Increased Polymerase Fidelity of E89G, a Nucleoside Analog-Resistant Variant of Human Immunodeficiency Virus Type 1 Reverse Transcriptase

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Nucleoside analog resistance in human immunodeficiency virus type 1 results from mutations in reverse transcriptase (RT) that allow the enzyme to discriminate against such analogs. To evaluate the possible impact of such mutations on the ability of human immunodeficiency virus RT to selectively incorporate Watson-Crick base-paired deoxynucleotide triphosphates (dNTPs) over incorrectly paired dNTPs, we have measured the fidelity of dNTP insertion by the E89G variant of RT in *in vitro* reaction mixtures containing synthetic template primers. The E89G RT was previously shown to be resistant to several ddNTPs and to phosphonoformic acid. Our results show that the mutant enzyme displays a lower level of efficiency of misinsertion than did the wild-type RT for every mispair tested (ranging from 2- to 17-fold).

The appearance of drug-resistant variants of human immunodeficiency virus (HIV) has impeded antiviral therapies aimed at reducing the viral burden in patients afflicted with AIDS (18, 19, 33). The generation of variants of HIV, facilitated by an overall low polymerase fidelity of viral reverse transcriptase (RT) (12, 29, 30, 32, 34, 41), provides a pool from which resistant variants can readily be selected during antiviral therapy. A means to control the rate of genetic variation in HIV, which is currently unavailable, might reduce the frequency or delay the appearance of drug-resistant viruses in AIDS patients undergoing therapy.

Nucleoside analogs, together with nonnucleoside inhibitors, comprise the anti-HIV-1 RT drugs in vogue. Examples of nucleoside analog drugs include 2', 3'-dideoxy-3'-azidothymidine (AZT [zidovudine]), 2', 3'-dideoxycytidine (ddC), 2', 3'-dideoxyinosine (ddI), 2', 3'-dideoxy-2', 3'-didehydrothymidine (d4T), and (–) 2' 3'-dideoxy-3'-thiacytidine (3TC). These inhibitors compete with the deoxynucleotide triphosphate (dNTP) substrate (7, 14, 31). Mutations that decrease susceptibility do so by altering the ability of the enzyme to bind or incorporate nucleoside analogs, while minimally affecting the incorporation of normal dNTP substrates (18, 38).

The ability of nucleoside analog-resistant RTs to preferentially incorporate dNTP substrates in the presence of a competitive analog is suggestive of a pocket better suited to bind the normal dNTP substrate. In an extension of this concept, one can view a correctly base-paired dNTP as a normal substrate and incorrectly base-paired dNTPs as analogs. Thus, with an ability to selectively incorporate dNTPs, some of the nucleoside analog-resistant RTs are likely to polymerize with a greater fidelity by favoring the insertion of the correct dNTPs. To examine this hypothesis, a nucleoside analog-resistant variant of HIV-1 RT, the E89G RT, was employed. The E89G variant is a ddGTP-resistant mutant first identified by phenotypic screening in bacteria (27, 28) and subsequently observed in a 3TC-resistant HIV variant isolated under selective pressure in cell culture (11). This mutant is also cross-resistant to

several dNTP analogs, including ddTTP, ddCTP, ddATP, AZTTP, 3TCTP, and phosphonoformic acid (28). In this communication, we report results showing that in addition to its decreased sensitivity to dNTP analogs, the E89G variant of HIV-1 RT displays a higher fidelity of dNTP insertion than the wild type.

Methods available to study the *in vitro* fidelity of HIV-1 RT include amber codon reversion assays (30, 40), *lacZ* forward mutation assays (2, 4, 34), and kinetic assays for nucleotide misinsertion (29, 30, 32, 41) or mispair extension (25, 41). We have used a gel-based steady-state kinetic assay described by Goodman and colleagues (5, 22, 23, 26) because it allows determination of misinsertion efficiency (f_{ins}) for all mispairs without the need to perform large-scale sequencing. Nucleotide insertion reactions, in which a 5'-³²P-labeled DNA primer is extended by the addition of a dNTP, were performed with purified HIV-1 wild-type RT or the E89G RT in the presence of a single dNTP. The recombinant, purified wild-type and E89G mutant RTs (15, 21) used were nuclease free and had a specific activity of approximately 800 U/mg of protein each [1 U is defined as 1 nmol of dTTP incorporated into poly(rA)·oligo(dT) in 10 min at 37°C]. The primers used in these reactions (Fig. 1) had partial homology to the cognate primer for HIV RT, tRNA_{Lys}³ and were hybridized to an oligodeoxynucleotide (45-mer) corresponding to the sequence around the primer binding site (PBS) of the HIV-1 genome. (Primers of variable lengths were used to measure misinsertion opposite all four template bases.) The oligodeoxynucleotides used as the template and primers were purified as described before (39). The primers were end labeled with ³²P with T4 polynucleotide kinase (40 U) (New England Biolabs) and [γ -³²P]ATP (3,000 Ci/mmol). The primed templates were generated by combining 74 pmol of ³²P-end-labeled primer with 94 pmol of template in 50 mM Tris-Cl (pH 8.0)–50 μ g of acetylated bovine serum albumin–2 mM β -mercaptoethanol (total volume, 1 ml), heating the mixture to 100°C for 3 min, and allowing the mixture to slowly cool to room temperature over 60 min. The annealed status of the primers was confirmed by their complete conversion into extended product (data not shown).

The misinsertion reactions, performed according to the procedures described by Goodman and colleagues (5, 22, 23, 26), were initiated by combining equal volumes (5 μ l) of enzyme-

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PBS-G	3' ACCGCGGGCTTGTCCCTGGACTTTCGC5' 5' GTGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACCTGAAAGCG3'	45/27
PBS-A	3' CACCGCGGGCTTGTCCCTGGACTTTCGC5' 5' GTGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACCTGAAAGCG3'	45/28
PBS-C	3' TCACCGCGGGCTTGTCCCTGGACTTTCGC5' 5' GTGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACCTGAAAGCG3'	45/29
PBS-T	3' TCGTCACCGCGGGCTTGTCCCTGGACTTTCGC5' 5' GTGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACCTGAAAGCG3'	45/32

FIG. 1. Sequences of the oligonucleotides used as template-primer pairs. Oligonucleotides used in the determination of the K_m and $V_{max, rel}$ for the insertion of dNTPs opposite G, A, C, and T (indicated by boldface letters) are shown. The template length is constant (45-mer), and the primers are of variable lengths.

template-primer solution and dNTP-salts solution (dNTP at a concentration of 0 to 10 mM in 160 mM KCl–100 mM Tris-Cl [pH 8.0]–20 mM dithiothreitol–12 mM MgCl₂). The enzyme-template-primer solution contained purified RT at enzyme concentrations of 0.21, 4.2, or 36 nM, corresponding to enzyme/template primer molar ratios of 1:170, 1:8.5, or 1:1, respectively (in general, an excess of template primer was used to ensure that all enzyme molecules were in a bound state). A set of reactions was carried out at 37°C with increasing concentrations of dNTP for an empirically determined reaction time to allow the conversion of about 25% of the primer to extended products. Reactions were terminated by the addition of 30 μ l of stop solution (95% formamide, 20 mM EDTA). Two to 8 μ l of boiled terminated reaction mixture was loaded onto a 12 to 18% polyacrylamide–urea gel and electrophoresed for 1.75 to 2.5 h at 30 W. The gels were then autoradiographed within the linear response range of the film (Fig. 2). From the densitometric quantitations of unextended and extended primers from each reaction set, the initial velocities of product formation were plotted against the dNTP concentrations. The initial, linear portion of the curve was employed to obtain accurate relative V_{max} ($V_{max, rel}$) and K_m values via the use of Cleland's FORTRAN program (8). The enzyme concentration factor was taken into account when the V_{max} was calculated, since variable amounts of the enzyme were used, depending upon the variation in the rates of formation of different mispairs. The f_{ins} for each of the 16 possible insertion events, i.e., 4 correct base pairs and 12 mispairs, was then derived from these $V_{max, rel}$ and K_m values as described before (5, 22, 23, 26, 39).

Nucleotide misinsertion rates are important parameters contributing to the overall polymerase fidelity of HIV-1 RT, especially since HIV-1 RT has no proofreading activity. Through kinetic analysis of reactions leading to the formation of all 12 mispairs by the two enzymes, measured at the same sites under the same reaction conditions, we were able to discern the relative fidelities of wild-type and E89G RTs. As evident from the f_{ins} values, the E89G mutant displays a greater fidelity of insertion than does the wild-type HIV-1 RT (Tables 1 and 2 and Fig. 3). While a higher fidelity was observed in the formation of every mispair tested, a much higher increase in insertion fidelity was observed in the formation of certain mispairs (e.g., T·G, T·T, and G·A were formed 11- to 17-fold less efficiently than in the wild type). It is interesting to note that the increase in fidelity of the E89G mutant was achieved either by increasing the K_m for the dNTP being misinserted and/or by decreasing the V_{max} . For example, K_m discrimination was the basis for higher fidelity for some mispairs (e.g., A·G and A·A mispairs) and V_{max} discrimination (a lower $V_{max, rel}$ for the mispair formation) for other mispairs (e.g., T·G and C·T mispairs), while the increase in fidelity of a third set of mispairs (e.g., A·C, T·C, T·T, and G·A) was achieved by both K_m

and V_{max} discrimination. The overall increase in fidelity displayed by the E89G RT over that of the wild type, determined by a comparison of the average misinsertion efficiencies, was found to be 6.25-fold. The range of misinsertions by the E89G RT was 3.2×10^{-3} to 1.2×10^{-5} , while that of wild type was 1.9×10^{-2} to 5.7×10^{-5} .

The relative f_{ins} for various mispairs by the wild-type HIV-1 RT have been established with other template sequences (29, 32, 41). For example, the misinsertion of dATP and dCTP opposite template base A appears to be more efficient than the misinsertion of dGTP (29). Similarly, the insertion of dTTP opposite G (G·T mispair) is known to be much more efficient than that of either dGTP or dATP (G·G or G·A). The patterns of misinsertion efficiencies by wild-type HIV-1 RT observed here (Fig. 3) are in close agreement with data reported by others. Furthermore, the E89G mutant also displays generally the same relative efficiencies of misinsertion as the wild type for various mispairs, even though the overall rates at which it misinserts are significantly reduced (Table 1 and Fig. 3).

Insights into the molecular basis of the decreased susceptibility of the E89G variant of HIV-1 RT to nucleoside analogs have come from biochemical studies of recombinant mutant enzymes (6, 15, 37) and X-ray structural information about the wild-type enzyme (6, 13, 16). For example, structural studies of wild-type HIV-1 RT complexed with double-stranded DNA show that the E89 residue makes contact with the template DNA at the penultimate base pair near the 3'-hydroxyl of the primer DNA (13). Molecular modeling, mutagenesis, and biochemical studies suggested that the glycine substitution at residue 89 induces subtle changes in the geometry of the dNTP-binding pocket via interactions with the template (6, 15, 37). The basis for decreased susceptibility to nucleoside analogs caused by several of the mutations appears to involve interactions with the template primer which modulate dNTP binding or directly influence the incoming dNTP. The E89G and the ddI resistance mutation L74V are thought to alter susceptibility via template repositioning (6, 37); the M184V alteration responsible for 3TC resistance may affect interactions of RT with the primer terminus and incoming dNTP (38), and the position of the Q151M alteration (found in patients receiving combination therapy with ddI-AZT or ddC-AZT (35, 36) and

FIG. 2. Autoradiogram showing the effect of dNTP concentration on the rate of misinsertion by wild-type (WT) and E89G mutant RTs. The reactions were run as described in the text, and 6 μ l of the reaction mixture was separated on a 16% polyacrylamide–urea gel. The template-primer used was PBS-G (Fig. 1). Note that the correct dNTP (dCTP) is in the micromolar range and the incorrect dNTP (dTTP) is in the millimolar range.

TABLE 1. $V_{\max, \text{rel}}$, K_m , and f_{ins} for the wild-type^a and E89G RTs

Base pair ^b	$V_{\max, \text{rel}}$ ^c (%/min)		K_m ^c (μM)		f_{ins} ^d	
	WT	E89G	WT	E89G	WT	E89G
A · T	3.90 ± 1.00*	6.20 ± 0.19*	3.20 ± 0.55*	4.20 ± 0.13*	1	1
A · C	1.05 ± 0.01	0.52 ± 0.10	810 ± 57.20	2600 ± 590	1.10 × 10 ⁻³	1.30 × 10 ⁻⁴
A · G	0.02 ± 0.00	0.03 ± 0.00	150 ± 31	1000 ± 0.01	1.10 × 10 ⁻⁴	2.00 × 10 ⁻⁵
A · A	1.10 ± 0.02	1.50 ± 0.24*	620 ± 140	4400 ± 2500*	1.40 × 10 ⁻³	2.30 × 10 ⁻⁴
T · A	5.20 ± 0.05	5.70 ± 0.07	0.77 ± 0.11	1.60 ± 0.14	1	1
T · G	1.96 ± 0.28	0.08 ± 0.00	490 ± 70	640 ± 35	5.90 × 10 ⁻⁴	3.50 × 10 ⁻⁵
T · C	0.67 ± 0.22	0.10 ± 0.03	1100 ± 220	2400 ± 70	9.00 × 10 ⁻⁵	1.20 × 10 ⁻⁵
T · T	0.63 ± 0.08	0.10 ± 0.00	420 ± 80	1500 ± 190	2.20 × 10 ⁻⁴	1.90 × 10 ⁻⁵
G · C	2.70 ± 0.21	11.10 ± 0.01	1.20 ± 0.26	3.80 ± 1.20	1	1
G · T	6.70 ± 2.70	5.10 ± 1.80	160 ± 45	540 ± 170	1.90 × 10 ⁻²	3.20 × 10 ⁻³
G · A	0.94 ± 0.09	0.20 ± 0.03	710 ± 200	1800 ± 20	5.90 × 10 ⁻⁴	3.80 × 10 ⁻⁵
G · G	0.24 ± 0.05	0.22 ± 0.08	350 ± 60	510 ± 30	3.00 × 10 ⁻⁴	1.50 × 10 ⁻⁴
C · G	3.40 ± 0.57	3.20 ± 0.26*	0.43 ± 0.12	0.97 ± 0.25*	1	1
C · A	0.68 ± 0.09	0.14 ± 0.02*	330 ± 30	740 ± 110*	2.60 × 10 ⁻⁴	5.70 × 10 ⁻⁵
C · T	0.23 ± 0.04*	0.05 ± 0.01	510 ± 110*	770 ± 25	5.70 × 10 ⁻⁵	2.00 × 10 ⁻⁵
C · C ^e	ND	ND	ND	ND	ND	ND

^a Data for wild-type (WT) RT are reproduced from reference 39 to permit comparison.^b Base pairs are shown with the template base first.^c Both $V_{\max, \text{rel}}$ and K_m are relative values. The standard deviations presented are derived from two or three independent measurements. Values obtained from three independent measurements are indicated by an asterisk.^d Evaluated from $V_{\max, \text{rel}}/K_m$ ratios as detailed in reference 22.^e The misinsertion of dCTP opposite template C was undetectable (ND) under the conditions used.

shown to confer resistance to multiple dNTP analogs) suggests that the mutation may exert its effect via contact with the first template nucleotide ahead of the 3'-hydroxyl of the primer. Since the structural elements that form the dNTP-binding pocket must largely overlap those promoting base pairing between the incoming dNTP and the template, the changes induced by such mutations could also affect fidelity. The increased dNTP insertion fidelity indicated by our results for the nucleoside analog-resistant variant E89G supports this view. Recently, we and others have demonstrated an increased fidelity for the M184V RT in assays similar to those used here (24, 39). Studies to determine the influence of substitutions L74V and Q151M are currently in progress.

The influence of mutations that affect enzyme-template-

primer interactions on the fidelity of polymerization by HIV-1 RT is further supported by studies reported by Wilson and Kunkel and their coworkers (1, 3). Variant RTs with substitutions in the residues forming the primer grip displayed elevated dissociation rate constants for template primer, a decreased sensitivity to inhibition by AZTTP, and reduced fidelity for template-primer slippage errors.

Changes in fidelity are not associated with all mutations that confer nucleoside analog resistance. For example, RT mutations leading to viral AZT resistance (that appear during therapy) do not cause any changes in the fidelity of the mutant RTs (17). This is in agreement with the fact that not all of the affected residues are at the active site (6, 38) and that no biochemical resistance is observed with such mutants (17, 20).

The extent to which the increases in fidelity measured here in vitro will affect the mutation rate in vivo are currently not clear. In addition to misinsertions, mutations can also be established by mispair extension or slippage (3, 4, 10, 41). The influence of the E89G mutation on these types of errors is not known at present. Thus, the increase in fidelity reported here could be a minimal estimate. The in vivo mutation rate will also be affected by the relative rate of replication and fitness of the mutant virus (9). Most drug-resistant viruses are thought to display suboptimal fitness compared with the wild-type virus (9), which could translate into slower rates of replication despite a selective value in the presence of the drug. Therefore, we speculate that the time required for the outgrowth of a new variant from a relatively homogeneous population (with respect to the genotype of RT) of increased-fidelity, drug-resistant virus should be considerable. This is in fact supported by a substantial delay seen in the outgrowth of drug-resistant variants during replication of the M184V variant under selective pressure in cell culture (39).

Moreover, our results with E89G and previous studies with M184V (39) mutants of HIV-1 RT suggest that drug-resistant variants of HIV could serve a beneficial role in anti-HIV ther-

TABLE 2. Fold increases in the insertion fidelity of mutant RTs

Mispair	Fold increase in insertion fidelity of E89G RT ^a
A · C	8.5
A · G	5.5
A · A	6.1
T · G	16.8
T · C	7.5
T · T	11.6
G · T	5.9
G · A	15.5
G · G	2.0
C · A	4.6
C · T	2.8
C · C	ND ^b

^a The decrease in the f_{ins} s by E89G RT over those by the wild type was calculated from data in Table 1.^b ND, not determined.

FIG. 3. Graphic representation of f_{ins} s of wild-type and E89G RTs. A comparative plot of f_{ins} s experimentally determined for 11 of the 12 possible mispairs for wild-type and E89G RTs (from Table 1) is shown.

apeutics. For example, in patients undergoing long-term treatment, the drug-resistant virus typically constitutes the majority in the virus population in that patient. If the drug-resistant mutant is indeed of a higher polymerase fidelity, it affords two types of possible benefit to the patient. On the one hand, the lack of variation might slow down the rate of production of immunological escape mutants, allowing the immune system to raise an effective antiviral response. On the other hand, if, after confirming that the variant HIV is indeed the major species, a second drug directed to a different viral component (such as an antiprotease drug) is administered, the virus population would be both sensitive to the new drug and armed with an increased-fidelity polymerase. The low mutagenic frequency of the viruses should limit the rapid development of resistance to the new drug.

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