Insertions into the β3-β4 Hairpin Loop of HIV-1 Reverse Transcriptase Reveal a Role for Fingers Subdomain in Processive Polymerization*

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Yvonne Kew† ‡‡, Laurence R. Olsen¶§, Anthony J. Japour¶, and Vinayaka R. Prasad¶**

From the Departments of ¶Microbiology & Immunology and §Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461 and ¶Beth Israel Hospital, Boston, Massachusetts 02215

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) displays a characteristic poor processivity during DNA polymerization. Structural elements of RT that determine processivity are poorly understood. The three-dimensional structure of HIV-1 RT, which assumes a hand-like structure, shows that the fingers, palm, and thumb subdomains form the template-binding cleft and may be involved in determining the degree of processivity. To assess the influence of fingers subdomain of HIV-1 RT in polymerase processivity, two insertions were engineered in the β3-β4 hairpin of HIV-1NL4-3 RT. The recombinant mutant RTs, named FE20 and FE103, displayed wild type or near wild type levels of RNA-dependent DNA polymerase activity on all templates tested and wild type or near wild type-like sensitivities to dideoxy-NTPs. When polymerase activities were measured under conditions that allow a single cycle of DNA polymerization, both of the mutants displayed 25–30% greater processivity than wild type enzyme. Homology modeling the three-dimensional structures of wild type HIV-1NL4-3 RT and its finger insertion mutants revealed that the extended loop between the β3 and β4 strands protrudes into the cleft, reducing the distance between the fingers and thumb subdomains to ~12 Å. Analysis of the models for the mutants suggests an extensive interaction between the protein and template-primer, which may reduce the degree of superstructure in the template-primer. Our data suggest that the β3-β4 hairpin of fingers subdomain is an important determinant of processive polymerization by HIV-1 RT.

The human immunodeficiency virus type 1 (HIV-1)1 life cycle is dependent on the functions of the virally encoded polymerase, reverse transcriptase (RT). In addition to its being a target for drug development, HIV-1 RT is unusual in its structural features, versatile use of both RNA and DNA templates, high error rates (1–3), and poor processivity (4). HIV-1 RT is a heterodimer containing 66- and 51-kDa polypeptides, termed p66 and p51 (5, 6). The p51 is generated by the proteolytic cleavage of the p66 subunit (7, 8). Because of its likeness to a right hand, the subdomains of HIV-1 RT are named fingers, palm, and thumb which are joined to the RNase H domain via a connection subdomain (9, 10). The fingers, palm, thumb, and connection subdomains of p66 constitute the polymerase domain of RT and form a cleft for template-primer binding and for the polymerase active site (9–12). The p51 subunit lacks the RNase H domain, and its three-dimensional structure, in contrast to that of p66, is more globular with no cleft (13).

The precise roles played by the various subdomains in the polymerase function of HIV-1 RT are unknown. The palm subdomain contains the catalytic triad of aspartates and therefore plays a key role in catalysis (9, 10, 14). In the three-dimensional x-ray crystal structures of the apo-RT and of RT complexed with template-primer or a non-nucleoside inhibitor, the thumb subdomain has been shown to occupy different positions with respect to the fingers (9–12). Based on these observations, and the fact that the thumb domain intimately interacts with the template-primer (10), it is proposed that the thumb subdomain mediates the translocation of the enzyme along template (12). The palm, thumb, and fingers subdomains are all thought to contact the template-primer, and together they constitute the template-primer cleft of HIV-1 RT.

To understand the role of the fingers subdomain of HIV-1 RT on the polymerase processivity, we engineered two gross alterations into the β3-β4 hairpin creating insertions within the flexible loop connecting the β strands. Both mutants were characterized for polymerase activity, sensitivity to nucleotide triphosphate analogs, kinetic constants, and finally processivity. The results indicate that the fingers subdomain plays an essential role in determining the processivity of polymerization by HIV-1 RT. Modeling the three-dimensional structure of RT with the extended fingers provided insight into the possible role of the β3-β4 hairpin in increasing the processivity of RT.

EXPERIMENTAL PROCEDURES

Bacteria and Plasmids—The Escherichia coli strain DH5αF’IQ (Life Technologies, Inc.) was used for expression of HIV-1 RT. The sequences of the RT employed in this study were derived from the molecular clone NL4-3 (15). The expression vector used is pRT6H-NB/PROT containing an RTkR362 p66 cassette and a separate HIV-1 PR expression cassette (16) containing NotI at the 5′-end. pl66-PROT is a version of pRT6H-NP/PROT in which the RT sequences are replaced by a polylinker sequence.

Construction of RT Mutants—First, the RTkR362 sequences of pRT6H-NP/PROT were replaced with corresponding sequences from NL4-3 via polymerase chain reaction followed by digestion of the products by NotI and BgII and ligation of the fragment into the corresponding sites in pl66-PROT. The deletion and insertion mutants were created by cassette mutagenesis as described previously (17, 18). Briefly, a gap was

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created in RT fingers region lacking the codons 60–79 to facilitate both insertions and deletions into the region. This intermediate clone was digested with BspMI to release the central fragment, and double-stranded adaptors were ligated to the BspMI sites to generate the deletion and insertion mutants (Fig. 1). The mutant RTs were analyzed by subcloning the RT, NotI/BglII insert into the expression construct, pL6H-PROT, which places a hexahistidine tag at the carboxyl terminus of p66.

**Bacterial Expression, Lysis, and Purification of RTs—**Bacteria carrying the appropriate expression vector were induced for expression by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (Sigma) as described previously (19). The pellets were resuspended in 0.5 ml of lysis buffer with lysozyme (1 mg/ml, for 20 min on ice), sonicated, and lysates cleared by centrifugation at 15,000 rpm (Sorvall SS-34 rotor). The purification of the finger extension mutants was essentially similar to the procedure we used previously (19), except that all binding, washing, and elutions were done by the batch method. The specific activities of the wild type, FE20, and FE103 RTs were 3030, 1515, and 3333 units/mg, respectively (1 unit is defined as the activity equivalent to incorporation of 1 mmol of dTMP in 10 min at 37 °C using poly(rA)oligo(dT)).

**RNA-dependent DNA Polymerase Assays—**The RNA-dependent DNA polymerase (RDDP) assays on heteropolymeric templates were performed in a 16-mM reaction mix containing 50 mM S rRNA template-22-mer primer (5′AGTGGATTGGTTGTTGTCACCAT3′) and 1.5 units of RT enzymes, and 10 nM ddNTPs. The ddNTP concentration in each case (Boehringer Mannheim) was at 5 μM, while the template residues 12–15, and all primer residues were fixed. Additional substitutions were incorporated into the model to change the HXB2 sequence used for the x-ray structure determination to the wild type NL4-3 sequence used here. Residues that were modeled as alamines in the crystal structure because of weak electron density were also changed to conform to the actual wild type NL4-3 RT sequence. The loop in question was subcloned into FE20 and FE103 and the coordinates were transferred to the model of wild type NL4-3 RT using the loop-building function in the Homology package. The models of the three proteins, which included all atoms of their respective residues (including the inserted residues), were minimized using steepest descents with a CVFF forcefield in two steps using the Discover package (Bioystm). In the first minimization, all residues present in the original crystalllographic coordinates were constrained, and in the second, all non-hydrogen atoms were fixed. Next, a model of A-type DNA was made using the Builder package (Bioystm). This model includes the 5 base pairs of A-form DNA that were positioned based on the phosphorous positions available from the RT-DNA co-crystal coordinates (1HMI) (10). In addition, a 10-base overhang in the template strand was modeled assuming that the single-stranded extension maintained an A-type conformation. Although the phosphate backbone of the single-stranded region is flexible, it is plausible that this DNA is in the A-form and such an assumption has been made by others (22) attempting to model template extensions. The sequence of the template strand is thus 5′-AAAAAATGGCC-3′. The overhang modeled is not in the original coordinates. The double-stranded region positioned based on the coordinates of RT bound to the template. For the calculations, residues involved in the interactions were fixed throughout the minimization and dynamics calculations served as an anchor for the single-stranded region. Each protein-DNA model was minimized in sequential rounds, in which residues 105–112 and 178–191 (which together include the catalytic triad of aspartates) were fixed along with residues 12–15 of the template DNA and all 5 residues of the primer strand. The protein residues were fixed to prevent unreasonable distortions from occurring in the polymerase active site, particularly at or near Met-184 which is part of a turn containing unusual ϕ and ψ angles. The remaining portions of the model were restrained during minimization. Restraints were gradually lowered as minimization proceeded. Minimizations used a steepest descents algorithm initially and then switched to conjugate gradients when a maximum derivative limit of 5 kcal/mol Å2 was reached. A had been reached. A maximum derivative of 0.001 kcal/mol Å2. Next, the structures were submitted to a dynamics calculation. Again, amino acid residues 105–112, 178–191, template residues 12–15, and all primer residues were fixed. Additionally, residues 388–560 and all 440 residues of p51 subunit were fixed. This simplification reduced computational times. Residues 388–560 are too far away to interact with the model DNA or residues binding the template. Dynamics calculations were carried out on an IBM 370 155/286 at 298 K using a canonical ensemble. Following the dynamics calculation, coordinates were minimized using caff91 forcefield with the pH set to 7.5. These structures were again minimized, with residues 105–112, 178–191, and 388–560 of p66, all residues of p51, and template residues 12–15 and primer residues 1–5 fixed as before. Minimization was implemented to a derivative cut-off of 0.001 kcal/mol Å2, before being resubmitted to further 11 ps of dynamics. The resulting coordinates were then minimized in an Amber forcefield to allow simulation of the effects of bulk solvent. A distance-dependent dielectric was employed with an r–4 dependence for the dielectric constant, and 25-ps dynamics simulations were calculated. The root mean square deviations for the wild type HIV-1RT(NL4-3), FE20, and FE103 models with respect to 1HNI starting coordinates were 1.6, 1.7, and 1.7 Å, respectively, when the palm residues were superpositioned. Superpositioning the fingers region yields root mean square deviations of 2.7, 3.0, and 3.6 Å, and root mean square deviations of 2.4, 2.1, and 2.8 Å from superimposing the thumb residues of the wild type, FE20, and FE103 models on 1HNI coordinates. The only differences seen between model residues and template is that between tyrosine 183 (wild type numbering) and template residues 12 (2 position with respect to the template base that base pairs with incoming dNTP). The distance between Tyr-183 Ca and N-2 of the template residue 12 is 2.5 Å. This is a result of the constraints against movement imposed on these atoms to keep the active site aspartates or the template anchor residues from moving. No collisions are observed between finger residues and the extended template.

**RESULTS**

**Engineering Alterations in the β3-β4 Hairpin—**Mutations were introduced into the fingers subdomain of recombinant pRT(NL4-3)6H-BiOT expression construct which contains separate expression cassettes for HIV-1NL4-3 RT p66 and HIV-1 protease (PR). Insertions in the β3-β4 hairpin were based on
the structure reported by Jacobo-Molina et al. (10). In the original design of the finger extension, we wished to simply increase the bulk of the flexible loop. In the absence of suitable foreknowledge, we did not want to introduce electrostatic interactions which might adversely affect translocation and/or processivity as a result of altered enzyme-template-primer interactions, leading to an inactive state. Since serine residues avidly form hydrogen bonds and glycine residues have a high degree of main chain flexibility, we inserted a sequence that is rich in these residues to extend the size of the flexible loop (FE103, Fig. 1).

During the process of mutagenesis to generate this insertion mutant, a fortuitous base deletion-insertion event within the inserted sequence led to the formation of an unintended mutant with an altered sequence in the insert (FE20, see Fig. 1).

Insertions into Fingers Subdomain Do Not Affect the Gross Properties of HIV-1 RT—To assess the impact of alterations in the β3-β4 region on the polymerization function, lyssates of bacteria infected for RT expression were initially tested for RNA-dependent DNA polymerase (RDDP) activity on both heteropolymeric and homopolymeric RNA templates. RDDP assays on heteropolymeric RNA template revealed that the activities of two insertion mutants were very similar to that of wild type RT. The assays for each RT were performed using poly(rA) as template, dTTP as template-primer (summarized in Table III). The kinetic constants for insertion mutants of HIV-1 NL4-3 RT were determined in RDDP assays utilizing 16 S rRNA. The “ratio” represents the fold increase or decrease with respect to wild type (WT) RT.

TABLE I

RNA-dependent DNA polymerase (RDDP) activities of finger insertion mutants

| Enzyme     | RNA-dependent DNA polymerase activity |
|------------|---------------------------------------|
|            | Poly(rA) | Poly(rC) | 16 S rRNA |
|            | pmol     | pmol     | pmol     |
| WT         | 41       | 1.0      | 3.0      | 3.0      | 1.5      | 1.0      |
| FE20       | 28       | 0.68     | 2.4      | 0.8      | 0.9      | 0.6      |
| FE103      | 39       | 0.95     | 2.7      | 0.9      | 1.6      | 1.1      |

The RDDP activities were measured on three different RNA templates as indicated. The levels of activity were determined via picomoles of dNMP incorporated (dTMP for poly(rA) and dGMP for poly(rC) and 16 S rRNA). The “ratio” represents the fold increase or decrease with respect to wild type (WT) RT.

TABLE II

Sensitivities of the finger insertion mutants to ddNTPs

Concentrations (μM) of ddNTP resulting in 50% inhibition (IC50) of RDDP activity, when measured on 16 S RNA, are shown for each ddNTP. The ratio indicates the fold increase or decrease over wild type (WT) inhibition concentration.

| RT         | ddATP | ddCTP | ddTTP |
|------------|-------|-------|-------|
|            | IC50  | Ratio | IC50  | Ratio | IC50  | Ratio |
| WT         | 3.5   | 1     | 1     | 1     | 1     | 1     |
| FE20       | 3.0   | 0.86  | 0.5   | 1     | 0.9   | 1.1   |
| FE103      | 3.2   | 0.91  | 0.75  | 1.5   | 3.1   | 1.5   |

TABLE III

The kinetic constants for insertion mutants of HIV-1 NL4-3

The assays for each RT were performed using poly(rA) as template and the kinetic constants determined as described in text, WT, wild type.

| HIV-1 RT(NL4-3) | Km, dTTP | Vmax | Vmax/Km |
|-----------------|----------|------|---------|
| WT              | 8.8 ± 0.4| 2.7 ± 0.05| 0.31   |
| FE20            | 12.1 ± 0.3| 2.1 ± 0.03| 0.17   |
| FE103           | 10.6 ± 0.6| 2.3 ± 0.05| 0.22   |
ciencies were not significantly compromised by the large insertions in the β3-β4 hairpin.

To test the hypothesis that the overall size of the β3 and β4 strands (including the connecting loop) correlates with processivity of polymerization, RDDP assays were performed in the presence of a polymerase trap (heparin). A heteropolymeric RNA template consisting of u3, r, and u5 regions of the long terminal repeat and the primer binding site was prepared by in vitro transcription. A 5’-end-labeled oligodeoxyribonucleotide (PBS-G) complementary to the primer binding site was used as primer. RT was first allowed to bind to preannealed template-primer, and polymerization was initiated by simultaneous addition of dNTP substrates and an excess of heparin. The presence of the heparin trap ensures a single cycle of polymerase binding, DNA synthesis, and dissociation during the reaction.

Analysis of the products generated during the processive synthesis indicates that both FE20 and FE103 were able to generate longer DNA products than the wild type RT (Fig. 3, A and B). Interestingly, FE103 produced more of the larger products than did FE20. The effectiveness of the trap was confirmed by mixing the template-primer with excess heparin before adding RT and initiating the reaction with dNTP substrates. This reaction produced no polymerization products (data not shown). When heparin was omitted from the reaction, allowing detection of products synthesized during multiple rounds of DNA synthesis, no differences were observed in the intensities of full-length product and the intermediate products produced by the wild type and mutant RTs (Fig. 3A). Furthermore, we used the influenza virus M1 RNA as template to assess whether the enhanced processivity of the mutants is a unique property of the template sequences. The mutant RTs were again able to produce larger products than wild type (Fig. 3, C and D) showing that the increased processivity is not limited to HIV-1 genomic RNA template. The FE103 RT still produced more of the larger products in comparison with the wild type and the FE20 RTs.

The processivity of FE20 and FE103 mutants appears to be increased by 25–30% as indicated by the size of the products generated on either HIV-1 or M1 RNA template. Plots of individual band intensities as a percentage of total band intensities (Fig. 3, B and D) illustrate the positions at which the RT molecules dissociated from the template. These represent the pause sites on the template due to secondary structures (4) or runs of the same nucleotide on the template (23). In processive synthesis with the wild type RT on HIV-1 RNA, two strong pause sites (at 59 and 97 bases from the primer terminus) and several weaker ones (between positions 100 and 150) are observed (Fig. 3B). Most of the weak pause sites are absent or reduced in intensity for the two mutant RTs. In addition, FE20 RT shows significantly reduced pausing at position 97, whereas FE103 RT displays significant reduction at both positions 59 and 97 (Fig. 3B). On M1 template, the three major pause sites corresponding to positions 86, 106, and 130 were observed with wild type and FE20 RT; however, with FE103 RT, stalling at these sites was significantly reduced (Fig. 3D). Altogether, FE20 and FE103 RTs appear to be more processive than wild type RT on both templates, and FE103 RT is clearly the most processive.

Modeling of the Finger Extension Mutants—To obtain structural insight into the role of HIV-1 RT finger extensions, we generated a computer model of the three-dimensional structure of NL4-3 RT and its two finger extension derivatives, FE20 and FE103. The coordinates from the structure of HIV-1BH10 RT bound with α-APA R 95845 refined to 2.8 Å (21) were used as a
and also shows extensive movement of the loop between the latter. In addition, a small YASSPA routine from the program O (24) shows that FE103 main (Fig. 4). Secondary structure assignment made using the changes in the overall secondary structure of the finger subdomain that the insertions are accommodated without gross energy states, and the final conformation may not be the global minimum. It is important to recognize that the flexible loop in our models for the two mutants represents one of several low-energy states. Modeling experiments in which the residues of the loop extensions were changed to conform to the sequence of the companion loop extension mutant show that FE103 can adopt a conformation like that seen here for FE20 but not vice versa. FE20 cannot form the saddle-like loop structure seen for FE103 because of restrictions in backbone conformation imposed by substitution of the Gly residues of FE103 with non-Gly residues of FE20. Dynamics trajectories run on the FE103 model starting from the FE20 conformation do not lead to an interconversion of this conformation with that seen in the original FE103, although the total energy is higher for the FE20-like conformation. This suggests that individual conformations are separated by significant energies, and the final conformation may not be the global minimum.

The models, in conformity with the biochemical data, indicate that the insertions are accommodated without gross changes in the overall secondary structure of the finger subdomain (Fig. 4). Secondary structure assignment made using the YASSPA routine from the program O (24) shows that FE103 retains the β strands 3 and 4 with only a slight shortening of the latter. In addition, a small β strand at residues 70–72 (wild type numbering) is inserted in the FE103 model. In the case of FE20, β strand 3 is retained, but the region corresponding to β strand 4 assumes an irregular secondary structure, becoming an extension of the loop region introduced by the mutation, with only a 3-residue strand (75–78) being retained. Both models show some additional distortions. In FE20, helix A is interrupted and β8 is shortened due to distortion in the loop between β7 and β8. FE103 is distorted in the region around β1 and also shows extensive movement of the loop between β7 and β8 but with only a 1-residue shortening of β8.

All three models show a rotation of the fingers and thumb region toward one another (see Fig. 5). The slight rotation is roughly the same in all three models and leaves the DNA quite solvent-exposed. The fact that the modified β3-β4 loops appear to pinch the DNA from the sides rather than wrap around the DNA is consistent with our inability to detect differences in nuclease protection of the single-stranded portion of the template (data not shown). The models show that the insertion constricts the passage of single-stranded template between the thumb and fingers subdomains. The distance across the gap between the tip of the β3-β4 loop and the closest thumb residue narrows from 20 Å (in wild type HIV-1 NL4-3 RT) to as little as 12 Å for both FE20 and FE103 RTs (Fig. 6), presumably forcing the secondary structures in the template to melt prior to translocation to the dNTP addition site. The models indicate that the altered β3-β4 loops interact with the single-stranded template and extensively distort the phosphate backbone in the cases of FE20 and FE103 RTs. The exact nature of the interactions seen in the model are biased by the initial assumption that the template extension adopts the A conformation. Nonetheless, the resulting distortion seen in the A-form model suggests that only a DNA with a distorted geometry could be accommodated, although an exact geometry cannot be garnered from the present models alone. The distortion of the single-stranded overhang region seen here primarily involves rotation about a number of glycosidic bonds, particularly those at the +4 and +5 template positions. These have χ (glycosidic torsion) angles of −138 and −132, respectively, in the HIV-1 NL4-3 RT model. Unfavorable torsion angles avoid steric clash of these two bases which are forced toward one another as a result of a kink introduced into the single-stranded region by the adoption of a C1′-exo conformation by the deoxyribose at the +3 template position. The kink prevents steric clash with the protein residues of the β4 strand. Similar distortions are seen in the other two models. In FE20, it is the second base that has an unusual deoxyribose pseudorotation angle. The larger FE20 finger forces it to adopt a C4′-exo conformation. Interestingly, the FE103 is the least distorted of all of the models. The flexibility of the loop allows it to exact minimal interference with the A-form model, although the deoxyribose at the +3 template position is C1′-exo. It is noteworthy that the template residues most distorted by interaction with the fingers are the +3 through +6 residues which are shown by binding studies to have a significant influence on the dissociation constant with RT (25). The extended finger β3-β4 loop is positioned ahead of the 3′ terminus of the primer similar to wild type HIV-1 RTexo. The models indicate that most of the interactions of the fingers subdomain with the template extension are via the inserted amino acid residues in the fingers subdomain for both FE20 and FE103 (Fig. 7), and the residues flanking the insertion cannot engage in interactions analogous to those they display in the wild type enzyme. This suggests that the role of
HIV-1 RT is known to display a moderate to poor degree of polymerase processivity. On heteropolymeric RNA templates, the HIV-1 RT is known to synthesize up to 105 nucleotides (26) in a single processive cycle of DNA synthesis. Although much larger products (300 bases long) can be obtained under certain conditions, these involve the use of homopolymeric RNA templates such as poly(rA) (27). Structural elements that control processivity of HIV-1 RT are currently not defined. The work reported here reveals an important role for the HIV-1 RT fingers subdomain in processive polymerization. The two insertion mutations created in the fingers subdomain, described in this report, did not lead to any gross changes in RT function other than increasing the polymerase processivity.

Previous work from several laboratories has implicated fingers β3-β4 loop in the following two functions: positioning the template-primer (28, 29) and nucleoside analog sensitivity (14). When an extended template overhang was modeled into the three-dimensional structure of HIV-1 RT double-stranded DNA complex, the flexible loop of the β3-β4 hairpin was shown to contact the template approximately three nucleotides ahead of the primer terminus (22). Nuclease footprinting experiments have also confirmed that such an interaction is likely (30). Biochemical support for this comes from studies that showed greater affinity of RT to double-stranded DNAs with 6-base or longer overhangs as compared with those with 1-base overhang (25). The β3-β4 loop of the fingers subdomain is also a hotspot for nucleoside analog resistance mutations (14). The fact that the β3-β4 loop is distal to the dNTP-binding pocket makes this intriguing. However, when combined with the fact that the β3-β4 loop contacts the template-primer (22), the ability of β3-β4 mutations to confer nucleoside analog resistance is consistent with an indirect role played by template-primer contacting residues in determining the conformation of the dNTP-binding pocket. This effect has been termed “template repositioning” (22).

Interestingly, despite the large perturbation, we observed only a small change (ranging from –0.14- to +3.1-fold) in sensitivity of the finger insertion mutations to ddNTPs (Table II). A large number of nucleoside analog resistance mutations have been identified for HIV-1 RT, and a significant data base on their biochemical properties is available. In a majority of the cases, the levels of decrease in sensitivity are in the range of 10–100-fold (31–33). Thus, a change of 3-fold appears very close to background or wild type levels. It is unclear why large insertions that appear to increase the interaction between fingers subdomain and the template-primer have no effect on nucleoside analog sensitivity.

Although the mutant FE103 displayed levels of RDDP activity that were comparable to that of wild type, there was a 40% decrease in the activity on heteropolymeric RNA for the insertion mutant FE20 (Table I). However, a decrease of only 20 or 30% was observed on other templates for the same mutant (Table I). It is interesting that FE20 displays an increase in processivity in light of the fact that its overall activity is consistently lower than wild type. The fact that the finger insertions had either a small or no effect on the $K_m$ and $V_{\text{max}}$ values for RDDP activity or the sensitivities to the ddNTPs suggest that the insertions at this locus of the fingers subdomain, unlike the nucleoside analog resistance mutations known to arise during treatment, did not affect the conformation of the dNTP-binding pocket.

Although a poor processivity for HIV-1 RT is well recognized, mutations that alter this property are rarely observed. The (-)-2',3'-dideoxy,3'-thiacytidine (3TC)-resistant M184V variant (34) and site-directed mutations in the thumb subdomain

![FIG. 5. Comparisons of the starting protein coordinates (1HNI (22)) with the wild type HIV-1 NL4-3 RT model and of the wild type model with those of the insertion mutants. For the insertion mutants the β3-β4 loop is colored white. The proteins were superimposed on the Ca 1HNI coordinates for the palm region. A, overlay of the wild type model (red) on 1HNI (yellow). B, overlay of the wild type model and FE20 insertion mutant model (magenta). C, overlay of the wild type model and the FE103 insertion mutant model (cyan). The figure was prepared using InsightII (Biosym).](image-url)
(35) decrease the processivity of HIV-1 RT. Significant increases in processivity were also reported for azidothymidine-resistant variant of HIV-1 RT (including D67N, K70R, T215Y, and K219Q alterations) (36) and a didanosine-resistant variant with the K65R alteration (37). Interestingly, the K65R, D67N, and K70R mutations found in these mutant RTs also map to the $\beta_3$-$\beta_4$ loop.

The method of preparation of the RT heterodimers employed here will result in the presence of the insertion mutations in both p66 and p51 subunits. In the wild type HIV-1 RT heterodimer structure published, the $\beta_3$-$\beta_4$ hairpin will face the template-primer duplex only as a part of the p66 subunit. The corresponding segment of p51 is tucked within the fingers subdomain of that subunit, well away from the template-interacting surfaces and appears to be clearly distal to the dimerization interface. Similarly, when the models of the two insertion mutations were examined, the extensions appeared to be also tucked away in the same manner (with the exception, for FE103 alone, of some contacts with residues 401 and 427 in the p66 connection subdomain). Thus, it is unlikely that the inser-

**FIG. 6.** Stereo view of the molecular surface of the fingers (blue), palm (red), and thumb (green) subdomains of p66 in each of the three models. The atoms of the template (magenta) and the primer (cyan) are shown. For clarity, the surface covering the insertion in FE20 and FE103 is colored white. The figure was generated using GRASP (38). Identical views are shown for the wild type HIV-1NL4-3 (top), FE20 (middle), and FE103 (bottom) RTs. The figure shows greater interaction of the altered fingers with the bases of DNA as a result of the greater spatial constraint imposed by the presence of 15 additional amino acid residues in the binding cleft. Both FE20 and FE103 cradle the single-stranded DNA, reducing the size of the cleft through which the template must thread into the active site.
tions in the p51 play a role in the increased processivity of these mutant RTs. These mutations also did not have an impact on the formation or the stability of the heterodimer as shown by the stoichiometry of the two subunits during purification via an affinity tag present only on the p66 subunit (Fig. 2).

The absence of crystallographic data for the HIV-1 \(1_{\text{NL4-3}}\) RT and its finger extension forms makes it difficult to interpret the data on processivity. Modeling suggests that the inserted residues of the mutant RTs reduce the size of the channel within which the template is held, effectively making a tighter clamp out of the fingers and thumb subdomains (see Figs. 4 and 5). This may confer greater processivity by improving the template-RT interactions or by helping melt structured regions of template better than wild type RT. Analysis of the accessible surface area for the modeled finger domains of wild type and mutant RTs shows that the occluded surface area, that is the solvent-accessible surface area lost as a result of complex formation, increases by 12% for \(\text{FE}20\) and by 14% for \(\text{FE}103\) RTs relative to wild type HIV-1 \(1_{\text{NL4-3}}\) RT. This is consistent with the idea that the mutant RTs may form a stronger complex with template than wild type RT and may be partially responsible for their greater processivity. Analysis of the electrostatic potential, using the program GRASP (38), indicates that the surface of the fingers subdomain in proximity to the template overhang of the mutant \(\text{FE}103\) possesses a more positive potential. This may allow \(\text{FE}103\) RT fingers to form a stronger complex with template than \(\text{FE}20\) RT by interacting more strongly with the template's phosphate backbone and is consistent with the greater processivity observed for \(\text{FE}103\) RT than for \(\text{FE}20\) RT. Does further reduction in the distance between the fingers and the thumb subdomains result in a greater increase in processivity? To examine this issue, we are currently in the process of creating a series of insertions of progressively increasing sizes in the \(\beta\)-\(\beta\) loop.

The structural elements controlling processivity and translocation of RT along the template are poorly understood. Work reported here reveals that the fingers subdomain, which is already implicated in template-primer contact and in nucleoside analog sensitivity, may play an important role in determining processivity. Several factors might contribute to the low level of processivity of wild type HIV-1 RT as follows: weak template-polymerase interactions, secondary structures, and other sequence-specific structural variations along the template. Our results suggest one of two factors may be responsible for the increased processivity of the insertion mutants as follows: a shorter distance between the fingers and the thumb subdomain, or an increased contact between the single-stranded portion of the template and the fingers subdomain. Further studies are required to delineate the factors that influence this important aspect of RT function.

The processive polymerization is an important aspect of DNA replication. HIV RT displays a characteristic low processivity that may have been evolutionarily conserved to facilitate the strand transfer reactions, a key step in retroviral DNA synthesis (39). It has been proposed that the poor processivity may be the principal cause for the high rate of nucleotide substitutions, deletions, insertions, as well as recombination observed for HIV-1 RT (39). However, during viral replication, other viral proteins such as nucleocapsid or integrase may modulate the processivity of HIV-1 RT. A positive influence of Ncp7 on the processivity of HIV-1 RT \(\text{in vitro}\) has been reported earlier (40). Additional studies are required to investigate the influence of integrase and other viral proteins on the processivity of HIV-1 RT.

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Yvonne Kew, Laurence R. Olsen, Anthony J. Japour and Vinayaka R. Prasad

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