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Mutants of Human Immunodeficiency Virus Type 1 (HIV-1) Reverse Transcriptase Resistant to Nonnucleoside Reverse Transcriptase Inhibitors Demonstrate Altered Rates of RNase H Cleavage That Correlate with HIV-1 Replication Fitness in Cell Culture

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Three mutants of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (V106A, V179D, and Y181C), which occur in clinical isolates and confer resistance to nonnucleoside reverse transcriptase inhibitors (NNRTIs), were analyzed for RNA- and DNA-dependent DNA polymerization and RNase H cleavage. All mutants demonstrated processivities of polymerization that were indistinguishable from wild-type enzyme under conditions in which deoxynucleoside triphosphates were not limiting. The V106A reverse transcriptase demonstrated a three- to fourfold slowing of both DNA 3′-end-directed and RNA 5′-end-directed RNase H cleavage relative to both wild-type and V179D enzymes, similar to what was observed for P236L in a previously published study (P. Gerondelis et al., J. Virol. 73:5803–5813, 1999). In contrast, the Y181C reverse transcriptase demonstrated a selective acceleration of the secondary RNase H cleavage step during both modes of RNase H cleavage. The relative replication fitness of these mutants in H9 cells was assessed in parallel infections as well as in growth competition experiments. Of the NNRTI-resistant mutants, V179D was more fit than Y181C, and both of these mutants were more fit than V106A, which demonstrated the greatest reduction in RNase H cleavage. These findings, in combination with results from previous work, suggest that abnormalities in RNase H cleavage are a common characteristic of HIV-1 mutants resistant to NNRTIs and that combined reductions in the rates of DNA 3′-end- and RNA 5′-end-directed cleavages are associated with significant reductions in the replication fitness of HIV-1.

Infection with human immunodeficiency virus (HIV) is the cause of AIDS and affects over 30 million people worldwide (64). The primary targets of therapy for HIV infection include the viral protease and reverse transcriptase (RT). HIV type 1 (HIV-1) RT is a heterodimer consisting of 66- and 51-kDa subunits (p66 and p51, respectively) (3). p66 contains both the polymerase and the RNase H active sites of the enzyme (34, 37, 39). The RNase H domain is present in the carboxy-terminal third of p66. Although p51 is derived from p66 by proteolytic cleavage, it assumes a very different tertiary structure and does not contain a catalytic site (37, 39). The function of p51 is not known, but it may play a role in binding the $tRNA_3^{Lys}$-template complex (3, 39) and in maintaining the structural integrity of the heterodimer (1).

RNase H cleavage is essential for HIV-1 replication (61; for a review see reference 11). Two modes of RNase H cleavage have been described (Fig. 1). "Polymerase-dependent" cleavage is thought to occur in concert with DNA polymerization to degrade the genomic RNA during minus strand DNA synthesis (26, 46). The position of the primary DNA 3′-end-directed cleavage occurs 15 to 18 nucleotides (nt) from the recessed 3′ end of the DNA (26, 33); we have referred to this mode of cleavage as DNA 3′-end-directed RNase H cleavage. A second mode of RNase H cleavage occurs independently of DNA polymerization. The position of the primary RNA 5′-end-directed RNase H cleavage occurs 15 to 18 nt from the 5′ end of the recessed RNA and can occur with RNA-DNA templates in which the DNA is circular (i.e., it has no free end to direct cleavage) (18, 42–44). RNA 5′-end-directed RNase H cleavage is thought to degrade plus-strand genomic RNA fragments left behind after DNA 3′-end-directed cleavage (18) and appears to play an important role in the formation and removal of the polypurine tract (48, 53, 63), which primes plus-strand synthesis. In addition, RNA 5′-end-directed RNase H cleavage is thought to expose the R region of minus-strong-stop DNA, which is essential for translocation of minus-strong-stop DNA to the 3′ end of the genome (30, 60). In addition to the primary cleavage event, a secondary cleavage, which occurs at a slower rate than does the primary cleavage, makes a cut approximately 5 to 7 nt from the end of the strand directing cleavage (see Fig. 1) during both modes of RNase H cleavage (18, 42, 47).

Resistance of HIV-1 to antiretroviral drugs is a major factor that limits the efficacy of current antiretroviral regimens (reviewed in references 10 and 35). There are three classes of RT inhibitors: nucleoside analogs, nucleotide analogs, and nonnucleoside reverse transcriptase inhibitors (NNRTIs). NNRTIs inhibit HIV-1 RT by binding a specific region of the p66 subunit of RT that is adjacent to the polymerase active site of the enzyme (13, 39, 55). This binding causes an allosteric change of the polymerase active site which inhibits DNA polymerization (21, 22, 39, 55, 57). NNRTIs currently licensed for

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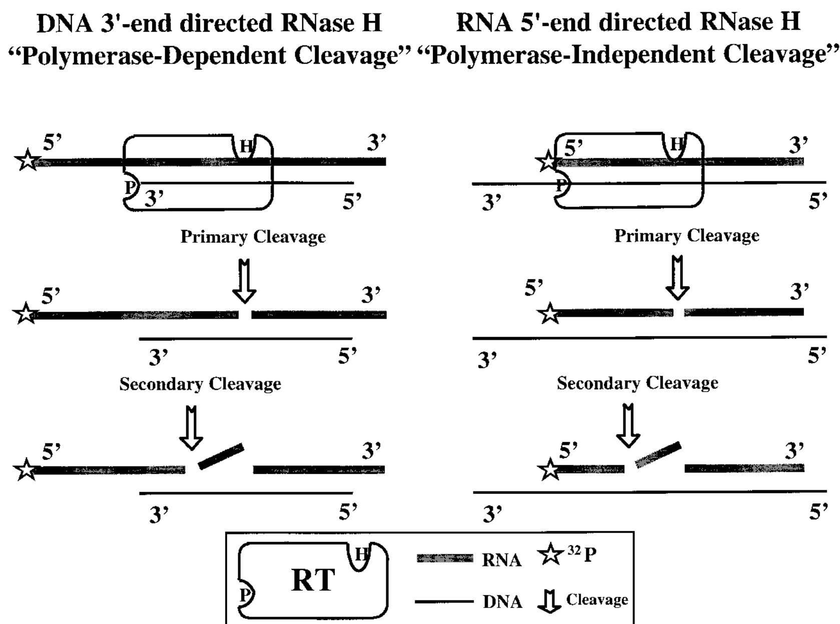


FIG. 1. Diagram of substrates used to measure DNA 3′-end-directed and RNA 5′-end-directed RNase H cleavage. RNA is represented by a thick line; DNA is represented by a thin line. The stars represent the radiolabeled 5′ end of the RNA. The arrows represent the position at which cleavage of the RNA occurs. The polymerase active site of RT is denoted by a "P," and the RNase H active site is denoted by an "H." DNA 3′-end-directed RNase H activity is assayed using a 41-nt 5′-end-radiolabeled RNA hybridized to a complementary DNA such that the 3′ end of the DNA is recessed. Cleavage is monitored by measuring the size of labeled RNA products. RNA 5′-end-directed RNase H activity is assayed using the same 41-nt radiolabeled RNA, hybridized to a long complementary DNA, such that the 5′ end of the RNA is recessed.

clinical use include nevirapine, delavirdine, and efavirenz (DMP-266) (6, 20, 24, 25, 41, 45, 51, 59, 65).

Resistance of HIV-1 to NNRTIs is mediated by mutations in residues that line the NNRTI binding pocket of the viral RT. The most common mutations that occur in clinical isolates of patients treated with currently approved NNRTIs are Y181C and K103N (17, 52) (L. T. Bacheler, B. Anton, D. Baker, et al., Abstr. 6th Conf. Retroviruses Opportunistic Infect., abstr. 109, 1999). A variety of other mutations of residues in the NNRTI binding pocket can be seen less commonly in clinical isolates. These mutations include L100I, K101E, V106A, V179D, Y188H or Y188C, P225H, and P236L (16, 17, 52, 58) (Bacheler et al., Abstr. 6th Conf. Retroviruses Opportunistic Infect.).

A number of studies have examined the effects of drug resistance mutations on HIV-1 RT function. These studies have demonstrated changes in the processivity and/or fidelity of RTs that contain mutations conferring resistance to nucleoside analogue inhibitors (4, 5, 19, 36, 50, 54, 62). In addition, zidovudine-resistant mutants have been shown to have increased rates of pyrophosphorolysis (2, 40). In contrast, little is known about the effects of NNRTI resistance mutations on the biochemical activity of HIV-1 RT. Studies with the Y181C mutant have shown that it has a k_{cat} similar to that of wild-type enzyme (14), but it has reduced affinity for deoxynucleoside triphosphates (56). The G190E mutant demonstrates reductions in polymerase activity and processivity (8, 23), as well as in RNase H activity (23). We have shown that NNRTI-resistant RTs with the P236L or K103N mutations each are slowed in the rate of specific modes of RNase H cleavage with no detectable effect on RNA-dependent DNA polymerization (29). In this study, we have extended our original observations and demonstrated that abnormalities in rates of RNase H cleavage are commonly seen with this class of drug-resistant mutants and that combined slowing of both DNA 3′-end- and RNA 5′-end-directed RNase H cleavage is associated with a significant reduction in the replication fitness of HIV-1.

MATERIALS AND METHODS

Reagents and cell lines. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: the infectious molecular clone pNL4-3 was obtained from Malcolm Martin, and the HeLa-CD4-LTR-β-Gal cell line was obtained from Michael Emmerman. H9 and 293 cell lines were obtained from ATCC. All cell lines were propagated as described previously (29). The expression vector pRSET was from Invitrogen. *Msc*I, *Spe*I, and *Age*I were obtained from New England Biolabs. Metal affinity columns (Talon) were obtained from Clontech, and Sepharose columns were from Pharmacia. T4 polynucleotide kinase, poly(rA), and oligo(dT) were purchased from Boehringer Mannheim. Oligonucleotides were purchased from Oligos, Etc.; radiolabeled chemicals were from New England Nuclear; and all other chemicals were from Sigma.

Site-directed mutagenesis. The V106A, V179D, and Y181C mutations were each introduced into the vector pRHA1, which contains a 4.1-kb region of pNL4-3 flanked by the *Sph*I and *Eco*RI restriction sites (29), using site-directed mutagenesis (QuikChange; Stratagene). The sequences of the mutagenic oligonucleotides used were 5′-GAA AAA ATC AGC AAC AGT ACT GGA TGT G-3′ (V106A), 5′-CAA AAT CCA GAC ATA GAC ATC TAT CAA TAC-3′ (V179D), and 5′-CAT AGT CAT CTG TCA ATA CAT-3′ (Y181C). A clone containing the desired mutation was sequenced on both strands across the entire length of HIV-1 sequence to verify that no extraneous mutations had been introduced during the process of mutagenesis.

HIV-1 RT expression and purification. HIV-1 RT sequences derived from pNL4-3 were expressed as separate p66 and p51 subunits in *Escherichia coli*, using the pRSET expression vector, as previously described (29). *Msc*I and *Age*I were used to subclone the region of *pol* containing the NNRTI mutations into the p66 and p51 expression vectors. HIV-1 RT p66 and p51 subunits were purified to >95% homogeneity, using metal affinity and ion-exchange chromatography, as previously described (29).

Measurement of specific activities of DNA polymerization and K_m for dTTP. Protein concentrations of RT preparations were determined by using the Bradford assay (9) (Bio-Rad, Hercules, Calif.). DNA polymerization activity of each RT preparation was measured in at least three independent experiments, using incorporation of [^{32}P]dTTP with a poly(rA)-oligo(dT) template-primer. A unit was defined as the amount of enzyme required to incorporate 1 nmol of dTTP into nucleic acid product in 10 min at 37°C using the poly(rA)-oligo(dT) template-primer. The specific activities of RT preparations of different mutant RTs

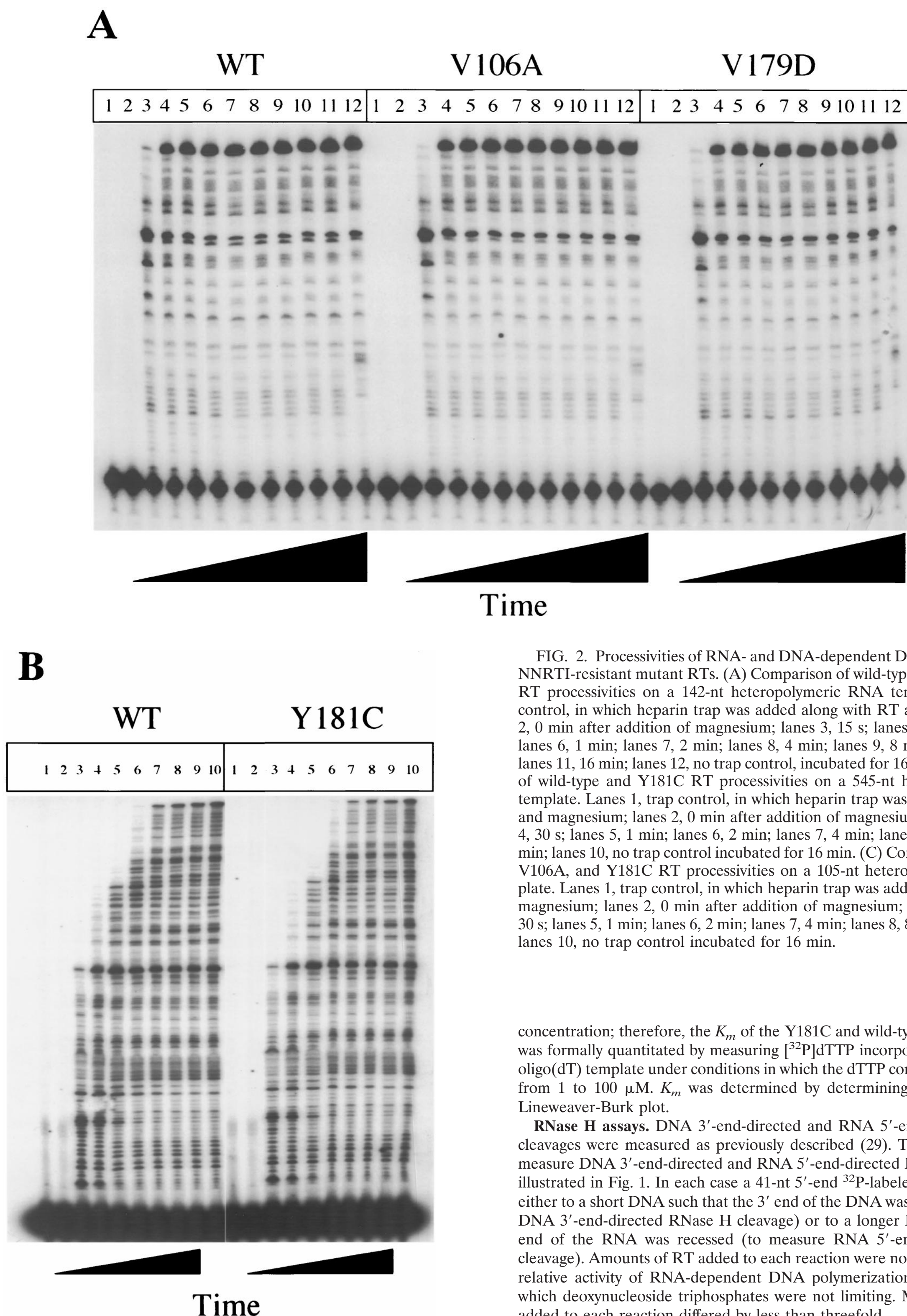


FIG. 2. Processivities of RNA- and DNA-dependent DNA polymerization by NNRTI-resistant mutant RTs. (A) Comparison of wild-type, V106A, and V179D RT processivities on a 142-nt heteropolymeric RNA template. Lanes 1, trap control, in which heparin trap was added along with RT and magnesium; lanes 2, 0 min after addition of magnesium; lanes 3, 15 s; lanes 4, 30 s; lanes 5, 45 s; lanes 6, 1 min; lanes 7, 2 min; lanes 8, 4 min; lanes 9, 8 min; lanes 10, 12 min; lanes 11, 16 min; lanes 12, no trap control, incubated for 16 min. (B) Comparison of wild-type and Y181C RT processivities on a 545-nt heteropolymeric RNA template. Lanes 1, trap control, in which heparin trap was added along with RT and magnesium; lanes 2, 0 min after addition of magnesium; lanes 3, 15 s; lanes 4, 30 s; lanes 5, 1 min; lanes 6, 2 min; lanes 7, 4 min; lanes 8, 8 min; lanes 9, 16 min; lanes 10, no trap control incubated for 16 min. (C) Comparison of wild-type, V106A, and Y181C RT processivities on a 105-nt heteropolymeric DNA template. Lanes 1, trap control, in which heparin trap was added along with RT and magnesium; lanes 2, 0 min after addition of magnesium; lanes 3, 15 s; lanes 4, 30 s; lanes 5, 1 min; lanes 6, 2 min; lanes 7, 4 min; lanes 8, 8 min; lanes 9, 16 min; lanes 10, no trap control incubated for 16 min.

differed by less than threefold, and were reproducible in independent experiments. Relative activities of polymerization by the different mutants were used to normalize amount of RT added to both processivity and RNase H reactions. Preliminary experiments showed that the specific activity of the Y181C mutant relative to wild-type RT differed depending on the deoxynucleoside triphosphate concentration; therefore, the K_m of the Y181C and wild-type enzymes for dTTP was formally quantitated by measuring [^{32}P]dTTP incorporation on a poly(rA)-oligo(dT) template under conditions in which the dTTP concentration was varied from 1 to 100 μM. K_m was determined by determining the x intercept of a Lineweaver-Burk plot.

RNase H assays. DNA 3′-end-directed and RNA 5′-end-directed RNase H cleavages were measured as previously described (29). The substrates used to measure DNA 3′-end-directed and RNA 5′-end-directed RNase H cleavage are illustrated in Fig. 1. In each case a 41-nt 5′-end ^{32}P-labeled RNA was annealed either to a short DNA such that the 3′ end of the DNA was recessed (to measure DNA 3′-end-directed RNase H cleavage) or to a longer DNA such that the 5′ end of the RNA was recessed (to measure RNA 5′-end-directed RNase H cleavage). Amounts of RT added to each reaction were normalized based on the relative activity of RNA-dependent DNA polymerization under conditions in which deoxynucleoside triphosphates were not limiting. Molar amounts of RT added to each reaction differed by less than threefold.

Processivity assays. Processivities of the different RTs on heteropolymeric 142- or 545-nt RNA substrates (29) and a heteropolymeric 105-nt DNA substrate (5′-TGA TTA CGC CAA GCT CGG AAT TAA CCC TCA CTA AAG GGA ACA AAA GCT TGC ATG CCT GCA GGT CGA CTC TAG AGG ATC CCC GGG TAC CGA GCT CGA ATT CGC CCT-3′) were measured by annealing 40 fmol of a 5′-end ^{32}P-labeled DNA oligonucleotide, PBS-1281 (5′-TCGCTTTC AAGTCCCTGTT), to 20 fmol of the RNA template or the labeled DNA oligonucleotide PBS-21 (5′-GGG CGA ATT CGA GCT CGG TAC-3′) to the

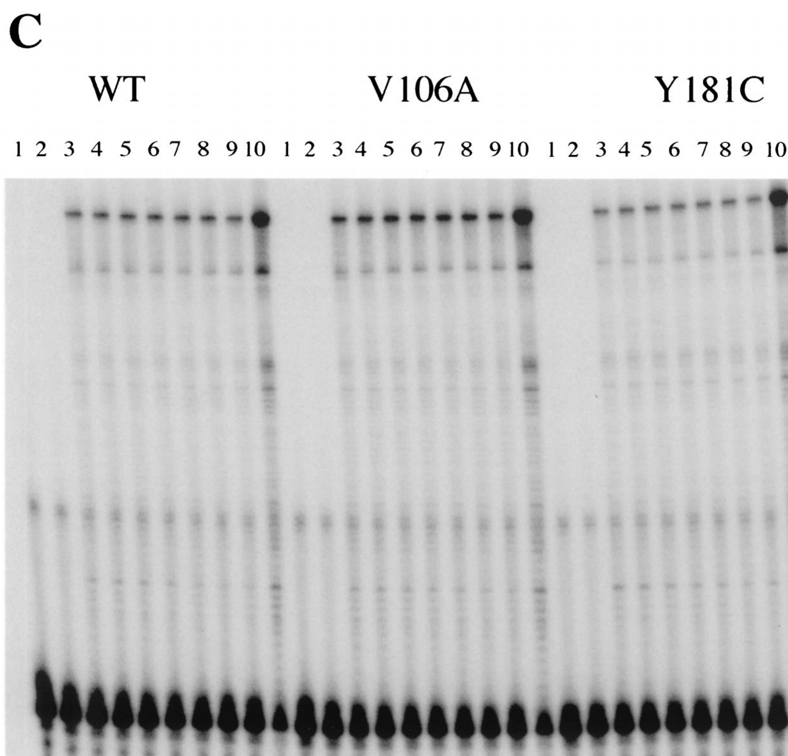


FIG. 2—*Continued.*

DNA template, as previously described (29). Reactions were carried out in the presence of a heparin trap and sampled at times ranging from 15 s to 16 min.

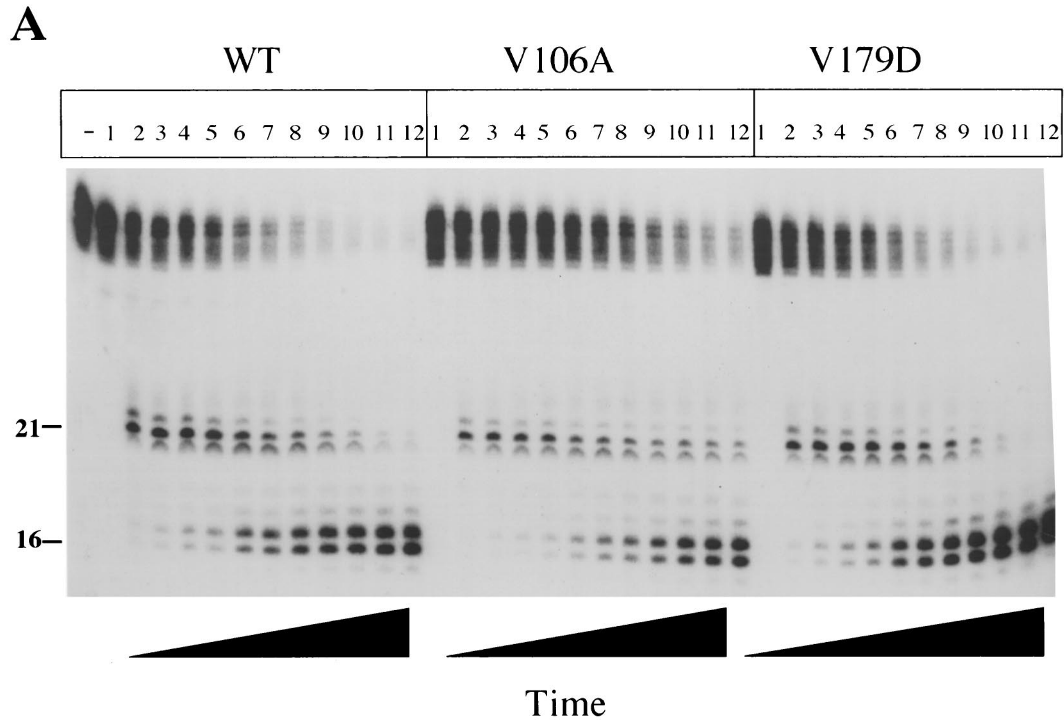
Replication kinetics of wild type and NNRTI-resistant mutants of HIV-1 in parallel cultures. The *pol* region of HIV-1, containing the V106A, V179D, or Y181C mutations, was subcloned from pRHA1 into pNL4-3 using *Spe*I and *Age*I. Individual clones of each mutant were isolated and sequenced to verify the integrity of the cloning sites and the presence of the appropriate RT resistance mutation. 293 cells were transiently transfected with each pNL4-3 mutant construct using lipofection (SuperFect; Qiagen, Santa Clarita, Calif.), as previously described (29). A total of 0.2×10^6 H9 cells were infected separately with each virus stock at a multiplicity of infection (MOI) of 0.001, as previously described (29). Infections were performed in triplicate and assayed for p24 antigen content. At least two independently generated virus stocks were used in separate experiments with similar results.

Growth competition assays for relative replication fitness of wild type and NNRTI-resistant mutants of HIV-1. Relative replication fitness of NNRTI-resistant mutants of NL4-3 was measured using growth competition experiments in H9 cells. H9 cells (10^6) were infected with a pair of virus stocks at an MOI of 0.0005 each, in a final volume of 1 ml at 37°C for 1 h. After washing, infected cells were resuspended in 2 ml of medium and incubated at 37°C in 5% CO_2. Each growth competition experiment between a given pair of mutants was performed in triplicate. After 7 days (1 passage), 10 μl of culture supernatant was used to infect an additional 10^6 H9 cells. Cultured cells were harvested and pelleted at time zero and at the termination of each passage. Genomic DNA was harvested from cell pellets using the QIAamp DNA Blood Mini kit (Qiagen). A region of the *pol* gene encompassing codons 106, 179, and 181 was amplified using PCR (Expand; Roche Molecular Systems) and the following primers: antisense, 5′-CTA TTC CAT CTA GAA ATA GTA CTT TCC TGA TTC C-3′; and sense, 5′-AAA GCC CGG GAT GGA TGG CCC AAA AG-3′ (underlined sequences represent *Xba*I and *Xma*I restriction sites, respectively). Cycling conditions were those recommended by the manufacturer, using an annealing temperature of 65°C. Direct sequencing of PCR products was performed using BigDye fluorescent-labeled terminators and AmpliTaqFS (PE Biosystems, Foster City, Calif.). The sequencing primers used were RT060 (sense, 5′-CTG AAA ATC CAT ACA ATA CTC C-3′) and 215D (antisense, 5′-TCT GTA TGT CAT TGA CAG TCC AGC-3′). The relative prevalence of mutant variants at each passage was quantitated by averaging the relative peak heights from the sense and antisense sequencing reactions of a minimum of two replicate infections.

RESULTS

RNA- and DNA-dependent DNA polymerization by NNRTI-resistant HIV-1 RTs. We found that the Y181C mutant RT demonstrated an approximately threefold increase in K_m for dTTP (10.8 μM for Y181C versus 3.25 μM for wild-type RT) using a homopolymeric RNA template. When measured under conditions in which the deoxynucleoside triphosphate concentrations were not limiting (100 μM dTTP), the RNA-dependent DNA polymerase specific activities of wild-type, V106A, V179D, and Y181C RTs were similar to each other (5,830 ± 223 U/mg for wild-type RT, 4,760 ± 862 U/mg for V106A RT, 4,720 ± 502 U/mg for V179D RT, and 2,810 ± 651 U/mg for Y181C RT). We measured the processivities of wild-type, V106A, V179D, and Y181C RTs on heteropolymeric RNA templates under conditions in which deoxynucleoside triphosphates were not limiting and found that the distribution of primer extension lengths was similar for the wild-type and each mutant RT (Fig. 2A and B). We also found no differences among wild-type, V106A, and Y181C RTs in their processivities of polymerization on a heteropolymeric DNA template (Fig. 2C).

DNA 3′-end-directed RNase H cleavage by V106A and V179D RTs. Because we had observed slowing of DNA 3′-end-directed RNase H cleavage by both the P236L and K103N RTs in previous studies (29), we asked whether the other NNRTI-resistant RT mutants also demonstrated altered kinetics of this mode of RNase H cleavage. We first tested the V106A and V179D mutant RTs, using an RNA-DNA hybrid with a recessed DNA 3′ end (Fig. 3A). Input amounts of each RT were adjusted, based on the relative activity of DNA polymerization using a homopolymeric RNA template. The time course of RNase H activity using this substrate was measured in the



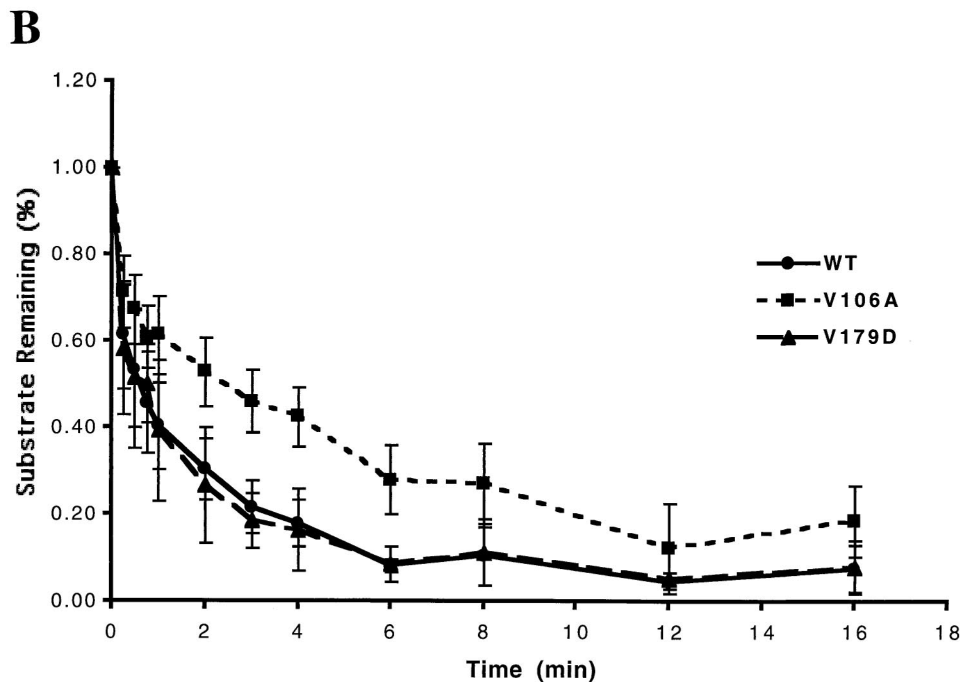


FIG. 3. DNA 3′-end-directed RNase H activity of wild-type, V106A, and V179D RTs. (A) Autoradiogram of a representative experiment. Lane "−," no RT control. Lanes 1, 0 min; lanes 2, 15 s; lanes 3, 30 s; lanes 4, 45 s; lanes 5, 1 min; lanes 6, 2 min; lanes 7, 3 min; lanes 8, 4 min; lanes 9, 6 min; lanes 10, 8 min; lanes 11, 12 min; lanes 12, 16 min. Numbers to the left of the autoradiogram represent the length in nucleotides. Bands of approximately 21 nt represent primary cleavage products (13 nt from the recessed DNA 3′ end), and bands of approximately 16 nt represent secondary cleavage products (8 nt from the recessed DNA 3′ end). (B) DNA 3′-end-labeled RNase H cleavage. A graph of the quantitation data, obtained by using phosphorimaging, is shown. Datum points are means of five independent experiments; vertical bars represent standard deviations. The vertical axis represents the fraction of substrate remaining relative to time zero.

absence of deoxynucleoside triphosphates, from 15 s to 16 min. The time required for the V106A mutant to degrade half of the substrate was approximately three- to fourfold slower than for the wild-type enzyme (Fig. 3B). The slowed DNA 3′-end-directed RNase H cleavage by the V106A mutant was reproducible in five independent experiments. The formation of the primary (14 to 18 nt) and secondary (6 to 7 nt) cleavage products by the V106A mutant appeared to be affected to a

similar extent (Fig. 3A and data not shown). In contrast, there was little detectable effect of the V179D mutation on 3′-end-directed RNase H cleavage (Fig. 3). No aberrantly sized cleavage products were seen with either mutant.

RNA 5′-end-directed RNase H cleavage by V106A and V179D RTs. We next examined whether the V106A or V179D mutations affected RNA 5′-end-directed cleavage, using the same 5′-end-labeled RNA, which was hybridized to a complementary DNA such that the 5′ end of the RNA was recessed. Similar to what was seen with DNA 3′-end-directed RNase H cleavage, the V106A mutant demonstrated a four- to sixfold slowing of RNA 5′-end-directed RNase H cleavage (Fig. 4). Formation of both primary and secondary cleavage products appeared to be similarly affected (Fig. 4A), and no aberrant cleavage products were detected. No consistent abnormality in RNase H cleavage by the V179D mutant was detected using this substrate, although in one experiment a slight acceleration of cleavage was detected (data not shown).

RNase H cleavage by the Y181C mutant RT. When we studied RNA 5′-end-directed RNase H cleavage by the Y181C mutant RT, we observed that the relative accumulation of cleavage products by this mutant differed from that observed with either wild-type or other NNRTI-resistant mutants (Fig. 5). Overall, cleavage of the 41-nt RNA substrate was unchanged relative to the wild-type enzyme (Fig. 5). However, we did observe a reduction in accumulation of the first cleavage product and an acceleration of formation of the second cleavage product by the Y181C mutant relative to the wild-type enzyme (Fig. 5). Similar results were seen when the Y181C mutant was assayed for DNA 3′-end-directed RNase H cleavage (data not shown).

Replication kinetics of NNRTI-resistant mutants. The replication fitness of these three NNRTI-resistant mutants were compared in parallel infections of H9 cells (Fig. 6). Y181C and V179D each had a 1.5- to 2-fold reduction in accumulation of p24 antigen relative to the wild-type virus. In contrast, V106A, showed 5- to 10-fold-lower p24 antigen concentration than with the wild-type virus and consistently replicated more slowly than either Y181C or V179D. The replication kinetics of V106A were similar to those of P236L (data not shown). The relative order of replication fitness (wild type > V179D, Y181C > V106A, P236L) was seen in three independent experiments using two independently generated sets of virus stocks.

Growth competition experiments of wild-type and NNRTI-resistant HIV-1. The relative replication fitness of wild type and NNRTI-resistant mutants of NL4-3 was also assessed using growth competition experiments in H9 cells. Pairwise comparisons of fitness included wild type versus V106A, wild type versus V179D, wild type versus Y181C, V106A versus V179D, V106A versus Y181C, and V179D versus Y181C. H9 cells were infected with each pair of virus stocks at an MOI of 0.0005 each, as measured in the MAGI assay (38). At day 7, a fraction of the culture supernatant was used to infect fresh H9 cells. Relative amounts of each genotypic variant were measured by quantitating the relative peak heights of mixed position bases after direct sequencing of PCR product amplified from proviral DNA. Figure 7 summarizes the proportion of each mutant over time for each pairwise growth competition experiment. Panel A shows the relative prevalence of the V106A mutant compared with wild-type, V179D, or Y181C viruses. Of note is that the direct sequencing method we used was more sensitive for the presence of the V106A mutant than would have been predicted based on relative inputs of infectious virus. By the first passage, there was a dramatic decrease in V106A relative to the other three genotypes, with essentially complete loss of the V106A mutant after only two passages. These findings are consistent with the results from parallel infections, which showed that the V106A mutant replicated more poorly than the wild type or the other two NNRTI-resistant mutants.

The reduced replication fitness of the V106A mutant is supported by the results shown in panels B and C, which demonstrate a parallel rise in the prevalence of V179D and Y181C, respectively, when these mutants are each competed with the V106A mutant. We found that direct sequencing was relatively insensitive to the presence of the V179D mutant (Fig. 7B), resulting in an apparent proportion of V179D at baseline of only 10%, despite infecting with equal amounts of each stock based on infectivity assays. We have observed this relative insensitivity of direct sequencing methods for detecting the V179D mutant in a previous study of clinical isolates (15). Despite the apparent low prevalence of the V179D mutant at baseline in the competition assays, it quickly overgrew the Y181C and V106A mutants (Fig. 7B). Although there appeared to be a slight growth disadvantage of the V179D mutant relative to wild-type NL4-3 (Fig. 7B), we are unable to definitively establish the relative replication fitness of the V179D and wild-type viruses in these competition experiments because of the relative insensitivity of the direct sequencing assay for this NNRTI-resistant mutant.

DISCUSSION

It is important to determine the effects of drug resistance mutations on HIV-1 RT function in order to better understand the consequences of selection for drug resistance on HIV-1 replication fitness and pathogenic potential. NNRTIs are now commonly used as part of combination antiretroviral regimens and appear to provide a similar antiviral effect to protease inhibitors with fewer long-term toxicities (reviewed in reference 28). A major disadvantage of NNRTIs is that the genetic barrier to the development of drug-resistant HIV is relatively low, and only one to two mutations are usually required for high-level drug resistance. In order to design drug regimens with more durable antiviral effects, it will be necessary to understand what factors impact on the emergence of drug-resistant mutants and how resistance mutations affect HIV replication fitness and RT function.

Mutations conferring drug resistance are unlikely to result in severe impairment of HIV-1 replication fitness, since they develop readily in clinical isolates during therapy. One might anticipate that any biochemical abnormalities associated with such mutations would be subtle and difficult to detect in biochemical assays. Remarkably, we found that measurable biochemical abnormalities in NNRTI-resistant mutants of RT are quite common. The most commonly observed effect of NNRTI resistance mutations is a change in the ratio of RNase H to polymerase activities.

Mutants of HIV-1 resistant to NNRTIs all line the NNRTI binding pocket, which is adjacent to the polymerase active site, and are thought to confer resistance through a reduction in drug binding. Despite their proximity to the polymerase active site, the mutants analyzed in this and our previous studies (29) demonstrated no significant alterations in RNA- or DNA-dependent DNA polymerization. We found no consistent differences in polymerization activities or processivities of these mutants, although the Y181C mutant did demonstrate a modest increase in K_m for dTTP, a finding consistent with previous observations by another group (56). The fact that these NNRTI-resistant mutants all demonstrated processivities of DNA polymerization equivalent to wild-type RT strongly suggests that the polymerization reaction cycle, which consists of

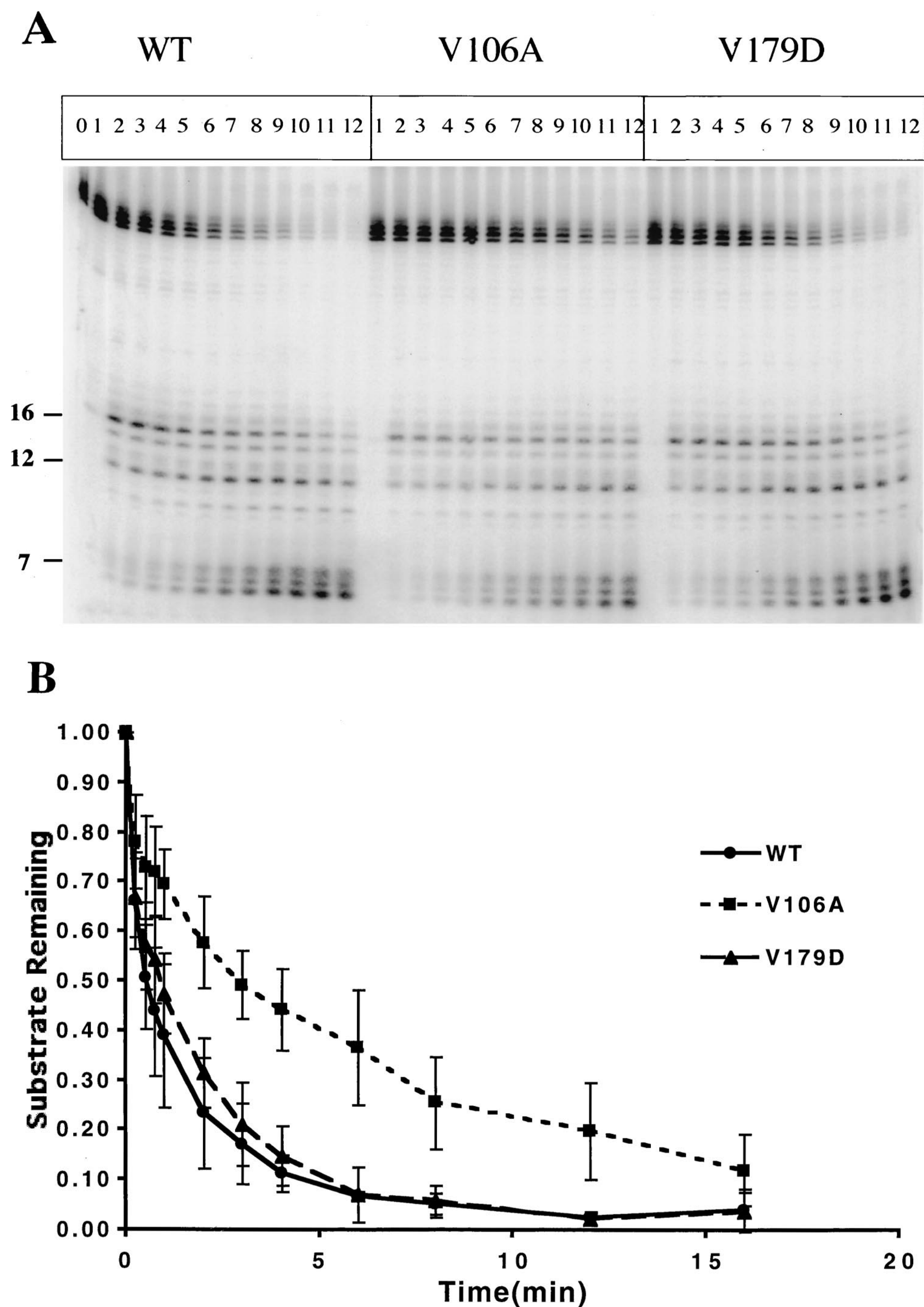


FIG. 4. RNA 5′-end-directed RNase H activity of wild-type, V106A, and V179D RTs. (A) Autoradiogram of a representative experiment. Assays were performed as described in Materials and Methods. Lane −, control in which RT was not added; lanes 1, 0 min; lanes 2, 15 s; lanes 3, 30 s; lanes 4, 45 s; lanes 5, 1 min; lanes 6, 2 min; lanes 7, 3 min; lanes 8, 4 min; lanes 9, 6 min; lanes 10, 8 min; lanes 11, 12 min; lanes 12, 16 min. Numbers to the left of the autoradiogram represent the length in nucleotides. Bands migrating at a position approximately 16 nt from the 5′ end represent primary cleavage products, and bands migrating at a position approximately 7 nt from the 5′ end represent secondary cleavage products. (B) RNA 5′-end-directed RNase H activity. A graph of the quantitation data for substrate degradation, obtained by using phosphorimaging, is shown. Datum points are means of four independent experiments; vertical bars represent standard deviations. The vertical axis represents the fraction of substrate remaining, relative to time zero.

primer binding, nucleotide addition, and dissociation after primer elongation, is not significantly altered. It should be noted that we did not exhaustively assay other activities associated with the polymerase active site, such as fidelity of nucleotide incorporation or binding affinity for template-primer. Thus, we cannot definitively rule out the possibility that these mutants affect aspects of the polymerization cycle not directly assayed in our studies.

We have demonstrated that four of the five NNRTI-resistant mutants tested in this and a previous study (29) exhibit repro-

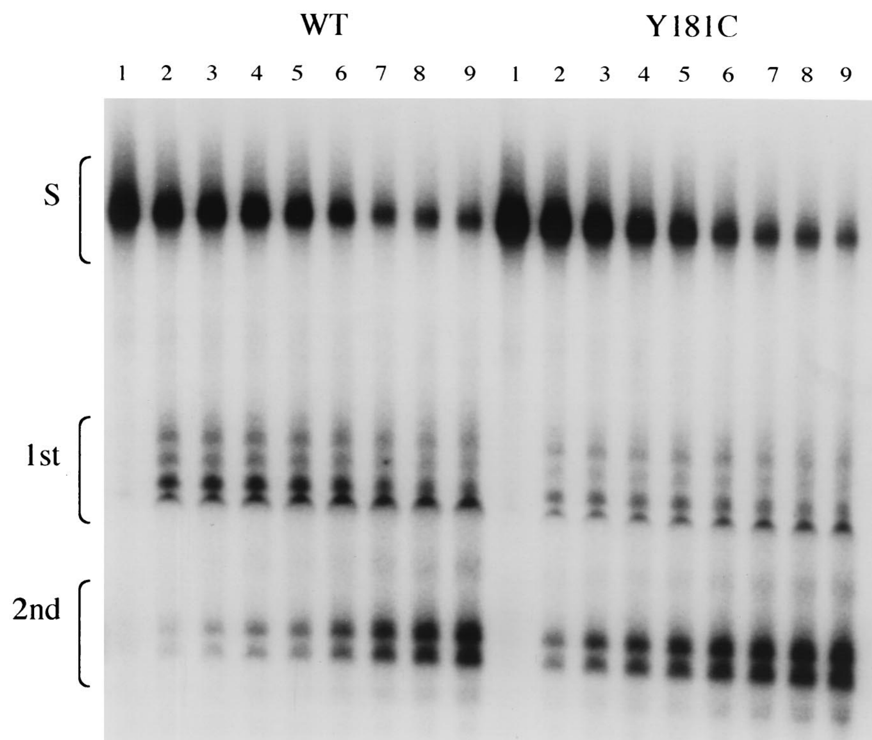


FIG. 5. RNA 5′-end-directed RNase H activity of wild-type and Y181C RTs. Autoradiogram of a representative experiment. Lanes 1, 0 min; lanes 2, 15 s; lanes 3, 30 s; lanes 4, 45 s; lanes 5, 1 min; lanes 6, 2 min; lanes 7, 4 min; lanes 8, 8 min; lanes 9, 16 min. Brackets denote substrate (S), first cleavage products (1st), and second cleavage products (2nd).

ducible alterations in rates of RNase H cleavage. It should be emphasized that the relative amounts of mutant and wild-type enzymes were carefully normalized to each other, so that any differences in specific activities of the different preparations could not account for the results that were observed.

When analyzing overall substrate degradation, three mutant RTs (K103N, V106A, and P236L) demonstrated a reduction in the ratio of RNase H to polymerase activities. Apparently, HIV-1 can tolerate some alteration in the ratio of RNase H to polymerase activities of RT, although our results suggest that the greater reductions in the ratio of RNase H to polymerase activities seen with the V106A and P236L mutants have an adverse effect on HIV-1 replication fitness as measured in cell culture. Reductions in this ratio could adversely impact viral replication fitness by impairing the degradation of the RNA genome and the initiation of plus-strand DNA synthesis. If there is a disadvantage to reducing the ratio of RNase H to polymerase activities of RT, it does not necessarily follow that increasing this ratio would prove to be an advantage to the virus, since wild-type RT is likely to have evolved an optimal ratio of these activities.

Four of the NNRTI resistance mutations also had specific effects on the different modes of RNase H cleavage. The V106A mutant demonstrated slowing in RNase H cleavage when assayed for both DNA 3′-end- and RNA 5′-end-directed modes of cleavage. The biochemical phenotype of the V106A mutant is similar to that of the P236L mutant, which demonstrated a replication defect in cell culture, relative to both wild-type virus and the K103N mutant (29). The K103N mutant, which has a more subtle replication abnormality in cell culture than P236L, has slowed DNA 3′-end-directed cleavage but normal RNA 5′-end-directed cleavage (29). We have postulated that the combined abnormalities in DNA 3′-end- and RNA 5′-end-directed cleavages contribute to the decreased replication fitness of the P236L mutant. Our current replication studies support this hypothesis, suggesting that a combined reduction in both DNA 3′-end- and RNA 5′-end-directed modes of RNase H cleavage is associated with a significant reduction in the replication fitness of HIV-1.

One resistance mutation, Y181C, showed an alteration in the relative distribution of primary and secondary RNase H cleavage products. Previous work has shown that the primary and secondary cleavages occur 14 to 20 nt and 5 to 7 nt, respectively, from the end of the strand directing cleavage (18, 26, 33, 42–44, 47, 48, 64). Studies of the rates of accumulation of these cleavage products suggest that the primary cleavage occurs much more rapidly than the secondary cleavage, although there are no published data on whether the primary cleavage event is required for the secondary cleavage to occur.

Y181C, which along with K103N is one of the two most common NNRTI-resistant mutants that develop in clinical isolates, showed a selective increase in the accumulation of the secondary RNase H cleavage product during both RNA 5′-end- and DNA 3′-end-directed RNase H cleavage. The interpretation of these findings depends on whether the primary and secondary cleavage events can occur independently of each other. If the secondary cleavage can only occur after the primary cleavage, then the finding of a decrease in primary and an increase in secondary product formation relative to substrate with no change in the rate of substrate degradation is most compatible with a selective acceleration by the Y181C mutant of the secondary cleavage event, with little effect on the rate of primary cleavage. However, if the secondary cleavage event can occur independently of the primary cleavage, then a slowing of the primary cleavage with an acceleration of the

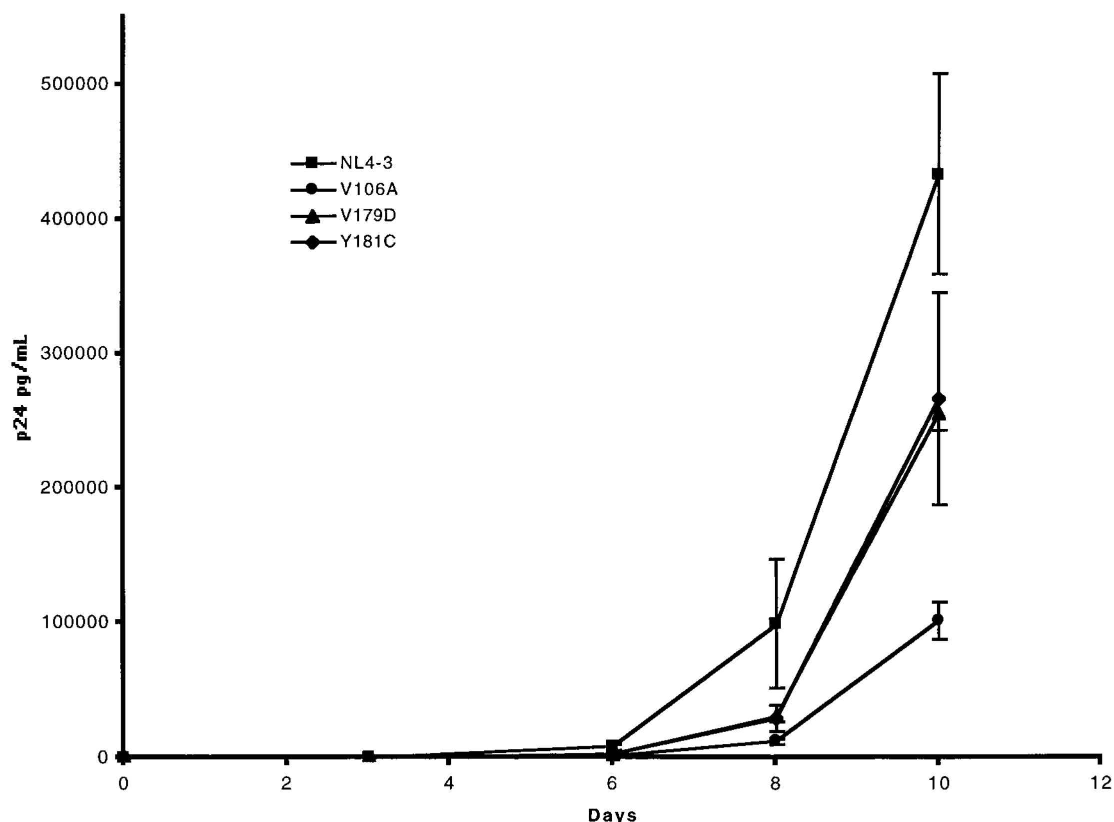


FIG. 6. Replication kinetics of NNRTI-resistant mutants. Separate cultures of H9 cells were infected with HIVNL43 (■), HIVNL43/V106A (●), HIVNL43/V179D (▲), and HIVNL43/Y181C (◆), as described in Materials and Methods. Viral replication was monitored over a period of 10 days by measuring HIV-1 p24 antigen concentration. Error bars show the standard deviation of the mean p24 antigen concentration from three independent infections.

secondary cleavage would be the most likely explanation of these findings.

It is uncertain to what extent these alterations in RNase H activity contribute to the modest reduction in replication fitness of Y181C relative to wild-type virus, in part because this mutant also demonstrates a reduction in affinity for nucleotide substrate. This reduction in substrate affinity is likely to be a disadvantage to the virus and could explain the modest reduction in replication kinetics of Y181C relative to wild-type virus that was seen in our study. In addition, we still do not completely understand what parameters of RNase H cleavage best correlate with HIV replication fitness. The P236L and V106A mutants, which show marked reductions in replication fitness relative to wild-type and other NNRTI-resistant viruses, demonstrate both a reduction in the rate of substrate degradation and a reduction in the rate of secondary product formation. Overall, the Y181C mutant showed no alteration in the rate of RNase H degradation of full-length substrate. If this measure of RNase H activity best correlates with replication fitness, then one might expect that the altered RNase H activity of the Y181C mutant would have little or no impact on replication fitness. However, it is also possible that the formation of the secondary RNase H cleavage product could be the parameter that most influences viral replication fitness, since the secondary product is most likely to spontaneously dissociate from the RNA-DNA hybrid. Potential advantages resulting from an accelerated secondary cleavage event could include increased rates of plus-strand DNA initiation and synthesis resulting from increased dissociation of genomic RNA fragments and/or accelerated rates of minus-strong-stop-DNA transfer due to increased cleavage near the 5′ end of genomic RNA.

Our studies have not yet convincingly demonstrated a biochemical abnormality that could account for the slightly reduced replication fitness of the V179D mutant. Although we are still in the process of establishing correlations between HIV-1 replication fitness in cell culture and RT function, our experience with the Y181C and K103N mutants leads us to expect that the V179D mutant should demonstrate an abnormality in RT function. It is possible that this mutant has an abnormality in RNase H cleavage that is too subtle to consistently detect in our assays (an acceleration of cleavage was observed in only one of four experiments). Alternatively, V179D may have an abnormality in RT function that we have not assayed for, such as DNA-dependent polymerization, fidelity of nucleotide incorporation, or strand transfer.

The magnitude of difference in RNase H activities of these NNRTI-resistant mutants relative to wild-type enzyme was relatively modest (ca. fourfold). These differences, however, were consistently observed in replicate experiments. It is not surprising that the drug-resistant mutants examined here would demonstrate modest alterations in biochemical function, since these mutants are selected for in patients during therapy and likely have relatively well-preserved replication fitness. The presence of detectable biochemical abnormalities in four of these five mutants is likely to have some subtle effect on replication fitness and is consistent with the theory that drug-resistant mutants rarely predominate in the absence of drug

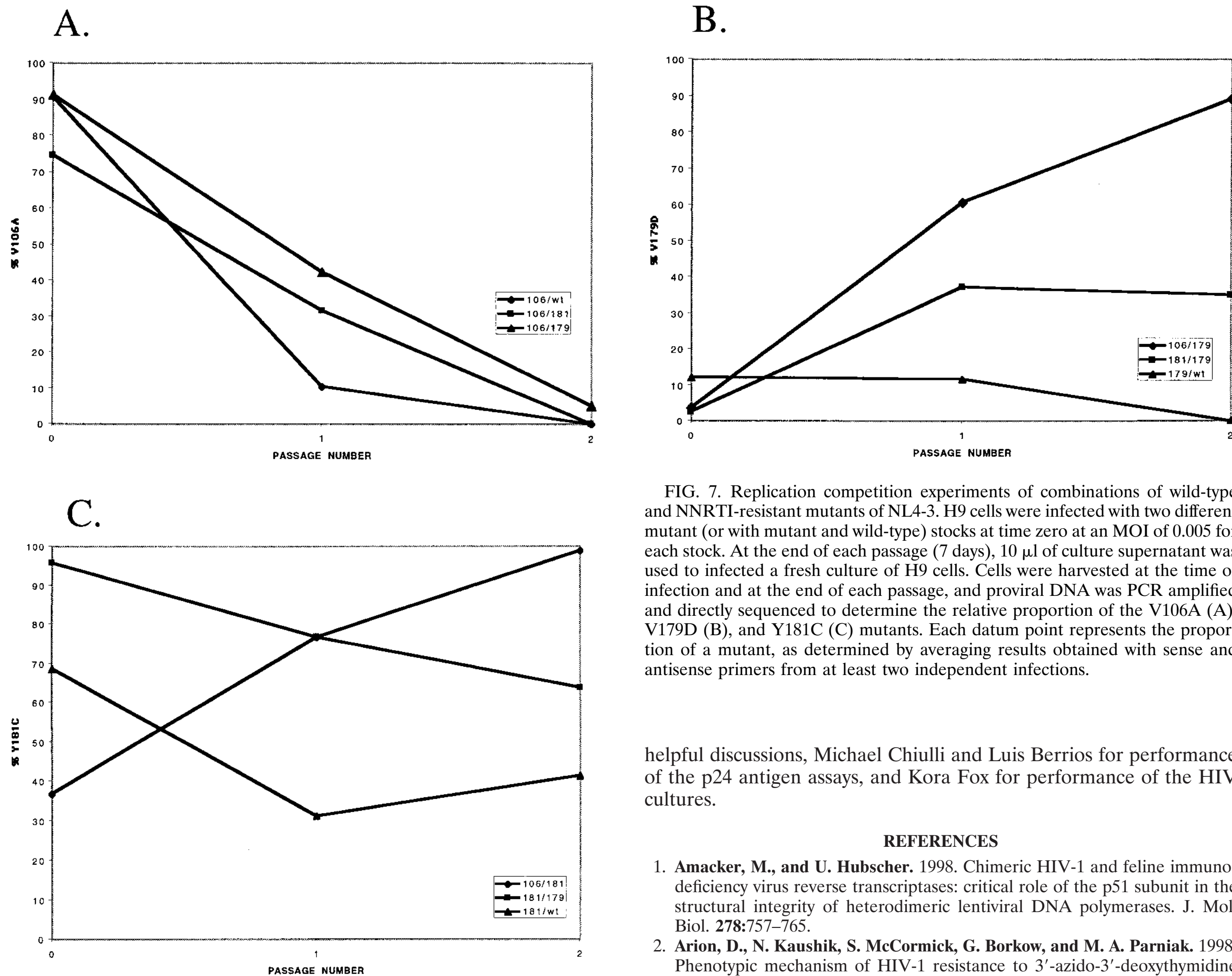


FIG. 7. Replication competition experiments of combinations of wild-type and NNRTI-resistant mutants of NL4-3. H9 cells were infected with two different mutant (or with mutant and wild-type) stocks at time zero at an MOI of 0.005 for each stock. At the end of each passage (7 days), 10 μl of culture supernatant was used to infected a fresh culture of H9 cells. Cells were harvested at the time of infection and at the end of each passage, and proviral DNA was PCR amplified and directly sequenced to determine the relative proportion of the V106A (A), V179D (B), and Y181C (C) mutants. Each datum point represents the proportion of a mutant, as determined by averaging results obtained with sense and antisense primers from at least two independent infections.

selection pressure because they have reduced replication fitness relative to wild-type virus (12).

The mechanism of how these NNRTI-resistant mutants, which are remote from the RNase H active site, affect RNase H cleavage remains to be elucidated. There is extensive precedent in the literature that residues in the polymerase domain of retroviral RTs can have significant effects on the positioning of the RNase H domain and therefore affect the efficiency of cleavage. Mutants in the fingers, palm, and thumb subdomains of RT can affect the specificity and/or efficiency of RNase H cleavage (7, 27, 31, 32, 44, 48, 49). The alterations in RNase H cleavage seen with these NNRTI-resistant mutants may adversely impact on the replication fitness of HIV-1 through a number of mechanisms, including reducing rates of genomic RNA degradation, slowing rates of minus-strong-stop-DNA transfer, and reducing rates of PPT formation and removal. Studies to assess whether these mutants affect these different steps of reverse transcription in vivo and in vitro are in progress in our laboratory.

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