleavage site. Q475 contacts the RNA strand 1 nucleotide upstream of the cleavage site (through the ribose ring) and also contacts the RNA strand through a base-specific contact with the exocyclic amine of G5. This is the only base-specific contact that RT makes near the RNase H active site. We have mutated each of the Gs in the G-tract of the PPT and assessed the effects of the mutations on viral replication and on PPT cleavage (Julias et al., 2004). Mutations in G2 and G5 (especially G to T transversions, these mutants are called T-2 and T-5) had the

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**Fig. 1.** The sequence of the PPT, the numbering of the G-tract in the PPT and the contacts between Y501 and Q475 in HIV-1 RT and an RNA/DNA duplex that contains the PPT. (A) The sequence of the PPT and flanking sequences are shown. The PPT is underlined, and the positions of the G-tract are numbered (1 through 6) in subscript. The RNase H cleavage site defines the PPT/U3 junction and is designated by the arrow. (B) The sequences of the PPT and the minus-strand DNA are shown. The small green circles between the bases in the PPT (RNA) represent the ribose-phosphate backbone; the small blue circles between the bases in the (−) strand DNA represent the deoxyribose-phosphate backbone. Solid black arrows represent contacts between the amino acids and the ribose-phosphate backbone in the PPT (RNA) when RT is bound for cleavage at the PPT/U3 junction. In this mode, the RNA strand is the template strand and the polymerase domain contacts the RNA strand 5' of the RNase H domain (to the left in the drawing). These types of contacts exist for Q475 (pale bluish-green). Gray arrows represent contacts between Q475 or Y501 (yellow) and the deoxyribose backbone of the minus-strand. The red arrow represents the (major groove) contact between Q475 and the guanine base at position G5.
greatest effects on titer and on proper PPT cleavage. A double mutant (T-2-5, in which both of the Gs were converted to Ts) had a greater effect than either single G to T mutation. These in vivo data are in reasonably good (but not perfect) agreement with published data derived from in vitro RNase H cleavage assays (Dash et al., 2004; Julias et al., 2002; Kvaratskhelia et al., 2002; McWilliams et al., 2003; Pullen et al., 1993). The in vivo data suggest that the contacts involving the G2 and G5 nucleotides and Y501 and Q475 might be particularly important. Alternatively, Y501 and Q475, and G2 and G5, might have particularly important roles in determining the overall structure of the protein/nucleic acid complex that directs the proper PPT cleavages.

To try to resolve these possibilities, we constructed complex mutants that have the Y501A mutation combined with a G to T mutation in G2 and G5 (T-2 and T-5) and also made the equivalent mutants by combining a Q475A mutation and these same G-to-T mutations. Because the effects of a mutant (T-2-5) with two positions changed in the PPT were stronger than either of the single T substitutions, we also made mutants in which the Y501A mutation and the Q475A mutation were each combined separately with the T-2-5 mutation. If the critical issue is the contacts between the individual amino acids and the respective nucleotides, then mutating the nucleic acid and amino acid involved in an important contact (for example, Y501A combined with T-2) which would involve only a single contact should not have as great an effect on viral replication as would combining Y501A with T-5 because the latter combination would disrupt two contacts. In contrast, if the mutations have some important effect on the overall structure, combining the PPT and RT mutations will have a profound effect whether or not the mutations in the PPT and the RT are involved in a specific contact. The data show that combining the RT mutations with the PPT mutations has powerful effects on the titer of the virus and on the specificity and the rate of PPT cleavage in all cases, whether the combined RT and PPT mutations do or do not involve a single contact. This suggests that the RT and/or the PPT mutations affect the structure of protein and/or the PPT in the RT/nucleic complex in ways that go beyond disrupting individual contacts between RT and the PPT.

Results

The one-round HIV-1-based viral vector pNLNgoMIVR-E, HSA has been described ([Julias et al., 2001] see also Materials and methods). Basically, the vector expresses the murine heat-stable antigen (HSA, CD24) from the nef open reading frame. HSA is a surface protein that can easily be detected on the surface of infected cells with a specific monoclonal antibody. The mutations in RT (Y501A and Q475A) have been described, as have the mutations in the G-tract region of the PPT: T-2, T-5 and T-2-5 (Julias et al., 2002, 2004). Mutants were constructed in which each of the mutations in RT was paired with the mutations in the PPT. Viral stocks were generated by transfecting 293 cells (see Materials and methods); viruses were harvested and used to infect HOS cells. The infected cells were labeled with antibody directed against CD24, and the virus titers were measured by FACS. The titers of the combined RT + PPT mutants were lower than the titer of the single mutants from which they were constructed (Fig. 2). However, in contrast to the data obtained with the PPT mutations alone, when the PPT mutations were combined with either the Y501A or the Q475A RT mutations, the effect of changing two of the Gs in the G-tract (T-2-5) was not markedly worse than having either of the single changes in the G-tract (T-2 or T-5). In fact, the lowest titers were obtained with the Y501A + T-2 double mutant (0.01 of wild-type); this is lower than either the titer of Y501A + T-5 (0.14 of wild-type) or Y501A + T-2-5 (0.08 of wild-type). The addition of the Y501A mutation did not significantly affect the titer of the T-2-5 mutation (0.08 vs. 0.10). The titers of the Q475A + PPT mutants ranged from 0.05 of wild-type (for the Q475A + T-2 mutant) to 0.10 (for the Q475A + T-5). A mutant that had the Q475A mutation combined with the double G-tract mutation, T-2-5, had a titer (0.07) which is slightly lower than the Q475A mutant (0.20) and similar to the T-2-5 mutant (0.10).

The mutations in the RT and the PPT (either individually or in combination) could affect either the specificity of PPT cleavage, or the rate of PPT cleavage, or both. Altering both the specificity of PPT cleavage and the extent of cleavage would affect the generation of a proper PPT primer and, by extension, the amount of linear viral DNA with ends that can be integrated successfully. Thus, the observed effects on the titer represent the sum of the effects of mutations on the specificity of PPT cleavage and the rate/extent of PPT cleavage.

**Combining RT mutations with PPT mutations affects the specificity of RNase H cleavage**

The ends of the linear viral DNA are determined by the RNase H cleavages that remove the tRNA and PPT primers.

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Fig. 2. Sequences and relative titers of RT and PPT mutants. The sequences of the mutant PPTs are shown in the left column. Mutations in the PPT are in bold-type face. The right column shows the relative titers of the mutants viruses. The virus titers were determined by labeling virus-infected cells with antibody directed against the cell-surface HSA followed by FACS. The relative virus titers were calculated from the linear range of virus dilution (see Materials and methods).
the sequences of the ends of the linear DNA provide information about the specificity of RNase H cleavage. The ends of the linear DNA can be ligated to form 2-LTR circles (Coffin et al., 1997; Li et al., 2001; Whitcomb et al., 1990). We have used the sequence of the 2-LTR circle junction as a surrogate for the ends of the linear viral DNA; however, in a normal infection, the linear viral DNA has several possible fates. The vast majority of the linear viral DNAs that have consensus ends are integrated into the host genome and form proviruses; a portion of the remaining unintegrated DNA is converted into 1-LTR and 2-LTR circles. Because integration greatly enriches for the linear viral DNAs with aberrant ends (those linear DNAs that cannot integrate), it is simpler to screen for aberrant circle junctions in an IN+ infection. If integration is blocked, then the whole population of linear DNAs is available to form circles; consequently, a more accurate measurement of the percentage of linear DNAs with consensus ends is measured by monitoring the 2-LTR circle junctions formed in an IN− background. Because we wanted to accurately measure both the percentage of aberrant ends and understand the exact nature of the aberrant RNase H cleavages, we measured the percentage of consensus 2-LTR circle junctions using an infection with an IN− vector, then we measured the different types of errors made by each of the mutants with an IN+ vector (Fig. 3). The percentage of consensus circle junctions measured with an IN− vector did not correlate with the titer, suggesting that the combination of mutations might affect the rate/extent of PPT cleavage (discussed below). When compared to wild-type IN− (0.95 consensus), the mutants with either the Y501A or the Q475A mutant paired either with the T-2 or the T-5 mutations have approximately 0.5 consensus circle junctions \( P < 0.0001 \) mutants versus wt). However, the fraction of consensus circle junctions dropped to 0.24 for the Q475A + T-2-5 triple mutant and to 0.16 for the Y501A + T-2-5 triple mutant \( P < 0.0001 \) either (triplet) mutant vs. wt; \( P < 0.0001 \) Q475A + T-2-5 vs. Q475A + T-2 or Q475A + T-5 \( P < 0.0001 \) Y501A + T-2-5 vs. Y501A + T-2]. This effect is particularly obvious for the Y501A + T-2 mutant; however, a similar effect is seen (to a lesser extent) with the Q475A + T-2 mutant and the Y501A + T-5 mutant. One explanation of the low titers seen with these complex mutants is that RNase H cleaves the PPT inefficiently and the relatively small amount of PPT primer means that only a small amount of full-length linear viral DNA is synthesized (discussed below).

We also determined the sequences of the circle junctions obtained from an infection with IN+ vectors (Fig. 4). As expected, the fraction of consensus circle junctions dropped DNA is available to form circles; consequently, a more accurate measurement of the percentage of linear DNAs with consensus ends is measured by monitoring the 2-LTR circle junctions formed in an IN− background. Because we wanted to accurately measure both the percentage of aberrant ends and understand the exact nature of the aberrant RNase H cleavages, we measured the percentage of consensus 2-LTR circle junctions using an infection with an IN− vector, then we measured the different types of errors made by each of the mutants with an IN+ vector (Fig. 3). The percentage of consensus circle junctions measured with an IN− vector did not correlate with the titer, suggesting that the combination of mutations might affect the rate/extent of PPT cleavage (discussed below). When compared to wild-type IN− (0.95 consensus), the mutants with either the Y501A or the Q475A mutant paired either with the T-2 or the T-5 mutations have approximately 0.5 consensus circle junctions \( P < 0.0001 \) mutants versus wt). However, the fraction of consensus circle junctions dropped to 0.24 for the Q475A + T-2-5 triple mutant and to 0.16 for the Y501A + T-2-5 triple mutant \( P < 0.0001 \) either (triplet) mutant vs. wt; \( P < 0.0001 \) Q475A + T-2-5 vs. Q475A + T-2 or Q475A + T-5 \( P < 0.0001 \) Y501A + T-2-5 vs. Y501A + T-2]. This effect is particularly obvious for the Y501A + T-2 mutant; however, a similar effect is seen (to a lesser extent) with the Q475A + T-2 mutant and the Y501A + T-5 mutant. One explanation of the low titers seen with these complex mutants is that RNase H cleaves the PPT inefficiently and the relatively small amount of PPT primer means that only a small amount of full-length linear viral DNA is synthesized (discussed below).

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relative to the fraction seen in an IN− background; this reduction reflects the successful integration of consensus (and some near-consensus) linear DNAs. T2−5 appears to be the exception to this rule. The fraction of consensus sequences is, if anything, lower with the IN− vector (0.06 vs. 0.03). However, some non-consensus linear DNAs can be integrated. In particular, linear DNAs whose sequences differ from consensus by only a single (extra) nucleotide can also be used as substrates for integration in infected cells. This is relevant because the mutations in the PPT (alone) give rise to one-nucleotide insertions; the effect is particularly important for T-5 and T-2-5 (see Fig. 5). This affects, in the opposite way, the proportion of both consensus and non-consensus 2-LTR junctions. In addition, integration events that derive from the linear viral DNAs that have the extra nucleotide contribute to the overall titer (Julias et al., 2004).

The Q475A + T-2, the Y501A + T-5 and the Y501A + T-2-5 mutants all showed an increase in the number of PPT + flanking sequence insertions at the 2-LTR circle junction (P < 0.05). However, the addition of the RNase H mutations to the PPT mutations did not cause a significant increase in the number of simple PPT insertions. This suggests that three of the complex mutations (RT + PPT) are deficient in their ability to generate and/or remove the normal PPT primer, implying that these mutants had some problems with cleavage specificity as well as with cleavage efficiency. However, the complex mutant Q475 + T-5 showed only a modest increase in the insertion of a PPT + flanking sequence at the 2-LTR junction, which is not statistically significant.

One reason to pair the T-2, T-5 and T-2-5 mutations with the Q475A and Y501A mutations is that the T-2, T-5 and T-2-5 each cause, by themselves, a substantial increase in a particular type of RNase H cleavage error, the one-nucleotide insertion (Fig. 5). This insertion could be caused because RNase H miscleaves the PPT initially; alternatively, RNase H (mis)cleavage can cause a one-nucleotide insertion if RNase H fails to remove the entire PPT after the PPT is used as the plus-strand primer. In either case, the one-nucleotide insertion results from an alteration in the specificity of RNase H cleavage rather than simply from an overall loss of cleavage specificity. We wanted to know how the addition of the RT mutations, either Y501A or Q475A, would affect the specificity of cleavage, in particular, whether the presence of these RT mutations would alter the number of one-nucleotide insertions caused by the T-2, T-5 and T-2-5 mutations. In every case, the addition of either the Q475A or the Y501A reduced the percentage of one-nucleotide insertions seen at the circle junction. For example, in the case of the T-5 mutation, which, when present by itself, causes almost half the circle junctions (0.48) to contain a one-nucleotide insertion, the addition of either the Q475A or the Y501A mutation significantly reduced the number of one-nucleotide insertions (0.06 and 0.12 respectively, P < 0.001 either mutant RT vs. wt RT). In the case of the T-2 mutation, the percentage of one-nucleotide insertions is lower (0.15) and the reduction seen with the Q475A + T-2 mutant (to 0.04) is not statistically significant. The fraction of one-nucleotide insertions seen for T-2-5 alone (0.49) was reduced significantly by the addition of either the Q475A or the Y501A mutation (0.23 and 0.29 respectively, P < 0.01). This reduction in one base insertions is statistically significant for both the Q475A + T-2-5 and the Y501 + T-2-5 mutants; however, the relatively large number of one-nucleotide insertions seen with the RT + PPT complex mutants is also significantly higher than the number of one-nucleotide insertions seen with the wild-type virus (0.04, wt vs. Q475A + T-2-5 P < 0.0003, wt vs. Y501A + T-2-5 P < 0.0001). These results suggest that the mutations in the protein (Q475A and Y501A) not only reduce the specificity of the cleavage of the normal PPT, but also alter the cleavage pattern of mutant PPTs that the wild-type RT frequently miscleaves by one nucleotide. These data reinforce the idea that the addition of the RNase H mutations reduces the (altered) specificity of cleavage caused by the mutations within the PPT.

### Combining RT mutations with PPT mutations affects the rate of PPT cleavage

Although the mutations affected the specificity of PPT cleavage, this effect did not fully explain the effects of the mutations on the titer, and we also measured the rate and extent of DNA synthesis. The amounts of viral DNAs specific for the initiation of DNA synthesis (RU5), for minus-strand DNA transfer (U3) and for plus-strand DNA transfer were measured using real-time PCR at 2 h, 4 h, 6 h and 24 h after infecting cells with virus (Fig. 6). If the mutations affect the rate at which the PPT primer is generated and used by RT, the amounts plus-strand DNA transfer products would decrease relative to the amounts of RU5 or U3 viral DNA, particularly at early times after infection. Cells were infected with viruses containing WT RT, WT RT combined with the T-2-5 PPT mutation, Q475A mutation combined with the WT PPT, the T-2 mutation, the T-5 mutation, or the T-2-5 double-mutation in the PPT. To ensure that the DNA used in the transfection process did not significantly contribute to the amounts of DNA products detected in infected cells, virions that contained the D110E mutation in the polymerase active site of RT were prepared and used as a control. Virions containing this defective RT are not

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Fig. 5. Frequency of 1-nucleotide and 2-nucleotide insertions at the 2-LTR circle junction for the RT-PPT mutants. The PPT mutations are listed in the leftmost column, the RT mutations are listed in the columns on the right. Below each RT are the proportions of 1-nucleotide and 2-nucleotide insertions. nd = not determined.
able to synthesize viral DNA; consequently, any DNA measured in these samples resulted from plasmid carryover from the transfection process used to generate the virus stocks. The viral DNA copy numbers from the D110E control viruses were dramatically lower (by more than 1000-fold) than the viral DNA copy numbers in the experimental samples (data not shown). We wanted to determine if the RNase H primer grip mutant Q475A affects the rate of generation of the PPT primer when combined with the PPT mutations T-2, T-5 and T-2-5 (Fig. 6A). The amounts of the RU5 viral DNA products produced 2 h, 4 h and 6 h after infection were reduced no more than 10-fold relative to WT for all of the combinations containing Q475A with the wild-type and mutant PPTs, or WT RT combined with the wild-type PPT or the T-2-5 mutations. In all of these samples, the relative amounts of U3 were similar to the amounts of RU5 at each of the different time points, indicating that none of the mutations substantially impaired minus-strand DNA transfer. The amount of viral DNA specific for plus-strand DNA transfer was reduced, and its rate of accumulation was delayed slightly when WT RT was paired with the T-2-5 PPT. When the Q475A mutation was paired with the normal PPT, the amount of reverse transcription products specific for plus-strand DNA transfer was lower than wild-type at 2 h, 4 h and 6 h after infection. However, at the 24 h time point, the amount of plus-strand DNA transfer was similar for wild-type and Q475A. This indicated that the Q475A mutation delayed the generation of the PPT primer, plus-strand initiation and/or plus-strand DNA transfer. When combined with the PPT mutations, the Q475A mutation in RT markedly delayed the accumulation of plus-strand DNA.

We also wanted to determine if the Y501A mutation affected the accumulation of viral DNA products specific for plus-strand DNA transfer (Fig. 6B). The amount of DNA products specific for RU5 were within 10-fold of the wild-type control with the T-2-5 mutant, and for the Y501A mutant and for Y501A with T-5, and T-2-5. The amount of RU5 DNA measured for the Y501A combined with the T-2 mutation was about 100-fold lower than for WT at the earliest time point (2 h); however, the level of RU5 was similar at 24 h. The rate of accumulation of U3 DNA was similar to the amount of RU5 for all of the mutants that carried

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Fig. 6. Levels of viral DNA synthesis intermediates at various time points after infection. Minus-strand DNA synthesis was monitored by measuring the amount of RU5; minus-strand transfer was monitored by measuring the amount of U3; (+) strand DNA transfer was monitored using PCR primers that span the primer binding site (pbs). The viral DNA copy numbers are shown on the y axis, and the time points when DNA was harvested are shown on the x axis. (A) Viral DNA synthesis for mutant viruses containing the Q475A mutation in RT. (B) Viral DNA synthesis for mutant viruses containing the Y501A mutation in RT.
Y501A. The generation and utilization of the PPT primer, as measured by plus-strand DNA transfer, were modestly delayed when the T-2-5 mutation was paired with WT RT; however, the Y501A mutation dramatically delayed plus-strand DNA transfer when combined with either the normal PPT or the T-2 mutation. The T-2-5 mutation in the PPT appeared to enhance the rate of plus-strand priming by the Y501A RT mutant; Y501 showed faster priming with T-2-5 than with a wild-type PPT or with either the T-2 or the T-5 mutations. In particular, the combination of the Y501A mutation and the T-2 mutation reduced the amount of plus-strand DNA dramatically, even at the 24 h time point (Fig. 6B). These effects of the complex mutations on the rate of plus-strand DNA transfer, when taken together with the more modest affects on the specificity of PPT cleavage, can explain the viral titer.

Discussion

The specificity of the RNase H cleavages that generate and remove the PPT primer is remarkable when one considers that the enzyme makes only one base-specific contact with its nucleic acid substrate and that, outside the PPT, RNase H cleavage appears to be relatively nonspecific. The specificity with which RNase H cleaves the PPT depends on the interactions of the nucleic acid and the enzyme; more specifically, it depends on the structure of the nucleic acid when it is in a complex with RT. This structure can be affected (and the specificity of the PPT cleavage altered and/or the generation of the PPT decreased) by making mutations in either the nucleic acid or the protein (Coffin et al., 1997; Dash et al., 2004; Julias et al., 2002, 2004; McWilliams et al., 2003; Powell and Levin, 1996; Pullen et al., 1993; Rausch et al., 2002; Smith et al., 1999; Snyder and Roth, 2000). There are critical contacts between the protein and the nucleic acid involving the amino acids Y501 and Q475. When RT is aligned to cleave the PPT/U3 junction, the corresponding contact points for these amino acids are at G2 and G5 in the PPT. The importance of these contacts is illustrated by the observation that mutations in either the amino acids involved in these contacts (Q475A and Y501A) or mutations that convert G2 and G5 to Ts (T-2 and T-5) affect the titer of the virus and the ability of the RNase H of RT to appropriately cleave the PPT. One simple model to explain these data would suggest that the contacts between G2 and Y501 and between G5 and Q475 are critical. In a model of this type, a double mutant in which both sides of an RT/PPT contact, for example, G2 and Y501 (or G5 and Q475) are mutated, would not be significantly more impaired than mutants in which either G2 or Y501 was mutated. In contrast, the model predicts combining mutations at Y501 and G5 (or Q475 and G2) would have a much more profound effect. Alternatively, if the mutations in the protein (or in the PPT) have significant effects on the structure of the complex that go beyond the loss of a simple contact, then the effects of combining the mutations are less clearly predictable. There are reasons to anticipate the more complex result. The effects of the corresponding RT and PPT mutations do not exactly match (the effects of T-2 differ from the effects of Y501A, and the effects of T-5 differ from the effects of Q475A). One possible explanation could be that the additional contacts between Q475 and Y501 and the nucleic acid are important in the interactions between RT and the PPT. However, we showed that mutating the other contact points in the PPT do not profoundly affect either the titer of the virus or the specificity of RNase H cleavage (McWilliams et al., 2003). In addition, one would expect mutations that interrupt the run of purines in the PPT (like T-2, T-5 and T-2-5) would affect the structure of the nucleic acid both in solution and in a complex with RT, which could affect the efficiency of cleavage, the specificity of cleavage or both.

In terms of the effect on the titer, pairing the T-2 with Y501 had the greatest effect. The Y501A + T-2-5 mutant actually had a higher titer than Y501A + T-2. Moreover, the titer of all the complex mutants that involve T-5 and T-2-5 was fairly similar. The effects of combining the mutants on the titer cannot be explained solely by an effect on cleavage specificity. The 2-LTR circle junctions, done in the absence of a functional integrase, showed that the mutants combining either Y501A or Q475A with T-2-5 produced fewer consensus circle junctions than did the mutants in which T-2 or T-5 were combined with Q475A or Y501A. Analysis of the rate and extent of minus-strand and plus-strand viral DNA synthesis provides the explanation that, although mutations in the PPT (alone) or in RT (alone) did not have a profound effect on the rate or final amount of plus-strand DNA synthesis, combining the PPT mutants and the RT mutants did profoundly affect the synthesis of plus-strand DNA. This is most easily explained as an affect on the rate (and extent) of PPT cleavage. These data also help to explain the effects of the double mutants on viral titer. The mutants that had the lowest titers (for example, Q475A + T-2) cleaved the PPT with moderate accuracy, but this cleavage was much slower than normal. Although the synthesis of plus-strand DNA did catch up to some extent at later time points, there was an effect on the amount of plus-strand viral DNA, particularly in the case of the Y501A + T-2 double mutant.

Given the profound delay in the synthesis of plus-strand DNA, it is somewhat surprising that the titers of the double (and triple) mutants are not lower than they are. By the 24 h time points, the amount of plus-strand viral DNA recovered to a greater or lesser extent depending on the mutant. This shows that the reverse transcription complex is relatively stable and that a substantial delay in the process is tolerated reasonably well. This agrees with data showing that, if viral DNA synthesis was not completed because the host cell is serum-starved, the viral DNA intermediates are stable and viral DNA synthesis could be efficiently completed when serum was added back to the starved cells (Fritsh and Temin, 1977; Varmus et al., 1977).

Taken together, our data argue that the interactions between RT and the PPT are complex and multifaceted and that the mutations in the PPT and/or the RT affect the overall structure of the complex (and RNase H cleavage), thus affecting both the rate and specificity of PPT cleavage in ways that cannot be explained simply by a small number of critical contacts between the protein and the PPT. This conclusion is supported by the data that show that, when the mutations in the PPT that cause
the specific insertion of a single nucleotide (T-5 and T-2-5) are paired with Y501A or Q475A, there is a reduction in the number of 2-LTR circle junctions that have a single nucleotide insertion. This means that the addition of the mutations in the protein to the mutation(s) in the PPT causes an additional loss of specificity, not only in terms of the ability of the RNase H to make the exactly correct cleavage, but also in terms of RNase H making a specific, if incorrect, cleavage. Moreover, the addition of either the Q475A mutation or the Y501A mutation had a profound affect on the ability of T-5 mutation in the PPT to cause the specific one-nucleotide insertion error, reinforcing the idea that the mutations have effects that go well beyond simply disrupting the contacts between G2 and Y501 and between G5 and Q475. Combining mutations in RNase H and the PPT appeared to have a greater effect on the initiation of plus-strand DNA synthesis than on the specificity of cleavage. This translated into an effect on the ability of the mutant viruses to complete viral DNA synthesis at the 24 h time point and, in turn, into an effect on viral titer.

This idea is supported by the fact that combining either the Q475A or the Y501A mutations with the double PPT mutant, T-2-5, had either a similar, or a smaller, effect on the titer of the mutant viruses than did combining the Q475A with either the T-2 or the T-5 mutant, or Y501A with T-5. This result was initially somewhat surprising both because the T-2-5 double mutant by itself had a more profound effect on the titer and the circle junctions than did either the T-2 or -T-5 single mutations (by themselves) and because, in the absence of integration, the Q475A + T-2-5 and the Y501A + T-2-5 mutants gave rise to the smallest numbers of consensus circle junctions. One factor that contributed to the higher than expected titer of these complex mutants is that the mutants generate a significant number of linear DNAs that have one-nucleotide insertions; these slightly aberrant linear DNAs can be successfully integrated (Julias et al., 2004). However, even when this is taken into account, the titer of the Y501A + T-2-5 and Q475A + T-2-5 mutants is somewhat higher than would be expected.

In the context of wild-type RT, the double mutation (T-2-5) has a more profound effect on the overall structure (and cleavage) of the PPT than do either the T-2 or the T-5 single mutations. Thus, the T-2-5 mutation is more deleterious when the mutant PPTs are cleaved by the RNase H of wild-type RT, but the T-2-5 mutation is somewhat beneficial when the PPTs are cleaved either by the Q475A or by the Y501A mutants. One possible explanation is that the mutant PPTs appear to be cleaved relatively slowly by all the RNases H (both wild-type RT and the mutants). Although the double (T-2-5) mutations in the PPT may reduce the specificity of cleavage, this double mutation may disrupt the specialized structure conferred on the PPT by the run of purines in the G-tract. This greater alteration in the structure of the PPT could help the mutant RTs overcome a severe problem in the rate of PPT cleavage. In this way, the presence of two mutations in the G-tract could help an RNase H with a mutation in an important contact (either Q475 or Y501) cleave more rapidly even if there is a loss in the specificity of the cleavage, an idea that is supported by the experiments that measured the rates of plus-strand initiation. Viewed in this light, it appears to be more important, in terms of viral titer, for the RNase H to cleave the PPT more rapidly and less specifically than for the RNase H to retain greater specificity if the overall rate of cleavage is low.

**Materials and methods**

**Construction of the HIV-1 mutants**

The one-round HIV-1 based viral vector pNLNgoMIVR-E-HSA has been described (Julias et al., 2001). Briefly, the vector contains frameshift mutations that inactivate env and expresses the murine cell surface marker CD24 from the nef reading frame. Mutations in the PPT of the HIV-1 based vector pNLNgoMIVR-E-HSA were generated using a BspMI-cassette strategy as previously described (McWilliams et al., 2003). The mutations Q475A and Y501A in the RNase H primer grip of RT were previously described (Julias et al., 2002). The vector-encoding plasmids were analyzed by restriction endonuclease digestion and DNA sequencing to confirm that only the desired mutations were present in the vectors. Vectors containing the D116N mutation in the integrase coding region were generated by site-directed mutagenesis using the Quick-change kit (Stratagene). Briefly, DNA oligonucleotides (Biosource International) containing the necessary mutations were synthesized and used in the site-directed mutagenesis protocol. The mutagenesis substrate was pKS containing the Asp718 to SacI fragment of pNLNgoMIVR-E-HSA. The resulting plasmid was analyzed by restriction endonuclease digestion, and DNA sequence analysis was used to confirm that only the desired mutation in IN was present; this plasmid is called pKSINTD116N. The Asp718 to SacI fragment from pKSINTD116N was cloned into pNLNgoMIVR-E-HSA vectors containing the desired PPT mutations, using Asp718 and SacI (these sites are each present once in the plasmid that encodes the vector). The resulting plasmids were analyzed by restriction endonuclease digestion and DNA sequence analysis. An NgoMIV restriction site is present near the 3′ end of the RT coding region of the single-cycle vectors derived from pNLNgoMIVR-E-HSA; another NgoMIV site is present 3′ of the downstream LTR. The NgoMIV fragment of the plasmid containing the desired mutant PPTs in the context of wild-type or mutant HIV-1 integrase (D116N) was ligated into the HIV-1 vector-encoding plasmid containing the Y501A or the Q475A mutation in RT. The vectors containing mutations in the PPT were previously described in both IN+ and IN− backgrounds. The NgoMIV restriction sites were used to combine the PPT (or PPT plus IN) mutations with the mutations in RT.

**Cells**

The human embryonal kidney cell line 293 was obtained from the American Type Culture Collection (ATCC). The human osteosarcoma cell line HOS was obtained from Dr. Richard Schwartz (Michigan State University; Lansing, MI). 293 and HOS cells were maintained in Dulbecco’s modified
Eagle’s medium (Invitrogen) supplemented with 5% fetal bovine serum, 5% newborn calf serum and penicillin (50 units/ml) plus streptomycin (50 μg/ml) (Quality Biological).

**Transfection, infection and phenotyping protocol**

293 cells were transfected with 3 μg of pNLNgoMIVR-E-.HSA and 1.0 μg of pHCMV-g (obtained from Dr. Jane Burns, UCSD) using the calcium phosphate method. pHCMV-g expresses the VSV-glycoprotein and can be used to generate high-titer viral stocks (Bartz and Vodicka, 1997; Yee et al., UCSD) using the calcium phosphate method. pHCMV-g using the calcium phosphate method. Forty-eight h supernatants were harvested, clarified by low-speed centrifugation, and an aliquot was used to infect HOS cells. The amount of p24 in the supernatant was determined using the Coulter HIV-1 p24 antigen assay (Beckman Coulter); the p24 concentration was used to control for the amount of virus in the samples. HOS cells were plated in 60-mm diameter dishes at a density of 1.0 × 10⁵ cells per plate on the day prior to transfection. At this plating density, the cells were approximately 25% confluent on the day of transfection. The precipitate was added to the 293 cells dropwise. Fresh medium was added 16 h after transfection. The 48-h supernatants were harvested, clarified by low-speed centrifugation, and an aliquot was used to infect HOS cells. The amount of p24 in the supernatant was determined using the Coulter HIV-1 p24 antigen assay (Beckman Coulter); the p24 concentration was used to control for the amount of virus in the samples. HOS cells were plated in 60-mm diameter dishes at a density of 1.5 × 10⁵ cells per plate on the day prior to infection. The virus was allowed to absorb to the cells for 4 h, then fresh medium was added. Forty-eight hours after infection, the cells were harvested from the plate by treatment with 1.0 ml of versene (Invitrogen), an additional 3 ml of PBS was added, and then the cells were collected by centrifugation, washed and resuspended in 200 μl PBS. The cells were labeled with phycoerythrin-conjugated rat anti-mouse CD24 monoclonal antibody (Pharmingen) using standard procedures, fixed with paraformaldehyde and subjected to fluorescence-activated cell sorting to determine viral titer.

**Transfections, infections and nucleic acid extraction for 2-LTR circle junction analysis**

293 cells were transfected with 5 μg of plasmid DNA encoding the pNLNgoMIVR-E-.HSA vector and 3 μg of pHCMV-g using the calcium phosphate method. The medium on the cells was changed 16 h after infection. The 48 h supernatants were harvested and clarified by centrifugation, and 4 ml of virus-containing supernatant was used to infect HOS cells. The supernatants were left on the cells for 4 h, then fresh medium was added. The total DNA was isolated from the HOS cells approximately 24 h after infection using the viral blood DNA kit (Qiagen).

**PCR amplification, cloning and sequencing of 2-LTR circle junctions**

The 2-LTR circle junctions were amplified in 100 μl reactions using an upstream PCR primer that anneals near the RU5 junctions and a downstream primer that anneals in the U3 region of the LTR. The sequence of the upstream primer was 5'-CGATGAATTCGCTAACTAGGGAACCCCACCTGCT-3'; the sequence of the downstream primer was 5'-GC CATTCTA-

GAGTTCTCTCTTTATTGGGCCTC-3'. Ten microliters of DNA from cells infected with the PPT mutants and 0.25 μl each of the forward and reverse primers (100 nM final concentration) were used with 90 μl of Platinum PCR Supermix (Invitrogen) in each PCR reaction. The expected product is approximately 350 bp long and has EcoRI and XbaI cleavage sites introduced by the primers. The PCR products were digested with EcoRI and XbaI and cloned into SK (Stratagene). The 2-LTR circle junction clones were analyzed using restriction enzyme digestion and DNA sequence analysis.

**Determination of consensus 2-LTR circle junctions in an IN− background**

Transfections, infections, DNA isolation, PCR amplification and cloning of the 2-LTR circle junction product were done identically for the IN− viruses (containing the D116N mutation) as for the integration-competent viruses. Consensus 2-LTR circle junctions contain an ScaI recognition sequence. The percentage of consensus 2-LTR circle junctions was determined by digesting DNA from individual clones with ScaI and fractionating the digestion products by agarose gel electrophoresis. The SK plasmid into which the PCR products were cloned also contains an ScaI recognition sequence; plasmids containing a consensus 2-LTR circle junction will produce 2 bands upon ScaI digestion and agarose gel electrophoresis.

**Quantitative PCR**

293 cells were transfected with the 3 μg of HIV-based vectors and 2 μg of pHCMV-g (expresses the VSV envelope glycoprotein) using the calcium phosphate method. Forty-eight hour supernatants (8 ml) were harvested, centrifuged at low speed to remove cell debris, filtered through 45 μm syringe filter (Millipore) and treated for 30 min with RNase-free DNase I [final concentration 10 units/ml (Roche)] at room temperature. Six milliliters of the DNase-I-treated supernatants was concentrated to 0.5 ml using 20 ml 300,000 MWCO concentrators (Vivascience). DMEM supplemented with 10% fetal bovine serum was added to the concentrated virus to increase the volume to 4.1 ml per sample. 0.5 ml of each virus was then added to the HOS cells for 2 h. HOS cells were washed with 4 ml PBS, and DNA was isolated using the EZ-1 DNA tissue kit (QIAGEN). The elution volume was 100 μl for each sample. Real-time PCR reactions were performed using the 2× Universal Taqman Master Mix (Applied Biosystems) in 50 μl reaction volumes using previously described Taqman reagents and conditions (Julias et al., 2001). Viral DNA copy numbers were determined using the ABI 7700 (Applied Biosystems) and normalized for the amount of p24 antigen in the supernatant.

**Statistical methods**

Data were analyzed by log linear categorical analysis, contingency table analysis and related methods. I × J Circle Junction by Mutant tables were decomposed through the use of the likelihood ratio chi-square statistics into independent
partitions to show associations between circle junction and/or mutant groupings and categories. Subsets of pertinent groupings were followed up with traditional $2 \times 2$ chi-square analyses and Fisher's Exact tests.

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