Abacavir has been shown to select for multiple resistant mutations in the human immunodeficiency type 1 (HIV-1) pol gene. In an attempt to understand the molecular mechanism of resistance in response to abacavir, and nucleoside analogs in general, a set of reverse transcriptase mutants were studied to evaluate their kinetics of nucleotide incorporation and removal. It was found that, similar to the multidrug-resistant mutant reverse transcriptase (RT) containing the mutations L74V, M184V, and a triple mutant containing L74V/Y115F/M184V all caused increased selectivity for dGTP over the active metabolite of abacavir (carbovir triphosphate). However, the magnitude of resistance observed in cell culture to abacavir in previous studies was less than that observed to other compounds. Our mechanistic studies suggest that this may be due to carbovir triphosphate decreasing the overall effect on its efficiency of incorporation by forming strong hydrophobic interactions in the RT active site. Unlike RTAZTR, no increase in the rate of ATP- or PPi-mediated chain terminator removal relative to RTWT could be detected for any of the mutants studied. Marked decreases in the steady-state rate may serve as a mechanism for increased removal of a chain-terminating carbovir monophosphate by increasing the time spent at the primer terminus for some of the mutants studied. The triple mutant showed no advantage in selectivity over RTM184V and was severely impaired in its ability to remove a chain terminator, giving no kinetic basis for its increased resistance in a cellular system. Biochemical properties including percentage of active sites, fidelity, and processivity may suggest that the triple mutant’s increased resistance to abacavir in cell culture is perhaps due to a fitness advantage, although further cellular studies are needed to verify this hypothesis. These data serve to further the understanding of how mutations in RT confer resistance to nucleoside analogs.

Human immunodeficiency virus (HIV), the causative agent of AIDS, requires reverse transcriptase (RT) to copy its single-stranded RNA genome into a double-stranded DNA copy for integration into the host cell genome. Although almost all aspects of the HIV-1 life cycle have been targeted (1–3), a majority of the drugs that have been effective in clinical trials are nucleoside reverse transcriptase inhibitors (NRTIs). However, treatment with NRTIs is limited by their toxicity to the host (often through their interaction with mitochondrial DNA polymerase γ (4–7) and the ability of the virus to mutate and gain resistance (8). Other factors that affect the ability of these inhibitors to reduce viral replication are uptake, transport, metabolism, and incorporation of the drug. All clinically used nucleoside analogs lack 3’-hydroxyl groups and are metabolically activated by host cellular kinases to their triphosphate forms. Compounds currently approved by the Food and Drug Administration are β-D-(+)-3’-azido-3’-deoxythymidine (AZT or zidovudine), β-D-(+)-2’-3’-dideoxy-3’-deoxythymidine (D4T or stavudine), β-L-(-)-2’-3’-dideoxy-3’-thiacytidine (3TC or lamivudine), β-L-(+)-2’-3’-dideoxythycytidine, β-D-(+)-2’-3’-dideoxynosine, (1S,4R)-4-[2-amino-6-(cyclopropyl-aminio)-9H-purin-9-yl]-β-l-2-cyclopentene-1-methanol succinate (abacavir or zidovudine), and (R)-(9-2-phosphonylmethoxy-propyl)adenine (PMPA).

HIV-1’s high rate of replication and the lack of proofreading by RT during viral replication leads to frequent mutations (9–12). Distinct mutations occur in the presence of different NRTIs, and their temporal occurrence is often predictable (13–18). Initial mutations are often responsible for resistance to the compound, whereas later mutations increase the fitness of the mutant virus (19).

The mutations that are responsible for conferring nucleoside drug resistance are primarily clustered in three regions of the protein as illustrated in Fig. 1. These include the dNTP binding site (region II), the site near the n + 1 templating base (region III), and the putative ATP binding site (region I). These mutations have been shown to cause resistance by two distinct mechanisms.

The first mechanism of resistance appears to be related to a change in the incorporation of the activated drug into the replicating viral genome. These mutations are often found to be in direct contact with the incoming dNTP in the active site of RT and impede nucleotide analog incorporation and thereby show altered reaction kinetics (Fig. 1, region II) (20–25, 42). This has been best illustrated by steric hindrance interfering with 3TCTP binding in the active site of HIV-1 RT containing a Met184 to Val mutation (20, 26, 27). Additionally, other mutations have also been noted that contact the n + 1 templating base and appear to cause resistance at the level of incorpora-
tion by repositioning the active site in an unfavorable orientation for analog incorporation (Fig. 1, region III) (28–32).

The second mechanism of resistance appears to involve removal of the chain-terminator. RT does not contain 3' → 5' exonuclease activity in the same sense as traditional polymerases, but the reverse of the forward reaction catalyzed by the polymerase active site can serve to remove a 3' chain-terminating compound. The largest amount of work done to understand this mechanism of resistance is in the case of AZT resistance. AZT-resistant mutations occur in a pocket that is connected to the triphosphate binding region of the active site (Fig. 1, region I) (33, 34, 43). Reports have shown that AZT-resistant mutations, in the absence of causing large changes in incorporation (25, 35), may cause an increase in the rate of pyrophosphorolysis (36) and ATP-mediated removal (34, 37). There are some conflicting reports on whether the kinetic rate of pyrophosphorolysis is in fact increased by AZT-resistant mutations, and mounting evidence suggests that there is no increase in the rate of removal by PP_i (34). It may be argued, however, that this does not mean that PP_i does not play a role in AZT resistance. Studies have shown that there is a slower rate of primer-template release by the AZT-resistant mutant (38) and a tendency to form a stable complex, less sensitive to inhibition by the presence of the next correct nucleotide, at the incorporated AZTMP nucleotide (39). Accordingly, this may increase the overall amount of removal by prolonging the time RT spends at the 3' terminus no matter what the removing agent (PP_i or ATP) and in the absence of any marked change in the kinetic rate of removal. These results taken together suggest that both modes of removal may be active in vivo (40), although further studies are needed to clarify inconsistencies in the literature.

To better understand resistance to the Food and Drug Administration-approved compound abacavir and the mechanism of resistance to NRTIs in general, transient kinetic methodology was employed with abacavir's active metabolite (CBVTP, Fig. 2) (41) and a subset of mutants found in an in vitro drug selection study (17). Conclusions were further tested using a clinically relevant multidrug-resistant mutant (Q151M (16)) and an AZT-resistant mutant (RTAZTR, D67N/K70R/T215Y/K219Q (14, 15)). Our study looked at both incorporation and removal (by PP_i and ATP) along with other biochemical properties of these mutants. Results showed that the resistance of mutants seen in cell culture could not always be explained by incorporation alone. None of the abacavir-selected mutants showed increases in PP_i- or ATP-mediated removal rates, but some had significantly slower primer-template release rates, which is discussed as a possible mode of facilitating nucleotide removal. Abacavir's resistance profile, in light of the large number of mutations coupled with the relatively small changes in biological and kinetic behavior, may best be described as “minimization of resistance rather than avoidance.” This, in part, may be the result of CBVTP's inability to effectively mimic dGTP and a dampening of the overall reaction kinetics by strong hydrophobic interactions in the RT active site.

**Fig. 1. Location of the three major regions where mutations causing resistance to NRTIs occur.** This figure was generated using the crystal structure of the ternary complex of HIV-1 RT, dTTP, and primer-template (33).
Mechanism of Resistance Progression to Abacavir

40481

EXPERIMENTAL METHODS

Preparation of HIV-1 RT—RTWT, RT115F, RTL74V, and RTM184V clones were generously provided by Stephen Hughes, Paul Boyer, and Amanda Periris (NCI-Frederick). RT115F and RTM184V clones were co-anteated from the Hughes clone and kindly provided by Phillip Furman, Joy Feng, and Jerry Jeffrey (Triangle Pharmaceuticals). The triple mutant L74V/Y115F/M184V (RTCBVR) was made by sequentially adding mutations to the Hughes RTM184V clone using the Stratagene QuikChange kit. All RT clones were sequenced to verify correct sequence.

Purification of HIV-1 RT—The N-terminal histidine-tagged heterodimeric p66/p51 enzymes were purified as previously described (47, 48).

Nucleoside Triphosphates—dGTP was purchased from Amersham Biosciences. The (~)-CBVTP was generously provided by Dr. William B. Parker (Southern Research Institute, Birmingham, AL). The compound was further purified by high pressure liquid chromatography utilizing a gradient from 20 to 60% Et3NH(OH)(HCO3)2 in water and an Amersham Biosciences ion exchange column (mono Q HR 5/5). Its identity was verified using liquid chromatography/electrospray ionization mass spectrometry. The concentration of purified CBVTP was determined using the extinction coefficient ε280 = 13,260 M-1 cm-1 (49).

Oligonucleotides—Primers and templates used for incorporation and removal studies are shown in Table I, and all of the DNA oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer (Keck DNA synthesis facility, Yale University) and purified using 20% polyacrylamide denaturing gel electrophoresis. The 45-mer was synthesized and purified by New England Biolabs. D30-CBVMP was made using RTWT as previously described (6). 30- and 31-mer DNAs and 45-mer RNA were 5'-32P-labeled with T4 polynucleotide kinase (New England Biolabs) as previously described (50). [γ-32P]ATP was purchased from Amersham Biosciences. Biotin primers for the removal of excess [γ-32P]ATP were purchased from Bio-Rad.

Annealing of the DNA primers (D23, D30, D31, or D30-CBVMP) and 45-mer DNA and RNA templates were carried out by adding a 1:1.4 molar ratio of purified primer to 45-mer at 90 °C for 5 min, 50 °C for 10 min, and ice for 10 min. The annealed primer and template were then analyzed using 15% nondenaturing polycrylamide gel electrophoresis to ensure complete annealing. Concentrations of the oligonucleotides were estimated by UV absorbance at 260 nm using calculated extinction coefficients.

Pre-steady-state Burst and Single-turnover Experiments—Rapid chemical quench experiments were performed as previously described with a KinTek Instruments model RQF-3 rapid quench-flow apparatus (47, 50).

A pre-steady-state kinetic analysis was used to examine the incorporation of a nucleotide of dGMP or CBVTP into a DNA/DNA or DNA/RNA duplex. To analyze rates of incorporation of less than 2 s-1, single-turnover experiments were used. The reactions were carried out by rapid mixing of a solution containing the preincubated complex of 250 nM HIV-1 RT (active site concentration) and 50 nM 5'-labeled DNA/DNA duplex with a solution of 10 mM MgCl2 and varying concentrations of the next correct dNTP in the presence of 50 mM Tris-Cl, 50 mM NaCl, at pH 7.8 and 37 °C (all concentrations represent the final concentration after mixing). Single-turnover experiments were also used to study removal, misincorporation, and processivity. Misincorporation was studied in the same manner, except the DNA 31-mer primer was used to allow for the observation of incorporation of dGMP opposite a templating dTMP. In processivity experiments, elongation of the 23-mer DNA with the 45-mer DNA or RNA template was followed in the presence of 125 μM (final) of all four dNTPs. Polymerization was quenched at various time points by the addition of 0.3 mM (final) EDTA. Single-turnover conditions were also used to study the removal of chain-terminating nucleotides. Either 2 mM PPi (J.T. Baker catalogue no. 3850-1) or 3.2 mM ATP (Sigma catalogue no. A2383) were mixed with a solution containing 250 nM HIV-1 RT prebound to 50 nM 5'-labeled chain-terminated DNA/DNA duplex with 10 mM MgCl2 in the presence of 50 mM Tris, 50 mM NaCl, at pH 7.8 and 37 °C (all concentrations represent the final concentration after mixing). Removal studies using the original D45/D31 template-primer were unsuccessful in that no removal of a chain-terminating CBVMP and the D31-mer (containing dGMP at the 3' terminus) was only extended under these conditions. The observed incorporation may have been due to ribonucleotide incorporation or a small amount of deoxynucleotide contamination. The inefficient nucleotide-dependent removal when the next correct incorporation is complementary to the removing nucleotide has been previously noted (51). In order to alleviate this problem, the templating base was changed from dTMP to dGMP in the D45-mer template for removal studies.

To obtain observed rates of incorporation for faster reactions and to measure the steady-state rate of incorporation, pre-steady-state burst experiments were used. Pre-steady-state bursts were done under the same conditions as those described for a single-turnover experiment, except the amount of primer-template (300 nM final) was in 3-fold excess of enzyme (100 nM active sites final). Products were analyzed by 20% polyacrylamide gel electrophoresis and quantified using a Bio-Rad GS825 molecular imager.

Data Analysis—Data were fit by nonlinear regression using the program KaleidaGraph version 3.09 (Synergy Software, Reading, PA). Results from pre-steady-state burst experiments were fit to a burst equation: [Product] = A1(1 - exp(-kobsd) + kpol), where A represents the amplitude of the burst that correlates with the concentration of active enzyme, kobsd is the observed first-order rate constant for dNTP or analog incorporation, and kpol is the observed steady-state rate constant. Data from single-turnover incorporation experiments were fit to a single exponential equation: [Product] = A1(1 - exp(-kobsd)), where A1 is the observed steady-state rate constant.

RESULTS

In the current study, we compare the activities of RTWT and a number of drug-resistant mutants in terms of incorporation and removal of dGMP and CBVMP (CBVTP shown in Fig. 2) into model oligonucleotide substrates (Table I). Experiments were also done to further characterize the effects of some of the mutations on biochemical properties such as misincorporation and processivity. A series of pre-steady-state bursts and single-turnover experiments were conducted in order to determine the kinetic parameters for correct and incorrect single nucleotide incorporation directed by a DNA or RNA template. Kinetic constants determined include the maximum rate of incorpora-
tion \((k_{\text{pol}})\) and the equilibrium dissociation constant \((K_d)\). From these values, the incorporation efficiency was calculated \((k_{\text{pol}}/K_d)\) and used as a means for comparing different substrates and enzymes. The observed rate of dNMP removal from the end of a terminated chain was also determined at a single concentration of PP, \((k_{\text{pyro}})\) and ATP \((k_{\text{ATP}})\). This information was used as a quantitative basis for understanding these RT mutants and how their presence may confer resistance to abacavir.

**Active Site Concentrations of Mutant RT during DNA- and RNA-directed Single Nucleotide Incorporation**—When primer-template is in slight excess over RT, the kinetics of nucleotide incorporation during the first enzyme turnover as well as multiple turnovers can be examined. During the incorporation of all natural dNMPs by RT, this results in the observation of a burst of product formation, as prebound substrate is turned over, followed by a linear phase reflecting the overall rate-limiting step of product release (50). All of the RT mutants showed a burst of product formation during incorporation of dGMP (an example of a burst of CBVMP incorporation is shown in Fig. 4A). The amplitude of the fast phase of product formation can be directly correlated to the amount of active enzyme, and when compared with the total protein concentration (determined by absorbance at 280 nm), the percentage of active protein can be calculated. These analyses showed that none of the mutants had a significantly lower active site concentration than RT\(^{WT}\) (25%) and that many had different activity depending on the primer-template (Fig. 3). During DNA-directed polymerization, RT\(^{L74V}\) and RT\(^{CBVR}\) (L74V/Y115F/M184V) showed the highest percentage of active sites (around 40%). During RNA-directed polymerization, RT\(^{M184V}\) and RT\(^{CBVR}\) showed the highest level of activity (around 50%). RT\(^{CBVR}\) was the only protein with at least a 10% higher percentage of active sites during both DNA- and RNA-directed polymerization than RT\(^{WT}\).

**Pre-steady-state Kinetics of Incorporation by Wild Type and Mutant Forms of RT**—The presence of a pre-steady-state burst during the incorporation of an analog suggests that it is being incorporated by a similar kinetic mechanism as natural nucleotides. A pre-steady-state burst of product formation was observed for the incorporation of CBVMP by RT\(^{WT}\) and all mutants studied (CBVMP incorporation by RT\(^{CBVR}\) shown in Fig. 4A).

To better understand the effect of mutations at positions 74, 115, and 184 both alone and in combination and a multidrug-resistant mutation at position 151 (locations of mutations shown in Fig. 1), pre-steady-state incorporation studies were carried out. By fitting the observed rates of nucleotide incorporation \((k_{\text{introl}})\) for DNA- and RNA-directed polymerization at different concentrations of dGTP for each of the RT mutants to hyperbolic curves, the maximum rates of dGMP incorporation \((k_{\text{pol}})\) and the binding constants for dGTP \((K_d)\) were obtained. Fig. 4 shows the typical kinetic data obtained and how it is quantitated during transient kinetic studies (in this case the DNA-directed incorporation of CBVMP by RT\(^{CBVR}\)). First, a pre-steady-state burst is done to determine whether, like a natural nucleotide, the rate-limiting step follows chemistry (Fig. 4A). A set of single-turnover experiments were then done at varying nucleotide concentrations to determine the observed rate (Fig. 4B), and the observed rates were then plotted against nucleotide concentration and fit to a hyperbolic curve to determine the \(K_d\) and \(k_{\text{pol}}\) (Fig. 4C). In general, the \(k_{\text{pol}}\) values for different mutants were all equal to or faster than those obtained for RT\(^{WT}\) incorporation of dGMP; however, the \(K_d\) values varied markedly between different mutants and substrates (Fig. 5A, summarized in Table II). Comparing the efficiencies of incorporation \((k_{\text{pol}}/K_d)\) showed that all mutants had similar efficiencies during DNA-directed polymerization except for RT\(^{Q151M}\), which showed a 2-fold higher efficiency (Table II). During RNA-directed polymerization, RT\(^{L74V}\), RT\(^{Y115F}\), and RT\(^{CBVR}\) all showed significantly lower efficiencies of incorporation due to weak binding of dGTP (reflected by an increase in the \(K_d\)) at their active sites. RT\(^{M184V}\) and RT\(^{Q151M}\) showed similar efficiencies of incorporation to RT\(^{WT}\).

In contrast to dGMP incorporation, all mutants during DNA- and RNA-directed CBVMP incorporation showed a decrease in \(k_{\text{pol}}\) relative to values obtained with RT\(^{WT}\) (Fig. 5B; summarized in Table II). The slowest \(k_{\text{pol}}\) values were obtained with RT\(^{CBVR}\) and RT\(^{Q151M}\) (10-fold less than those observed with RT\(^{WT}\)). \(K_d\) values for CBVTP binding varied unpredictably in response to mutation during both DNA- and RNA-directed incorporation by RT\(^{WT}\) and all mutants studied (CBVMP incorporation by RT\(^{CBVR}\) shown in Fig. 4A).

### Table I

**Sequence of primers and templates used to study CBVMP incorporation and removal**

Templating bases for single nucleotide incorporation and removal studies are shown in boldface italic type, and sites of expected RNase H cleavage during incorporation into D30/R45 at 18 and 21 base pairs from the site of incorporation are underlined.

| Name          | Sequence                                      |
|---------------|-----------------------------------------------|
| DNA 23-mer    | 5’-GCC TCG CAG CGG TCC AAC ACA CT             |
| DNA 30-mer    | 5’-GCC TCG CAG CGG TCC AAC ACA CTC            |
| DNA 31-mer    | 5’-GCC TCG CAG CGG TCC AAC ACA CTC            |
| DNA 30-CBVMP  | 5’-GCC TCG CAG CGG TCC AAC ACA CTC V\(^*\)   |
| DNA 45-mer    | 3’-CCG AGG CCC AGG UAG UAG UAG GAG            |
| DNA 45-mer (removal) | 3’-CCG AGG CCC AGG UAG UAG UAG GAG | CBVMP.  

\(^*\) V, CBVMP.
incorporation. During DNA-directed incorporation, RT<sup>M184V</sup> was the only mutant to show weaker binding than RT<sup>WT</sup>. In contrast, RT<sup>Y115F</sup> was found to bind in the submicromolar range (20-fold tighter than RT<sup>WT</sup>). RT<sup>L74V</sup> showed unique incorporation kinetics with two exponential phases each accounting for 50% of the total incorporation under both single-turnover and pre-steady-state burst conditions (data not shown).

The concentration dependence on the rate for both phases was determined and is summarized in Table II. During RNA-directed incorporation, RT<sup>P24V</sup>, RT<sup>M184V</sup>, RT<sup>Q151M</sup>, and RT<sup>CBVR</sup> all bound with weaker affinities than RT<sup>WT</sup>. Similar to the higher affinity observed with a DNA/DNA primer-template, RT<sup>Y115F</sup> had a 2-fold tighter <i>K<sub>d</sub></i> than RT<sup>WT</sup>. By comparing the efficiencies of incorporation in the presence of dGTP and the reaction was quenched at indicated times and analyzed by 16% sequencing gel electrophoresis. The solid line represents a fit to a burst equation with an amplitude (<i>A</i>) equal to 188 ± 10 nM, an observed first-order rate constant for the burst phase (<i>k<sub>burb</sub></i>) equal to 0.061 ± 0.006 s<sup>-1</sup>, and an observed rate for the linear phase (<i>k<sub>lin</sub></i>) equal to 0.0012 ± 0.0004 s<sup>-1</sup>.

B. pre-steady-state single-turnover kinetics of incorporation of CBVMP into a DNA/DNA primer-template by RT<sup>CBVR</sup> were measured by mixing a preincubated solution of RT (250 nM) and DNA/DNA primer-template (50 nM) with various concentrations of CBVTP (1 μM (●), 5 μM (○), 10 μM (△), 25 μM (×), 100 μM (+), and 200 μM (●)) and MgCl<sub>2</sub> (10 mM) under rapid quench conditions (all concentrations are final after mixing). The <i>k<sub>burb</sub></i> values ranged from 0.01 s<sup>-1</sup> at 1 μM to 0.072 s<sup>-1</sup> at 200 μM. Data were used to generate the <i>K<sub>D</sub></i> curve in C, where the observed rate (<i>k<sub>obd</sub></i>) is plotted against CBVTP concentration. The hyperbolic fit to the data gives a maximum rate of incorporation (<i>k<sub>pol</sub></i>) of 0.074 ± 0.004 s<sup>-1</sup> and an equilibrium binding constant (<i>K<sub>D</sub></i>) of 7.1 ± 2 μM.
CBVTP, a selectivity value can be calculated (efficiency dGTP / efficiency CBVTP), which provides an estimate of how much dGTP is favored over CBVTP as a substrate. All mutants except RTY115F showed a higher selectivity for dGTP over CBVTP than RT WT. The order of selectivity values during DNA-directed incorporation was RT CBVR/H11005, RTM184V/H11022, RTL74V/H11022, RTQ151M/H11022, RTWT/H11022, and RTY115F/H11022. For RNA-directed incorporation, it was RTCBVR/H11005, RTM184V/H11022, RTQ151M/H11022, RTL74V/H11022, RTY115F/H11022, and RTWT/H11005 (Table II).

The linear phase of a pre-steady-state burst experiment gives a measurement of the steady-state rate of incorporation ($k_{ss}$ or $k_{off}$; Fig. 4), which is thought to be equal to the rate of elongated primer-template release (50). During DNA-directed synthesis, the $k_{off}$ values were found to be similar during the incorporation of dGMP by all mutants. In contrast, many of the mutants had significantly slower $k_{off}$ rates during CBVMP incorporation (Table III). Similarly, no significant difference in steady-state rates for dGMP were found during RNA-directed incorporation, whereas CBVMP incorporation rates varied with similar relative decreases for certain mutants as those seen during DNA-directed incorporation (data not shown).

Pre-steady-state Kinetics of PPi- and ATP-mediated Removal by Wild Type and Mutant Forms of RT—To see if removal of a chain-terminating CBVMP could play a role in the resistance observed by these mutations, studies were carried out looking at removal by PPi (2 mM) or ATP (3.2 mM). Studies were also conducted using AZT-resistant RT (RTAZTR) containing mutations at positions 67, 70, 215, and 219. Mutations at these sites have been implicated in causing resistance to AZT by PPi-mediated (36) and ATP-mediated (34, 37) removal.

In general, removal reactions were found to be very inefficient, with rates greater than 1000-fold less than incorporation and reactions not proceeding to completion. The removal of dGMP from the end of a 31-mer DNA primer annealed to a 45-mer DNA template by PPi showed very similar rates by all mutants ($k_{pyro}$ values varying by no more than 2-fold), and no mutant was significantly faster than RTWT (Fig. 6A and Table III). A larger amount of variation was noted in the ability of various mutants to catalyze the removal of dGMP by ATP ($k_{ATP}$ values varying by 20-fold). RTAZTR showed the highest rate of ATP-mediated removal, and RTQ151M also showed a slight elevation of rate compared with that of RTWT, whereas RTM184V and RTCBVR were impaired in their ability to remove dGMP when compared with other RTs (Fig. 6B). In contrast to dGMP removal by PPi, there was a large variation (130-fold) in the mutants’ ability to remove CBVMP. No mutant showed a faster $k_{pyro}$ than RTWT, but RTY115F, RTQ151M, and RTAZTR all had similar rates. RTL74V, RTM184V, and RTCBVR were all impaired in their rate of CBVMP removal by pyrophosphorolysis (Fig. 6C). Similar to the PPi-mediated removal of CBVMP, a marked difference (>100-fold) was noted in $k_{ATP}$ for CBVMP removal by different mutants. RTAZTR showed the highest rate, and all other proteins showed very low removal activity using ATP as a substrate (Fig. 6D; all values summarized in Table III).
In order to estimate the rate of removal under physiological conditions, by mutants relative to RTWT, a removal index was calculated. A greater than physiological concentration of PPi was used in order to amplify possible differences in pyrophosphorylase rates between mutants (2 mM was used, and the cellular concentration has been reported to be 125 \mu M (53)). To estimate the rate of kATP under physiological conditions (assuming the K_{pol} for pyrophosphate to be 2 mM (54)), the rate was divided by 10. No correction was necessary for kATP measurements, because cellular concentrations of ATP have been measured to be around 3.2 mM (55). In considering the forward and reverse reactions, the sum of the rates of PPi and ATP-mediated removal at physiological concentrations was divided by the steady-state rate to obtain a measure of the amount of removal per incorporation and normalized to obtain an estimate of the removal index relative to a value equal to 1 for RTWT. This analysis showed that RTM184V and RTCBVVR would be expected to be impaired in removal and that RTL74V, RTY115F, and RTQ151M would be expected to have elevated removal levels compared with RTWT (summarized in Table III).

### Table II

| Primer-template | RT   | dNTP  | k_{pol} | K_{d}  | k_{pol}/K_{d} | Selectivity^a |
|-----------------|------|-------|---------|--------|---------------|---------------|
| DNA/DNA         | WT^b | dGTP  | 24 ± 1  | 14 ± 0 | 1.7 ± 0.3     | 30            |
| DNA/DNA         | L74V | CBVTP | 1.0 ± 0.06 | 21 ± 3 | 0.05 ± 0.01   |               |
| DNA/DNA         | Y115F| dGTP  | 0.9 ± 0.05 | 9.6 ± 0.8 | 2.3 ± 0.2 | (20, 200)^d |
| DNA/DNA         | Q151M| CBVTP | 0.066 ± 0.002 | 5.2 ± 0.7 | 0.12 ± 0.002 |               |
| DNA/DNA         | M184V| dGTP  | 22 ± 2  | 5.5 ± 2 | 3.0 ± 0.3     | (60)          |
| DNA/DNA         | CBVTP| dGTP  | 0.11 ± 0.01 | 1.9 ± 0.2 | 0.13 ± 0.07 |               |
| DNA/RNA         | WT^b | dGTP  | 24 ± 1  | 9.1 ± 2.5 | 2.6 ± 0.7     | 10            |
| DNA/RNA         | L74V | dGTP  | 0.31 ± 0.02 | 0.89 ± 0.26 | 0.35 ± 0.10 |               |
| DNA/RNA         | CBVTP| dGTP  | 0.3 ± 1  | 8.5 ± 2 | 4.6 ± 0.3     |               |
| DNA/RNA         | Y115F| dGTP  | 0.11 ± 0.01 | 1.4 ± 0.2 | 0.078 ± 0.013 |               |
| DNA/RNA         | Q151M| dGTP  | 35 ± 1  | 16 ± 2 | 2.2 ± 0.3     | 200           |
| DNA/RNA         | M184V| dGTP  | 0.45 ± 0.02 | 51 ± 5 | 0.009 ± 0.001 |               |
| DNA/RNA         | CBVTP| dGTP  | 0.074 ± 0.004 | 7.1 ± 2 | 0.01 ± 0.002 |               |
| DNA/RNA         | L74VY115F/M184V| dGTP | 44 ± 4 | 29 ± 9 | 1.5 ± 0.3 | 200            |

^a Selectivity = efficiency_{dGTP}/efficiency_{analog}.

^b Refs. 21 and 22.

^c Two phases of CBVMP incorporation were observed that were responsible for producing 50% of the product during DNA-directed incorporation (Ref. 21 and 22).

### Table III

| Primer-template | RT   | dNTP  | k_{pol} | k_{pyro} | k_{ATP} | Removal index^c |
|-----------------|------|-------|---------|----------|---------|---------------|
| DNA/RNA         | WT^b | dGMP  | 0.27 ± 0.05 | 1.6 ± 0.1 | 0.14 ± 0.02 |               |
| DNA/RNA         | L74V | dGMP  | 0.093 ± 0.009 | 0.61 ± 0.02 | 0.0099 ± 0.003 | 1            |
| DNA/RNA         | Y115F| dGMP  | 0.0049 ± 0.0013 | 0.26 ± 0.02 | 0.0051 ± 0.0006 | 8            |
| DNA/RNA         | Q151M| dGMP  | 0.28 ± 0.07 | 1.4 ± 1 | 0.15 ± 0.02 |               |
| DNA/RNA         | M184V| dGMP  | 0.010 ± 0.002 | 0.58 ± 0.06 | 0.012 ± 0.001 | 9            |
| DNA/RNA         | CBVMP| dGMP  | 0.23 ± 0.02 | 0.98 ± 0.07 | 0.039 ± 0.004 |               |
| DNA/RNA         | CBVMP| CBVMP | 0.046 ± 0.002 | 0.0099 ± 0.0029 | <0.001 | 0.03           |
| DNA/RNA         | CBVMP| dGMP  | 0.24 ± 0.02 | 0.99 ± 0.15 | 0.053 ± 0.001 |               |
| DNA/RNA         | CBVMP| CBVMP | 0.012 ± 0.001 | 0.0047 ± 0.0008 | <0.001 | 0.06           |
| DNA/RNA         | Q151M| dGMP  | 0.31 ± 0.05 | 1.5 ± 2 | 0.32 ± 0.04 |               |
| DNA/RNA         | CBVMP| CBVMP | 0.0016 ± 0.0004 | 0.68 ± 0.15 | 0.00789 ± 0.0002 | 65          |
| DNA/RNA         | AZTR | dGMP  | ND^d | 1.8 ± 0.3 | 0.85 ± 0.12 |               |
| DNA/RNA         | CBVMP| ND   | 0.46 ± 0.02 | 0.11 ± 0.02 | ND            |

^c Removal index = ((k_{pyro}/10) + k_{ATP}/K_{pol} × 0.00667). k_{pyro}/10 approximately equals the rate of pyrophosphorylation at a physiological concentration of PPi (125 \mu M (53)).

^d ND, not determined.

Misincorporation by RTWT, RTY115F, and RTCBVVR during DNA-directed Single Nucleotide Incorporation—A very interesting observation was made during the evaluation of RTY115F in that it was the only protein to show detectable levels of misincorporation during pre-steady-state incorporation of dGMP into the D30-mer primer at concentrations as low as 50 \mu M dGTP (as observed by the appearance of a D32-mer product, data not shown). In order to gain a quantitative understanding of misincorporation by RTY115F, pre-steady-state incorporation studies were carried out by examining the DNA-directed dGMP incorporation into a D31-mer primer opposite a templating dTMP (representing a G-T misincorporation). For comparison with RTY115F, RTWT and the Y115F-containing triple mutant RTCBVVR were also examined. This analysis showed that...
**RTY115F** and **RTCBVR** had 1 order of magnitude tighter binding to dGTP than **RTWT** but that **RTCBVR** also had a much slower rate of incorporation (Fig. 7 and Table IV). **RTY115F** showed both a tighter binding and a more rapid rate of misincorporation than **RTWT**, combining to make it 10-fold more efficient at dGMP misincorporation. The decrease in the maximum rate of misincorporation seen for **RTCBVR** may explain the lack of observed misincorporation during DMP incorporation studies into the D30-mer primer (data not shown).

**Processivity of RTWT, RTL74V, and RTCBVR during DNA- and RNA-directed Polymerization—**A recent transient kinetic study reported that the V75T mutation of RT, present in the same “template grip” region of RT (Region III, Fig. 1) as the L74V mutation, showed increased processivity when compared with **RTWT**, and this change was suggested to play a possible role in low level resistance to D4TTP (32). In order to gain a better understanding of the processivity of RTL74V, incorporation studies were done in the presence of all four natural dNTPs during both DNA- and RNA-directed polymerization by **RTWT**, **RTL74V**, and the L74V-containing triple mutant **RTCBVR**. Polyacrylamide gel analysis showed that the three RTs had similar processivity. During DNA-directed polymerization, **RTL74V** was slightly better than **RTWT**, and **RTCBVR** had the greatest accumulation of elongated products. However, during RNA-directed polymerization, both of these mutants caused decreased processivity when compared with **RTWT** (Fig. 8).

**DISCUSSION**

Initial studies on incorporation in the presence of CBVTP showed that it was a poor substrate for RT, and comparison of its utilization to that previously obtained for D4TTP (56) suggested that the oxygen in the ribose ring (which is present in D4TTP but absent in the carbocyclic ring of CBVTP) might be important in defining high efficiency incorporation by **RTWT** and resistance by **RTM184V** (21). A recent report directly tested this hypothesis, since D4GTP was synthesized and found to be a superior substrate for **RTWT**, and **RTM184V** conferred no resistance at the level of incorporation. These results suggest that key interactions with the protein active site and nucleotide structural features, both presumably affected by the hydrophobic nature of the carbocyclic ring of CBVTP, may play a role in defining the differences in incorporation between CBVMP and D4GMP by **RTWT** and **RTM184V** (22). D4GTP’s highly efficient utilization by RT and the possible implications that this could have on the development of drug resistance prompted the synthesis of an acid-stable D4G prodrug, cyclo-D4G (57). In this current report, the mechanism of resistance progression to abacavir was studied by looking at the kinetics of incorporation and removal of CBVMP by mutants selected for in a cell culture study (17), and conclusions were strengthened by studying **RTQ151M** and **RTAZTR**.

**Selection of Mutants with Reduced Maximum Rates of CBVMP Incorporation during Cell Culture Drug Selection Studies—**The set of three mutations chosen for detailed kinetic studies in this paper (L74V, Y115F, and M184V both as single mutants and in combination) were selected for in an in *vitro* drug selection study (17) in which unphysiologically high intracellular concentrations of CBVTP were most likely formed. Under these experimental conditions, it would be expected that the greatest selection pressure would be for RT mutants with decreased maximum rates of incorporation ($k_{pol}$), because at concentrations significantly higher than the $K_d$, binding of the nucleotide is saturated and perhaps less of a contributing factor. Review of the kinetic data shows that all of the mutants selected for during this study had decreases in $k_{pol}$ values with less predictable changes in binding ($K_d$). In fact, **RTL74V**, **RTY115F**, and **RTCBVR**’s tighter binding constants during DNA-directed incorporation may make them more sensitive to CBVTP at low concentrations than **RTWT** (illustrated by Fig. 5B). **RTY115F** was unique, being the only mutant to incorporate CBVMP more efficiently during RNA-directed synthesis and as efficiently during RNA-directed synthesis as **RTWT** (Table II). **RTY115F** was only selected for in late passages (17), suggesting that it is only effective at less physiologically relevant concentrations of CBVTP.
the reason that the Y115F mutation has rarely been observed in vivo (58). RTY115F was the only mutant (including Q151M) resistant during both DNA- and RNA-directed incorporation at the level of both incorporation and binding, possibly explaining its early appearance and prevalence both in vitro (17) and in vivo (58).

Interaction between CBVTP and the “Steric Gate” of HIV-1 RT—Tyr115 is in close proximity to the deoxyribose ring and has been shown to be responsible for the “north” deoxyribose ring bias of RT (59) (ribose ring conformations reviewed in Ref. 60) and may be important in the exclusion of 2'-hydroxyl-containing ribose nucleotides (61). We have suggested that CBVTP’s loose relative binding during DNA-directed incorporation and correspondingly tight relative binding during RNA-directed incorporation by RTWT may be due to its interaction with Tyr115 (22). This binding may be related to favorable hydrophobic interaction when the carbocyclic ring of CBVTP is bound over the aromatic ring and negative electrostatic interactions when it is positioned closer to the hydroxyl group of Tyr115. If this is indeed the case, the mutation of Y115F would be expected to increase the binding of CBVTP by increasing the hydrophobicity of the nucleotide binding pocket. As predicted, data summarized in Table II show that RTY115F has a very tight association (Kd) with CBVTP during both DNA- and RNA-directed incorporation. As discussed above, this mutation also caused a decrease in the kpol, possibly reflecting the tight binding of CBVTP shifting the equilibrium away from incorporation. The steady-state rate (kcat) was also decreased, suggesting that RTY115F remains bound to the elongated, chain-terminated primer-template longer than wild type (Table III). Given the lack of a selectivity of RTY115F against CBVMP incorporation, it is assumed that these features somehow confer the slight resistance in drug susceptibility assays (2-fold) (17).

Role for the Hydrophobic Nature of CBVTP’s Ribose Ring in RTWT may be due to its interaction and correspondingly tight relative binding during RNA-CBVTP containing ribose nucleotides (61). We have suggested that RTY115F has a very tight association (Kd) with CBVTP during both DNA- and RNA-directed incorporation and correspondingly tight relative binding during RNA-directed incorporation by RTWT may be due to its interaction with Tyr115 (22). This binding may be related to favorable hydrophobic interaction when the carbocyclic ring of CBVTP is bound over the aromatic ring and negative electrostatic interactions when it is positioned closer to the hydroxyl group of Tyr115. If this is indeed the case, the mutation of Y115F would be expected to increase the binding of CBVTP by increasing the hydrophobicity of the nucleotide binding pocket. As predicted, data summarized in Table II show that RTY115F has a very tight association (Kd) with CBVTP during both DNA- and RNA-directed incorporation. As discussed above, this mutation also caused a decrease in the kpol, possibly reflecting the tight binding of CBVTP shifting the equilibrium away from incorporation. The steady-state rate (kcat) was also decreased, suggesting that RTY115F remains bound to the elongated, chain-terminated primer-template longer than wild type (Table III). Given the lack of a selectivity of RTY115F against CBVMP incorporation, it is assumed that these features somehow confer the slight resistance in drug susceptibility assays (2-fold) (17).

### Table IV

| RT         | kpol s⁻¹ | kcat μM | kpolKcat μM⁻¹ s⁻¹ |
|------------|----------|--------|------------------|
| WT         | 0.043 ± 0.001 | 1400 ± 100 | 0.31 ± 0.02 |
| Y115F      | 0.068 ± 0.001 | 150 ± 10 | 4.5 ± 0.3 |
| CBVR       | 0.023 ± 0.001 | 190 ± 40 | 1.2 ± 0.3 |

Abacavir’s Resistance Profile—The 10-fold resistance to abacavir found in cell culture (17) is minimal compared with the 100–2000-fold resistance isolated to AZT and 3TC, respectively (15, 44). Abacavir’s favorable resistance profile appears to be second only to that of D4T, which selects for 2-fold resistance in cell culture (46). Although both D4T and abacavir have excellent resistance profiles in cell culture, there is a marked distinction in the number of mutations selected for. D4T only selects for one mutation in cell culture (V75T) (46), whereas abacavir selects for four single mutations (K65R, L74V, Y115F, and M184V), which are mixed in seven different combinations of multiply mutated virus (17). In this sense, D4T can be described as avoiding resistance caused by mutation, whereas abacavir minimizes it.

The biochemical mechanism for D4T’s lack of selection of resistant mutations has been suggested to be due to D4TTP’s ability to mimic dTTP in the RT active site by a study that showed D4TTP to be incorporated as efficiently as dTMP by RTWT (56). Data presented here clearly illustrate that this is not the case for CBVTP, which is a 10–30-fold worse substrate than dGTP. In fact, abacavir’s selection of a large number of mutations could be attributed to CBVTP’s inability to effectively mimic dGTP. Inspection of the data shows that with all of the mutants except M184V, CBVTP has a tighter association with the mutant than with RTWT during either DNA- or RNA-directed incorporation, minimizing the overall effect of the mutation on the efficiency of CBVMP incorporation. These results may suggest that CBVTP can reduce the effects of a mutation by taking advantage of hydrophobic contacts in the active site during elongation of one of the many substrates utilized by RT. In effect, this would serve to circumvent high levels of resistance through hydrophobic interactions. Support for this hypothesis can be found in results with RTQ151M. In this kinetic study, RTQ151M only showed a 2-fold increase in selectivity over RTWT during DNA-directed incorporation and a more substantial increase of 8-fold during RNA-directed incorporation of CBVMP. The reduced effect during DNA-directed incorporation was due to a 20-fold tighter binding of CBVTP by RTQ151M compared with that of RTWT (summarized in Table II). These kinetic observations may translate into the less dramatic sensitivity of abacavir compared with AZT or D4T to clinical isolates of Q151M-mutated virus (62), although the level of resistance to all of these compounds is sufficient to

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**Fig. 7.** The dependence of the observed rate of misincorporation on dGTP concentration. Misincorporation of dGMP opposite a templating dTMP by RTWT (●), RTY115F (○), and RTCBV (△) is shown.
leave them almost completely ineffective against HIV in a patient.

Mutations in the “Template Grip” Affecting the Substrate Specificity of RT—The leucine at position 74 is present in the region of RT that contacts the templating base and is responsible for flipping out the next templating base (Fig. 1, region III) (33). The movement of the $n + 2$ templating base is thought to play an important role in polymerase fidelity by allowing for more contact with the recently formed base pair (between the incoming dNTP and the $n + 1$ base), and a resulting kink causes the primer-template strand to take a more A-form character near the active site (63). Many mutations conferring resistance to nucleoside analog drugs have been reported in this region (Fig. 1, region III). A62V, V75I, and F77L are associated with Q151M in a multidrug-resistant complex (16), and, as previously discussed, V75T causes slight resistance to D4T (46). Besides abacavir, L74V has been associated with resistance to ddi and ddC (18, 45). Despite their importance, a mechanistic understanding of these mutations is for the most part lacking.

Similar to a previous steady-state study on ddATP (64), RT$^{L74V}$ showed a 3-fold increase in selectivity to CBVTP during both DNA- and RNA-directed incorporation over RT$^{WT}$ (Table II). As has been suggested, this reduction in incorporation may be related to a movement of the active site in such a way that distinguishes between the natural substrate and analog (28–31). Part of this reorientation of the active site may be reflected in a change in the interaction between the amino acid at position 74 and the flipped out template base. This hypothesis is supported by a fidelity study that showed that RT$^{L74V}$ has an increased tendency to cause frameshift mutations (65). In a recent report, where similar resistance was observed to D4TPP by the V75T mutation, it was suggested that residues in the “template grip” region may interact with Gln$^{151}$ in a manner that can increase the selectivity of RT to nucleotide analogs (32). In this study, many of the kinetic parameters for resistance by RT$^{L74V}$ and RT$^{Q151M}$ were found to be similar, lending support to this hypothesis.

One similarity between RTs containing the L74V and Q151M mutations is that a switch in the preferred primer-template combination from that observed with RT$^{WT}$ occurs. Both RT$^{L74V}$ and RT$^{Q151M}$ incorporate dGMP 2-fold more efficiently during DNA-templated incorporation than RNA-templated. This is in contrast to RT$^{WT}$, which has been shown in this (Table II) and previous studies (20, 35, 48, 56) to have a higher efficiency during incorporation into DNA/RNA primer-templates. Surprisingly, the reason for this bias toward DNA-directed incorporation by RT$^{L74V}$ is due to a 2-fold increase in the efficiency of incorporation over that seen with RT$^{WT}$. This increased efficiency of incorporation may be related to HIV-1 strains that contain the Q151M mutation being more fit than WT under cell culture conditions (66). Whereas RT$^{L74V}$ shows a similar efficiency as RT$^{WT}$ during DNA-directed incorporation, it was found to have a marked reduction in the efficiency of incorporation during RNA-directed incorporation. These results suggest that this area of the protein is a key site in the bias of RT toward an RNA template that should be explored further both for an understanding of drug resistance and the fundamental mechanism of nucleotide incorporation by RT.

Removal as a Mode of Resistance to Abacavir—A recent report showed that hepatitis B virus RT catalyzes the pyrophosphorolytic removal of dNMPs with an efficiency comparable to that of their incorporation (67). Data presented in this paper show that HIV-1 RT is very poor at nucleotide removal, suggesting that this process may not play as great a role in NRTI sensitivity with HIV as it does with hepatitis B virus. Our results show that none of the abacavir-selected mutations had an increased rate of PPi- or ATP-mediated removal over those observed with RT$^{WT}$. It was found that in most cases there was an inverse relationship between selectivity and the rate of removal. In other words, a mutation that decreased the efficiency of incorporation also decreased the rate of removal with ATP and PPi. This makes sense, considering the intrinsic relationship between the two processes and would suggest that a mutant that mediates resistance by removal would not show dramatic reductions in the incorporation of an analog. In support of this hypothesis, studies on RT$^{AZT^R}$, which appears to confer resistance by removing chain-terminating AZTMDP (34, 36, 37, 40) and D4TMP (39, 68, 69), have shown very little or no reduction in the incorporation of AZTMP (25, 35). Consistent with previous reports suggesting that AZT resistance mutations cause resistance by increasing the rate of dNMP removal.
mediated by ATP (34, 37, 39), RT<sub>AZTR</sub> showed the fastest $k_{ATP}$ rate with both dGMP and CBVMP chain-terminated primers. The elevated rate of CBVMP removal may be related to the association between AZT resistance mutations and decreased abacavir activity in vitro (70) and in vivo (71). Interestingly, RT<sub>Q151M</sub> also showed an elevated rate of ATP-mediated removal of dGMP; however, it was slightly slower than RT<sub>WT</sub> at removing CBVMP. This suggests that removal may play a role in Q151M-mediated resistance to some compounds, although studies have suggested removal to be unimportant in its resistance to AZT (40) or D4T (68).

A reduction in the steady-state rate, which in most cases reflects the rate of product release ($k_{cat}$) for an incorporation catalyzed by RT (80), could cause resistance by increasing the amount of removal in the absence of major increases in $k_{prod}$ or $k_{ATP}$. By summing the estimated rates of removal under physiological conditions and dividing by the rate of release, a removal index was calculated, which relates removal to incorporation (Table III). In this study, three of the mutants (RT<sub>L74V</sub>, RT<sub>Y115F</sub>, and RT<sub>Q151M</sub>) had removal indexes higher than that of RT<sub>WT</sub> suggesting that removal may play a role in their resistance to abacavir. Although these results suggest that removal may be important in resistance to abacavir, recent reports have proposed that the most important factor in removal is the creation of a stable complex centered on the chain-terminating analog (34, 39), an issue that this study does not directly address. However, it is still tempting to suggest that removal may play a role in the slight resistance conferred by the Y115F mutation in cell culture (17), which showed no increase in selectivity in our kinetic studies (Table II). Possibly, the strong hydrophobic interactions between incorporated CBVMP and Phe<sup>115</sup> cause a stalled complex poised for pyrophosphorylation or ATP-mediated removal.

Consistent with a previous report, the M184V mutation impaired the removal of both dGMP and CBVMP (Table III) (72). RT<sub>CBVR</sub>, which contains the M184V mutation, also had diminished ability to remove dGMP or CBVMP. The other two mutations present in RT<sub>CBVR</sub> (L74V and Y115F) only showed slight effects on CBVMP removal and did not impair dGMP removal. Taken together, these results suggest that the M184V mutation alone or in combination causes a decrease in the rate of removal. Consistent with our results, the effectiveness of AZT/3TC combination therapy (73) has been hypothesized to be due to the M184V mutation selected for by 3TC, decreasing the ability of AZT resistance mutations to rescue AZTMP chain-terminated viral transcripts (72). Boyer et al. recently published a report with data supporting this hypothesis and suggesting that the negative effect of M184V on removal may be associated with the movement of the active site relative to the hypothesized ATP binding site reducing the ability of ATP to be utilized in chain terminator removal (74). However, another report showed that under their experimental conditions, M184V did not effect rescue of AZT chain-terminated primer-templates in the presence of AZT resistance mutations (75). Clearly, a careful kinetic study is needed to resolve these conflicting findings.

During this study, it was found that none of the removal reactions went to completion. During PP<sub>i</sub>-mediated removal, the inability of the reaction to go to completion is probably related to the reaction quickly attaining equilibrium because of the rapid relative reincorporation of the triphosphate product of the reaction (76). This, however, is an unlikely explanation for the inability of the ATP-catalyzed reaction to go to completion, because the resulting product is presumed to be a poor substrate for polymerization. Perhaps a majority of the ATP binds to the enzyme in a noncatalytically competent complex for removal. Precipitation may also be a problem with these reactions because of the interaction between magnesium and the high concentration of phosphate-containing compounds (either ATP or PP<sub>i</sub>). If either ATP- or PP<sub>i</sub>-mediated removal reactions are not going to completion because they reach equilibrium, this would in fact exaggerate the rate of these slow reactions, and values reported should thus be considered as upper estimates on the rate of removal.

**Possible Reasons for Selection of RT<sub>CBVR</sub> in Cell Culture**

RT<sub>CBVR</sub> shows no advantage over RT<sub>M184V</sub> at the level of selectivity, and it is severely impaired at catalyzing removal reactions using both ATP and PP<sub>i</sub>. Why then is RT<sub>CBVR</sub> more resistant than any of the single mutants in cell culture? From the available data, it would appear that the reason RT<sub>CBVR</sub> is more resistant to abacavir is that the presence of the three mutations in combination leads to greater fitness than any of the single mutations alone.

Evidence suggests that each of the abacavir-selected single mutants is less fit than WT. Viral particles containing either the L74V or M184V mutation have been shown to be less fit than WT in in vitro viral selection assays (77, 78). The fitness defect of M184V-containing viruses has been attributed to decreased processivity by RT<sub>M184V</sub> (77). Data presented here would suggest that L74V mutant virus may be less fit because of a decrease in the efficiency of natural nucleotide incorporation and a decreased processivity by RT<sub>L74V</sub> during RNA-directed incorporation (Table II and Fig. 8). Although no fitness data are available on RT<sub>Y115F</sub>, its rare appearance in vivo (58) and the 1-order of magnitude increase in its efficiency of misincorporation (Table IV and Fig. 7) would suggest that its presence could be detrimental to the virus.

Although the biochemical data presented here are not sufficient to draw solid conclusions about fitness, there is evidence that RT<sub>CBVR</sub> may be more fit than any of the single mutants. RT<sub>CBVR</sub> was more processive than RT<sub>L74V</sub> during DNA-directed synthesis (Fig. 8) and was found to be less likely to misincorporate due to a large drop in the maximum rate of misincorporation compared with RT<sub>Y115F</sub> (Table IV and Fig. 7). The percentage of active sites has been suggested to be related to the ability of RT to orient itself at the 3′-end of an elongating primer (79, 80). The percentage of active sites for RT<sub>CBVR</sub> was also found to be higher than other mutants during both DNA- and RNA-directed incorporation, suggesting that it can position itself more effectively (Fig. 3). Studies on multidrug resistance mutations (Q151M, V75I, A62V, F77L, and F116Y) would also suggest that advanced mutagenesis results in mutant viruses more fit than the initial single mutant (Q151M) (66).

**Conclusions**—A better understanding of how RT gains resistance may allow for the future design of compounds with better resistance profiles that are more effective at long term suppression of HIV. Despite a mechanistic understanding of M184Vs resistance to 3TC (20, 24, 26, 72) and a growing amount of information on the AZT resistance mutations, resistance to AZT (24, 34–37, 40) and D4T (39, 68, 69), the mechanisms of many mutations and combinations of mutations are still poorly understood. In this report, we show that all but one of the tested mutations selected for by abacavir in cell culture increase selectivity against CBVTP. However, CBVTP’s highly hydrophobic ribose ring and its resulting high affinity to the HIV-1 RT active site appear to be able to lessen the overall effects of these mutations on incorporation of CBVMP during distinct stages of HIV replication (represented here by DNA- and RNA-directed incorporation). Although, unlike RT<sub>AZTR</sub>, none of the mutants showed an increase in the rates of removal by ATP or PP<sub>i</sub>, the marked decreases in the release rates of elongated primer leave open the suggestion that removal plays
a role in resistance to abacavir. Evidence presented here sug-
gests that RT<sup>691AS</sup> is more resistant to abacavir because it is
more fit than any of the single mutants. This interaction be-
tween resistant single mutations may serve to broaden the
definition of “compensatory mutations,” and further cellular
studies are warranted.

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REFERENCES

1. De Clercq, E. (1995) J. Med. Chem. 38, 2491–2517
2. De Clercq, E. (1997) Clin. Microbiol. Rev. 10, 674–693
3. Mitsuya, H., Yarchoan, R., Kageyama, S., and Broder, S. (1991) FASEB J. 5, 2369–2381
4. Parker, W. B., and Cheng, Y.-C. (1994) J. Biol. Chem. 269, 2369–2376
5. Martin, J. L., Brown, C. E., Matthews-Davis, N., and Reardon, J. E. (1994) Antimicrob. Agents Chemother. 38, 2743–2749
6. Johnson, A. A., Ray, A. S., Hanes, J. W., Suo, Z., Colacino, J. M., Anderson, R. S., and Johnson, K. A. (2001) J. Biol. Chem. 276, 40847–40857
7. Feng, J., Johnson, A., Johnson, K., and Anderson, K. (2001) J. Biol. Chem. 276, 23832–23837
8. Larder, B. A. (1994) J. Gen. Virol. 75, 951–957
9. Preston, B. D., Pizzur, B., and Loeb, L. A. (1998) Science 282, 1166–1167
10. Roberts, J. D., Barbehek, K. and Kunkel, T. A. (1988) Science 244, 1171–1173
11. Ji, J., and Loeb, L. A. (1992) Biochemistry 31, 954–958
12. Coffin, J. M. (1995) Science 267, 483–489
13. Shirasaka, T., Yarchoan, R., O'Brien, M. C., Husson, R. N., Anderson, B. D., Kejima, E., Shimada, T., Broder, S., and Mitsuya, H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 562–566
14. Larder, B. A., and Kemp, S. D. (1989) Science 246, 1155–1158
15. Kellam, P., Boucher, C. A., and Larder, B. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1934–1938
16. Shafer, R. W., Kozal, M. J., Winters, M. A., Iversen, A. K., Katzenstein, D. A., Ragn, M. V., Meyer, W. A., III, Gupta, P., Bashheed, S., Coombs, R. et al. (1994) J. Infect. Dis. 169, 722–729
17. Tisdale, M., Alnafad, T., and Coussens, D. (1997) Antimicrob. Agents Chemother. 41, 1094–1098
18. Moyle, G. J. (1997) J. Antimicrob. Chemother. 39, 6135–6139
19. Thrall, S. H., Barchi, J. J., Jr, and Marquez, V. E. (2000) Biochemistry 39, 23605–23611
20. Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2000) Eur. J. Biochem. 267, 4618–4624
21. Ray, A. S., and Anderson, K. S. (2001) Antimicrob. Agents Chemother. 45, 217–221
22. Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3056–3061
23. Miller, M. D., Motag, N. A., Lamad, P. F., Fuller, M. D., Anton, K. E., Mulato, J. S., and Charrington, T. M. (2001) J. AIDS 27, 450–459
24. Patel, P. H., Suzuki, M., Adaman, B., Shinkai, A., and Loeb, L. A. (2001) Mol. Biol. 308, 823–837
25. Martin, J. L., Wilson, J. E., Hayner, R. L., and Furman, P. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6135–6139
26. Jonckheere, H., De Clercq, E., and Anne, J. (2000) Eur. J. Biochem. 273, 2688–2686
27. Kasalasaka, P., Kovalik, M. F., Maroun, V., Le, R., and Mitsuya, H. (1999) J. Virol. 73, 5536–5536
28. Urban, S., Fischer, K. P., and Tyrrrell, D. L. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4894–4899
29. Lennardt, J., Stammers, D. K., and Larder, B. A. (2001) Antimicrob. Agents Chemother. 45, 2114–2146
30. Isel, C., Ehresmann, C., Walter, P., Ehresmann, B., and Marquet, R. (2001) J. Biol. Chem. 276, 48725–48729
31. Walter, H., Schmidt, B., Werwein, M., Schwingel, E., and Korn, K. (2002) Antimicrob. Agents Chemother. 46, 89–94
32. Kliina, N., Klinkait, T., Schiller, V., Irigoyen, J., Telenti, A., and Hirschel, B. (2000) AIDS 14, 791–799
33. Geiss, M., Arien, D., Parniai, M. A., and Wainberg, M. A. (2000) J. Virol. 74, 3579–3585
34. Larder, B. A., Kemp, S. D., and Harrigan, P. R. (1995) Science 269, 696–699
35. Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2002) J. Virol. 76, 3248–3256
36. Naeger, L. K., and Graham, C. B. (2000) Science 292, 1460–1461
37. Thiel, F. and Wirth, B. M. (2000) Science 292, 1462–1463
38. Enke, N. and Klinkait, T. (2000) J. Biol. Chem. 275, 2369–2371
39. Boucher, C. A., Oude Essink, B. B., van Kruenberg, A. P. B., van Gennip, A. H., and Berkhourt, B. (1996) EMBO J. 15, 4040–4049
40. Shkarapin, S. T. (2000) J. Virol. 75, 8432–8434
41. Boucher, C. A., Oude Essink, B. B., van Kruenberg, A. P. B., and van Gennip, A. H. (1996) EMBO J. 15, 4040–4049
42. Shkarapin, S. T. (2000) J. Virol. 75, 8432–8434
43. Boucher, C. A., Oude Essink, B. B., van Kruenberg, A. P. B., and van Gennip, A. H. (1996) EMBO J. 15, 4040–4049