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The Interaction of Vpr with Uracil DNA Glycosylase Modulates the Human Immunodeficiency Virus Type 1 In Vivo Mutation Rate

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The Vpr protein of human immunodeficiency virus type 1 (HIV-1) influences the in vivo mutation rate of the virus. Since Vpr interacts with a cellular protein implicated in the DNA repair process, uracil DNA glycosylase (UNG), we have explored the contribution of this interaction to the mutation rate of HIV-1. Single-amino-acid variants of Vpr were characterized for their differential UNG-binding properties and used to *trans* complement *vpr* null mutant HIV-1. A striking correlation was established between the abilities of Vpr to interact with UNG and to influence the HIV-1 mutation rate. We demonstrate that Vpr incorporation into virus particles is required to influence the in vivo mutation rate and to mediate virion packaging of the nuclear form of UNG. The recruitment of UNG into virions indicates a mechanism for how Vpr can influence reverse transcription accuracy. Our data suggest that distinct mechanisms evolved in primate and nonprimate lentiviruses to reconcile uracil misincorporation into lentiviral DNA.

Retroviral RNA is copied into DNA by the virus-encoded enzyme reverse transcriptase via a process called reverse transcription (2, 42). The error-prone nature of reverse transcription greatly contributes to the high level of genetic diversity observed within populations of retroviruses (4, 14, 18, 40, 41). Several variables define the diversity of human immunodeficiency virus type 1 (HIV-1) and retrovirus populations: (i) the rate of mutation per replication cycle, (ii) the number of replication cycles, (iii) the fixation rate of mutations, and (iv) the rate of recombination (6, 26). The high rate of HIV-1 replication is an important determinant in driving HIV-1 evolution (5, 13, 47).

A genetically engineered system has been developed for HIV-1 to measure the in vivo rate of forward mutation per replication cycle (27). The mutation rate of HIV-1 in this system was determined to be 3×10^{-5} to 4×10^{-5} mutation per target base pair per cycle (24, 27), where base substitution mutations (G-to-A and C-to-T transitions) and frameshift mutations (−1 frameshifts in runs of T's and A's) were most commonly detected. Replication of HIV-1 in the presence or absence of the auxiliary protein Vpr indicated that the mutation rate was as much as fourfold higher in the absence of Vpr (25, 27). This indicated that Vpr could influence the in vivo mutation rate of HIV-1. The *vpr* gene encodes a 96-amino-acid (aa) nonstructural protein which is associated with HIV-1 particles at a level comparable to that of the Gag precursor and then accumulates in the nuclei of infected cells (7, 22, 30). Incorporation of Vpr into particles requires a direct interaction with the p6 region of Gag (1, 35). In addition to influencing the mutation rate, Vpr has been implicated in the nuclear translocation of the preintegration complex and in cell arrest in the G_2 phase of the cell cycle (11, 12, 17, 33). A recent report has indicated that Vpr alone could decrease the frequency of deletion mutations which occur following introduction of UV-damaged plasmid DNA into cells (16). This phenotype does not appear to be related to Vpr's role in the process and accuracy of reverse transcription (25).

The HIV-1 Vpr protein has been found to interact with several cellular partners (32, 51), in particular with two proteins involved in the DNA repair process, uracil DNA glycosylase (UNG) and the human homologue of the yeast RAD23 protein (HHR23A) (3, 10, 49). UNG is an enzyme involved in the base excision repair pathway which specifically removes the RNA base uracil from DNA (19). Uracil appears in DNA by misincorporation during its synthesis when the dUTP pool level is high or by cytosine deamination of dCMP. When cytosine deamination occurs and is not repaired, the result is a C-to-T transition mutation in that DNA strand (and a G-to-A transition in the opposite strand) in the next round of replication. The human *ung* gene contains two promoters that are required for generation of the mitochondrial (UNG1) and nuclear (UNG2) forms of the enzyme by alternative splicing (28). UNG1 and UNG2 have 35 and 44 unique N-terminal aa, respectively, while the C-terminal 269 aa are identical and contain the catalytic domain. The Vpr-binding site was mapped within the common C-terminal part of UNG, but the interaction did not perturb in vitro UNG enzymatic activity (3). While recent results suggest that the HHR23A protein is a mediator of Vpr-induced cell cycle arrest (10, 34), a detailed mutational analysis of Vpr revealed that the interaction with UNG is genetically separable from the ability of Vpr to perturb the cell cycle (49). A Trp residue located in position 54 of Vpr was found to be critical for the interaction with UNG, but replacement of this residue did not disrupt the G_2 arrest activity. It has been observed that Vpr from simian immunodeficiency virus of sooty mangabeys, but not Vpx, associates with UNG (36).

Based on these observations, we tested the hypothesis that the interaction of Vpr with UNG could influence the in vivo mutation rate of HIV-1. We found that binding of Vpr to UNG correlates with the influence of Vpr on the mutation rate. We demonstrate that Vpr recruits the nuclear form of UNG into HIV-1 virions to influence the in vivo mutation rate. These data indicate a mechanism by which Vpr can influence reverse

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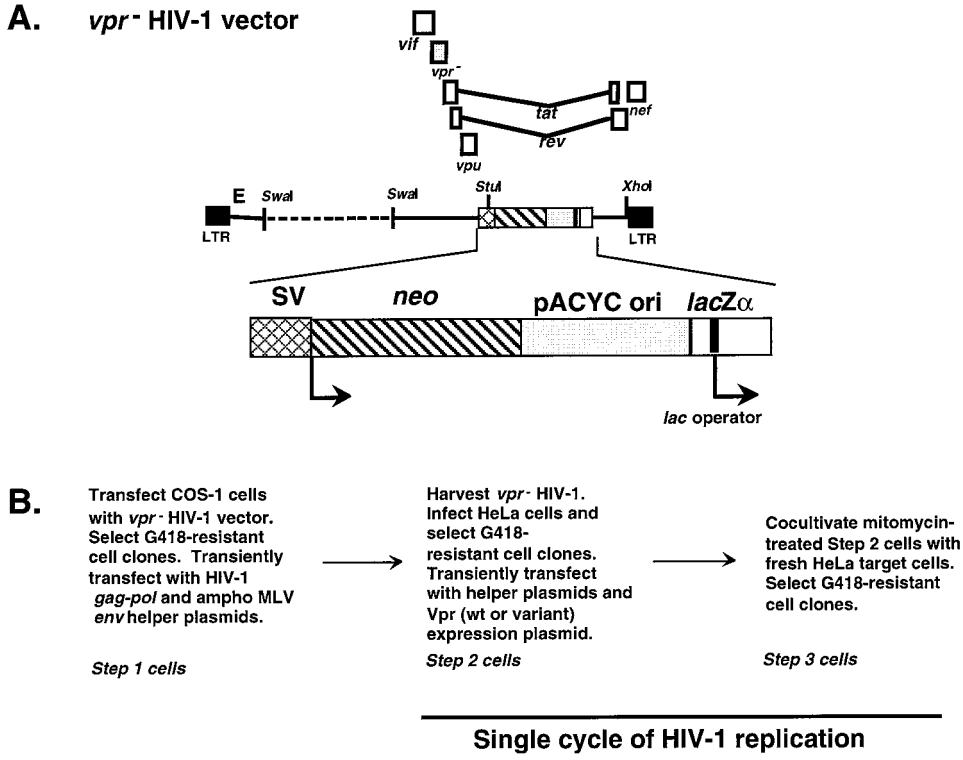

FIG. 1. HIV-1 vector used for in vivo forward mutation rate studies. (A) *vpr* null mutant HIV-1 vector. The vector is shown in the proviral DNA form and has been previously described (25). (B) Protocol for one cycle of HIV-1 vector replication. The steps going from a parental shuttle vector provirus in the step 2 cell to a vector provirus in the step 3 cell constitute a single cycle of replication. LTR, long terminal repeat; SV, simian virus 40; ampho MLV, amphotropic murine leukemia virus.

transcription accuracy and suggest the evolution of distinct mechanisms in primate and nonprimate lentiviruses to reconcile uracil misincorporation into lentiviral DNA.

MATERIALS AND METHODS

HIV-1 vectors and expression plasmids used for mutation rate studies. The HIV-1 vector used in the in vivo forward mutation rate assay (Fig. 1A) was constructed as previously described (27). A *vpr* null mutant derivative of this vector was made by a primary-combinatorial two-step PCR protocol (25). Plasmids pSVgagpol-rre-r and pSV-A-MLV-env have been previously described (25). The vectors used for expression of wild-type (wt) Vpr or Vpr variants (pCMVvpr) were constructed by amplifying the wt or mutated *vpr* gene by PCR and inserting it into plasmid pCR3 (Invitrogen). The Vpr variants (Vpr*W54R and Vpr*R90K) were selected by two-hybrid screening from an HIV-1 Vpr mutant library generated by random error-prone PCR (34).

Cell culture, transfections, infections, and cocultivations. HeLa and COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum or 10% fetal bovine serum, respectively. HIV-1 vectors and expression plasmids were transfected into COS-1 or HeLa cells by use of dimethyl sulfoxide-Polybrene (27). HeLa cells were infected in the presence of Polybrene. Infection of HeLa target cells was done by cocultivation of virus-producing cells with target cells (25). The 293T cells used in the virion packaging assay were maintained in DMEM supplemented with 10% fetal calf serum, and 25 mM HEPES was added during virus production. They were transfected with the 22-kDa polyethylenimine (Euromedex) as previously described (35).

Cell culture strategy used to generate a single cycle of HIV-1 replication. The experimental protocol developed to assay a single cycle of HIV-1 shuttle vector replication is shown in Fig. 1 (25). 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 mutation was introduced.

Recovery of HIV-1 vector proviral DNA and sequence analysis of the *lacZ*α peptide region. Purified genomic DNA from pools of step 3 clones was digested with the restriction enzymes *Stu*I and *Xho*I to release the *neo*, pACYC origin of replication, and *lacZ*α peptide gene sequences from the HIV-1 vector. Proviral DNA was purified with the Lac repressor protein as previously described (25). The ratio of the number of white plus light blue bacterial colonies to the total number of colonies observed provided the forward mutation rate for a single retroviral replication cycle. Plasmid DNA was purified and sequenced in the *lacZ*α peptide gene region by an automated DNA sequencer (Applied Biosystems). Mutation rates were calculated as previously described (25).

Yeast two-hybrid assay. The construction of the HIV-1 Vpr mutant library fused to the DNA-binding domain of the LexA repressor (LexABD) and the two-hybrid screening procedure of the library have already been described (34). Vectors for expression of wt Vpr or the Vpr*W54R and Vpr*R90K variants fused to LexABD were described previously (34), while vectors for expression of UNG1, UNG2, and a truncated form of UNG without the N-terminal part of the protein (UNG57/66) fused to the Gal4 activation domain (Gal4AD) were constructed in the pGAD1318 plasmids (3). The L40 yeast strain was cotransformed with the indicated LexABD and Gal4AD hybrid expression vectors and plated on selective medium. Double transformants were then assayed for β-galactosidase activity and histidine auxotrophy as previously described (34).

Analysis of Vpr and UNG incorporation into HIV-1 virions. Incorporation of Vpr and UNG was analyzed using a packaging assay in which Vpr and UNG were expressed in *trans* and incorporated into virions (35). The HIV-1-based packaging vectors pCMVΔR8.9 (lacking the *env* and auxiliary genes) and pCMVΔR8.2 (lacking only the *env* gene) and the pMD.G plasmid for expression of the vesicular stomatitis virus G protein were kindly provided by D. Trono (Geneva, Switzerland) (53). Vectors for expression of wt or mutated Vpr and UNG1, UNG2, and UNG57/66 fused to the epitope tag from the influenza virus hemagglutinin (HA) were constructed in pAS1B as previously described (35). For analysis of Vpr-dependent incorporation of UNG, cells were cotransfected with 10 μg of pCMVΔR8.9, 5 μg of pMD.G, 10 μg of pAS1B-UNG57/66, and the indicated amounts of pAS1B-Vpr (wt or mutated). For incorporation analysis of the distinct UNG forms, cells were cotransfected with 10 μg of pCMVΔR8.2, 5 μg of pMD.G, and 10 μg of either pAS1B-UNG1, -UNG2, or -UNG57/66. Cell culture supernatants were collected 48 h after transfection and filtered through 0.45-μm-pore-size filters, and an aliquot was assayed for CAp24 antigen. Virions were collected by ultracentrifugation for 1 h at 100,000 × *g* and suspended in ice-cold lysis buffer (10 mM Tris [pH 7.6], 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100). For preparation of cell lysates, cells were trypsinized, collected by centrifugation, and suspended in ice-cold lysis buffer. Cell and virion lysates were incubated on ice for 5 min and clarified by centrifugation. The protein concentration of the cell lysates was measured (Bio-Rad). Proteins from cell (50 μg of total protein) and virion (50 ng of CAp24) lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed

TABLE 1. Incorporation of Vpr into virions is required to influence the mutation frequency of HIV-1 vectors[a]

Virus variant	No. of mutants/total no. of bacterial colonies	Mutation frequency (no. of mutations/cycle)
vpr null mutant HIV-1	24/1,107	0.022
	19/1,183	0.016
vpr null mutant HIV-1 *trans* complemented with wt Vpr in producer cells	10/1,520	0.007
	7/1,264	0.006
p6$^-$ *vpr* null mutant HIV-1	27/1,329	0.020
	14/943	0.015
p6$^-$ *vpr* null mutant HIV-1 *trans* complemented with wt Vpr in producer cells	33/1,775	0.019
	28/1,181	0.024
	19/1,227	0.015
vpr null mutant HIV-1 *trans* complemented with wt Vpr in target cells	27/1,329	0.020
	14/943	0.015
	30/1,673	0.018

[a] The average mutation frequency of *vpr* null mutant HIV-1 in the absence of *trans* complementation was 0.019 mutation/cycle and was 0.006 mutation/cycle in the presence of Vpr *trans* complementation. The average mutation frequency of p6$^-$ *vpr* null mutant HIV-1 was 0.018 mutation/cycle. Complementation with wt Vpr of p6$^-$ *vpr* null mutant HIV-1 resulted in an average mutation frequency of 0.019 mutation/cycle (standard deviation of 0.005 mutation/cycle). Complementation with wt Vpr in target cells resulted in an average mutation frequency of 0.018 mutation/cycle (standard deviation of 0.003 mutation/cycle).

by Western blotting with anti-HA 3F10 (Boehringer) or anti-CAp24 antibodies (39).

RESULTS

Replication of a *vpr* null mutant HIV-1 vector and genetic *trans* complementation with Vpr. The *vpr* null mutant HIV-1 vector (25) used in these studies was derived from the HIV-1 shuttle vector 3.12 (Fig. 1A). In order to complement this *vpr* null mutant HIV-1 vector, a plasmid expressing wt Vpr or a Vpr variant was transiently transfected along with *gag-pol* and *env* expression plasmids (25). The HIV-1 vector produced from either COS-1 or HeLa cells was used to infect fresh HeLa target cells (Fig. 1B). Cocultivation of mitomycin C-treated step 2 cells with fresh HeLa target cells to produce step 3 cells led to titers that were typically about 8×10^2 to 3×10^3 CFU/2.5×10^5 HeLa target cells. The steps going from a parental shuttle vector provirus in step 2 cells to a vector provirus in step 3 cells constitute a single cycle of replication (Fig. 1B). Southern analysis of total DNA from each step 2 cell clone was done to ensure that each clone contained only one provirus copy (data not shown). Proviral DNA from at least 5×10^5 cells of each step 2 cell clone was purified using the Lac repressor protein and introduced into *Escherichia coli* to screen for mutations in the *lacZ*α gene region.

Vpr virion incorporation is required to influence the in vivo mutation rate. The virion incorporation of Vpr into HIV-1 particles requires direct interaction with the p6 region of the Gag precursor (1, 35). In order to extend the observation that Vpr virion incorporation is required to influence the HIV-1 mutation rate, we used the assay described in Fig. 1B to compare the effects of Vpr *trans* complementation on the mutation frequencies of *vpr* null mutant HIV-1 (expressing a wt *gag* gene) and p6$^-$ *vpr* null mutant HIV-1 (expressing a mutant *gag* gene lacking the p6-encoding region). As indicated in Table 1, complementation with Vpr had no influence on the mutation rate of p6$^-$ *vpr* null mutant HIV-1 and resulted in an average mutation frequency comparable to that of noncomplemented *vpr* null mutant HIV-1 (chi square, 0.009; $P > 0.95$) but significantly higher (chi square, 20; $P < 0.01$) than that of *vpr* null mutant HIV-1 complemented with Vpr. Levels of Vpr expression were comparable in cells expressing the wt and p6-truncated forms of the Gag precursor (data not shown). These data support the requirement of Vpr incorporation into virions in order for Vpr to influence the HIV-1 mutation rate.

To test whether *trans* complementation of Vpr could be provided in the infected target cells rather than in the virus-producing cells, *vpr* null mutant HIV-1 was replicated with *trans* complementation of Vpr in step 3 target cells. Complementation of target cells with Vpr resulted in a mutation frequency significantly higher (chi square, 15; $P < 0.01$) than that observed by complementation in virus-producing cells and equivalent (chi square, 0.033; $P > 0.5$) to the mutation frequency of noncomplemented *vpr* null mutant HIV-1 (Table 1). Expression of Vpr in target cells was comparable to that in the virus-producing cells (not shown). These data indicate that *trans* complementation with Vpr in the target cells does not complement *vpr* null mutant HIV-1 in the mutation rate assay. This further supports the conclusion that Vpr virion incorporation is required to influence the HIV-1 mutation rate.

Vpr binding to UNG correlates with the influence on the HIV-1 in vivo mutation rate. In order to analyze the potential correlation between the Vpr influence on the HIV-1 mutation frequency and the interaction with UNG, the effect of Vpr variants was analyzed in the mutation rate assay. We had previously reported that a single substitution of the Trp residue in position 54 (Vpr*W54R variant) was sufficient to abolish binding to UNG but did not disrupt the Vpr-induced G_2 arrest activity. These data demonstrated the critical role of this residue in the maintenance of Vpr binding to UNG and indicated that this interaction is not involved in the perturbations of the cell cycle (34). The Vpr*R90K variant, containing a conservative substitution of Arg90 located in the C-terminal basic domain of the protein, was included in our analysis to study the relationship between the G_2 arrest activity and the influence on the HIV-1 mutation rate. This mutant interacted with UNG as efficiently as wt Vpr but was unable to induce G_2 arrest in HeLa cells (34).

Vpr*W54R and Vpr*R90K were analyzed in parallel for their influence on the HIV-1 mutation rate. The *vpr* null mutant HIV-1 vector was replicated in the absence of Vpr or *trans* complemented with a wt Vpr, Vpr*W54R, or Vpr*R90K expression plasmid. The proviral DNA from pooled step 3 cells, representing over 50,000 different clones for each experiment, was purified and introduced into *E. coli* to screen for mutations in the *lacZ*α gene.

Three thousand seven hundred thirty-four bacterial colonies were screened in three replicates where *vpr* null mutant HIV-1 was *trans* complemented with Vpr*W54R. Fifty-nine of these colonies had a white or light blue colony color phenotype (Table 2). The average mutation frequency in these experiments was 59 to 3,734 or 0.016 mutation per cycle. The mutation frequency of *vpr* null mutant HIV-1 complemented with Vpr*W54R was significantly different from that found when it was complemented with wt Vpr (chi square, 17; $P < 0.01$) but not from the mutation frequency of noncomplemented *vpr* null mutant HIV-1 (chi-square, 0.4; $P > 0.5$). This indicates that expression of Vpr*W54R leads to a mutation frequency phenotype comparable to that of *vpr* null mutant HIV-1 alone and therefore does not influence the in vivo mutation rate. Vpr*W54R interacted with the Gag precursor (data not shown) and was incorporated into HIV-1 particles as efficiently as wt Vpr (see Fig.

TABLE 2. Mutation frequency in recovered proviruses of *vpr* null mutant HIV-1 complemented in *trans* with Vpr variants with differential binding to UNG[a]

trans-Vpr variant	No. of mutants/total no. of bacterial colonies	Mutation frequency (no. of mutations/cycle)
No *trans* complementation	13/928	0.014
	18/773	0.023
wt Vpr	10/2,029	0.005
	12/1,726	0.007
Vpr*W54R	23/1,639	0.014
	19/1,285	0.015
	17/810	0.021
Vpr*R90K	11/1,340	0.008
	3/709	0.004
	8/1,233	0.007

[a] The average mutation frequency of *vpr* null mutant HIV-1 in the absence of *trans* complementation was 0.018 mutation/cycle. Complementation with wt Vpr resulted in an average mutation frequency of 0.006 mutation/cycle. Complementation with Vpr variants Vpr*W54R and Vpr*R90K resulted in average mutation frequencies of 0.016 mutation/cycle (standard deviation of 0.004 mutation/cycle) and 0.007 mutation/cycle (standard deviation of 0.002 mutation/cycle), respectively.

3C and reference 35), indicating that the mutation frequency phenotype of Vpr*W54R was not related to inefficient virion incorporation.

Three thousand two hundred eighty-two bacterial colonies were screened in three replicates where *vpr* null mutant HIV-1 was complemented with Vpr*R90K. Twenty-two of these colonies had a white or light blue colony color phenotype (Table 2). The average mutation frequency was thus 22 to 3,282 or 0.007 mutation per cycle. The mutation frequency of *vpr* null mutant HIV-1 complemented with Vpr*R90K was not significantly different from that of *vpr* null mutant HIV-1 complemented with wt Vpr (chi square, 0.02; $P > 0.5$) but was significantly different from the mutation frequency of noncomplemented *vpr* null mutant HIV-1 (chi square, 15; $P < 0.01$). We have previously shown that Vpr*R90K bound to the Gag precursor and was efficiently incorporated into HIV-1 particles (35). These results indicate that the expression of Vpr*R90K influences the in vivo mutation rate in a manner comparable to that of wt Vpr. Since Vpr*R90K completely fails to induce a G_2 arrest (34), they indicate that the effects of Vpr on the cell cycle are genetically separable from those on the reverse transcription process.

Complementation of *vpr* mutant HIV-1 with a Vpr*W54R UNG binding-deficient mutant protein leads to a mutation phenotype similar to that observed with *vpr* null mutant HIV-1 alone. In order to compare the mutation phenotype of *vpr* mutant HIV-1 complemented with Vpr*W54R to that of noncomplemented *vpr* null mutant HIV-1, the types of mutations that led to the white or light blue colony color phenotype were determined by DNA sequencing of the *lacZ*α gene (Fig. 2). Twenty (34%) of the 59 mutants sequenced from *vpr* null mutant HIV-1 complemented with Vpr*W54R had a single G-to-A base pair transition mutation, which was the predominant substitution observed. This percentage is comparable to what was observed for single G-to-A substitution mutations in *vpr* null mutant HIV-1 alone (11 [37%] of 30). Six hypermutants that each contained multiple mutations were observed for *vpr* null mutant HIV-1 complemented with Vpr*W54R (Fig. 2), and five of them contained at least one G-to-A transition. Therefore, 25 (44%) of the 59 mutants sequenced for *vpr* null mutant HIV-1 complemented with Vpr*W54R had G-to-A mutations. In comparison, two hypermutants (Fig. 2) were observed for *vpr* null mutant HIV-1 alone and both had at least one G-to-A mutation, indicating that 13 (43%) of the 30 mutants had G-to-A mutations. These data indicate that the rates of G-to-A mutation in both *vpr* null mutant HIV-1 complemented with Vpr*W54R and *vpr* null mutant HIV-1 alone are

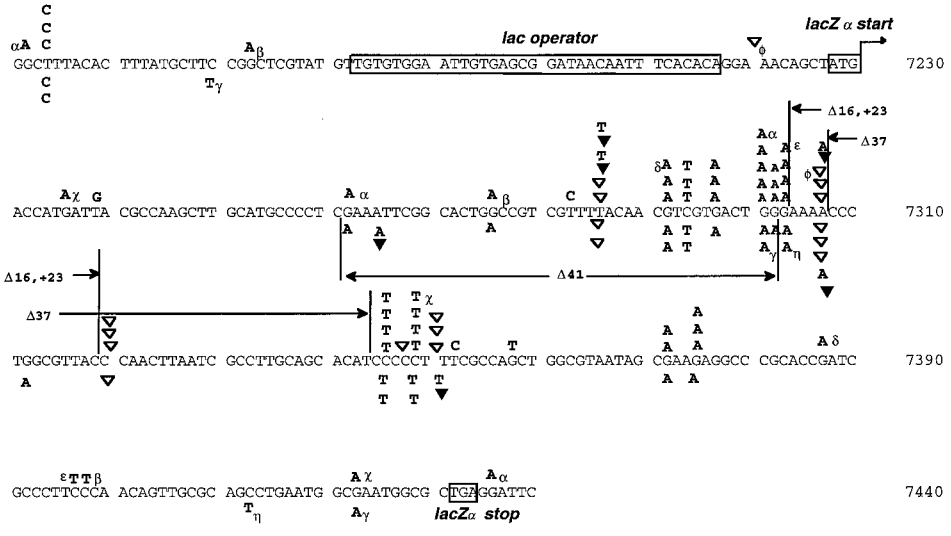

FIG. 2. Plus strand nucleotide sequence of the *lacZ*α gene region in a *vpr* null mutant HIV-1 vector complemented with Vpr*W54R. The start for nucleotide numbering is the beginning of the 5′ long terminal repeat of the HIV-1 vector. The locations of base substitution, frameshift, and deletion mutations in *vpr* null mutant HIV-1 vectors from parallel experiments that were complemented with Vpr*W54R or noncomplemented are shown above and below the nucleotide sequence, respectively. The start and stop codons of the *lacZ*α open reading frame and the *lac* operator sequence are boxed. Nucleotide positions of base pair substitutions (letters above or below the sequence), +1 frameshifts (letters with ▼), −1 frameshifts (▽) and deletions (solid black lines with arrows and adjacent to the deletion names) are indicated. Mutations from the same mutant are designated with a Greek letter adjacent to each mutation; identical Greek letters indicate mutations from the same mutant.

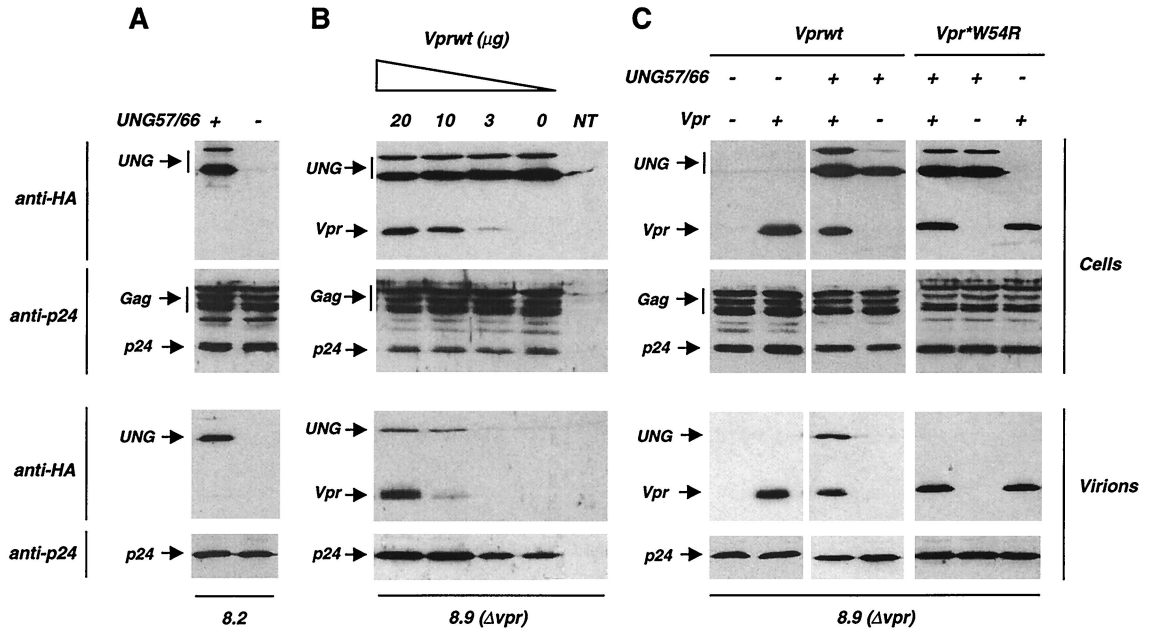

FIG. 3. Vpr-dependent recruitment of UNG into HIV-1 particles. (A) Virion incorporation of UNG57/66. 293T cells were cotransfected with pCMVΔR8.2 (containing an intact *vpr* gene), pMD.G, and either pAS1B-UNG57/66 (+) or pAS1B without an insert (−). (B) Vpr dose-dependent incorporation of UNG. Cells were cotransfected with pCMVΔR8.9 (lacking *vpr*) and pMD.G in combination with 10 μg of pAS1B-UNG57/66 and the indicated increasing amounts of pAS1B-Vprwt. NT, nontransfected cells. (C) Failure of Vpr*W54R to recruit UNG into virions. Cells were cotransfected with pCMVΔR8.9 and pMD.G with or without 10 μg of pAS1B-UNG57/66 in combination with 20 μg of either pAS1B-Vprwt, pAS1B-Vpr*W54R, or pAS1B without an insert (−). Proteins from cell and virion lysates were separated by SDS-PAGE and analyzed by Western blotting with anti-HA (cells and virions, upper panels) or anti-CAp24 (cells and virions, lower panels) antibody.

comparable and three- to fourfold higher than that of *vpr* null mutant HIV-1 complemented with wt Vpr (data not shown).

C-to-T transition mutations were the second most common type of mutation detected for both *vpr* null mutant HIV-1 complemented with Vpr*W54R and *vpr* null mutant HIV-1 alone (Fig. 2). For *vpr* null mutant HIV-1 complemented with Vpr*W54R, 10 (17%) of 59 mutants contained a single C-to-T mutation. Two of the six hypermutants had one C-to-T transition, for a total of 12 (20%) of 59 mutants having C-to-T transitions. For *vpr* null mutant HIV-1 alone, 6 (20%) of 30 mutants had single C-to-T transitions, both hypermutants had C-to-T transitions, and thus a total of 8 (27%) of the 30 mutants had C-to-T transitions. This indicates that the rates of C-to-T transitions are similar for both *vpr* null mutant HIV-1 complemented with Vpr*W54R and *vpr* null mutant HIV-1 alone and are over twofold higher than that of *vpr* null mutant HIV-1 complemented with wt Vpr (data not shown).

Single frameshift mutations as an entire group of mutations (but primarily −1 or +1 frameshifts in runs of T's and A's) were identified in 14 (24%) of 59 mutants for *vpr* null mutant HIV-1 complemented with Vpr*W54R, and 8 (27%) of 30 for *vpr* null mutant HIV-1 alone. One hypermutant from *vpr* null mutant HIV-1 complemented with Vpr*W54R had two −1 frameshifts in runs of A's. The rates of frameshift mutations observed for *vpr* null mutant HIV-1 complemented with Vpr*W54R and for *vpr* null mutant HIV-1 alone are comparable to what was observed in *vpr* null mutant HIV-1 complemented with wt Vpr (not shown). Similarly, the rates of deletion mutations detected for both *vpr* null mutant HIV-1 complemented with Vpr*W54R (1 [2%] of 59) and for *vpr* null mutant HIV-1 alone (1 [3%] of 30) are comparable to that observed in *vpr* null mutant HIV-1 complemented with wt Vpr (not shown). These data confirm that these two types of mutations are not influenced by expression of Vpr (25).

Based upon the characterization of the types of mutations that occurred, the calculated in vivo mutation rate for *vpr* null mutant HIV-1 complemented with Vpr*W54R is 13×10^{-5} mutation per target base pair per cycle, which is fourfold higher than that of *vpr* null mutant HIV-1 complemented with wt Vpr.

UNG is recruited into HIV-1 particles through Vpr incorporation. Since virion incorporation of Vpr is required to influence the in vivo mutation rate, we have explored whether UNG could be recruited into virus particles. Incorporation into virions was analyzed using a packaging assay in which UNG fused to the HA epitope (HA-tagged UNG) was expressed in *trans* in virus-producing cells (35). We first analyzed the incorporation of a truncated form of UNG containing a deletion of the N-terminal part of the protein (UNGΔ57/66) because it corresponded to the UNG clone initially isolated in the two-hybrid screening performed to identify Vpr-interacting proteins (3). 293T cells were transfected with the HA-tagged UNG expression vector in combination with a HIV-1-based packaging vector (pCMVΔR8.2) containing an intact *vpr* gene (53), and the virion- and cell-associated UNG was then assessed by Western blot analysis using an anti-HA monoclonal antibody (MAb) (Fig. 3A). The HA-tagged version of UNG57/66 was detected in the supernatant of transfected cells (lower panel), indicating that it is incorporated into virions. To determine if the recruitment of UNG into virions is dependent on Vpr incorporation, we used the same virion packaging assay described above but HA-tagged Vpr and UNG57/66 were both expressed in *trans* in virus-producing cells transfected with a HIV-1 packaging vector lacking the auxiliary genes (pCMVΔR8.9). Parallel experiments were performed in which 293T cells were cotransfected with pCMVΔR8.9 and a constant amount of the HA-tagged UNG57/66 expression vector, in combination with increasing amounts of the HA-tagged Vpr

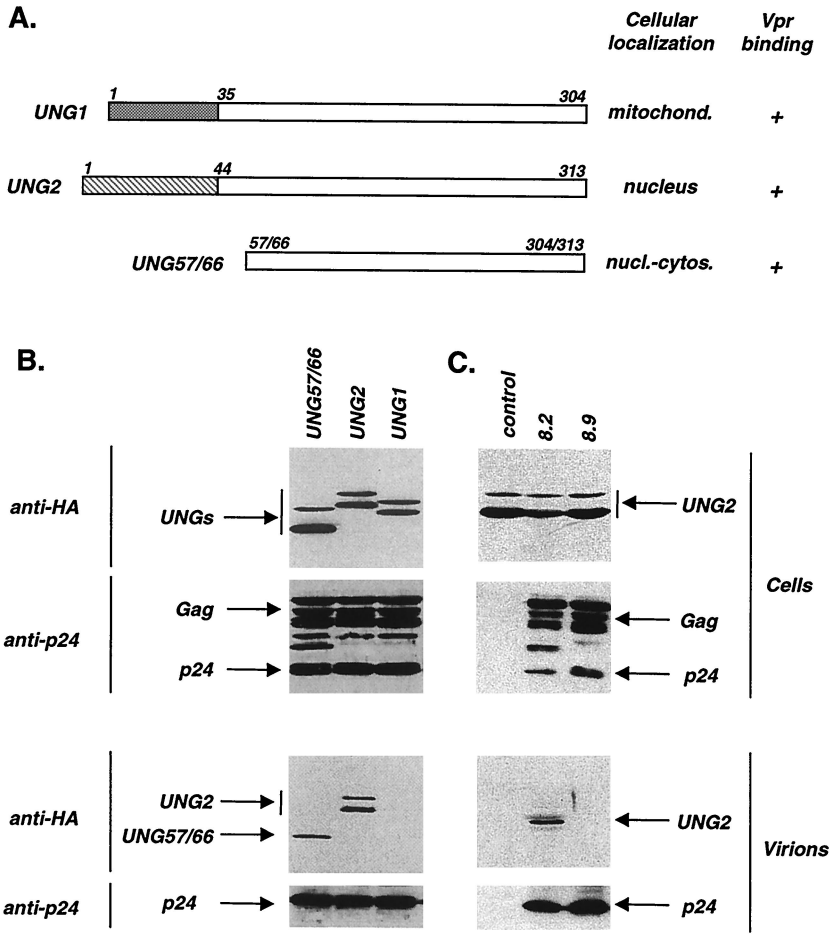

FIG. 4. Preferential incorporation of nuclear UNG2 into HIV-1 virions. (A) Cellular distribution and Vpr binding of UNG1, UNG2, and UNG57/66. UNG1 and UNG2 have 35 and 44 unique N-terminal aa, respectively, while the C-terminal 269 aa are identical. Cellular distribution was analyzed by indirect immunofluorescence assay of 293T cells transfected with plasmid pAS1B-UNG1, -UNG2, or -UNG57/66. Vpr binding was determined in a two-hybrid assay of L40 yeast cells expressing the LexABD-Vprwt hybrid and either UNG1, UNG2, or the UNG57/66 Gal4AD hybrid. mitochon., mitochondria; nucl., nucleus; cytos., cytoplasm. (B) Virion incorporation analysis of the two UNG forms. 293T cells were cotransfected with pCMVΔR8.2, pMD.G, and either plasmid pAS1B-UNG1, -UNG2, or -UNG57/66. (C) Vpr-dependent incorporation of UNG2 into virions. 293T cells were cotransfected with pMD.G, pAS1B-UNG2, and either plasmid pCMVΔR8.2 or pCMVΔR8.9. In panels B and C, proteins from cell and virion lysates were separated by SDS-PAGE and analyzed by Western blotting with anti-HA (cells and virions, upper panels) or anti-CAp24 (cells and virions, lower panels) antibody.

vector. The virion- and cell-associated UNG and Vpr were then assessed by Western blot analysis with the anti-HA MAb (Fig. 3B). No HA-UNG57/66 was detected in virions when Vpr was not expressed in virus-producing cells, indicating that UNG is incorporated in a Vpr-dependent manner. In contrast, UNG57/66 was found in virions when the amount of Vpr expressed in cells was increased. Both HA-Vpr and UNG were simultaneously detected in a range of 10 to 20 μg of Vpr plasmid that was transfected into cells. The same blots were probed with an anti-CAp24 MAb to verify that similar amounts of virions were produced in all of the transfections. These results demonstrate that Vpr incorporation is required to recruit UNG into HIV-1 particles.

We then analyzed whether the mutation frequency phenotype of the Vpr*W54R UNG binding-deficient variant was related to a defect in UNG incorporation into virions. Cells were thus transfected with pCMVΔR8.9 and the HA-tagged UNG57/66 expression vectors, in combination with the HA-tagged wt Vpr or Vpr*W54R expression plasmid (Fig. 3C). As previously, UNG57/66 was detectable in virions only when wt Vpr was coexpressed in virus-producing cells, and both UNG and wt Vpr were thus incorporated. In contrast, no UNG was detected in virions produced from cells expressing high levels of Vpr*W54R, even though this variant was incorporated as efficiently as wt Vpr. These data demonstrate that a UNG binding-deficient Vpr variant does not recruit UNG into virions, suggesting that the mutation frequency phenotype of Vpr*W54R results from its inability to allow the recruitment of UNG into HIV-1 particles.

The nuclear form of UNG is preferentially incorporated into HIV-1 particles. Since UNG exists as mitochondrial (UNG1) and nuclear (UNG2) isoforms whose N-terminal sequences differ (see Fig. 4A), we have explored whether both forms of UNG could be recruited into virions. Each of these UNG forms fused to Gal4AD bound to LexABD-Vpr as efficiently as did UNG57/66 in a yeast two-hybrid assay (Fig. 4A), confirming that the N-terminal portions of UNG1 and UNG2 do not influence Vpr binding. The incorporation of UNG1 and UNG2 into virions was analyzed by transfection of 293T cells with the HIV-1 pCMVΔR8.2 vector and the HA-tagged UNG1, UNG2, or UNG57/66 expression vector. Virion- and cell-associated UNGs were then assessed by Western blot analysis using the

anti-HA MAb (Fig. 4B). UNG2 and UNG57/66 were efficiently incorporated into virions, since the HA-tagged version of each form was detected in the supernatants of transfected cells (lower panel). In contrast, mitochondrial UNG1 was not incorporated into virions despite detectable level of the protein in transfected cell lysate (upper panel). UNG2 was incorporated in a Vpr-dependent manner, since it was detected in virions when 293T cells were transfected with pCMVΔR8.2 but not when cells were transfected with pCMVΔR8.9 lacking the *vpr* gene (Fig. 4C). These results indicate that nuclear UNG2 is the preferential form incorporated into HIV-1 particles.

DISCUSSION

This work focused on the functional characterization of the interaction of HIV-1 Vpr with UNG, a cellular protein that is implicated in the DNA repair process. The results indicate the contribution of the binding of Vpr to UNG to the in vivo mutation rate of HIV-1. Further data are presented which show that the Vpr recruitment of the nuclear form of UNG into HIV-1 particles is required for Vpr to influence the in vivo mutation rate. The correlation between the capacity of Vpr to interact with UNG and its ability to both influence the HIV-1 mutation rate and mediate virion packaging of UNG supports this conclusion. In contrast, Vpr binding to HHR23A, the other Vpr-interacting DNA repair protein, does not correlate with the influence of Vpr on the mutation rate (L.M.M. and S.B., unpublished results).

Two Vpr variants were tested for their influence on the in vivo mutation rate of HIV-1 in order to assess whether the interaction of Vpr with the DNA repair protein UNG could be correlated with the influence of Vpr on the mutation rate. The Vpr*W54R variant does not bind UNG, does interact with the Gag precursor, is efficiently incorporated into virus particles, and causes cell cycle arrest. This indicates that Vpr*W54R displays a wt phenotype, with the exception of its inability to bind UNG. When Vpr*W54R was used for *trans* complementation of *vpr* null mutant HIV-1 in a single cycle of replication, it was found that the rate of mutation was comparable to that of noncomplemented *vpr* null mutant HIV-1 alone. In addition, *vpr* null mutant HIV-1 complemented with Vpr*W54R had a spectrum of mutations similar to that of noncomplemented *vpr* null mutant HIV-1. This provides genetic evidence in support of the conclusion that the inability of Vpr*W54R to interact with UNG influences the in vivo mutation rate of HIV-1. In contrast, Vpr*R90K influences the in vivo mutation rate in a manner comparable to that of wt Vpr but completely fails to induce a G_2 arrest (34). Therefore, the Vpr effects on the cell cycle are genetically separable from those on the HIV-1 mutation rate since they are related to distinct regions of Vpr. Alpha-helical region II of the protein contributes to reverse transcription accuracy, while the C-terminal basic domain is crucial for the G_2 arrest activity (8, 52).

The absence of UNG in HIV-1 particles that have efficiently incorporated Vpr*W54R indicates that the failure of Vpr*W54R to interact with UNG not only prevents virion incorporation of UNG but also affects the influence of Vpr on the HIV-1 mutation rate. The Vpr dose dependence for incorporation of UNG into HIV-1 particles also provides evidence for a Vpr-specific mechanism of UNG incorporation. These observations indicate that for Vpr to influence the in vivo mutation rate of HIV-1, both Vpr and UNG must be efficiently incorporated into HIV-1 particles. Nuclear UNG2 is the predominant form of UNG that is incorporated into virions, whereas the mitochondrial UNG1 form is not efficiently incorporated. Like UNG2 (28), HIV-1 Vpr displays evident karyophilic properties (15, 31, 46), suggesting that the Vpr-UNG2 complex takes place in the nuclei of infected cells, migrates to the cytoplasm, and then is incorporated into virions through the interaction of Vpr with the Gag precursor protein. The inability of Vpr*W54R to mediate UNG incorporation is not related to a defect of nuclear import of this Vpr variant, since it localizes to the nucleus as efficiently as the wt protein (not shown). Alternatively, the Vpr-UNG2 complex could be formed in the cytoplasm and targeted to the plasma membrane before nuclear import of both Vpr and UNG2. Since the Trp54 residue located in C-terminal alpha-helical region II of Vpr is crucial for the maintenance of UNG binding (34), it appears that Vpr may also simultaneously interact with the Gag precursor through N-terminal alpha-helical region I (8, 23, 35, 50). In contrast, the UNG1 form sequestered into mitochondria fails to access the core of HIV-1 virions although it displays the ability to physically interact with Vpr. It was recently reported that UNG was detected in HIV-1 virions in the absence of Vpr, requiring the presence of the viral integrase protein when Vpr is absent for UNG incorporation (48). Determination of UNG incorporation with a Vpr mutant that was deficient in UNG binding but was efficiently incorporated into HIV-1 particles was not analyzed in this study. Our data indicate that when Vpr is not present in HIV-1 particles, there is no detectable UNG incorporation. While we cannot formally exclude the possibility that integrase also contributes to UNG incorporation in the virion packaging assay used in the present study, our results suggest that the interaction of UNG with Vpr is the major pathway for UNG incorporation into HIV-1 particles.

The observation that Vpr binding to UNG correlates with the in vivo mutation rate of HIV-1 implies a role for UNG in the accuracy of the reverse transcription process. UNG functions in cells as a DNA repair enzyme that specifically removes from DNA the RNA base uracil, which appears by misincorporation during DNA synthesis when the dUTP pool is high or by cytosine deamination of dCMP. When cytosine deamination occurs, the result is a C-to-T transition mutation in that DNA strand and a G-to-A transition in the opposite strand in the next round of replication. The data presented in Fig. 2 indicate that the predominant types of mutations detected in *vpr* null mutant HIV-1 both alone and *trans* complemented with Vpr*W54R were G-to-A and C-to-T transition mutations. Based upon what is known of UNG function, a G-to-A transition mutation in the HIV-1 plus-strand DNA, in the absence of functional UNG activity, could be an indication of cytosine deamination in the minus strand DNA made during reverse transcription. In the presence of UNG activity, the uracil created by cytosine deamination would be in the DNA strand of a DNA-RNA hybrid, assuming that the cytosine deamination occurred during the minus strand DNA synthesis step of reverse transcription. Little is known regarding the function of UNG in removing uracil from DNA that is in a DNA-RNA hybrid. The presence in HIV-1 particles of other repair enzymes which participate in the uracil excision repair pathway could help to support the specific role in virion packaging of UNG and the HIV-1 mutation rate. However, we failed to detect in HIV-1 virions the apurinic/apyrimidinic (AP) endonuclease (known as HAP, APEX, or Ref-1), the second enzyme involved in this pathway (19), suggesting that the other enzymes are recruited after viral entry into the target cells. Although the data presented here indicate that UNG enzymatic activity directly influences the HIV-1 mutation rate, UNG may also influence the mutation rate by other mechanisms, such as modulation of the access of deoxynucleoside triphosphates to reverse transcriptase or interaction of the

Vpr-UNG complex with the reverse transcriptase to influence its enzymatic fidelity.

Most nonprimate lentiviruses are known to encode and package into virus particles a dUTPase, an enzyme that regulates the levels of dUTP in cells and therefore influences the potential misincorporation of uracil into viral DNA (9, 20, 21, 38, 43–45). Replication of nonprimate lentiviruses that lack functional dUTPase activity leads to misincorporation of uracil into viral DNA, a reduced level of replication in macrophages (i.e., nondividing cells), and an increased level of G-to-A transition mutations. The inhibition of dUTPase activity leading to an increased level of G-to-A transitions appears to have a phenotype similar to that of *vpr* null mutant HIV-1 or *vpr* null mutant HIV-1 *trans* complemented with Vpr*W54R in the mutation rate assay. Both primate and nonprimate lentiviruses have the ability to replicate in nondividing cells, which are presumed to have low levels of S-phase cellular enzymes involved in DNA synthesis and repair, such as dUTPase and UNG (19, 29, 37). The encoding of dUTPase by nonprimate lentiviruses and the incorporation of UNG by primate lentiviruses such as HIV-1 by its interaction with Vpr support the hypothesis that these different mechanisms evolved in order for these viruses to remove uracil from their DNA when replicating in nondividing cells.

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