Mechanistic Understanding of an Altered Fidelity Simian Immunodeficiency Virus Reverse Transcriptase Mutation, V148I, Identified in a Pig-tailed Macaque*

Tracy L. Diamond‡, George Sourroullas§§, Kellie K. Weiss‡, Kwi Y. Lee‡, Robert A. Bambara¶, Stephen Dewhurst‡‡, and Baek Kim***

From the ‡Department of Microbiology and Immunology, the ¶Department of Biochemistry and Biophysics, and §¶Cancer Center, University of Rochester, Rochester, New York 14642

We have recently reported that the reverse transcriptase (RT) of SIVMNE 170 (170), which is a representative viral clone of the late symptomatic phase of infection with the parental strain, SIVMNE CL8 (CL8), has a largely increased fidelity, compared with the CL8 RT. In the present study, we analyzed the mechanistic alterations of the high fidelity 170 RT variant. First, we found that among several 170 RT mutations, only one, V148I, was responsible for the fidelity increase over the CL8 RT. This V148I mutation lies near the Gln-151 residue that we recently found is important to the low fidelity of RT and the binding of incoming dNTPs. Second, we compared dNTP binding affinity (K<sub>0</sub>) and catalysis (k<sub>pol</sub>) of the CL8 RT and the CL8-V148I RT using pre-steady state kinetic analysis. In this experiment, the high fidelity CL8-V148I RT has largely decreased binding to both correct and incorrect dNTP without altering k<sub>pol</sub>. The fidelity increase imparted by the V148I mutation is likely because of the major reduction seen in RT binding to dNTPs. This parallels our findings with the Q151N mutant. Third, site-directed mutagenesis targeting amino acid residue 148 has revealed that a valine amino acid at this position is essential to RT infidelity. Based on these findings, we discuss possible structural impacts of residue 148 (and mutations at this site) on the interaction of RT with incoming dNTPs and infer how alterations in these properties may relate to viral replication and fitness.

One of the most unique enzymatic properties of lentiviral DNA polymerases (i.e. human and simian immunodeficiency virus (HIV-1 and SIV) reverse transcriptases (RT)) is their highly error-prone mode of DNA synthesis (1, 2). The anti-viral immune selection drives the viral diversity that may result from the fast replication of lentiviruses (3–5) and the efficient mutation synthesis catalyzed by lentiviral RTs (2). As recently reported, it is increasingly apparent that infidelity of lentiviral RTs provides the raw material for the viral genomic hyper-mutation that allows lentiviruses to efficiently evolve and escape from various anti-viral selective pressures (6). Unlike DNA polymerases involved in the cellular genomic replication process, lentiviral RTs lack the 3’ to 5’ proofreading nuclease activity, which would normally help prevent mutagenic DNA synthesis. However, this is not the only factor contributing to the low fidelity of lentiviral RTs because RTs of onco-retroviruses lacking the proofreading exonuclease activity, such as murine leukemia virus and avian myeloblastosis virus, have 10–18-fold higher fidelity than lentiviral RTs (7). This suggests that lentiviral RTs harbor DNA polymerase active sites with unique molecular characteristics responsible for their unfaithful DNA synthesis.

Kinetic studies with DNA polymerases suggest that mutation synthesis, which generates a mismatch at the 3’ end of newly synthesized DNA, interrupts processive DNA polymerization (8). HIV-1 RT is highly efficient in the incorporation of incorrect dNTPs (misinsertion). In addition, compared with other known DNA polymerases involved in genomic replication, HIV-1 RT more efficiently extends the mismatched primer created by a misinsertion (9). Possibly, the high capability of HIV-1 RT to extend a mismatched primer may compensate for the disruption of processive DNA synthesis caused by frequent misinsertion events during viral genomic replication by HIV-1 RT.

The interaction of DNA polymerases with incoming dNTPs is one of the key elements that determines DNA polymerase fidelity. Mutations in the dNTP binding domain, O helix, of pol DNA polymerase family members, such as the Klenow fragment of Escherichia coli DNA polymerase (10, 11) and the thermostable Taq polymerase (12–15), alter their fidelity. Mutations in several dNTP-binding residues of HIV-1 RT were also found to affect RT fidelity. For example, alterations in the Arg-72 residue of HIV-1 RT, which interacts with the triphosphate moiety of incoming dNTPs, increase RT fidelity; however, these alterations can dramatically decrease RT activity (16). In contrast, mutations in the Tyr-115 residue that interacts with base-sugar moieties of the incoming dNTP reduce RT fidelity (17, 18).

Kinetic parameters indicating the dNTP binding affinity and chemical catalysis of DNA polymerases have been determined under reaction conditions where DNA polymerase molecules prebound to the template-primer complex (T/P), incorporate a single nucleotide; this is referred to as the pre-steady state.
condition (8). We recently reported a pre-steady state kinetic study with another high fidelity HIV-1 RT mutant, Q151N. The Gln-151 residue, encoded in the β-α-β loop of RT, directly interacts with the 3’ OH of the incoming dNTP. Molecular modeling suggested that the Q151N mutation disconnects the interaction of the RT with the 3’ OH of the incoming dNTP. Our pre-steady state kinetic study demonstrated that the Q151N mutant specifically slows down the binding step (kₐ) for all incoming dNTPs (correct and incorrect) but not the chemistry step (kₐ). In fact, the binding of the Q151N mutant to incorrect dNTP was reduced to such an extent that we were not able to determine an accurate kₐ value (19). Therefore, this kinetic study suggests that the Gln-151 RT residue interacts with the 3’ OHs of both correct and incorrect incoming dNTPs, thereby providing support for the existence of a common mechanism involved in the binding of both correct and incorrect dNTPs.

Met-184 RT mutations of HIV-1 RT, M184V and M184I, are the only in vivo lentiviral RT mutations previously found to have moderately increased fidelity. These mutations were initially isolated from virus strains that exhibited resistance to a nucleoside RT inhibitor, 3TC. HIV-1 RTs containing the M184V and M184I mutations showed 1.8- and 3.5-fold increased fidelities, respectively, compared with wild type HIV-1 RT, as determined by the M13 lacZa forward mutation assay (20). A pre-steady state kinetic study with M184V RT showed reduced binding affinity to both correct and incorrect dNTPs (21). Structural studies have proposed that the β-branched side chain of the valine (or isoleucine) at position 184 in HIV-1 RT blocks the entry of incorrect dNTPs, including 3CTCP and incorrect dNTP, into the active site (22).

We found recently that an in vivo SIV RT variant, called SIVMNE 170 (170) RT, has an enhanced replication fidelity. SIVMNE 170 virus is a molecular clone isolated at the late stage of infection of a pig-tailed macaque (M57004) initially infected with a molecular clone of SIVMNE CL8 (CL8) (23, 24). The 170 RT has ~11-fold higher fidelity than the initial low fidelity CL8 RT. Sequence analysis revealed that the 170 high fidelity RT has six mutations in the DNA polymerase domain of RT, compared with the low fidelity CL8 RT. Identification of the 170 high fidelity variant suggests that RT fidelity may change during the course of viral infection. Indeed, the 170 RT is the first in vivo RT variant isolated during the course of natural infection (no antiretroviral drug treatment) with such an increase in fidelity (25).

In the present study, we investigated kinetic and structural properties of the high fidelity 170 RT protein. First, we identified a 170 RT mutation solely responsible for the fidelity increase of the 170 RT. Second, we determined the reaction step affected by the 170 high fidelity mutation by employing the pre-steady state kinetic assay. Third, we examined the effect of other types of mutations at the 148 residue of CL8 RT on RT fidelity. Finally, based on these kinetic and mechanistic analyses, we proposed a model for the structural impact made by the 170 high fidelity 170 RT mutation.
n-galactopyranosidase (X-gal) and isopropyl-1-thio-β-n-galactopyranoside with CHS950 lawn cells. The mutant frequency was determined as the ratio of mutant (pale blue and clear) plaques to mutant plus wild type (dark blue) plaques as described.

**Misincorporation Assay with DNA or RNA Templates—** Procedures were modified from those of Preston et al. (1). The RNA T/P was prepared by annealing a 40-mer RNA (5′-AAGCGGCGCCGCAAGAUUGCGCGAAGAUAUGCGCCGCG-3′, Dharmacon Research) to the 17-mer A primer (5′-CGCGGCGAATTCGCCCG-3′; template-primer, 2:5:1, Invitrogen) 32P-labeled at the 5′ end by T4 polynucleotide kinase (New England Biolabs). The RNA T/P was prepared by annealing a 40-mer DNA (5′-AAGCTTGGCTGCAGAATATTGCTAGCGG-3′), which encodes the same sequence as the 40-mer RNA template, to the 17-mer used for DNA template-preparation. Assay mixtures (20 µl) contained 10 mM T/P, RT proteins as specified in figure legends, or exon−T7 DNA polymerase (Sequenase version 2.0 DNA polymerase, U. S. Biochemical Corp.), 3 or 4 dNTPs (250 µM each), 25 mM Tris-HCl, pH 8.0, 100 mM KCl, 2 mM DTT, 5 mM MgCl2, 10 µM (dATP)20, and 0.1 mg/ml bovine serum albumin (New England Biolabs). Reactions were incubated at 37 °C for 5 min and terminated by 10 µl of 40 mM EDTA, 99% formamide. Reaction products were immediately denatured by incubating at 95 °C for 5 min and analyzed by electrophoresis in 14% polyacrylamide-urea gels.

**Extension of Mismatched Primers—** To measure the capability of RT to extend mismatched primer, we used two different mismatched primers annealed to either the RNA or DNA template used in misincorporation assay. 32P-labeled 16-mer G/T mismatched primer (5′-CGCGCGAATTCGCCCG-3′, mismatch underlined) or 19-mer C/A primer (5′-CGCGCGGCGATTCGCCCG-3′, mismatch underlined) was annealed to the 40-mer RNA or DNA templates (see above), respectively. For control matched primer, we used 32P-labeled C primer (5′-CGCGCGAATTCGCCCG-3′) annealed to the 40-mer RNA or DNA templates (see above). Extension conditions for this extension assay were the same as those used for the misincorporation assay above, except for the different T/Ps. The reactions were also analyzed by electrophoresis on a 14% denaturing sequencing gel.

**Pre-steady State Kinetic Assay—** Pre-steady state burst and single-turnover experiments were performed to examine the transient kinetics associated with a single nucleotide incorporating onto the 3′ end of a 32P-labeled 17-mer A primer (see above) annealed to a 40-mer RNA template (see above). We used 20 µl of T/P pre-incubated with purified SIV RT protein and reaction buffer (25 mM Tris-HCl, pH 8.0, 40 mM KCl, 2 mM DTT, 5 mM MgCl2, 0.1 mg/ml bovine serum albumin). This mixture was injected into one sample tube of the rapid quench machine (Kinetek). An equal volume of dNTP pre-incubated with Mg2+ (10 mM) was injected into the other sample tube. The polymerization reaction was initiated by rapidly mixing the two reagents and was terminated by adding 0.25 M EDTA at different time points.

In the pre-steady state burst experiments, T/P (150 nM) was present in excess over active RT (50 or 150 nM purified protein), and the reaction was initiated by addition of 800 µM dATP (correct). These experiments were used to determine the active site concentrations of the RT proteins (see data analysis Refs. 8 and 28). The pre-steady state single-turnover experiments were used to determine the dNTP concentration dependence of the purified SIV RT proteins. In the presence of various dNTP concentrations (in the range of 600 nM to 2 mM), active RT (100 nM) was used in slight excess of T/P (90 nM). In reactions involving incorrect dNTPs, the experiments were performed manually at longer time points and used a higher concentration of RT (700 nM) (10, 28).

**Product Analysis—** The reactions were analyzed by 14% denaturing sequencing gel electrophoresis. The extended product in each reaction was quantified by the Cyclone PhosphorImager (PerkinElmer Life Sciences).

**Data Analysis—** Pre-steady state kinetic data was analyzed using nonlinear regression. Equations were generated with the program Kaleidagraph version 3.51 (Synergy Software, Reading, PA). Data points obtained during the burst experiment were fit to the burst Equation 1,

\[
A \approx e^{-ktobs} + kobs \cdot [\text{product}]
\]

The value A is the amplitude of the burst, which reflects the actual concentration of enzyme that is in active form, \(k_{obs}\) is the observed first-order rate constant for dNTP incorporation, whereas \(k_{f+}\) is the observed steady state rate constant (8, 10, 28). Data from single turnover experiments were fit to a single exponential equation that measures the rate of dNTP incorporation (\(k_{obs}\)) per given dNTP concentration (dNTP). These results can then be used to determine \(k_{f+}\), the dissociation constant for dNTP binding to the RT-T/P binary complex. This was done by fitting the data to the following hyperbolic Equation 2,

\[
k_{obs} = k_{f+} \cdot [\text{dNTP}] / (Kf + [\text{dNTP}])
\]

A Naturally Occurring RT Fidelity Mutant

**TABLE I. Fidelity of SIVMNE RT proteins determined by M13 lacZα forward mutation assay**

| SIVMNE RT proteins | Total plaques | Mutant plaques | Mutant frequency (×10−5) | Fold decrease |
|--------------------|--------------|----------------|--------------------------|--------------|
| CL8 RT             | 7,920        | 141            | 17.8 ± 1.3               | ×8.2         |
| CL8-V148I RT       | 7,580        | 17             | 2.2 ± 0.07               | ×7.4         |
| 170 RT             | 9,240        | 22             | 2.4 ± 0.2                | ×7.4         |
| 170-1148V RT       | 6,750        | 130            | 19.5 ± 1.6               | ×9.2         |

* Numbers represent pooled totals from at least two independent fill-in reactions.
In addition, the other five SIVMNE 170 RT mutations (I73M, K173R, S211G, Y303F, and N332S) are unlikely to be involved in the fidelity increase. The CL8 K173R mutant RT, for example, had identical fidelity to CL8 RT by misincorporation assay (data not shown).

Next, we confirmed the effect of the V148I mutation on RT fidelity by testing whether reversion of the V148I mutation in the high fidelity SIVMNE 170 RT reduces its fidelity. We constructed and purified an SIVMNE 170 RT derivative containing five of the six SIVMNE 170 RT mutations except for the V148I mutation, called 170-I148V RT. As shown in Table I, the 170-I148V RT showed an 10-fold reduced fidelity, compared with the high fidelity 170 RT. In addition, similar low mutation frequencies were seen between the 170-I148V and CL8 RTs (Table I). Therefore, these results suggest that the V148I mutation is responsible for the high fidelity of the 170 RT.

Misincorporation and Mismatched Primer Extension of SIV RT Proteins—We also confirmed the effect of the Ile-148 and Val-148 residues on the fidelity of the CL8 and 170 RTs, respectively, using a primer extension assay that employs only three kinds of dNTPs; this is called a misincorporation assay (1). In this assay, DNA polymerases with low fidelity can easily misinsert at stop sites where missing dNTPs should be incorporated, resulting in the extension of the primer beyond the stop sites. Therefore, the more primer extension beyond the stop sites, the higher the misincorporation efficiency (lower fidelity) of the RT. As seen in Fig. 1, the CL8-V148I RT showed a lower level of primer extension beyond the stop sites than the CL8 RT. In addition, the 170-I148V RT showed higher primer extension beyond the stop sites than the 170 RT, thus confirming the importance of the 148 residue in contributing to the RT fidelity change.

As a control, we also performed this assay with RT and T7 DNA polymerase lacking 3’-5’-exonuclease activity (exo-T7 DNA polymerase), which show similar steady state kinetic incorporation of correct dNTP and similar dNTP binding affinity in pre-steady state kinetic assays (see Fig. 1C) (30). However, it has been shown that the exo-T7 DNA polymerase (31) discriminates correct and incorrect dNTPs much more efficiently than HIV-1 RT (32), suggesting that the exo-T7 DNA polymerase has higher fidelity than RT. As shown in Fig. 1C, the exo-T7 polymerase showed much less primer extension beyond the stop site than the CL8 RT, likely due to the difference in fidelity. If the primer extension beyond the stop site was due to the incorporation of contaminating correct dNTPs (i.e. dCTP in the minus dCTP reaction), these two polymerases
would show comparable primer extension beyond the stop sites because their steady state incorporation of correct dNTP is very similar. The inability of the exo- T7 DNA polymerase to extend past the first stop sites indicates that contamination of the missing dNTP under our reaction conditions is not an issue. Furthermore, other assays, including the M13 lacZa forward mutation assay, indicate that the differences seen in extension beyond the stop sites in our misincorporation assays using the V148I mutant are likely the result of a fidelity change.

Next, we tested the mismatched primer extension capability of these SIV RT proteins. In this assay, using the G/T- and C/A-mismatched primers, a high fidelity RT protein will show a lower level of mismatched primer extension. This assay analyzes fidelity at the second step of mutation synthesis, misextension. The G/T and C/A primers represent misextension after the most prevalent lentiviral mutation (G→A) during both first strand and second strand synthesis, respectively. We found that the CL8-V148I protein shows a reduced capability to extend the mismatched primer, compared with the CL8 RT (for detailed description see below and Fig. 7C). When this assay was completed with the 170 and 170-I148V RTs the 170-I148V RT showed an increased capability to extend the mismatched primer, compared with the high fidelity 170 RT (data not shown). Therefore, these data, together with the results of the M13 lacZa forward mutation assay, clearly revealed that the V148I mutation is responsible for the fidelity increase of the 170 RT obtained from the late stage of infection.

Initial Burst and Active Site Concentration of CL8 and CL8-V148I Proteins (Fig. 2)—Next, we investigated kinetic alterations made by the V148I mutation employing the single nucleotide incorporation assay under pre-steady state conditions. This assay determines the dNTP binding affinity \( (K_d) \) and chemical catalysis rate \( (k_{pol}) \) of DNA polymerases after defining the amount of active DNA polymerases bound to T/P and capable of dNTP incorporation \( (8) \). First of all, we determined the active site concentrations of the purified CL8 and CL8-V148I RT proteins. For this, the single nucleotide (correct dATP) incorporation reaction with RTs prebound to T/P was performed using a T/P concentration in molar excess over the enzyme concentration. As shown in Fig. 2, we observed both fast single turnover events associated with the pre-steady state burst, and slow multiple rounds of DNA polymerization occurring during steady state kinetics. Reactions ranging from 10 ms to 2 s were performed using the rapid quench machine.

We used 150 nM of purified CL8 or CL8-V148I protein and 800 μM dATP in these initial burst experiments. By using Equation 1 (see "Materials and Methods ", we were able to determine the active site concentration, the rate of dNTP incorporation under pre-steady state conditions \( (k_{obs}) \), and the rate of dNTP incorporation during the steady state \( (k_{ss}) \) for both proteins. We calculated that 52 nM (35%) of CL8 RT and 30 nM (20%) of CL8-V148I was actually active in the 150 nM purified proteins (Fig. 2). These calculated active site concentrations of two SIV RT proteins were used to determine their dNTP binding and chemical catalysis parameters (see below). For the CL8 RT, \( k_{obs} \) was \( 14 \times 10^{-3} \) ms\(^{-1} \) and \( k_{ss} \) was \( 0.24 \times 10^{-2} \) ms\(^{-1} \). For the CL8-V148I protein, the values for \( k_{obs} \) and \( k_{ss} \) were \( 16 \times 10^{-3} \) and \( 0.36 \times 10^{-2} \) ms\(^{-1} \), respectively (Fig. 2).

The faster reaction rates calculated for \( k_{obs} \) compared with \( k_{ss} \) have been noted previously \( (8, 32) \) and suggest that reaction rates are increased in pre-steady state kinetic conditions \( (k_{ss}) \) over steady state conditions \( (k_{obs}) \). Hypothetically, the steady state reaction rate is slower due to a number of rate-determining steps (i.e. T/P binding and product release) that occur during multiple rounds of replication.

Pre-steady State Incorporation of Correct dATP by SIVMNE RT Proteins (Table II and Fig. 3)—Once we determined the active site concentrations of CL8 and CL8-V148I RT, we performed experiments to determine the binding affinity \( (K_d) \) of the RTs for correct dNTP (dATP) and their maximum rate of incorporation of correct dNTP onto the T/P \( (k_{pol}) \). We were able to calculate \( K_d \) and \( k_{pol} \) by analyzing the dependence of reaction rate \( (k_{obs}) \) on dNTP concentration (Fig. 3 and Equation 2, see "Materials and Methods "). We and others reported \( (19, 21, 28) \) that the \( K_d \) value of wild type HIV-1 RT to correct dNTPs is on the order of 1–10 μM, which is slightly higher than that of the CL8 RT. We found that the CL8-V148I RT had a 700-fold increase in \( K_d \) over CL8 RT, whereas the presence of the V148I mutation had little effect on the maximum rate of correct dATP incorporation \( (k_{pol}) \). Table II, suggesting that this mutation specifically alters the interaction of RT with incoming dNTP. This significant change in binding affinity translates to an almost 2000-fold reduction in the pre-steady state incorporation efficiency \( (k_{pol}/K_d) \) of CL8-V148I relative to CL8 RT (Table II). Our recent study with the Q151N HIV-1 RT high fidelity mutant reported that the Q151N mutation also affects only the binding of correct dNTP with little effect on \( k_{pol} \). CL8-V148I RT, however, appears to have a larger reduction in the effi-
iciency of incorporation of correct dATP than the HIV-1 Q151N RT mutant when they are compared with their wild type RTs on the same template-primer (19).

As shown in Fig. 3, although CL8 RT is able to polymerize at rates close to its maximum reaction rate \( k_{\text{pol}} \) even at low dNTP concentrations (i.e. 2.5 \( \mu M \)), the reactions with CL8-V148I RT are much slower, even at 100 \( \mu M \) dNTP. This result suggests that the dNTP binding of the CL8-V148I protein becomes a rate-limiting step in the DNA polymerization reaction at these low dNTP concentrations.

Pre-steady State Incorporation of Incorrect dCTPs by SIVMNE RT Proteins (Table I and Fig. 4) — Next, we measured the kinetic parameters associated with the incorporation of an incorrect dNTP (dCTP). These reactions were performed manually at high concentrations of RT and incorrect dCTP because incorporation of incorrect dNTPs is very slow and inefficient (see “Methods and Materials” (21)). As shown in Table II, the CL8 RT showed a 220 times lower binding to incorrect dCTP than to correct dATP. In addition, \( k_{\text{pol}} \) of the CL8 RT to incorrect dCTP was 1800 times lower than that to correct dATP. In our recent study with identical template and primer, wild type HIV-1 RT showed 150 times lower binding and 2600 times lower \( k_{\text{pol}} \) with incorrect dCTP than with correct dATP (19), suggesting that wild type HIV-1 RT and CL8 RT have very similar misincorporation kinetic values under pre-steady state conditions.

CL8-V148I RT was very inefficient at incorporation of incorrect dCCTP at every dCTP concentration tested. Because the values for \( k_{\text{pol}} \) and \( K_d \) are determined by assessing the dependence of reaction rate on dNTP concentration (Equation 2), and the reaction rates with CL8-V148I RT and dCTP (up to 2 mM) were linear with respect to dNTP concentration (Fig. 4), we could not make these calculations accurately. Misincorporation of dCTP was much lower in reactions with CL8-V148I RT than with CL8 RT (Fig. 4A). Although saturating concentrations of dCTP are greater than 2 mM, higher dNTP concentrations (>2 mM) could not be used because they inhibit RT activity (reducing \( k_{\text{obs}} \)) (31). We were, however, able to estimate a minimum \( k_{\text{pol}} \) value \( k_{\text{obs}} \) at 2 mM incorrect dNTP. 0.01 \( s^{-1} \) for the CL8-V148I RT with incorrect dCTP is apparently higher than 2 mM. Therefore, we were not able to obtain a true value for CL8-V148I misincorporation efficiency \( k_{\text{obs}}/K_d \). Our study with the Q151N HIV-1 RT mutant also demonstrated that the binding affinity of the Q151N protein to the incorrect dNTP was so severely reduced that we could not accurately determine the pre-steady state kinetic parameters of the Q151N protein (19). The alterations in \( K_d \) for incorrect dNTP seen with the CL8-V148I and Q151N HIV-1 RTs compared with CL8 and HIV-1 RTs, respectively, is likely responsible for the fidelity increases observed in the misincorporation and M13 lacZa forward mutation assays.

\[(\text{dNTP})\text{-dependent Primer Extension Capability of CL8, CL8-V148I RT Proteins (Fig. 5) — Because the pre-steady state kinetic data described above demonstrated that, due to the reduced dNTP binding affinity, the dNTP binding of the CL8-}

\[\text{RT + T/P}_{n} \rightleftharpoons \text{RT} \cdot \text{T/P}_{n} \rightleftharpoons \text{RT} \cdot \text{dNTP} \rightleftharpoons \text{RT}\text{-T/P}_{n+1} \cdot \text{PP}_{i} \rightleftharpoons \text{RT} + \text{T/P}_{n+1} + \text{PP}_{i} \]
V148I becomes a rate-limiting step, especially at the lower dNTP concentrations tested (i.e., 2.5 μM, where CL8 RT is still near maximal reaction rate; Fig. 3), we tested the effect of the dNTP concentration on the multiple rounds of DNA synthesis catalyzed by the CL8 and CL8-V148I SIVMNE RT proteins. The 17-mer primer A, bound to the 40-mer RNA template, was extended by the CL8 and CL8-V148I RT proteins in the presence of various concentrations of all four dNTPs. An active RT concentration (12.5 nM CL8 RT or 25 nM CL8-V148I RT) giving 50% primer extension at 1 mM dNTP under these experimental conditions was used in this assay. As shown in Fig. 5, the CL8 RT maintained a high rate of primer extension ability even at a concentration of 250 nM of each dNTP, whereas the CL8-V148I RT showed dramatically decreased productive DNA synthesis when the dNTP concentration was reduced beyond 1 μM. These data demonstrate that the V148I mutation reduced the capability of the RT for efficient primer elongation under conditions with reduced dNTP concentration. This could be the result of the large decrease in the dNTP binding affinity caused by the V148I mutation. Therefore, the dNTP binding step became a rate-limiting step during the multiple rounds of primer extension catalyzed by the CL8-V148I RT. Similarly, our studies of the Q151N HIV-1 RT mutant with the same primer extension reactions also revealed a reduced capability for productive DNA synthesis at low dNTP concentrations, compared with wild type HIV-1 RT.3

AZTTP Sensitivity of CL8 and CL8-V148I RT Proteins (Fig. 6)—We and others (33, 34) previously demonstrated that the Q151N mutation mildly decreases the sensitivity of HIV-1 RT to AZTTP, a chain terminator used for inhibiting RT activity and viral replication. We therefore examined the AZTTP sensitivity of the CL8 and CL8-V148I SIV RT proteins. The A primer annealed to the 40-mer RNA template was extended by RT proteins in the presence of all four dNTPs and different concentrations of AZTTP. In this assay AZTTP inhibited CL8 RT more significantly at lower concentrations of drug than it inhibited the CL8-V148I RT. This suggests that, like the Q151N mutation, the V148I mutation also mildly decreases AZTTP sensitivity.

Random Site-directed Mutagenesis of Residue 148 of the CL8 RT—Next, we performed random site-directed mutagenesis of residue 148 of the CL8 RT and were able to construct 11 different predicted amino acid substitutions at this position. Among these mutant RT proteins, 10 were obtained at protein purities higher than 90% (and thus were suitable for use in biochemical analyses). These are as follows: Ala-148, Ser-148, Cys-148, Thr-148, Leu-148, Arg-148, His-148, Lys-148, and Ile-148. The Ala-148, Ser-148, Cys-148, Thr-148, Leu-148, Arg-148, His-148, and Ile-148 RT mutants all showed high specificity activity (30–100% of the CL8 RT), whereas the Leu-148, His-148, and Lys-148 mutants showed largely reduced activity. We compared the misincorporation capability of the 10 active residue 148 CL8 mutants using both the misincorporation assay with biased dNTP pools and the mismatched primer extension assay. As seen in Fig. 7B, the 148 mutants shown have reduced primer extension beyond the stop sites in the misincorporation assay (similar results were also observed with the five mutants not represented in this figure; data not shown). The same effect was seen when the mismatched primer extension assay was performed on the five mutants (Fig. 7C). These data suggest that the Val residue at position 148 is important for maintaining primer extension beyond

2 K. K. Weiss, R. Chen, K. Y. Lee, R. A. Bambara, L. M. Mansky, and B. Kim, unpublished data.
Effect of V148I Mutation on HIV-1 RT—Misincorporation and mismatched primer extension assays were also performed to compare the fidelity of HIV-1 RT and HIV-1 RT containing the V148I mutation. As seen in Fig. 8, primer A was bound to an RNA template, and extension was completed by each of the RTs in the presence of only three of the four natural dNTPs. It is evident that there is significantly more extension past the first stop site when extension is mediated by the wild type HIV-1 RT than when it is performed by the RT containing the V148I mutation. In addition, similar results were seen when this assay was performed on a DNA template or the two RTs were used in the mismatched primer extension assay. Altogether, the results of these assays parallel the findings obtained in the extension assays with the CL8 and CL8-V148I RTs.

**Fig. 6. AZTTP sensitivity assay.** The $^{32}$P-labeled 17-mer primer annealed to the 40-mer RNA template was extended by SIVMNE CL8 and SIVMNE CL8-V148I RT proteins showing the same levels of the primer extension with all four dNTPs (no drug) at 37 °C for 5 min. The extension reactions were performed in the presence of four dNTPs ($20 \mu M$ dGTP, dATP, dCTP, and 0.6 $\mu M$ TTP) and increasing concentrations of AZTTP. The reactions were analyzed by PhosphorImager quantification of a 14% polyacrylamide-urea denaturing gel. The fraction full-length at each concentration of AZTTP was calculated by measuring the ratio of full-length product to the amount of product in the entire lane; each data point is derived from two separate reactions.

**Fig. 7. Misincorporation and mismatched primer extension assays with the SIVMNE CL8 V148X RT proteins.** A, the $^{32}$P-labeled 17-mer primer ($S$: A primer) annealed to 40-mer RNA template was extended by two quantities of SIVMNE RT proteins ($4 \times$ (lane 1) and $1 \times$ (lane 2)) showing the same levels of the primer extension with all four dNTPs at 37 °C for 5 min. B, the extension reactions were also performed by the $4 \times$ and $1 \times$ quantities of the RT proteins in the presence of only three complementary dNTPs (minus TTP). The sequence of the extended part of the primer is shown. In this misincorporation assay, the lower fidelity RT generates more extended products beyond the first stop site (*), which corresponds to the site before the missing dNTP would normally be incorporated. C, the $^{32}$P-labeled mismatch primer $GT$ bound to the RNA template was also extended by the SIV RTs in the presence of all four natural dNTPs. In this assay, low fidelity RTs are expected to generate more products that have extended past the mismatched primer ($M$). The reactions were analyzed by 14% polyacrylamide-urea denaturing gel electrophoresis. $1 \times$ concentrations of SIVMNE RTs are as follows: CL8 RT, 12.5 nM; CL8-Ala-148 RT, 41.8 nM; CL8-Ser-148 RT, 31.3 nM; CL8-Cys-148 RT, 25 nM; CL8-Thr-148 RT, 20 nM; and CL8-Ile-148, 25 nM. The single letter amino acid codes are used to depict each mutant. $F$, fully extended products; $S$, un-extended primer.

the stop site, which is indicative of low fidelity.
fidelity of the parental high fidelity 170 RT.

In order to investigate the kinetic effects of the V148I high fidelity mutation, we employed a pre-steady state kinetic assay and determined kinetic parameters for the CL8 and CL8-V148I proteins, $K_d$ and $k_{pol}$, that indicate dNTP binding affinity and chemical catalysis of RT, respectively. As shown in Table II, SIVMNE CL8 RT exhibited a $K_d$ of 0.5 μM for the correct dNTP (dATP). As reported by us and others, HIV-1 RT has a $K_d$ of 1–10 μM for correct dNTPs (19, 21, 28). In addition, like HIV-1 RT, SIVMNE CL8 RT also has a lower $K_d$ to incorrect dNTPs (dCTP), which indicates that the tight binding of lentiviral RTs to incorrect dNTPs may contribute to the unique low fidelity of these RTs. $k_{pol}$ values of the CL8 RT to both correct and incorrect dNTPs were also similar to those published for HIV-1 RT. It was reported previously (8) that both tight binding (low $K_d$ with incorrect dNTP) and efficient incorporation (high $k_{pol}$ with incorrect dNTP) contribute to the low fidelity of HIV-1 RT. This also appears to be the case for SIV RT.

In contrast to the CL8 RT, the CL8-V148I RT showed a largely reduced binding affinity (700-fold) to the correct dNTP (dATP, Table II and Fig. 3). However, the $k_{pol}$ values of both CL8 and CL8-V148I RT proteins were similar. These data suggest that the V148I mutation affects mainly the dNTP binding step, but not the chemical reaction step, during the DNA polymerization reaction. As shown in Fig. 3, the reaction rate of the CL8-V148I RT was significantly slower than that of the CL8 RT at dNTP concentrations where CL8 RT is still at its maximum reaction rate (i.e. 2.5 μM). This suggests that the dNTP binding affinity becomes a rate-limiting step in the reaction with the CL8-V148I protein at low dNTP concentrations. In our recent kinetic study, we also observed that the Q151N mutation exclusively affects the dNTP binding affinity ($K_d$) of HIV-1 RT (19). As shown in Fig. 4A, the CL8-V148I RT had much lower incorporation of incorrect dNTP (dCTP), compared with the CL8 RT. The reaction rate of the CL8-V148I RT increased in a linear fashion, up to 2 mM dCTP. In addition, the incorporation rate started decreasing at dCTP concentrations higher than 2 mM, which is likely due to the inhibitory effects of high dNTP concentrations, as reported previously (31). Although the $K_d$ of the CL8-V148I RT for an incorrect dNTP (dCTP) cannot be accurately determined, it is clear that it is much higher than 2 mM. Because the reaction rate of the V148I, protein with an incorrect dNTP (dCTP) still continues to increase at 2 mM dCTP (Fig. 4), it is possible that the maximum rate of catalysis with incorrect dNTPs is not significantly altered between the two proteins. Similar data with incorrect dNTPs were observed in our recent study (19) with the Q151N HIV-1 RT mutant. Collectively, these data suggest that the binding affinity of the CL8-V148I to incorrect dNTP must be greatly reduced. This reduction of binding to the incorrect dNTP may be the key element for the high fidelity effect of the V148I mutation.

Because the pre-steady state kinetic analysis demonstrated that the dNTP binding step (to correct dNTP) becomes a rate-limiting step of the DNA synthesis reaction at lower dNTP concentrations (Fig. 3), we tested whether the V148I mutation affects the steady state DNA synthesis capability of the CL8 RT at low dNTP concentrations. As seen in Fig. 5, this indicates not only that CL8-V148I RT is dependent on high levels of dNTPs for multiple rounds of DNA synthesis but also that CL8-V148I RT has reduced primer extension ability compared with the CL8 RT at low dNTP concentrations. These observations indicate that the V148I mutation makes the dNTP binding step a rate-limiting step during DNA polymerization only at low dNTP concentrations. Unlike at low dNTP concentrations, the dramatic pre-steady state kinetic alteration made by the V148I mutation with correct dNTP (Table II) does not contribute to steady state DNA synthesis at high dNTP con-
These findings suggest that the ability of viruses containing the V148I RT mutant to replicate productively may depend on the cell type being infected. For example, replicating cells (i.e. activated T cells and transformed cell lines) have high cellular dNTP concentrations compared with resting cells (8, 28) are rate-limiting. This assay was also performed with the 170 and 170-1148V RTs, and the extension levels were identical to the CL8-V148I and CL8 RTs, respectively.

These results again parallel what is seen in studies of the SIV-1 Q151N mutant RT and lend further credence to the contention that Val-148 and Gln-151 residues regulate RT fidelity in a similar manner.

As discussed earlier, two different mechanistic steps, misinsertion and mispair extension, contribute to mutation synthesis. In fact, it has been shown that the misinsertion step, which consists of dNTP binding (Kd) and catalysis (kcat), simlar to the misinsertion step, is very efficient for HIV-1 RT, contributing to its low fidelity (9). Because our kinetic data showed that the V148I mutation significantly reduces dNTP binding during matched primer extension, it is a reasonable prediction that the reduction of the dNTP binding affinity is likely to affect the mispair extension step as well. This prediction is supported by our preliminary data that in transformed cell lines, with high concentrations of dNTPs, there is not a significant difference in the replication rates of SIV containing the CL8 or CL8-V148I RT during multiple rounds of infection.

Finally, we examined the structural location of the conserved Val-148 residue using the HIV-1 RT crystal structure (35) (Fig. 9), which has a high degree of amino acid sequence identity and an identical number of amino acids to SIV RT. The conserved

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Val-148 residue is located near the border between the fingers and palm domain of RT and does not directly interact with the RT substrates, such as the incoming dNTP or template-primer (TP). Furthermore, this 148 residue is relatively distant from the DNA polymerization catalytic center. Interestingly, as shown in Fig. 9, which was made using the PyMol Molecular Graphics System, the Val-148 residue (blue) is present in the same local domain, 98, with the Gln-151 residue (red) that interacts with the 3' OH in the sugar moiety of the incoming dNTP (purple) (35). We demonstrated previously that the Q151N mutation largely enhances RT fidelity, suggesting that the interaction between the Gln-151 residue and the incoming dNTP plays an important role in regulating the incorporation of an incorrect dNTP and mutation synthesis (19, 34). Possibly, the V148I mutation mimics the role of the Q151N high fidelity mutation inducing the loss of the interaction of RT with the 3' OH in the sugar moiety of the incoming dNTP. More specifically, as shown in Fig. 9, the side chain of the Val-148 residue (blue) makes contact with the peptide backbone between residues Ser-117 (yellow) and Val-118 (orange) of RT. Therefore, the high fidelity V148I mutation, containing a longer side chain than the wild type Val-148, may push the 98 region of RT containing the Gln-151 residue away from the dNTP-binding site (see dashed arrow in Fig. 9). As we noted previously, when the Q151N mutant RT incorporates an incorrect dNTP both the interaction between the RT 151 residue and the 3' OH of the incoming dNTP, and base-pairing between the template nucleotide and the incoming incorrect dNTP are absent. Similarly, the structural changes in RT that are produced by the V148I mutation may result in disconnection of the side chain of the Gln-151 residue from the 3' OH of the incoming dNTP (see dashed arrow in Fig. 9). This may destabilize the binding of the incoming incorrect dNTP and reduce the efficiency of misincorporation (thereby increasing RT fidelity). However, it is unlikely that the V148I mutation induces a large structural change in the RT active site because both CL8 and CL8-V148I RT proteins showed similar active site concentrations (30–40%) at a given RT protein concentration (Fig. 2). As shown from our site-directed mutagenesis (Fig. 7), not only the V148I original mutation but also other residue 148 mutations with side chains that are smaller than the wild type Val sequence increased CL8 RT fidelity. Presumably, these alterations in the side chain length of residue 148 (see arrow in Fig. 9) may contribute to the destabilization of the Gln-151 interaction with the incoming dNTP. The fidelity of other residue 148 mutations with charged (i.e. V148K) and bulky (i.e. V148H) also increased RT fidelity (data not shown). This suggests that the wild type Val residue is critical for maintaining SIV RT fidelity by positioning the Gln-151 residue to bind to incorrect dNTP. It is possible that mutation of residue 148 from a Val to any other amino acid shifts the position of residue 151 so it is either too far or too close to the 3' OH of the incoming dNTP. Confirmation of these predictions would have to be done using crystallographic structure studies. Furthermore, as shown in Fig. 8, the V148I mutation identified in SIV RT also increased the fidelity of HIV-1 RT. Therefore, it is likely that the Val-148 residue also contributes to error-prone DNA synthesis by HIV-1 RT.

It remains to be determined whether the V148I mutation decreases the generation of new viral mutants during the course of viral infection. First, we were able to observe the production of infectious viruses containing only the V148I mutation in both SIV and HIV-1 backgrounds (data not shown). As discussed above, the V148I mutation reduced polymerization only about 5-fold in the multiple round incorporation experiment (Fig. 5), which is likely to be the case for viral replication process. As described previously, the induced fit conformation change step (RT-dNTP-TP* → RT*-dNTP-TP), not the dNTP-binding step, is a rate-limiting step during the pre-steady state kinetic single dNTP incorporation (8, 37). Furthermore, the other rate-limiting steps such as TP binding and product release play significant roles in the multiple round process of dNTP incorporation (8). Due to minimal contribution of the dNTP binding step (Kd) in the overall processive DNA synthesis, the 700-fold reduction of Kd might lead to the small reduction of the multiple round DNA synthesis (Fig. 5) and allowed the production of infectious viruses containing only the V148I mutation.

In addition, our preliminary studies using a pseudotyped virus-based mutagenesis assay suggest that a HIV variant containing the V148I mutation exhibits reduced mutation frequency. This suggests that HIV or SIV variants that contain the V148I mutation with decreased binding to incorrect dNTP may have a reduced capability to produce new viral genomic mutations. This could conceivably have biological significance in vivo, in light of the fact that the V148I containing SIV RT (SIVMNE 170) was detected only at the late stage of virus infection, when host immune function was significantly impaired. The fidelity increase could be related to the previously described inverse correlation between lentivirus population diversity and host immune function (38). It is possible that high fidelity RT mutations such as V148I only occur during the late stage of immunodeficiency virus infections, when the virus no longer needs to generate large populations of new viral mutants, in order to evade host immune responses. In addition, as discussed above for CL8-V148I RT the binding affinity for correct dNTP may also play a role in the appearance of the V148I mutation late in infection. Further studies will be required to address these hypotheses and to explore more fully the pathobiological significance of high fidelity lentivirus mutants.

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Tracy L. Diamond, George Souroullas, Kellie K. Weiss, Kwi Y. Lee, Robert A. Bambara, Stephen Dewhurst and Baek Kim

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