The Mutation Rate of Human Immunodeficiency Virus Type 1 Is Influenced by the vpr Gene

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A system has been designed to study the in vivo forward rate of mutation of human immunodeficiency virus type 1 (HIV-1) during one round of replication. A HIV-1 shuttle vector was used that contained the lacZA peptide gene as a reporter for mutations. The forward mutation rate of HIV-1 was found to be $3 \times 10^{-5}$ mutations per target base pair per cycle, or about 20-fold lower than the error rates reported for purified HIV-1 reverse transcriptase with sense-strand RNA and DNA templates of the lacZA peptide gene in a cell-free system. To test the hypothesis that the vpr gene product might, at least in part, account for the lower mutation rate observed in vivo, a HIV-1 vector was replicated to determine if the mutation rate was higher in the absence of the wild-type vpr gene product. A vpr- shuttle vector had an overall mutation rate as much as 4-fold higher than that of the parental vector. A shuttle vector with an amino acid substitution in Vpr that prevents efficient incorporation of Vpr into virus particles was found to have a mutation frequency similar to that of the vpr- vector, and was interpreted to indicate a requirement for Vpr incorporation into the virus particle in order to observe the influence of vpr on the mutation rate. Replication of a vpr- shuttle vector in the presence of a wild-type vpr expression plasmid led to a mutation frequency similar to that of the parental vector, suggesting that the vpr mutation could be complemented in trans. Immunoprecipitation analysis indicated that Vpr virion incorporation coincided with the influence of vpr on the mutation rate.

INTRODUCTION

The Retroviridae family of RNA viruses replicate through a DNA intermediate (Baltimore, 1970; Temin and Mitzutani, 1970). The viral RNA is copied into DNA by the viral-encoded enzyme reverse transcriptase in a process known as reverse transcription. The process of reverse transcription is known to be error-prone, contributing to the high genetic variability of retroviruses (Coffin, 1992; Holland et al., 1992; Katz and Skalka, 1990; Temin, 1961). The error-prone nature of reverse transcription has been proposed to be due to abnormal strand transfers that can occur during this process (Temin, 1993). The level of genetic variation of human immunodeficiency virus type 1 (HIV-1) is high (Alizon et al., 1986; Balfe et al., 1990; Cheng-Mayer et al., 1991; Hahn et al., 1986; Levy, 1993; Starich et al., 1986; Wong-Staal, 1990) relative to some retroviruses in other genera (Dube et al., 1993; Ratner et al., 1991; Willems et al., 1993). Genetic variation of retroviruses can be viewed as the composite of several variables: (1) the mutation rate per replication cycle, (2) the number of replication cycles, (3) the fixation rate of mutations, and (4) the rate of recombination (Coffin, 1990; Hu and Temin, 1990; Zhang and Temin, 1993). It has been argued that HIV-1 completes about 300 or more cycles per year (Coffin, 1995), based on the reports that the average lifetime of an HIV-1-infected cell is less than 1 to 2 days (Ho et al., 1995; Wei et al., 1995).

A system has been developed to measure the forward mutation rate of HIV-1 with a vector containing the lacZA peptide gene as a reporter for mutations (Mansky and Temin, 1995). This system allows for the study of mutations that occur during a single round of HIV-1 replication. The mutation rate of HIV-1 in this system was determined to be $3 \times 10^{-5}$ mutations per target base pair per cycle in HeLa cells (Mansky and Temin, 1995) and $4 \times 10^{-5}$ mutations per target base pair per cycle in a T-lymphoid cell line (Mansky, 1996). 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 Ts and As). This mutation rate is maximally 5% of the reported error rates for purified HIV-1 reverse transcriptase with a sense-strand RNA and DNA template in cell-free studies, indicating that the mutation rate of HIV-1 is less than that predicted by the measured fidelity of purified HIV-1 reverse transcriptase (Boyer et al., 1992).

The difference between the in vivo mutation rate of HIV-1 and the error rate of purified HIV-1 reverse transcriptase may be due to several possibilities. One hypothesis is that, in vivo, protein factors and reverse transcriptase together contribute to the accuracy of reverse transcription; in the absence of these factors, the error rate of purified reverse transcriptase is higher. Protein factors that are viral proteins would be predicted, like
FIG. 1. HIV-1 shuttle vector used for mutation rate studies. The HIV-1 shuttle vector is shown in the proviral DNA form. Solid black boxes represent the HIV-1 long terminal repeats (LTRs). Solid black lines indicate viral sequences. Rectangular boxes above 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 vif, vpr, tat, rev, vpu, and nef. Bent lines in between the coding regions for tat and rev show reading frames joined by splicing events. E indicates the location of the encapsidation signal. 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 lacZa peptide gene is represented as an open rectangular box with a black band representing the lac operator sequence. Dashed lines indicate deleted viral coding sequence removed by digestion with SwaI. The StuI and XhoI sites were used in the purification of the vector proviral DNA containing the neo gene, the pACYC origin of replication, and the lacZa peptide gene.

reverse transcriptase, to be present in virus particles. To test the hypothesis that viral proteins contribute to the accuracy of reverse transcription, the HIV-1 vpr gene was mutated in order to determine the influence of vpr on the rate of mutation. The vpr gene encodes a 96-amino-acid, nonstructural protein that is associated with virus particles (Cohen et al., 1990) and that requires the p6 region of the gag gene for incorporation (Kondo et al., 1995; Lu et al., 1995, 1993; Paxton et al., 1993). The incorporation of Vpr into virus particles implies a role either early in the HIV-1 replication cycle (prior to the synthesis of new viral proteins) or late in the replication cycle (during assembly and budding). Specific roles of vpr include the nuclear localization of the preintegration complex and prevention of cell proliferation during chronic infection (He et al., 1995; Heinzinger et al., 1994; Jowett et al., 1995; Planelles et al., 1995; Rogel et al., 1995). This study has found that the mutation rate of a vpr vector mutant was as much as fourfold higher than that of the parental vector. This observation supports an early role of vpr in the HIV-1 replication cycle. These data support the conclusion that the vpr gene partially accounts for predicted in vivo mutation rate of HIV-1.

MATERIALS AND METHODS

HIV-1 vectors and expression plasmids

The plasmid pHIV shuttle 3.12 (Fig. 1) was constructed as described (Mansky and Temin, 1995). A vpr derivative of pHIV shuttle 3.12, pHIV shuttle 3.12 vpr ATG-, was made by a primary/combinatorial two-step polymerase chain reaction (PCR) protocol (Ito et al., 1991). Oligonucleotide primers that changed the start codon of the vpr gene from ATG to ATT, but did not affect the overlapping vif open reading frame, were used as internal primers. The PCR product from this reaction, containing the vpr gene with the mutation of the start codon, was cloned into the pCRII cloning vector (Invitrogen Corp., San Diego, CA). The cloned DNA was sequenced to verify the introduced mutations using a nonradioactive DNA sequencing kit (Silver Sequencing kit; Promega Corp., Madison, WI) and digested with PflMI and SalI, and the fragment containing the vpr gene was cloned into the PMI and SalI sites of pHIV shuttle 3.12.

The construction of the Vpr virion-incorporation mutant was made using a similar PCR mutagenesis protocol used for pHIV shuttle 3.12 vprATG- (as described above). Nucleotide changes in the vpr gene of HIV shuttle 3.12 were made that led to changes in the coding for amino acid 30 (alanine to phenylalanine; Vpr mutant A30F); this residue is near the amino-terminal end of Vpr in the predicted α-helical region of the protein (residues 17–34). Mutagenesis of this domain has been found to affect Vpr stability and the efficiency of Vpr incorporation into virions (Mahalingam et al., 1995a,b,c). This particular amino acid substitution mutation in the vpr gene has been previously reported to limit the incorporation of Vpr into virus particles (Yao et al., 1995).

The HIV-1 gag-pol expression plasmid used, PSVgag-pol-rre-r (Smith et al., 1990), was kindly provided by David...
Rekosh, University of Virginia. The amphotropic murine leukemia virus env expression plasmid used, pSV-A-MLV-env (Landau and Littman, 1992), was provided by Dan Littman, University of California, San Francisco. The vpr expression plasmid, pCMVvpr, was constructed by amplifying the vpr gene from HIV shuttle 3.12 by PCR and inserting it into a eukaryotic expression vector that contains the CMV promoter (pCR3; Invitrogen).

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 (Temin, 1968) containing 10% calf serum or 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 DMSO/polybrene (Kawai and Nishizawa, 1984). HeLa cells were infected in the presence of polybrene (Mansky, 1994). Infection of HeLa target cells was done by cocultivation of virus-producing cells with target cells (Mansky et al., 1995; Mansky and Temin, 1994). Briefly, virus-producing cells (typically $2.5 \times 10^5$ cells per 60-mm petri dish, $5 \times 10^5$ cells per 100-mm petri dish, or $7.5 \times 10^5$ cells per 150-mm petri dish) were treated with mitomycin C (10 $\mu$g/ml), an inhibitor of host cell DNA synthesis, for 2 hr at 37°C. The cells were then washed three times with fresh medium, and HeLa target cells equivalent to the number of treated virus-producing 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, until the formation of colonies (typically about 3 weeks).

Strategy for generating a single cycle of retrovirus replication

The experimental protocol developed to assay 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 pSVgagpol-re-r and pSV-A-MLV-env along with a HIV-1 tat expression plasmid, pSVtat (Kao et al., 1987; Peterlin et al., 1986) (to aid in expression of the accessory genes from the shuttle vector). In Step 2, vector virus was harvested 48 hr posttransfection from Step 1 cells and used to infect fresh HeLa cells. G418-resistant cell clones were transiently transfected with the helper plasmids and the HIV-1 tat expression plasmid (Step 2 cells); in complementation experiments, pCMVvpr was transiently transfected along with these plasmids. Step 2 clones were tested by Southern analysis to ensure that only a single vector proviral DNA was present. The lacZa peptide gene in the vector proviral DNA of Step 2 clones was sequenced to confirm that no mutations were introduced. In Step 3, vector virus was transferred to fresh HeLa target cells by cocultivation 24–48 hr after transient transfection of helper plasmids; cells were then placed under G418 selection (Step 3 cells). Cocultivation was used to produce Step 3 cells to maximize the number of Step 3 cells for analysis of the mutation rate.

Recovery of shuttle vector proviral DNA and DNA sequencing of the lacZa peptide region

Purified genomic DNA (Sambrook et al., 1989) from pools of Step 3 clones was digested with the restriction enzymes Stul and Xhol, to release the neo, pACYC origin of replication, and lacZa peptide gene sequences from the HIV-1 shuttle vector proviral DNA (Fig. 1). Proviral DNA was purified with the Lac repressor protein (Promega) as previously described (Mansky and Temin, 1994). The purified proviral DNA was treated with the Klenow fragment of DNA polymerase to fill in single-stranded ends, and 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 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 (Sambrook et al., 1989) and sequenced in the lacZa peptide gene region with a nonradioactive DNA sequencing kit (Promega Corp.) and by an automated DNA sequencer (Applied Biosystems).

Cell radiolabeling and immunoprecipitation analysis

Step 2 cells with HIV shuttle 3.12 or derivatives with vpr mutations were metabolically labeled with $^{35}$S-methionine (100 $\mu$Ci/ml) for approximately 15 hr after transfection with helper plasmids. Virus particles produced during the labeling were pelleted through a 20% sucrose cushion in a Beckman SW28 rotor for 90 min at 4°C. Cells and pelleted virions were lysed in immunoprecipitation buffer (1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, and 0.2 mM phenylmethylsulfonyl fluoride in phosphate-buffered saline) and then immunoprecipitated with a rabbit anti-Vpr serum (deposited by L. Ratner in the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, Bethesda, MD) absorbed to protein A–agarose beads (Bethesda Research Laboratories, Gaithersburg, MD).
FIG. 2. Experimental protocol for studying one round of HIV-1 shuttle vector virus replication. (A) Experimental protocol. In Step 1, COS-1 cells were transfected with a pHIV shuttle 3.12 or a derivative and were placed under G418 selection. These cells were transiently transfected with the HIV-1 gag-pol expression plasmid, pSVGAPOL-re-r, the amphotropic murine leukemia virus env gene, pSV-A-MLV-env, and a HIV-1 tat expression plasmid, pSVtat (to aid in expression of the accessory genes from the shuttle vector). Forty-eight hours posttransfection, virus was harvested and used to infect HeLa cells. G418-resistant cell clones were transiently transfected with pSVGAGPOL-re-r, pSV-A-MLV-env, and the tat expression plasmid (Step 2 cells). Twenty-four to 48 hr 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). (B) One round of HIV-1 shuttle vector virus replication. The steps going from a parental shuttle vector provirus in the Step 2 cell to a vector provirus in the Step 3 cell constitute a single cycle of replication. 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.

RESULTS

Replication of the HIV-1 shuttle vectors containing mutations of the vpr gene

The HIV-1 shuttle vectors used in these studies were based on HIV shuttle vector 3.12 (Fig. 1), which contains a deletion 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 lacZa peptide gene. These vectors replicate in mammalian cells as a virus and can be selected with the neomycin analog G418. The vectors can replicate in E. coli as a plasmid and are selected using the drug kanamycin. To be packaged into a virus particle, these vectors are complemented into 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). 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. Cocultivation of mitomycin C-treated Step 2 cells of HIV shuttle 3.12 or derivatives (typically 2.5 × 10⁵ cells per 60-mm petri dish, 5 × 10⁵ cells per 100-mm petri dish, or 7.5 × 10⁵ cells per 150-mm petri dish; mitomycin C is an inhibitor of host cell DNA synthesis and led to cell death of Step 2 cells as described under Materials and Methods) with fresh HeLa target cells to produce Step 3 cells led to titers of 8 × 10² - 3 × 10³ CFU/2.5 × 10⁵ HeLa target cells. 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 (after transient transfection with the helper plasmids) of HIV shuttle 3.12 or derivatives was done to ensure that each cell clone used contained only one provirus copy (data not shown). Proviral DNA from at least 5 × 10⁵ cells of each Step 2 cell clone was purified using the Lac repressor protein and introduced into E. coli to screen for mutations in the lacZa gene region; blue colonies were observed for each Step 2 clone (data not shown), indicating that the bacterial colonies containing vector proviral DNA had the wild-type lacZa gene phenotype.
The lacZα gene region in the vector proviral DNA of each Step 2 clone was sequenced to confirm that no mutations had been introduced into the lacZα gene region in the steps leading to the vector provirus in the Step 2 cell. The templates used for DNA sequencing were from proviral DNA purified from Step 2 cells using the Lac repressor protein and proviral DNAs from at least three blue colonies (from the above phenotypic analysis in E. coli) for each Step 2 clone used; DNA sequencing indicated that each Step 2 clone contained the wild-type lacZα gene sequence (data not shown). The vpr gene in the vector proviral DNA of the parental Step 2 clones 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 a vpr− HIV-1 shuttle vector

A HIV-1 shuttle vector containing a mutation of the vpr gene start codon was used (i.e., HIV shuttle 3.12 vpr ATG−). This vector was tested in parallel with the parental vector (Table 1). The proviral DNA from pooled Step 3 cells representing over 70,000 different cell clones of the vpr− mutant was purified with the Lac repressor protein and introduced into E. coli to screen for mutations in the lacZα gene region. Vector DNAs with mutations at target nucleotides, previously determined to lead to a phenotypic change (Bebenek et al., 1989; Pathak and Temin, 1990b), were detectable as bacterial colonies with a white or light-blue colony color phenotype. Three thousand two hundred fifty-four bacterial colonies were screened for vpr− shuttle vector proviral DNAs containing mutations in the lacZα peptide gene (Table 1). Fifty of these colonies had a white or light-blue colony color phenotype. The mutation frequency was 50/3254 or 15 × 10⁻³ mutations per cycle. This mutation frequency was about four times that of the mutation frequency of the vpr+ parental vector (24/6462 or 4 × 10⁻³ mutations per cycle) in parallel experiments (Table 1). The mutation frequencies of the vpr− mutant and the parental shuttle vector were significantly different (χ² = 38; P < 0.01).

To determine the types of mutations in the lacZα 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. Forty-two of the 50 mutants sequenced from the vpr− shuttle vector were found to contain a single mutation in the lacZα peptide gene region (Fig. 3). Similar kinds of mutations were observed with the vpr+ parental vector (Fig. 3).

Eight of the 50 mutants sequenced from the vpr− shuttle vector had multiple mutations in the lacZα peptide gene region. Most of the mutants contained multiple G to A transition mutations. These mutants are likely to have resulted from proviral DNA synthesis catalyzed by an error-prone reverse transcriptase. Since the forward mutation rate of HIV-1 is 3 × 10⁻³ mutations per base pair per cycle (Mansky and Temin, 1995), the observation of 2 or more mutations in the 280-base lacZα peptide gene region is indicative of hypermutation (Pathak and Temin, 1990a).

Effect of a mutation that limits incorporation of Vpr into virus particles on the mutation rate

A mutation in the vpr gene was made that would limit incorporation of Vpr into virus particles, without resulting in large structural changes to the protein. Specifically, a vpr mutant based on HIV shuttle 3.12 was made that would express Vpr with a single amino acid substitution at residue 30 (changing alanine to phenylalanine) in the α-helical domain located near the amino terminus of the protein; this mutant has been reported to limit the incorporation of Vpr into virus particles (Yao et al., 1995). Vector virus produced from COS-1 cells was used to infect fresh HeLa target cells. Step 2 cells containing this shuttle vector were transfected with the helper plasmids, and were cocultivated with fresh HeLa target cells to produce Step 3 cells in parallel with cocultivation of fresh HeLa cells with the vpr+ parental shuttle vector.

The proviral DNA from pooled Step 3 cells representing over 50,000 different cell clones was purified with the Lac repressor protein and introduced into E. coli to screen for mutations in the lacZα gene region. Three thousand ninety-two bacterial colonies were screened for shuttle vector proviral DNAs containing mutations in the lacZα peptide gene (Table 2). Forty-four of these

### Table 1

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Step 2 clone No.</th>
<th>No. of mutants/total No. of bacterial colonies</th>
<th>Mutation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV shuttle 3.12 vpr ATG−</td>
<td>1</td>
<td>10/795</td>
<td>13 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17/1025</td>
<td>17 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12/881</td>
<td>14 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>11/553</td>
<td>20 × 10⁻³</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>50/3254</td>
<td>15 × 10⁻³</td>
</tr>
<tr>
<td>Parental, vpr+ HIV shuttle vector 3.12</td>
<td>1</td>
<td>8/1383</td>
<td>6 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13/3724</td>
<td>4 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3/1355</td>
<td>2 × 10⁻³</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>24/6462</td>
<td>4 × 10⁻³</td>
</tr>
</tbody>
</table>

* a Average mutation frequency with a standard deviation of 3 × 10⁻³ mutants per cycle.
* b Average mutation frequency with a standard deviation of 2 × 10⁻³ mutants per cycle.
colonies had a white or light-blue colony color phenotype. The mutation frequency was 44/3092 or $14 \times 10^{-3}$ mutations per cycle. The mutation frequency of the vpr$^+$ parental vector in parallel experiments was 10/2450 or $4 \times 10^{-3}$ mutations per cycle. The mutation frequency of the shuttle vector containing the amino acid substitution in Vpr was significantly different from that obtained with the parental vector ($\chi^2 = 14; P < 0.01$), but was not different than the mutation frequency of the vpr$^-$ vector ($\chi^2 = 0.14; P > 0.1$). These results indicate the requirement of efficient Vpr incorporation in order to observe the influence of the vpr gene on the rate of mutation.

Complementation in trans of mutations in the vpr gene

HIV shuttle 3.12 vpr ATG$^-$ was used to test for complementation in trans of the vpr mutations made in HIV shuttle vector 3.12. Complementation was determined based on the mutation rate of the vector. Step 2 cells containing HIV shuttle 3.12 vector vpr ATG$^-$ were transfected with the helper plasmids and the vpr expression plasmid.

These cells were then cocultivated with fresh HeLa target cells to produce Step 3 cells along with parallel cocultivation experiments using the vpr$^+$ parental vector.

The proviral DNA from pooled Step 3 cells representing over 50,000 different cell clones was purified with the Lac repressor protein and introduced into E. coli to screen for mutations in the lacZ$\alpha$ gene region. Two thousand ninety-two bacterial colonies were screened for mutations in the lacZ$\alpha$ gene (Table 3). Nineteen( $\chi^2 = 0.14; P > 0.1$) of these colonies had a white or light-blue colony color phenotype. The mutation frequency for the vpr$^+$ vector was not significantly different from the mutation frequency of the parental vector ($\chi^2 = 11; P < 0.01$). This provides evidence that a vpr$^+$ vector can be complemented in trans with a vpr expression plasmid.
Vpr was inefficiently expressed in nontransfected Step 2 cells from HIV shuttle 3.12 (Fig. 4A, lane 1) relative to Vpr in cells transiently transfected with the helper plasmids and the tat expression plasmid (Fig. 4A, lane 2). The relatively low expression of Vpr from HIV shuttle 3.12 in the untransfected Step 2 cells provides an explanation as to why these cells are able to proliferate in the presence of the wild-type vpr gene. Vpr was detected in virions only from the transiently transfected cells producing virus particles (Fig. 4B, lane 2). In total, these data support the correlation between Vpr virion incorporation and the influence of the vpr gene on the mutation rate.

**DISCUSSION**

The HIV-1 accessory gene vpr was mutated to determine whether the vpr gene could, at least in part, account for the lower than predicted in vivo mutation rate of HIV-1. Mutation of the vpr gene start codon was made and introduced into a HIV-1 shuttle vector that has been used to determine the in vivo mutation rate of HIV-1 (Mansky and Temin, 1995). The mutation rate of this vpr– vector was determined using the lacZα peptide gene as a reporter for mutations. The mutation rate of a vpr– HIV-1 was determined to be $12 \times 10^{-5}$ mutations per target base pair per cycle (Table 4). This mutation rate is fourfold higher than that of the parental vector, $3 \times 10^{-5}$ mutations per target base pair per cycle (Table 4). Two additional experiments supported the influence of vpr on the mutation rate of HIV-1. First, a HIV-1 shuttle vector containing a mutation in the vpr gene that limits incorporation of Vpr into virus particles had a mutation frequency similar to that of the vpr– vector. Second, the mutation frequency of the vpr– vector was found to be comparable to the vpr+ parental vector when complemented in trans with a wild-type vpr expression plasmid. Mutation of the vpr gene did not significantly influence the proportion of base-substitution or frameshift mutations to the total number of mutants recovered, when compared to similar proportions of the parental vector.

**TABLE 2**

Mutation Frequency in Recovered Proviruses of a HIV-1 Shuttle Vector Containing a Mutation in the vpr Gene That Limits Vpr Incorporation into Virus Particles

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Step 2 clone No.</th>
<th>No. of mutants/ total No. of bacterial colonies</th>
<th>Mutation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV shuttle 3.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vpr A30F</td>
<td>1</td>
<td>5/1041</td>
<td>$5 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6/778</td>
<td>$8 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8/1173</td>
<td>$7 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>19/2992</td>
<td>$6 \times 10^{-3}$a</td>
</tr>
<tr>
<td>Parental, vpr+ HIV shuttle vector 3.12</td>
<td>1</td>
<td>4/967</td>
<td>$4 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7/1112</td>
<td>$6 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>11/2079</td>
<td>$5 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

* Average mutation frequency with a standard deviation of $1 \times 10^{-3}$ mutants per cycle.
The HIV-1 Vpr protein has been found to have several different influences and interactions. Vpr has been found to act intracellularly to influence productive infection and latency (Levy et al., 1994, 1995), to influence HIV-1 transcription (Cohen et al., 1990), to inhibit proliferation and activation of cell differentiation in a human muscle cell line (Levy et al., 1993), to interact with cellular proteins (Bouhamdan et al., 1996; Refaeli et al., 1995; Zhao et al., 1994), to prevent cell proliferation during chronic infection (Rogel et al., 1995), and to be involved in the nuclear localization of HIV-1 DNA in nondividing cells (Heinzinger et al., 1994).

The importance of vpr in viral persistence and pathogenesis is unclear. Reversion of an experimentally introduced mutation into the initiation codon of the SIV vpr gene was associated with disease progression (Lang et al., 1993); however, progression to AIDS can occur in the absence of vpr (Gibbs et al., 1995). Nonsense mutations in the vpr gene have been characterized in isolates obtained from HIV-1-seropositive individuals (Nakaya et al., 1994), suggesting that virus persistence is associated with the accumulation of nonsense mutations in the vpr gene.

The in vivo mutation rate of HIV-1 is 20-fold lower than the error rates of purified HIV-1 reverse transcriptase with RNA and DNA templates of the lacZa peptide gene (Boyer et al., 1992; Mansky and Temin, 1995). Mutation of the vpr gene leads to a HIV-1 mutation rate that is fivefold lower than the in vitro error rates. This indicates that other protein factors may also influence the in vivo mutation rate. The nucleocapsid protein, which is closely associated with the genomic RNA, is part of the viral core particle, and is involved in the initiation of reverse transcription (Lapadat-Tapolsky et al., 1993), could also influence the accuracy of reverse transcription. Viral replication accessory proteins that affect the accuracy of replication have been described for other viruses. For example, bacteriophage T4 encodes two proteins, gene 32 protein (a helix-destabilizing protein) (Hurley et al., 1993) and gene 45 protein, that are part of the replication complex and have been shown to influence the accuracy of replication (Mufti, 1979, 1980; Topal and Sinha, 1983; Watanabe and Goodman, 1978).

The imbalance of deoxynucleotide triphosphate pools has been suggested to influence the efficiency and accuracy of HIV-1 replication and the fidelity of purified HIV-1 reverse transcriptase (Bebenek et al., 1992; Martinez et al., 1994; Meyerhans et al., 1994; Vartanian et al., 1994). A possible effect of the vpr gene on HIV-1 reverse transcription could be to influence the access of deoxynucleotide triphosphates to reverse transcriptase.

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