The Y181C Mutant of HIV-1 Reverse Transcriptase Resistant to Nonnucleoside Reverse Transcriptase Inhibitors Alters the Size Distribution of RNase H Cleavages†

Richard H. Archer,‡ Michele Wisniewski,§ Robert A. Bambara,§,||,⊥ and Lisa M. Demeter*,‡,||,⊥

Departments of Medicine, Biochemistry and Biophysics, and Microbiology and Immunology, and the Cancer Center, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Received October 4, 2000; Revised Manuscript Received January 29, 2001

ABSTRACT: We investigated the effects of the nonnucleoside reverse transcriptase inhibitor-resistant mutant Y181C on RNA 5′-end-directed RNase H cleavage by HIV-1 reverse transcriptase, using an RNA·DNA hybrid in which a radiolabeled RNA 5′ end was recessed. Y181C produced a higher ratio of secondary (9 nucleotide long) to primary (18 nucleotide long) products than wild type. When the RNA was 3′-end-labeled, Y181C generated a long product, which results when secondary cleavage precedes the primary. When using an RNA·DNA hybrid in which the labeled RNA 5′ end and DNA 3′ end were flush, formation of secondary product by both enzymes was inhibited. Under these conditions, Y181C cleaved closer to the RNA 5′ end than wild type. Studies with this substrate labeled at the RNA 3′ end showed that Y181C is no more likely than wild type to cleave toward the RNA 3′ end. Thus, Y181C RT has a strong preference to cleave in the direction of the RNA 5′ end even when secondary cleavage is prevented, resulting in a disruption of the normal sequence of primary followed by secondary cleavages.

HIV-1 reverse transcriptase (RT)[1] is an essential enzyme in the virus life cycle and a major target for antiretroviral therapy of HIV-1 infection. HIV-1 RT converts the RNA genome of the virus to a double-stranded DNA, which is ultimately integrated into the host chromosome (*1*). This enzyme catalyzes several activities during the replication cycle of the virus, including RNA- and DNA-dependent polymerization, RNase H cleavage, strand transfer, and strand displacement. HIV-1 RT is a heterodimer consisting of 66 and 51 kDa subunits (p66 and p51, respectively) (*2*, *3*). p66 contains both the polymerase and RNase H active sites of the enzyme (*4*−*6*). The RNase H domain is present in the carboxy-terminal third of p66 (*7*−*9*). p51, which is derived from p66 by proteolytic cleavage, may play a role in facilitating binding to and initiation from the $tRNA^{Lys3}$ primer (*10*, *11*).

RNase H cleavage is required for several steps during reverse transcription and is essential for HIV-1 replication (reviewed in ref *12*). RNase H cleavage degrades the viral genome during and after synthesis of minus-strand DNA. RNase H cleavage of the 5′ R region of the HIV-1 genome is required for translocation of minus strong stop DNA to the 3′ end of the genome (*13*, *14*). Specific RNase H cleavages are also required for formation of the polypurine tract (PPT) RNA primer (*15*−*18*) that primes plus-strand synthesis and for removal of the PPT (*15*−*18*) and tRNA primers (*19*−*22*) after extension.

Two modes of RNase H cleavage have been described (Figure 1). "Polymerase-dependent" cleavage, which we have referred to as DNA 3′-end-directed cleavage, is thought to occur in concert with DNA polymerization to degrade the genomic RNA during minus-strand DNA synthesis (*19*, *23*, *24*). The position of the initial, or primary, DNA 3′-end-directed cleavage occurs approximately 18 NT from the recessed 3′ end of the DNA (*19*, *24*). A second mode of RNase H cleavage, in which the position of the primary cleavage occurs approximately 18 NT from a recessed RNA 5′ end, occurs independently of DNA polymerization (*25*, *26*). This mode of cleavage, which we have referred to as RNA 5′-end-directed RNase H cleavage, can occur with RNA·DNA hybrids in which the DNA is circular (i.e., has no free end to direct cleavage) (*26*) and may contribute to the degradation of larger genomic RNA fragments left behind after DNA 3′-end-directed cleavage (*25*, *26*). RNA 5′-end-directed cleavage also appears to play an important role in the formation and removal of the polypurine tract (PPT) (*15*−*17*).

After the initial endonucleolytic cleavage approximately 18 NT from the end of the nucleic acid directing cleavage, the enzyme shifts and makes an additional cleavage, approximately 7−9 NT from that end (see Figure 1) (*25*−*27*). We have referred to this RNA 3′ → 5′ directional cleavage or processing as the secondary RNase H cleavage. Measurement of the rate of secondary cleavage by wild-type RT shows that it is approximately 3−6-fold slower than the

† This work was supported primarily by NIH Grant R01-AI-041387 and in part by Grant R01-GM-49573.

* To whom correspondence should be addressed: University of Rochester Infectious Diseases Unit, 601 Elmwood Ave., Box 689, Rochester, NY 14642. Telephone: 716-275-4764. Fax: 716-442-9328. E-mail: Lisa_Demeter@urmc.rochester.edu.

‡ Department of Medicine.

§ Department of Biochemistry and Biophysics.

|| Department of Microbiology and Immunology.

⊥ Cancer Center.

[1] Abbreviations: DNA, deoxyribonucleic acid; dNTP, deoxyribonucleoside triphosphate; HIV-1, human immunodeficiency virus type 1; kDa, kilodalton; NNRTI, nonnucleoside reverse transcriptase inhibitor; NT, nucleotide; PPT, polypurine tract; RNA, ribonucleic acid; RNase, ribonuclease; RT, reverse transcriptase; tRNA, transfer RNA; Y181C, tyrosine to cysteine mutation at codon 181.

10.1021/bi002328a CCC: $20.00
Published on Web 03/09/2001

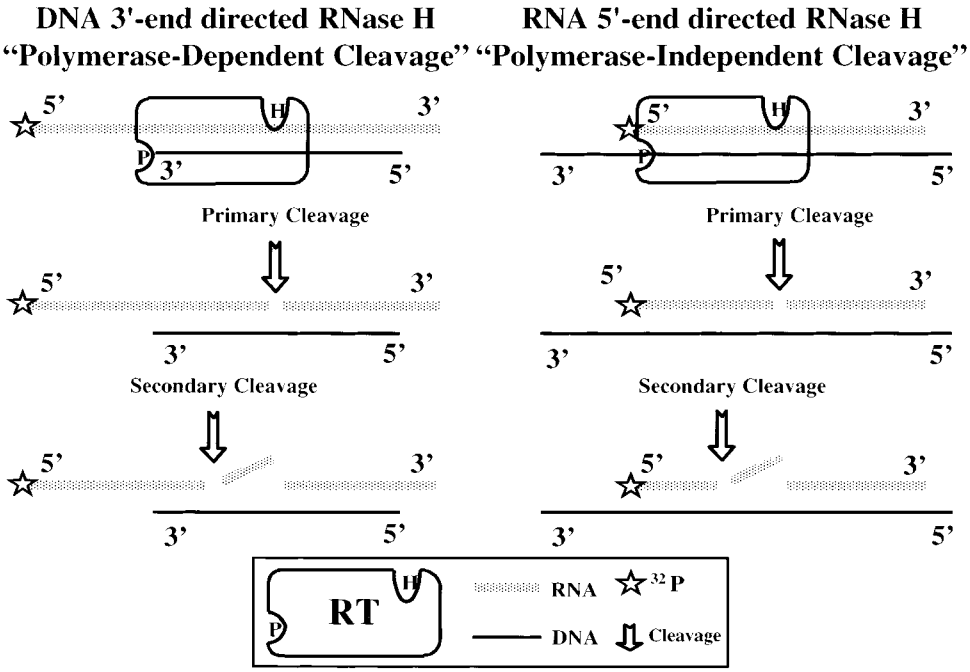

FIGURE 1: Diagram illustrating the two modes of RNase H cleavage carried out by HIV-1 RT. RNA is represented by a stippled line and DNA by a solid line. DNA 3′-end-directed RNase H cleavage is thought to occur in concert with DNA polymerization and is diagramed on the left-hand side of the figure. During this mode of cleavage, the polymerase active site of the enzyme (represented by the letter P) is positioned at the 3′ end of the DNA, which is recessed. The RNase H active site (represented by the letter H) is positioned approximately 18 NT from the polymerase active site, and its position from the 3′ end of the DNA is thought to determine the position of the primary cleavage. After the primary cleavage, wild-type RT then repositions and makes a secondary cleavage 7−9 NT from the recessed 3′ end of the DNA. A second mode of RNase H cleavage, which has been referred to as RNA 5′-end-directed RNase H cleavage, or polymerase-independent cleavage, is assayed with a RNA·DNA hybrid in which the RNA 5′ end is recessed. The RT is positioned to make the primary cleavage, which is approximately 18 NT from the 5′ end of the RNA. Of note is that the position of RT during this mode of cleavage would not allow polymerization to occur at the 3′ end of the DNA. The RT then repositions to make a secondary cut 7−9 NT from the 5′ end of the RNA.

initial primary cleavage (*28*). Mutations that reduce secondary RNase H cleavages prevent strand transfer of minus strong stop DNA to the 3′ end of the genome that is required for viral replication (*13*, *29*), as well as internal strand transfers that likely contribute to HIV-1 recombination (*23*, *30*). Reduction in secondary RNase H cleavages most likely prevents strand transfer because the resulting larger genomic RNA fragments form more stable hybrids and are less likely to dissociate from minus-strand DNA.

Nearly all combination antiretroviral regimens recommended for the initial therapy of HIV-1 infection include inhibitors of the HIV-1 reverse transcriptase (RT) (*31*). A widely used group of RT inhibitors are the nonnucleoside RT inhibitors (NNRTIs), which are highly active, specific inhibitors of HIV-1 RT that inhibit polymerization by binding a specific region of p66 adjacent to the polymerase active site of the enzyme (*8*, *32*, *33*). Drug binding induces an allosteric change of the polymerase active site which results in nonproductive binding of the incoming dNTP (*8*, *33*−*36*). NNRTIs in combination with nucleoside analogues can provide antiviral effects that are equal to or greater than protease inhibitor-containing regimens (*37*) and may have fewer long-term toxicities (*31*).

Development of drug resistance is a major factor limiting the efficacy of antiretroviral therapies for HIV-1 infection (reviewed in ref *38*). Resistance of HIV-1 to NNRTIs is mediated by mutations of residues that line the NNRTI binding pocket of the viral reverse transcriptase and reduce drug binding (*32*). The most common mutations that occur in clinical isolates of patients treated with currently approved NNRTIs are Y181C and K103N (*39*−*41*).

It has been postulated that drug-resistance mutations, because they rarely arise in the absence of drug selection pressure, reduce the replication fitness of HIV-1 (*42*). A number of studies have examined the effects of RT inhibitor resistance mutations on HIV-1 replication fitness and RT function. These studies have demonstrated that reductions in HIV-1 replication fitness can be associated with changes in RT processivity and/or fidelity of nucleoside analogue-resistant mutants (*43*−*50*). Zidovudine-resistant mutants have been shown to have increased rates of pyrophosphorolysis (*51*, *52*), which accelerate the removal of incorporated nucleoside analogues from chain-terminated DNAs. We have shown that NNRTI-resistant RTs with the P236L, K103N, Y181C, or V106A mutations each specifically alter the rate of one or both modes of RNase H cleavage with no significant effects on RNA- or DNA-dependent DNA polymerization (*53*, *54*). In addition, we found that greater reductions in RNase H activity were associated with greater reductions in replication fitness (*53*, *54*).

In our previous study, the Y181C mutant, which is one of the two most common NNRTI-resistant variants that emerge during therapy with NNRTIs, demonstrated an increase in the ratio of secondary to primary RNase H cleavage products during both RNA 5′-end- and DNA 3′-end-directed modes of cleavage (*54*). In this study, we further explore the effects of the Y181C mutant on RNA 5′-end-directed RNase H cleavage and demonstrate that this mutant cleaves prefer-

entially toward the RNA 5′ end of the RNA·DNA hybrid. This cleavage preference results in smaller RNase H cleavage products, which should be more likely to dissociate from plus-strand DNA and may lead to increased rates of minus strong stop DNA transfer and recombination.

EXPERIMENTAL PROCEDURES

Reagents. The expression vector pRSET was obtained from Invitrogen. *Msc*I, *Spe*I, and *Age*I were obtained from New England Biolabs. Metal affinity columns (Talon) were obtained from Clontech. The *O*-methyl-substituted RNA oligonucleotide was purchased from Dharmacon Research, Inc.; all other oligonucleotides were purchased from Oligos Etc.

HIV-1 RT Expression and Purification. HIV-1 RTs with and without the Y181C mutation were expressed as separate p66 and p51 subunits in *Escherichia coli*, using pRSET expression vectors, as previously described (*53*, *54*). HIV-1 RT p66 and p51 subunits were purified to greater than 95% homogeneity, using metal affinity and ion-exchange chromatography, as previously described (*53*, *54*).

Measurement of Specific Activities of DNA Polymerization. Protein concentrations of RT preparations were determined in triplicate, using the Bradford assay, and averaged (*55*). DNA polymerization activity of each RT preparation was determined in at least four independent experiments, by measuring incorporation of ^{32}P-dTTP into a poly(rA)−oligo(dT) template-primer. The specific activities of the wild-type and Y181C RTs used in these experiments were 5830 and 2810 units/mg of RT, respectively, where a unit is defined as the amount of dTTP (in nanomoles) incorporated in 10 min. Relative activities of polymerization by the different mutants were used to normalize the amounts of wild-type and mutant RTs used in assays of RNase H activity.

Preparation of Oligonucleotide Substrates. The substrates used in the RNase H assays are diagramed in Figures 2−8. A 41 nucleotide (NT) RNA was labeled either at its 5′ end as previously described, using T4 polynucleotide kinase (*53*), or at its 3′ end, using Sequenase. For 3′-end labeling of RNA, the RNA was annealed to a 77 NT long DNA oligonucleotide MB-8 (5′-TCG ATC GCT GCA GGT CGA CTC TAG AGG ATC CCC GGG TAC CGA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AA-3′, in which the underlined base is annealed to the 3′-most end of the RNA). 3′-End-labeling reactions contained 100 pmol each of the 41 NT RNA and MB-8, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 25 mM NaCl, 13 units of Sequenase, and 75 μCi of α-^{32}P-dATP in a final volume of 30 μL. Extension products were purified using 2-propanol precipitation followed by separation and elution from a denaturing 20% polyacrylamide gel. An internally labeled 36 NT long oligonucleotide with the same sequence as the 5′ end of the 41 NT substrate was created by 5′-end labeling a 22 NT RNA oligonucleotide with the same sequence as the 15−36th NT of the 41 NT long substrate, and annealing it, along with a synthetic 14-mer RNA corresponding to the first 14 NT of the 41 NT RNA, to the 77 NT long MEW-3 DNA (*53*). T4 DNA ligase was then used to ligate the two RNA oligonucleotides. The resultant internally labeled RNA 36 NT substrate was then denatured, gel purified, quantitated, and annealed to MEW-3.

RNase H Assays. To measure DNA 3′-end-directed RNase H cleavage, the labeled RNA described above was annealed to the 26 NT long DNA oligonucleotide PG003 (*53*) such that the 3′ end of the DNA was recessed. To measure RNA 5′-end-directed RNase H cleavage, the same RNA was annealed to the 77 NT long DNA MEW-3 (*53*) such that the 5′ end of the RNA was recessed. Annealing the RNA to the oligonucleotide 50pbs-41bl (5′-GCC TGC AGG TCG ACT CTA GAG GAT CCC CGG GTA CCG AGC TCG AAT TCG CCC-3′) resulted in an RNA·DNA hybrid in which the 5′ RNA and 3′ DNA ends were flush; the presence of a blunt end results in significant inhibition of the secondary RNase H cleavage event (*56*). RNase H assays were carried out as previously described for times ranging from 15 s to 16 min (*53*), using a 2−4-fold molar excess of RT relative to substrate. Cleavage products were resolved using denaturing 10% polyacrylamide gels and quantitated using PhosphorImaging (Molecular Devices).

RESULTS

RNase H Cleavage by Y181C RT Leads to an Increased Ratio of Secondary to Primary Cleavage Products. We have previously reported that RNase H cleavage by the Y181C mutant results in an increased ratio of secondary to primary RNase cleavage products during both DNA 3′-end- and RNA 5′-end-directed cleavage (*54*). The substrate used to measure DNA 3′-end-directed cleavage is a 5′-end-labeled 41 NT long RNA annealed to a shorter complementary DNA, such that the DNA 3′ end is recessed (Figure 2A). During DNA 3′-end-directed cleavage, wild-type RT positions with its polymerase domain at the 3′ end of the DNA and produces an initial cut 18 NT from the 3′ end of the DNA (resulting in an approximately 26 NT long labeled product), followed by a subsequent accumulation of a 17 NT long labeled product resulting from a secondary cleavage approximately 9 NT from the 3′ end of the DNA (Figure 2A).

Previous studies have demonstrated that the initial primary RNase H cleavage, whose position is determined by the distance between the polymerase active site (positioned at the end of the nucleic acid strand directing cleavage) and the RNase H active site, is followed by a subsequent shifting of the RT by a distance of approximately 9 NT toward the 5′ end of the RNA (*25*, *28*, *57*, *58*). The primary product is then converted into the shorter secondary product. The time course of accumulation of these two products suggests either that the secondary cleavage can only occur after the primary or that it occurs at a significantly slower rate than the primary cleavage. The factors that influence the location of the secondary RNase H cleavage are not well understood. Y181C RT produces the same size primary and secondary cleavage products as wild-type RT but produces an increased ratio of secondary to primary cleavage products (Figure 2A).

The substrate used to measure RNA 5′-end-directed cleavage is a 5′-end-labeled 41 NT long RNA annealed to a longer complementary DNA such that the RNA 5′ end is recessed (Figure 2B). During RNA 5′-end-directed cleavage, we have postulated that the wild-type RT positions with its polymerase domain at the 5′ end of the RNA and produces an initial cut 18 NT from the 5′ end of the RNA (resulting in a 18 NT long labeled product), followed by a subsequent accumulation of an approximately 9 NT long labeled

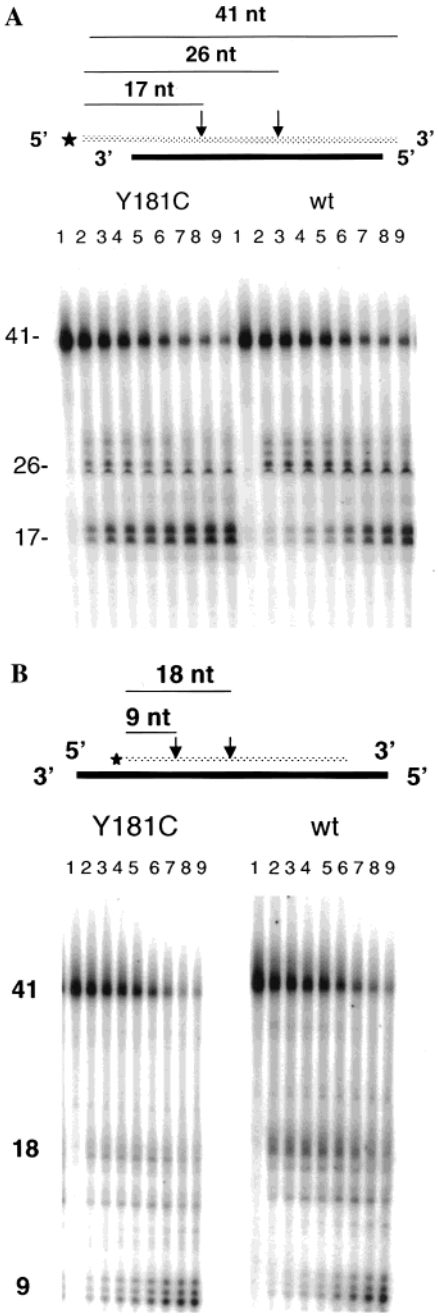

FIGURE 2: RNase H cleavage of RNA·DNA hybrids by wild-type and Y181C RTs. Panel A: Polymerase-dependent or DNA 3′-end-directed RNase H cleavage. The substrate, as diagramed here, is a 41 NT long 5′-end-labeled heteropolymeric RNA (stippled line), hybridized to a DNA (black line), such that the DNA 3′ end is recessed. RT was allowed to prebind to the substrate before initiation of the reaction with magnesium. Degradation of the starting material into primary (26 NT long) and secondary (17 NT long) cleavage products can be seen to accumulate over time. Lanes: 1, 0 min; 2, 15 s; 3, 30 s; 4, 45 s; 5, 1 min; 6, 2 min; 7, 4 min; 8, 8 min; 9, 16 min. Panel B: Polymerase-independent or RNA 5′-end-directed RNase H cleavage. The substrate is a 41 NT long 5′-end-labeled heteropolymeric RNA (stippled line), hybridized to a DNA (black line), such that the RNA 5′ end is recessed. Reaction conditions and time points were otherwise the same as in panel A. Accumulation of the approximately 18 NT primary products and the 9 NT secondary products can be seen over time.

secondary product resulting from a second cleavage approximately 9 NT from the 5′ end of the RNA (Figure 2B). With a substrate in which the 5′ end of the RNA is recessed, incubation with wild-type RT results in an initial accumulation of the expected 18 NT primary product, followed by subsequent accumulation of a 9 NT secondary product (Figure 2B). Similar to what was seen during DNA 3′-end-directed cleavage, Y181C RT produces a greater ratio of secondary to primary cleavage products during RNA 5′-end-directed RNase H cleavage compared to wild-type RT (compare the relative ratios of the 18 and 9 NT products for Y181C and wild-type RT in Figure 2B).

We next quantitated the rates at which the substrate was degraded, and the rates at which the different RNase H cleavage products were formed during RNA 5′-end-directed RNase H cleavage, using PhosphorImaging. When the amount of substrate degradation during RNA 5′-end-directed RNase H cleavage was plotted over time, there were no significant differences in the overall rate of RNase H degradation between wild-type and Y181C RTs (Figure 3A). However, when looking at the accumulation of specific cleavage products, there was a decrease in the extent to which Y181C produced primary cleavage products (Figure 3B) and an increase in the extent to which it produced secondary cleavage products (Figure 3C). The initial rate at which the secondary product accumulated was estimated by measuring the linear slope of the curve representing accumulation of the secondary cleavage product during the first minute of the reaction (Figure 3D). The rate of accumulation of secondary product by Y181C RT was 80% more rapid than wild-type RT for the RNA 5′-end-directed mode of cleavage.

The reduced accumulation of the primary cleavage product with Y181C RT could be due solely to a more rapid secondary cleavage event (which would degrade the primary product more rapidly) or could reflect an additional defect resulting in a decrease in the rate of the primary cleavage event. Although there was a slight slowing of the initial rate of accumulation of primary product with Y181C relative to wild-type RT (Figure 3B), we were not able to precisely measure the rate of primary product formation, since it is being degraded to the secondary product as it is being produced. We thus cannot rule out a direct effect of Y181C on the rate of primary cleavage.

The Formation of Secondary RNase H Cleavage Products by Y181C RT Can Occur Independently of Primary Cleavage. We asked whether secondary RNase H cleavage by the Y181C RT could occur independently of primary cleavage. To study this question, we utilized the same RNA·DNA hybrid substrate but with the radiolabel on the 3′ end of the RNA (Figure 4). A primary cleavage 18 NT from the RNA 5′ end will result in a 24 NT long product. Because the primary cleavage occurs between the secondary cleavage site and the radiolabel, the 33 NT long product of secondary cleavage will only be observed if it precedes the primary cleavage. We did not observe the 33 NT long product with wild-type RT but did detect this product using Y181C RT (Figure 4). The interpretation of these findings depends on whether wild-type RT requires that the primary cleavage occur before it can catalyze the secondary cleavage. If this is the case, then the Y181C mutation could inhibit or alter the process by which the two cleavages are coupled. If the earlier accumulation of the primary product with wild-type RT merely reflects the faster rate of the primary relative to the secondary cleavage, the effect of Y181C may instead be to sufficiently increase the rate of the secondary cleavage

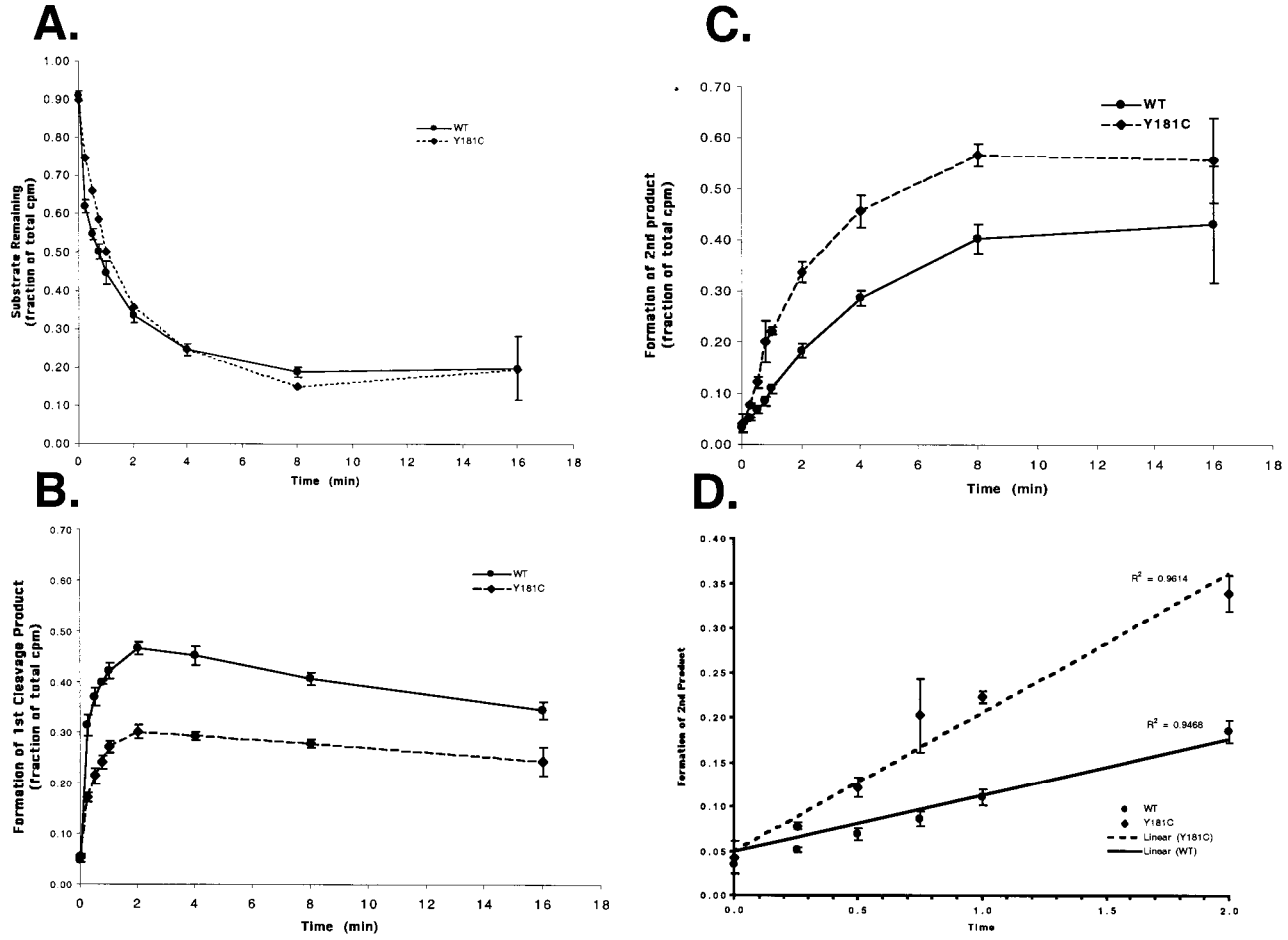

FIGURE 3: Kinetics of substrate loss (panel A), primary product formation (panel B), and secondary product formation (panel C) during RNA 5′-end-directed RNase H cleavage by wild-type and Y181C RTs. *Y*-axes represent the amount of each band that remains, expressed as a fraction of total counts in each lane. Wild-type RT, solid line; Y181C RT, dashed line. Panel D: Linear regression fit of the initial accumulation of secondary RNase H cleavage product by wild-type (solid line) and Y181C (dashed line) RTs. The rate of accumulation of secondary product with Y181C RT is 80% greater than with wild-type RT.

relative to the primary cleavage, leading to an increased probability of the secondary cleavage preceding the primary cleavage.

Inhibition of Primary Cleavage Does Not Alter the Ability of Y181C To Make Secondary Cleavages More Rapidly Than Wild-Type RT. We studied the effect of inhibiting the primary RNase H cleavage, using an RNA substrate in which nucleotides 12 through 22 (counting from the 5′ end of the RNA) were replaced with *O*-methyl-substituted ribonucleotides. As expected, study of the 3′-end-labeled *O*-methyl-substituted RNA hybridized to a DNA such that the RNA 5′ end was recessed showed complete inhibition of the primary RNase H cleavages by both wild-type and Y181C RTs (Figure 5). Interestingly, despite this inhibition of the primary RNase H cleavage, the Y181C RT still showed a strong preference, relative to wild-type RT, for making an isolated secondary cut, represented by the 33−35 NT long bands. Thus, the effect of Y181C on the order of primary and secondary cleavages cannot be explained solely by a reduction in the rate of primary RNase H cleavage, since inhibition of the ability of wild-type RT to make the primary cleavage does not result in similar cleavage patterns for the wild-type and mutant enzymes.

Primary RNase H Cleavages Can Occur before or after Secondary Cleavages with the Y181C RT. We wanted to determine whether the Y181C mutant RT always performed the secondary cleavage before the primary. To study this, we created an RNA·DNA hybrid in which a specific internal label was placed in the RNA at a position between the primary and secondary cleavages (Figure 6). The RNA included the first 36 NT of the 41 NT RNA studied in the previous experiments. Using the substrate with an internal label placed 14 NT from the 5′ end of the RNA resulted in cleavage products representing isolated primary (18 NT) or secondary (27 NT) cleavages (Figure 6). The 27 NT product representing the secondary cleavage alone was only seen with the Y181C mutant but appeared to represent a minority of the cleavage products produced by Y181C RT. Cleavage products representing the primary cleavage alone were also seen with the mutant enzyme, although their relative prevalence was lower than with the wild-type enzyme (Figure 6). The mutant enzyme also demonstrated an increase relative to wild type in products between 7 and 14 NT long that were most prominent at later times (between 2 and 16 min; see lanes 5−8 in Figure 6). The selective formation of these intermediate size products by the Y181C mutant may represent an additional type of initial primary cut that occurs after the first secondary cleavage but whose position is still determined by the original 5′ end of the RNA despite the presence of a nick at the site of the first secondary cleavage.

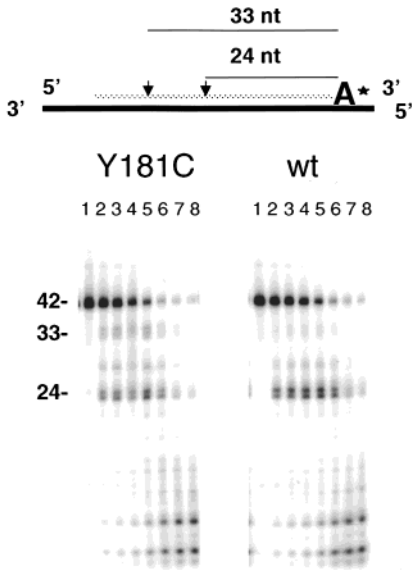

FIGURE 4: RNA 5′-end-directed cleavage of a 3′-end-labeled RNA·DNA hybrid by wild-type and Y181C RTs. Substrate, diagramed at the top of the figure, consists of a 41 NT RNA (stippled line) to which a ^{32}P-labeled dATP was incorporated at its 3′ end (represented by the letter A), hybridized to a DNA (solid line), such that the RNA 5′ end is recessed. Primary RNase H cleavage will yield an approximately 24 NT long product, whereas an isolated secondary cleavage will yield an approximately 33 NT long product. Products resulting from a secondary cleavage following a primary cleavage will not be visualized. Results from a representative time course of RNA 5′-end-directed cleavage for Y181C and wild-type RTs are shown below. Lanes: 1, 0 min; 2, 15 s; 3, 30 s; 4, 1 min; 5, 2 min; 6, 4 min; 7, 8 min; 8, 16 min. The appearance of a 33 NT long band, which represents isolated secondary cleavages, is seen with the Y181C mutant, whereas little if any of this product accumulates with wild-type enzyme. The approximately 9 NT long products are thought to result from the second primary cleavage event and accumulate to a similar extent with each enzyme.

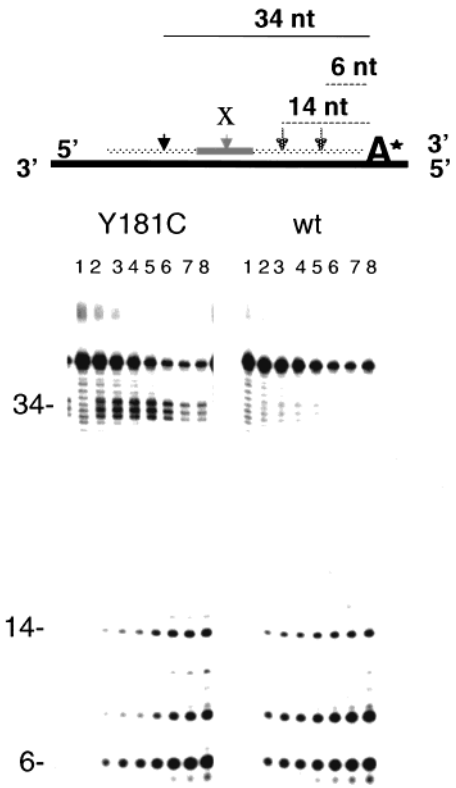

FIGURE 5: Effect of inhibition of the primary cleavage event on RNase H cleavage by wild-type and Y181C RTs. The substrate used was a 41 NT long synthetic RNA (stippled line) containing 2-*O*-methyl substitutions at nucleotide positions 12−22 (solid gray line), which was labeled at its 3′ end as described in Figure 4, and hybridized to a DNA (black line), such that the 5′ end of the RNA was recessed. Results from a representative time course are shown below. Lanes: 1, 0 min; 2, 15 s; 3, 30 s; 4, 1 min; 5, 2 min; 6, 4 min; 7, 8 min; 8, 16 min. The effective inhibition of primary RNase H cleavages can be seen by the virtual absence of 16−30 NT long cleavage products. Under conditions in which the primary cleavages are inhibited, wild-type RT is still less likely than Y181C to produce the 33−35 NT long products, which represent independent secondary cleavages.

Inhibition of Secondary Cleavage Results in a Smaller Size Distribution of Cleavage Products by Y181C RT Compared with Wild-Type RT. We postulated that the increased accumulation of secondary RNase H cleavage products that occurred with Y181C RT could result from an increased preference of the mutant enzyme to slide toward the 5′ end of the RNA after making the initial primary cleavage. We wanted to determine what impact removing the single-stranded 3′ DNA tail, a structural change that inhibits secondary cleavages by wild-type RT, would have on the secondary cleavage by Y181C RT. We therefore studied the size distribution of cleavage products resulting from wild-type and Y181C RTs cleaving a blunt-ended RNA·DNA hybrid (Figure 7). This substrate inhibits the accumulation of the 9 NT secondary cleavage product, presumably because the absence of a single-stranded DNA tail limits the enzyme's ability to bind with its RNase H active site 9 NT from the 5′ end of the RNA. We found that, when using this RNA 5′-end-labeled substrate, the formation of the 9 NT product by both wild-type and Y181C RTs was severely inhibited (Figure 7). However, the size distribution of primary cleavages was smaller for the Y181C compared to wild-type RT. The wild-type and Y181C RTs still made a primary cut 18 NT from the RNA 5′ end, but the Y181C made an additional cut 14 NT from the 5′ end of the RNA to a much greater extent than wild-type RT (Figure 7).

Y181C Increases the Preference of RT for Cleaving RNA·DNA Hybrids Closer to the RNA 5′ End. It appeared that the preference for the Y181C RT to make smaller RNase H cleavages reflected an increased ability to position toward the 5′ end of the RNA. We then asked whether the Y181C RT only showed a preference for positioning toward the 5′ end of the RNA or whether the mutant enzyme could also reposition away from the point of primary cleavage toward the 3′ end of the RNA. We compared the size distribution of RNase H cleavage products with the wild-type and Y181C RTs, using the same blunt-end RNA·DNA hybrid, now labeled at the 3′ end of the RNA (Figure 8). Primary RNase H cleavage of this substrate results in a 24 NT product. Any increased tendency of Y181C to cleave toward the 3′ end of the RNA would result in an increase in cleavage products smaller than 24 NT. Using this substrate, we found no significant difference in the prevalence of smaller size products between wild-type and Y181C RTs (Figure 8), suggesting that Y181C preferentially cleaves toward the RNA 5′ end of an RNA·DNA hybrid.

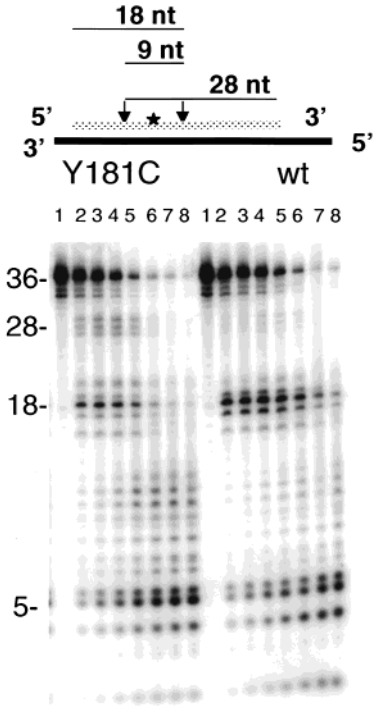

FIGURE 6: Cleavage of an internally labeled RNA 5′-end-recessed RNA·DNA hybrid by wild-type and Y181C RTs. The substrate, diagramed at the top of the figure, was a 36 NT long RNA (stippled line) that was internally labeled at nucleotide 14 (position noted with a star) as outlined in Experimental Procedures. Results from a representative time course are depicted in the bottom half of the figure. Lanes: 1, 0 min; 2, 15 s; 3, 30 s; 4, 1 min; 5, 2 min; 6, 4 min; 7, 8 min; 8, 16 min. Y181C RT results in less accumulation of 18 NT primary products and more 27−29 NT long products (which represent isolated secondary cleavages) than wild-type RT. The origin of the 7−14 NT long products, which are more prevalent with the Y181C RT, is not known. These products could represent an attempt of the Y181C RT to create a primary cut after the first secondary by measuring from the original 5′ end of the nicked RNA.

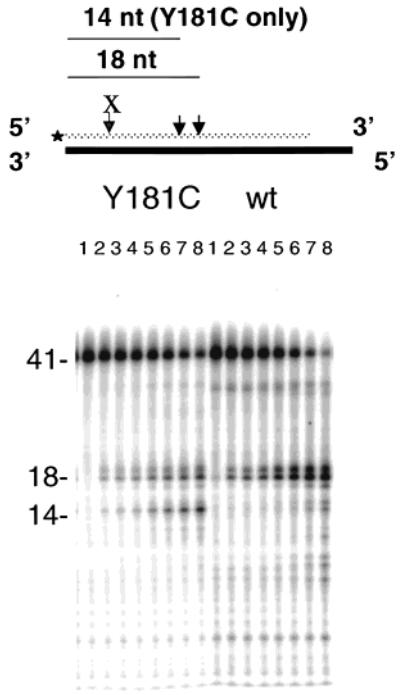

FIGURE 7: Cleavage of a flush 5′-end-labeled RNA·DNA hybrid by wild-type and Y181C RTs. The substrate, diagramed at the top of the figure, was a 41 NT long RNA (stippled line) that was labeled at its 5′ end and hybridized to a complementary DNA such that the 5′ end of the RNA and the 3′ end of the DNA were flush with each other. This substrate inhibits secondary RNase H cleavages and would be expected to yield approximately 18 NT long products. Results from a representative time course are shown below. Lanes: 1, 0 min; 2, 15 s; 3, 30 s; 4, 1 min; 5, 2 min; 6, 4 min; 7, 8 min; 8, 16 min. The preference for Y181C RT to cleave closer to the 5′ end of the RNA is reflected in the accumulation of a smaller 14 NT product which is not seen with wild-type RT.

DISCUSSION

Our studies have demonstrated that the presence of the Y181C mutation in HIV-1 RT increases the prevalence of secondary RNase H cleavage products. Determination of relative activities of RNA-dependent DNA polymerization of the mutant and wild-type enzymes were carefully calculated, using several replicate determinants. Thus, it is unlikely that this difference in RNase H activity between the Y181C and wild-type RTs is due to differences in relative input of mutant and wild-type enzymes. In addition, the finding of an alteration in size preference of RNase H cleavage products suggests an intrinsic alteration in the mechanism of RNase H cleavage rather than an overall alteration in the ratio of RNase H to polymerase activities. Interestingly, another group has demonstrated that the Y232A mutant, which is located in the primer grip region near the NNRTI binding pocket, also demonstrates a qualitatively similar, although much stronger, preference for performing the secondary RNase H cleavage (*59*).

We found that Y181C RT produced a higher proportion of secondary cleavage products independent of the mode of RNase H cleavage, during both DNA 3′-end-directed as well as RNA 5′-end-directed RNase H cleavage. This finding suggests that the event(s) in RNase H cleavage altered by the Y181C mutant is (are) common to both modes of RNase H cleavage. Because the Y181C RT alters the size distribution of products resulting from both modes of RNase H cleavage, it may have wide-ranging effects on reverse transcription. Studies of other mutants that affect the relative ratio of secondary and primary cleavage products have provided support for the hypothesis that the secondary cleavage event is required for efficient strand transfer (*29*, *59*), although potential effects on other steps in reverse transcription have not been fully examined.

Our studies have focused on examining the underlying biochemical mechanism for the Y181C RT's preference to make secondary cleavages. We have demonstrated that the usual order of primary followed by secondary RNase H cleavages is disrupted by the Y181C mutation. When assaying a 3′-end-labeled RNA·DNA hybrid, we observed a long product corresponding to an isolated secondary cleavage that was unique to the Y181C mutant. Inhibition of primary cleavage using an RNA·DNA hybrid in which the positions of primary RNase H cleavage were substituted with *O*-methyl nucleotide derivatives showed that wild-type RT did not produce more rapid secondary cleavages, suggesting that the effect of Y181C on the order of RNase H cleavages is not due solely to an inhibition of primary RNase H cleavage. When using a substrate in which the radiolabel was placed between the position of the primary and secondary cuts, we found that, although the secondary

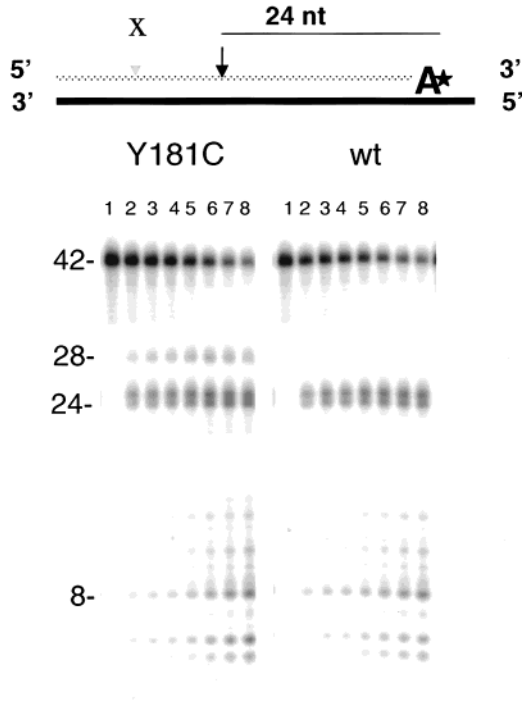

FIGURE 8: Cleavage of a flush 3′-end-labeled RNA·DNA hybrid by wild-type and Y181C RTs. The substrate, diagramed at the top of the figure, was a 41 NT long RNA (stippled line) that was labeled at its 3′ end and hybridized to a complementary DNA such that the 5′ end of the RNA and the 3′ end of the DNA were flush with each other. The expected product, which would result from a primary RNase H cleavage, is approximately 24 NT long. If the apparent tendency of Y181C RT to cleave toward the 5′ end of the RNA is merely due to a more distributive pattern of cleavages by the Y181C mutant, one would expect to see a smaller size distribution of products with this 3′-end-labeled substrate, compared to wild-type RT. Results from a representative time course for Y181C and wild-type RT are depicted below. Lanes: 1, 0 min; 2, 15 s; 3, 30 s; 4, 1 min; 5, 2 min; 6, 4 min; 7, 8 min; 8, 16 min. Y181C RT produces a larger 28 NT long product, which results from cleavages closer to the RNA 5′ end than wild-type RT, but Y181C shows no increase in the production of smaller cleavage products, suggesting that the increased cleavage preference of this mutant is directional.

cut could precede the primary cut with the Y181C enzyme, there still were a substantial proportion of events in which the primary cut preceded the secondary. A recently published study has demonstrated that, under certain circumstances, the primary and secondary cleavages by wild-type HIV-1 RT can also be uncoupled (*28*). Taken together, these findings support the hypothesis that cleavage at the primary position is not normally required for the secondary cut to occur. Thus, the apparent strict ordering of primary followed by secondary cleavages during RNase H activity with wild-type HIV-1 RT is likely due to the fact that the rate of primary cleavage is normally much faster than that of the secondary cleavage. Thus, the most plausible explanation for our findings is that the Y181C mutation increases the rate of secondary cleavages enough that the uncoupling of primary and secondary cleavages becomes more apparent, resulting in the secondary cleavage preceding the primary a certain fraction of the time.

Of interest is that the preference of the Y181C RT to produce smaller RNase H cleavage products persists even when the formation of secondary RNase H products is inhibited. Thus, when assaying a blunt-end RNA·DNA hybrid in which the RT is physically unable to make the 9 NT secondary cut, there is still a strong preference of the Y181C RT to make a 14 NT cut in addition to an 18 NT cut, whereas the wild-type RT only makes an 18 NT cut. We postulate that it is this tendency for the Y181C RT to position toward the 5′ end of the RNA that leads to a preference to produce secondary over primary RNase H cleavage products. This hypothesis is further supported by the finding that products resulting from cleavages in the direction of the 3′ end of the RNA are not increased with the Y181C RT. Thus, this mutant enzyme appears to have a strong directional preference to position and cleave toward the 5′ end of the RNA.

The effect of Y181C RT's RNase H abnormality on viral replication cannot be predicted with certainty. The fact that Y181C RT also demonstrates a reduced affinity for nucleotide substrate (*54*) complicates any attempt to correlate an increase in the ratio of secondary to primary cleavage products with the replication fitness of Y181C. Our previous studies have demonstrated that, although Y181C HIV-1 replicates less well than wild-type virus, it clearly has an improved replication fitness relative to other drug-resistant mutants, such as V106A, which are associated with significant reductions in the ratio of RNase H to polymerase activities (*54*). It seems quite likely that a reduced affinity for nucleotides would reduce the replication fitness of HIV-1 with the Y181C mutation. If an increase in the rate of secondary RNase H cleavages by Y181C increased the efficiency of reverse transcription (for example, by increasing rates of minus strong stop DNA transfer), it may partially overcome the negative impact of the mutant's reduced affinity for its nucleotide substrate. If an increase in the rate of secondary RNase H cleavages reduces the efficiency of reverse transcription, the magnitude of the effect is likely modest, since Y181C replicates more efficiently than other drug-resistant mutants and can readily emerge during natural infection. Studies to address the effects of the Y181C mutation on specific steps in reverse transcription are in progress in our laboratories.

In summary, we have demonstrated that a common, clinically occurring, drug-resistant mutant of HIV-1 alters the ratio of secondary to primary RNase H cleavage products. This finding is likely due to an increase in the rate of secondary RNase H cleavages that results from a tendency of the mutant enzyme to cleave toward the RNA 5′ end of an RNA·DNA hybrid. Potential consequences of this alteration in cleavage preference could include increases in rates of strand transfer and recombination. These findings have important implications for HIV pathogenesis in humans infected with this drug-resistant strain and may lead to the development of strategies to selectively inhibit this mutant.

ACKNOWLEDGMENT

We thank Mini Balakrishnan and Carrie Dykes for many helpful discussions.

REFERENCES

1. Gilboa, E., Mitra, S. W., Goff, S., and Baltimore, D. (1979) *Cell 18*, 93−100.
2. di Marzo Veronese, F., Copeland, T. D., DeVico, A. L., Rahman, R., Oroszlan, S., Gallo, R. C., and Sarngadharan, M. G. (1986) *Science 231*, 1289−1291.

3. Lightfoote, M. M., Coligan, J. E., Folks, T. M., Fauci, A. S., Martin, M. A., and Venkatesan, S. (1986) *J. Virol. 60*, 771−775.
4. Le Grice, S. F., Naas, T., Wohlgensinger, B., and Schatz, O. (1991) *EMBO J. 10*, 3905−3911.
5. Hostomsky, Z., Hostomska, Z., Fu, T. B., and Taylor, J. (1992) *J. Virol. 66*, 3179−3182.
6. Johnson, M. S., McClure, M. A., Feng, D. F., Gray, J., and Doolittle, R. F. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7648−7652.
7. Hostomsky, Z., Hostomska, Z., Hudson, G. O., Moomaw, E. W., and Nodes, B. R. (1991) *Proc. Natl. Acad. Sci. U.S.A 88*, 1148−1152.
8. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992) *Science 256*, 1783−1790.
9. Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., et al. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6320−9324.
10. Arts, E. J., Ghosh, M., Jacques, P. S., Ehresmann, B., and Le Grice, S. F. (1996) *J. Biol. Chem. 271*, 9054−9061.
11. Jacques, P. S., Wohrl, B. M., Howard, K. J., and Le Grice, S. F. (1994) *J. Biol. Chem. 269*, 1388−1393.
12. Champoux, J. J. (1993) in *Reverse Transcriptase, Cold Spring Harbor monograph series; 23* (Skalka, A. M., and Goff, S. P., Eds.) p 492, Cold Spring Harbor Laboratory Press, Plainview, NY.
13. Ghosh, M., Howard, K. J., Cameron, C. E., Benkovic, S. J., Hughes, S. H., and Le Grice, S. F. (1995) *J. Biol. Chem. 270*, 7068−7076.
14. Tanese, N., Telesnitsky, A., and Goff, S. P. (1991) *J. Virol. 65*, 4387−4397.
15. Wohrl, B. M., and Moelling, K. (1990) *Biochemistry 29*, 10141−10147.
16. Powell, M. D., Beard, W. A., Bebenek, K., Howard, K. J., Le Grice, S. F., Darden, T. A., Kunkel, T. A., Wilson, S. H., and Levin, J. G. (1999) *J. Biol. Chem. 274*, 19885−19893.
17. Schultz, S. J., Zhang, M., Kelleher, C. D., and Champoux, J. J. (1999) *J. Biol. Chem. 274*, 34547−34555.
18. Huber, H. E., and Richardson, C. C. (1990) *J. Biol. Chem. 265*, 10565−10573.
19. Furfine, E. S., and Reardon, J. E. (1991) *J. Biol. Chem. 266*, 406−412.
20. Smith, J. S., and Roth, M. J. (1992) *J. Biol. Chem. 267*, 15071−15079.
21. Pullen, K. A., Ishimoto, L. K., and Champoux, J. J. (1992) *J. Virol. 66*, 367−373.
22. Smith, J. S., and Roth, M. J. (1993) *J. Virol. 67*, 4037−4049.
23. Peliska, J. A., and Benkovic, S. J. (1992) *Science 258*, 1112−1118.
24. Gopalakrishnan, V., Peliska, J. A., and Benkovic, S. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10763−10767.
25. DeStefano, J. J., Mallaber, L. M., Fay, P. J., and Bambara, R. A. (1993) *Nucleic Acids Res. 21*, 4330−4338.
26. Palaniappan, C., Fuentes, G. M., Rodriguez-Rodriguez, L., Fay, P. J., and Bambara, R. A. (1996) *J. Biol. Chem. 271*, 2063−2070.
27. Post, K., Guo, J., Kalman, E., Uchida, T., Crouch, R. J., and Levin, J. G. (1993) *Biochemistry 32*, 5508−5517.
28. Wisniewski, M., Balakrishnan, M., Palaniappan, C., Fay, P. J., and Bambara, R. A. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 11978−11983.
29. Cirino, N. M., Cameron, C. E., Smith, J. S., Rausch, J. W., Roth, M. J., Benkovic, S. J., and Le Grice, S. F. (1995) *Biochemistry 34*, 9936−9943.
30. DeStefano, J. J., Mallaber, L. M., Rodriguez-Rodriguez, L., Fay, P. J., and Bambara, R. A. (1992) *J. Virol. 66*, 6370−6378.
31. Carpenter, C. C., Cooper, D. A., Fischl, M. A., Gatell, J. M., Gazzard, B. G., Hammer, S. M., Hirsch, M. S., Jacobsen, D. M., Katzenstein, D. A., Montaner, J. S., Richman, D. D., Saag, M. S., Schechter, M., Schooley, R. T., Thompson, M. A., Vella, S., Yeni, P. G., and Volberding, P. A. (2000) *J. Am. Med. Assoc. 283*, 381−390.
32. De Clercq, E. (1998) *Antiviral Res. 38*, 153−179.
33. Smerdon, S. J., Jager, J., Wang, J., Kohlstaedt, L. A., Chirino, A. J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3911−3915.
34. Esnouf, R., Ren, J., Ross, C., Jones, Y., Stammers, D., and Stuart, D. (1995) *Nat. Struct. Biol. 2*, 303−308.
35. Esnouf, R. M., Ren, J., Hopkins, A. L., Ross, C. K., Jones, E. Y., Stammers, D. K., and Stuart, D. I. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3984−3989.
36. Spence, R. A., Kati, W. M., Anderson, K. S., and Johnson, K. A. (1995) *Science 267*, 988−993.
37. Staszewski, S., Morales-Ramirez, J., Tashima, K. T., Rachlis, A., Skiest, D., Stanford, J., Stryker, R., Johnson, P., Labriola, D. F., Farina, D., Manion, D. J., and Ruiz, N. M. (1999) *N. Engl. J. Med. 341*, 1865−1873.
38. Hirsch, M. S., Brun-Vezinet, F., D'Aquila, R. T., Hammer, S. M., Johnson, V. A., Kuritzkes, D. R., Loveday, C., Mellors, J. W., Clotet, B., Conway, B., Demeter, L. M., Vella, S., Jacobsen, D. M., and Richman, D. D. (2000) *J. Am. Med. Assoc. 283*, 2417−2426.
39. Richman, D. D., Havlir, D., Corbeil, J., Looney, D., Ignacio, C., Spector, S. A., Sullivan, J., Cheeseman, S., Barringer, K., Pauletti, D., et al. (1994) *J. Virol. 68*, 1660−1666.
40. Bacheler, L. T., Anton, E. D., Kudish, P., Baker, D., Bunville, J., Krakowski, K., Bolling, L., Aujay, M., Wang, X. V., Ellis, D., Becker, M. F., Lasut, A. L., George, H. J., Spalding, D. R., Hollis, G., and Abremski, K. (2000) *Antimicrob. Agents Chemother. 44*, 2475−2484.
41. Demeter, L. M., Shafer, R. W., Meehan, P. M., Holden-Wiltse, J., Fischl, M. A., Freimuth, W. W., Para, M. F., and Reichman, R. C. (2000) *Antimicrob. Agents Chemother. 44*, 794−797.
42. Coffin, J. M. (1995) *Science 267*, 483−489.
43. Back, N. K., Nijhuis, M., Keulen, W., Boucher, C. A., Oude Essink, B. O., van Kuilenburg, A. B., van Gennip, A. H., and Berkhout, B. (1996) *EMBO J. 15*, 4040−4049.
44. Back, N. K., and Berkhout, B. (1997) *Antimicrob. Agents Chemother. 41*, 2484−2491.
45. Wainberg, M. A., Drosopoulos, W. C., Salomon, H., Hsu, M., Borkow, G., Parniak, M., Gu, Z., Song, Q., Manne, J., Islam, S., Castriota, G., and Prasad, V. R. (1996) *Science 271*, 1282−1285.
46. Drosopoulos, W. C., and Prasad, V. R. (1996) *J. Virol. 70*, 4834−4838.
47. Hsu, M., Inouye, P., Rezende, L., Richard, N., Li, Z., Prasad, V. R., and Wainberg, M. A. (1997) *Nucleic Acids Res. 25*, 4532−4536.
48. Rezende, L. F., Curr, K., Ueno, T., Mitsuya, H., and Prasad, V. R. (1998) *J. Virol. 72*, 2890−2895.
49. Sharma, P. L., and Crumpacker, C. S. (1999) *J. Virol. 73*, 8448−8456.
50. Caliendo, A. M., Savara, A., An, D., DeVore, K., Kaplan, J. C., and D'Aquila, R. T. (1996) *J. Virol. 70*, 2146−2153.
51. Meyer, P. R., Matsuura, S. E., Mian, A. M., So, A. G., and Scott, W. A. (1999) *Mol. Cell 4*, 35−43.
52. Arion, D., Kaushik, N., McCormick, S., Borkow, G., and Parniak, M. A. (1998) *Biochemistry 37*, 15908−15917.
53. Gerondelis, P., Archer, R. H., Palaniappan, C., Reichman, R. C., Fay, P. J., Bambara, R. A., and Demeter, L. M. (1999) *J. Virol. 73*, 5803−5813.
54. Archer, R. H., Gerondelis, P., Dykes, C., Fox, K., Fay, P., Reichman, R. C., Bambara, R. A., and Demeter, L. M. (2000) *J. Virol. 74*, 8390−8401.
55. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
56. Fu, T. B., and Taylor, J. (1992) *J. Virol. 66*, 4271−4278.
57. Schatz, O., Mous, J., and Le Grice, S. F. (1990) *EMBO J. 9*, 1171−1176.
58. DeStefano, J. J., Buiser, R. G., Mallaber, L. M., Bambara, R. A., and Fay, P. J. (1991) *J. Biol. Chem. 266*, 24295−24301.
59. Ghosh, M., Jacques, P. S., Rodgers, D. W., Ottman, M., Darlix, J. L., and Le Grice, S. F. (1996) *Biochemistry 35*, 8553−8562.

BI002328A