Purifying Selection Masks the Mutational Flexibility of HIV-1 Reverse Transcriptase*

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DNA and RNA polymerases share a core architecture composed of three structurally conserved motifs: A, B, and C. Although the amino acid sequences of these motifs are highly conserved between closely related organisms, variation across broader evolutionary distances suggests that only a few residues in each motif are indispensable for polymerase function. To test this, we constructed libraries of human immunodeficiency virus type-1 (HIV-1) containing random single amino acid replacements in motif B of reverse transcriptase (RT), and we used selection in culture to assess RT function. Despite the nearly absolute constancy of motif B in vivo, virus replicating in culture tolerated a range of conservative and nonconservative substitutions at 10 of the 11 amino acid positions examined. These included residues that are invariant across all retroviral subfamilies and highly conserved in diverse retroelements. Several mutants retained wild type infectivity, and serial passage experiments revealed replacements that were neutral or even beneficial to viral fitness. In addition, a number of the selected variants exhibited altered susceptibility to the nucleoside analog inhibitors AZT and 3TC. Taken together, these data indicate that HIV-1 tolerates a range of substitutions at conserved RT residues and that selection against slightly deleterious mutations (purifying selection) in vivo masks a large repertoire of viable phenotypic variants. This mutational flexibility likely contributes to HIV-1 evolution in response to changing selection pressures in infected individuals.

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1 The abbreviations used are: RT, reverse transcriptase; RDRP, RNA-dependent RNA polymerase; HIV, human immunodeficiency virus; DMEM, Dulbecco’s modified Eagle’s medium; FFU, focus-forming units; PBS, phosphate-buffered saline; 3TC, (−)-3′-azido-3′-deoxythymidine; LTR, long terminal repeat; nt, nucleotide(s).

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positions Gly152, Lys154, Pro157, and Gln161, separate mutagenesis reactions were conducted for each mutant oligonucleotide preparation, and the products of these reactions were maintained as separate libraries and pools in subsequent cloning and virus production steps. For the remaining amino acid positions, single mutagenesis reactions were conducted by mixing equal amounts of each oligonucleotide preparation prior to mutagenesis. Products from the mutagenesis reactions were electroporated into ElectroMAX DH10B E. coli (Invitrogen), plasmids were isolated from pools of 10^4 independent transfectants, and ApaI/EcoRI fragments from the pools were cloned into pR9apoL (Fig. 2A). The resulting full-length pR9 mutant libraries were purified from pools of 10^4 independent transfectants using the Endo-Free Maxiprep kit (Qagen). Mutant plasmid libraries derived from pBS-pol,ival were additionally digested with ClaI, transformed back into E. coli DH10B, and reisolated. This step increased the proportion of mutants in the final plasmid pools from 5 to 40%. 

**Transfections**—To prepare wild type virus stocks and random virus pools, CaPO_{4} pR9 DNA coprecipitates were prepared with 10 μg of plasmid DNA as described (41) except that the 2× PBS buffer was replaced with 2× HEPES-buffered saline (270 mm NaCl, 10 mm KCl, 1.5 mm Na_{2}HPO_{4}/2H_{2}O, 11 mm dextrose, 40 mm HEPES, pH 7.05). 293T cultures were seeded into 10-cm plates and grown to 25% confluence prior to the addition of the CaPO_{4}/DNA coprecipitates. Following a 3-h incubation, culture supernatants were aspirated, 2 ml of 10% glycerol in phosphate-buffered saline (PBS) were added, and the cells were incubated at 37 °C for 15 min at 37 °C. Cells were then placed in 37 °C for 42 h after transfection, filtered through 0.4-μm syringe filters, and stored in 1-ml aliquots in the vapor phase of liquid nitrogen for subsequent analysis. Stocks of individual HIV-1 mutants were produced by transfection of 293T cells as described above, except that the glycerol shock step was omitted. Cultures were instead treated with chloroquine at a final concentration of 25 μM in DMEM immediately before adding the DNA/CaPO_{4} mixtures. Supernatants were aspirated and replaced with fresh medium 1 day later and then harvested and frozen the following day as described above. The titers of wild type stocks produced by either protocol were measured from 2× 10^4 to 10^5 focus-forming units (FFU)/ml frozen stocks and 1× 10^4 to 4× 10^6 FFU/ml fresh preparations.

**Infectivity Assay**—Single-round infectivities were determined using HeLa-P4 indicator cells (27). HeLa-P4 cells were seeded into 96-well plates at 0.5× 10^5 cells/well 1 day prior to infection. Serial dilutions of virus were prepared in DMEM containing 20 μg/ml DEAE-dextran (Sigma), and 25 μl of each dilution were added to separate culture wells. Following a 3-h incubation at 37 °C, 175 μl of DMEM were added to each well, and the plates were returned to the incubator. Stocks containing low titers were assayed in 24-well plates to increase the accuracy of the assay. In this case, each well received 2× 10^4 cells, 200 μl of medium containing the virus was added, and the cultures were returned to the incubator. Culture supernatants were directly sequenced as mixed pools without further lysis of infected cells. Sequences were performed on ice to prevent possible strand jumping. Viral RNA was precipitated with 300 μl of 100% isopropanol followed by incubation on ice for 30 min. Samples were pelleted by centrifugation at 15,500 × g, washed with 300 μl of 70% ethanol, resuspended in 20 μl distilled H_{2}O, and stored at −20 °C.

**RT-PCR**—RT-PCR was performed in thin-walled tubes (Robbins) using the Access RT-PCR system (Promega) in 50-μl reactions containing a 200 μM concentration of each dNTP, 1× mM MgSO_{4}, 5 units of avian myeloblastosis virus reverse transcriptase, 5 units of T7 DNA polymerase, 10 μl of 5× avian myeloblastosis virus/T7 buffer, 50 pmol each of primers H3 and BH1 (see below), and 1 μl of extracted viral RNA. Primers BH1 (5′-TATGGAAGCTTATAAGCCTGGTGTCCGGTGGCTTT-3′) and H3 (3′-CGGAATTCATGGATTAATTC-5′) were designed to amplify a 0.8-kb fragment corresponding to nucleotides 2597-3438 of HIV-1 NL4-3. Thermocycling conditions in an MJ Research PTC-100 thermocycler were as follows: 48 °C for 45 min, 94 °C for 2 min; then 40 cycles of 94 °C for 30 s, 60 °C for 1 min, and 68 °C for 2 min; and with 68 °C for 7 min. Control reactions lacking RT were performed and analyzed in parallel to ensure that amplification was RNA-dependent. When required, viral RNA samples were subjected to a second DNase I treatment to remove contaminating plasmid or proviral DNA (see above). RT-PCR products were sequenced either directly (to quantify fitness) or after subcloning (to identify individual mutants) using primer pNL1 (5′-CTTTACAGGAGTAGTACTGCT-3′) and Big Dye Terminator chemistry (Applied Biosystems). Molecular Cloning and Sequencing—RT-PCR products were digested with BamHI and HindIII (Promega), ligated into pBluescript II Ks− (Stratagene), and electrotransfected into ElectroMAX DH10B E. coli (Invitrogen) for blue/white screening. Individual white colonies were picked and mixed into 25 μl of RT-PCR mixture as described above, but without avian myeloblastosis virus RT, and the 0.8-kb pol fragment (nt 2597-3438) was amplified using the Access RT-PCR system (Promega) and primers H3 and BH1. Thermocycling conditions were as follows: 94 °C for 3 min; then 30 cycles of 94 °C for 30 s, 60 °C for 1 min, and 68 °C for 2 min; and ending with 68 °C for 7 min. The resulting PCR products (5 μl) were purified using QIAquick PCR purification kit (QIAGEN), resuspended in a final volume of 50 μl of distilled H_{2}O, and sequenced using primer pNL1.

**Quantitation of Relative Fitness**—RT-PCR products derived from culture supernatants were directly sequenced as mixed pools without subcloning, and the resulting chromatograms were used to calculate the relative frequency of mutant and wild-type-expressing viruses at different passage intervals. Peaks that unambiguously represented the wild type sequence were identified at specific nucleotide positions for each motif B codon (except Gly152, Trp155, and Gln161; see legend to Fig. 4B). Wild type and mutant peak heights were measured at the informative nucleotide position of each codon and used to calculate relative fitness, f_{i}/f_{W} = M_{i}/M_{W}, where f_{i}/f_{W} is the relative frequency of mutants at passage n, W represents the wild type for the wild type nucleotide, and M is the sum of all mutant peak heights at the informative nucleotide position. f_{i}/f_{W} ratios were plotted as a function of passage number, and the slopes of the resulting fitness
The HIV-1 CD4 receptor and therefore cannot be reinfected by progeny virions, enabling us to examine the infectious potential of the virus pools prior to biologic selection (see below). All cloning steps and library manipulations were conducted with >10^5 independent clones or viruses to minimize sampling bias and ensure population diversity.

Sequence analysis of 180 arbitrarily isolated clones from the Gly152, Lys154, Pro157, and Gln161 mutant libraries confirmed that the targeted motif B positions contained a diverse array of amino acid substitutions (Fig. 5 and data not shown). Of the 20 possible amino acids, codons for 12, 16, 17, and 14 were observed in this sampling of the Gly152, Lys154, Pro157, and Gln161 mutant pools, respectively (average = 15). Our approach limited but did not exclude wild-type codons from the libraries, which were present at frequencies of 5–12%. Less extensive sequencing of the other libraries revealed similar mutant diversity and wild-type levels (data not shown). Thus, we estimate that the 11 mutant libraries together represent a minimum of 150 different mutations in motif B.

**HIV-1 Tolerates Mutations at Conserved Residues in Motif B**—To determine the effects of motif B mutations on virus infectivity, each of the mutant HIV-1 pools were titered on HeLa-P4 indicator cells (Fig. 3A). Randomization of positions Pro150 or Gly152 resulted in a dramatic reduction in infectivity (0.2 and 0.1% of wild-type, respectively), indicating that these residues are essential for HIV-1 replication. In contrast, pools randomized at positions Leu149, Gln151, or Trp156 showed low but significant levels of infectivity (1% of wild type), and the remaining six mutant populations exhibited 15–70% of wild-type infectivity, indicating that these pools contained a high proportion of replication-competent variants.

Replication-competent mutants were selected from the random virus pools by subjecting the pools to a single passage in HeLa-P4 cells (Fig. 2B). The spectra of infectious variants in these selected populations were then determined by sequencing individual clones derived from each mutant pool (Fig. 3B). Among 368 clones sequenced from the passage 1 pools, 345 (95%) were replication-competent as evidenced by their persistence in subsequent passages and/or infectivity as purified mutant virus (see below). Altogether, 53 different amino acid substitutions in motif B supported virus replication. Two categories of sites were identified based on the range of substitu-
tions observed in the passage 1 populations. The first group, represented by 7 of the 11 motif B positions examined (Val 148, Leu 149, Gln 151, Lys 154, Gly 155, Pro 157, and Gln 161), tolerated both conservative and nonconservative amino acid substitu-
tions. The second group (Pro 150, Trp 153, and Ser 156) tolerated only a limited range of replacements. Only one residue (Gly 152) failed to yield any replication-competent variants.

Relative Fitness of Motif B Variants—To further quantify the effect of motif B mutations on HIV-1 replication, we performed serial passages of each mutant virus pool (Fig. 2B, right) and determined the relative frequencies of variant and wild type progeny virus at several passage intervals (Fig. 4). This provides a measure of viral “fitness” (44), here defined as the overall ability of HIV-1 variants to survive and replicate in HeLa-P4 cells relative to wild type HIV-1.

Substitutions in motif B were generally deleterious to viral fitness, as evidenced by the progressive loss of sequence diversity (Fig. 4A) and emergence of wild type virus in most of the

Fig. 3. Mutations in motif B support viral replication in culture. A, relative infectivities of the random virus pools. Titers from HeLa-P4 cultures were normalized to the amount of HIV-1 capsid p24 in the supernatant to determine the infectivity of each mutant pool relative to wild type virus. See Table I and “Experimental Procedures” for details. B, motif B variants identified in the random pools following a single passage in culture. The subscripts indicate the number of clones containing wild type (top line) or variant sequences (lower lines) and are the combined results of all mutant pools (see “Experimental Procedures” for pool descriptions). The black letters indicate mutants shown to be replication-competent based on their persistence in subsequent passages and/or infectivity in the single cycle assay (Table I and data not shown). Mutants in gray letters were detected only in passage 1 supernatants and were not assayed for replication competence. However, most of these mutants are probably infectious, because they were present at frequencies similar to other replication-competent mutants in the passage 1 pools. K154Q was absent in passage 1 but detected in passage 2 (1 of 32 clones sequenced).

Fig. 4. Relative fitness of motif B variants. A, sequencing chromatograms showing changes in the relative frequencies of wild type and mutant genotypes during serial passage. Viral RNA in culture supernatants was amplified by RT-PCR, and the products were analyzed by automated DNA sequencing. The wild type nucleotide sequence for each randomized codon is shown in parentheses. Green, black, red, and blue peaks correspond to A, G, T, and C, respectively. Data are shown in order of increasing variant fitness (top to bottom). Data for Lys 154 are from a pool randomized with primer 154BNN as described under “Experimental Procedures.” B, fitness vectors for motif B variants. The slope of each vector indicates the rate of change in the proportion of mutant HIV-1 genotypes relative to wild type during serial passage of a single random virus pool (Fig. 2B). Each pool contains a mixture of wild-type HIV-1 and mutant viruses with substitutions at the indicated RT residue (see Fig. 3B). The relative mutant frequencies in each population (except Gln 161) were determined from chromatogram peak heights (see Fig. 3A) as described under “Experimental Procedures.” Variants for Gln 161 were identified by sequencing 25–54 clones per passage (see Fig. 5), since none of the chromatogram peaks at this codon could be unambiguously assigned to the wild type sequence. Outgrowth of wild type virus in the Gly 152 and Trp 153 pools was too rapid to measure relative variant fitness. The neutral case (slope = 0) is shown with a dotted line.
random mutant pools during the course of serial passage (Fig. 4B). Similar rates of wild type outgrowth were observed in independent serial transfers of Lys\textsuperscript{154} mutant pools, illustrating the reproducible nature of this assay. Sequencing of individual clones from each passage of the Lys\textsuperscript{154}, Pro\textsuperscript{157}, and Gln\textsuperscript{161} mutant pools confirmed a steady increase in the proportion of wild type virus and the persistence of variants at low levels in these populations through passage 4 (Fig. 5, A–C).

Although there was a general trend toward reduced fitness, several mutant pools exhibited slow or undetectable outgrowth of wild type virus (Fig. 4). Only a gradual increase in the relative proportion of wild type virus was apparent in the Lys\textsuperscript{154} and Gln\textsuperscript{161} pools, whereas no wild type outgrowth was detected in the pool randomized at position Val\textsuperscript{148} (vector slope not statistically different from zero; \( p = 0.42 \), F test). Thus, several amino acid substitutions at Val\textsuperscript{148} were neutral under these culture conditions. Surprisingly, a single alanine variant emerged from the Ser\textsuperscript{156} random pool that increased in frequency relative to wild type during the course of serial passage. The positive slope of the resulting fitness vector indicates that the S156A mutation improved the relative replication rate of HIV-1 in HeLa-P4 cell culture (slope \( 0.05 \pm 0.005; p < 0.001 \), F test).

To determine whether sampling errors or the presence of wild type virus affected variant selection in our protocol, we performed a second serial passage experiment using a Lys\textsuperscript{154} mutant pool that contained no detectable wild type sequences (<1%). After 13 passages, wild type virus remained undetectable (0 of 27 clones sequenced) (Fig. 5C, bottom), indicating that Lys\textsuperscript{154} variants replicate in HeLa-P4 cells as a relatively stable mutant virus population. Sequencing of individual clones from passages 1–4 showed that the spectrum of replication-competent mutants in this second experiment (Fig. 5C, bottom) was similar to that observed in the original experiment (Fig. 5C, top). Thus, sampling errors did not contribute significantly to the outcome of these serial passage experiments, and the presence of wild type virus did not influence the diversity of emergent genotypes. Mutagenesis strategies that stringently exclude wild type sequences are required to perform similar analyses at other amino acid positions.

**Motif B Variants Exhibit Unique Phenotypes**—To characterize the phenotypic properties of variants selected from the virus pools, we first examined the relative infectivities of individual mutants selected in passage 1 (Fig. 3B and Table I). We tested 23 different mutant clones, each containing a single amino acid replacement in motif B. These were constructed by site-directed mutagenesis or by subcloning PCR products that contained only the desired RT mutation (confirmed by DNA sequencing). Among the 23 mutant clones tested, only one (L149E) failed to produce infectious virus (data not shown). Approximately half of the viable mutants (12 of 22 clones)
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TABLE I
Infectivities and drug sensitivities of motif B variants

| Variant          | Relative infectivitya | EC50b (μM) | 3TC (μM) |
|------------------|-----------------------|------------|----------|
| WT               | 1.00 ± 0.00 (1)       | 0.017 ± 0.01 (0.1) | 0.09 ± 0.1 (1) |
| V148R            | 2.2 ± 1               | 0.017 ± 0.01 (0.1) | 2.1 ± 0.6 (2) |
| Q151A            | 94 ± 30               | 0.013 ± 0.005 (0.1) | 3.4 ± 0.6 (4) |
| Q151V            | 4.3 ± 1               | 0.0028 ± 0.0005 (0.02) | 9.3 ± 1.1 (10) |
| Q151M            | 61 ± 13               | 0.64 ± 0.04 (4) | 1.4 ± 0.3 (2) |
| K154S            | 19 ± 3                | 0.035 ± 0.002 (0.2) | 0.77 ± 0.4 (1) |
| K154R            | 79 ± 6                | 0.14 ± 0.05 (1) | 1.9 ± 0.1 (2) |
| G155N            | 100 ± 9               | 0.053 ± 0.008 (0.2) | 0.29 ± 0.1 (0.3) |
| P157G            | 67 ± 16               | 0.056 ± 0.02 (0.3) | 3.7 ± 0.5 (4) |
| P157S            | 87 ± 20d              | 0.078 ± 0.02 (0.5) | 5.9 ± 0.9 (7) |
| Q161G            | 75 ± 8                | 0.025 ± 0.003 (0.1) | 1.1 ± 0.2 (1) |
| Q161E            | 60 ± 20               | 0.10 ± 0.03 (0.5) | 5.3 ± 1.2 (6) |
| M184V            | 120 ± 30              | 0.049 ± 0.007 (0.3) | >100 (100) |
| Q151M complexa   | 70 ± 7                | >10 (>100) | 6.3 ± 2.4 (7) |

a Relative infectivity was determined on HeLa-CD4-LTR-lacZ (HeLa-P4) cells as FFU/ng of p24 antigen. Values are the averages ± S.D. of three or more independent experiments, with two determinations of titer and one determination of p24 concentration per experiment. WT, wild type.

b Drug susceptibility was determined by measuring the dose-dependent reduction of infected foci in HeLa-P4 microcultures. Values are the means ± S.E. of three or more independent experiments, with five or more drug treatments (plus untreated control wells) performed in duplicate per experiment. EC50 values were obtained by linear regression of the resulting dose-response curves. The numbers in parentheses indicate EC50 values relative to wild type. Values in boldface type indicate variants with >4-fold increases or decreases in EC50.

c Infectivity value not significantly different from wild type (p > 0.05, analysis of variance).

d Previously published (65).

Multinucleoside-resistant mutant Q151M/A62V/V75I/F77L/F116Y (46).

Retained at least 50% of wild type infectivity, and several of these were not statistically different from wild type (Table I, column 2, and data not shown). Only two variants (V148R and Q151V) exhibited substantial replication defects (<5% of wild type infectivity). Thus, the majority of mutants observed in the passage 1 pools retained relatively high replication capacity and did not require second-site mutations for this viability.

To further evaluate the phenotypes of the selected variants, we determined their susceptibilities to two nucleoside analog inhibitors of HIV-1 RT that function as chain terminators of DNA synthesis (45): AZT and 3TC (Table I, columns 3 and 4). In agreement with previous studies (46, 47), the Q151M mutation in RT conferred low level resistance to AZT, and the addition of four other amino acid substitutions to the Q151M background (A62V, V75I, F77L, and F116Y) increased the level of resistance >100-fold. Other substitutions at Gln151 and mutations at Val148, Lys154, Gly155, and Gln161 had the opposite effect, resulting in 4–50-fold hypersensitivity to AZT. We also observed substitutions that affected virus susceptibility to 3TC. Replacements at Gln151, Pro157, and Gln161 increased virus resistance to 3TC 5–10-fold, whereas G155N conferred a slight (3-fold) hypersensitivity to the drug.

In total, mutations at 7 of the 11 motif B sites analyzed resulted in a significant change in EC50 for AZT or 3TC. Three of the Gln151 substitutions (Ala, Val, and Ile) conferred both AZT hypersensitivity and 3TC resistance. Taken together, these data indicate that many residues in RT motif B influence nucleoside analog susceptibility.

DISCUSSION

Motifs A, B, and C form a core structure conserved in all RTs and RNA-dependent viral polymerases (Fig. 1A) (2–6). Although the amino acid sequences of these motifs are conserved within a particular viral family, alignments of sequences from evolutionarily distant RNA viruses suggest that only a few residues in each motif are absolutely indispensable for polymerase function and viral replication (Fig. 1B). To test this idea, we performed random mutagenesis of motif B in HIV-1 RT and identified the range of replacements that preserve viral replication in culture (Fig. 2). Replication-competent viruses selected from the random pools contained amino acid substitu-
Mutational flexibility of conserved structural elements is a general property of polymerases from diverse organisms.

Similar to the in vivo situation, we observed that amino acid substitutions in motif B were generally deleterious to viral fitness (Fig. 4). We note, however, that most of the mutants retained 50% or more of wild type infectivity (Table I and data not shown), and randomization only slightly impacted the relative fitness of several mutant pools (Fig. 4). The Leu149Gln, Val151Lys, Gly155Pro, and Gln161Phe pools exhibited rates of variant loss relative to wild type virus of <4% per replication cycle (assuming a wild type replication rate of 1–2 days–1) (/2). These data suggest that motif B variants would also replicate in vivo, based on previous studies of patient-derived isolates (45, 50). Perhaps most striking were the observations that several Val149 substitutions appear neutral and that the S156A mutant replicates faster than wild type virus in culture (0.5–1% S156A outgrowth per replication cycle). There are other examples of HIV-1 mutants replicating better than wild type in culture, although the majority of these involve multiple substitutions in RT or protease (51–54). We conclude that a number of RT motif B mutations are only slightly deleterious for HIV-1 replication and that some are nearly neutral or even adaptive.

Mutations throughout motif B conferred significant changes in the susceptibility of HIV-1 to the nucleoside analog inhibitors AZT and 3TC (Table I). Generally, these involved hypersensitivity to AZT or resistance to 3TC; mutants Q151A and Q151V exhibited both phenotypes. Our observation that Gln151 mutations result in AZT hypersensitivity was unexpected, since a methionine substitution at this site confers weak AZT resistance in clinical isolates, and the combination of four additional mutations with Q151M results in high level resistance to AZT and several other nucleoside analogs (46, 47) (Table I). Analyses of purified RTs show that the Q151M and Q151N mutations confer resistance to nucleoside analogs as well as mild to moderate increases in the fidelity of nucleotide incorporation in cell-free assays (11, 55, 56). Substitutions of alanine or asparagine at the equivalent position of murine leukemia virus RT (Gln190) also confer increased fidelity but render the enzyme hypersensitive to dideoxynucleoside triphosphates (57). Thus, mutations at this position have similar effects on HIV-1 and murine leukemia virus RT fidelity but opposite effects on nucleoside analog susceptibility, suggesting that differences in local protein environment modulate the effects of Gln151 substitutions. Our data also show that Gln151 mutations can either increase or decrease drug sensitivity, depending on the nature of the amino acid substitution and the structure of the drug (Table I) and that some Gln151 variants are hypersensitive to a number of nucleoside inhibitors.2 Studies of purified mutant RTs are required to identify biochemical mechanisms that contribute to these viral phenotypes (45, 58).

In patients receiving antiretroviral therapy, the development of nucleoside analog resistance is commonly associated with specific mutations in motifs A–D or the “fingers” region of RT (4, 59). The discovery of a significant number of replication-competent mutants with altered drug sensitivity in our relatively small survey of RT variants (encompassing only 11 amino acid residues) suggests that the range of resistance mutations observed clinically represents a fraction of the total number of mutations with the potential to confer drug resistance. By extension, it is likely that other HIV-1 proteins exhibit similar mutational flexibility and that a large repertoire of viable phenotypic variants continuously arise in replicating HIV-1 populations in vivo.

The mutational flexibility of RT has important implications for the evolution of HIV-1 variants. Although variation at conserved RT positions is rarely observed in isolates from infected patients (see above), the persistence of variants during serial passage in culture (Figs. 4 and 5) suggests that motif B and other RT mutants are present at low frequencies in the complex mixtures of variants that comprise HIV-1 populations in vivo (44). Components of these mutant “swarms” or “quasispecies” can emerge as the dominant member of a population as a result of changing environmental demands (52, 44, 60). This is clearly evident in the development of resistance to antiviral therapy, where positive selection often results in the outgrowth of rare, drug-resistant variants that preexist in drug-naïve patients (42, 50, 61–64). There is accumulating evidence that random genetic drift also contributes to HIV-1 RT variation (61), and our observation that S156A mutants replicate faster than wild type virus in culture (whereas the reverse must be true in vivo) demonstrates the importance of slightly deleterious mutations when negative selection barriers are lifted or changed (23). The fixation of new RT variants in HIV-1 populations by all of these mechanisms (positive selection, genetic drift, and relaxed negative selection) hinges on the ability of the virus to tolerate RT mutations without severe fitness loss. The plasticity of RT observed in our study suggests that substitutions at the majority of amino acid sites are only slightly deleterious to virus replication, underscoring the tremendous evolutionary potential of HIV-1 RT.

In summary, we show that a conserved region of HIV-1 RT tolerates a number of mutations that preserve virus replication capacity. Several of the variants selected in culture retain sufficient infectivity to support viral replication in vivo (42, 50). A number of the selected mutants also exhibit significant hypersensitivity to AZT and/or resistance to 3TC, indicating that many sites in motif B contribute to nucleoside analog susceptibility. We conclude that, in addition to HIV-1 population size, diversity, and turnover (60), the plasticity of RT is an important contributor to escape from selective pressures in infected individuals.

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