Structural Determinants of Slippage-mediated Mutations by Human Immunodeficiency Virus Type 1 Reverse Transcriptase*

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Single-base deletions at nucleotide runs or −1 frameshifting by human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) result from template slippage during polymerization. In crystal structures of HIV-1 RT complexed with DNA-DNA template-primer, the palm subdomain in the template cleft contacts the template backbone near the proposed site of slippage via the Glu⁸⁹ side chain. We investigated the role of Glu⁸⁹ in frameshifting by perturbing this interaction. Substitutions with Asp, Gly, Ala, Val, Ser, Thr, Asn, or Lys were created in recombinant HIV RT, and frameshift frequencies of the resulting mutant RTs were measured. All substitutions led to reduced −1 frameshifting by HIV-1 RT (2–40-fold). Interestingly, the suppression of −1 frameshifting frequently coincided with an enhancement of +1 frameshifting (3–47-fold) suggesting that Glu⁸⁹ can influence the slippage of both strands. Glu⁸⁹ substitutions also led to reduced rates of dNTP misincorporation that paralleled reductions in −1 frameshifting, suggesting a common structural mechanism for both classes of RT error. Our results reveal a major influence of Glu⁸⁹ on slippage-mediated errors and dNTP incorporation fidelity. The crystal structure of HIV-1 RT reveals a salt bridge between Glu⁸⁹ and Lys¹⁵⁴, which may facilitate −1 frameshifting; this concept is supported by the observed reduction in −1 frameshifting for K154A and K154R mutants.

Human immunodeficiency virus, type 1 (HIV-1)⁵ reverse transcriptase (RT), which converts HIV-1 genomic RNA to a double-stranded DNA form, displays an intermediate level of fidelity among DNA polymerases (1–3). The relative error proneness of HIV-1 RT is recognized as one of the factors responsible for high genetic variation in HIV, which ultimately leads to the rapid emergence of drug-resistant HIV variants. The broad structural basis of HIV-1 RT fidelity is reasonably well understood. The first structural insights into HIV-1 RT fidelity came from biochemical studies on nucleoside analog RT inhibitor-resistant mutants, in which the residues affected tend to be located at or near the polymerase active site. For example, it was shown that the (−)−2′-deoxy-3′-thiacytidine-resistance mutation M184V, affecting a residue located near the incoming dNTP, conferred an increased fidelity of dNTP incorporation on HIV-1 RT (4–6). The first crystal structure of HIV-1 RT complexed with double-stranded DNA template-primer showed specific structural elements termed primer grip and template grip, which correctly position the templating base and the primer 3‘ terminus for the insertion of incoming dNTP (Fig. 1) (7). The structure of HIV-1 RT complexed with template-primer and dNTP showed that the β3–β4 hairpin of the fingers subdomain provides key contacts for the templating nucleotide and the incoming dNTP (8). The importance of this region for dNTP selection and fidelity is further supported by reports of several mutations in the β3–β4 hairpin that affect RT fidelity (9–12).

Whereas residues proximal to the dNTP-binding pocket can directly influence polymerase fidelity, the contribution of residues that contact template-primer duplex to fidelity is presumably indirect. Such residues primarily influence processes related to template-primer interaction such as the RT-template-primer affinity, polymerase translocation along the template during synthesis, and processivity (13–15). Mutations at such residues can also affect fidelity. For example, two well-studied structural elements, αH of the thumb subdomain and the template grip β5a in the palm subdomain of HIV-1 RT, which are known to affect polymerase processivity (15, 16) also affected fidelity (16, 17). Alanine substitutions of residues in the αH helix that form a part of the minor groove-binding track in the thumb have been shown to alter frameshift fidelity (16, 18). The E89G variant in β5a of the template grip increases dNTP incorporation fidelity of HIV-1 RT (17).

Glu⁸⁹ is of particular interest because of its unique location in the RT template grip (see below) and its established importance in HIV-1 replication and drug resistance. The E89G variant was first identified in an in vitro phenotypic screen of a random library of RT mutations (19, 20). HIV-1 virions containing the E89G mutation are replication competent and exhibit multidideoxynucleoside analog resistance (20). Although the E89G mutation is rarely observed alone in natural settings, it has been observed in association with M184V in (−)−2′-deoxy-3′-thiacyti-
Template Grip of HIV-1 RT and −1 Frameshifting Mutations

Table 1

Sequences of template and primer oligonucleotides used in fidelity assays

| Mispair extension | Single dNTP exclusion |
|-------------------|-----------------------|
| G:C               | 5'-CCGTTTCAGGTCCTGGTGCAGCCACTGC-3' |
|                   | 55-mer                |
| G:T               | 3'-CCGAAATCGGCCAGAAGCCGGCGCGTACATCTCTAAGAGGCTGACTGATTTT-5' |
|                   | 55-mer                |
| G:G               | 3'-CCGAAATCGGCCAGAAGCCGGCGCGTACATCTCTAAGAGGCTGACTGATTTT-3' |
|                   | 31-mer                |
| G:A               | 5'-CCGTTTCAGGTCCTGGTGCAGCCACTGAG-3' |
|                   | 55-mer                |

Enzymes, dNTPs, and Oligonucleotides—HIV-1 RT is a heterodimer of p66 and p51 subunits. RT mutants were prepared by introducing the mutation in the catalytic subunit, p66. The mutant p66 RTs were expressed from the expression plasmid pRT and reconstituted with the wild-type RT p51 expressed from p6HRT51, followed by purification as described before (29). The purified RTs were found to be free of DNase activity even after incubation of excess RT with radiolabeled DNA substrate for up to 8 h. Purification of K154A, K154R, and P157S mutants was as described previously (30, 31). Deoxynucleotide triphosphates (dNTPs) were purchased from Amersham Biosciences. The oligonucleotides used in this study are listed in Table 1.

Measuring Frameshift Mutagenesis—Frameshift fidelities were measured via a phage-based gapped duplex assay described by Bebenek et al. (26). The M13mp2 DNA used as a substrate for the assay contained a single-base (T) insertion in the run of Thr at position 70 of lacZα for scoring −1 frameshifts (5T-1 substrate) and a single-base deletion for scoring +1 frameshifts (7T+1 substrate) (32). Both M13mp2-gapped DNA substrates were generous gifts of T. A. Kunkel (National Institutes of Health, NIEHS, Research Triangle Park, NC). M13-gapped DNA substrates were filled in by wild-type and mutant RTs as described previously (26). Complete filling in was ensured by agarose gel electrophoresis and the successfully filled-in gapped duplex DNA was electroporated into Escherichia coli strain MC1061. Electroporations were performed on separate days for each of three trials for each DNA synthesis reaction. Cells were allowed to recover for 10 min, then mixed with E. coli CSH50 cells (Δpro-lac, thi, ara, strA/F (pro), lacZ_M15, trd36) in the presence of 0.25 mM isopropyl β-thiogalactopyranoside (Labscientific) and soft agar containing 0.2 mM X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; Labscientific), and plated on M9 plates. Plates were incubated at 37°C for 15 h and the plaques were screened for β-galactosidase activity by looking for those displaying the dark blue reversion phenotype. Such revertant plaques were picked for sequencing and stored in 1 ml of 0.9% saline at 4°C. Reversion frequencies were determined by dividing the number of revertants by the total number of plaques screened. All reversion frequencies were corrected for background reversion frequency of the substrates (1.30 × 10⁻⁵ for the 5T-1 substrate and 8.3 × 10⁻⁵ for the 7T+1 substrate (32)). The isolation of single-stranded DNA from revertant plaques and the determination of nucleotide sequences were as described previously (33). The forward mutation frequencies were determined using a wild-type lacZα-gapped duplex DNA as described previously (24).

Single dNTP Exclusion Assay—The “single dNTP exclusion” or the “minus dNTP” assay were performed on DNA-DNA template (55-mer) and primer (28-mer) oligonucleotides (Table 1). A 5'-32p-labeled primer oligonucleotide was extended by purified wild-type or the eight Glu89 mutant RTs in the presence of all four combinations of three dNTPs.
The DNA template-primers were prepared by annealing the template (at a molar ratio of 2 template to 1 primer) to the end-labeled primer. Reactions were initiated by combining equal volumes (10 μl) of enzyme-template-primer solution and dNTP salt solution in which all four dNTPs were present, or in which one of the dNTPs was omitted. The primer concentrations used for the wild-type, E89A, E89V, E89D, and E89G were selected to be at equal levels of DNA-dependent DNA polymerase activities on the template-primer used here. Thus, the {1 × and 2.5 ×}. The 1× concentrations for wild-type, E89A, and E89V were, respectively, 0.68, 0.09, and 0.18 nM. Enzyme concentrations for wild-type and mutant RTs were selected to be at equal levels of DNA-dependent DNA polymerase activities on the template-primer used here. Thus, the 1× concentration for each enzyme is different in terms of protein input or unit activity as measured on poly(rA) oligo(dT) template-primer, but similar with respect to their enzyme activity on the DNA-DNA template-primer used. After mixing, reactions contained 100 nM template-primer, increasing concentrations of wild-type, and mutant RTs as described above, 250 μM dNTPs, 50 mM Tris-HCl, pH 8, 60 mM NaCl, 20 mM dithiothreitol, 0.05% IGEPAL (surfactant), and 10 mM MgCl2. The reactions were terminated after 6 min by the addition of 20 μl of stop solution (95% formamide, 20 mM EDTA). Eight microliters of boiled reaction mixture were subjected to electrophoresis in a 10% urea polyacrylamide gel for 2.5 h at 100 W. Autoradiograms were analyzed by densitometry, and band intensities were quantitated via ImageQuant. Total band intensity was calculated as the total intensity of the band immediately below the barrier site plus all the bands above it. Extension was calculated as the sum of all bands at and above the barrier site. Percentage extension as a fraction of the total band intensity was then calculated from total band intensity and extension.

**Mispair Extension Reactions**—Primer extension reactions were performed using four different 5′-32P-labeled DNA 31-mers (G-C, G-T, G-G, G-A; see Table 1) annealed to a 55-mer DNA template using wild-type and mutant RTs. As before, two different concentrations of each RT (selected to be equal in DNA-dependent DNA polymerase activity between different enzymes as tested on a DNA-DNA template-primer) were tested for each enzyme at 1× and 2.5× concentrations. The 1× concentrations used for the wild-type, E89A, E89V, E89D, and E89G were 0.68, 0.12, 0.18, 0.09, and 2.3 nM, respectively.

Reactions and buffer conditions were described as above for the dNTP exclusion assay, but in the presence of dGTP, dATP, and dTTP, each at 25 μM. Reactions were terminated after 10 min and analyzed as previously described for dNTP exclusion assay.

**RESULTS**

**Single-base Deletion Errors**—Glu89 of the β5a strand in the palm subdomain of HIV RT is located near the site of template strand slippage.
Single-base Insertion Errors—Although HIV-1 RT can also produce +1 frameshifts, which are mediated by the primer slippage events, the residue Glu<sup>89</sup> does not contact the primer, which is located on the other side of the point of Glu<sup>89</sup> contact with template-primer duplex. Therefore, it was of interest to determine whether Glu<sup>89</sup> would have any influence on primer slippage events. We measured the +1 frameshifting frequency of Glu<sup>89</sup>-substituted RT mutants using a different M13 substrate reporter gene, which contained a single-base deletion in lacZ<sub>α</sub> such that the insertion of a base within a run of 7 Ts results in the reversion phenotype. Although both +1 and −2 (deletion of 2 bases via template slippage) mutations could restore a functional lacZ<sub>α</sub> gene, previous analysis of a large number of clones has revealed no −2 mutations in this construct.<sup>6</sup>

In contrast to the results obtained for −1 frameshift frequency measurements, all Glu<sup>89</sup> substitutions (except the Glu to Val substitution) led to an increase in the frequency of +1 frameshift errors (Table 2). The wild-type +1 frameshift frequency was about five times lower than the −1 frameshift frequency. The substitutions led to a 2.6–47-fold increase in +1 frameshift frequency over that of wild-type (Table 2). Thus, the majority of frameshift mutations by wild-type RT were of the −1 type, whereas the majority of frameshift mutations by all of the variant RTs were of the +1 type. Some of the substitutions, such as Ser, Thr, and Asn caused increases in total frameshifting (7-, 2-, and 6-fold, respectively) while maintaining the inverse ratio of +1 to −1 frameshifting compared with wild-type RT (Table 1).

Base Substitution Fidelity—It was surprising that the wild-type enzyme displayed the highest −1 frameshift mutation frequency, as one would expect the wild-type enzyme to have minimal slippage-mediated mutations, which have little evolutionary benefit for protein coding sequences. Therefore, we investigated the influence of wild-type Glu<sup>89</sup> on base substitution fidelity. Base substitution errors (or misincorporation) result from two events during polymerization. First the insertion of an incorrectly paired dNTP (misinsertion) must occur. Such events result in a mispaired primer terminus and are generally not preferred by most DNA polymerases. For the misinserted base to become a misincorporated base, the mispaired primer terminus must be extended by correct insertion of a base opposite the next template base (mispair extension).

To investigate the effect of Glu<sup>89</sup> on both misinsertion and mispair extension, we utilized a gel-based single dNTP exclusion assay or minus dNTP assay (1), which provides a gross estimate of the efficiency of base substitution for a polymerase without proofreading function. This assay involves copying a heteropolymeric DNA template using a 5'-end-labeled primer in the presence of only 3 dNTPs (absence of a single dNTP). Primer extension past a template site for which the complementary dNTP is missing is indicated by a large dot to the left. The nucleotide sequence of the DNA product synthesized is indicated to the left and the major band at the bottom of panels represents unincorporated primer. 8, quantitative plot of extension products in dNTP exclusion assay for all the mutants for reactions missing dATP, dCTP, and dGTP. Because reactions missing dTTP produced no extension, we were unable to plot data for dTTP exclusion. Percentage extension as a fraction of total band intensities are shown for wild-type and all Glu<sup>89</sup> RT mutants.

<sup>6</sup>T. A. Kunkel, personal communication.
We then compared primer extension by wild-type and mutant RTs in reactions missing each of the three dNTPs. In reactions where one dNTP is missing, little or no full-length products were generated as expected, but different RTs extended primers to different extents (Fig. 2A). Fig. 2A shows the results of a dNTP exclusion assay for three of the RTs; wild-type, E89A, and E89V. As seen in the second panel, which displays products of the reactions lacking dATP (Minus dATP panel), E89V RT does not extend the primers beyond the polymerization block imposed by the missing dNTP as readily as does wild-type RT, whereas E89A RT polymerizes to an intermediate extent. E89A and E89V RTs therefore display higher levels of fidelity with respect to dNTP incorporation and extension than does wild-type RT, with E89V displaying the highest fidelity. This trend in fidelity was also apparent in reactions in which dCTP or dGTP were excluded. We note some exceptions. E89A for example, displayed site-specific increase in misincorporation at one site (Fig. 2A, −dATP panel). However, in general, there is a decreased misincorporation compared with wild type. Extension reactions by other Glu89 mutants revealed similar increases in fidelity (gels not shown). We do not believe that synthesis above the barrier is because of contamination with the correct dNTP for two reasons. One, the dNTPs we used were high pressure liquid chromatography purified by the manufacturer (Amersham Biosciences). Two, in primer extension experiments, addition of different dNTPs leads to a differential mobility shift (data not shown). Therefore, variations in extension synthesis beyond the barrier site, despite the use of equal inputs of activity for different mutant RTs, suggests changes in the efficiency of misincorporation.

To estimate differences in base substitution efficiencies of the Glu89 mutant panel, we quantified the DNA synthesis above the first barrier for all mutants. The sum of intensities of all products above the “no substrate” barrier for which the complementary dNTP is missing (presented as a fraction of the sum of the intensities of the barrier band plus those above it) shows that all substitutions at Glu89 decrease the efficiency of base misinsertion by RT (Fig. 2B). Quantitation of extension products representing polymerization beyond the barrier revealed that the trend in increased fidelity observed for misinsertion frequency mimics the trend for −1 frameshifting fidelity (Table 2 and Fig. 2B). For example, E89V substitution resulted in the largest decrease in both −1 frameshifting and base substitution errors (Fig. 2), whereas E89A, E89S, and E89N displayed fidelities most similar to wild-type in both cases. In fact, graphical representations of changes in fidelity in comparison to wild-type for the eight Glu89 substitutions for −1 frameshifting and base substitution are superimposable (compare Table 2 and Fig. 2B). Although, at first, this seems to suggest that the decreased efficiency of misincorporation may be because of slippage-mediated synthesis, we believe this to be unlikely because of the fact that none of the barrier sites being studied (the first in each case) are in the nucleotide runs in the heteropolymeric DNA template used in this assay.

In addition to its error proneness for internal misinsertions and frameshifting events, HIV-1 RT has been shown to be capable of inserting non-templated dNTPs at blunt ends of both RNA-RNA and DNA-DNA template-primer (upon reaching the end of copying of a long template) at a high efficiency (35). Interestingly, we observed that some of the Glu89 mutants also display an absence of non-templated addition of nucleotides. For example, E89V showed a complete lack of non-templated addition (as shown by the light band at the top of the lane, above the dark band representing the full-length product), but E89A retained this activity. Similarly, E89D, E89G, E89K, and E89T all showed a complete lack of non-templated dNTP incorporation, whereas E89N and E89S retained levels of non-templated dNTP incorporation that were comparable with wild-type (data not shown).
the overall mutation frequencies of RT mutants. To examine this, we selected two mutants, E89K and E89V, displaying the largest decreases in −1 frameshifting and mutant E89S, which showed the largest increase in +1 frameshifting. Using a forward mutation frequency assay, we measured the overall mutation frequency of these mutants and compared it to that of wild-type HIV-1 RT. As shown in Table 3, the mutation frequencies of E89K, E89V, and E89S were altered minimally, bringing about reductions of 1.2-, 1.3-, and 1.6-fold from that of wild-type RT, respectively (which correspond to reductions of 16, 25, and 38%, respectively).

Other Template Grip Residues Interacting with Glu89 or the Template Strand—We wished to investigate the role of other amino acid residues in the vicinity of Glu89 in frameshifting. Pro157 in αE is also part of the template grip. Interestingly, this residue contacts the same sugar moiety of the template as Glu89 approaching it from the other side of the deoxyribose ring. A P157S mutation in HIV-1 RT has been shown to confer resistance to inhibition by nucleotide analogs such as 3TCnTP ((−)-2'-deoxy-3'-thiacytidine triphosphate) and FTCnTP (2',3'-dideoxy-5-fluoro-3'-thiacytidine triphosphate) (30). More importantly, the sequence specificity in sensitivity to inhibition by chain terminators displayed by the wild-type HIV-1 RT was altered by the P157S substitution, further confirming its ability to modulate template-primer conformation via interaction with the template strand. Therefore, we tested the effect of the P157S mutation on −1 frameshift fidelity. Our results show that the effect was minimal with a 1.8-fold reduction in frameshifting (Table 4). When compared with the effect of Glu89, revealed by Glu89 substitution mutants (2.5–40-fold decrease in −1 frameshifting), the role of Pro157 appears to be minor. However, one cannot exclude the possibility that more pronounced effects could result from substitutions with residues other than serine.

In HIV-1 RT complexed with DNA and dNTP, in addition to its contact with the sugar-phosphate backbone, Glu89 forms a salt bridge with the side chain of Lys154, also part of the template grip element αE. This contact between Glu89 and Lys154 is likely to be transient and may contribute to structural constraints that may directly or indirectly affect the stability of frameshifted complexes of RT and template-primer. To investigate this hypothesis, we tested the influence Lys154 on −1 frameshifting. Lys154 is the only positively charged residue in the VLPQGWK motif in the β8–αE loop at the junction of the fingers and palm subdomains. Among several substitutions of Lys154 that were previously tested for the effect on HIV-1 RT function (31), we selected two that retained wild-type-like polymerase activities, K154A and K154R substituted mutant RTs, and tested them for −1 frameshifting. Interestingly, as seen in Table 4, both mutations led to significant reductions in −1 frameshifting, with 10- and 7.5-fold reductions, respectively (Table 4). These results suggest that Lys154 is involved in the template-slippage-mediated frameshifting events.

**DISCUSSION**

Our results demonstrate an important role for the Glu89 of template grip β5a in frameshift mutagenesis by HIV-1 RT. In the wild-type enzyme, Glu89 is positioned to allow a relative high frequency of −1 frameshifting (i.e. template slippage) with a 5 times lower frequency of +1 frameshifting (primer slippage). Substitutions at Glu89 appear to disturb this ratio resulting in greater +1 frameshifting events. Our analysis of wild-type and eight different substitutions at position 89 reveals a key role for Glu89 in many aspects of polymerase fidelity tested, including −1 and +1 frameshifting, dNTP misincorporations, and mispair extensions. It appears that all types of RT errors except +1 frameshifting are at a maximal level when the wild-type Glu residue is present.

Because of the proximity of Glu89 with the template backbone, it is not surprising that substitution of this residue leads to a change in frameshifting because of either direct or indirect effects on template slippage. However, the decreased frequency of −1 frameshifting for all Glu89 substitutions was unexpected.

The characteristic high frequency of −1 frameshifting errors in homopolymeric runs may be because of the ability of HIV-1 RT to stabilize intermediate nucleic acid structures that result in non-copying of template nucleotides that would yield slipped, mismatched, or unpaired bases. Such structural aberrations are likely to disrupt the positioning of the ribose and phosphate moieties of one or more template base(s). The relatively high −1 frameshifting frequency of wild-type RT suggests that Glu89 may directly or
indirectly facilitate the proper conformation of the template grip leading to stabilization of aberrant template-primer duplexes in the template-primer cleft of RT. In a typical, heteropolymeric template sequence, transient misalignments between template and primer nucleotides can be resolved, at some probability, by sequence-specific Watson-Crick base pairing, resulting in correct copying of the sequence. However, within a run of identical nucleotides, the partial misalignment could resolve incorrectly with base pairing to the previous or following primer base.

An opposite effect was observed for +1 frameshifting fidelity; all substitutions led to increased +1 frameshifting. It appears that the template- and primer-slippage events are inter-related and are apparently mutually exclusive. For example, wild-type Glu89 appears to facilitate template slippage (−1 frameshifting), but not primer slippage (+1 frameshifting). In contrast, all substitutions tested here appear to disfavor the template-slippage but promote primer-slippage events. The structural aberration(s) of nucleic acid during primer slippage will be different from that during template slippage and by extension, contacts with the enzyme should also be different. The effect of Glu89 mutations on the ability of RT to accommodate non-canonical nucleic acids is also likely to be different. Structural studies with the relevant mutants and/or relevant RT-DNA complexes may provide insights into the precise role of residue 89 in +1 frameshifting. It is also possible that suppression of template slippage leading to an increase in the frequency of primer slippage may be because of an innate property of the homopolymeric nucleic acid duplex, such as an unusual conformation, that forces slippage events at these sites. HIV-1 RT is reportedly sensitive to minor groove compression caused by homopolymeric runs of adenosine (36). The fact that several substitutions at Glu89 cause a reduction in −1 frameshifting suggests that whether the homopolymeric stretches of sequence leads to template or primer-slippage events is in part determined by the nature of the interaction of the residue 89 or the surrounding residues of template grip β3a with the nucleic acid.

Strucutral Insights into Frameshifting Determinants—To rationalize the influence of RT mutations on frameshifting, we modeled the various substitutions into the structure of HIV-1 RT complexed with DNA and dNTP. As mentioned before, the crystal structure of HIV-1 RT reveals an interaction between Glu89 and Lys154 residues (Fig. 4, A and B). Glu89 is located in the template grip, contacting the template sugar phosphate backbone near the n-2 base (where the templating base is denoted n). Lys154 is located within the β8–αE loop forming the junction between the fingers and palm subdomains. Residues other than lysine or arginine at that position are known to significantly affect RT-DNA-dNTP complex formation (31). Interestingly, Lys154 is in a position to form a hydrogen bond with Glu89 (8) (Fig. 4B). We hypothesize that Lys154 and Glu89 act in concert to maintain the integrity of the template grip region and by extension the polymerase active site.

We hypothesize that +1 and −1 frameshifting rates can be explained by movements that are influenced by the nature of the protein-DNA contacts present along the two strands. Furthermore, contacts on the template and primer strand are correlated, in the sense that modifying the contacts along one strand may influence the precise pattern of contacts along the other strand because of the necessity for the duplex DNA to pass between the template and primer grips. Thus, substitutions at position 89 would affect interactions with the template strand.

It is common to visualize the precise change in DNA conformation during slippage events as a bulge in the duplex. However, for frameshifting to occur, RT must accommodate this bulge. Because an extra nucleotide can occur in either strand, specific structural ‘pockets’ should be present in both template and primer grip elements, neither pockets are observed. In fact, when we attempted modeling a slippage intermediate, a bulge could not be accommodated near the Glu89 contact, but only 5–6 bases away from the primer 3′ terminus near the end of the polymerase module part of the template cleft. Furthermore, if there was an extrahelical pocket, frameshifts mediated by HIV-1 RT should not be exclusively limited to nucleotide runs.

An alternate possibility is suggested by base sharing observed in other nucleic acid-protein interactions. For example, in MutS complexed with mismatched DNA (37), a strand containing an extra nucleotide can overwind around the other strand thus keeping the extra base within the duplex and base pairs shear. Base pair sharing (or shearing) can occur most readily in a single nucleotide repeat and for both insertion and extension steps. A similar base sharing is observed in the case of the RNA/DNA duplex containing the polyurine tract sequence when bound to the ribonuclease H domain of HIV-1 RT (38). This possibility is shown schematically in Fig. 5. Thus, base sharing would imply that Glu89 mutants vary in their ability to accommodate the kinked backbone of the template or primer DNA for −1 and +1 frameshifting events, respectively.

Multiple Effects of Glu89 Substitutions—All tested substitutions at Glu89 led to an increase in base substitution fidelity, as demonstrated by the results of dNTP exclusion assay results (Fig. 2). The pattern of changes observed among various mutants for −1 frameshifting was similar to that of base substitutions. It has been proposed that subtle varia-
tion in the conformation and/or position of the template-primer duplex can influence the dNTP-binding pocket, as shown by altered nucleoside analog sensitivity of the E89G variant (25) and the impact of P157S mutant on sequence-specific susceptibility to chain terminators (30). Our results from the dNTP exclusion assay further corroborate that Glu89, in addition to affecting frameshifting, also influences the efficiency of dNTP misinsertion consistent with the previous report of a E89G mutant (17). These results are in agreement with the notion that template-contacting residues influence the geometry of the dNTP binding pocket. The effects of Glu89 substitutions on mispair extension were milder and did not correlate with the pattern of variation observed for −1 frameshifting. Thus, the contacts by Glu89 or the nearby residues that apparently affect DNA conformation and/or positioning, do not appear to influence mispair extension frequencies to a considerable degree.

The fact that Glu89 substitutions led to decreases in both −1 frameshifting frequency and base substitution frequency, and that the trends in fidelity for Glu89 mutants were strikingly similar suggests that these distinct mutational events are linked and probably result in a particular enzyme-template-primer conformation. It appears that the status of the duplex DNA that favors primer slippage also results in lower rates of misinsertion and mispair extension and that the wild-type RT (with a Glu at this position) seems to perform more template-slippage events as well as higher rates of misinsertion and mispair extension events. It is possible that the interaction of non-Glu amino acid residues with the template-primer duplex can influence the dNTP-binding pocket, as shown by altered template-primer conformation and/or positioning.

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REFERENCES

1. Preston, B. D., Poiesz, B. J., and Loeb, L. A. (1988) Science 242, 1168–1171
2. Roberts, J. D., Bebenek, K., and Kunkel, T. A. (1988) Science 242, 1171–1173
3. Kunkel, T. A. (2004) I. Biol. Chem. 279, 16895–16898