Retroviral resistance to AZT and 3TC has been associated with two different mechanisms. The M184V mutation in the reverse transcriptase (RT) of the human immunodeficiency virus, type 1 (HIV-1) diminishes the incorporation of 3TC-monophosphate (3TC-MP), whereas AZT resistance-conferring mutations were shown to facilitate the phosphorolytic excision of incorporated AZT-MP in the presence of ATP. Both mechanisms show a certain degree of incompatibility; however, previous clinical data revealed that mutations E44D and V118I, when present in a background of classical AZT mutations (M41L, D67N, L210W, and T215Y), confer dual resistance to AZT and 3TC. We have purified RT enzymes that contain E44D and V118I either alone or in a background of different combinations of AZT mutations to study the underlying biochemical mechanisms. We found that enzymes containing E44D in a background of these latter mutations increase the efficiency of excision of 3TC-MP. Unexpectedly, V118I-containing enzymes show dramatic reductions in rates of incorporation of AZT-MP and 3TC-MP. The V118I mutant is also associated with diminished rates of ATP-dependent primer unblocking. The additional presence of mutations M41L, D67N, L210W, and T215Y can partially neutralize this deficit, which helps to explain the concurrent presence of these changes in resistant isolates. These biochemical data make clear that mutations E44D and V118I play distinct mechanistic roles in dual resistance to AZT and 3TC. Our findings are consistent with an increasing number of clinical studies suggesting that the V118I cluster constitutes a novel pathway for HIV resistance to multiple nucleotide analogue RT inhibitors.

Nucleoside analogue reverse transcriptase inhibitors (NRTIs) represent an important class of compounds to treat infection with the human immunodeficiency virus, type 1 (HIV-1).

Seven different NRTIs are in clinical use: 3’-azido-3’-deoxythymidine (zidovudine or AZT), 2’,3’-didehydro-2’,3’-dideoxythymidine (stavudine or D4T), 2’,3’-dideoxynosine (didanosine or ddI), (-)-β-L-2’,3’-dideoxy-3’-thacytidine (lamivudine or 3TC), 2’,3’-dideoxycytidine (zalcitabine or ddC), and, more recently, the carbonyl nucleoside abacavir, and the acyclic inhibitor tenofovir. These compounds are intracellularly phosphorylated and compete with natural dNTP pools for incorporation into the growing DNA chain. The relevant metabolites of ddI and abacavir are ddATP and carbocvir triphosphate, respectively. Although the clinical use of these chain terminators in combination with non-nucleoside analogue RT inhibitors (non-NRTIs), viral protease inhibitors (PIs), can sometimes reduce the viral burden to levels below the limit of detection, the development of drug resistance cannot be prevented. The emergence of resistance-conferring mutations is a major cause for viral rebound and treatment failure, and certain mutational patterns are associated with decreased susceptibility to multiple NRTIs, which can dramatically reduce remaining options for the management of the infection. Here we studied reaction pathways involved in dual resistance to AZT and 3TC. Both drugs are frequently administered together in combination with a non-NRTI or a protease inhibitor.

Recent studies have shown that resistance to the individual drugs involves different biochemical mechanisms that show a certain degree of incompatibility (1, 2). The M184V mutation confers high level (>100-fold) resistance to 3TC (3, 4) (5). This mutation caused reductions in rates of incorporation of 3TC-MP, whereas rates of incorporation of dCTP remained largely unaffected (6). In contrast, mutant enzymes containing classical AZT-associated amino acid substitutions, i.e. M41L, D67N, K70R, L210W, T215/F/Y, and K219Q, were shown to incorporate AZT-MP with similar efficiency as seen with wild-type RT (6, 7). However, both wild-type HIV-1 RT and AZT-resistant mutant enzymes are capable of removing the incorporated nucleotide analogue in the presence of physiologically relevant concentrations of pyrophosphate or ATP (8, 9). The latter was shown to act as a pyrophosphate donor. Rates of ATP-dependent rescue of chain-terminated DNA synthesis are significantly increased with mutant enzymes containing AZT resistance-conferring mutations, which provides an important mechanism for resistance to the drug (10, 11).

Combinations of at least two to three of the classical AZT mutations can also reduce susceptibility to other NRTIs. Accordingly, these amino acid substitutions have been named nucleoside-associated mutations (NAMs). Biochemical studies have shown that corresponding mutant enzymes can indeed increase rates of excision of AZT-MP, D4T-MP, ddAMP, and carbocvir monophosphate (12). The rate of excision depends on both the chemical nature of the chain terminator and the
specific mutational pattern. Unblocking of AZT-terminated primers is relatively efficient, whereas the removal of 3TC-MP is difficult to detect. We have demonstrated previously (13) that M184V-containing mutant enzymes are literally unable to remove 3TC-MP, and the rescue of AZT-terminated DNA synthesis was also compromised in this context. These results help to explain previous tissue culture and clinical data showing that the M184V mutation can increase susceptibility to AZT, which can sometimes lead to resensitization of formerly AZT-resistant viruses (1). Such phenotypes are of potential clinical benefit; however, drug hypersusceptibility and resensitization effects may occur only transiently, as shown for AZT/3TC combination therapy (14). The evolution toward dually resistant viruses only transiently, as shown for AZT/3TC combination therapy (14). The evolution toward dually resistant isolates is seen in the vast majority of clinical cases.

Dual resistance is often found in the context of multidrug resistance. The Q151M mutation, which is commonly accompanied with changes at residues 62, 75, 77, and 116, confers resistance to multiple NRTIs including AZT and 3TC. A family of rearrangements, i.e. insertions or deletions, located between codons 67 and 70 in the RT gene, is also tightly associated with decreased susceptibility to a variety of NRTIs (15). However, the frequency of occurrence of these two patterns is relatively low (~2%) among clinical isolates with moderate or high level resistance to 3TC (16). By far the most prevalent pattern associated with reduced susceptibility to both AZT and 3TC are mutations E44D or E44A and/or V118I in conjunction with two or more NAMs (17-23). These mutations were initially found in isolates showing moderate levels of resistance to 3TC in the absence of M184V (19). Recent analyses of HIV treatment and resistance databases showed a prevalence of ~12% (V118I), ~5% (E44D), and ~2% (E44A) (22). The prevalence of these mutations is significantly increased in NRTI pretreated patients, as compared with treatment of naive individuals. The V118I mutation has also been identified through in vitro selection under the pressure of drug combinations containing AZT (24).

The previous independent studies, showing that dual phenotypic resistance to AZT and 3TC requires the additional presence of at least two classical NAMs, suggest that E44D or E44A and V118I exert their effects through a unifying mechanism that may involve ATP-dependent excision of both 3TC-MP and AZT-MP. To test this hypothesis, we generated various mutant enzymes that contained the two mutations alone or together in a background of various combinations of NAMs and studied these mutant enzymes in regard to their abilities to incorporate and to excise AZT-MP and 3TC-MP, respectively. Unexpectedly, we found that Asp-44 and Ile-118 play distinct roles in HIV resistance to nucleotide analogues. The former increases rates of excision of 3TC-MP, whereas the latter diminishes the excision of chain terminators and causes decreased rates of incorporation of multiple nucleotide analogues including AZT-MP and 3TC-MP.

### EXPERIMENTAL PROCEDURES

**Enzymes and Nucleic Acids—Wild-type (WT) HIV-1 RT and mutant enzymes were expressed and purified essentially as described previously (25).** Mutant constructs were generated by site-directed mutagenesis (QuickChange kit; Stratagene). As in our previous studies (15, 26), we utilized the following heteropolymers and oligonucleotide analogues (Invitrogen) as DNA/DNA primer/template substrates: 5′-CTTGGTAGTTA-GCCCTTCAGTCCTCCTTTCCTTTAAATGGGTTCAA-G3′ (termed 57D) served as a DNA template, and 5′-TAAAAGAAAAGG-GCCCTCCAGCACCTCCTCTTTCTTTTTAGTGGTACAA-G3′ (termed 17D), 5′-TTAAAAGAAAAAGG-GCCCTCCAGCACCTCCTCTTTCTTTTTAGTGGTACAA-G3′ (termed 18D), and 5′-TTAAAAGAAAAAGG-GCCCTCCAGCACCTCCTCTTTCTTTTTAGTGGTACAA-G3′ (termed 19D) were used as primer strands. 5′-End labeling of the primer and its subsequent gel purification were conducted following standard procedures (26). The homopolymeric DNA/RNA substrates, i.e. (dT)15/poly(rA), are products from Invitrogen and Amersham Biosciences, respectively. 3TC-TP was kindly provided by Dr. R. C. Bethell (Shire), AZT-TP was obtained from TriLink Biotechnologies, Inc., dNTPs and ddNTPs were purchased from Roche Applied Science, and radiolabeled nucleotides ([3H]dATP and [32P]dATP) are products from PerkinElmer Life Sciences and ICN, respectively.

### Table 1

Inhibition of DNA synthesis in the presence of AZT-TP: wild-type HIV-1 RT versus resistant mutant enzymes

| Enzyme mutated positions | IC<sub>50</sub> -Fold resistance | K<sub>m</sub> | K<sub>i</sub> | K<sub>i</sub>K<sub>m</sub> -Fold resistance | IC<sub>50</sub> -Fold resistance |
|--------------------------|-------------------------------|-------|-----|----------------------------|----------------------------|
| WT                       | 38<sup>a</sup>                  | 0.8   | ND  | ND                         | 83                         |
| 44                       | 40                            | 1.1<sup>b</sup> | 9.7 | 0.030                      | 83                         |
| 41/215                   | 41                            | 1.1   | 8.5 | 0.031                      | 1.0                        |
| 41/215/210               | 66                            | 1.7   | ND  | ND                         | 222                        |
| 41/215/210/44            | 31                            | 0.8   | ND  | ND                         | 380                        |
| 41/215/210/44/67         | 47                            | 1.2   | 7.0 | 0.053                      | 1.6                        |
| 41/215/69S-8S            | 39                            | 1.0   | 7.2 | 0.016                      | 0.6                        |
| 1122                     | 315                           | 8.3   | 1.8 | 0.192                      | 4.5                        |
| 44/118                   | 327                           | 8.6   | 11.1| 0.207                      | 5.4                        |
| 41/215/210/67/44/118     | 1109                          | 29.2  | 15.2| 0.122                      | 19.2                       |
| 41/215/44/118            | 656                           | 17.3  | 14.4| 0.635                      | 12.6                       |
| 41/215/44/118/184        | 473                           | 12.5  | 20.0| 0.763                      | 10.9                       |

<sup>a</sup> Values are averages of three to six independent measurements. Standard deviations are within a range of ±30%.

<sup>b</sup> Fold resistance is highlighted in bold.

<sup>ND</sup> Not determined.

<sup>IC<sub>50</sub></sup> corresponds to the concentration of ATP that results in 50% inhibition of DNA synthesis.

**Inhibition of DNA Synthesis with AZT-TP—Inhibitory effects of AZT-TP were screened with wild-type HIV-1 RT and mutant enzymes through use of filter-based assays. 50-μl reactions contained 10 ng of RT (mutant or WT RT), 10 ng of (dT)15, 500 ng of poly(rA) and [3H]dTTP, and varied concentrations of AZT-TP and dNTP-TP in 50 mM Tris-HCl (pH 8.0) and 50 mM NaCl. DNA synthesis was initiated by the addition of MgCl₂ (5 mM). Reactions were allowed to proceed for 10 min at 37 °C and quenched with 250 μl of 10% trichloroacetic acid. Samples were filtered, and the remaining radioactivity was measured through scintillation analysis. IC<sub>50</sub> measurements were performed at constant concentrations of dTTP and varied concentrations of AZT-TP. The data were analyzed and quantified using the program Prism. The determination of the kinetic parameters (K<sub>m</sub>, K<sub>i</sub>) was performed following standard procedures (27). Reactions were conducted in either the absence or presence of ATP to assess the impact of changes that affect both the incorporation and the excision of the inhibitor. Stock solutions of ATP (MBI Fermentas) were pretreated with 0.5 units of inorganic pyrophosphatase (Roche Applied Science) in a buffer containing 1 mM dithiothreitol, 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 6 mM MgCl₂.

**Gel-based Assay to Assess the Efficiency of Chain Termination—**The primer/template duplex was prepared as described previously (13). 5 pmol of the heteropolymeric substrate was incubated with 10 pmol of HIV-1 RT in a buffer containing 50 mM Tris-HCl (pH 7.8), 50 mM KCl, followed by the addition of 10 μl of each of the four dNTPs and varying concentrations of AZT-TP. Reactions were initiated with 6 mM MgCl₂ and allowed to proceed for 15 min at 37 °C. Samples were finally precipitated with ethanol and resolved on 8% polyacrylamide-7.5 M urea gels. Data were analyzed through autoradiography or through use of a PhosphorImager. IC<sub>50</sub> values were determined essentially as described previously (28).

** Primer Unblocking and Rescue of DNA Synthesis—**Excision and the
ensuing rescue of chain-terminated DNA synthesis was monitored in
time course experiments after the addition of 3.5 mM ATP and the
appropriate dNTP mixtures as indicated in the figures. Reactions were
performed at 37°C and stopped at different times by adding 1-
H9262 aliquots of reaction mixture to 9-
H9262 l of 95% formamide. Samples were
heat-denatured for 5 min at 95°C and finally resolved on 8% polyacryl-
amide-7 M urea gels.

RESULTS

Experimental Design—Two distinct AZT resistance path-
ways have been characterized in vivo (29). T215Y is often found
in a background of M41L and L210W, and T215F is often found
in background of K219Q and K70R. Several clinical studies
have shown that mutations E44D or E44A and Ile-118 occur
predominantly in a background of mutations that belong to the
Tyr-215 pathway (M41L, D67N, L210W, and T215Y) (22, 29).
We concentrated our biochemical study on the analysis of the
two most prevalent mutations, E44D and V118I.

Effects on Inhibition of DNA Synthesis with AZT-TP—To
study the impact of these mutations on biochemical mecha-
nisms involved in dual resistance to AZT and 3TC, we gener-
ated RT enzymes that contained mutations E44D or V118I
either alone or together with different combinations of amino
acid substitutions at positions 41, 67, 210, and 215. These
enzymes were initially screened for their abilities to diminish

![Table II](https://example.com/tables/table2.png)

| Inhibitor | WT RT | 44D | 118I | NAMs/44D/118I | 184V |
|-----------|-------|-----|-----|---------------|-----|
| IC50 μM (Fold resistance) |       |     |     |               |     |
| AZT-TP    | 2.7<sup>a</sup> | 2.9 (1.1) | 14.8 (5.4) | 31.2 (11.5) | ND<sup>b</sup> |
| 3TC-TP    | 5.53  | 3.6 (0.7) | 120.2 (21.7) | 110.8 (20.0) | 696 (>50) |
| ddATP     | 1.96  | 2.2 (1.1) | 10.6 (5.4) | 3.9 (2.0) | ND |

<sup>a</sup> Values are averages of two to three independent measurements. Standard deviations are within a range of ±30%.

<sup>b</sup> ND, not determined.

the inhibitory effects of AZT-TP using homopolymeric
poly(A)/dT substrates (Table I). The experiments were con-
ducted in the absence and in the presence of ATP to assess the
impact of changes in regard to both incorporation and excision
of AZT-MP. Differences among the various mutant enzymes
are expressed as -fold resistance, as compared with wild-type
HIV-1 RT, on the basis of both IC50 measurements and K<sub>i</sub>/K<sub>m</sub>
determinations. In agreement with previous reports, we found
that enzymes with classical NAMs (M41L, D67N, L210W, and
T215Y) show 3- to 4-fold decreases in AZT-mediated inhibition
when reactions were monitored in the presence of ATP (11).
Stronger effects are seen with an enzyme containing mutations

---

Footnotes:

<sup>a</sup> Values are averages of two to three independent measurements. Standard deviations are within a range of ±30%.

<sup>b</sup> ND, not determined.
M41L and T215Y together with the T69S-SS insertion (30, 31). E44D, either alone or in a background of classical NAMs, does not appear to elevate levels of ATP-dependent primer unblocking. None of these enzymes show significant effects in the absence of ATP.

The level of AZT-mediated inhibition of DNA synthesis is dramatically decreased with Ile-118-containing enzymes, even in the absence of ATP (up to 29-fold). Both IC_{50} measurements and K_{m}/K_{n} determinations yielded very similar results. The data suggest that V118I, unlike classical NAMs, exerts its resistance-conferring effects through effective discrimination between AZT-TP and its natural counterpart, dTTP. Importantly, mutant enzymes containing both V118I and the classical 3TC resistance-conferring mutation M184V showed very similar effects. Thus, it appears that the negative effect of M184V on rates of excision of AZT-MP may be compensated by the diminished rates of incorporation of the chain terminator.

We noted slight increases in K_{m} values associated with Ile-118-containing mutant enzymes; however, differences with respect to wild-type RT are rather small (<2-fold). These results suggest that the polymerase properties per se are not significantly affected by this mutation. Increases in K_{m} values follow the trend WT < NAMs ~ E44D < V118I < M184V-containing enzymes. Subtle decreases in regard to the polymerase activity associated with M184V-containing enzymes have been reported earlier (6). However, the aforementioned data also raise several questions. In contrast to tissue culture and clinical data, our results show that V118I alone, in the absence of NAMs, can diminish the inhibitory effects of AZT-TP. Moreover, the function of E44D is not evident from these experiments, and it remains unclear how E44D and Ile-118 affect susceptibility to 3TC.

Effects of Mutations E44D and V118I on the Efficiency of Incorporation of AZT-MP—To address these issues, we employed heteropolymeric primer/template substrates and analyzed the reactions through the use of gel-based assays (Fig. 1A). DNA synthesis was first monitored in the presence of increasing concentrations of AZT-TP to analyze the ability of the mutant enzymes to discriminate between the nucleotide analogue and its natural counterpart, dTTP. Fig. 1B shows a comparison between WT RT and the V118I mutant as an example. The results are in good agreement with the data shown in Table I. We found that the Ile-118 mutant diminishes the efficiency of chain termination, whereas the Asp-44 mutant is indistinguishable from the wild-type enzyme in this regard (Fig. 1C). IC_{50} determinations revealed 5- to 11-fold decreases...
in regard to the efficiency of incorporation of AZT-MP associated with V118I (Table II). The value is higher when using an enzyme that contains V118I in a background of NAMs, which is also evident from the results obtained with the homopolymeric RNA/DNA substrate (Table I).

**Effects on the Efficiency of Excision of AZT-MP**—We next studied the ability of the mutant enzymes to excise the incorporated AZT-MP from the primer terminus. The different enzymes were incubated with a primer/template substrate that contained an AZT-terminated primer and a mixture of ATP and nucleotides to promote primer unblocking and to allow the ensuing addition of three more residues (Fig. 2A). The rescue of chain-terminated DNA synthesis was then analyzed in time course experiments. We found that the ATP-dependent unblocking of the AZT-terminated primer and the continuation of DNA synthesis is severely compromised with the Ile-118 mutant, whereas the Asp-44 mutant did not show any discernable difference when compared with the WT RT (Fig. 2, B and C, top). Thus, the V118I mutation inhibits the incorporation of AZT-MP, as well as its excision. This provides a possible explanation for clinical and cell culture studies that do not show any noticeable change in susceptibility to AZT in a context of viruses that contain the V118I substitution alone. It appears that the diminished efficiency of primer unblocking may neutralize the effects of the diminished rates of incorporation of AZT-MP.

Consistent with data published previously, we show that NAMs containing mutant enzyme can increase levels of rescue of DNA synthesis. The additional presence of Asp-44 does not appear to increase or decrease this effect (Fig. 2, B and C, middle). However, the presence of NAMs/Asp-44/Ile-118- and M184V-containing mutant enzymes. IC_{50} values are shown in Table II.

**Effects on Both Incorporation and Excision of 3TC-MP**—The efficiency of chain termination with 3TC-TP was studied using the same primer/template as described in Fig. 1A. Fig. 3A depicts the substrate and shows possible sites of incorporation of 3TC-MP. As for AZT-MP, we found that V118I decreased the efficiency of incorporation of 3TC-MP (Fig. 3B, top), whereas the E44D mutant behaved like WT RT (Fig. 3B and C, bottom). Thus, it appears that Ile-118 exerts its resistance-conferring effects predominantly through substrate discrimination at the level of binding and/or incorporation, and the background of NAMs is required to compensate for defects in regard to the ATP-dependent unblocking reactions.

**Mechanisms Involved in Dual HIV-1 Resistance to AZT and 3TC**

Fig. 3. **Efficiency of chain termination with 3TC-TP.** A, primer/template sequences used in this experiment. Possible sites of incorporation of 3TC-MP are underlined. B, comparison of incorporation of 3TC-MP between WT RT and the V118I mutant. Lanes 2 to 14 show reactions in the presence of 1 μM dCTP and 10 μM of the three other dNTPs and different concentrations of 3TC-TP (0, 0.49, 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, and 500 μM and 1 mM). Reactions were as described in Fig. 1. C, graphic representation of data shown in B and data obtained with Asp-44-, NAMs/Asp-44/Ile-118-, and M184V-containing mutant enzymes. IC_{50} values are shown in Table II.
susceptibility to 3TC is always associated with the additional presence of at least two or three NAMs. Thus, as suggested for AZT, it is conceivable that simultaneous reductions in rates of excision may neutralize the diminished rates of incorporation of 3TC-TP. The assessment of the efficiency of these reactions is, however, hampered by the fact that the removal of 3TC-MP is already severely compromised with WT RT.

To study rescue of 3TC-terminated DNA synthesis, we used an equivalent assay as described in Fig. 2A (Fig. 4A). As expected, the ATP-dependent rescue of 3TC-terminated DNA synthesis is almost undetectable with the wild-type enzyme, the V118I mutant, and the Asp-44 mutant (Fig. 4, B and C, top). Rates of rescue are slightly increased in the presence of the four NAMs, and a mutant enzyme containing Asp-44 in a background of NAMs caused a significant increase in the efficiency of removal of 3TC-MP and the following rescue of DNA synthesis (Fig. 4, B and C, middle). Such an increase is the first clear effect that can be attributed to the E44D mutation. Although the efficiency of the reaction is by far not as pronounced as seen with AZT-terminated primers (Fig. 2), the increase in rates of excision may be sufficient to mediate moderate levels of resistance to the drug as seen in clinical samples. This is further supported by the fact that the efficiency of incorporation of 3TC-MP is relatively poor, which, in turn, implies that DNA synthesis may not need to be frequently rescued. The additional presence of V118I can almost completely reverse this effect, which demonstrates the negative impact of this mutation on the excision reaction (Fig. 4, B and C, bottom).

Effects on the Incorporation and Excision of ddNTPs—To further verify our findings, we also included 2',3'-dideoxynucleotides in this study. We found that the efficiency of chain termination with ddAMP is also diminished with Ile-118-containing mutant enzymes. We measured a 2- to 5-fold increase in IC_{50} values, as compared with wild-type HIV-1 RT (Table II). Similar levels of decreased rates of incorporation have also been detected with the other ddNMPs (data not shown). We finally tested the efficiency of excision of ddAMP with mutant enzymes containing NAMs together with Asp-44 and NAMs together with Asp-44/Ile-118. The results are consistent with trends seen with 3TC-terminated primer strands. The NAMs/Asp-44-containing enzyme caused an increase in rescue of DNA synthesis, and the V118I substitution in the same mutational background can partially reverse this effect (data not shown). Thus, HIV resistance in the presence of E44D and V118I may not be restricted to AZT and 3TC. The data make clear that both mutations play distinct mechanistic roles; although the former can increase rates of excision in the presence of NAMs, the latter refines the discrimination between nucleotide analogue inhibitors and their natural dNTP pools.

DISCUSSION

Prolonged use of antiretroviral drugs can result in complex mutational patterns and viral phenotypes that include resistance to multiple drugs, and, on the other side of the spectrum,
Mechanisms Involved in Dual HIV-1 Resistance to AZT and 3TC

**Fig. 5.** Qualitative comparison of enzymatic properties and viral phenotypes associated with mutations Asp-44 and Ile-118. NAMs refers to the cluster of the four mutations M41L, D67N, L210W, and T215Y. Alterations regarding the efficiency of incorporation of the nucleotide analogue and its excision in the presence of ATP are listed separately. We refer to substrate discrimination and primer unblocking, respectively. Previous studies have shown that both types of reactions are important factors associated with changes in HIV drug susceptibility. Relative changes, as compared with WT RT, are listed in three categories to facilitate the comparison with the viral phenotype: resistant (red), i.e. diminished rates of incorporation or increased rates of excision; sensitive (green), i.e. indistinguishable from wild-type properties; and hypersusceptible (blue), i.e. increased rates of incorporation or diminished rates of excision. Viral phenotypes are based on drug susceptibility assays with mutant viruses generated by site-directed mutagenesis. The relevant references are listed accordingly. Mixed colors (green/red) take into account that the additional presence of NAMs and Asp-44 improve rates of excision; however, the efficiency of the reaction is still diminished as compared with WT RT. Mixed colors (green/blue) take into account that classical NAMs have been associated with low level resistance to 3TC (19) (32), and the relevant mutant enzyme cause slight increases in rescue of DNA synthesis. The question mark points to problems in the determination of changes regarding the efficiency with which the 3TC-MP is excised. Because the reaction with wild-type RT is already severely compromised, it is difficult to determine further putative decreases of the reaction.

| Drug | Mutational pattern | Substrate Discrimination | Primer Unblocking | Viral Phenotype |
|------|-------------------|--------------------------|------------------|----------------|
| AZT  | NAMs              |                          |                  |                |
|      | E44D              |                          |                  |                |
|      | NAMs/E44D         |                          |                  |                |
|      | V118I             |                          |                  |                |
|      | NAMs/V118I/E44D   |                          |                  | (13)           |
|      | M184V             |                          |                  |                |
|      | NAMs/M184V/E44D/V118I |                  |                  |                |
| 3TC  | NAMs              |                          |                  |                |
|      | E44D              |                          |                  |                |
|      | NAMs/E44D         |                          |                  |                |
|      | V118I             |                          |                  | ?              |
|      | NAMs/V118I/E44D   |                          |                  |                |

Drug hypersusceptibility and resensitization effects. The study of biochemical mechanisms associated with changes in drug susceptibility became an important tool in attempts to categorize resistance-conferring mutations. Here we studied biochemical mechanisms associated with dual resistance to AZT and 3TC by mutations E44D and V118I. We characterized mutant enzymes in regard to their abilities to incorporate and to excise AZT-MP and 3TC-MP. Relative changes in both types of reactions, as compared with the wild-type enzyme, can change susceptibility to a given NRTI. However, the relationships between enzymatic properties and viral phenotypes turn increasingly complex with an increasing number of mutations that may even exert opposing effects on the activities of HIV-1 RT. To facilitate a comparison with published viral phenotypes, we summarized our biochemical data in schematic fashion (Fig. 5). The results with respect to the efficiency of both incorporation and excision of chain terminators are qualitatively expressed in three categories: resistant, sensitive, and hypersusceptible.

In agreement with previous studies (10, 11), we found that different combinations of M41L, D67N, L210W, and T215Y facilitate the ATP-dependent unblocking of AZT-terminated primers. These results suggested that the excision of incorporated AZT-MP is a major factor in viral resistance to AZT, whereas substrate discrimination appears to be negligible in this regard. Here we show that resistance to AZT can involve both reaction pathways, and the presence or absence of a single amino acid substitution, i.e. V118I, can determine whether substrate discrimination or increased rates of excision of the chain terminator is dominant in this regard. The V118I mutation alone decreases the efficiency of both incorporation and excision of AZT-MP. The ability to discriminate between natural dNTP pools and the nucleotide analogue at the level of binding or incorporation would decrease phenotypic susceptibility to AZT; however, a simultaneous decrease in efficiency of excision is detrimental to this effect. Thus, it appears that the effects of reductions of both incorporation and excision of AZT-MP are, in this case, equilibrated, which helps to explain why mutant viruses that contain Ile-118 alone are not associated with changes in drug susceptibility. The viral phenotype will critically depend on the additional presence of NAMs that augment levels of ATP-dependent primer unblocking. However, our data suggest that substrate discrimination is the dominant mechanism associated with V118I, because the efficiency of excision never increased the levels seen with WT RT.

The refined substrate discrimination may also help to explain why viruses containing NAMs in a background of both Val-184 and Ile-118 have not been associated with AZT resensitization effects (19, 22, 32), although each of the latter two mutations can reduce the efficiency of rescue of AZT-terminated DNA synthesis. It appears that the Ile-118-mediated reductions with respect to the incorporation of AZT-MP are sufficient to compensate for these deficits. Although the biochemical data are consistent with drug susceptibility assays in this case, it may be very difficult to assess the contribution of both reactions on the resulting phenotype in other cases, because the rates of nucleotide incorporation are significantly higher as compared with rates of excision. It is conceivable that viruses containing V118I in the absence of NAMs may also be associated with hypersusceptibility to other drugs, provided that the diminished rates of excision are not sufficiently compensated by diminished rates of incorporation of the inhibitor. V118I-containing enzymes are also associated with diminished
Mechanisms Involved in Dual HIV-1 Resistance to AZT and 3TC

rates of incorporation of 3TC-MP, which provides a mechanism for dual resistance to AZT and 3TC. In contrast, E44D-containing enzymes are literally indistinguishable from WT RT in this context; however, this mutation caused increases in rates of excision of incorporated 3TC-MP, when present in a background of NAMs. Thus, dual resistance to AZT and 3TC is based, in this latter case, on ATP-dependent unblocking. As for AZT, the additional presence of Ile-118 diminishes the excision reaction, and the refinement in substrate discrimination becomes dominant, which is also notably consistent with cell culture data showing that E44D and V118I do not appear to exert any additive or synergistic effects (22). Together these data make clear that mutations E44D and V118I are mechanistically uncoupled and play distinct roles in dual resistance to AZT and 3TC.

These findings are in accordance with the different locations of positions 44 and 118 in the structure of HIV-1 RT (33). V118I lies in close proximity to residues Tyr-115 and Phe-116, which are mechanistically uncoupled and play distinct roles in substrate discrimination becomes dominant, which is not also notably consistent with cell culture data showing that E44D and V118I do not appear to exert any additive or synergistic effects (22). Together these data make clear that mutations E44D and V118I are mechanistically uncoupled and play distinct roles in dual resistance to AZT and 3TC.

Acknowledgment—We thank Dr. Mark A. Wainberg for continuous support.

REFERENCES
1. Larder, B. A., Kemp, S. D., and Harrigan, P. R. (1996) Science 270, 686–699.
2. Gotte, M., and Wainberg, M. A. (2000) Drug Resist. Updat. 3, 30–58.
3. Schinazi, R. F., Lloyd, R. M., Jr., Nguyen, M. H., Cannon, D. L., McMillan, A., Ilkosy, N., Chu, C. K., Liotta, D. C., Bazmi, H. Z., and Mellors, J. W. (1993) Antimicrob. Agents Chemother. 37, 875–881.
4. Boucher, C. A., Cammack, N., Schipper, P., Schuurman, R., Rousse, P., Wainberg, M. A., and Cameron, J. M. (1993) Antimicrob. Agents Chemother. 37, 2231–2234.
5. Gao, Q., Gu, Z., Parniak, M. A., Cameron, J., Cammack, N., Boucher, C., and Wainberg, M. A. (1995) Antimicrob. Agents Chemother. 39, 1396–1399.
6. Krebs, R., Immenderfer, U., Thrall, S. H., Wehrli, B. M., and Goody, R. S. (1997) Biochemistry 36, 10392–10390.
7. Lacey, S. F., Reardon, J. D., Furfine, E. S., Enkkel, T. A., Bebeneck, K., Eckert, K. A., Kemp, S. D., and Larder, B. A. (1992) J. Biol. Chem. 267, 15789–15794.
8. Arion, D., Kaushik, N., McCormick, S., Borkow, G., and Parniak, M. A. (1998) Antimicrob. Agents Chemother. 42, 1540–1545.
9. Naege, L. K., Magee, N. A., and Miller, D. B. (2002) Antimicrob. Agents Chemother. 46, 2179–2184.
10. Gotte, M., Arion, D., Parniak, M. A., and Wainberg, M. A. (2000) J. Virol. 74, 3578–3585.
11. Miller, V., Phillips, A., Rottmann, C., Chasanides, S., Pauwels, R., Hertogs, K., de Bethune, M. P., Kemp, S. D., Bloor, S., Harrigan, P. R., and Larder, B. A. (1996) J. Infect. Dis. 173, 1521–1532.
12. Larder, B. A., Bloor, S., Kemp, S. D., Hertogs, K., Desmet, R. L., Miller, V., Sturmer, M., Staszeszki, S., Ren, J., Stammers, D. K., Stuart, D. I., and Pauwels, R. (1999) Antimicrob. Agents Chemother. 43, 1961–1966.
13. Van Gaerenbergh, K., Van Laethem, K., Albert, J., Boucher, C., Cabet, B., Floridia, M., Gerstorf, J., Heijlman, B., Nielsen, C., Cauwesse, C., Ferrin, L., Pirlot, M. F., Ruiz, L., Schmitz, J. C., Schneider, F., Scholmerich, A., Schuurman, R., Stellbrink, H. J., Stuyver, L., Van Lunzen, J., Van Remoortel, B., Van Wijngaerden, E., Vella, S., Vetvurk, M., Yerly, S., De Clercq, E., Destemery, J., and Vandamme, A. M. (2000) Antimicrob. Agents Chemother. 44, 2169–2177.
14. Delaguerre, C., Mouroux, M., Yvon-Groussin, A., Simon, A., Angleraud, F., Huraux, J. M., Agut, H., Katlama, C., and Calvez, V. (2001) Antimicrob. Agents Chemother. 45, 948–949.
15. Gallego, O., Briones, C., Corral, A., and Soriano, V. (2000) J. Acquired Immune Defic. Syndr. 25, 99–105.
16. Hertogs, K., Bloor, S., De Vroey, V., and Van Denuye, C., Deheort, B., van Caenenberge, A., Stummem, T., Wegner, S., van Houtte, M., Miller, V., and Larder, B. A. (2000) Antimicrob. Agents Chemother. 44, 568–573.
17. Martinez-Picado, J., DePasquale, M. P., Kartsonis, N., Hanna, G. J., Wong, J., Finzi, D., Rosenberg, E., Gunthard, H. F., Sutton, L., Savara, A., Petropoulos, C. J., Hellmann, N., Walker, B. D., Richman, D. D., Siliciano, R., and Montes, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10458–10463.
18. Gallego, O., Briones, C., Corral, A., and Soriano, V. (2000) J. Med. Virol. 69, 299–303.
19. Romano, L., Venturi, G., Bloor, S., Harrigan, R., Larder, B. A., Majer, C. J., and Sarghi, M. (2002) J. Infect. Dis. 185, 898–904.
20. Rossner, S., Uy, J., Duon, C., Lu, J., Shugarts, D., and Kuritzkes, D. R. (2002) Antimicrob. Agents Chemother. 46, 4000–4003.
21. Boucher, G., Arion, D., Wainberg, M. A., and Parniak, M. A. (1999) Antimicrob. Agents Chemother. 43, 259–268.
22. Le Grice, S. F., and Gruninger-Leitch, F. (1990) Eur. J. Biochem. 187, 307–314.
23. Metz, C. S., and Segondy, M. (2001) J. Infect. Dis. 183, 1059–1063.
24. Kedder, P. S., Abbas, J., Korns, R., Lesia, K. T., and Voorhees, P. H. (1999) Biochemistry 29, 3603–3611.
25. Kallergi, G. J., Smith, R. A., Schinini, R. F., North, T. W., and Preston, B. D. (2000) J. Biol. Chem. 275, 359–366.
26. Hanna, G. J., Johnson, V. A., Kuritzkes, D. R., Richman, D. D., Brown, A. J., and Silliman, A. V. (2000) J. Biol. Chem. 275, 2109–2117.
27. Martin-Hernandez, A. M., Domingo, E., and Menendez-Arias, L. (1996) EMBO J. 15, 4433–4442.
28. Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3056–3061.
29. Delaigre, C., Bertolet, J., Rofield, M. F., Guerreiro, C., Sarfati, S., and Canard, B. (2002) J. Biol. Chem. 277, 4297–4302.
30. Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2001) J. Virol. 75, 4832–4842.
31. Chamberlain, P. F., Ben, J., Nichols, C. E., Douglas, L., Leenstra, J., Larder, B. A., Stuart, J., and Stammers, D. K. (2002) J. Virol. 76, 10015–10019.
32. Isel, C., Ehrensien, C., Walter, P., Ehrensien, B., and Marquet, R. (2001) J. Biol. Chem. 276, 48725–48732.