

# Molecular Mechanisms of Resistance to Human Immunodeficiency Virus Type 1 with Reverse Transcriptase Mutations K65R and K65R+M184V and Their Effects on Enzyme Function and Viral Replication Capacity

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**Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) resistance mutations K65R and M184V result in changes in susceptibility to several nucleoside and nucleotide RT inhibitors. K65R-containing viruses showed decreases in susceptibility to tenofovir, didanosine (ddI), abacavir, and (–)-β-D-dioxolane guanosine (DXG; the active metabolite of amdoxovir) but appeared to be fully susceptible to zidovudine and stavudine in vitro. Viruses containing the K65R and M184V mutations showed further decreases in susceptibility to ddI and abacavir but increased susceptibility to tenofovir compared to the susceptibilities of viruses with the K65R mutation. Enzymatic and viral replication analyses were undertaken to elucidate the mechanisms of altered drug susceptibilities and potential fitness defects for the K65R and K65R+M184V mutants. The relative inhibitory capacities ( $K_i/K_m$ ) of the active metabolites of tenofovir, ddI, and DXG were increased for the RT containing the K65R mutation compared to that for the wild-type RT, but the relative inhibitory capacity of abacavir was only minimally increased. For the mutant viruses with the K65R and M184V mutations, the increase in tenofovir susceptibility compared to that of the mutants with K65R correlated with a decrease in the tenofovir inhibitory capacity that was mediated primarily by an increased  $K_m$  of dATP. The decrease in susceptibility to ddI by mutants with the K65R and M184V mutations correlated with an increase in the inhibitory capacity mediated by an increased  $K_i$ . ATP-mediated removal of carbovir as well as small increases in the inhibitory capacity of carbovir appear to contribute to the resistance of mutants with the K65R mutation and the mutants with the K65R and M184V mutations to abacavir. Finally, both the HIV-1 K65R mutant and, more notably, the HIV-1 K65R+M184V double mutant showed reduced replication capacities and reduced RT processivities in vitro, consistent with a potential fitness defect in vivo and the low prevalence of the K65R mutation among isolates from antiretroviral agent-experienced patients.**

Human immunodeficiency virus (HIV) type 1 (HIV-1) reverse transcriptase (RT) is the primary target for antiretroviral drugs such as nucleoside and nucleotide RT inhibitors (NRTIs). Cellular enzymes metabolize the nucleoside analogs zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), abacavir, and amdoxovir (DAPD) to their triphosphate forms and the nucleotide analog tenofovir to its diphosphate form. Their mechanisms of action are as competitive inhibitors of natural deoxynucleoside triphosphates (dNTPs), and upon incorporation, they terminate the polymerizing viral DNA chain. Although effective at reducing viral loads in HIV-1-infected patients, NRTI use has resulted in the development of mutations in RT that contribute to HIV-1 drug resistance. A mutation from lysine to arginine at residue 65 (K65R) can be selected through in vitro selection experiments by ddC, abacavir, tenofovir, and (–)-β-D-dioxolane guanosine (DXG) (6, 18, 42, 45). The K65R mutation confers reduced susceptibilities to ddI, ddC, DXG, tenofovir, abacavir, and 3TC, with mutants showing greater than three-

fold decreased susceptibilities to these drugs in in vitro susceptibility assays (6, 22, 42, 45). However, this mutation does not result in cross-resistance to the thymidine analogs AZT and d4T (18). At present, K65R is rarely observed in vivo and was found in <1% of HIV-1-infected patients in a large survey (7).

Several mechanisms are known to contribute to decreased drug susceptibility (34). Mutations that alter the binding affinities for inhibitors or natural substrates can result in decreased levels of incorporation of the inhibitor. The relative binding affinity can be measured as the ratio of the inhibition constant ( $K_i$ ) to the Michaelis constant ( $K_m$ ). An example of this mechanism is the RT from mutants with the K65R mutation (the K65R RT), which has an increased  $K_i$  for ddATP, the active metabolite of ddI (17), and this is likely to contribute to the three- to fourfold reduced susceptibilities of K65R mutant viruses to ddI. Mutations that increase the rate of removal of chain-terminating inhibitors by pyrophosphorolysis or ATP-mediated removal may also contribute to drug resistance. The rate of removal of AZT and d4T through pyrophosphorolysis and ATP-mediated drug removal from RT mutants containing multiple thymidine analog mutations (TAMs) is increased compared to that from wild-type strains, which is likely to contribute to AZT and d4T resistance in these mutants (2, 9, 28, 32, 33). Mutations that affect the fitness of viruses by

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impairing enzyme function might also contribute indirectly to altered drug susceptibility and the outgrowth of resistant viruses in vivo.

The K65R substitution occurs in the conserved IKKK region consisting of amino acids 63 to 66 located in the fingers subdomain of HIV-1 RT. Molecular modeling based on crystal structures of HIV-1 RT suggest that during dNTP incorporation, part of the fingers subdomain rotates toward the palm subdomain and inward toward the primer-template and the polymerase active site. As a result of this movement, lysine 65 makes contact with the incoming dNTP and forms a salt bridge between the  $\epsilon$ -amino group of the lysine and the  $\gamma$ -phosphate of the incoming dNTP (24, 37). The change of lysine to arginine at amino acid 65 is likely to affect the interaction between the enzyme and the triphosphate moieties of dNTPs and NRTIs and might therefore alter the nucleotide-binding specificity or the phosphodiester bond formation efficiency of RT (24, 37).

Due to the frequent use of 3TC in antiretroviral therapy, the 3TC resistance mutation M184V is highly prevalent in treatment-experienced HIV-1-infected patients (7). HIV-1 isolates with the M184V substitution are highly resistant to 3TC, and resistance is mediated primarily by a marked decrease in the level of incorporation of this drug (30, 32, 35). The M184V substitution confers low-level cross-resistance to ddI, ddC, and abacavir in vitro (19, 36). M184V has been shown to result in no or low-level cross-resistance to DXG (19, 20, 27). In contrast, M184V induces an increase in susceptibility to tenofovir, AZT, and d4T in vitro (8, 26, 27, 30, 44, 45; J. M. Whitcomb, E. E. Paxinos, W. Huang, M. Marana, K. Limoli, C. Chappey, N. T. Parkin, N. S. Hellmann, and C. J. Petropoulos, *Abstr. 9th Conf. Retrovir. Opportun. Infect.*, abstr. 569-T, 2002). M184V, either alone or in the context of other mutations, can induce binding changes for selected NRTIs and results in decreased removal of 3TC and decreased enzymatic processivity when it is present. However, there are conflicting reports describing its impact on removal of AZT or other NRTIs when it is present with AZT resistance mutations (4, 16, 32, 40). In patients, the K65R and M184V substitutions can occur together (31), and their impact on NRTI resistance is under investigation.

Tenofovir disoproxil fumarate, the oral prodrug of tenofovir, has recently been approved for the treatment of HIV-1 infection. Tenofovir is an acyclic nucleotide phosphonate analog of AMP that requires two phosphorylation steps by cellular kinases to become the active metabolite tenofovir diphosphate (tenofovir-PP). The primary mutation selected for by tenofovir in vitro is K65R (45). Although tenofovir does not select for TAMs, the presence of specific combinations of TAMs resulted in reduced susceptibility to tenofovir in vivo (26). We have previously shown that recombinant HIV-1 isolates containing K65R displayed three- to fivefold reduced susceptibilities to tenofovir in vitro and that K65R-containing HIV-1 isolates showed three- to fourfold reduced susceptibilities to tenofovir in patients (26, 45). One mechanism explaining this decreased susceptibility is the decreased ability of the K65R RT to bind to and incorporate tenofovir-PP, measured by a 3.5-fold increase in  $K_i$  (45). However, the contribution of chain-terminator removal to this process has not been previously investigated. Interestingly, recombinant viruses contain-

ing the M184V mutation alone or in combination with K65R show wild-type or slightly increased sensitivities to tenofovir in vitro antiviral susceptibility experiments (45), but enzymatic analyses describing the mechanism responsible for this observation have not been reported.

Abacavir, a carbocyclic guanosine analog, is converted by a unique intracellular activation mechanism to its active metabolite, carbovir triphosphate (carbovir-TP), which is a potent inhibitor of HIV-1 RT (11, 14). Viruses with the K65R or the M184V mutation demonstrate threefold reduced susceptibilities to abacavir; when these two mutations are present together, the viruses demonstrate sevenfold reduced susceptibilities to abacavir (42). Both of these mutations can be selected together in vitro and in vivo by abacavir or other combinations of drugs (22, 31).

DXG is phosphorylated by cellular kinases to DXG triphosphate (DXG-TP) and demonstrates activity against both HIV-1 and hepatitis B virus (25, 41). DAPD, a bioavailable prodrug of DXG, is converted to DXG by the action of adenosine deaminase and is in development for the treatment of HIV-1 and hepatitis B virus infections (15). The K65R mutant virus has a five- to eightfold reduced susceptibility to DXG in vitro (6, 19). The role of the M184V substitution in DXG resistance is unclear on the basis of the findings of wild-type susceptibility (27) versus findings of two- to fivefold reduced susceptibility to DXG in vitro (19, 20).

To investigate the effects of resistance mutations on RT function and the molecular mechanisms of the altered susceptibilities of these mutants to tenofovir, ddI, abacavir, and DXG, we evaluated the susceptibilities, replication capacities, and enzymatic processivities of HIV-1 isolates with RTs containing the K65R mutation (the K65R RTs) and HIV-1 isolates with RTs containing the combination of the K65R and M184V mutations (the K65R+M184V RTs). We also determined the  $K_m$  and  $K_i$  for natural substrates and NRTIs, respectively, and examined removal of NRTIs from chain-terminated primers by the ATP-mediated removal mechanism.

## MATERIALS AND METHODS

**Recombinant HIV-1 production and antiviral susceptibility assay.** PCR fragments corresponding to the first 1,000 bp of HIV-1 RT were amplified from plasma HIV-1 and cotransfected with HIV-1 proviral molecular clone pHXB2 $\Delta$ 2-261RT (a gift from C. Boucher, Utrecht University, Utrecht, The Netherlands), from which the RT gene was deleted, as described previously (8, 29). Replication-competent viruses generated by homologous recombination were harvested after 8 to 18 days, when the cultures contained notable syncytia. The genotypes of the recombinant viruses were determined by RT-PCR of viral supernatant followed by sequencing with an ALF Express automated DNA sequencer (Amersham Pharmacia Biotech, Piscataway, N.J.). Three patient-derived viruses containing the K65R mutation were isolated. The M184V mutation was added to each of these viruses by oligonucleotide-based site-directed mutagenesis. The three patient viruses contained additional RT mutations and polymorphisms, as follows: patient 1, K103N, V108I/V, E122K, D123E, D177E, Y181C, Q207K/Q, R211K, L214F, and L228R; patient 2, V90I, K101E, E122K, Y181C, G190A, Q207E, R211K, L214F, and V245T/K; and patient 3, R83K, I178M, Y181C, T200A, Q207E, R211K, and L214F. Recombinant viruses expressing only the K65R or K65R+M184V mutation were also constructed by site-directed mutagenesis of HXB2D and homologous recombination.

The susceptibilities of the recombinant mutant viruses and wild-type HIV-1 molecular clone HXB2D to tenofovir (Gilead Sciences, Foster City, Calif.), AZT, d4T, and ddI (Sigma, St. Louis, Mo.), abacavir (GlaxoSmithKline, Research Triangle Park, N.C.), and DXG (Triangle Pharmaceuticals, Durham, N.C.) were evaluated by a modified 2,3-bis(2-methoxy-4-nitro-5-sulfonyl)-2H-tetrazolium-5-carboxanilide (XTT)-based viability assay in MT-2 cells, as

described previously (10). All infections were performed with  $1.2 \times 10^6$  cells at a multiplicity of infection of approximately 0.001, which resulted in equal levels of cell killing in the absence of drug over the 5-day assay period. Fifty percent effective concentrations ( $EC_{50}$ s) were calculated as an average from two to eight experiments. Statistical significance ( $P < 0.05$ ) was determined by two-tailed Student's  $t$  tests.  $t$  tests were performed on the array of  $EC_{50}$ s from all experimental data for each drug for K65R and K65R+M184V patient-derived viruses or viruses with mutations inserted by site-directed mutagenesis compared to the experimental data for each drug for the wild-type or K65R viruses.

**Recombinant RT construction, purification, and kinetic analyses.** The wild-type RT expression construct pRT66 (18) was a gift from M. Wainberg, McGill University, Montreal, Quebec, Canada. The *pol* sequences were amplified by PCR from the HXB2D molecular clone of HIV-1 and cloned into the expression vector pKK223-3 (Amersham Pharmacia Biotech). K65R RT was a gift from M. Wainberg, K65R+M184V RT was generated by oligonucleotide-based site-directed mutagenesis, and the constructs were completely sequenced. *Escherichia coli* strain JM109 was transformed with the wild-type or mutant constructs and induced with 1.5 mM isopropyl- $\beta$ -D-thiogalactopyranoside. RT purification was performed essentially as described previously (21) by using sequential separations of DEAE-Sephacel in batches, phosphocellulose column chromatography, and poly(rU)-Sephacel 4B column chromatography. Active fractions were identified by RT assay as described below and then concentrated by using Centrifuil YM-30 columns (Millipore Corporation, Bedford, Mass.). The protein concentration was determined by comparison to bovine serum albumin standards by using Coomassie-stained sodium dodecyl sulfate-polyacrylamide gels. For all RT preparations, the majority of protein was the p66 form, but the p51 form was also present and was present at similar levels among the enzymes. The enzyme kinetic analyses were performed as described previously (30). Carbovir-TP and DXG-TP were synthesized by Sierra Bioresearch, Tucson, Ariz. The reaction mixtures for detection of the DNA-dependent DNA polymerase function contained 50 mM Tris-HCl (pH 7.8), 60 mM KCl, 10 mM  $MgCl_2$ , 5 mM dithiothreitol (DTT), 50  $\mu$ M each dNTP, 150  $\mu$ g of activated calf thymus DNA (Amersham Pharmacia Biotech) per ml, and 30 to 100  $\mu$ Ci per mmol of the appropriate [ $^3H$ ]dNTP (Amersham, Arlington Heights, Ill.). Kinetic constants were determined by plotting the substrate concentration ( $S$ ) versus the initial rate ( $v$ ) data in a Michaelis-Menten hyperbolic relationship curve and fitting by nonlinear regression to the equation  $v = V_{max} \times S / (K_m + S)$  by using SigmaPlot (version 4.01). In these experiments, the template was present in excess, and competitive inhibition was observed on the basis of a constant  $V_{max}$  when increasing concentrations of NRTIs were assayed.

**ATP-mediated chain-terminator removal assay.** A 16-nucleotide (nt) primer (primer prPBS; 5'-GTC CCT GTT CGG GCG C-3') and an 18-nt primer (primer prPBS; 5'-GTC CCT GTT CGG GCG CCA-3') corresponding to the natural tRNA primer binding site (3) were 5' end labeled with [ $\gamma$ - $^{33}P$ ]ATP and chain terminated to produce 18-mers for ddA and tenofovir and 21-mers for DXG and carbovir essentially as described previously (2, 32). End-labeled, chain-terminated primers were gel purified twice on an 8 M urea-16% polyacrylamide sequencing gel, excised, eluted in elution buffer (0.5 M ammonium acetate-10 mM Tris-HCl (pH 7.5)-0.1 mM EDTA-2 mM  $MgCl_2$ -0.01% sodium dodecyl sulfate), and desalted by filtration through NAP10 columns (Amersham Pharmacia Biotech). Purified, chain-terminated primers were annealed to pHIV-1-PBS RNA template prepared as described previously (2). The template-chain-terminated primer (2.5 nM) was incubated with approximately 25 nM wild-type recombinant RT enzyme or mutant enzymes for 5 min at 37°C in RT buffer, and then ATP (final concentration, 3.2 mM) was added to start the reaction. The amount of each enzyme used in this assay was equalized for activity in a short polymerization reaction, performed as described previously (16, 32). Reactions were stopped by the addition of an equivalent amount of formamide loading buffer at 0.1, 5, 10, 20, and 30 min. Samples were heated at 95°C and run on 8 M urea-16% polyacrylamide sequencing gels, and the bands were quantified with a Storm860 PhosphorImager (Amersham Pharmacia Biotech) and ImageQuant software (version 5.0; Molecular Dynamics, Sunnyvale, Calif.). The percentage of the chain terminator removed was determined by quantification of the band intensities as the ratio of the sum of the bands minus the chain-terminated primer to the sum of all the bands. Degradation of the primer band did not occur when each RT enzyme was incubated with the primer-template for 15 to 30 min at 37°C in the absence of ATP, thus ruling out exonuclease activity as a source of the removal. In previous experiments with D67N-K70R-T215Y RT, these or longer (24-nt) chain-terminated primers showed evidence of removal, although the levels were low for tenofovir and ddA (33).

**HIV-1 replication capacity.** Replication capacity was measured by a single-cycle virus growth assay as described previously (13). A retrovirus vector capable of measuring changes in replication capacity resulting from mutations within the

protease and RT regions was constructed by using the NL4-3 infectious molecular clone of HIV-1. The vector contains a luciferase expression cassette that replaces the HIV-1 envelope gene. NL4-3 protease and RT sequences (wild type) and HXB2D HIV-1 containing site-directed drug resistance mutations (K65R and K65R+M184V) derived by RT-PCR from patient-derived and recombinant viruses were inserted into the vector by using defined restriction enzyme sites. Vectors prepared from these samples were prepared as libraries (or sequence pools).

Recombinant virus stocks were generated by cotransfecting 293 cells with retroviral vector DNA and an expression vector that produces the murine leukemia virus envelope protein. Two days after transfection, virus stocks were harvested and used to inoculate fresh cell cultures, which were incubated for an additional 2 to 3 days. The virus input at infection was normalized for transfection efficiency, as measured by luciferase expression in transfected cells. After normalization, the amount of luciferase activity in infected cells was used as a direct measure of replication capacity, i.e., the ability of the virus to complete a single cycle of replication. Relative replication capacity was assessed by comparing the amount of luciferase activity produced by mutant recombinant viruses to the amount of luciferase activity produced by a wild-type NL4-3-based recombinant virus that was produced in parallel to the mutant viruses.

**Processivity assays.** Homopolymeric poly(rA) and oligo(dT)<sub>15</sub> labeled at the 5' end with [ $\gamma$ - $^{33}P$ ]ATP (ratio, 14:1) was used as a template for RT processivity assays as described previously (5). The reaction mixtures contained an excess of primer-template compared to the amount of RT enzyme, and heparin was used as a quencher so that only a single round of polymerization was measured. The final reaction mixture contained 1  $\mu$ g of poly(rA)-oligo(dT)<sub>15</sub>, 10  $\mu$ M DTT, 50 mM Tris-HCl (pH 6.8), 60 mM KCl, 1 mM EDTA, 10 mM  $MgCl_2$ , 9 nM wild-type or K65R RT, and 15 nM K65R+M184V RT (to achieve equivalent activities of the RTs). The RT was allowed to associate to the primer-template for 5 min at 37°C, and the reaction was started by adding 5, 0.5, or 0.05  $\mu$ M dTTP with or without 0.2 mg of heparin per ml. The reaction mixtures were incubated at 37°C for 15 min, the reactions were stopped with 4 $\times$  formamide loading buffer, and the extension products were separated through an 8 M urea-8% polyacrylamide gel. We performed a series of control experiments. The lengths of the extension products seen in our assay were not limited by use of a 14:1 template-primer ratio or a 15-min reaction time, and the amount of enzyme added affected the intensities but not the lengths of the extension products (data not shown). Bands were quantified in groups of 10 nt (using boxes spanning bands at 10-nt intervals) with ImageQuant software (Molecular Dynamics), and the median and the peak (the mode of the distribution) were determined.

Heteropolymeric HIV-1 RNA template was prepared from a linearized pHIV-1-PBS plasmid and oligonucleotide primer (18-nt primer prPBS). Processivity experiments were performed as described previously (32). Briefly, 3 to 6 nM wild-type or mutant RT was incubated at room temperature for 10 min with 2 pmol of heteropolymeric primer-template (calculated in moles of primer) in a reaction buffer consisting of 10  $\mu$ M DTT, 50 mM Tris-HCl (pH 6.8), 60 mM KCl, 1 mM EDTA, and 10 mM  $MgCl_2$ . The reactions with the 50- $\mu$ l reaction mixtures were initiated by adding dTTP, dCTP, and dGTP to the final concentration of 50  $\mu$ M; 50 nM [ $\alpha$ - $^{33}P$ ]dATP (500 Ci/mmol); and 450 nM cold dATP, with or without heparin (final concentration, 0.2 mg/ml). After 30 min at 37°C, the total level of incorporation of radiolabel in unquenched reactions was assessed by a filter-based assay. Quenched reactions were stopped with 4 $\times$  formamide loading buffer, and the products were electrophoresed in an 8 M urea-8% polyacrylamide gel. The processivities of the mutant and the wild-type RT enzymes were determined by normalizing the amount of full-length product from quenched reactions obtained by PhosphorImager analysis with the total activity determined from the filter-based assay in order to control for the amount of active enzyme for each RT. The efficiency of quenching, as determined by the addition of the heparin to the primer-template prior to the addition of RT and filter-based analysis, was greater than 95%.

## RESULTS

**Susceptibility in cell culture.** Cell-based antiviral drug susceptibility assays were performed with three patient-derived viruses containing K65R by using tenofovir, ddI, abacavir, DXG, AZT, and d4T (Fig. 1). The K65R-containing viruses were three- to sixfold less susceptible to tenofovir, ddI, abacavir, and DXG than wild-type virus HXB2D. These patient-derived viruses showed less than twofold changes in susceptibilities to AZT and d4T compared to that of the wild type.

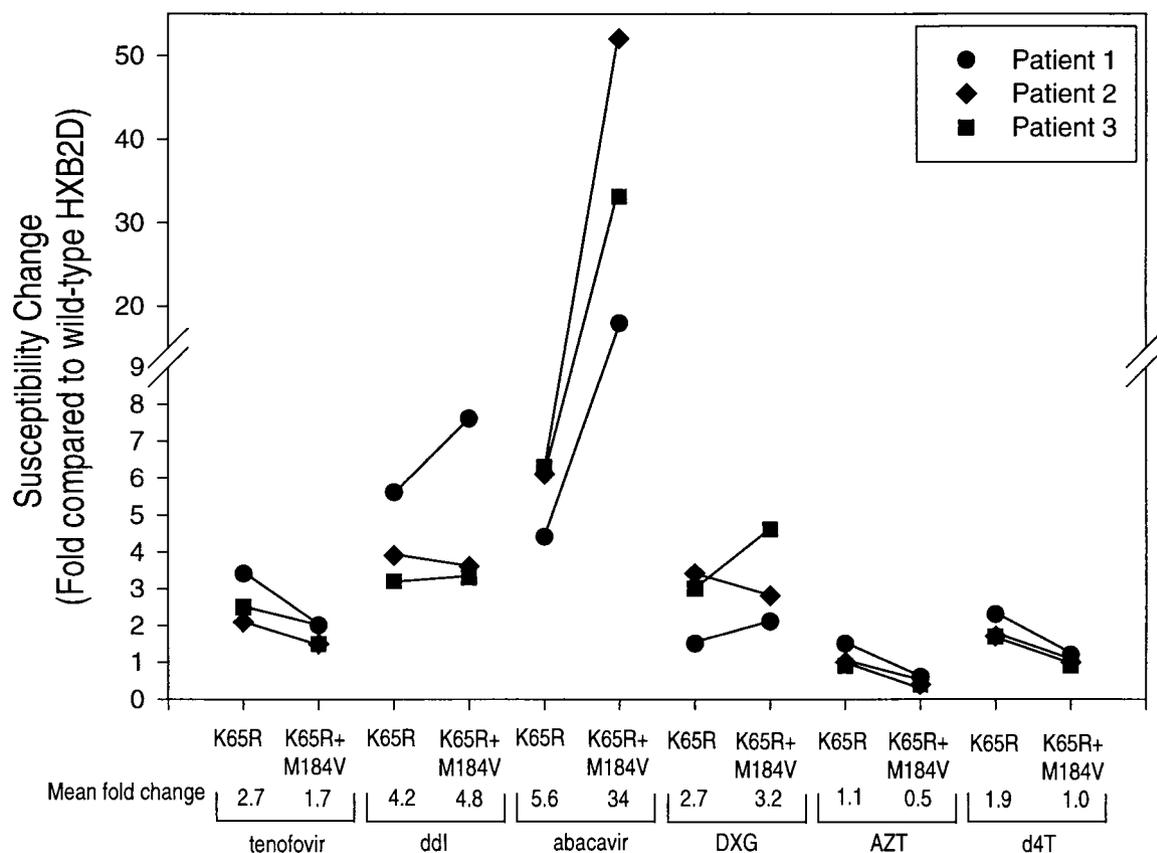


FIG. 1. Susceptibilities of three patient-derived HIV-1 pairs containing the K65R mutation with or without the M184V mutation introduced by site-directed mutagenesis. MT-2 cells were infected with wild-type and mutant viruses and treated with tenofovir, ddI, abacavir, DXG, AZT, or d4T; and the cytopathic effect was quantified by a modified XTT-based assay. The datum points are mean values from two to eight experiments and are shown as fold changes in susceptibility compared to that of the wild-type HXB2D virus, with individual patient-derived virus pairs connected by lines and with the mean fold changes shown in the *x*-axis legend. The  $EC_{50}$ s of tenofovir, ddI, abacavir, DXG, AZT, and d4T for wild-type virus were 3.1, 4.3, 0.3, 1.9, 0.14, and 7.5  $\mu$ M, respectively.

Approximately 34% of the K65R-containing viruses also contain the M184V substitution (Stanford HIV RT and Protease Sequence Database [http://hivdb.stanford.edu]), suggesting that M184V frequently occurs in viruses containing K65R. To determine the effect of the M184V mutation on K65R-containing viruses, we constructed double mutants by adding the M184V mutation to these viruses by site-directed mutagenesis. The addition of the M184V mutation to K65R-containing viruses resulted in statistically significant increases in susceptibility to tenofovir compared to the susceptibilities of K65R viruses ( $P < 0.001$ ), resulting in an overall mean change from the susceptibility of the wild-type virus of less than twofold (Fig. 1). Viruses containing the K65R and M184V mutations also showed significant increases in their susceptibilities to AZT and d4T compared to the susceptibilities of viruses with the K65R mutation ( $P = 0.01$  and  $P < 0.001$ , respectively). For ddI and DXG, the K65R+M184V viruses were on average less susceptible than K65R viruses, but these values were not statistically different from those for K65R viruses ( $P = 0.6$  and  $P = 0.5$ , respectively). The K65R+M184V viruses displayed 6.1-fold decreases in abacavir susceptibilities compared to those of K65R viruses ( $P < 0.001$ ). Additional mutations or polymorphisms present in the patient-derived viruses may have con-

tributed to drug susceptibility patterns, and it should be noted that the patient-derived viruses studied contained Y181C, a nonnucleoside RT inhibitor mutation.

Susceptibilities to tenofovir, ddI, abacavir, DXG, d4T, and AZT were also determined for recombinant HIV-1 RT mutant viruses that contained the site-directed K65R mutation alone or in combination with M184V in an HXB2D background and that therefore lacked the additional amino acid substitutions present in the patient-derived viruses (Table 1). These mutant viruses yielded susceptibility values similar to those for the patient-derived viruses described above. The K65R mutant virus was threefold less susceptible to tenofovir than wild-type virus, while the K65R+M184V mutant had only twofold decreased susceptibility compared to that of the wild type. The K65R virus was four- to fivefold less susceptible to ddI, abacavir, and DXG than the wild-type virus. For the virus with the site-directed K65R and M184V mutations, the susceptibility to DXG was slightly increased compared to that of the K65R virus; however, this was not a significant change ( $P = 0.6$ ). For ddI and abacavir, there were further decreases in susceptibility of 2- and 14-fold, respectively, for the K65R+M184V double mutant compared with that for the K65R mutant ( $P = 0.09$  and  $P < 0.001$ , respectively). Consistent with data obtained for the

TABLE 1. Drug susceptibilities for recombinant HIV with site-directed mutations

Drug	EC <sub>50</sub> (μM) <sup>a</sup>		
	HXB2D (wild type)	K65R	K65RM184V
Tenofovir	3.1 ± 0.14	9.8 ± 1.2 <sup>b</sup> (3.2) <sup>c</sup>	6.3 ± 0.94 <sup>b</sup> (2.0)
ddI	4.3 ± 0.41	15.4 ± 2.7 <sup>b</sup> (3.6)	29.5 ± 1.77 <sup>b</sup> (6.9)
Abacavir	0.3 ± 0.02	1.1 ± 0.2 <sup>b</sup> (3.8)	15.0 ± 0.001 <sup>b</sup> (52.0)
DXG	1.9 ± 1.8	10.2 ± 2.2 <sup>b</sup> (5.4)	8.0 ± 1.2 <sup>b</sup> (4.2)
AZT	0.14 ± 0.02	0.07 ± 0.01 (0.5)	0.06 ± 0.01 (0.4)
d4T	7.5 ± 2.2	13.2 ± 2.5 <sup>b</sup> (1.8)	10.4 ± 1.6 <sup>d</sup> (1.4)

<sup>a</sup> EC<sub>50</sub> for recombinant viruses were determined by an XTT-based viability assay with MT-2 cells, and averages ± standard errors for three to eight experiments are shown.

<sup>b</sup>  $P < 0.001$  compared to the EC<sub>50</sub> for the wild-type by two-tailed Student's *t* test.

<sup>c</sup> The values in parentheses are the fold change in the EC<sub>50</sub> for the mutant compared to that for the wild type.

<sup>d</sup>  $P < 0.05$  compared to the EC<sub>50</sub> for the wild type by two-tailed Student's *t* test.

patient-derived viruses, the HXB2D mutants with site-directed mutations remained susceptible to d4T and AZT.

**Relative binding affinity measurements.** RT mutations can induce resistance to chain-terminating inhibitors by altering the binding to or the incorporation of inhibitors to their respective natural substrates (dNTPs). We examined the ability of the K65R and K65R+M184V RT enzymes to selectively bind to and incorporate the natural dNTPs versus their ability to bind to and incorporate the inhibitors as a possible explanation for the decreases in susceptibilities. K65R and K65R+M184V mutants obtained by site-directed mutagenesis and wild-type RT enzymes were purified from *E. coli*, and their relative binding affinities were measured by determining the  $K_m$  values for natural dNTP substrates, the  $K_i$  values, and the relative inhibitory capacities ( $K_i/K_m$ ) for the active metabolites of tenofovir, ddI, abacavir, and DXG (tenofovir-PP, ddATP, carbovir-TP, and DXG-TP, respectively).

We measured the  $K_m$  values for dATP, the natural substrate mimicked by the analogs tenofovir-PP and ddATP, and dGTP, the natural substrate mimicked by carbovir-TP and DXG-TP (Table 2). The  $K_m$  values of the wild-type RT for dATP and dGTP were 0.333 and 0.172 μM, respectively, consistent with the values from previous reports (17, 32). The K65R RT showed slightly elevated  $K_m$  values of 1.2- and 1.5-fold for dATP and dGTP, respectively, compared to those of the wild-type RT ( $P = 0.27$  and  $P = 0.05$ , respectively). The K65R+M184V mutant showed statistically significant  $K_m$  increases of 1.7-fold for dATP and 1.8-fold for dGTP compared

TABLE 2.  $K_m$  values of wild-type and mutant HIV-1 RT enzymes

HIV-1 RT	$K_m$ (μM) <sup>a</sup>	
	dATP	dGTP
Wild type	0.333	0.172
K65R	0.392	0.254 <sup>b</sup>
K65R+M184V	0.554 <sup>c</sup>	0.314 <sup>c</sup>

<sup>a</sup>  $K_m$  values are averages for multiple experiments, with an average standard error of 0.063 for dATP and 0.03 for dGTP.

<sup>b</sup>  $P \leq 0.05$  compared to the  $K_m$  for the wild type by two-tailed Student's *t* test.

<sup>c</sup>  $P \leq 0.01$  compared to the  $K_m$  for the wild type by two-tailed Student's *t* test.

to those of the wild-type RT ( $P = 0.01$  and  $P = 0.004$ , respectively). These data suggest that K65R RT binds to and incorporates natural substrates with an efficiency similar to or slightly reduced compared with that of the wild-type RT, but the addition of the M184V mutation to K65R RT more notably reduces the levels of binding to and incorporation of natural dNTPs.

We determined the  $K_i$  values of the wild-type and the mutant RT enzymes for the active metabolites of nucleotide and nucleoside analogs (Table 3). The K65R RT yielded  $K_i$  values for tenofovir-PP, ddATP, and DXG-TP that were significantly elevated ( $P < 0.01$ ) 6.7-, 7.2-, and 4.1-fold, respectively, compared to those of the wild-type RT. There was a twofold increase in the  $K_i$  for carbovir-TP that did not achieve statistical significance ( $P = 0.08$ ). Therefore, the decreased susceptibilities to tenofovir, ddI, and DXG may be attributed to a reduced binding affinity and/or incorporation by the K65R mutant enzyme into these analogs, but the decreased susceptibility to abacavir cannot be fully attributed to a change in  $K_i$ . The  $K_i$  values for all four drugs were significantly increased for the K65R+M184V double mutant compared to those for the wild-type RT, with changes ranging from 4- to 16-fold. When the  $K_i$  of the K65R+M184V RT was compared to that of the K65R RT, the  $K_i$  was increased for carbovir-TP ( $P = 0.001$ ) and ddATP ( $P = 0.004$ ), but no change was noted for tenofovir-PP ( $P = 0.14$ ) or DXG-TP ( $P = 0.95$ ). These data show that the addition of the M184V mutation to viruses with the K65R RT decreases the relative binding affinity and/or the level of incorporation of carbovir-TP ( $P = 0.001$ ) and ddATP ( $P = 0.004$ ), consistent with the antiviral susceptibility results for the mutant viruses with site-directed mutations in Table 1.

To determine the inhibitory capacities for tenofovir-PP, ddATP, carbovir-TP, and DXG-TP, the  $K_i/K_m$  ratios were calculated for the wild-type and the mutant RT enzymes (Table 3). The K65R RT showed 5.8- and 6.1-fold increases in  $K_i/K_m$  compared to those of the wild-type RT for tenofovir-PP and ddATP, respectively, demonstrating that the K65R mutation decreases the relative binding affinity of RT to these inhibitors. The  $K_i/K_m$  for DXG-TP was elevated 2.8-fold but was not changed for carbovir-TP. Changes in the  $K_i/K_m$  of the K65R+M184V RT for ddATP and tenofovir-PP correlated with changes in susceptibility, in which the  $K_i/K_m$  values were decreased for tenofovir-PP and increased for ddATP compared to the  $K_i/K_m$  of the K65R RT. The  $K_i/K_m$  for DXG-TP was not significantly different for the K65R+M184V RT compared to that for the K65R RT. The  $K_i/K_m$  for carbovir-TP, however, was only slightly elevated (2.3-fold) (Table 3), whereas 30- to 50-fold decreased susceptibility was seen in culture (Fig. 1 and Table 1). These data suggest that alterations in the relative binding affinities of the mutant RTs for carbovir-TP relative to those for dGTP do not fully explain the reduced susceptibilities seen in cell culture.

**Removal of chain terminators by the ATP-mediated removal mechanism.** We analyzed ATP-mediated removal of the chain terminators tenofovir, ddA, carbovir, and DXG in order to determine what contribution this mechanism of resistance makes to the susceptibility profiles of viruses containing K65R or K65R+M184V. Using ATP at the physiological concentration of 3.2 mM (23, 43), we detected minimal removal (<4% at 30 min) of tenofovir, ddA, and DXG by wild-type, K65R, and

TABLE 3.  $K_i$  and  $K_i/K_m$  of wild-type and mutant HIV-1 RT enzymes

HIV-1 RT	Tenofovir-PP		ddATP		Carbovir-TP		DXG-TP	
	$K_i$ ( $\mu\text{M}$ ) <sup>a</sup> (fold) <sup>b</sup>	$K_i/K_m$ (fold) <sup>c</sup>	$K_i$ (fold)	$K_i/K_m$ (fold)	$K_i$ ( $\mu\text{M}$ ) (fold)	$K_i/K_m$ (fold)	$K_i$ ( $\mu\text{M}$ ) (fold)	$K_i/K_m$ (fold)
Wild type	0.155 (1.0)	0.46 (1.0)	0.024 (1.0)	0.072 (1.0)	0.181 (1.0)	1.05 (1.0)	0.008 (1.0)	0.05 (1.0)
K65R	1.040 <sup>d</sup> (6.7)	2.67 (5.8)	0.173 <sup>c</sup> (7.2)	0.441 (6.1)	0.357 (2.0)	1.42 (1.3)	0.033 <sup>c</sup> (4.1)	0.13 (2.8)
K65R+M184V	0.824 <sup>d</sup> (5.3)	1.62 (3.5)	0.383 <sup>d</sup> (16)	0.690 (9.6)	0.807 <sup>d</sup> (4.5)	2.45 (2.3)	0.033 <sup>c</sup> (4.1)	0.10 (2.2)

<sup>a</sup>  $K_i$  values are averages for three to six experiments, with average standard errors of 0.067 for tenofovir-PP, 0.013 for ddATP, 0.059 for carbovir-TP, and 0.003 for DXG-TP.

<sup>b</sup> Fold change in  $K_i$  value from that for the wild type.

<sup>c</sup> Fold change in  $K_i/K_m$  value from that for the wild type.

<sup>d</sup>  $P < 0.001$  compared to the  $K_i$  for the wild type by two-tailed Student's  $t$  test.

<sup>e</sup>  $P < 0.05$  compared to the  $K_i$  for the wild type by two-tailed Student's  $t$  test.

K65R+M184V RTs after 30 min (Fig. 2). In contrast, we detected increased removal of carbovir by the mutant RTs compared to that by the wild-type RT, illustrated by the appearance of several bands with molecular weights lower than that of the upper, chain-terminated primer band (Fig. 2). At 30 min, 6.3 and 5.8% of carbovir was removed by the K65R and K65R+M184V RTs, respectively. For comparison, the efficiency of removal by these mutants is less than the efficiency of removal of AZT observed for a mutant RT containing multiple thymidine analog mutations, in which 25% of the drug is removed after 15 min (32). These data suggest that ATP-mediated removal may contribute to the decreased susceptibilities of K65R and K65R+M184V mutants to abacavir. Removal of chain terminators by ATP does not appear to significantly contribute to changes in susceptibility to tenofovir, ddI, or

DXG, for which altered inhibitor binding or incorporation appears to be the mechanism.

**Replication capacity.** One component of viral fitness is the relative replication capacity of the virus in the absence of drug. To determine this component of fitness, we measured the relative single-cycle replication capacities of recombinant viruses containing the K65R or the K65R+M184V mutation. In these experiments, 100% replication capacity was defined as the replication capacity of wild-type HIV-1-derived, drug-sensitive reference virus (Fig. 3). The K65R virus with a site-directed mutation yielded a relative replication capacity of 53% of that for the wild-type controls. The replication capacity of the K65R+M184V virus was further decreased to 24% of that for the reference virus. Therefore, the addition of the M184V mutation reduced the replication capacity compared to that of the virus with the K65R mutation. Patient isolates that lack resistance mutations have replication capacities similar to the replication capacity of this reference virus. For all six patient-derived viruses, the replication capacities were reduced

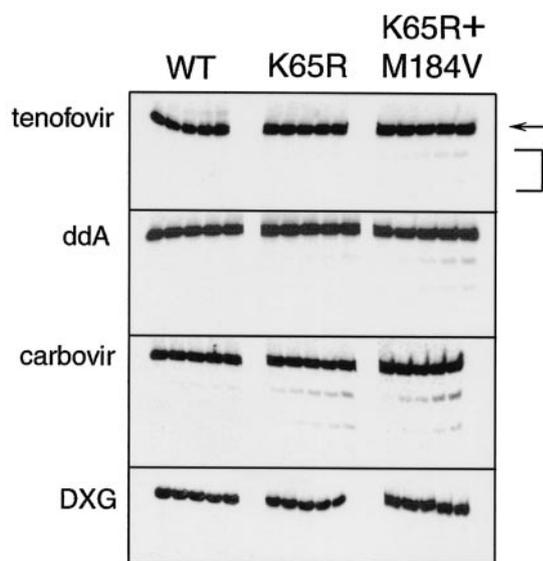


FIG. 2. Removal of chain terminators by the ATP-mediated removal mechanism. 5'-end-labeled primer-templates terminated with tenofovir-PP, ddATP, carbovir-TP, or DXG-TP were incubated with wild-type (WT), K65R, or K65R+M184V RT; and removal of tenofovir, ddA, carbovir, and DXG was evaluated following the addition of 3.2 mM ATP at 0.1, 5, 10, 20, and 30 min (left to right) and subsequent separation through 8 M urea–16% polyacrylamide sequencing gels and exposure to a PhosphorImager screen. For tenofovir, the chain-terminated primers are shown with an arrow and the shorter removal products are indicated with a bracket.

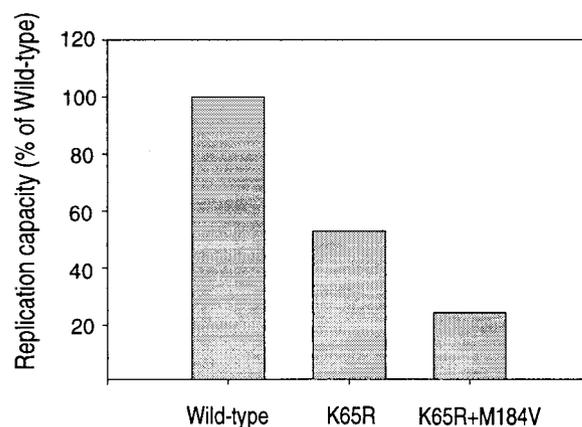


FIG. 3. Relative replication capacity was determined by measuring the single-cycle growth of recombinant viruses containing a luciferase reporter gene. 293 cells were infected with equivalent amounts of reference virus (NL4-3) or recombinant viruses in which the K65R mutation or the K65R and M184V mutations were inserted by site-directed mutagenesis. Luciferase activity was quantified after 48 h, and relative virus growth was normalized to that of an NL4-3 reference virus by using p24 assay values obtained for each virus in a parallel experiment. One hundred percent replication capacity was defined as the replication capacity of wild-type HIV-1-derived, drug-sensitive reference virus.

compared to the replication capacity of the wild-type virus. The replication capacities for these patient-derived viruses ranged from 10 to 70% for those with the K65R mutation and from 10 to 40% for those with the K65R and M184V mutations compared to the replication capacity of the reference virus (data not shown).

**Processivity.** To investigate a potential explanation for the reduced replication capacities of the K65R and K65R+M184V viruses, we analyzed wild-type and mutant RT processivities on homopolymeric and heteropolymeric RNA templates. Single-cycle processivity can be measured as the number of nucleotides added to a growing DNA chain by a polymerase prior to dissociation from the template or as the amount of full-length product formed under conditions that do not permit reassociation. With a homopolymeric poly(rA) template, the K65R enzyme showed processivity similar to that of the wild-type RT in the presence of 0.5  $\mu$ M dTTP, whereas the K65R+M184V enzyme showed decreased processivity in this assay (Fig. 4A, lanes 1 to 3). The median and peak (mode of the distribution) lengths of cDNAs were determined by quantification of bands measured at 10-nt intervals. The medians of the product lengths for the wild-type, K65R, and K65R+M184V enzymes were 50, 52, and 38 nt, respectively (Table 4). Similar results were observed for the peak lengths of the cDNA products, with 40-, 40-, and 35-nt extensions measured for the wild-type, K65R, and K65R+M184V enzymes, respectively. The magnitude of the processivity defects observed for these mutants was dependent on the substrate concentration used in the assay. At more limiting substrate concentrations of 0.05  $\mu$ M, the defects were more pronounced, in which the K65R mutant displayed decreased processivity (Fig. 4A and Table 4). At a higher substrate concentration of 5  $\mu$ M, both mutants showed processivities similar to that of the wild type (data not shown), consistent with what has previously been reported for the K65R mutant enzyme when high substrate concentrations were used (2).

The second assay measured processivity by using a more biologically relevant heteropolymeric template consisting of HIV-1 RNA annealed to a 16-nt DNA primer complementary to the primer binding site. Single-cycle processivity was measured by quantification of the 191-nt full-length product produced from this HIV-1 template in the presence of a quenching agent and was normalized to the total unquenched enzyme activity determined by filter-binding assays. Under single-cycle quenching conditions, when the values were normalized to those for total unquenched activity, both mutants showed decreased abilities to efficiently synthesize full-length cDNA (Fig. 4B). Quantification of the full-length bands shows that K65R RT had 69% of the processivity of the wild type (Table 4). As seen for the homopolymeric template, the addition of the M184V mutation further reduced the processivity of K65R RT to 31% of that of the wild type.

These results suggest that the K65R RT has a processivity defect under limiting substrate conditions with both templates, and this defect is more pronounced when M184V is also present. The decreases in enzyme processivity observed for these mutant enzymes are consistent with the increases in  $K_m$  values for the natural substrates dATP and dGTP and the decreases in the single-cycle replication capacities of viruses containing mutations at these sites.

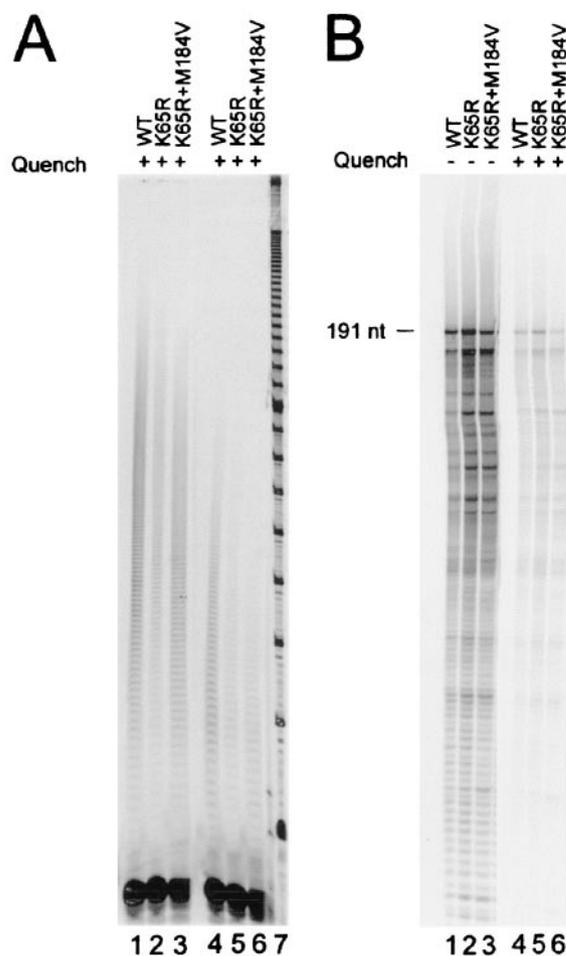


FIG. 4. Analysis of processivity of wild-type (WT) RT and recombinant RT mutants with site-directed mutations. The single-cycle processivities of each of the recombinant RT enzymes with poly(rA)-oligo (dT)<sub>15</sub> (A) or HIV-1 RNA-16-mer PBS primer (B) as primer-templates are shown as PhosphorImages of the extension products after separation through a 8 M urea-16% polyacrylamide sequencing gel from processivity experiments. (A) Homopolymeric template with wild-type RT (lanes 1 and 4), K65R RT (lanes 2 and 5), or K65R-M184V RT (lanes 3 and 6) under single-cycle processivity in the presence of 200  $\mu$ g/ml of heparin and 0.5  $\mu$ M dTTP (lanes 1 to 3) or 0.05  $\mu$ M dTTP (lanes 4 to 6); (B) heteropolymeric template with wild-type RT (lanes 1 and 4), K65R RT (lanes 2 and 5), or K65R-M184V RT (lanes 3 and 6) under continuous processivity (lanes 1 to 3) or single-cycle processivity in the presence of 200  $\mu$ g/ml heparin (lanes 4 to 6). A 10-bp ladder was used as a size marker (lane 7). The full-length product for the HIV-1 RNA template is 191 nucleotides.

## DISCUSSION

HIV-1 resistance to NRTIs is mediated by multifactorial and complex mechanisms that are being defined at the molecular level. Two primary mechanisms of resistance consist of altered relative binding and incorporation that can be measured as alterations in the natural substrate and inhibitor binding constants and the amounts of inhibitors removed after incorporation into the polymerizing DNA chain mediated by either ATP or pyrophosphate. As mechanisms of resistance are not fully understood at this time, it is possible that other alterations of RT enzymatic function such as polymerase processivity, the

TABLE 4. Processivity of HIV-1 wild type and mutant RT enzymes

HIV-1 RT	Processivity with poly(rA)-oligo(dT) <sub>15</sub> template				Processivity with HIV RNA template (%) <sup>c</sup>
	Median length extended <sup>a</sup>		Peak length extended <sup>b</sup>		
	0.5 μM dTTP	0.05 μM dTTP	0.5 μM dTTP	0.05 μM dTTP	
Wild type	50	30	40	20	100
K65R	52	24	40	10	69
K65R+M184V	38	21	35	10	31

<sup>a</sup> Median length extended (in nucleotides) measured as the median length of extension products determined from regression analysis of band intensity measured by using 10-nt intervals.

<sup>b</sup> Peak of the percentage of products generated versus length of product (in nucleotides) measured by using 10-nt intervals.

<sup>c</sup> Percentage of full-length cDNA in the presence of a quenching reagent/total activity without a quenching reagent normalized to the value for the wild type (WT), which was set at 100%.

rate of polymerization ( $k_{\text{pol}}$ ), interactions with metal ions, binding and dissociation constants for RNA and DNA templates, RNase H activity, strand transfer activity, and fidelity may indirectly contribute to HIV-1 resistance, in addition to their direct effects on RT function and viral replication. HIV-1 RTs containing the K65R mutation and the combination of the K65R and M184V mutations were investigated in the present study to assess their contributions to resistance and other RT enzymatic functions, i.e., alterations in natural substrate binding ( $K_m$ ), alterations in inhibitor binding ( $K_i$ ), removal of inhibitors by the ATP-mediated removal mechanism, viral replication capacity, and RT processivity. The K65R RT showed a 5.8-fold increased  $K_i/K_m$  for tenofovir-PP and dATP, demonstrating the decreased ability of this mutant to incorporate tenofovir-PP over its ability to incorporate the natural substrate. The increase in  $K_i$  reported here compared to that of the wild type (6.7-fold) was higher than the value of 3.5-fold that Wainberg et al. (45) reported previously. Addition of the M184V mutation to the K65R mutation resulted in no further change in the  $K_i$  for tenofovir-PP. Consistent with an increase in the  $K_m$  of the M184V mutant alone (32), the  $K_m$  for dATP of the mutant with the double RT mutation was significantly increased, which resulted in a relative inhibitory capacity for tenofovir-PP that was increased by only 3.5-fold relative to that of the wild-type RT. The contribution of ATP-mediated removal of tenofovir from chain-terminated primers to resistance was minimal for the K65R and K65R+M184V viruses. These data suggest that an altered ability to bind to or incorporate tenofovir is the major factor contributing to the reduced susceptibilities of K65R-containing viruses to this drug and that removal by the ATP-mediated removal mechanism does not significantly contribute to this process. When the M184V mutation is present with the K65R mutation, the enzyme binds to natural substrates less efficiently (increased  $K_m$ ) and tenofovir can compete more effectively than it can when only the K65R mutation alone is present. This may contribute to the observed increase in the susceptibility of the double mutant to tenofovir.

The K65R substitution conferred reduced susceptibility to ddI that was further reduced in the presence of the M184V mutation. The  $K_i/K_m$  of the K65R mutant was increased 6-fold compared to that of the wild type, and  $K_i/K_m$  of the

K65R+M184V mutant was increased almost 10-fold. Similar increases in the  $K_i$  values of the K65R mutant RT have been reported for ddATP (17, 44), and we observed further binding and incorporation deficiencies with the addition of the M184V mutation. We detected only minimal removal of ddATP by the ATP-mediated removal mechanism. These data suggest that, similar to the mechanism of resistance to tenofovir, a defect in binding to or incorporation of ddATP and not its removal is the primary mechanism of resistance to ddI for these mutants. However, in contrast to tenofovir, the addition of the M184V mutation results in a further reduction in the level of binding to and incorporation of ddATP.

The mechanisms of resistance of the K65R and K65R+M184V viruses to the guanosine analog DXG were investigated. Two- to fivefold decreases in the susceptibilities of viruses containing these mutations were detected, similar to the data that other investigators (18, 20) obtained for K65R. The data suggest that the M184V substitution does not appear to further decrease susceptibility to DXG in the context of K65R and supports the findings of Mewshaw et al. (27) for viruses containing M184V alone. When binding to this inhibitor was examined, equivalent increases in  $K_i$  of fourfold for both the K65R and the K65R+M184V RTs compared to that for the wild-type RT were measured. The  $K_m$  of the K65R mutant RT for dGTP relative to that of the wild-type RT was slightly increased, but that of the K65R+M184V RT was increased notably more, suggesting that the RT double mutant also has a defect in binding to dGTP, as was observed for dATP. An increased  $K_m$  for several dNTPs has also been shown for RT with the M184V mutation alone (46) or in the context of multiple thymidine analog mutations (32), suggesting that this mutation may broadly induce changes in the  $K_m$  of RT in the context of multiple genetic backgrounds. However, the relative inhibitory capacities of DXG-TP for the two mutants were not significantly different from each other. As with tenofovir and ddATP, we detected minimal ATP-mediated removal of DXG, suggesting that the minor decreases in the susceptibilities to DXG of the K65R and K65R+M184V mutant viruses are mediated by decreased levels of binding to or incorporation of the inhibitor.

Decreased susceptibility to abacavir was shown for viruses containing the K65R mutation and was further increased with the addition of the M184V mutation. The 20- to 50-fold decreased susceptibilities to abacavir for viruses containing K65R and M184V mutations appear to be greater than those observed previously (42) and those expected from an additive effect from each mutation alone, as the reduced susceptibilities are 3.8- and 2.2-fold for viruses with the K65R mutation alone (Table 1) and the M184V mutation alone (11), respectively. The  $K_i/K_m$  value of the K65R RT was similar to that of the wild-type RT, but that of the RT double mutant showed a 2.3-fold increase. However, in contrast to tenofovir, ddI, and DXG, removal of carbovir from terminated primers by ATP was readily detected, with approximately 6% of the inhibitor removed after 30 min by the mutants, whereas 2.6% was removed by the wild type. Although this level of removal of carbovir is less than that observed for AZT in the presence of TAMs (33), our results suggest that increased removal may contribute to the decreased susceptibility of the K65R mutant to abacavir and that both decreased inhibitor binding and

increased carbovir removal may play a role in the decreased susceptibility observed for the K65R+M184V double mutant. Other mechanisms that remain undefined may also contribute to the significant reduction in the susceptibility of the K65R+M184V mutant virus to abacavir.

The replication capacities and RT processivities of the K65R and K65R+M184V mutants were also determined. The rarity of K65R in antiretroviral agent-treated patients (<1%) (7) suggests that HIV-1 isolates with this mutation may have decreased fitness *in vivo*. In the present study, the decreased fitness of the K65R mutant was supported by (i) decreased replication capacity in a single-cycle virus growth assay and (ii) decreased RT processivity at limiting substrate concentrations for the K65R RT compared to that for the wild-type RT. Previous reports suggested no change or slightly increased processivity for the K65R RT (1, 30, 44); however, the increased processivity was observed in assays conducted with high substrate concentrations (50  $\mu$ M) (1). At these high concentrations, we also failed to detect a defect in processivity for the K65R RT. For the K65R+M184V double mutant, the processivity defect and reduction in replication capacity were more pronounced and were also observed at high dNTP concentrations. RT enzymes containing the M184V mutation alone have also been shown to have decreases in their processivities (4), and this defect was maintained when the M184V mutation was added to RT containing other mutations such as thymidine analog mutations (32). When dNTP concentrations are low, defects in processivity appear to be greatest. Since dNTP levels in cells can vary between different cell types and during the cell cycle (43), the defects in processivity and replication capacity would be exacerbated in cells with low dNTP concentrations, which could result in reduced fitness for K65R and K65R+M184V mutant viruses. The slight increases in the  $K_m$  values of the K65R RT for dATP and dGTP and the significant increases in the  $K_m$  values of the K65R+M184V RT for dATP and dGTP may contribute to the decreased processivity and the subsequent decrease in virus production when substrate concentrations drop below certain threshold levels. Alterations in other enzymatic parameters such as  $k_{pol}$  or  $K_d$  for the template or the fidelity of reverse transcription may also play a role in the decreased replication capacities of these mutant viruses and overall viral fitness (38, 39).

The mechanisms involved in drug resistance may differ for each NRTI, for each pattern of RT mutations, and for combinations of these variables. The K65R mutation confers reduced susceptibility to a number of NRTIs including ddI, abacavir, tenofovir, DXG, 3TC, and ddC. However, mutants with this mutation remain susceptible to AZT and d4T *in vitro*. In the present work, we have demonstrated that K65R can confer resistance through different mechanisms depending on the NRTI. Interestingly, the K65R RT displayed defects in processivity and single-cycle replication capacity which may contribute to a reduced overall level of fitness of viruses containing this mutation. The addition of the M184V mutation to isolates with the K65R mutation results in a further decrease in viral replication capacity and processivity, further decreased susceptibility to ddI and abacavir, but increased susceptibility to tenofovir. These results emphasize the complex nature of resistance and how multiple factors contributing to reduced fitness may result from the development of resistance. Furthermore,

these results may also explain the low frequency of the K65R mutant among antiretroviral agent-experienced patients (7) as well as its infrequent development among ddI- and tenofovir-treated patients (12, 26).

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