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Mutations at position 184 of human immunodeficiency virus type-1 reverse transcriptase affect virus titer and viral DNA synthesis

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Abstract

Methionine at position 184 of human immunodeficiency virus type-1 (HIV-1) reverse transcriptase (RT) was changed to valine, isoleucine, threonine, or alanine in an HIV-1-based vector. The vectors were analyzed for replication capacity and for resistance to the nucleoside analog 2′,3′-dideoxy-3′-thiacytidine (3TC) using a single-cycle assay. Viruses containing the valine or isoleucine mutations were highly resistant to 3TC and replicated almost as well as the wild-type virus. The virus containing the threonine mutation was resistant to 3TC, but replicated about 30% as well as the wild-type. The alanine mutation conferred partial resistance to 3TC, but replicated poorly. The amounts of viral DNA synthesized decreased in 3TC-treated cells when the cells were infected with wild-type virus and the M184A mutant. The effect of these mutations on the generation of the ends of the linear viral DNA was determined using the sequence of the 2-LTR circle junctions. The M184T mutation increased the proportion of 2-LTR circle junctions containing a tRNA insertion, suggesting that the mutation affected the RNase H activity of RT.
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Keywords: HIV-1; 3TC; Real-time PCR; 2-LTR circle junction; RNase H activity

Introduction

The inhibitors currently approved for the treatment of human immunodeficiency virus type-1 (HIV-1) infections target viral entry and the viral enzymes protease and reverse transcriptase (RT) (Coffin et al., 1997; Kilby et al., 1998). RT inhibitors block the synthesis of viral DNA in infected cells. There are two general classes of reverse transcriptase inhibitors, nucleoside analogs (NRTIs) and nonnucleoside inhibitors (NNRTIs). Viruses resistant to either class of drug emerge after the initiation of drug treatment.

3TC is an NRTI; both structural and biochemical data suggest that the mechanism of resistance to 3TC by HIV-1 RT involves steric hindrance (Gao et al., 2000; Huang et al., 1998; Sarafianos et al., 1999). 3TC rapidly selects for RT variants that have either valine or isoleucine substituted for the methionine normally found at position 184, which is the second amino acid in the conserved YXDD motif at the polymerase active site (Schinazi et al., 1993). Both valine and isoleucine are β-branched amino acids. 3TC contains an oxathiolane ring in place of the deoxyribose ring found in dNTPs; the L or (−) entantiomer of 3TC is used in the treatment of HIV-1 infections. When 3TC-TP is bound at the active site of HIV-1 RT, the oxathiolane ring of 3TC is closer to the amino acid at position 184 than the deoxyribose ring of a normal triphosphate. If a β-branched amino acid is present at position 184, the oxathiolane ring will clash with the β-branch; this steric hindrance interferes with the incorporation of 3TCTP, which leads to the high-level drug resistance associated with M184V and M184I variants (Gao et al., 2000; Huang et al., 1998; Sarafianos et al., 1999).

There are data to suggest that the same mechanism for resistance to 3TC occurs in other viral RTs. 3TC can block the replication of FIV; the resistant viruses that are selected have a methionine to threonine (threonine is a β-branched

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amino acid) substitution in the second position of the YXDD motif of the polymerase active site in FIV RT (this position is equivalent to 184 in HIV-1 RT) (Smith et al., 1997). However, this mutation is not usually selected in HIV-1 RT (Schinazi et al., 2000). Treatment of hepatitis B virus (HBV) with 3TC selects for isoleucine or valine in place of methionine at the second position of the YXDD motif (Tipples et al., 1996). Not all wild-type retroviruses have a methionine at the second position of the YXDD motif; for example, Moloney murine leukemia virus (MLV) has valine (YVDD). The steric hindrance model predicts that MLV should be resistant to 3TC; as predicted MLV is relatively resistant to 3TC (Halvas et al., 2000). However, a mutant MLV with methionine at the second position of the YXDD motif was relatively resistant to 3TC. In vitro experiments with purified MLV RT showed that wild-type MLV RT was resistant to 3TCTP; an MLV RT with a methionine at the second position of the YXDD motif was more sensitive to 3TCTP than the wild-type enzyme with valine at this position (Boyer et al., 2001). However, the MLV RT with methionine in the YXDD motif was more resistant to 3TCTP than wild-type HIV-1 RT. In addition, substitution of alanine for methionine in the YXDD motif of HIV-1 RT caused partial resistance to 3TCTP in vitro. Alanine is not a β-branched amino acid and the MLV, MLV RT, and HIV-1 RT data suggest that other factors, for example, the exact position of the template primer in the RT active site, may contribute to 3TC (3TCTP) resistance (Boyer et al., 2001).

The effects of various amino acid substitutions in the YXDD motif have been studied using both purified HIV-1 RT and in the context of the replicating virus. Several groups have reported that the V and I mutations affect the in vitro processivity of RT (Avidan and Hizi, 1998; Back and Berkhout, 1997; Boyer and Hughes, 1995; Sharma and Crumpacker, 1999). The M184V mutation has also been reported to affect the initiation of viral DNA synthesis (Diallo et al., 2003; Wei et al., 2003). These studies modeled the initiation of plus- or minus-strand DNA in vitro with RTs containing the M184V (or L74V) mutation in RT. The mutant enzymes were deficient in the utilization of the cognate tRNA primer or ppt in the initiation of DNA synthesis (Diallo et al., 2003). Mutations at position 184 can also affect viral replication. Wakefield et al. (1992) reported that there was a correlation between polymerase activity (measured on a homopolymeric template) and viral replication measured by the production of p24 antigen. DNA synthesis was profoundly impaired in cells infected with viruses containing the M184V mutation in RT that also contain a deletion in an A-rich loop upstream of the primer binding site (Wei et al., 2003), suggesting the M184V mutation may impair interactions of RT with its primer-template that are involved in the initiation of reverse transcription. These studies describe important characteristics of the M184 mutants; however, they do not directly address why certain amino acid substitutions (V and I) in the YXDD motif of HIV-1 RT are selected by 3TC, and others (in particular A and T) are not. To understand why the V and I mutations are selected in HIV RT and T and A mutations are not, we examined the effects of these mutations on viral replication and drug sensitivity. We compared the wild-type virus to mutants that had methionine normally present at 184 changed to valine, isoleucine, threonine, or alanine. As expected, the M184V and M184I mutations caused high-level resistance to 3TC and had a minimal effect on the efficiency of replication in a single round of viral replication. The M184A and M184T viruses replicated poorly; the M184T virus was quite resistant to 3TC, the M184A mutation caused a slight decrease in susceptibility to 3TC. Real-time PCR was used to monitor viral DNA synthesis in infected cells. The results indicated that viruses containing the M184T and M184A mutations synthesize less DNA than the wild-type virus. In the absence of 3TC, the M184T and M184A mutations decreased the initiation of DNA synthesis. The amounts of viral DNA synthesized by the wild-type virus and virus with the M184A mutation decreased when cells were treated with 3TC. We also examined the effects of these mutations on the generation of the ends of the linear viral DNA using the 2-LTR circle junction as a surrogate for the ends of the linear viral DNA. The M184T mutation affected the generation of the ends of the linear viral DNA; there was a significant increase in the number of tRNA primer-derived sequences in circle junctions from infections with the M184T mutant. The M184V, M184I, and M184A mutations did not cause statistically significant increases in the proportion of circle junctions containing sequences derived from the tRNA primer. These results suggest that the M184T mutation affects the level of RNase H activity. The presence of PPT-derived sequences at the circle junctions suggests that the M184T mutation could affect the efficiency of RNase H cleavage. The effects of M184T on RNase H activity provide additional support for the idea that the enzymatic activities (polymerase and RNase H) of HIV-1 RT are interdependent (Dudding and Mizrahi, 1993; Dudding et al., 1991; Gao et al., 1998; Hughes et al., 1998; Julias et al., 2001, 2002, 2003; Mizrahi et al., 1990, 1994).

Results

Vectors and replication assay

The vector pNLNgoMIVR-E-.LUC is based largely on the NL4-3 isolate of HIV-1 (see Fig. 1 and Materials and methods for information on the construction of the vector). The vector expresses *gag* and *pol*, but the envelope gene has been inactivated. The luciferase gene has been introduced into the *nef* open reading frame. Mutations in the YXDD motif of RT were introduced into the vector as *Sma*I to *Asp*718 fragments from plasmids that express recombinant HIV-1 RT in *Escherichia coli* (Boyer et al., 1992). Viral

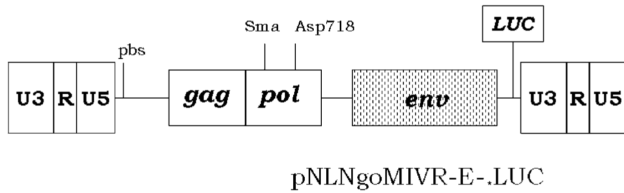


Fig. 1. The HIV-1-based vector used to measure viral infectivity. The HIV-1 vector pNLNgoMIVR$^-$E$^-$.LUC was created from the vector pNLNgoMIV.R$^-$E$^-$.HSA as described in Materials and methods. The vector expresses the firefly luciferase gene from the *nef* open reading frame. The *env* gene and the *vpr* gene have been inactivated in this vector. The vector is packaged into virions using the VSV-g envelope glycoproteins (Bartz and Vodicka, 1997; Yee et al., 1994). The drawing is not to scale. The vector undergoes only a single cycle of replication.

vectors were constructed encoding valine, isoleucine, alanine, and threonine in place of the methionine normally found at position 184 of RT. The vector was packaged into virions by co-transfecting 293 cells with the plasmid containing the vector and with pHCMV-g, a plasmid that expresses the VSV-g envelope (see Materials and methods).

3TC resistance

Virions containing wild-type RT, and RTs with the valine, isoleucine, threonine, and alanine substitutions at position 184 were generated and used to infect HOS cells. The effects of 3TC on the replication of the viruses were determined by treating cells with 0, 5, 10, and 20 μM 3TC (Fig. 2). Data from a representative experiment are shown. The relative titers and drug resistance profiles were highly reproducible between experiments (variation of about ±5% or less after normalization). As expected, the wild-type virus was sensitive to 3TC; 5 μM of 3TC reduced virus replication by approximately 90%. The RT mutants containing valine and isoleucine at position 184 had virus titers approximately 90% of the wild-type titer in the untreated sample; as expected, these mutants displayed high-level resistance to 3TC and were not measurably inhibited by 20 μM 3TC. In the absence of 3TC, the mutant containing threonine at position 184 had a titer of about 30% of the wild-type virus. Threonine containing the mutant also displayed high-level resistance to 3TC. The mutant containing alanine at 184 had the lowest relative titer, approximately 15% the titer of wild type. The alanine mutation caused modest resistance to 3TC; 5 μM inhibited the wild-type virus by 90%, but M184A was inhibited by approximately 50%.

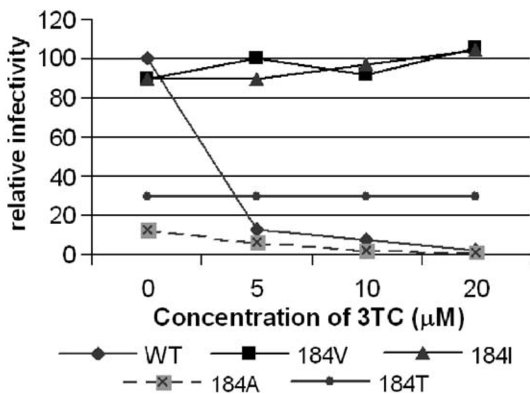


Fig. 2. The relative infectivity of the virus and drug resistance of viruses containing mutations at position 184 in RT. The relative infectivity is shown on the *y*-axis, the concentration of 3TC is shown on the *x*-axis.

Synthesis of viral DNA in cells infected with WT virus and viruses containing the M184T and M184A mutations in the presence and absence of 3TC

The effects of mutations at position 184 on viral DNA synthesis were determined by infecting HOS cells with wild-type virus and viruses containing the M184T or M184A mutations. Total DNA was isolated from cells 24 h after infection. Viral DNA synthesis in infected cells was monitored using quantitative real-time PCR (see Fig. 3). The DNA copy number is shown on the *y*-axis and the RT mutant is shown on the *x*-axis. Virions were generated that contained wild-type RT and the M184T, M184A, and the D110E (polymerase active site) mutants. The D110E mutation abolishes polymerase activity; consequently, viruses containing this mutation cannot synthesize viral DNA. This mutant was included as a control to monitor residual DNA carryover from the transfection process used to generate the virions. The DNA copy number for the D110E sample was approximately 100 copies for RU5, U3, or Gag. These data indicate that there is minimal plasmid carryover from the

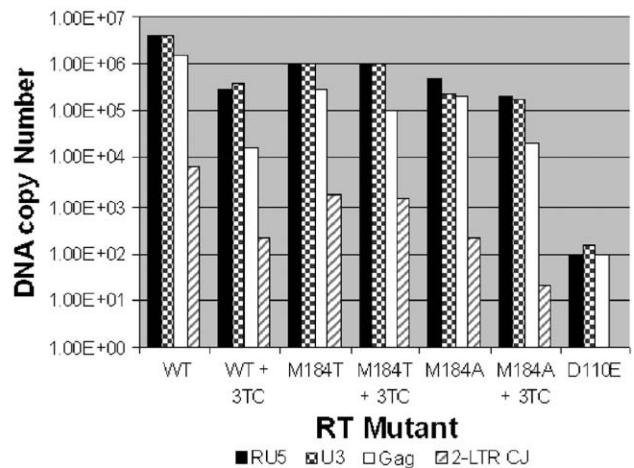


Fig. 3. Determination of viral DNA copy number in infected cells. The *x*-axis designates the virus and indicates the presence or absence of 3TC treatment. The *y*-axis shows the DNA copy number on a logarithmic scale. Taqman probe and primer and primer sets were used in quantitative PCR reactions to determine the viral DNA copy number in infected cells (see Materials and methods). Quantitative PCR was used to measure RU5 (black bars) to measure initiation of DNA synthesis, U3 (checkered bars to measure first strand transfer), Gag (white bars) to measure elongation, and 2-LTR circles (diagonally striped bars) were measured as a surrogate for the completion of viral DNA synthesis.

transfection process. There were no 2-LTR circle junctions detected in samples derived from cells infected with virions containing the D110E mutation. The plasmid used to generate the virus stock does not contain a 2-LTR circle junction; retroviral reverse transcription is required to generate the linear viral DNA that is joined to form the 2-LTR circles.

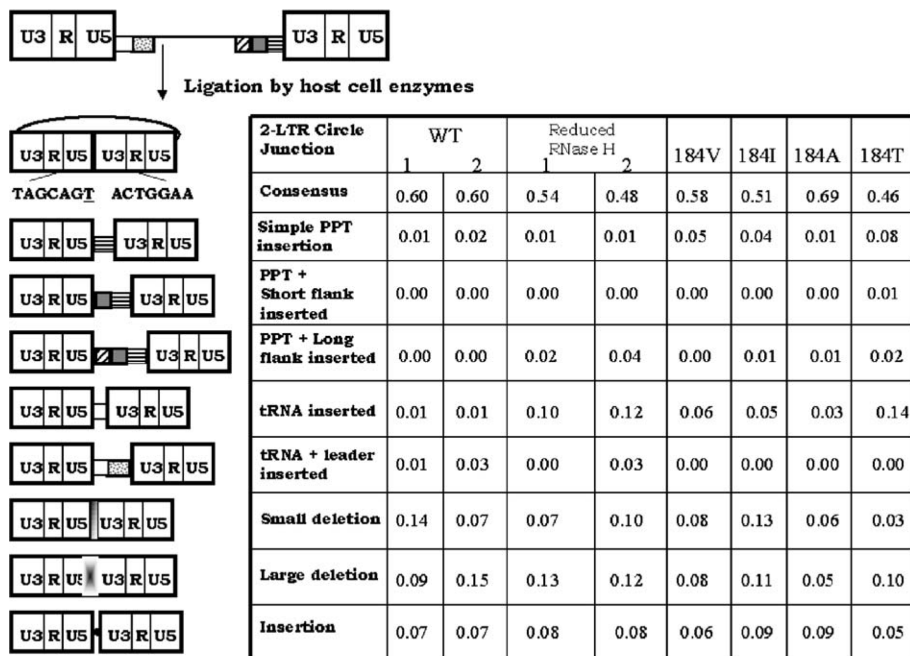
The viral DNA copy numbers in cells infected with the wild-type virus were approximately 4×10^6 for RU5 and U3 (Fig. 3). About 40% of DNAs undergoing first strand transfer was elongated through the *gag* gene. There were 7×10^3 copies of the 2-LTR circle junction present in the sample. When the cells infected with the wild-type virus were treated with 20 μM 3TC, no effect was seen on the amounts of DNA synthesized from RU5 or U3; however, there was a 10-fold decrease in the amount of *gag* DNA. 3TC treatment caused an approximately 30-fold reduction in the number of 2-LTR circles. These results are consistent with the decrease in virus titer observed when 20 μM of 3TC was added to cells infected with the wild-type virus.

When cells were infected with viruses containing the M184T mutation, there were approximately 1×10^6 copies of DNA for RU5 and U3, indicating a decrease in the initiation of DNA synthesis (Fig. 3). There were 3×10^5 copies of *gag* DNA indicating that the M184T mutant and wild-type RTs carry out minus-strand elongation with equal efficiency. There were 1.7×10^3 copies of the 2-LTR circle junction measured. The viral DNA copy numbers were similar to samples treated with 20 μM 3TC and the samples that did not receive 3TC, consistent with the observation that viruses containing the M184T mutation are highly resistant to 3TC.

The M184A mutation caused a decrease in the amount of viral DNA synthesized. There were 5.2×10^5 copies of RU5, indicating a decrease at the level of initiation of DNA synthesis. There were 2.3×10^5 copies of U3, 2.0×10^5 copies of *gag*, and 2×10^2 copies of 2-LTR circles. When the cells were treated with 20 μM 3TC, a 10-fold decrease in the amount of gag DNA was observed. There was also a 10-fold decrease in the amount of 2-LTR circle junctions. These results are consistent with the partial resistance to 3TC conferred by the M184A mutation.

M184T affects RNase H cleavage

The removal of tRNA and PPT primers by RNase H defines the ends of linear viral DNA. The removal of the tRNA defines the right end; the removal of the PPT defines the left end. A portion of the linear viral DNAs is ligated, by host cell ligases, to form 2-LTR circles (Li et al., 2001). To determine if mutations at position 184 in RT alter the efficiency or specificity of RNase H, we used the sequence of the 2-LTR circle junction as a surrogate for the ends of the genome (Fig. 4). 2-LTR circle junctions were amplified from cells infected with viruses containing wild-type RT, with viruses containing reduced RNase H activity (viruses generated by co-transfecting 293 cells with a mixture in which 10% of the plasmids encoded wild-type RT and 90% of the plasmids encoded RT with the active site mutation E478Q in RNase H), and with viruses with mutations at



2-LTR Circle Junction	WT 1	WT 2	Reduced RNase H 1	Reduced RNase H 2	184V	184I	184A	184T
Consensus	0.60	0.60	0.54	0.48	0.58	0.51	0.69	0.46
Simple PPT insertion	0.01	0.02	0.01	0.01	0.05	0.04	0.01	0.08
PPT + Short flank inserted	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
PPT + Long flank inserted	0.00	0.00	0.02	0.04	0.00	0.01	0.01	0.02
tRNA inserted	0.01	0.01	0.10	0.12	0.06	0.05	0.03	0.14
tRNA + leader inserted	0.01	0.03	0.00	0.03	0.00	0.00	0.00	0.00
Small deletion	0.14	0.07	0.07	0.10	0.08	0.13	0.06	0.03
Large deletion	0.09	0.15	0.13	0.12	0.08	0.11	0.05	0.10
Insertion	0.07	0.07	0.08	0.08	0.06	0.09	0.09	0.05

Fig. 4. The sequence of the 2-LTR circle junction. The sequence of 2-LTR circle junctions was determined from cells infected with wild-type virus, from virions with reduced levels of RNase H, and from cells infected with vectors containing mutations at position 184 in RT. The numbers in the table represent the fraction of 2-LTR circle junctions containing the given property (1.00 = 100%, 0.60 = 60%, etc.). The data for the wild-type RT and virions with reduced RNase H activity are from previously published experiments (Julias et al., 2003).

amino acid 184 in RT. The frequency of defects in the 2-LTR circle junction was determined by sequencing approximately 100 2-LTR circle junction clones from infections with the wild-type virus, the virus with reduced RNase H activity, and each of the 184 mutants. A summary of the sequence analysis is shown (Fig. 4). When the sequence of the 2-LTR circle junction is correct (the complete sequence from the ends of the linear viral DNA is present), this is defined as a consensus 2-LTR circle junction. The proportion of 2-LTR circle junctions having a consensus sequence is shown in the top row of Fig. 4. We previously generated two independent sets of data after infecting cells with wild-type virus and virions with reduced RNase H activity. The fraction of consensus 2-LTR circle junction sequences was 0.60 from infections with viruses containing wild-type RT in both experiments and 0.54 and 0.48 with virions containing reduced RNase H activity (Julias et al., 2003). The fraction of consensus junctions was 0.58, 0.51, 0.69, and 0.46 for the viruses containing the M184V, M184I, M184A, and M184T mutations, respectively.

When cells were infected with the wild-type virus, the fraction of 2-LTR circles that contained tRNA insertions was 0.01. Some mutations at 184 caused a small increase in the fraction of circle junctions containing tRNA insertions. The fraction containing tRNAs was 0.06, 0.05, and 0.03 for the M184 V, I, and A mutants, respectively. This is not statistically different than wild type. However, the 2-LTR circle junctions derived from infecting cells with the M184T mutant had a larger increase in the fraction of circle junctions containing tRNA; the fraction was 0.14. This is statistically significant ($P < 0.05$) and suggests that a threonine at position 184 caused a decrease in RNase H activity.

If the PPT is properly generated, but is not removed (or is incompletely removed) by RNase H, then a 2-LTR circle junction containing a simple PPT insertion is generated. Simple PPT insertions can also be generated if an improper primer is used to initiate plus-strand DNA synthesis. Two independent sets of data were analyzed after infecting HOS cells with wild-type virus and virions with reduced RNase H activity; the frequency of simple PPT insertions was 0.01 and 0.02 for wild-type and 0.01 in both experiments with virions with reduced RNase H activity (Fig. 4). The 2-LTR circle junctions derived from infecting cells with the 184A mutant contained simple PPT insertions at a fraction of 0.01; the same fraction of simple PPT inserts as the circle junctions derived from infection with wild-type virus or virions with reduced RNase H activity. The presence of V or I at position 184 increased the fraction of circle junctions containing PPT inserts to 0.04 and 0.05 (for 184 V and I, respectively). These increases are not statistically significant. However, the increase in the proportion of 2-LTR circle junctions containing a PPT seen after infecting cells with the 184T virus (0.08) is statistically significant ($P <$ 0.05). The fact that there is no increase in PPT insertions when the RNase H activity is reduced by 90% suggests that the increase in PPT insertions seen with the M184T mutants is caused by a change in RNase H specificity, and not simply by a decrease in RNase H activity.

2-LTR circle junctions containing PPTs with short flanking sequences could arise by improper generation of PPT. If the aberrant PPT is then retained because RNase H fails to remove the aberrant primer, a PPT plus a short flank (the U-tract) will be found at the 2-LTR circle junction. This type of insertion can also arise from plus-strand priming upstream of the normal site of initiation. PPTs with short flanking sequences were not observed in 2-LTR circle junctions derived by infecting cells with virions containing wild-type RT or virions with reduced RNase H activity. Aberrant 2-LTR circle junctions containing short flanking sequences were observed at low frequency (0.01) after infecting cells with viruses containing the M184T mutation in RT; this is not statistically significant.

A PPT with a long flanking sequence can arise if RT uses (and removes) a primer upstream of the PPT to initiate plus-strand DNA synthesis, or if an aberrant primer is used, but not removed. The resulting 2-LTR circle junction contains the PPT with a considerable amount of flanking sequence (typically >20 nt upstream of the PPT). This type of aberrant 2-LTR circle junction was observed at a frequency of 0.02 when RNase H activity was reduced in the virions. Insertions of this type were also observed at approximately the same frequency in the circle junctions derived from infections with the M184I, A, and T mutants. The frequency of PPTs with long flanks was 0.01, 0.01, and 0.02, respectively.

If RNase H fails to correctly recognize the 3′ end of the PPT and cleaves within U3 to generate the 3′ end of the RNA primer, then the PPT primer is removed, there will be a small deletion in the 5′ end of U3. We analyzed the frequency of small deletions in U3 or U5 to determine if the 184 mutants incorrectly generate (and remove) the U3 primer. We previously showed that the RNase H primer grip mutants caused a dramatic increase in the frequency of 5-bp deletions in the U3 region (Julias et al., 2002); however, no similar increase in short deletions in U3 was observed with the M184V, M184I, M184A, or M184T mutants (data not shown).

Discussion

Treatment of HIV-1 with 3TC results in the emergence of M184I and M184V mutations in RT (Schinazi et al., 1993). These amino acid changes at the polymerase active site substitute a β-branched amino acid for methionine. During a single-cycle assay, the replication capacity of these variants was about 90% of wild type, and high-level resistance to 3TC was observed. Because this assay is limited to a single cycle of retroviral replication, we would not expect to see a dramatic effect on viral replication. If viruses containing these mutations were allowed to undergo several rounds of replication and the viruses had a replication capacity of 90% of the wild-type virus in each cycle of replication, a larger

effect would be seen. Although it is less sensitive to small changes in replication capacity, a single-cycle assay has the advantage that the exact number of replication cycles is known and reversion of the mutations is not a concern. The M184T mutation also replaces methionine with a β-branched amino acid; as expected, the virus is highly resistant to 3TC. However, the virus has a decreased replication capacity; the relative titer is about 30% of wild type. M184A replicates poorly and confers only minimal resistance to 3TC.

The analysis of viral DNA synthesis in cells infected with wild-type virus and viruses containing the M184T and M184A mutations was consistent with the results of the effects of these mutations on virus titer. Both mutations caused a decrease in the initiation of DNA synthesis. In contrast to valine and isoleucine, the β-branch of threonine contains a hydroxyl group (OH). The initiation of DNA synthesis is a difficult step for HIV-1 RT both in vitro and in vivo (Isel et al., 1996; Julias et al., 2002; Launchy et al., 2000). One possible explanation is that it is difficult for the enzyme to bend the RNA–RNA substrate used to initiate DNA synthesis to conform to the structures seen with RNA–DNA and DNA–DNA substrates. This would make the initiation step particularly vulnerable to changes that affect the binding of the nucleic acid or the regions around the polymerase and RNase H active sites. It is also possible that it is the presence of the hydroxyl group on the side chain of threonine that makes it more difficult for RT to appropriately extend an RNA–RNA duplex during the initiation of reverse transcription. The OH group may affect the positioning of the dNTP substrate or the template primer. The β-branched amino acids V and I narrow the sugar-binding pocket of HIV-1 RT, which blocks the incorporation of 3TCTP and reduces the incorporation of other nucleosides with L-sugars (Gao et al., 2000; Huang et al., 1998; Sarafianos et al., 1999). However, V and I affect the incorporation of normal dNTPs to a minimal extent. It is possible that the presence of the OH of threonine narrows the sugar pocket enough to interfere with the incorporation of normal dNTPs. However, there is no inherent problem with a threonine at this position in the active site of an RT: 3TC-resistant FIV has a T in the second position of the YXDD motif. Although alanine does not have a β-branch, the presence of alanine at position 184 affects polymerization and gives rise to low-level resistance to 3TC. 184 is close to both the sugar ring of the incoming dNTP and the template primer. An alanine at position 184 could affect the incorporation of dNTPs and 3TCTP either directly or by repositioning the template primer (Boyer and Hughes, 1995).

When M184V, M184I, and M184A mutants were analyzed in vitro, processivity defects were observed for the mutant RTs (Back et al., 1996; Back et al., 1997; Boyer and Hughes, 1995; Wei et al., 2003; Sharma and Crumpacker, 1999). The data we obtained with a one-round vector do not reveal any obvious elongation defects for these mutants during viral replication. Previously, when we made phenotypically mixed virions with reduced levels of polymerase activity, we saw defects at the level of the initiation of DNA synthesis and in some aspects of plus-strand DNA synthesis (Julias et al., 2001). Elongation was not measurably affected by limiting the amount of polymerase in the virion. It is possible that we did not observe elongation defects when the M184 mutations were tested using a one-round vector replicating in cultured cells because the cells were growing rapidly and presumably have relatively high levels of dNTPs. When HIV-1 infects primary cells, where the levels of dNTPs are lower, elongation defects are more pronounced and the rate of viral DNA synthesis is affected (Back et al., 1997).

There are reports that mutations at 184 affect virus replication (Miller et al., 1999; Newstein and Desrosiers, 2001). When E89G and M184V mutations were introduced into the RT of SIVmac, there was extensive reversion of the mutation at E89, but the M184V mutation persisted (Newstein and Desrosiers, 2001). The authors suggest that reversion of M184V to wild type was not observed because two nucleotide substitutions are needed for the reversion. The persistence of the M184V mutation in virus replicating in the absence of 3TC suggests that viruses carrying this mutation replicate relatively well, however the P272S mutation was present in a high proportion of the samples. It is possible that this mutation affected the replication capacity of the M184V virus. HIV-1 containing the M184V mutation has a decreased replication capacity in PBMCs (Miller et al., 1999); similarly, SIV with mutations at 184 shows reduced replication capacity (Van Rompay et al., 2002).

The emergence of M184I and M184V viruses during 3TC treatment is rapid, and the viruses are highly resistant to 3TC; however, the M184T virus, which is also 3TC resistant, is seen only rarely (Cho et al., 2002). Because the M184T virus is highly resistant to 3TC, the relatively low replication capacity of the M184T virus accounts for its rarity in patients. The M184A virus has a greatly reduced replication capacity and is only marginally resistant to 3TC; consequently, this virus has not been found in patients.

The observation that the M184T mutation, which is in the polymerase active site, not only affects DNA synthesis and drug resistance, but also RNase H cleavage, suggests that M184T affects the positioning of the nucleic acid during viral replication. The fact that M184T affects RNase H cleavage provides support for the idea that the M184T mutation affects DNA synthesis because it alters the positioning of the nucleic acid.

The enzymatic activities of HIV-1 RT (polymerase and RNase H) are interdependent. The isolated RNase H domain of HIV-1 RT has little enzymatic activity because the RNase H domain, when expressed separately from the polymerase domain, binds nucleic acid substrates poorly. The addition of part of the polymerase domain (the connection subdomain) restores RNase H activity (Smith et al., 1994). This indicates that the polymerase domain helps RNase H properly bind its

substrate. The interdependence is mutual. Mutations in the RNase H domain of HIV-1 can affect the initiation of DNA synthesis and mutations in the RNase H domain of MLV and HIV can decrease the fidelity of DNA synthesis during reverse transcription (Zhang et al., 2002). Because the active sites of polymerase and RNase H are separated by 17–18 nucleotides, the simple explanation is that amino acids in both domains play important roles in binding and positioning the nucleic acid relative to both of the active sites of RT. The proper positioning of the nucleic acid is critical for the precise RNase H cleavages that are needed to generate linear viral DNAs with correct ends. Analysis of the 2-LTR circle junctions from cells infected with mutants at position 184 in HIV-1 RT demonstrated that the M184T mutation affects the RNase H cleavages that generate the ends of the linear viral DNA during reverse transcription. This result indicates that polymerase active site mutations can affect RNase H activity; this strongly supports the notion that the two enzymatic activities of RT are interdependent.

Materials and methods

Vector construction

The construction of the parental vector pNLNgoMIVR$^-$E$^-$.HSA has been described previously (Julias, 2001). Briefly, this vector expresses the HIV-1 *gag-pol* gene, but the *env* gene is inactivated in the vector. Unique restriction endonuclease cleavage sites for *Sma*I and *Asp*718 are present in the pol coding region to facilitate the cloning of mutant RTs from previously described HIV-1 RT expression plasmids (Boyer et al., 1992). The murine heat stable antigen gene (CD24 or *hsa*) is expressed from the *nef* reading frame in this vector (Wegner et al., 1993). The *hsa* gene is flanked by *Xho*I and *Not*I restriction endonuclease cleavage sites. These sites were used to introduced the luciferase gene to generate pNLNgoMIVR-E-.LUC series of vectors.

Cells, transfection, and infection

The human embryonal kidney cell line 293 was obtained from American Type Culture Collection (ATCC). The human osteosarcoma cell line HOS was obtained from Dr. Richard Schwartz (Michigan State University, Lansing, MI). HOS and 293 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies) 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 luciferase protocol

293 cells were transfected with 2 μg of pNLNgoMIVR$^-$E$^-$.LUC DNA containing different mutations at position 184 in RT and 2 μg of pHCMV-g (obtained from Dr. Jane Burns, UCSD) using the calcium phosphate method. 293 cells were plated in 100-mm diameter dishes at a density of 1.8 × 10^6 cells per plate on the day before transfection; at this density, the cells are approximately 30% confluent on the day of transfection. The precipitate was added to 293 cells dropwise. Eight hours after transfection, the cells were washed once with 8 ml of Dulbecco's phosphate-buffered saline (PBS) (Gibco BRL/Life Technologies) and fresh medium was added. The medium was changed again 24 h after transfection. The 48-h supernatants were harvested, clarified by low-speed centrifugation, filtered through a Millex-GS 0.22-μm filter (Millipore), and used to infect HOS cells. The amount of p24 in the samples was determined using the HIV-1 p24 antigen capture assay kit (AIDS Vaccine Program, SAIC; Frederick, MD); this information was used to control for the amount of virus in the samples. The effects of 3TC (Moravek Biochemical) were determined by pretreating cells with 3TC for 4 h before the infection. The virus was allowed to adsorb to the cells for 2 h, then fresh medium (with 3TC) was added. Forty-eight hours after infection, the cells were washed once with 2 ml PBS, and then lysed using 0.5 ml of Glo-Lysis Buffer (Promega). The luciferase activity was determined using the Steady-Glo reagent system (Promega). The luciferase activity was normalized to the amounts of p24 antigen present in the supernatants and to the protein concentration of the lysate.

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

293 cells were transfected with 5 μg of wild-type pNLNgoMIVR$^-$E$^-$.HSA vector DNA or with DNA for vectors encoding amino acid substitutions V, I, T, or A at position 184 in RT and 3 μg of pHCMV using the calcium phosphate method. The 48-h supernatants were harvested, clarified by centrifugation, and filtered. Two microliters of virus-containing supernatant was used to infect HOS cells plated at a density of 1 × 10^6 cells per 100-mm diameter plate the day before infection. The supernatants were left on the cells for 4 h and fresh medium was added. Total DNA was isolated from 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 to the RU5 junction and a downstream primer that anneals to the U3 region of the LTR as previously described (Julias et al., 2002). The expected product is approximately 350 bp long and is flanked by *Eco*RI and *Xba*I sites that were introduced by the PCR primers. The PCR product was digested with *Eco*RI and *Xba*I and cloned into the cloning

plasmid SK (Stratagene). The 2-LTR circle junction clones were analyzed using restriction endonuclease digestion and DNA sequence analysis.

Determination of viral DNA copy number using real-time PCR

HOS cells receiving 3TC treatment were incubated with 20 μM 3TC (final concentration) for 16 h before infection and 24 h following infection. Transfections used to generate the virus, infections, and DNA isolation were performed as previously described (Julias et al., 2001). Quantitative real-time PCR used to determine viral DNA copy number was performed with the ABI 7700, 2× Universal Master Mix, and Taqman probe DNA primer sets as previously described (Julias et al., 2001).

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