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The Antiretrovirus Drug 3′-Azido-3′-Deoxythymidine Increases the Retrovirus Mutation Rate

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It was previously observed that the nucleoside analog 5-azacytidine increased the spleen necrosis virus (SNV) mutation rate 13-fold in one cycle of retrovirus replication (V. K. Pathak and H. M. Temin, J. Virol. 66:3093–3100, 1992). Based on this observation, we hypothesized that nucleoside analogs used as antiviral drugs may also increase retrovirus mutation rates. We sought to determine if 3′-azido-3′-deoxythymidine (AZT), the primary treatment for human immunodeficiency virus type 1 (HIV-1) infection, increases the retrovirus mutation rate. Two assays were used to determine the effects of AZT on retrovirus mutation rates. The strategy of the first assay involved measuring the in vivo rate of inactivation of the *lacZ* gene in one replication cycle of SNV- and murine leukemia virus-based retroviral vectors. We observed 7- and 10-fold increases in the SNV mutant frequency following treatment of target cells with 0.1 and 0.5 μM AZT, respectively. The murine leukemia virus mutant frequency increased two- and threefold following treatment of target cells with 0.5 and 1.0 μM AZT, respectively. The second assay used an SNV-based shuttle vector containing the *lacZ*α gene. Proviruses were recovered as plasmids in *Escherichia coli*, and the rate of inactivation of *lacZ*α was measured. The results indicated that treatment of target cells increased the overall mutation rate two- to threefold. DNA sequence analysis of mutant proviruses indicated that AZT increased both the deletion and substitution rates. These results suggest that AZT treatment of HIV-1 infection may increase the degree of viral variation and alter virus evolution or pathogenesis.

Retrovirus populations demonstrate high levels of variation (3, 49). Genetic variation in retrovirus populations depends on the mutation and recombination rates per replication cycle, the replication rate (replication cycles/time), and the selective forces that act on the population (4–6, 17, 32, 33, 39–41). Recent studies of human immunodeficiency virus type 1 (HIV-1) dynamics in vivo suggest that approximately 10^4 to 10^5 mutations arise at each nucleotide position every day in an HIV-1-infected patient (5, 16, 54). The enormous degree of variation present in each HIV-1-infected individual has frustrated efforts to develop vaccines (8).

HIV-1 variation is associated with a number of clinically significant observations. Drug resistance to 3′-azido-3′-deoxythymidine (AZT), (−) 2′-deoxy-3′thiacytidine (3TC), 2′,3′-dideoxycytidine, and 2′,3′-dideoxyinosine monotherapy or combination therapy is associated with specific mutations in the virally encoded reverse transcriptase (RT) (9, 15, 28, 47, 48, 50). Accumulation of the specific mutations results in the outgrowth of drug-resistant virus refractory to the effects of the antiviral drugs (27). Once drug-resistant HIV-1 arises in the patient, virus loads can increase in the presence of the drug, causing an increased risk of clinical progression to AIDS (18). Resistance to drugs targeting other viral enzymes such as protease is also conferred by specific mutations in HIV-1 (7, 43). Additionally, HIV-1 cell tropism is associated with variation in the virus envelope, since specific mutations in the V3 loop of the envelope protein can confer the ability to infect T cells or macrophages (36). Thus, variation in HIV-1 populations plays a critical role in the course of the disease and treatment.

One important source of mutations in retrovirus genomes is the error-prone polymerization by RT (1, 19, 32, 33, 39–41, 44, 45, 49). RT lacks exonucleolytic proofreading; consequently, the fidelity of RT is low because the accuracy of polymerization depends solely on the discrimination of incoming nucleotides. Two host cell polymerases could also introduce mutations in retrovirus genomes. First, the host cell DNA polymerases that replicate the provirus through each cell division may introduce mutations in the virus genomes, but their error rate is 10^5- to 10^6-fold lower than that for RT, and so their contributions to retrovirus mutation rates are negligible (13, 23). Second, the host cell RNA polymerase II, which transcribes the provirus, may generate mutations in the retrovirus genomic RNA. Although evidence exists for proofreading during transcription by RNA polymerase in prokaryotes (29), the error rate of the eukaryotic RNA polymerase II has not been measured, and its contribution to the retrovirus mutation rate remains unknown.

The in vivo forward mutation rate for a single replication cycle was first determined for spleen necrosis virus (SNV) (40, 41). Subsequently, the in vivo forward mutation rates have been measured for the bovine leukemia virus (32), HIV-1 (33), and Moloney murine leukemia virus (MLV) (39). In these studies, the retrovirus mutation rates for a single replication cycle have ranged from 0.48×10^5 to 3.4×10^{-5}/bp/replication cycle.

Previously, it was shown that the nucleoside analog 5-azacytidine increased the retrovirus mutation rate 13-fold in a single cycle of retrovirus replication (42). The results suggested that 5-azacytidine increased retrovirus mutation rates through incorporation into RNA or DNA; alternatively, secondary effects on nucleotide pools may have increased the error rate. Based on these observations, we hypothesized that nucleoside analogs used as antiviral drugs may increase retrovirus mutation rates by disturbing nucleotide pools. We sought to determine the effects of the dideoxynucleoside analog AZT, the primary antiviral drug used for treatment of HIV-1 infection, on ret-

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rovirus mutation rates in a single retrovirus replication cycle. Two different assays were used to examine the effects of AZT on in vivo retrovirus mutation frequencies. These assays used SNV- or MLV-based retrovirus vectors encoding two mutational target genes (*lacZ* and *lacZ*α) and a drug resistance gene for selection of infected cells. Following infection, selected cells were analyzed for mutant phenotypes. The rates of inactivation of either of the mutational target genes were determined as an indicator of the retrovirus mutation rate in a single cycle of retrovirus replication. These experiments demonstrate that the antiretroviral drug AZT increases the retrovirus mutation rates of SNV and MLV.

MATERIALS AND METHODS

Plasmid construction. Standard molecular cloning procedures were used for construction of retrovirus vectors (46). SNV-based retrovirus vector pLW-1 was derived from pWH450 (gift from W.-S. Hu). pWH450 contains the long terminal repeats (LTRs), E site, primer binding site, and polypurine tract from SNV. An internal ribosomal entry site (IRES), present upstream of the hygromycin phosphotransferase B gene (*hygro*), allows the expression of *hygro* (14). pWH450 was digested with *Sma*I and then treated with calf intestinal phosphatase. The *lacZ* gene was inserted into this site as a blunt-ended 3.5-kb *Not*I-*Hin*dIII fragment from plasmid pSVβ (Clontech). MLV-based retrovirus vector pGA-1 was derived from pWH390 (gift from W.-S. Hu). pWH390 was generated by digesting pLAEN, an MLV-based retrovirus vector, with *Eco*RI, resulting in the deletion of the adenosine deaminase gene (gift from A. D. Miller). pWH390 contains the LTRs, ψ site, primer binding site, and polypurine tract from MLV. Additionally, an IRES is present upstream of the neomycin phosphotransferase gene (*neo*) from Tn*5* (20). pWH390 was digested with *Eco*RI and treated with Klenow enzyme and calf intestinal phosphatase. The *lacZ* gene was inserted as a blunt-ended 3.5-kb *Not*I fragment from pSVβ (Clontech). SNV-based retrovirus vector plasmid BK-2 was derived from vector VP254. Both of these vectors have been previously described (22, 42). Briefly, BK-2 contains the SNV *cis*-acting elements. The promoter in the LTR expresses the *hygro* gene. The *neo* gene is present and is expressed only in bacterial cells from a prokaryotic promoter. The pBR origin of replication allows BK-2 to replicate as a plasmid in bacteria. *lacZ*α is present in the 3′ LTR and is expressed only in bacterial cells. A detailed description of all cloning steps is available upon request.

Cells, transfections, and infections. D17 and C3A2 cells (obtained from the American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (ICN) supplemented with 6% bovine calf serum (HyClone Laboratories), penicillin (50 U/ml; Gibco), and streptomycin (50 μg/ml; Gibco). D17 is a dog osteosarcoma cell line that can be infected with SNV. C3A2 and DSDh are D17-derived reticuloendotheliosis virus-based helper lines that can be used to package SNV (17, 53). Hygromycin B (Calbiochem) was present in the media at a final concentration of 120 μg/ml (0.23 mM) for DSDh, C3A2, and D17 cells. DSDh and C3A2-derived helper cells were propagated in the presence of polyclonal anti-SNV antibodies. The antibodies were generated at the University of Wisconsin by infecting young chickens with reticuloendotheliosis virus. These antibodies have been used previously to suppress SNV reinfection (17, 22, 40–42). PG13 and PA317 cells (American Type Culture Collection) are MLV-based helper cell lines. PG13 and PA317 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). G418 was present at final concentrations of 600 μg/ml (0.79 mM) and 400 μg/ml (0.53 mM) in the media for PG13 and PA317 cells, respectively.

Helper cell clones producing LW-1, GA-1, and BK-2 viruses were derived by infecting fresh helper cells at a low multiplicities of infection (<0.00005). Therefore, the probability of obtaining cell clones with more than one provirus was less than 0.0005.

Cells were transfected by the previously described dimethyl sulfoxide-Polybrene method (21). D17 cells were plated at densities of 2×10^5 cells on 60-mm-diameter plates or 10^6 cells on 100-mm-diameter plates and subsequently infected with viruses. Twenty-four hours later, cells plated on 60-mm-diameter dishes were infected with 0.2 ml of virus and cells plated on 100-mm-diameter dishes were infected with 1 or 2 ml of virus by using Polybrene (50 μg/ml [final concentration]) as previously described (17). Infections using LW-1 or GA-1 were performed for 1 h with 1 ml of virus. Infections using BK-2 were performed for 4 h with 2 ml of virus. Transfected or infected cells were subjected to G418 or hygromycin selection 24 h later.

Staining LW-1 or GA-1 cells for β-galactosidase activity. Cells containing LW-1 or GA-1 were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) 14 days after selection was initiated, using previously described protocols (2). Briefly, following selection of cells containing LW-1 or GA-1, cells were fixed by using 1 ml of 0.05% glutaraldehyde in phosphate-buffered saline for 10 min at room temperature. The cells were rinsed three times with 4 ml of phosphate-buffered saline; the first rinse was for 1 min, the second rinse was for 10 min, and the final rinse was for 1 min. After the final rinse was removed, 1.25 ml of a solution containing 20 mM $K_3Fe(CN)_6$ (Sigma), 20 mM $K_4Fe(CN)_6$ (Sigma), 1.5 mM $MgCl_2$ (Fisher), and 1 mg of X-Gal (American Bioinorganics, Inc.) per ml was added to the plates. The plates were then sealed with Parafilm and incubated at 37°C for 24 h. The numbers of blue and white colonies were determined by viewing the cells under a light microscope at a magnification of ×40.

Recovery, restriction mapping, and DNA sequencing analysis of proviruses. Proviruses derived from infection of D17 cells with BK-2 were recovered by using the previously described Lac repressor protein-mediated affinity purification (22, 40). Transformed ElectroMax DH10B bacterial cells (Bethesda Research Laboratories/Life Technologies) had either blue, light blue, or white mutant colony phenotypes. Recovered mutant proviruses were analyzed by restriction digestions, gel electrophoresis, and DNA sequencing. Plasmid DNA sequencing was performed by using either a Sequenase kit and protocols (United States Biochemical) or AutoRead sequencing reactions and A.L.F. Express sequence analysis (Pharmacia).

RESULTS

A rapid in vivo assay to determine the effects of AZT on SNV and MLV mutant frequencies. A rapid in vivo assay was developed to measure the effects of antiretrovirus drugs on SNV and MLV mutant frequencies. The assay used the bacterial β-galactosidase gene (*lacZ*) as a reporter of mutations. An SNV-based retrovirus vector (LW-1) and an MLV-based retrovirus vector (GA-1) were constructed (Fig. 1A). Both vectors express the *lacZ* gene from the LTR promoter and a drug resistance gene from the IRES. The general strategy was to allow LW-1 and GA-1 to undergo one round of retrovirus replication, select for drug-resistant cell clones, and then determine the rate of inactivation of *lacZ*. β-Galactosidase activity in infected cells can be easily assayed by staining with X-Gal. In the presence of X-Gal, cells containing phenotypically wild-type LW-1 or GA-1 provirus express a functional β-galactosidase and stain blue; cells containing LW-1 or GA-1 provirus with inactivating mutations in *lacZ* fail to stain and appear white.

Experimental protocol used to determine the effects of AZT on retrovirus mutant frequencies. Helper cell clones producing LW-1 were established by first transfecting DSDh or C3A2 helper cells with pLW-1 (Fig. 1B). Viruses were harvested from these helper cells and used to infect fresh DSDh or C3A2 helper cells. After hygromycin selection, individual drug-resistant cell clones were isolated and expanded in the presence of anti-SNV antibodies to suppress reinfection of the virus-producing cells. The LW-1 vector was introduced into the helper cells by infection to avoid any mutations that may have occurred during transfection. In addition, helper cell clones were stained with X-Gal to verify that the *lacZ* gene was functionally active (data not shown).

A similar procedure was used to establish helper cell clones producing GA-1 virus. First, PA317 helper cells were transfected with pGA-1 (Fig. 1B). Viruses were harvested from the PA317 helper cells and used to infect PG13 helper cells. After selection for G418 resistance, individual cell clones were isolated and expanded. PG13 cells package vector RNA with gibbon ape leukemia virus envelope. PG13 cells are derived from mouse fibroblasts which do not express the receptor for the gibbon ape leukemia virus envelope; therefore, these virus-producing cells cannot reinfect themselves (37).

Next, a single cycle of retrovirus replication was carried out by harvesting virus from either the LW-1 or the GA-1 helper cell clones and infecting D17 target cells. The effects of AZT on the retrovirus mutant frequencies were determined by treating virus-producing cells or target cells with AZT and analyzing infected cells for inactivation of *lacZ*. Pretreatment refers to maintaining the helper cells in medium supplemented with AZT for 24 h before harvesting virus. AZT pretreatment could alter retrovirus mutation rates by affecting the accuracy

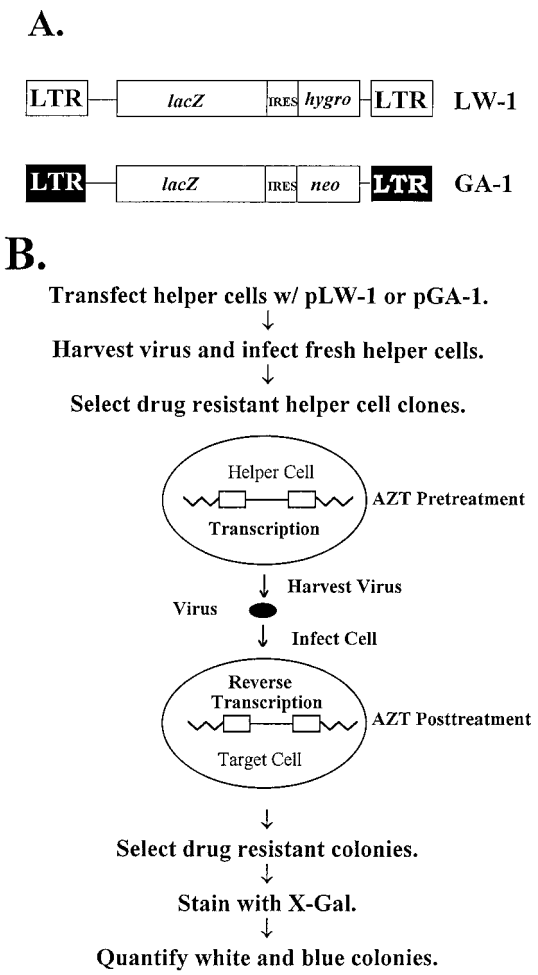

FIG. 1. Rapid in vivo assay to determine the effects of AZT on retrovirus mutation rates. (A) SNV-based retrovirus vector LW-1 and MLV-based retrovirus vector GA-1. White boxes, SNV LTRs; black boxes, MLV LTRs. LW-1 contains the LTRs and *cis*-acting elements from SNV. LW-1 transcribes the *E. coli lacZ* gene and the *hygro* gene from the promoter in the LTR. The *hygro* gene is expressed from the IRES of encephalomyocarditis virus. GA-1 contains the LTRs and *cis*-acting elements from MLV. GA-1 transcribes the *lacZ* gene and the *neo* gene from the promoter in the LTR. The *neo* gene is expressed from the IRES. (B) Experimental protocol. Helper cell clones producing LW-1 or GA-1 (DSDh or C3A2 for LW-1 and PG13 for GA-1) were generated by transfecting LW-1 or GA-1 plasmid DNA into helper cells, harvesting viruses, and infecting fresh helper cells. Following drug selection of cells infected with LW-1 or GA-1, individual colonies were isolated and expanded. Pretreatment is defined as maintaining virus-producing helper cells in 0.1 μM AZT supplemented medium for 24 h before harvesting virus. Posttreatment is defined as maintaining target cells in medium supplemented with various concentrations of AZT for 3 h before and 24 h following infection. Pre- plus posttreatment refers to performing both treatments in the same experimental groups. Proviral sequences are shown in the helper cell and target cell as boxes connected by a line; the zigzag lines represent host cell DNA. After hygromycin or G418 selection of infected cells, the drug-resistant colonies were stained with X-Gal and analyzed for β-galactosidase-deficient phenotype. The numbers of blue (wild-type) and white (mutant) colonies were determined, and the forward mutant frequency was calculated as the ratio of white to total (blue plus white) colonies.

of transcription of the provirus into RNA. Posttreatment refers to maintaining D17 target cells in media supplemented with various concentrations of AZT for 3 h before infection as well as 24 h after infection. Posttreatment with AZT may affect the mutation rate during only reverse transcription. These treatment times were used to ensure that AZT was present in the helper cells during transcription of the provirus into RNA in the pretreated cells and during the process of reverse transcription in the posttreated cells. Pre- plus posttreatment refers to maintaining both the helper cells and the target cells in medium supplemented with AZT. After drug selection, D17 cell colonies were stained with X-Gal. The numbers of blue and white colonies were determined 24 h after staining. The forward mutant frequency was calculated as the ratio of white colonies to the total number of colonies.

AZT increases the SNV mutant frequency in a dose-dependent manner. To determine whether AZT increases the retrovirus mutant frequency, a rapid in vivo assay was performed. C3A2 and DSDh helper cell clones producing LW-1 viruses were used. Viruses were harvested and used to infect D17 target cells in the presence of 0 to 0.50 μM AZT. The infected D17 cells were selected for hygromycin resistance, and the mutant frequencies were determined. Analysis of one DSDh helper cell clone (P1C1[d]) and two C3A2 helper cell clones (P1C1 and P5C1) is shown in Table 1. In the absence of AZT treatment, a mutant frequency of 0.05 (102 of 2,149) was observed. Posttreatment with 0.01 and 0.05 μM AZT resulted in mutant frequencies of 0.03 (28 of 915) and 0.07 (19 of 256), respectively. These mutant frequencies were similar to the control mutant frequency. In contrast, posttreatment with 0.10 and 0.50 μM AZT resulted in significantly higher mutant frequencies of 0.34 (742 of 2,188) and 0.51 (981 of 1,941), respectively. These mutant frequencies were 7-fold higher after 0.1 μM AZT treatment and 10-fold higher after 0.5 μM AZT treatment than the control mutant frequencies ($P < 0.0001$). These results indicated that the presence of AZT during reverse transcription increased the SNV mutant frequency in a

TABLE 1. Effects of AZT posttreatment on inactivation of the *lacZ* gene in LW-1-infected cells

AZT concn (μM)	LW-1 helper cell clone	No. of total colonies	No. of mutant colonies	Relative virus titer[a]	Mutant frequency[b]	Relative mutant frequency[c]
0.00	P1C1[d]	639	18		0.03	
	P1C1	926	47		0.05	
	P5C1	584	37		0.06	
	Total	2,149	102	1	0.05	1
0.01	P1C1[d]	915	28	0.7	0.03	1
0.05	P1C1[d]	256	19	0.2	0.07	1
0.10	P1C1[d]	117	26		0.22	
	P1C1	1,291	451		0.35	
	P5C1	780	265		0.34	
	Total	2,188	742	0.1	0.34	7
0.50	P1C1[d]	188	64		0.34	
	P1C1	546	297		0.54	
	P5C1	1,207	620		0.51	
	Total	1,941	981	0.01	0.51	10

[a] Virus titers for each experimental group were determined by serial dilution and infection. The relative virus titer represents a ratio of the viral titer of the treatment groups to the viral titer of the untreated group. The viral titer of the untreated group was 9.8×10^4 CFU/ml (average of three independent experiments).

[b] Ratio of mutant colonies to total colonies.

[c] Ratio of the mutant frequency of the treatment group to the mutant frequency of the untreated group. Statistical analysis of Fisher's least significant difference showed that the mutant frequencies after 0.1 and 0.5 μM AZT posttreatments were statistically different from the mutant frequency in the no-AZT treatment control ($P < 0.0001$). The mutant frequencies observed after 0.1 or 0.5 μM AZT posttreatments were also statistically different ($P = 0.01$).

[d] DSDh-derived helper cell clone. All other experiments were performed with C3A2-derived helper cell clones.

TABLE 2. Effects of pretreatment, posttreatment, and pre- plus posttreatment with 0.1 μM AZT on *lacZ* inactivation in LW-1

AZT treatment	Helper cell clones	No. of total colonies	No. of mutant colonies	Relative virus titer[a]	Mutant frequency[b]	Relative mutant frequency[c]
None	P1C1[d]	639	18		0.03	
	P1C1	189	13		0.07	
	P5C1	450	36		0.08	
	Total	1,278	67	1	0.05	1
Pretreatment	P1C1[d]	258	9		0.03	
	P1C1	116	9		0.08	
	P5C1	468	38		0.08	
	Total	842	56	0.80	0.07	1.4
Posttreatment	P1C1[d]	519	103		0.20	
	P1C1	610	224		0.37	
	P5C1	392	148		0.38	
	Total	1,521	475	0.14	0.31	6
Pre- plus posttreatment	P1C1[d]	313	76		0.24	
	P1C1	504	225		0.45	
	P5C1	283	110		0.39	
	Total	1,100	411	0.09	0.37	7

[a] Virus titers for each experimental group were determined by serial dilution and infection. The relative virus titer represents a ratio of the viral titer of the treatment group to the viral titer of the untreated group. The viral titer of the untreated group was 2.0×10^5 CFU/ml (average of three independent experiments).

[b] Ratio of mutant colonies to total colonies.

[c] Ratio of the mutant frequency of the treatment group to the mutant frequency of the untreated group. Statistical analysis of Fisher's least significant difference showed that the mutant frequencies after no treatment and pretreatment were not statistically different ($P = 0.30$). After posttreatment and pre- plus posttreatment, statistically different mutant frequencies were observed in comparison to the no-treatment control ($P < 0.00001$) but not between posttreatment and pre- plus posttreatment ($P = 0.22$).

[d] DSDh-derived helper cell clone. All other experiments were performed with C3A2-derived helper cell clones.

dose-dependent manner. After 0.01 and 0.05 μM AZT posttreatments, the relative virus titers were reduced to 0.7 and 0.2 in comparison to control virus titers. After 0.10 and 0.50 μM AZT posttreatments, relative virus titers were decreased to 0.10 and 0.01 in comparison to control virus titers. Thus, concentrations of AZT that reduced the virus titer 90 and 99% substantially increased the mutant frequencies 7-fold and 10-fold, respectively.

AZT increases the SNV mutant frequency primarily during reverse transcription. To determine the effects of AZT on retrovirus mutant frequencies in different stages of retroviral replication, pretreatments, posttreatments, and pre- plus posttreatments with 0.10 μM AZT were performed. These experiments were performed with 0.1 μM AZT because treatment of target cells with this concentration reduced virus titers 10-fold, ensuring that AZT was entering cells and affecting virus replication. In addition, 0.1 μM AZT is clearly within the range of clinically achievable AZT concentrations (38). The results are shown in Table 2. Viruses were harvested from the DSDh helper cell clone (P1C1[d]) and the C3A2 helper cell clones (P1C1 and P5C1). Control experiments were again performed to ensure that the control mutant frequencies were reproducible. A mutant frequency of 0.05 (67 of 1,278) was obtained, generating the same mutant frequency as the previous independent experiment. Pretreatment resulted in an overall mutant frequency of 0.07 (56 of 842), which was similar to the control mutant frequency ($P = 0.30$). However, posttreatment and pre- plus posttreatment resulted in higher mutant frequencies of 0.31 (475 of 1,521) and 0.37 (411 of 1,100), respectively ($P < 0.00001$). Pretreatment, posttreatment, and pre- plus posttreatment with AZT resulted in reductions of the virus titer to 0.8, 0.14, and 0.09, respectively, relative to the control virus titers. These results indicated that pretreatment did not have a significant effect on the SNV mutant frequency. However, posttreatment and pre- plus posttreatment with AZT significantly increased the mutant frequency. Therefore AZT increases the mutant frequency primarily during the treatment of target cells. The large increase in the mutant frequency after posttreatment demonstrated that AZT most likely decreases the accuracy of reverse transcriptase.

AZT also increases the MLV mutant frequency in a dose-dependent manner. To determine whether AZT increases the mutant frequency of MLV RT, MLV-based PG13 helper cell clones that produce GA-1 virus were used (Fig. 1A). Viruses were harvested from two different PG13 helper cell clones that produce GA-1 virus (clones 14 and 22). The results are presented in Table 3. In the absence of AZT treatment, an overall mutant frequency of 0.06 (87 of 1,527) was observed. Thus, the MLV control mutant frequency was similar to that of SNV. Posttreatments with 0.05 and 0.10 μM AZT resulted in reductions of virus titers to 0.25 and 0.08 relative to the control virus titers. Therefore, 0.10 μM AZT reduced the virus titers of both SNV and MLV to approximately 10% of the control virus titers. However, posttreatment of MLV-infected cells with 0.10 μM AZT increased the MLV mutant frequency only 1.5-fold, to 0.09 (202 of 2,173), in contrast to the 7-fold increase in the mutant frequency observed for SNV. Posttreatments with 0.50 and 1.0 μM AZT resulted in modest two- and threefold increases in the mutant frequencies ($P = 0.001$) and a substantial 99% reduction in the virus titers. These results demonstrated that MLV displayed a similar sensitivity to AZT, as reflected by similar decreases in virus titers at the same concentrations of AZT. However, the mutant frequency of MLV did not increase as much as the mutant frequency of SNV at similar levels of virus inhibition.

In addition to infecting D17 cells, we also infected human thymidine kinase-negative (TK^-) cells with GA-1. Human TK^- cells treated with AZT were infected with GA-1, selected with G418, and stained with X-Gal. We observed neither a decrease in virus titer nor an AZT-induced increase in the

TABLE 3. Effects of AZT posttreatment on inactivation of the *lacZ* gene in GA-1-infected cells

AZT concn (μM)	Helper cell clone	No. of total colonies	No. of mutant colonies	Relative virus titer[a]	Mutant frequency[b]	Relative mutant frequency[c]
0	14	1,124	67		0.06	
	22	403	20		0.05	
	Total	1,527	87	1	0.06	1
0.05	14	692	62		0.09	
	22	587	38		0.06	
	Total	1,279	100	0.25	0.08	1
0.10	14	784	62		0.08	
	22	1,389	140		0.10	
	Total	2,173	202	0.08	0.09	1
0.50	14	585	78		0.13	
	22	677	82		0.12	
	Total	1,262	160	0.01	0.13	2
1.0	14	1,069	177		0.17	
	22	555	98		0.18	
	Total	1,624	275	0.01	0.17	3

[a] Virus titers for each experimental group were determined by serial dilution and infection. The relative virus titer represents a ratio of the viral titer of the treatment groups to the viral titer of the untreated group. The viral titer of the untreated group was 1.5×10^5 CFU/ml (average of two independent experiments).

[b] Ratio of mutant colonies to total colonies.

[c] Ratio of the mutant frequency of the treatment group to the mutant frequency of the untreated group. Statistical analysis using a contrast to test for a linear response to the concentration of AZT demonstrated that the increase in mutant frequency with increasing AZT concentration is significant ($P = 0.001$).

GA-1 mutant frequency. This result indicated that phosphorylation of AZT is required for the virus inhibition as well as the increase in the mutant frequency (data not shown).

An in vivo assay using an SNV-based shuttle vector allows characterization of AZT-induced mutant proviruses. Since the *lacZ* gene is 3,474 bp in length, DNA sequence analysis of the *lacZ*-inactivating mutations would be time-consuming and laborious. To facilitate DNA sequencing of AZT-induced mutations, we used the previously described (22) SNV-based shuttle vector BK-2 (Fig. 2A). BK-2 contains all of the SNV *cis*-acting sequences needed for virus replication. In addition, BK-2 expresses *hygro* from the 5′ LTR promoter, contains *neo* that is expressed only in *Escherichia coli* from a prokaryotic promoter in the vector, has a pBR322 origin of replication for propagation of proviral plasmids in *E. coli*, and has *lacZ*α inserted in the U3 region of the 3′ LTR to serve as a mutational target. During reverse transcription, the *lacZ*α in the 3′ LTR is duplicated and is present in both LTRs of the resulting provirus. Since *lacZ*α is only 280 bp in length, it is relatively easy to characterize *lacZ*α-inactivating mutations by DNA sequencing.

The protocol used to generate pools of BK-2-infected cells and to recover proviral plasmids is outlined in Fig. 2B. The BK-2 DNA was transfected into the C3A2 helper cells, and pools of hygromycin-resistant helper cells were selected; viruses were harvested and used to infect fresh C3A2 cells. Again, hygromycin-resistant cells were selected, and individual cell clones were isolated and expanded in the presence of anti-SNV antibodies to suppress reinfection of the virus-producing cells. The BK-2 retrovirus vector was introduced into the C3A2 cells by infection to avoid any mutations that could occur during transfection. Viruses were harvested from the expanded C3A2 helper cell clones and used to generate pools of infected D17 target cells. To determine the effects of AZT on retrovirus mutation rates in different stages of replication, pretreatments, posttreatments, and pre- plus posttreatments with 0.10 μM AZT were performed.

Genomic DNAs were isolated from infected D17 cells and digested with *Bam*HI (Fig. 2). The proviral DNA was purified by using the Lac repressor protein-mediated affinity purification as previously described (22, 40). The purified proviral DNA was ligated and transformed into *E. coli* by electroporation. The resulting transformants were grown on agar plates containing kanamycin (a neomycin analog), isopropylthiogalactopyranoside, and X-Gal. Mutations in the *lacZ*α were identified by the white or light blue colony phenotype. The ratio of mutant colonies to total colonies recovered provided the in vivo forward mutant frequency.

AZT increases the mutation rate of SNV-based shuttle vector BK-2. To determine whether AZT also increases the rate of

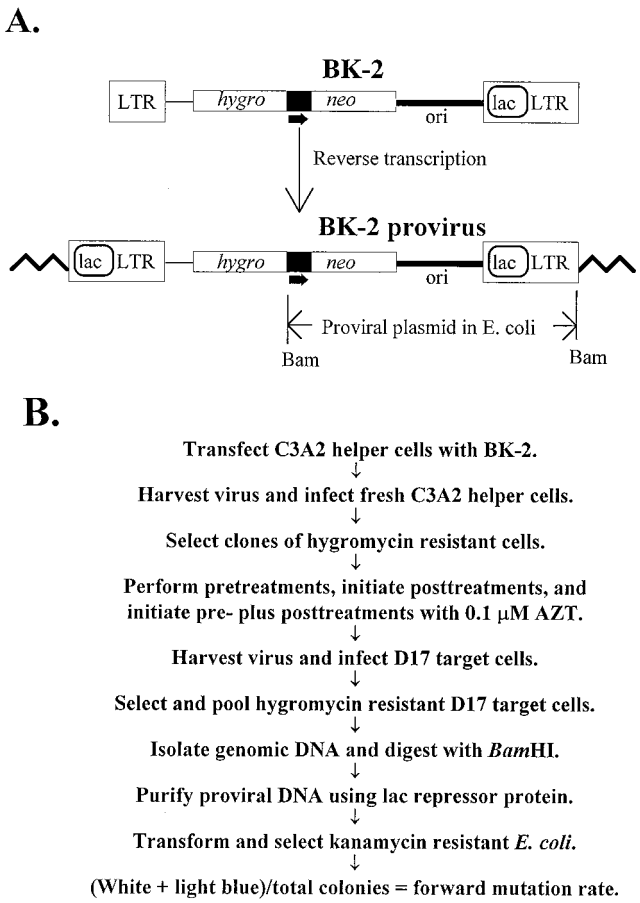

FIG. 2. An in vivo recovery assay to facilitate DNA sequence analysis of AZT-induced mutations. (A) The SNV-based vector BK-2 contains the LTRs, the *cis*-acting elements from SNV, *hygro*, *neo*, pBR322 bacterial origin of replication (ori), and *E. coli lacZ*α. The black square and solid black arrow represent a bacterial promoter that expresses *neo* in *E. coli* and the direction of transcription. The thick zigzag lines represent host chromosomal DNA adjacent to the integrated provirus, and Bam represents a *Bam*HI restriction site. *lacZ*α is inserted at the 5′ end of the U3 region of the 3′ LTR. The proviral plasmids recovered after *Bam*HI digestion (arrow below provirus) contain one *lacZ*α from the 3′ LTR. (B) Outline of the experimental approach for determining the effects of AZT on retrovirus mutation rates. Genomic DNA of infected D17 target cells was purified and digested with restriction enzyme *Bam*HI. Proviral plasmids were recovered by using Lac repressor protein-mediated affinity purification. The forward mutation rate was calculated by dividing the number of white plus light blue colonies by the total number of recovered colonies.

TABLE 4. Recovery of BK-2 proviral plasmids after 0.1 μM AZT treatments

Helper clone	No treatment		Pretreatment		Posttreatment		Pre- plus posttreatment	
	No. of mutants/total no. recovered[a]	Mutant frequency[b] (10^{-3})	No. of mutants/total no. recovered	Mutant frequency (10^{-3})	No. of mutants/total no. recovered	Mutant frequency (10^{-3})	No. of mutants/total no. recovered	Mutant frequency (10^{-3})
P1C2	27/8,843	3.05	22/6,101	3.61	22/3,959	5.56	30/3,250	9.23
P7C3	36/12,216	2.95	15/4,796	3.13	22/3,732	5.89	28/3,245	8.63
Total	63/21,059	2.99	37/10,897	3.40	44/7,691	5.72	58/6,495	8.93

[a] Number of white or light blue mutant colonies divided by the total number of colonies recovered (mutant plus wild type).
[b] Number of mutant colonies divided by the total number of colonies. Statistical analysis of Fisher's least significant difference showed that the mutant frequencies after no treatment and pretreatment were not statistically different ($P = 0.34$). In comparison to the no-treatment group, statistically different mutant frequencies were observed after posttreatment ($P = 0.002$) or pre- plus posttreatment ($P = 0.0004$). The mutant frequencies after posttreatment and pre- plus posttreatment were also significantly different ($P = 0.005$). Mutant frequencies after pretreatment were statistically different from the mutant frequencies following posttreatment ($P = 0.003$) and pre- plus posttreatment ($P = 0.0005$).

inactivation of the *lacZ*α reporter gene, the in vivo BK-2 mutant frequencies were determined (Table 4). Viruses were harvested from two C3A2-derived helper cell clones (P7C3 and P1C2) and used to infect D17 cells. Pretreatments, posttreatments, and pre- plus posttreatments with AZT were performed. If replication-competent viruses were present in the helper cell supernatants, the BK-2 vector may have undergone multiple rounds of replication by reinfection of the helper cells. Therefore, supernatants from the pools of infected cells were tested to rule of the presence of replication-competent SNV. No replication-competent viruses were detected, indicating that BK-2 underwent a single cycle of retrovirus replication (data not shown). Each treatment group contained at least 7×10^5 independent target cell colonies (data not shown). Genomic DNAs from each pool of D17 target cells were digested with *Bam*HI, and proviruses were recovered as plasmids. The phenotypes of the bacterial colonies were determined in the presence of X-Gal. From the control group (no AZT treatment), a total of 21,059 proviruses were recovered after *Bam*HI digestion (reported previously in reference 22); 63 of these colonies had a white or light blue mutant colony phenotype, providing a mutant frequency of 2.99×10^{-3} (1 of 334). A total of 10,897 colonies were recovered following pretreatment; 37 of these colonies displayed a mutant phenotype, resulting in a similar mutant frequency of 3.40×10^{-3} (1 of 295). This result indicated that pretreatment did not significantly increase the BK-2 mutant frequency ($P = 0.34$). A total of 7,691 colonies were recovered after posttreatment; 44 of these colonies displayed a mutant phenotype, providing a higher mutant frequency of 5.72×10^{-3} (1 of 175). This represented approximately a twofold increase in the mutant frequency ($P = 0.002$). A total of 6,495 colonies were recovered after pre- plus posttreatment; 58 of these displayed a mutant phenotype, providing the highest mutant frequency, 8.93×10^{-3} (1 of 112). This result indicated that pre- plus posttreatment increased the retrovirus mutant frequency approximately threefold ($P = 0.0004$). These results are consistent with those presented in Table 2 and indicate that AZT increases the retrovirus mutant frequencies of the *lacZ*α reporter gene during reverse transcription.

Characterization of mutant proviruses recovered after AZT treatment. To determine the types of mutations induced by AZT, DNA sequence analysis of the *lacZ*α genes of several mutant proviruses was performed. The results are presented in Table 5. A total of 37 of 63 mutants from the control group, 21 of 37 mutants after pretreatment, 22 of 44 mutants after post-

TABLE 5. In vivo mutation rates for SNV-based shuttle vector BK-2 following AZT treatments

Mutation class	Control[a]		Pretreatment		Posttreatment		Pre- plus posttreatment	
	No. of independent mutants/no. of mutants sequenced[b]	Mutation rate (10^{-6})[c]	No. of independent mutants/no. of mutants sequenced	Mutation rate (10^{-6})	No. independent mutants/no. of mutants sequenced	Mutation rate (10^{-6})	No. independent mutants/no. of mutants sequenced	Mutation rate (10^{-6})
Substitutions	12/20	14.3	12/12	17.2	11/12	27.6	10/21	46.1
Frameshifts	8/9	2.6	1/1	0.6	2/4	3.7	4/6	5.3
Simple deletions	3/3	0.9	6/6	3.5	2/4	3.7	5/6	5.3
Deletions with insertions	1/2	0.6	1/2	1.2	1/1	0.9	3/3	2.7
Duplications	1/1	0.3	0		0		0	
Hypermutations	2/2	0.6	0		1/1[d]	0.9	0	
Total	27/37	19.3	20/21	22.5	17/22	36.8	22/36	59.4

[a] The data represent a subset of the data previously described (22).
[b] The first number indicates the number of different mutants identified. If the same mutant was identified more than once in a pool, it was treated as one independent mutant. The second number indicates the total number of mutants sequenced.
[c] Number of mutations/base pair/replication cycle. Mutation rates were calculated as follows:

$$\frac{\text{no. of mutations identified} \times \text{total no. of mutants} \pm T}{\text{mutants sequenced total no. of colonies}}$$

Total number of mutants and total number of colonies recovered are listed in Table 4. For substitutions, $T = 113$ target nucleotides of the *lacZ*α (1). For frameshifts, simple deletions, deletions with insertions, duplications, and hypermutants, $T = 280$ target nucleotides (*lacZ*α and promoter region).
[d] The mutation rate within the hypermutated region was approximately 10^{-1}/bp (4 substitutions/41 nucleotides for hypermutant PO73:19, 21 substitutions/180 target nucleotides for P1C2B:73, and 8 substitutions/76 nucleotides for P7C3B:8).

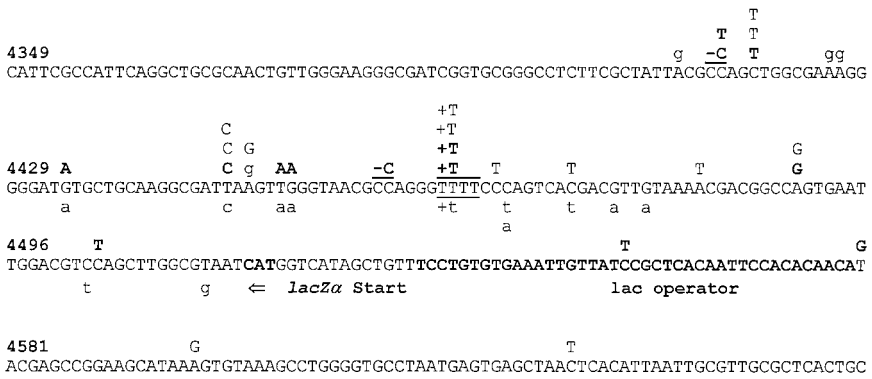

FIG. 3. Plus-strand nucleotide sequence of *lacZ*α and locations of substitutions, frameshifts, and A-to-G hypermutant mutations identified by DNA sequencing. Mutations identified in the pre- plus posttreatment group are shown as boldface uppercase letters above the sequence; mutations identified as the posttreatment group are shown as lightface uppercase letters above the sequence; mutations identified in the pretreatment group are shown as lightface lowercase letters below the sequence. The number above each line refers to the nucleotide number beginning at the start of the 5′ LTR. The runs of identical nucleotides in which frameshift mutations occurred are underlined. Lowercase g nucleotides shown above the sequence represent four A-to-G substitutions identified in one hypermutant provirus.

treatment, and 36 of 58 mutants after pre- plus posttreatment were analyzed. Most of the previously described types of mutations, including transition and transversion substitutions, frameshifts, simple deletions, deletions with insertions, duplications, and A-to-G hypermutations, were identified.

The rates of mutations for the variety of mutation types were calculated, and the overall rates of mutations were determined. The results suggested that pretreatment with AZT did not affect the mutation rate (22.5×10^{-6} mutations/bp/cycle versus 19.3×10^{-6} mutations/bp/cycle). In contrast, posttreatments and pre- plus posttreatments increased the mutation rates. Posttreatment with AZT increased the mutation rate approximately twofold (36.8×10^{-6} mutations/bp/cycle divided by 19.3×10^{-6} mutations/bp/cycle). Pre- plus posttreatment with AZT increased the mutation rate approximately threefold (59.4×10^{-6} mutations/bp/cycle divided by 19.3×10^{-6} mutations/bp/cycle). These results showed that the mutation rate of the *lacZ*α reporter gene was increased when AZT was present during reverse transcription.

The mutation rates of different types of mutations appeared to increase after AZT treatment. After pre- plus posttreatment with AZT, the rate of substitutions was approximately threefold higher (46.1×10^{-6} mutations/bp/cycle divided by 14.3×10^{-6} mutations/bp/cycle; $P < 0.0001$, chi square) and the rate of simple deletions was approximately sixfold higher (5.3×10^{-6} mutations/bp/cycle divided by 0.9×10^{-6} mutations/bp/cycle; $P < 0.05$, chi square). Posttreatment alone appeared to increase the rates of substitutions approximately twofold (27.6×10^{-6} mutations/bp/cycle divided by 14.3×10^{-6} mutations/bp/cycle), and simple deletions appeared to increase the rate of substitutions approximately fourfold (3.7×10^{-6} mutations/bp/cycle divided by 0.9×10^{-6} mutations/bp/cycle). Taken together, these results suggested that the presence of AZT during reverse transcription primarily increased the rates of these two types of mutations. No consistent increase in the mutation rates of frameshift mutations, deletions with insertions, hypermutations, and duplications were observed after AZT treatment.

Analysis of substitution mutations, frameshift mutations, and hypermutations. The locations of substitution mutations, frameshift mutations, and hypermutations are shown with the *lacZ*α sequence in Fig. 3. The majority (27 of 33) of the substitution mutations were transitions. C-to-T and G-to-A substitution mutations were the predominant substitutions identified in both the control and the AZT-treated groups. These two types of transitions represented 67% (8 of 12) of the substitutions following pretreatment, 54% (6 of 11) of the substitutions following posttreatment, and 60% (6 of 10) of the substitutions following pre- plus posttreatment. The ratio of transition substitutions to transversion substitutions was not altered by AZT treatment. Transition substitutions comprised 67% (8 of 12), 91% (10 of 11), and 80% (8 of 10) of the substitution mutations following pretreatments, posttreatments, and pre- plus posttreatments, respectively. Thus, the ratio of transition to transversion substitutions was similar to the ratios previously observed in the absence of AZT when *lacZ*α was used as a reporter of mutations (80%) (22, 40, 42). The nucleotides adjacent to substitution mutations were analyzed to see if dislocation mutagenesis during plus- or minus-strand DNA synthesis could account for the increase in the substitution frequency. Overall, 12 of 33 substitution mutations may have occurred by dislocation mutagenesis. The proportions of substitutions that may have occurred by dislocation mutagenesis were similar following pretreatment (4 of 12) and pre- plus posttreatment (3 of 10) but higher following posttreatment (6 of 11). Therefore, the apparent AZT-induced increase in the rate of substitutions did not result in any change in the type of substitutions or the mechanism by which the substitutions occurred.

At least four independent frameshift mutations were identified in the pre- plus posttreatment group (Fig. 3). One posttreatment mutant exhibited multiple A-to-G substitutions, constituting an A-to-G hypermutation. The hypermutant PO73:19 had a region of 41 bp in which 4 of 11 (36%) adenines were mutated to guanines. This A-to-G hypermutation was similar to two other hypermutations described previously (22).

Deletions and deletions with insertions. Analysis of proviral clones recovered after AZT pretreatments, posttreatments, and pre- plus posttreatments revealed 13 independent simple deletions and 5 independent deletions with insertions (Fig. 4). All 13 simple deletions contained short direct repeats at the deletion junctions ranging in size from 1 to 6 bp (Fig. 4A). Interestingly, a comparison of the average size of the direct repeats identified from the AZT-treated groups and the previously described untreated groups suggested that the AZT-induced simple deletions involved shorter direct repeats. The average size of the direct repeats identified from the untreated groups was 4.1 bp (10 simple deletions) (22, 41). In contrast,

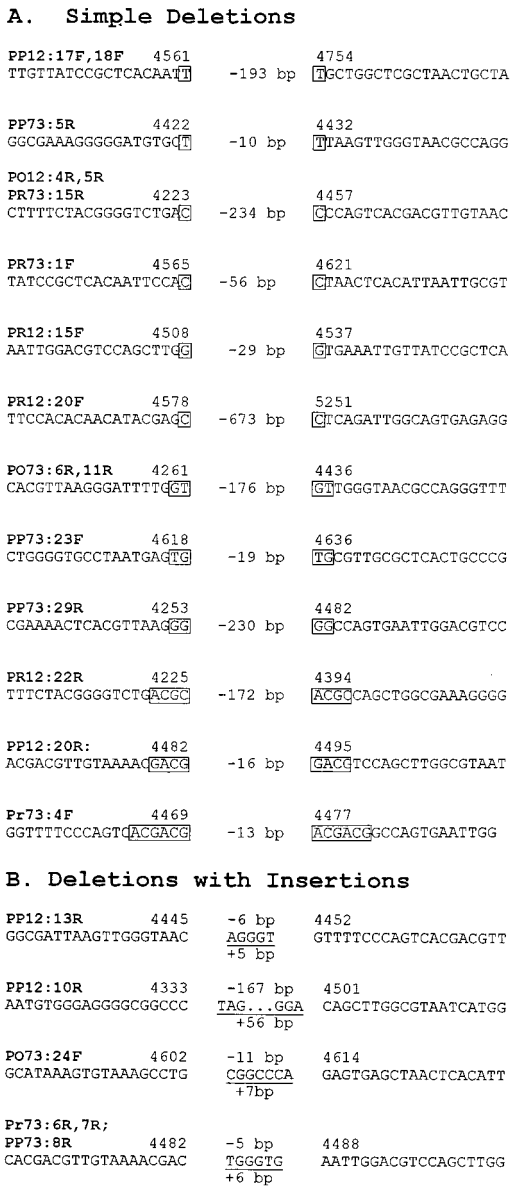

FIG. 4. Sequence analysis of deletions and deletions with insertions. Clone numbers are shown above the sequence in boldface. (A) Simple deletions. Short direct repeats at the deletion junctions are shown in boxes. The numbers of nucleotides deleted are indicated between the deletion junctions preceded by a minus sign. (B) Deletions with insertions. The numbers of nucleotides deleted are indicated between deletion junctions preceded by a minus sign. Inserted nucleotides are underlined, and the lengths of insertion are shown below the sequence.

the average size of the direct repeats identified from the AZT-treated groups was 2.1 bp (13 simple deletions). Therefore, AZT pre- plus post-treatment increased the rate of simple deletions which was associated with a decrease in the size of the direct repeats at the deletion junctions ($P < 0.05$, t test). This result suggested that AZT treatment reduced the processivity of RT.

Five independent deletions with insertions containing short insertions at the site of the deletions were identified (Fig. 4B). No differences were noted with the size of the deletion or insertion between mutants recovered with or without AZT treatment.

DISCUSSION

AZT increases the retrovirus mutation rate. Our experiments clearly indicate that the antiretrovirus drug AZT increases the mutation rates of type C retroviruses. The mutation rates were measured for SNV and MLV during a single cycle of retrovirus replication using two different mutation reporter genes. AZT affected the SNV mutation rate to a much greater extent than the MLV mutation rate, since 0.5 μM AZT increased the SNV mutation rate 10-fold, whereas 1.0 μM AZT increased the MLV mutation rate only 3-fold. The same reporter gene (*lacZ*) and target cells (D17) were used in the two studies, and the observed difference between the MLV and SNV mutation rates following 0.5 μM AZT posttreatment was statistically significant ($P = 0.01$, t test). The difference in sensitivities to the mutagenic effects of AZT may be related to the differences in intrinsic mutation rates of the two RTs (51). However, in our experiments, we observed similar rates of inactivation of the *lacZ* gene in GA-1 and LW-1, suggesting that the mutation rates of the two RTs are similar. Recently, it was observed that AZT treatment increased the emergence of host range mutants in a replicating feline immunodeficiency virus population (26). The studies did not directly measure mutation rates in a single replication cycle. Nevertheless, these two studies strongly suggest that AZT is likely to increase the mutation rates of most retroviruses.

Possible mechanisms for AZT-induced increase in the retrovirus mutation rates. Several potential mechanisms may be responsible for AZT-induced increase in the retrovirus mutation rates. One attractive hypothesis is that the increase in the retrovirus mutation rate results from the effect of AZT on target cell nucleotide pools. AZT has been shown to affect the balance of nucleotide pools by competing with thymidine for the host cell enzyme TK (10, 11). Ultimately, nucleotide pools change due to the complex mechanisms regulating the reduction of ribonucleotides to deoxyribonucleotides by ribonucleotide reductase (10). The pool imbalances result in increased levels of dCTP and decreased levels of dTTP and dGTP in the AZT-treated cells. It has been demonstrated that nucleotide pool imbalances can increase the mutation rates of eukaryotic polymerases in vivo (24, 25, 30, 35). Thus, nucleotide pool imbalances may also affect the in vivo mutation rates of RTs. In addition, in vitro mutation assays have shown that nucleotide pool imbalances can significantly increase the rates of G-to-A hypermutations (34). MLV RT may be less sensitive to nucleotide pool changes, which may explain the lower increase in the MLV mutation rate. Clearly, additional experiments are needed to determine whether AZT affects the retrovirus mutation rates by altering nucleotide pools. Another possible mechanism may involve incorporation of AZT into the plus-strand DNA; discontinuous plus-strand DNA synthesis may result in viral DNAs with proper ends that can integrate into the target cell DNA. Subsequently, host cell DNA repair mechanisms may carry out error-prone DNA repair and increase the virus mutation rate. It should be noted that the same target cells were used in the SNV and MLV studies. Finally, AZT may bind noncatalytically to RT and cause a conformational change that affects enzyme fidelity. If so, then AZT can bind noncatalytically to RTs as structurally diverse as those from SNV, MLV, and feline immunodeficiency virus.

Possible effects of AZT on the processivity of RT. AZT clearly increased the BK-2 mutation rate. When AZT pre- plus post-treatment was performed, the rate of substitutions was increased approximately threefold ($P < 0.0001$, chi square), and the rate of simple deletions was increased by approximately sixfold ($P < 0.05$, chi square). The increase in the rate

of simple deletions suggests the hypothesis that the presence of AZT during reverse transcription increases the rate of template switching and decreases the processivity of RT, perhaps indirectly through alterations in nucleotide pools. (Processivity is defined here as a rate of dissociation of RT-primer complex from the template and does not imply a dissociation of the RT from the primer-template complex.)

AZT treatment of HIV-1 infection may increase variation in virus populations. It is not clear how AZT treatment will affect the HIV-1 mutation rate or variation in HIV-1 populations. First, since the MLV mutation rate was affected to a lesser extent than the SNV mutation rate, the HIV-1 mutation rate may be affected to a lesser or greater extent than the SNV mutation rate. Second, if the hypothesis that nucleotide pool imbalances affect the RT mutation rate is correct, then the extent to which AZT affects the nucleotide pools of HIV-1 target cells will be a critical factor in determining the AZT effect on HIV-1 RT mutation rates. AZT concentrations used in this study (0.1 to 1 μM) were much lower than the concentrations that can be clinically achieved (>6 μM), suggesting that AZT treatment may have a greater impact on the nucleotide pools of HIV-1 target cells in patients (38).

It is arguable whether alterations in the mutation rates will affect variation in HIV-1 populations. A model has been proposed to ascertain the relative impact of mutation rates and selective forces on the virus population (5). Based on this model, it has been hypothesized that small changes in the selective growth advantage will have a greater impact on the mutant frequencies than large changes in the mutation rates. It was recently suggested that 3TC-resistant mutant isolates of HIV-1 have lower mutation rates and a delayed kinetics of development of drug resistance to other antiretroviral drugs (52). Further studies are needed to determine the role of HIV-1 mutation rates in viral evolution and pathogenesis.

Nucleotide pool imbalances induced by other drugs may also increase the mutation rate of RT. It has been demonstrated that hydroxyurea (HU) can inhibit HIV-1 replication (12, 31). HU inhibits ribonucleotide reductase and alters the nucleotide pools within the cell (30). It is possible that these alterations of the nucleotide pools also increase the mutation rate of the HIV-1 RT. The effects of HU and 3TC on the retrovirus mutation rates are currently being determined.

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