JOURNAL OF VIROLOGY, Apr. 1996, p. 2146–2153 Vol. 70, No. 4
0022-538X/96/$04.00+0

Effects of Zidovudine-Selected Human Immunodeficiency Virus Type 1 Reverse Transcriptase Amino Acid Substitutions on Processive DNA Synthesis and Viral Replication

ANGELA M. CALIENDO, ANU SAVARA, DONNA AN, KIMBERLY DEVORE, JOAN C. KAPLAN, AND RICHARD T. D'AQUILA*

Infectious Disease Unit, Massachusetts General Hospital, and Harvard Medical School, Boston, Massachusetts

Received 6 October 1995/Accepted 19 December 1995

Certain amino acid substitutions in the reverse transcriptase (RT), including D67N, K70R, T215Y, and K219Q, cause high-level resistance of human immunodeficiency virus type 1 (HIV-1) to zidovudine (3′-azidothymidine; AZT) and appear to approximate the template strand of the enzyme-template-primer complex in structural models. We studied whether this set of mutations altered RT-template-primer interaction as well as their effect on virus replication in the absence of inhibitor. When in vitro polymerization was limited to a single association of an RT with an oligodeoxynucleotide-primed heteropolymeric RNA template (a single processive cycle), recombinant-expressed mutant 67/70/215/219 RT synthesized 5- to 10-fold more high-molecular-weight DNA products (>200 nucleotides in length) than wild-type RT. This advantage was maintained as deoxynucleoside triphosphate (dNTP) concentrations were decreased to limiting levels. In contrast, no difference was seen between wild-type and mutant RTs under conditions allowing repeated associations of enzyme with template-primer. Because intracellular dNTP concentrations are low prior to mitogenic stimulation, we compared replication of mutant 67/70/215/219 virus and wild-type virus in peripheral blood mononuclear cells (PBMC) stimulated before and after infection. In the absence of inhibitor, mutant 67/70/215/219 virus had a replication advantage in PBMC stimulated with phytohemagglutinin and interleukin-2 after infection, but virus replication was similar in PBMC stimulated before infection in vitro. The results confirm that RT mutations D67N, K70R, T215Y, and K219Q affect an enzyme-template-primer interaction in vitro and suggest that such substitutions may affect HIV-1 pathogenesis during therapy by increasing viral replication capacity in cells stimulated after infection.

Human immunodeficiency virus type 1 (HIV-1) pathogenesis is driven by highly dynamic, ongoing virus replication (16, 54). HIV-1 plasma RNA can be partially and transiently suppressed by inhibitors which block virion entry into uninfected cells or reverse transcription in newly infected cells (16, 33, 54). The resurgence of virus plasma RNA during therapy is largely due to replication of drug-resistant mutant viruses selected from the virus population by antiretroviral therapy (7, 33, 46, 54). It has been theorized that in the absence of chemotherapeutic selection pressure, drug-resistant mutants have decreased replication capacity relative to wild-type virus in vivo (7). This idea is supported by the low frequency of preexisting drug-resistant mutants in virus populations of untreated individuals (37, 38) and comparisons of in vitro replication in proliferating, drug-free cells of wild-type and some drug-resistant mutant viruses (2, 6, 32). Zidovudine (3′-azidothymidine; AZT)-selected reverse transcriptase (RT) mutations T215Y (2) and T215Y and K219Q (6) slightly decreased replication in vitro compared with wild-type RT, but the effects of other AZT-selected RT mutations have not been reported.

Recent clinical observations suggest that some AZT-resistant mutants may have a replication advantage over wild-type virus in vivo in the absence of AZT therapy. The presence of highly AZT-resistant HIV-1 isolates has been associated with an increase in risk of death among patients switched from AZT to didanosine (ddI) monotherapy (8). Also, the increased risk of clinical progression or death associated with AZT resistance mutations in RT codons 41 and 215 was independent of randomization to either continued AZT or ddI therapy (23). Others have shown that addition of ddI or nevirapine to AZT therapy was unable to suppress the level of HIV-1 RNAs containing RT T215Y or F mutations in patients' plasmas, although it did suppress those containing a wild-type RT codon 215 (17). Finally, the proportion of AZT resistance mutations in the virus RNA population in serum continued to increase for months following AZT withdrawal in the absence of treatment with any other antiretroviral agent (33). Mechanistic explanations for all these observations have not been elucidated.

The enzymologic mechanisms underlying high-level AZT resistance also remain unclear. Highly AZT-resistant viruses containing multiple RT mutations, including D67N, K70R, T215Y, and K219Q (referred to as mutant 67/70/215/219), show a >100-fold increase in the 50% inhibitory concentration (IC_{50}) of AZT for viral replication in cell culture but exhibit only a minimal change in AZT-triphosphate (AZT-TP) binding to RT or inhibition of RT polymerase activity in cell-free assays (10, 29–31, 35). This suggests that a change in an enzyme function other than AZT-TP binding may result in high-level AZT resistance. In contrast, RT L74V-mediated ddI resistance is caused by decreased binding of the active inhibitor (ddATP) to the mutant enzyme (10, 35, 49), which is due to altered positioning of RT-bound template-primer (3).

In order to better characterize RT enzymology and the viral replicative capacity of AZT-resistant mutant 67/70/215/219, we compared wild-type and mutant 67/70/215/219 RTs in an assay system which limited processive RNA-dependent DNA poly-

* Corresponding author. Mailing address: Infectious Disease Unit, Massachusetts General Hospital, 149 13th St., Charlestown, MA 02129. Phone: (617) 726-5772. Fax: (617) 726-5411. Electronic mail address: daquila@helix.mgh.harvard.edu.

merization to a single association between an RT and a heteropolymeric primer-template by using heparin as a trap for free and dissociated RT molecules (44). We also compared a single processive cycle of DNA synthesis of wild-type and mutant 67/70/215/219 RTs in vitro over a range of decreasing deoxynucleoside triphosphate (dNTP) concentrations, since nonproliferating peripheral blood mononuclear cells (PBMC) have lower intracellular dNTP concentrations than mitogen-stimulated PBMC (13) and most circulating cells are not proliferating. In addition, the replication of wild-type and mutant 67/70/215/219 viruses was compared in drug-free PBMC stimulated by phytohemagglutinin (PHA) and interleukin-2 (IL-2) before and after in vitro infection.

MATERIALS AND METHODS

Molecular cloning. Site-directed mutagenesis was performed by the method of Kunkel et al. (28) on a plasmid subclone containing an HIV-1 *Sph*I-*Sal*I fragment (HxB2, nucleotides 1447 to 5786 [42]) to introduce the L74V substitution (mutant 74) and the T215Y and K219Q substitutions (mutant 215/219) in RT. The complete RT coding region of each resulting plasmid subclone was sequenced for confirmation (10). These mutant 74 and 215/219 plasmids were used as templates for separate PCR amplifications in order to construct mutant 74 (pK74) and mutant 215/219 (pK215/219) RT plasmid expression vectors. Stringent precautions to prevent carryover were used for these and all other amplifications. To control for potential contamination, multiple reagent controls lacking template DNA were included. Amplifications with *Taq* polymerase (Perkin Elmer Cetus) were performed with a 5′ primer containing an *Nco*I site (underlined, which introduced a translation initiation codon), 5′-TGCCATGGCCATT AGCCCTATTG AGACTGT-3′, and a 3′ primer containing a *Hin*dIII site (underlined, which introduced a translation termination codon), 5′-CGAAGCTTT ATAGTAT TTTCCTGATTCCAG-3′ (9). The amplification products were digested with both *Nco*I and *Hin*dIII and then cloned into the larger *Nco*I-*Hin*dIII restriction digestion fragment of an RT expression vector (pKRT2) (9, 10). DNA sequencing of the RT coding sequences confirmed the presence of the intended mutations in the appropriate plasmids and ruled out possible errors introduced during PCR.

The D67N and K70R mutations in RT were introduced into a wild-type RT expression plasmid by a modification of recombinant PCR (41, 57). A 430-bp fragment that included the relevant RT sequences (from the beginning of the coding sequences at nucleotide 2549 to the *Eco*RV restriction site at HxB2 nucleotide 2979 [42]) was amplified with *Taq* polymerase (Perkin Elmer Cetus) from wild-type pKRT2 (9). Two primer pairs were used, including overlapping, internal mutagenic primers which had dUMP incorporated in place of TMP and were designed to insert codon changes at D67N and K70R (mutations underlined in primer sequences below). The 5′-end primer pair was 5′-CUACUACU ACUACCATGGCCATTAGCCTATTG-3′ (5 CA 2550) and 5′-CUACTAATU TTCUCCAUCUAGUACUGTUTT-3′ (3 CA 67,70); the 3′-end primer pair was 5′-AAAACAGUACUAGAUGGAGAAAAUUAGUAG-3′ (5 CA 67,70) and 5′-CAUCAUCAUCAUACTGATATCTAATCCCGGGTG-3′ (3 CA 2986). The PCR products were mixed with pAMP vector DNA and digested with uracil deglycosylase (UDG; CloneAmp System, Gibco-BRL, Gaithersburg, Md.), which allowed annealing of the strands complementary to the internal mutagenic primers as well as annealing of the flanking ends of each PCR product to the complementary strands of pAMP vector DNA (40, 57). Bacteria were transformed with this mixture of annealed DNA. The 430-bp fragment derived from PCR was sequenced from the resulting plasmid (named pK67/70) to confirm the intended sequence. An RT expression clone with D67N, K70R, T215Y, and K219Q (mutant 67/70/215/219) was constructed by directional cloning of the *Nco*I-*Eco*RV restriction fragment from pK67/70 into the larger fragment of *Nco*I- and *Eco*RV-digested pK215/219 by standard ligase-mediated methods to yield an expression plasmid called pK67/70/215/219.

Mutant RT coding sequences from pK67/70/215/219 were amplified by PCR with a proofreading thermostable polymerase (Ultma; Perkin Elmer Cetus) and cloned into a 5′ half-genome plasmid, called p5′LTR, that contained pNL4-3 sequences from the *Stu*I site in 5′-flanking cellular sequences to the *Sal*I site at nucleotide 5785 (1). The resulting plasmid was called pRT2. DNA sequencing confirmed RT sequence throughout the PCR-derived region of pRT2. This plasmid was used, with a 3′ half-genome plasmid called p3′LTR derived from pNL4-3 (1), to generate molecular clone-derived RT mutant 67/70/215/219 virus. The p3′LTR plasmid contains pNL4-3 sequences from the *Sal*I site at nucleotide 5785 to the *Pst*I site in the 3′-flanking cellular sequences and includes the complete 3′ long terminal repeat (LTR). The construction of the 5′ half-genome plasmids p5′LTR and pRT2 as well as the 3′ half-genome plasmid p3′LTR from pNL4-3 will be detailed elsewhere (unpublished data). The half-genome plasmid DNAs pRT2 and p3′LTR were mixed together (5 μg of each) prior to electroporation (Gene Pulser; Bio-Rad) into MT-2 cells to yield infectious mutant 67/70/215/219 virus (14). pNL4-3 DNA was also electroporated into MT-2 cells to generate wild-type molecular clone-derived virus. Each resulting virus was passaged once in MT-2 cells. The infectivity of these cell-free (filtered) virus stocks was determined in PBMC cultures stimulated by PHA and IL-2 before infection by a standard method (22). The titers of the wild-type and mutant 67/70/215/219 virus stocks were equivalent.

Preparation of recombinant-expressed RT. *Escherichia coli* JM109 containing either a wild-type (pKRT2) or a mutant (either pK67/70/215/219 or pK74) RT expression vector was grown to late logarithmic phase at 37°C in 2×YT medium containing 100 μg of ampicillin (Sigma, St. Louis, Mo.) per ml. RT expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 12 to 16 h. Recombinant-expressed wild-type and mutant RTs were purified by the method of Kohlstaedt et al. (26). For experiments comparing wild-type with mutant 67/70/215/219 recombinant-expressed RTs, the enzymes were purified as follows. Cells were lysed by French press in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 6.0)–2 mM EDTA–0.02% 1-hexyl-β-D-glucopyranoside (βHG; Calbiochem–10% glycerol. After centrifugation, the pellet was resuspended in 50 mM MES (pH 6.0)–50 mM KCl–50 mM potassium phosphate–0.02% βHG–10% glycerol (HSE buffer). Polyethyleneimine was added to 0.05% to remove nucleic acid, and the RT activity was precipitated with 60% saturated ammonium sulfate. The ammonium sulfate precipitate was redissolved in HSE buffer and applied to a hydroxyapatite column (Bio-Rad) equilibrated in the same buffer. The column was eluted with a linear gradient from 50 to 250 mM potassium phosphate. Fractions containing RT were pooled, precipitated with ammonium sulfate, and redissolved in 50 mM 1,3-bis[tris(hydroxymethyl)methylamino]-propane (Bis-Tris propane, pH 7.0)–100 mM ammonium sulfate–0.02% βHG–10% glycerol (BTP buffer). The sample was applied to a heparin column (Pharmacia) equilibrated in the same buffer and eluted with a linear gradient from 0.1 to 1.0 M ammonium sulfate (26). For experiments comparing wild-type RT with mutant 74, the recombinant expressed RTs were purified with minor modifications of this procedure. The bacterial cells were lysed by gentle sonication. Both the lysis buffer and HSE buffers contained 0.02% Triton X-100 rather than βHG. Both the hydroxyapatite and the heparin columns were eluted with stepwise gradients.

Polymerase assays. Polymerase activity was measured on both heteropolymeric and homopolymeric template-primers. A 756-bp heteropolymeric RNA template was synthesized by T7 polymerase in vitro transcription with *Xmn*I-linearized pSP72 DNA as the template (Riboprobe Gemini II Core System; Promega). A 20-base deoxyoligonucleotide (nucleotides 1818 to 1838 of pSP72) complementary to the RNA template was 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (45). ^{32}P-labeled primer (64 nM), RNA template (60 nM), and purified RT (6 nM) were preincubated in 40 mM Tris (pH 8.0) and 8 mM dithiothreitol (DTT) for 5 min. Reactions were initiated with the addition of dNTP (final concentration, 150 μM each of dATP, dCTP, dGTP, and TTP; 600 μM total dNTP concentration unless otherwise noted) and 10 mM $MgCl_2$ with or without the simultaneous addition of heparin (0.15 mg/ml) as a trap for free and dissociated RT (44). Reactions were run for various times, and DNA products were separated on 6% polyacrylamide gels (8 M urea) and visualized by autoradiography. The autoradiographs were quantitated with a computerized scanning densitometer (Molecular Dynamics PhosphorImager). Total protein concentration and polymerase activity on a homopolymeric template-primer were quantitated for wild-type and mutant RTs to ensure that the same amount of wild-type and mutant RTs were used in each of the reactions on the heteropolymeric template-primer. Protein concentration was measured with the BCA (Pierce) enhanced protocol, with bovine serum albumin as the standard. Polymerase activity was measured by [^{3}H]TTP incorporation into poly(rA)-oligo(dT) template-primer (Pharmacia), as previously described (24). In each experiment involving synthesis from heteropolymeric template-primer, wild-type and mutant RTs had equivalent (±10%) protein concentration and polymerase activity on poly(rA)-oligo(dT).

AZT-TP IC_{50}s were determined under conditions in which DNA synthesis was limited to a single association of RT and primer-template. The 300-bp product band resulting from each synthesis at various AZT-TP concentrations (0 to 30 μM) was excised from a polyacrylamide gel. The band is indicated in Fig. 1, 3, and 4. Gel slices were hydrated in Soluene (Packard, Meriden, Conn.), and the amount of ^{32}P-labeled DNA product was quantitated by scintillation counting. The IC_{50} was calculated by nonlinear regression analysis of the median effect equation (5, 11).

Viral replication assays. PBMC from a single HIV-1-seronegative donor were obtained by Ficoll-Hypaque (Histopaque; Sigma) density gradient centrifugation of heparinized venous blood. Cells were stimulated with phytohemagglutinin (PHA; 2 μg/ml; Difco Laboratories) for 3 days and maintained in IL-2 (10%; Collaborative Biomedical Products) prior to in vitro infection, as described before (19, 25), a standard method in many HIV-1 virology laboratories to maximize viral replication in vitro (15). An aliquot of PBMC from the same sample of blood used to prepare stimulated PBMC was cultured in the absence of PHA or IL-2 for 3 days prior to infection; these are referred to as unstimulated cells. Cell proliferation of stimulated and unstimulated PBMC was compared at the time of infection by measuring incorporation of [^{3}H]thymidine into DNA with an automated multiwell harvester (Filtamate 196; Packard) (21).

In order to compare the kinetics of replication of molecular clone-derived wild-type and mutant 67/70/215/219 viruses, equivalent amounts of each virus (3,000 50% tissue culture infective doses [$TCID_{50}$s]) were used to infect separate

cultures (5×10^6 PBMC) that were either unstimulated, stimulated before infection (prestimulated), or stimulated after infection. The prestimulated PBMC were passaged and fed with medium alone (containing 10% IL-2) on days 4, 11, 17, and 24 after infection and wtih PHA- and IL-2-stimulated allogeneic PBMC on days 7, 14, 21, and 28 after infection. The unstimulated cells were maintained in the absence of PHA, IL-2, or added cells either throughout the experiment (unstimulated) or until day 10 after infection (stimulated after infection). For experiments with cells stimulated after infection, the cultures were fed with medium containing PHA (2 μg/ml) and IL-2 (10%) on day 10 and subsequent feedings on days 14, 17, 21, 24, and 28 after infection. No allogeneic cells were added to cells stimulated after infection at any time. Cell-free supernatant fluids were assayed by an HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) (NEN Research Products, Boston, Mass.) every 3 or 4 days after infection.

RESULTS

Comparison of RNA-dependent DNA polymerization by mutant 67/70/215/219 and wild-type RTs. When RT is preincubated with template-primer, a stable complex consisting of enzyme, primer, and template is formed. DNA synthesis is initiated from these complexes by the addition of dNTPs and magnesium chloride; synthesis can be limited to a single processive cycle by also adding a trap such as heparin to bind free and dissociating RT (18, 44). A representative experiment showing DNA synthesis of wild-type and mutant 67/70/215/219 RTs in the presence and absence of heparin is shown in Fig. 1A. Reactions were run for 0, 10, and 30 s and 1 and 5 min with a 756-bp RNA heteropolymer as the template and a complementary ^{32}P-end-labeled 20-bp DNA primer. The amount of DNA which accumulated at longer time points (5 min) was greater for mutant 67/70/215/219 RT than for the wild-type RT under conditions which limit DNA synthesis to a single processive cycle (Fig. 1A, reactions with heparin as the trap; compare lane E for mutant RT [M] with lane E for wild-type RT [W]). In the absence of heparin, there was no apparent difference between enzymes (Fig. 1A, reactions without heparin). When primer, template, and RT were preincubated with trap, no DNA synthesis occurred with either wild-type or mutant 67/70/215/219 RT (Fig. 1A, lanes 2 and 3). Since excess heparin (>2 mg/ml) could cause an artifactual reduction of primer usage (44), the minimum concentration of heparin that prevented DNA synthesis in the preincubation was used in these experiments (0.15 mg/ml). Similar results were obtained in two additional experiments that compared wild-type and mutant 67/70/215/219 RTs (data not shown). In contrast, there was no difference in the amount or length of DNA synthesized under either processive or nonprocessive conditions by mutant 74 RT in comparison to wild-type RT (Fig. 1B; second experiment not shown).

The increase in DNA synthesis by mutant 67/70/215/219 RT relative to that by wild-type RT in the presence of a trap appeared more pronounced for higher-molecular-weight products (Fig. 1A, reactions with heparin). In order to quantify this, a ratio of band intensity for mutant 67/70/215/219 RT versus wild-type RT was determined for each DNA product band. There was a 5- to 10-fold increase in DNA synthesis for mutant 67/70/215/219 versus wild-type RT for some DNA products of greater than 200 bp (peaks 1 to 9, Fig. 2). Smaller relative increases were found for mutant RT in lower-molecular-weight products. The most prominent product band in mutant reactions at a molecular size of about 300 bp (arrow in Fig. 1A and B) was quantified as a fourfold increase in intensity for mutant 67/70/215/219 RT versus wild-type RT (peak 6 in Fig. 2). However, when product bands were quantified from reactions performed in the absence of heparin, there was no difference in the amount or size of DNA synthesized by mutant 67/70/215/219 RT compared with wild-type RT (data not shown). Additional controls examined a longer (30 min) time course of DNA synthesis from poly(rA)-oligo(dT) (data not shown) and were consistent with the conclusion that a heparin trap limited DNA synthesis to a single processive cycle.

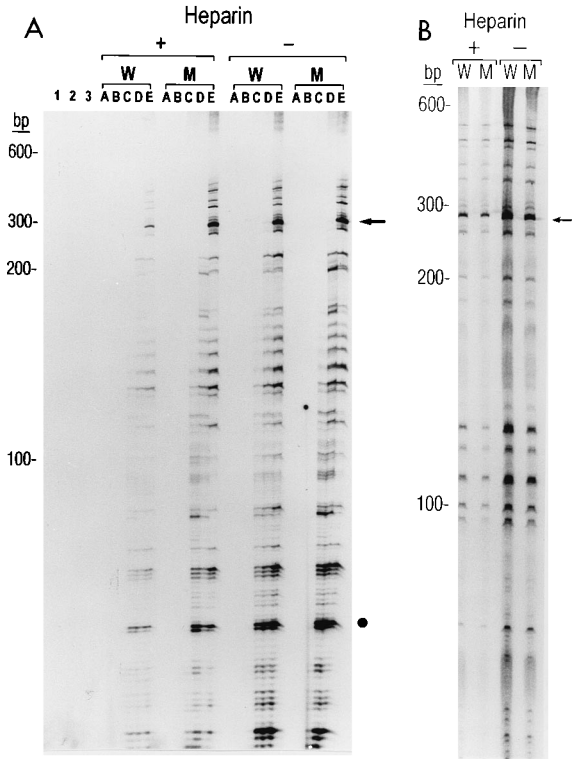

FIG. 1. DNA synthesis under processive and nonprocessive conditions. (A) DNA polymerization by wild-type (W) and mutant 67/70/215/219 (M) RTs was measured at various time points (0, 10, and 30 s and 1 and 5 min for lanes A to E, respectively). Reactions were run using a heteropolymer RNA template and a complementary DNA primer in the presence (+, processive conditions) and absence (−, nonprocessive conditions) of heparin (0.15 mg/ml) as a trap for free RT. Controls included (lane 1) no RT added, (lane 2) heparin added at the preincubation step for wild-type RT, and (lane 3) heparin added at the preincubation step for mutant 67/70/215/219. The intensities of the two most heavily labeled product bands of each enzyme in the presence of heparin were determined by densitometry; these bands are indicated by an arrow and a dot. (B) DNA synthesis for wild-type and mutant 74 RT. DNA synthesis by wild-type (W) and mutant 74 (M) RT was measured at 5-min time points under processive (+ heparin) and nonprocessive (− heparin) conditions. The mature product band is indicated by an arrow.

Determination of AZT-TP IC_{50}s under conditions limiting DNA synthesis to a single processive cycle. DNA synthesis was measured with mutant 67/70/215/219 and wild-type RTs in the

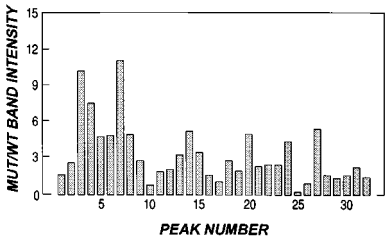

FIG. 2. Ratio of mutant 67/70/215/219 to wild-type product band intensity (MUT/WT) under conditions limiting synthesis to a single processive cycle. DNA products synthesized by wild-type and mutant 67/70/215/219 RTs in the presence of a heparin trap were quantified by scanning densitometry of the autoradiograph depicted in Fig. 1A. A ratio of mutant 67/70/215/219 to wild-type peak intensity was obtained.

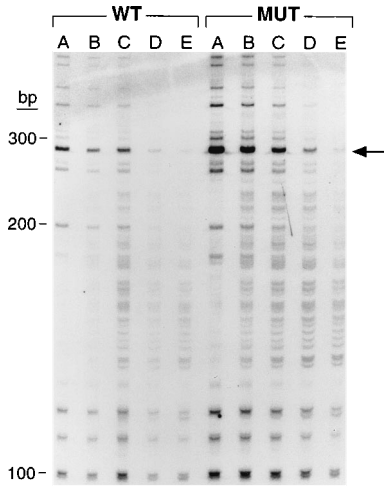

FIG. 3. In vitro DNA synthesis limited to a single processive cycle at various AZT-TP concentrations. DNA synthesis was determined for wild-type (WT) and mutant 67/70/215/219 (MUT) RTs in the presence of heparin (0.15 mg/ml) at various concentrations of AZT-TP. Reactions were run for 5 min. The AZT-TP concentrations were 0, 5, 10, 20, and 30 μM (lanes A to E, respectively). AZT-TP inhibition was quantified by measurements of the major product band, indicated by the arrow, as described in Materials and Methods.

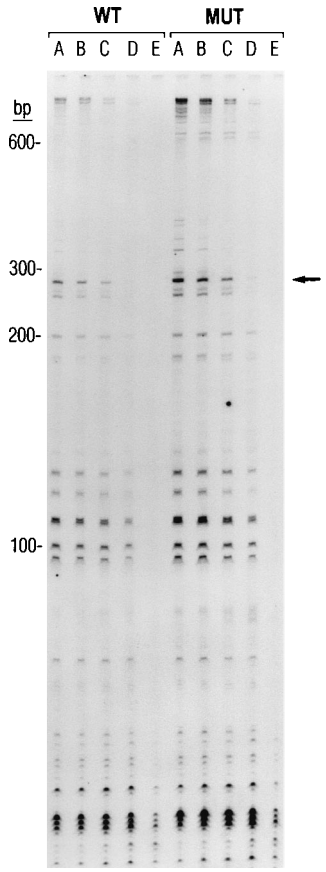

FIG. 4. In vitro DNA synthesis limited to a single processive cycle at various dNTP concentrations. DNA synthesis was determined for wild-type (WT) and mutant 67/70/215/219 (MUT) RTs in the presence of heparin (0.15 mg/ml) at various concentrations of dNTPs. Reactions were run for 5 min. The concentrations of each dNTP (total dNTP concentration) were 150 μM (600 μM), 50 μM 15 μM, 5 μM, and 1 μM (lanes A to E, respectively). The mature product band is indicated by an arrow.

presence of heparin and various concentrations of AZT-TP (0 to 30 μM). AZT-TP IC_{50}s were calculated by quantitating the most visually prominent band on the gel (shown by an arrow in Fig. 3; same product as indicated by the arrows in Fig. 1A and B), as described in Materials and Methods. At every AZT-TP concentration, more DNA was synthesized by mutant 67/70/215/219 than by wild-type RT (Fig. 3). However, in the presence of heparin, mutant 67/70/215/219 RT showed only a minimal increase in AZT-TP IC_{50} compared with wild-type RT, 14.3 ± 3.8 μM and 8.6 ± 0.85 μM, respectively. The results are the mean ± standard error of the mean (SEM) for three experiments, including two experiments in addition to the one depicted in Fig. 3.

Effect of decreasing concentration of dNTPs on DNA polymerization. DNA synthesis was also measured in the presence of heparin and various dNTP concentrations (1 to 150 μM each dNTP; 4 to 600 μM total dNTP concentration). With decreasing, equimolar concentrations of dNTPs, the amount of DNA synthesized was decreased to a similar degree for both wild-type and mutant 67/70/215/219 RTs (Fig. 4), based on densitometric quantitation of the prominent approximately 300-bp product (arrow, Fig. 4). There was no difference in the size distribution of DNA synthesized by mutant 67/70/215/219 RT compared with wild-type RT at lower equimolar dNTP concentrations (Fig. 4). However, mutant 67/70/215/219 RT synthesized more DNA than wild-type RT at every equimolar dNTP concentration from 150 to 5 μM (Fig. 4). At 1 μM dNTP, DNA synthesis appeared to be limited to an equivalent degree for both mutant and wild-type RTs (Fig. 4).

Comparison of mutant 67/70/215/219 and wild-type virus replication. In order to assess whether the RT mutations D67N, K70R, T215Y, and K219Q altered viral replication in the absence of an inhibitor, PBMC were infected in vitro with wild-type and mutant 67/70/215/219 molecular clone-derived viruses. Because we had noted differences from the wild type in RT polymerization that were limited to a single processive cycle in vitro over a wide range of dNTP concentrations, viral replication was compared in unstimulated PBMC, which contain lower intracellular dNTP concentrations than PHA-stimulated cells (13), as well as in PBMC stimulated with PHA and IL-2 3 days before infection. The proliferative response of unstimulated PBMC, as measured by [^{3}H]thymidine incorporation into DNA, was 2.0% ± 0.6% (mean ± SEM for three experiments) of that measured for PHA- and IL-2-stimulated PBMC. Unstimulated cells remained >80% viable, based on trypan blue dye exclusion, although they did not proliferate over the course of the experiment. HIV-1 p24 antigen levels in culture supernatant fluids decreased to the same degree for wild-type- and mutant virus-infected unstimulated cells over an 18-day period to below detectable levels (data not shown). This indicates that progeny virions were not produced from unstimulated cells by either wild-type or mutant virus.

Differences in replication between mutant 67/70/215/219 and wild-type virus were also measured in experiments involving stimulation of cells (from the same HIV-1-seronegative donor) either before or after infection. In PBMC which were stimulated with PHA and IL-2 on day 10 after infection, mutant virus produced significantly higher levels of supernatant HIV-1 p24 antigen than wild-type virus on days 7 to 21 after stimulation (for each measured time point, $P < 0.05$, Mann-Whitney U test) that peaked at sixfold higher p24 antigen levels for mutant virus over wild-type virus (Fig. 5). These differences persisted when p24 antigen production was expressed as nanograms per 10^6 viable cells. In contrast, the replication of mu-

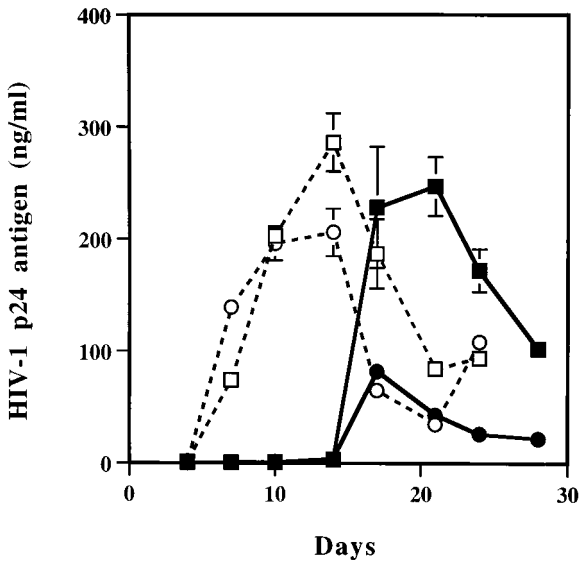

FIG. 5. Comparison of mutant 67/70/215/219 and wild-type virus replication in PBMC stimulated either before or after infection. Prestimulated (open symbols) and initially unstimulated (solid symbols) PBMC were infected with 3,000 $TCID_{50}$s of wild-type (circles) or mutant 67/70/215/219 (squares) molecular clone-derived HIV-1. Initially unstimulated PBMC were stimulated with PHA and IL-2 starting on day 10 after infection. Results are expressed as mean nanograms of HIV-1 p24 antigen per milliliter ± SE for independent cultures infected with each virus (two infections of prestimulated cells with each virus and three infections of initially unstimulated cells with each virus). On days 17, 21, 24, and 28 after infection, mutant 67/70/215/219 virus-infected cultures which were stimulated on day 10 after infection (solid squares) had a significantly higher concentration of HIV-1 p24 antigen in culture supernatant fluid than wild-type-virus-infected cultures stimulated on day 10 after infection (solid circles) ($P < 0.05$, Mann-Whitney U test). In cells stimulated before infection, there were no differences between mutant (open squares) and wild-type (open circles) viruses.

tant and wild-type viruses did not differ significantly in PHA- and IL-2-prestimulated PBMC isolated from the same donor (Fig. 5). In cultures stimulated 10 days after infection, p24 antigen production from wild-type virus peaked at less than half the amount produced in prestimulated cultures. However, peak p24 antigen production from mutant 67/70/215/219 virus in cultures stimulated 10 days after infection equalled the amount present in cultures stimulated before infection (Fig. 5).

These observations were confirmed in a second experiment with PBMC from a different HIV-1-seronegative donor. Similar differences in mean supernatant fluid p24 antigen levels (three cultures for each virus) for the wild-type (WT) and mutant (Mut) viruses were seen only in cultures stimulated after infection (expressed as nanograms of p24 antigen per milliliter ± SE): day 7 after stimulation, Wt = 312 ± 30 and Mut = 586 ± 27; day 10 after stimulation, Wt = 114 ± 13 and Mut = 510 ± 46; day 14 after stimulation, Wt = 56 ± 3 and Mut = 152 ± 10; day 17 after stimulation, Wt = 20 ± 2 and Mut = 53 ± 1; day 21 after stimulation, Wt = 8 ± 1 and Mut = 29 ± 3 (for each time point, $P < 0.05$, Mann-Whitney U test). The cultures stimulated 3 days before infection each reached similar peak levels of p24 antigen production on day 10 after infection (Wt = 541 ± 8 and Mut = 591 ± 67).

Mean cell counts per milliliter did not differ between mutant- and wild-type-infected cultures at any time point (data not shown), suggesting that differences in cellular proliferation did not explain the replication advantage of mutant virus in cells stimulated after infection. Sequencing of PCR products from infected PBMC confirmed the correct identity of viruses produced in cultures stimulated both before and after infection. DNA from cells infected with wild-type virus contained wild-type sequences, whereas DNA from cells infected with mutant virus contained mutant bases at RT codons 67, 70, 215, and 219. These analyses confirm that the similarity of replication of mutant and wild-type virus in prestimulated cells was not artifactual.

DISCUSSION

Mutant 67/70/215/219 RT synthesized 5- to 10-fold more high-molecular-weight DNA products (>200 nucleotides) from an oligonucleotide-primed heteropolymeric RNA template than did wild-type RT under conditions that limited RT to a single association with template-primer (Fig. 1A). This advantage for the mutant persisted even as dNTP concentrations were decreased (Fig. 4). In the absence of a trap for free RT, DNA was synthesized to the same degree by wild-type and mutant 67/70/215/219 RTs, as in a number of earlier studies that did not limit DNA synthesis to a single processive cycle (29, 31, 35). An important control for our experiments was an RT containing the ddI resistance mutation at codon 74 that synthesized amounts and sizes of DNA that were similar to those synthesized by the wild-type enzyme during a single processive cycle (Fig. 1B). Thus, an increase in DNA synthesis during a single processive cycle does not occur with every nucleoside resistance mutation in the "fingers" subdomain of RT p66.

In structural models of RT, amino acid residues 67, 70, and 215 as well as 74 are in close proximity to the RNA template (20, 27, 52). Therefore, the set of AZT resistance mutations D67N, K70R, T215Y, and K219Q has been hypothesized to alter interactions between RT and template-primer. A number of biochemical mechanisms could explain increased DNA synthesis by this mutant during a single processive cycle. Each would involve a change in an enzyme-template-primer interaction, although our data do not allow a conclusive choice to be made among the possible mechanisms. The processivity of the mutant RT, defined as the number of nucleotides incorporated into a growing DNA primer strand before polymerase dissociation, may be greater than that of the wild-type RT. Although the mutant and wild-type enzymes appear to synthesize products of similar size in the presence of heparin, the largest products (>600 bp) were only evident with mutant RT at the longest time point in some experiments (Fig. 1A, lanes A through E for both wild-type and mutant in the presence of heparin). In addition, densitometric analyses of the two most prominent products in the presence of heparin at the longest time point indicated that there was a different ratio of larger to smaller products for each enzyme (Fig. 1A, lanes E for both wild-type and mutant in the presence of heparin). The larger of the two major products (arrow in Fig. 1A) of the wild-type RT reaction was almost half as intense as the smaller product (dot in Fig. 1A). In contrast, the mutant RT synthesized approximately twofold more larger product than smaller product. In the absence of heparin, the ratio of these two products was similar for both enzymes. Differences between the enzymes in the ratio of larger to smaller products were less evident in comparisons of other product bands. A second possible biochemical explanation is based on earlier studies showing that HIV-1 RT switches to a processive mode of synthesis on a homopolymeric RNA template-primer only after one or a few distributive nucleotide incorporations have occurred (18, 34, 43). The mutant enzyme may differ from the wild-type enzyme in the switch from the distributive to the processive mode rather than in the number of incorporations during a single processive cycle. A third alternative is that the K_m for tem-

plate-primer may be altered so that mutant enzyme binds more tightly to the template-primer. However, this is not consistent with the lack of difference between the enzymes when heparin was absent. An earlier study also showed no difference between wild-type and 67/70/215/219 mutant HIV-1 RTs in a different type of gel electrophoretic primer extension assay with a heteropolymeric RNA template-oligodeoxynucleotide primer without a trap for free RT (29).

Increased DNA synthesis of the AZT-selected mutant 67/70/215/219 RT during a single processive cycle does not solve the previously studied enigma concerning the enzymologic basis of high-level (100-fold) AZT resistance of virus (10, 29, 30, 35). When DNA synthesis was measured in the presence of heparin and various concentrations of AZT-TP, there was little difference in the AZT-TP IC_{50} between wild-type and mutant 67/70/215/219 RTs.

We also investigated whether there were differences in replication between wild-type virus (NL4-3) and the 67/70/215/219 mutant virus (derived from NL4-3) in the absence of an inhibitor. In fact, replication of the two viruses was similar in drug-free PBMC stimulated with PHA and IL-2 before infection. We also compared replication of these viruses in unstimulated PBMC, since unstimulated PBMC have lower dNTP concentrations and permit less efficient synthesis of wild-type HIV-1 DNA than PHA-stimulated PBMC (13). HIV-1 p24 antigen levels did not differ when unstimulated cultures were infected with either wild-type or mutant viruses. PHA and IL-2 were added to initially unstimulated cultures on day 10 after infection because more efficient synthesis of reverse-transcribed replication intermediates by mutant virus RT in the presence of low dNTP concentrations might only be evident after mitogenic stimulation. In fact, replication of mutant 67/70/215/219 virus was significantly greater than that of wild-type virus under these conditions in PBMC derived from two different HIV-1-seronegative donors. Mutant virus replicated equally well in cells stimulated either before or after infection. The observed replication advantage of mutant virus in cells stimulated after infection could not be explained by differences in extent of proliferation of cells infected with either virus type. Preliminary data indicate a similar in vitro replication advantage for another AZT-resistant mutant virus containing RT mutations M41L, T215Y, and K219Q over wild-type virus in cells stimulated after infection (data not shown).

A number of studies have shown that wild-type HIV-1 can infect quiescent $CD4^+$ cells in vitro and in vivo, but proviral integration and virus production do not occur unless cells are subsequently activated with mitogen (4, 36, 39, 48, 50, 51, 55). Some data indicate that wild-type virus synthesizes complete, stable reverse transcripts in unstimulated cells (48, 50), while other data suggest incomplete reverse transcription of wild-type unintegrated DNA (51, 55, 56). In our experiments, peak production of wild-type virus was maximal in prestimulated cultures and decreased when cultures were stimulated 10 days after infection. In contrast, two studies that used higher multiplicities of infection reported the production of equivalent amounts of wild-type virus in cells stimulated before and after infection (48, 53).

A replication advantage for this AZT-resistant mutant virus in cells stimulated after infection in vitro in the absence of AZT may be consistent with the dominance of wild-type virus in HIV-1 populations of untreated patients (7, 37), because selection pressures change after initiation of AZT therapy. Unstimulated PBMC contain a lower ratio of the chain-terminating nucleoside competitor AZT-TP to the physiologic substrate TTP than do activated PBMC (12). AZT has also been shown to have less inhibitory activity against wild-type virus in PBMC stimulated after infection than in PBMC stimulated before infection (47; unpublished data). This may result in a selective advantage during AZT therapy for viruses with improved replication efficiency in cells stimulated after infection that was not present before initiation of AZT therapy. Such a selective advantage would not be expected during therapy with antiretroviral agents which accumulate similar levels of active inhibitor in both unstimulated and activated PBMC, including some other nucleosides (12, 47).

Clinical observations suggest that AZT-resistant mutant HIV-1 may have a replication advantage over wild-type virus in vivo, even in the absence of AZT selection pressure (8, 17, 23, 33). Our comparison of wild-type and mutant viruses raises the possibility that some AZT-selected mutants produce greater amounts of virus than the wild-type strain in cells stimulated after infection in the absence of AZT. Such mutants may increase the pool size of unstimulated, infected cells that harbor activatable, latent virus or the life span of HIV-1 replication intermediates in such cells. It will be of interest to determine if in vitro measures of HIV-1 replication in cells stimulated after infection are associated with the pathogenic potential of viruses selected during RT inhibitor therapy in vivo.

ACKNOWLEDGMENTS

This work was supported by NIH grants RO1 AI29193 and IT 32A107387.

We thank Glenn Lindwall for providing some wild-type and mutant 67/70/215/219 RTs purified from our expression clones, Thomas Steitz for support of that enzyme purification in his laboratory, and especially Lori Kohlstaedt for guidance in enzyme purification and many helpful discussions. Richard Dougherty and Martin Hirsch gave us useful advice. Cloned HxB2 DNA was generously provided by Lee Ratner, and pNL 4-3 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from its depositor, Malcolm Martin.

REFERENCES

1. **Adachi, A., H. E. Gendelman, F. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Boucher, C. A. B., R. Van Leeuwen, P. Kellam, P. Schipper, J. Tijnagel, J. M. A. Lange, and B. A. Larder.** 1993. Effects of discontinuation of zidovudine treatment on zidovudine sensitivity of HIV-1 isolates. Antimicrob. Agents Chemother. **37:**1525–1530.
3. **Boyer, P. L., C. Tantillo, R. G. Nanni, J. Ding, E. Arnold, and S. H. Hughes.** 1994. Sensitivity of wild type human immunodeficiency virus type 1 reverse transcriptase to dideoxynucleotides depends on template length: the sensitivity of drug resistant mutants does not. Proc. Natl. Acad. Sci. USA **91:** 4882–4886.
4. **Bukrinsky, M. I., T. Stanwick, L. M. P. Dempsey, and M. Stevenson.** 1991. Quiescent T lymphocytes as an inducible virus reservoir in HIV-1 infection. Science **254:**423–427.
5. **Chou, T.-C.** 1991. The median-effect principle and the combination index for quantitation of synergism and antagonism, p. 61–98. *In* T.-C. Chou and D. Rideout (ed.), Synergism and antagonism in chemotherapy. Academic Press, San Diego, Calif.
6. **Chow, Y.-K., M. S. Hirsch, D. P. Merrill, L. J. Bechtel, J. J. Eron, J. C. Kaplan, and R. T. D'Aquila.** 1993. Use of evolutionary limitations of HIV-1 multi-drug resistance to optimize therapy. Nature (London) **361:**650–654. (Correction, **364:**679.)
7. **Coffin, J. M.** 1995. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science **267:**483–489.
8. **D'Aquila, R. T., V. A. Johnson, S. L. Welles, A. J. Japour, D. R. Kuritzkes, V. DeGruttola, P. S. Reichelderfer, R. W. Coombs, C. S. Crumpacker, J. O. Kahn, D. D. Richman, AIDS Clinical Trials Group Protocol 116B/117 Team, and the Virology Committee Resistance Working Group.** 1995. Zidovudine resistance and HIV-1 disease progression during antiretroviral therapy. Ann. Intern. Med. **122:**401–408.
9. **D'Aquila, R. T., and W. C. Summers.** 1989. HIV-1 reverse transcriptase/ribonuclease H: high level expression in E. coli from a plasmid constructed using the polymerase chain reaction. J. Acquired Immune Defic. Syndrome **2:**579–587.
10. **Eron, J. J., Y.-K. Chow, A. M. Caliendo, J. Videler, K. DeVore, T. P. Cooley,**

H. A. Liebman, J. C. Kaplan, M. S. Hirsch, and R. T. D'Aquila. 1993. *pol* mutations conferring zidovudine and didanosine resistance with different effects in vitro yield multiply resistant human immunodeficiency virus type 1 isolates in vivo. Antimicrob. Agents Chemother. **37:**1480–1487.

11. **Eron, J. J., P. Gorczyca, J. C. Kaplan, and R. T. D'Aquila.** 1992. Susceptibility testing by polymerase chain reaction DNA quantitation: a method to measure drug resistance of human immunodeficiency virus type 1 isolates. Proc. Natl. Acad. Sci. USA **89:**3241–3245.
12. **Gao, W.-Y., R. Agbaria, J. S. Driscoll, and H. Mitsuya.** 1994. Divergent antihuman immunodeficiency virus activity and anabolic phosphorylation of 2′,3′-dideoxynucleoside analogs in resting and activated human cells. J. Biol. Chem. **269:**12633–12638.
13. **Gao, W.-Y., A. Cara, R. C. Gallo, and F. Lori.** 1993. Low levels of deoxynucleotides in peripheral blood lymphocytes: a strategy to inhibit human immunodeficiency virus type 1 replication. Proc. Natl. Acad. Sci. USA **90:** 8925–8928.
14. **Gibbs, J. S., D. A. Regier, and R. C. Desrosiers.** 1994. Construction and in vitro properties of HIV-1 mutants with deletions in "nonessential" genes. AIDS Res. Hum. Retroviruses **10:**343–350.
15. **Hammer, S., C. Crumpacker, R. D'Aquila, B. Jackson, J. Lathey, D. Livnat, and R. Reichelderfer.** 1993. Use of virologic assays for detection of human immunodeficiency virus in clinical trials: recommendations of the AIDS Clinical Trials Group Virology Committee. J. Clin. Microbiol. **31:**2557–2564.
16. **Ho, D. D., A. Neumann, U. A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature (London) **373:**123–126.
17. **Holodniy, M., L. Mole, D. Margolis, J. Moss, H. Dong, E. Boyer, M. Urdea, J. Kolberg, and S. Eastman.** 1995. Determination of human immunodeficiency virus RNA in plasma and cellular viral DNA: genotypic zidovudine resistance and viral load during zidovudine-didanosine combination therapy. J. Virol. **69:**3510–3516.
18. **Huber, H. E., J. M. McCoy, J. S. Seehra, and C. C. Richardson.** 1989. Human immunodeficiency virus 1 reverse transcriptase. J. Biol. Chem. **264:**4669–4678.
19. **Jackson, J. B., R. W. Coombs, K. Sannerud, F. S. Rhame, and H. H. Balfour.** 1988. Rapid and sensitive viral culture method for human immunodeficiency virus type 1. J. Clin. Microbiol. **26:**1416–1418.
20. **Jacobo-Molina, A., J. Ding, R. G. Nanni, A. D. Clark, X. Lu, C. Tantillo, R. L. Williams, G. Kamer, A. L. Ferris, P. A. Clark, A. S. Hizi, S. H. Hughes, and E. Arnold.** 1993. Crystal structure of HIV-1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. Proc. Natl. Acad. Sci. USA **90:**6320–6324.
21. **James, S. P.** 1994. Measurement of basic immunologic characteristics of human mononuclear cells, p. 7.10.1–7.10.10. *In* J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.), Current Protocols in Immunology. John Wiley and Sons Inc., New York.
22. **Japour, A. J., D. L. Mayers, V. A. Johnson, D. R. Kuritzkes, L. A. Beckett, J.-M. Arduino, J. Lane, R. J. Black, P. S. Reichelderfer, R. T. D'Aquila, and C. S. Crumpacker.** 1993. Standardized peripheral blood mononuclear cell culture assay for determination of drug susceptibilities of clinical human immunodeficiency virus type 1 isolates. Antimicrob. Agents Chemother. **37:**1095–1101.
23. **Japour, A. J., S. L. Welles, R. T. D'Aquila, V. A. Johnson, D. D. Richman, R. W. Coombs, P. S. Reichelderfer, J. O. Kahn, C. S. Crumpacker, D. R. Kuritzkes, the AIDS Clinical Trials Group 116B/117 Study Team, and the Virology Committee Resistance Working Group.** 1995. Prevalence and clinical significance of zidovudine resistance mutations in human immunodeficiency virus isolated from patients following long-term zidovudine treatment. J. Infect. Dis. **171:**1172–1179.
24. **Johnson, V. A., R. E. Byington, and J. C. Kaplan.** 1990. Reverse transcriptase (RT) activity assay, p. 98–102. *In* A. Aldovini and B. Walker (ed.), Techniques in HIV research. Stockton Press, New York.
25. **Johnson, V. A., D. P. Merrill, J. A. Videler, T.-C. Chou, R. E. Byington, J. J. Eron, R. T. D'Aquila, and M. S. Hirsch.** 1991. Two-drug combinations of zidovudine, didanosine, and recombinant interferon-alpha A inhibit replication of zidovudine-resistant human immunodeficiency virus type 1 isolates. J. Infect. Dis. **164:**646–655.
26. **Kohlstaedt, L. A., and T. A. Steitz.** 1992. Reverse transcriptase of human immunodeficiency virus can use either $tRNA_{Lys3}$ or *Escherichia coli* $tRNA_{Gln2}$ as a primer in an *in vitro* primer-utilization assay. Proc. Natl. Acad. Sci. USA **89:**9652–9656.
27. **Kohlstaedt, L. A., J. Wang, J. M. Friedman, P. A. Rice, and T. A. Steitz.** 1992. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science **256:**1783–1790.
28. **Kunkel, T. A., J. D. Robert, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
29. **Lacey, S. F., J. E. Reardon, E. S. Furfine, T. A. Kunkel, B. Katarzyne, K. A. Eckert, S. D. Kemp, and B. A. Larder.** 1992. Biochemical studies of the reverse transcriptase and RNase H activities from human immunodeficiency virus strains resistant to 3′-azido-3′-deoxythymidine. J. Biol. Chem. **267:** 15789–15794.
30. **Larder, B. A., G. Darby, and D. D. Richman.** 1989. HIV with reduced sensitivity to AZT isolated during prolonged therapy. Science **243:**1731–1734.
31. **Larder, B. A., and S. D. Kemp.** 1989. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to AZT. Science **246:**1155–1158.
32. **Larder, B. A., S. D. Kemp, and P. R. Harrigan.** 1995. Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. Science **269:**696–699.
33. **Loveday, C., S. Kaye, M. Tenant-Flowers, M. Semple, U. Ayliffe, I. V. Weller, and R. S. Tedder.** 1995. HIV-1 RNA serum-load and resistant viral genotypes during early zidovudine therapy. Lancet **345:**820–824.
34. **Majumdar, C., J. Abbotts, S. Broder, and S. H. Wilson.** 1988. Studies on the mechanism of human immunodeficiency virus reverse transcriptase. J. Biol. Chem. **263:**15657–15665.
35. **Martin, J. L., J. E. Wilson, R. L. Haynes, and P. A. Furman.** 1993. Mechanism of resistance of human immunodeficiency virus type 1 to 2′,3′-dideoxyinosine. Proc. Natl. Acad. Sci. USA **90:**6135–6139.
36. **McDougal, J. S., A. Mawie, and S. P. Cort.** 1985. Cellular tropism of the human retrovirus HTLV-III/LAV I: role of T cell activation and expression of the T4 antigen. J. Immunol. **135:**3151–3162.
37. **Najera, I., A. Holguin, M. E. Quinones-Mateu, M. A. Munoz-Fernandez, R. Najera, C. Lopez-Galindez, and E. Domingo.** 1995. *pol* gene quasispecies of human immunodeficiency virus: mutations associated with drug resistance in virus from patients undergoing no drug therapy. J. Virol. **69:**23–31.
38. **Najera, I., D. D. Richman, I. Olivares, J. M. Rojas, M. A. Peinado, M. Perucho, R. Najera, and C. Lopez-Galindez.** 1994. Natural occurrence of drug resistance mutations in the reverse transcriptase of human immunodeficiency virus type 1 isolates. AIDS Res. Hum. Retroviruses **10:**1479–1488.
39. **Patterson, B. K., M. Till, P. Otto, C. Goolsby, M. R. L. Furtado, J. McBride, and S. M. Wolinsky.** 1993. Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. Science **260:**976–979.
40. **Rashtchian, A., G. W. Buchman, D. M. Schuster, and M. S. Beringer.** 1992. Uracil DNA glycosylase-mediated cloning of PCR-amplified DNA: application to genomic and cDNA cloning. Anal. Biochem. **206:**91–97.
41. **Rashtchian, A., C. G. Thorton, and G. Heidecker.** 1992. A novel method for site-directed mutagenesis using PCR and uracil DNA glycosylase. PCR Methods Appl. **2:**124.
42. **Ratner, L., A. Fischer, L. L. Jagodzinski, H. Mitsuya, R.-S. Liog, R. C. Gallo, and F. Wong-Staal.** 1987. Complete nucleoside sequences of functional clones of the AIDS virus. AIDS Res. Human Retroviruses **3:**57–69.
43. **Reardon, J. E., and W. H. Miller.** 1990. Human immunodeficiency virus reverse transcriptase: substrate and inhibitor kinetics with thymidine 5′-triphosphate and 3′-azido-3′deoxythymidine 5′-triphosphate. J. Biol. Chem. **265:**20302–20307.
44. **Reddy, M. K., S. E. Weitzel, and P. H. von Hippel.** 1992. Processive proofreading is intrinsic to T4 DNA polymerase. J. Biol. Chem. **267:**14157–14166.
45. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
46. **Schuurman, R., M. Nijhuis, R. van Leewen, P. Schipper, D. de Jong, P. Collis, S. Danner, A. J. Mulder, C. Loveday, C. Christopherson, et al.** 1995. Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC). J. Infect. Dis. **171:**1441–1449.
47. **Shirasaka, T., S. Chokekijchai, A. Yamada, G. Gosselin, J.-L. Imbach, and H. Mitsuya.** 1995. Comparative analysis of anti-human immunodeficiency virus type 1 activities of dideoxynucleoside analogs in resting and activated peripheral blood mononuclear cells. Antimicrob. Agents Chemother. **39:** 2555–2559.
48. **Spina, C., A. J. C. Guatelli, and D. D. Richman.** 1995. Establishment of a stable, inducible form of human immunodeficiency virus type 1 DNA in quiescent CD4 lymphocytes in vitro. J. Virol. **69:**2977–2988.
49. **St. Clair, M. H., J. L. Martin, G. Tudor-Williams, M. C. Bach, C. L. Vavro, D. M. King, P. Kellam, S. D. Kemp, and B. A. Larder.** 1991. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science **253:**1557–1559.
50. **Stevenson, M., T. L. Stanwick, M. P. Dempsey, and C. A. Lamonica.** 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J. **9:**1551–1560.
51. **Tang, S., B. Patterson, and J. A. Levy.** 1995. Highly purified quiescent human peripheral blood $CD4^+$ T cells are infectible by human immunodeficiency virus but do not release virus after activation. J. Virol. **69:**5659–5665.
52. **Tantillo, C., J. Ding, A. Jacobo-Molina, R. G. Nanni, P. L. Boyer, S. H. Hughes, R. Pauwels, K. Andries, P. A. J. Janssen, and E. Arnold.** 1994. Locations of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase—implications for mechanisms of drug inhibition and resistance. J. Mol. Biol. **243:**369–387.
53. **von Schwedler, U., R. S. Kornbluth, and D. Trono.** 1994. The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T cells.

Proc. Natl. Acad. Sci. USA **91:**6992–6996.
54. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, M. S. Saag, and G. M. Shaw.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature (London) **373:**117–122.
55. **Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislip, and I. S. Chen.** 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile latent viral structure. Cell **61:**213–222.
56. **Zack, J. A., A. M. Haislip, and I. S. Y. Chen.** 1992. Incompletely reverse-transcribed human immunodeficiency virus type 1 genomes in quiescent cells can function as intermediates in the retroviral life cycle. J. Virol. **66:**1717–1725.
57. **Zhang, D., A. M. Caliendo, J. J. Eron, K. M. DeVore, J. C. Kaplan, M. S. Hirsch, and R. T. D'Aquila.** 1994. Resistance to 2′,3′-dideoxycytidine conferred by a mutation in codon 65 of the human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. **38:**282–287.