JOURNAL OF VIROLOGY, Aug. 1995, p. 5087–5094
0022-538X/95/$04.00+0
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Vol. 69, No. 8

Lower In Vivo Mutation Rate of Human Immunodeficiency Virus Type 1 than That Predicted from the Fidelity of Purified Reverse Transcriptase†

LOUIS M. MANSKY* AND HOWARD M. TEMIN‡

McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, Wisconsin 53706

Received 15 February 1995/Accepted 10 May 1995

The level of genetic variation of human immunodeficiency virus type 1 (HIV-1), a member of the lentivirus genus of the *Retroviridae* family, is high relative to that of retroviruses in some other genera. The high error rates of purified HIV-1 reverse transcriptase in cell-free systems suggest an explanation for this high genetic variation. To test whether the in vivo rate of mutation during reverse transcription of HIV-1 is as high as predicted by cell-free studies, and therefore higher than that rates of mutation of retroviruses in other genera, we developed an in vivo assay for detecting forward mutations in HIV-1, using the *lacZ*α peptide gene as a reporter for mutations. This system allows the rates and types of mutations that occur during a single cycle of replication to be studied. We found that the forward mutation rate for HIV-1 was 3.4×10^{-5} mutations per bp per cycle. Base substitution mutations predominated; G-to-A transition mutations were the most common base substitution. The in vivo mutation rates for HIV-1 are three and seven times higher than those previously reported for two other retroviruses, spleen necrosis virus and bovine leukemia virus, respectively. In contrast, our calculated in vivo mutation rate for HIV-1 is about 20-fold lower than the error rate of purified HIV-1 reverse transcriptase, with the same target sequence. This finding indicates that HIV-1 reverse transcription in vivo is not as error prone as predicted from the fidelity of purified reverse transcriptase in cell-free studies. Our data suggest that the fidelity of purified HIV-1 reverse transcriptase may not accurately reflect the level of genetic variation in a natural infection.

Retroviruses are RNA viruses that replicate through a DNA intermediate (4, 51). The viral RNA is copied into DNA by reverse transcription, a process that is catalyzed by the virus-encoded enzyme reverse transcriptase. The process of reverse transcription is error prone, contributing to the high genetic variability of these viruses (13, 22, 27, 48). Abnormal strand transfers during reverse transcription have been proposed to be responsible for the error-prone nature of this process (50). Genetic variation of retroviruses is the composite of the mutation rate per replication cycle, the number of replication cycles, and the fixation rate of mutations (i.e., the selective advantage or disadvantage possessed by the variant virus) (15). In addition, the rate of recombination of retroviruses is high and greatly contributes to genetic variation (24, 58).

The level of genetic variation of human immunodeficiency virus type 1 (HIV-1) is high (1, 3, 12, 20, 32, 45, 56) relative to that of some other members of the *Retroviridae* family (18, 40, 55). The high error rates of purified HIV-1 reverse transcriptase determined with various target sequences (2, 8, 25, 41, 57) have been used to suggest that the high genetic variation of HIV-1 may be due to errors occurring during the process of reverse transcription. In particular, error rates determined with a DNA template of the *lacZ*α peptide gene ranged from 5×10^{-4} to 6.7×10^{-4} (42). Error rates determined with an RNA template of the *lacZ*α peptide gene were similar to (26) or about 2.5-fold lower (9) than that determined with DNA templates. These observations have been used to hypothesize that the mutation rate for HIV-1 is comparable to the error rate of purified HIV-1 reverse transcriptase (35).

To test this hypothesis, we have developed a system to measure forward mutation rates with an HIV-1 vector containing the *lacZ*α peptide gene as a reporter for mutations. This system allowed us to study the mutations that occur during a single round of HIV-1 replication. The mutation rate of HIV-1 in this system was determined to be 3.4×10^{-5} mutations per bp per cycle. The most commonly detected mutations were base substitution mutations (G-to-A and C-to-T transition mutations) and frameshift mutations (−1 frameshifts in runs of T's and A's). The mutation rate was not affected by the orientation of the *lacZ*α peptide gene in the vector. The in vivo mutation rate is maximally 5% of the combined reported error rates for purified HIV-1 reverse transcriptase when sense-strand RNA and DNA templates of the *lacZ*α peptide gene are used. Our study indicates that the mutation rate of HIV-1 is less than that predicted by the measured fidelity of purified HIV-1 reverse transcriptase.

MATERIALS AND METHODS

Nomenclature. Plasmids containing vectors are designated "p" (e.g., pHIV shuttle 3.12). Names without the "p" (e.g., HIV shuttle 3.12) denote viruses (or proviruses) derived from this vector. All sequence coordinates are relative to the 5′ end of the 5′ long terminal repeat in the plasmid.

Vectors and expression plasmids. Plasmid pHIV shuttle 3.12 was constructed by using a derivative of a plasmid containing the proviral DNA of the NL4-3 strain of HIV-1 (Fig. 1A). This plasmid, pGB 107, contains a deletion in the *env* gene and the creation of a *Nhe*I site (10). A deletion in the *gag-pol* region was made by removal of the region bounded by restriction sites for *Swa*I, creating pGB107ΔSwa. A cassette containing the simian virus 40 promoter driving expression of the neomycin phosphotransferase gene (*neo*), an origin of replication from pACYC 184, and the *lacZ*α peptide gene from the vector BLV-SVNEO/ACYCLacZ (34) was amplified by PCR with primers containing the *Spe*I restriction site. The amplified DNA was digested with *Spe*I and inserted into the *Nhe*I site of pGB107ΔSwa to create pHIV shuttle 3.12 (Fig. 1B). The *lacZ*α peptide gene was originally from the vector pSU20 (5). An in-frame deletion was intro-

* Corresponding author. Mailing address: McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, 1400 University Ave., Madison, WI 53706. Phone: (608) 262-2024. Fax: (608) 262-2824. Electronic mail address: mansky@oncology.wisc.edu.

† This paper is dedicated to the memory of Howard M. Temin.

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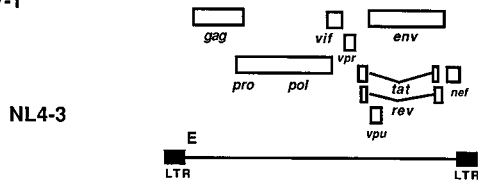

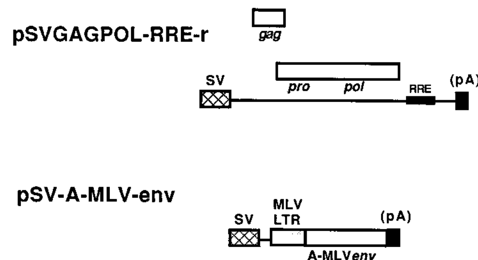

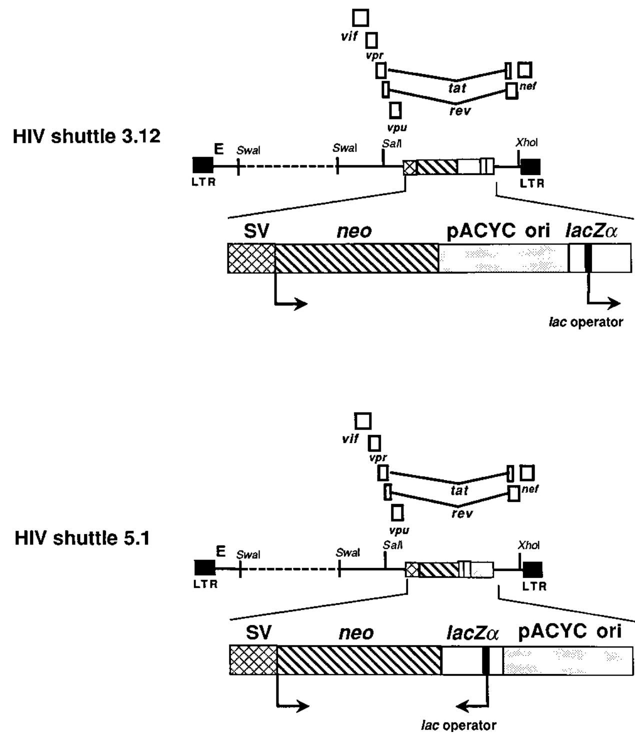

FIG. 1. HIV-1 and the HIV-1 shuttle vectors and helper plasmids used for the mutation rate studies. The HIV-1 and HIV-1 shuttle vectors are shown in the proviral DNA forms. Solid black boxes represent the HIV-1 long terminal repeats (LTRs). Solid black lines indicate viral sequences. Rectangular boxes above or below the solid black lines indicate viral coding sequences, with the relative locations of the boxes corresponding to the translational reading frame. Retroviral genes are indicated as *gag*, *pro*, *pol*, *env*, *vif*, *vpr*, *tat*, *rev*, *vpu*, and *nef*. Bent lines between the coding regions for *tat* and *rev* in panels A and B show reading frames joined by splicing events. E in panels A and B indicates the location of the encapsidation signal. In panels B and C, the simian virus 40 promoter (SV) is represented as a cross-hatched box, the *neo* gene is represented as a hatched rectangular box, the pACYC origin of replication is represented as the light gray box, and the *lacZ*α peptide gene is represented as an open rectangular box with a black band representing the *lac* operator sequence. Dashed lines in panel B indicate deleted viral coding sequence removed by digestion with *Swa*I. The *Sal*I and *Xho*I sites in panel B were used in the purification of the vector proviral DNA containing the *neo* gene, the pACYC origin of replication, and the *lacZ*α peptide gene. The light gray box in panel C represents the murine leukemia virus long terminal repeat (MLV LTR), the wide black box represents the HIV-1 *rev* response element (RRE), and the thin black box represents the simian virus 40 late gene polyadenylation signal (pA).

duced into the *lacZ*α peptide gene polylinker of pSU20 by digestion with *Xho*I and *Eco*RI, filling in ends with the Klenow fragment of DNA polymerase, and religation with T4 DNA ligase.

The construction of vector pHIV shuttle 5.1 was similar to the construction of pHIV shuttle 3.12, with the exception of the cassette inserted into pGB107ΔSwa. The cassette used for insertion into the *Nhe*I site of pGB107ΔSwa was from a bovine leukemia virus (BLV) shuttle vector, pBLV-SVNEO/LacZACYC (33a), with the pACYC origin of replication and the *lacZ*α peptide gene sequences in the opposite orientation relative to the *neo* gene (Fig. 1B).

The HIV-1 *gag-pol* expression plasmid used, pSVGAGPOL-RRE-r (Fig. 1C) (44), was kindly provided by David Rekosh, University of Virginia. This expression plasmid contains the simian virus 40 promoter driving expression of the HIV-1 *gag-pol* genes. The amphotropic murine leukemia virus *env* expression plasmid used, pSV-A-MLV-env (Fig. 1C) (31), was provided by Dan Littman, University of California, San Francisco.

Cells and media. The HeLa and COS-1 cell lines used were obtained from the American Type Culture Collection (Rockville, Md.) and were maintained in Temin's modified Eagle's medium (49) containing 10% calf serum and 10% fetal bovine serum, respectively.

Transfections, infections, and cocultivations. HIV-1 vectors and expression plasmids were transfected into COS-1 or HeLa cells by use of dimethyl sulfoxide-Polybrene (28). HeLa cells were infected in the presence of Polybrene (23). Infection of HeLa target cells was done by cocultivation of virus-producing cells with target cells (33, 34). Briefly, virus-producing cells (typically 2.5×10^5 cells in a 60-mm-diameter petri dish) were treated with mitomycin C (10 μg/ml), an inhibitor of host cell DNA synthesis, for 2 h at 37°C. The cells were then washed three times with fresh medium, and 2.5×10^5 HeLa target cells were added. Two days after cocultivation, selective medium containing G418 was added. Control experiments were done with each cocultivation experiment to ensure that mitomycin C-treated, virus-producing cells did not proliferate and no longer adhered to the surfaces of culture dishes. Cells expressing the *neo* gene were selected with the neomycin phosphotransferase analog G418.

Protocol for a single cycle of retrovirus replication. The experimental protocol developed to obtain a single cycle of HIV-1 shuttle vector replication is shown in Fig. 2. The protocol contains three steps. In step 1, the HIV-1 shuttle vector was introduced into COS-1 cells by transfection and placed under G418 selection. Cell clones were then transiently transfected with the helper plasmids pSVGAG POL-RRE-r and pSV-A-MLV-env. In step 2, vector virus was harvested 48 h posttransfection from step 1 cells and used to infect fresh HeLa cells. Step 2 clones were tested by Southern analysis to ensure that only a single vector proviral DNA was present. The *lacZ*α peptide gene in the vector proviral DNA of step 2 clones was sequenced to confirm that no mutations were introduced. G418-resistant cell clones were transiently transfected with the two helper plasmids (step 2 cells). In step 3, vector virus was transferred to fresh HeLa target cells by cocultivation; cells were then placed under G418 selection (step 3 cells). Cocultivation was used to produce step 3 cells because it was desirable to obtain the largest number of step 3 cells for analysis of the mutation rate.

Recovery of proviral DNA and DNA sequencing. Purified genomic DNA (43) from pools of step 3 clones was digested with the restriction enzymes *Sal*I and *Xho*I, to release the *neo*, pACYC origin of replication, and *lacZ*α peptide gene sequences from the HIV-1 shuttle vector proviral DNA (Fig. 1B). Proviral DNA was purified with the Lac repressor protein (Promega Corp., Madison, Wis.) by a modification (34) of a protocol developed by Pathak and Temin (36). The purified proviral DNA was ligated and used to electroporate competent *Escherichia coli* XLI Blue cells. Kanamycin-resistant bacterial colonies were selected in the presence of the 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (IPTG) inducer. The ratio of white plus light blue bacterial colonies to total bacterial colonies observed provided a forward mutation rate for a single retroviral replication cycle. Plasmid DNA was purified (43) and sequenced in the *lacZ*α peptide gene region with a nonradioactive DNA sequencing kit (Silver sequencing kit; Promega Corp.).

RESULTS

Replication of HIV-1 shuttle vectors. The HIV-1 shuttle vectors (Fig. 1) contained deletions in the *gag-pol* and *env* genes with an insertion, in the *env* gene, of a cassette containing the *neo* gene, the pACYC origin of replication, and the *lacZ*α peptide gene. These vectors replicate in mammalian cells as viruses and can be selected with the neomycin analog G418. The vectors can replicate in *E. coli* as plasmids and are

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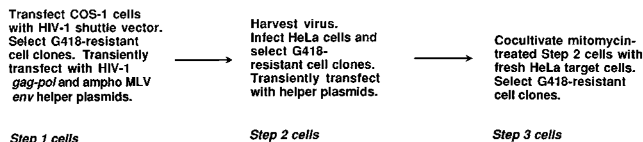

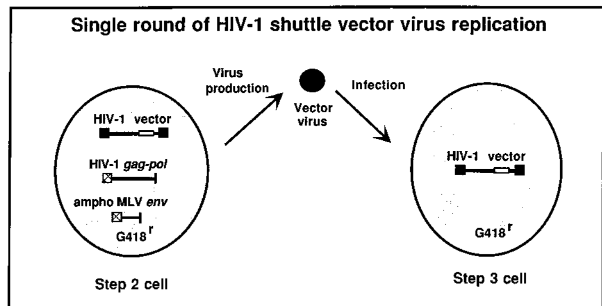

FIG. 2. Experimental protocols for studying one round of HIV-1 shuttle vector virus replication. In step 1, COS-1 cells were transfected with pHIV shuttle 3.12 or pHIV shuttle 5.1 and were placed under G418 selection. These cells were transiently transfected with the HIV-1 *gag-pol* expression plasmid, pSVGAGPOL-RRE-r, and the amphotropic murine leukemia virus (ampho MLV) *env* gene, pSV-A-MLV-env. Forty-eight hours posttransfection, virus was harvested and used to infect HeLa cells. G418-resistant cell clones were transiently transfected with pSVGAGPOL-RRE-r and pSV-A-MLV-env (step 2 cells). Forty-eight hours posttransfection, step 2 cells were treated with mitomycin C and cocultivated with fresh HeLa target cells, and all cells were placed under G418 selection (step 3 cells).

selected with the drug kanamycin. To be packaged into a virus particle, these vectors are complemented in *trans* by transient transfection of cells with an HIV-1 *gag-pol* expression plasmid and an amphotropic murine leukemia virus *env* expression plasmid.

Vector virus produced from either COS-1 or HeLa cells was used to infect fresh HeLa target cells (Fig. 2). Titers obtained from both COS-1 and HeLa cells were 120 CFU/ml (data not shown). Cocultivation of mitomycin C-treated step 2 cells (mitomycin C is an inhibitor of host cell DNA synthesis and led to cell death of step 2 cells as described in Materials and Methods) with fresh HeLa target cells to produce step 3 cells led to titers of 1×10^3 to 4×10^3 CFU/2.5×10^5 HeLa target cells (data not shown). Cocultivation was used to produce step 3 cells because it was desirable to obtain the largest number of step 3 cells for analysis of the mutation rate. The steps going from a parental shuttle vector provirus in the step 2 cells to a vector provirus in the step 3 cells constitute a single cycle of replication (Fig. 2). These steps include transcription of the proviral DNA by the cellular transcription machinery, packaging of the viral RNA, release of viral particles, infection of target cells, reverse transcription, and integration of newly synthesized viral DNA to generate a vector provirus. Southern analysis of total DNA from each step 2 cell clone was done to ensure that each cell clone used contained only one provirus (data not shown). The *lacZ*α gene region in the vector proviral DNA of each step 2 clone was sequenced to confirm that no mutations had been introduced in the steps leading to the vector provirus in the step 2 cell (data not shown).

Mutation frequency, type, and location observed with HIV-1 shuttle 3.12. The proviral DNA from pooled step 3 cells representing over 60,000 different cell clones was purified with the Lac repressor protein and introduced into *E. coli* to screen for mutations in the *lacZ*α gene region. Vector DNAs with mutations at target nucleotides previously determined to lead to a phenotypic change (6, 37) were detectable as bacterial colonies with a white or light blue colony color phenotype. We screened 8,678 bacterial colonies for HIV-1 shuttle 3.12 proviral DNAs containing mutations in the *lacZ*α peptide gene (Table 1). Thirty-eight of these colonies had a white or light blue colony color phenotype. The mutation frequency was 38/8,678, or 4.4×10^{-3} mutations per cycle. To determine the mutations in the *lacZ*α peptide gene and to calculate the mutations per base pair per cycle, plasmid DNA from these clones was analyzed by DNA sequencing in this region.

TABLE 1. Mutation frequencies in recovered proviruses for HIV shuttle 3.12

Step 2 clone no.	No. of mutants/total no. of bacterial colonies	Mutation frequency (10^{-3})
1	10/2,140	4.7
2	5/2,401	2.1
3	18/2,979	6.0
1	5/1,158	4.3
Total	38/8,678	4.4[a]

[a] The average mutation frequency with a standard error of 0.8×10^{-3} mutant per cycle.

Twenty-five of the thirty-eight mutants were found to have base substitution mutations, with the majority being transition mutations of G to A and C to T (15 and 6, respectively) (Table 2). Two of the mutants containing a G-to-A transition mutation contained an additional G-to-A transition. Inspection of the G-to-A mutations revealed that 13 of 17 occurred within a GpA dinucleotide; 3 of 17 G-to-A mutations occurred within a GpG dinucleotide. Twelve of the seventeen G-to-A mutations occurred within a 24-base region of the *lacZ*α peptide gene (bases 7283 to 7307 in HIV shuttle 3.12). Nine of the thirty-eight mutants had frameshift mutations. The majority (five of nine) of the frameshift mutations occurred in runs of four nucleotides, three in runs of T's and two in runs of A's. Four frameshifts occurred in runs of three nucleotides, two in runs of T's and one each in runs of A's and C's. Mutations in runs

TABLE 2. Base pair substitution, frameshift, and deletion mutations for HIV shuttle 3.12

Nucleotide change(s)	No. of recovered mutants
G to A[a]	15
C to T	6
T to C	2
T to G	1
T to A	1
TTT to TT	2
TTTT to TTT	2
TTTT to TTTTT	1
AAAA to AAA	2
AAA to AAAA	1
CCC to CC	1
Δ8	1
Δ21	1
Δ33	1
Δ4, +15	1
Total	38

[a] Two mutants each containing two G-to-A mutations were isolated, but only one mutation is shown, and only one mutation from each mutant was used to calculate the mutation rate.

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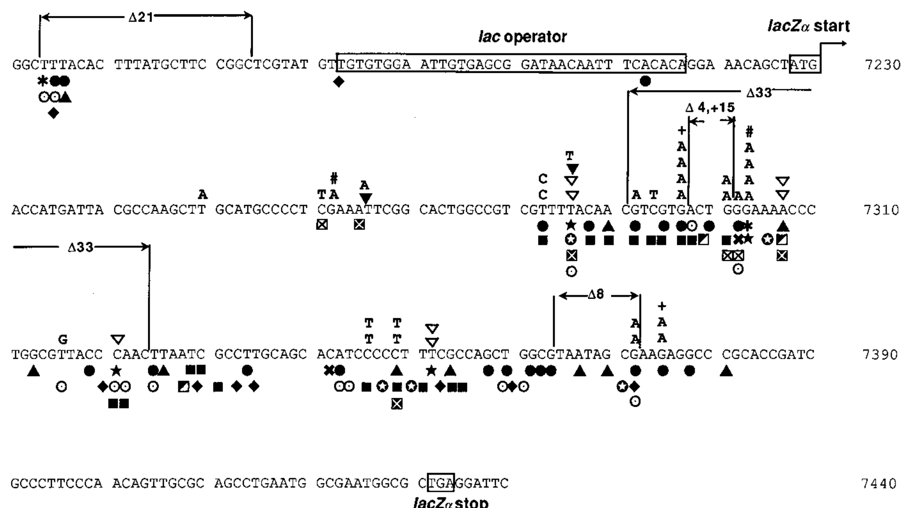

FIG. 3. Plus-strand nucleotide sequence of the *lacZα* gene region in HIV shuttle 3.12. The start for nucleotide numbering is the beginning of the 5′ long terminal repeat. The start and stop codons of the *lacZα* open reading frame (small boxed sequences) and the *lac* operator sequence (large boxed sequence) are shown. Nucleotide positions of base pair substitutions (letters above the sequence), +1 frameshifts (letters with ▼ above the sequence), −1 frameshifts (▽ above the sequence), deletions and deletions with insertions (solid black lines with arrows above the sequence and adjacent to the deletion names) are indicated above the sequence. Base substitution mutations occurring in the *lacZα* gene region of the same mutant are designated with a + or #, respectively. Symbols below nucleotides in the nucleotide sequence indicate the locations of base substitution and frameshift mutations that have been characterized in cell-free studies with purified HIV-1 reverse transcriptase, using the sense-strand of the *lacZα* gene region as an RNA template (9, 26) or a DNA template (6). Target sites for base substitution mutations in experiments using an RNA template are indicated by ⊙ for the study by Boyer et al. (9) and ■ and ✖ (hot-spot site for base substitutions) for the study by Ji and Loeb (26); target sites for frameshift mutations are indicated as ◆ and ✪ (hot-spot site for frameshift mutations) for the Boyer et al. study and ◩ for the Ji and Loeb study. Target sites for base substitution mutations in experiments using a DNA template (6) are indicated by ● and ✶ (hot-spot site for base substitutions), and frameshift mutations are indicated by ▲ and ★ (hot-spot sites for frameshift mutations). Additional symbols below nucleotides in the nucleotide sequence indicate the locations of base substitution and frameshift mutations that have been characterized in the BLV shuttle vector BLV-SVNEO/ACYCLacZ (34). Target sites for base substitution and frameshift mutations are indicated below the nucleotide sequence by ⊠ and ⊠, respectively.

of pyrimidines (six of nine) were more abundant than mutations in runs of purines (three of nine) in the plus-strand proviral DNA of HIV shuttle 3.12. Minus-one frameshift mutations occurred more often (seven of nine) than +1 mutations (two of nine). Four of the thirty-eight mutants contained deletion mutations. Three of these deletion mutants contained deletions ranging from 8 to 33 bases. One mutant contained a deletion of 4 bases and an insertion of 15 nucleotides. The locations of the mutations in the *lacZα* peptide gene are shown in Fig. 3. The forward mutation rate was calculated to be 3.5×10^{-5} mutations per target bp per cycle (calculated as described in Table 5, footnote *d*).

Effect of orientation of the *lacZα* peptide gene on the frequency and types of mutations. Pathak and Temin (36) had determined the forward mutation rate for spleen necrosis virus (SNV) by using a shuttle vector (pVP212) with the *lacZα* peptide gene in the orientation opposite that in HIV shuttle 3.12. To determine whether the orientation of the *lacZα* peptide gene had an effect on the mutation rate, we constructed an HIV-1 shuttle vector (HIV shuttle 5.1) with the *lacZα* peptide gene in the opposite orientation (Fig. 1B). The proviral DNA from pooled step 3 cells representing over 40,000 different cell clones was purified with the Lac repressor protein and introduced into *E. coli* to screen for mutations in the *lacZα* gene region. Twenty-eight mutants were recovered from screening 6,746 bacterial colonies (Table 3). The mutation frequency of 28/6,746, or 4.1×10^{-3} mutations per cycle is comparable to what was observed with HIV-1 shuttle vector 3.12 (38/8,678).

The majority of the mutants had base substitutions (Table 4). Seventeen of the twenty-eight mutants had base substitution mutations, mainly G-to-A and C-to-T transition mutations. Inspection of the G-to-A mutations revealed that 5 of 12 occurred within a GpA dinucleotide; 6 of 12 G-to-A mutations occurred within a GpG dinucleotide. Five of the twelve G-to-A mutations occurred within a 24-base region of the *lacZα* peptide gene (bases 6277 to 6301 in HIV shuttle 5.1). Eight of the twenty-eight mutants had frameshift mutations, which were mainly −1 frameshifts in runs of T's and A's. Four frameshift mutations were in runs of four nucleotides, including two each in runs of T's and A's. Four frameshifts occurred in runs of three nucleotides, two in runs of T's and one each in runs of A's and C's. Mutations in runs of pyrimidines (five of eight) were more abundant than mutations in runs of purines (three of eight) in the plus-strand proviral DNA of HIV shuttle 5.1. Minus-one frameshift mutations occurred more often (eight of eight) than +1 mutations (zero of eight). Three of the twenty-eight mutants had deletion mutations. Two deletion mutants had deletions of 16 and 31 bases, respectively; one mutant had a deletion of 7 bases and an insertion of 15 bases. The sites for some of the mutations were at the same nucleotide as for mutations that were observed with HIV shuttle 3.12 (Fig. 4). The forward mutation rate was calculated to be 3.2×10^{-5}

TABLE 3. Mutation frequencies in recovered proviruses of HIV shuttle 5.1

Step 2 clone no.	No. of mutants/total no. of bacterial colonies	Mutation frequency (10^{-3})
1	9/1,457	6.2
2	12/2,613	4.6
3	7/2,676	2.6
Total	28/6,746	4.2[a]

[a] The average mutation frequency with a standard error of 1.1×10^{-3} mutant per cycle.

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TABLE 4. Substitution, frameshift, and deletion mutations for HIV shuttle 5.1

Nucleotide change(s)	No. of recovered mutants
G to A[a]	11
C to T[b]	5
A to G	1
TTT to TT	2
TTTT to TTT	2
AAA to AA	1
AAAA to AAA	2
CCC to CC	1
Δ16	1
Δ31	1
Δ7, +15	1
Total	28

[a] One mutant containing two G-to-A mutations was isolated, but only one mutation from the mutant was used to calculate the mutation rate.

[b] One C-to-T mutant that also contained a G-to-A mutation was isolated, but only the C-to-T mutation was used to calculate the mutation rate.

mutations per target bp per cycle (calculated as described in Table 5, footnote *d*). The mutation rate and general types and frequencies of mutations characterized were not affected by the orientation of the *lacZ*α peptide gene.

Mutation site comparison with cell-free studies. Many of the sites at which base substitution and frameshift mutations were characterized in HIV shuttle 3.12 were identical to sites found using purified HIV-1 reverse transcriptase with a sense-strand RNA template of the *lacZ*α peptide gene (representing the fidelity of minus-strand DNA synthesis with an RNA template) (9, 26). Fifteen different nucleotide bases were sites for base substitution mutations with HIV shuttle 3.12 (Fig. 3). Of these, 10 sites were identical to sites of base substitution mutations with purified HIV-1 reverse transcriptase with a RNA template (Fig. 3). Two of the five runs of nucleotides where frameshift mutations were characterized were at identical runs where errors occurred when purified HIV-1 reverse transcriptase with a RNA template was used. A much larger number of mutants have been characterized in cell-free studies using a sense-strand DNA template of the *lacZ*α peptide gene (6), and these data were compared with the data for HIV shuttle 3.12 because of the large number of mutants characterized. However, data obtained with an antisense DNA strand would be the most appropriate for this shuttle vector (see Discussion). Seven of the fifteen sites of base substitution mutations were identical to sites characterized with a sense-strand DNA template (Fig. 3). Three of the five runs of nucleotides in HIV shuttle 3.12 where frameshift mutations were characterized were at identical runs where errors occurred with a sense-strand DNA template (Fig. 3). Twenty of the thirty-eight mutants (53%) had mutations that were located in a 24-base region (nucleotides 7283 to 7307) in HIV shuttle 3.12.

Several of the sites at which base substitution and frameshift mutations were characterized in HIV shuttle 5.1 are identical to sites found by using purified HIV-1 reverse transcriptase with a sense-strand DNA template of the *lacZ*α peptide gene (representing the fidelity of plus-strand DNA synthesis with a DNA template) (6). Twelve different nucleotide bases were sites for base substitution mutations (Fig. 4). Of these, four sites were identical to sites of base substitution mutations with a DNA template (Fig. 4). Four of the five runs of nucleotides where frameshift mutations were characterized were at identical runs where errors occurred with a DNA template in cell-free studies. Fifteen of the twenty-eight mutants (54%) had mutations that were located in a 24-base region (nucleotides 6277 to 6301) in HIV shuttle 5.1. The overall base pair substitution mutation rate, 2.4×10^{-5} (calculated as described in Table 5, footnote *d*), is about 1.5-fold lower than the substitution error rate by HIV-1 reverse transcriptase with a sense-strand RNA template of the *lacZ*α peptide gene and about 10-fold lower than the error rate with a sense-strand DNA template (9).

Mutation site comparison with in vivo mutation rate studies of SNV and BLV. Three sites of base substitution mutations and two of the runs of nucleotides at which frameshift mutations occurred with BLV in vivo (34) were at sites identical to those observed in HIV shuttle 3.12 (Fig. 3). Four sites of base substitution mutations and one of the runs of nucleotides at which frameshift mutations occurred with SNV in vivo (36, 38) were at identical sites in HIV shuttle 5.1 (Fig. 4). The deletion mutation of eight nucleotides found in this study with HIV shuttle 3.12 was the same as that of deletion mutant D2, which was characterized in the BLV study. The deletion mutations found in our study were different in size and location from those found in the SNV study.

Relative rates of mutation of HIV-1, SNV, and BLV. The characterized mutants allowed for the calculation of the mutation rate per replication cycle per base pair. Chi-square analysis of the mutation frequencies of HIV shuttle vector 3.12 (38/8,678) and HIV shuttle vector 5.1 (28/6,746) revealed no significant differences (chi square = 0.05, $P > 0.1$); therefore, the data from HIV shuttle 3.12 and HIV shuttle 5.1 were combined in order to calculate the overall mutation rate of HIV-1. The overall mutation rate for HIV-1 was calculated to be 3.4×10^{-5} mutations per target bp per cycle (Table 5). This rate is higher than the mutation rate of SNV (36) by a factor of 3 and higher than that of BLV (34) by a factor of 7. The mutation frequency for HIV-1 was significantly different from those of SNV (chi square = 11, $P < 0.005$) and BLV (chi square = 48, $P < 0.001$). Significant differences between the HIV-1 and SNV mutation frequency data sets were also indicated by the Wilcoxon rank sum test ($P < 0.05$).

DISCUSSION

We have found the forward mutation rate of HIV-1 in a single cycle of replication to be 3.4×10^{-5} mutations per bp per cycle. This rate was determined by using the *lacZ*α peptide gene as a mutational target. In comparison, the error rates of purified reverse transcriptase copying the sense strand of the *lacZ*α peptide gene have been reported to be 5×10^{-4} to 6.7×10^{-4} mutations per bp (42) with a DNA template and either comparable (26) or about 2.5-fold lower (9) with an RNA template. The in vivo mutation rate for HIV-1 is 6- to 15-fold lower than these error rates.

The mutations detected in our system may have occurred either during reverse transcription of minus-strand DNA with the genomic RNA template or during plus-strand DNA synthesis with a DNA strand as a template. The most appropriate comparison of our data from HIV shuttle 3.12 with the data from cell-free studies would be the error rate of purified HIV-1 reverse transcriptase obtained by using an RNA template with the sense strand of the *lacZ*α peptide gene and the error rate obtained by using a DNA template with the antisense strand of the *lacZ*α peptide gene. For comparison with the data from HIV shuttle 5.1, it would be most appropriate to compare data with the error rate of HIV-1 reverse transcriptase obtained by using an RNA template with the antisense strand of the *lacZ*α

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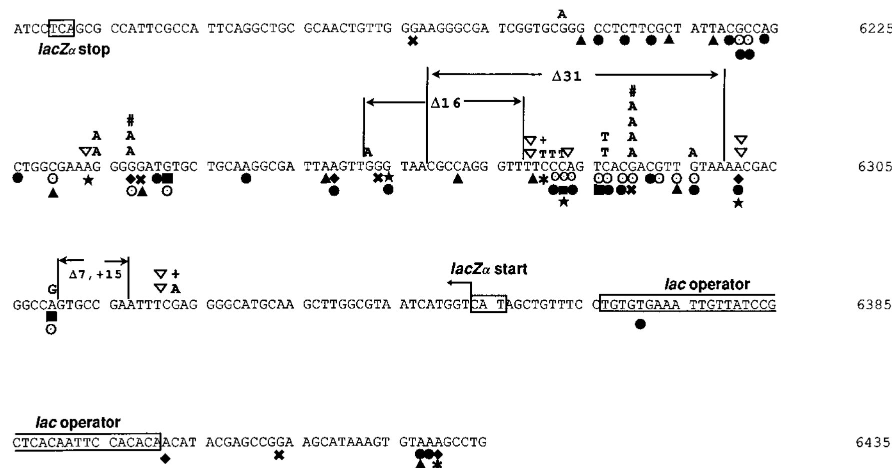

FIG. 4. Plus-strand nucleotide sequence of the *lacZα* gene region in HIV shuttle 5.1. The start for nucleotide numbering is the beginning of the 5′ long terminal repeat. The start and stop codons of the *lacZα* open reading frame (small boxed sequences) and the *lac* operator sequence (large boxed sequence) are shown. Nucleotide positions of mutations are indicated as in Fig. 3. Base substitution mutations occurring in the *lacZα* gene region of the same mutant are designated with a + or #, respectively. Symbols below nucleotides in the nucleotide sequence indicate the locations of base substitution and frameshift mutations that have been characterized in cell-free studies with purified HIV-1 reverse transcriptase that used the sense-strand of the *lacZα* gene region as a DNA template (6). Target sites for base substitution mutations are indicated by ● and ✶ (hot-spot site for base substitutions), and frameshift mutations are indicated by ▲ and ★ (hot-spot sites for frameshift mutations). Additional symbols below nucleotides in the nucleotide sequence indicate the locations of base substitution and frameshift mutations that have been characterized in the SNV shuttle vector VP212 (36, 38). Target sites for base substitution mutations are indicated by ⊙ (38) or ■ and ✖ (G-to-A transition mutation characterized in a hypermutant) (36); frameshift mutations are indicated by ◆ (36).

peptide gene and the error rate obtained by using a DNA template with the sense strand of the *lacZα* peptide gene. However, to date, there have been no published reports of cell-free studies using the antisense strand of the *lacZα* peptide gene. Comparison by adding the error rates determined on sense-strand RNA (9) and DNA (6) templates in cell-free studies (to account for both minus-strand and plus-strand DNA synthesis reactions) would estimate that the in vivo mutation rate is about 20-fold lower than the error rate of purified HIV-1 reverse transcriptase. The comparable mutation rates of HIV shuttle 3.12 and HIV shuttle 5.1 suggest that the estimated difference between the error rate of purified reverse transcriptase and the in vivo mutation rate is reasonable.

The in vivo mutation rate of HIV-1 is higher than the mutation rate of SNV by a factor of 3 and higher than that of BLV by a factor of 7 (16, 17, 34, 36, 37). Although the differences in these rates are statistically significant, the mutation rate of HIV-1 is within a factor of 10 of the mutation rates of both SNV and BLV. This finding indicates that HIV-1 reverse transcription is not especially error prone as had been previously predicted and that it is not dramatically different from that of SNV and BLV.

Genetic variation of retroviruses is the composite of the mutation rate per replication cycle, the number of replication cycles, and the fixation rate of mutations. It is not known how many replication cycles HIV-1 completes per year in an infected individual. However, it has been argued that HIV-1 completes about 300 or more cycles per year (14), given reports that the average lifetime of an HIV-1-infected cell is less than 1 to 2 days (21, 54). This implies that the number of replication cycles and the fixation rate for mutations have a greater influence on the genetic variation of HIV-1 than the mutation rate.

Base pair substitution mutations were the most common type of mutation detected in our system, representing 42

TABLE 5. Relative rates of mutation for HIV-1, SNV, and BLV

Mutation type or determination	No. of recovered mutant shuttle vector proviruses		
	HIV-1	SNV[a]	BLV[b]
Base pair substitution	42	11	3
Frameshift	17	5	4
Deletion	5	12	2
Deletion with insertion	2	7	2
Overall mutation frequency	66/15,424 (4.3×10^{-3}[c])	37/16,867 (2.5×10^{-3})	11/18,009 (6.1×10^{-4})
Overall mutation rate[d] (no. of mutations/bp/cycle)	3.4×10^{-5}	1.2×10^{-5}	4.8×10^{-6}

[a] Data from Pathak and Temin (36).
[b] Data from Mansky and Temin (34).
[c] The average mutation frequencies with a standard error of 0.6×10^{-3} mutant per cycle for HIV-1, 0.4×10^{-3} mutant per cycle for SNV, and 0.3×10^{-3} mutant per cycle for BLV.
[d] The rates of mutation were calculated as the sums of the rates of base pair substitution, frameshift, and deletion mutations per 15,424 total shuttle vector proviruses for HIV-1, per 16,867 total shuttle vector proviruses for SNV (36), and per 18,009 total shuttle vector proviruses for BLV (34), per 113 target nucleotides for substitutions, per 150 target nucleotides for frameshifts, or per 280 target nucleotides for deletion mutations. Target nucleotides for substitution and frameshift (6, 9) and deletion mutations (37) have been previously described.

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(64%) of 66 mutations characterized. Base substitutions in HIV shuttle 3.12 and HIV shuttle 5.1 were predominantly G-to-A (26 of 42) and C-to-T (11 of 42) transition mutations. The G-to-A transitions were mainly in GpA dinucleotides in both HIV shuttle 3.12 and HIV shuttle 5.1. Studies of the genetic heterogeneity of HIV-1 isolates have observed G-to-A hypermutation of *env* sequences within GpA and GpG dinucleotides (19, 53). Although our system does not presently allow us to determine whether the substitution mutations occurred during minus-strand or plus-strand DNA synthesis, cell-free studies provide some insight about the mechanism by which these mutations may have occurred. Transition mutations adjacent to runs of a single nucleotide have been implicated to occur by the mechanism of dislocation mutagenesis (6, 30). In this model, dislocation of the primer to the template produces an unpaired nucleotide base; realignment occurs between the primer and the template, resulting in a mismatch, followed by elongation beyond the mismatch. The G-to-A transition mutations that occurred at nucleotides 7301 to 7303 in HIV shuttle 3.12 and nucleotides 6235 and 6238 in HIV shuttle 5.1 occurred at sites adjacent to a run of nucleotides (Fig. 3 and 4), indicating that these mutations may have occurred via the dislocation mutagenesis model.

The frameshift mutations characterized in HIV shuttle 3.12 and HIV shuttle 5.1 were mainly −1 frameshifts in runs of T's and A's. These general trends are in agreement with those found for frameshift mutations made by purified HIV-1 reverse transcriptase (6). Plus-one frameshift mutations in runs of T's and A's occurred with SNV in vivo (11, 36). The frameshift mutations in homo-oligomeric runs suggest that these result from template primer slippage (7, 8, 29, 46, 47). The +1 frameshift mutations may have occurred during minus-strand DNA synthesis (11), while the −1 frameshift mutations could have occurred during either minus- or plus-strand DNA synthesis.

A total of seven deletion mutants were characterized. Two of these deletion mutants contained an insertion of DNA sequence of unknown origin. Similar mutants have been recovered with SNV, and the mechanisms by which they could have occurred have been proposed (37, 39). These mutations could have occurred during either minus- or plus-strand DNA synthesis.

The distributions of the different types of mutations observed for HIV-1 in our study (i.e., base substitution, frameshift, deletion, and deletion with insertion mutations) were the same as the types of mutations characterized with SNV (36, 37) and BLV (34). This is consistent with the hypothesis that abnormal strand transfers during reverse transcription are responsible for the error-prone nature of reverse transcription (50).

The mutation rate of a HIV-1 vector with the *lacZ*α peptide gene in the opposite orientation, HIV shuttle 5.1, was found to be comparable to the mutation rate of the parental vector, HIV shuttle 3.12. Certain nucleotides were targets for base substitution and frameshift mutations in both vectors, indicating that orientation did not affect mutations from occurring at these sites. This orientation independence suggests that any error-prone bias during reverse transcription of the *lacZ*α peptide gene was not detectable in our assay.

In an amber codon reversion assay, the rate of base substitution mutations occurring during the replication of a Moloney murine leukemia virus (MoMLV) vector was compared with the in vitro fidelity of MoMLV reverse transcriptase (52). The base substitution rate at a single locus was found to be 2×10^{-6} mutations per bp per cycle. The in vivo rate of base substitution of the MoMLV vector was 30-fold lower than the rate of formation of T G mispairs in vitro. The comparison of the mutation rate relative to the rate of formation of T G mispairs parallels our observation that the HIV-1 mutation rate is lower than the fidelity of purified HIV-1 reverse transcriptase.

In summary, we have found the in vivo mutation rate of HIV-1 to be lower than the error rate of purified reverse transcriptase by a factor of 20. This difference may be due to several factors, including the association of viral or nonviral accessory proteins during reverse transcription, the influence of cellular mismatch repair mechanisms, and/or differences between the reverse transcriptase produced in vivo with that assayed in vitro. We are currently testing the hypothesis that accessory proteins may influence the accuracy of reverse transcription.

ACKNOWLEDGMENTS

I thank Xiao-Juan Bi, Eric Hausmann, Tim Jacoby, Ann Krueger, Sarah Lulloff, Brad Seufzer, and Rebecca Wisniewski for superior technical assistance; Donal Kaehler for assistance in the BL-3 laboratory; Kathy Boris-Lawrie, Dawn Burns, Norman Drinkwater, Nito Panganiban, Gary Pulsinelli, Bill Sugden, and Shiaolan Yang for helpful discussions; Norman Drinkwater for help with statistical analyses; and Kathy Boris-Lawrie, Dan Loeb, and Nito Panganiban for critical reading of the manuscript.

This work was supported by grants CA22443 and CA07175 from the Public Health Service. L.M.M. was supported by NRSA Viral Oncology training grant CA0975-17 and by an NIH fellowship. H.M.T. was an American Cancer Society Research Professor.

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