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# Uracil DNA Glycosylase Specifically Interacts with Vpr of both Human Immunodeficiency Virus Type 1 and Simian Immunodeficiency Virus of Sooty Mangabeys, but Binding Does Not Correlate with Cell Cycle Arrest

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**The Vpr protein encoded by human immunodeficiency virus type 1 (HIV-1) is important for growth of virus in macrophages and prevents infected cells from passing into mitosis ($G_2$ arrest). The cellular target for these functions is not known, but Vpr of HIV-1 and the related Vpr from simian immunodeficiency virus of sooty mangabeys ($SIV_{SM}$) bind the DNA repair enzyme UNG, while the Vpx protein of $SIV_{SM}$ does not. Nonetheless, a mutational analysis of Vpr showed that binding to UNG is neither necessary nor sufficient for the effect of Vpr on the cell cycle.**

In addition to the structural genes *gag*, *pol*, and *env*, which are common to all replication-competent retroviruses, primate immunodeficiency viruses encode several auxiliary proteins. Two of these proteins, Vpr and Vpx, are efficiently incorporated into virions (5, 15, 30). Of the five major branches of primate lentiviruses, four of them, human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) strains $SIV_{AGM}$, $SIV_{MND}$, and $SIV_{SYK}$, encode only one of these proteins, while $SIV_{SM}$ and HIV type 2 (HIV-2) encode both (23).

The Vpr protein of HIV-1 has at least two separate roles in the virus life cycle (for a review, see reference 9). Vpr is important for efficient replication of HIV-1 in primary macrophages (1, 6, 29), and, in addition, Vpr causes infected T cells to arrest in the $G_2$ phase of the cell cycle (2, 12, 16, 21). These two functions appear to have been split in $SIV_{SM}$ such that the Vpr gene is capable of causing cell cycle arrest both in human and in simian cells but has no effect on the infection of macrophages, while Vpx has no effect on the cell cycle but is important for infection of macrophages (10).

The ability of HIV-1 Vpr and $SIV_{SM}$ Vpx to allow infection of macrophages has been attributed to their ability to target the preintegration complex into the nucleus in the absence of mitosis (10, 13). However, Vpr does not contain a classical nuclear localization signal and does not appear to use the common alpha-importin pathway to access the nucleus (11). In addition, Vpr may affect postintegration steps in macrophages (6). In any case, the cellular target that mediates these effects is not known. Likewise, the cellular target for the ability of HIV-1 and $SIV_{SM}$ Vpr to arrest cells in $G_2$ is unknown. Vpr prevents cells from passing into mitosis by preventing the activation of the cyclin B/p34$^{cdc2}$ kinase (2, 12, 16, 20) but does not bind to the kinase complex (20).

We recently reported that HIV-1 Vpr was able to bind tightly to and to be coimmunoprecipitated with UNG (3), the major uracil DNA glycosylase in human cells (24). Uracil DNA glycosylase cleaves DNA that contains uracil and is involved in base excision repair. Because UNG is a nuclear protein which could influence cell cycle progression through a role in checkpoint control at the level of DNA damage repair, it was possible that UNG is a target of either Vpr-mediated localization of the preintegration complex or Vpr-mediated cell cycle arrest. Therefore, in this study we investigated the relationship between the Vpr-UNG interaction and the known functions of Vpr.

**Binding of UNG to Vpr but not Vpx of SIV.** Because the two functions of HIV-1 Vpr, $G_2$ arrest and infection of macrophages, are segregated independently in $SIV_{SM}$ into the Vpr and Vpx genes, respectively (10), we first tested whether Vpr or Vpx from $SIV_{SM}$ was able to bind human UNG in the two-hybrid system as previously described (3) and used HIV Vpr as a positive control. HIV-1 Vpr, $SIV_{SM}$ Vpr, $SIV_{SM}$ Vpx, and $SIV_{AGM}$ Vpr were fused to the *lexA* DNA binding domain (LexABD) and tested against UNG fused to the Gal4 activation domain (Gal4AD). The liquid culture assay for quantitative β-galactosidase activity was performed by using the L40 strain as described previously (3), and each assay was performed in triplicate.

A simian UNG sequence has not yet been reported; however, the protein sequences of UNGs from different organisms are highly conserved (18). Moreover, $SIV_{SM}$ Vpr is able to cause cell cycle arrest in both human and simian cells, while $SIV_{SM}$ Vpx causes cell cycle arrest in neither (19, 26). In addition, we tested the ability of the divergent Vpr from $SIV_{AGM}$ to bind human UNG, because $SIV_{AGM}$ Vpr affects the cell cycle of simian but not human cells (19, 26).

The level of interaction of $SIV_{SM}$ Vpr with UNG was found to be more than twofold higher than that obtained with HIV-1 Vpr (Fig. 1). This was not expected, because $SIV_{SM}$ Vpr is less

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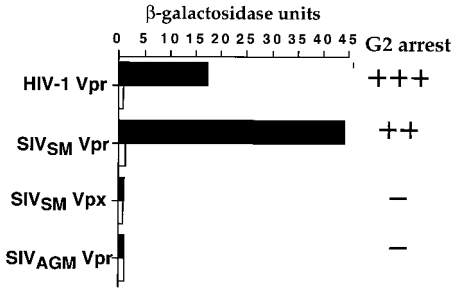


FIG. 1. UNG binding and $G_2$ arrest activity of Vpr and Vpx proteins from HIV-1, $SIV_{SM}$, and $SIV_{AGM}$. Vpr and Vpx proteins fused to LexABD in the pLex10 plasmid were analyzed for interaction with human UNG fused to Gal4AD in the pGAD1318 plasmid by quantitative β-galactosidase two-hybrid assaying (27) in the L40 yeast strain (solid bars). Values are the means of triplicate determinations made for two independent transformants. The background level is approximately 2 U and corresponds to yeast cotransformed with each LexABD hybrid and Gal4AD-RAF expression plasmids (white bars). HIV-1 is the LAI isolate (28), $SIV_{SM}$ is the Pbj1.9 isolate (7), and $SIV_{AGM}$ is the vervet isolate $SIV_{AGM}$ $SM^{9063}$ (14). $G_2$ arrest activity of Vpr and Vpx proteins from these alleles of HIV-1, $SIV_{SM}$, and $SIV_{AGM}$ is reported by us elsewhere in more detail (10, 26).

active than HIV-1 Vpr in human cells (26). On the other hand, no interaction was detected between $SIV_{SM}$ Vpx and UNG, and the more distantly related $SIV_{AGM}$ Vpr was also unable to bind human UNG (Fig. 1). Nonetheless, these results indicate that binding to UNG is a general property of related Vpr proteins (23).

**Abilities of Vpr mutants totally or partially deficient in cytostatic activity to bind to UNG.** In order to test the possible correlation between the cytostatic activity of HIV-1 Vpr and its ability to bind to UNG, we tested the abilities of HIV-1 Vpr mutants to bind UNG. Conservative point mutations were made in the Vpr coding sequence in amino acids that are identical between HIV-1 Vpr and $SIV_{SM}$ Vpr (Fig. 2A). These mutants were first screened for stability in human cells by Western blotting of transfected cells with a Vpr antibody (Fig. 2B). All of the mutants were expressed at approximately similar levels, with the exception of E24G and H33R, which were reduced by approximately threefold relative to wild-type Vpr. These mutants were then tested for their abilities to cause $G_2$ arrest in human cells (Fig. 3). Briefly, HeLa cells were cotransfected with humanized GFP (Life Sciences Technologies) and either wild-type Vpr, mutant Vpr, or vector alone. The cell cycle profile of cells that expressed GFP was determined as described elsewhere (21), except that the presence of the modified GFP greatly increased the signal-to-noise ratio noise relative to that of the nonmodified GFP (data not shown). At least 10,000 events were counted for each experiment. Two of the mutants, H33R and H71R, had lost the ability to cause $G_2$ arrest, while the remaining mutants, E24G, F34I, H78R, and S79A, retained this ability (Fig. 3A).

All these mutants were then fused to the Gal4 DNA binding domain (Gal4BD) and screened in the two-hybrid system for their abilities to interact with UNG fused to the Gal4AD. All Gal4BD-Vpr mutant fusion proteins were expressed at equivalent levels in yeast cells, as indicated by Western blotting with anti-Gal4BD antibodies (data not shown). The relative strengths of interaction between these Vpr mutants and UNG were again assessed by β-galactosidase quantitative tests.

Interestingly, the mutant S79A displayed a greater ability to interact with UNG than wild-type Vpr (induction of β-galactosidase activity was almost 1.5-fold higher than that with HIV-1 LAI Vpr). The two mutants H33R and H71R, which had lost their ability to cause $G_2$ arrest (Fig. 3A), on the other hand, had also lost most of their ability to interact with UNG (7 and 9%, respectively, of the interaction strength of HIV-1 LAI Vpr). However, other mutants with equivalent or slightly reduced cytostatic activities (E24G, F34I, and H78R) also interacted less well with UNG (23, 10, and 12%, respectively, of the interaction of HIV-1 LAI Vpr). These results indicate that several of the amino acids conserved between HIV-1 and $SIV_{SM}$ Vpr are involved in the UNG interaction. However, they also suggest either that UNG binding is not involved in Vpr-mediated $G_2$ arrest or that the amount of binding of F34I to UNG, although severely reduced (to 10% of wild-type levels), was sufficient to cause $G_2$ arrest.

To resolve this question, we generated a Vpr mutant library by random mutagenesis by error-prone PCR (4) to identify mutants of Vpr which would be totally deficient in binding to UNG in the two-hybrid system. To avoid isolation of mutants defective in UNG binding because of Vpr truncation due to premature stop codons, we took advantage of another Vpr-binding protein (clone 22) isolated from our previous cDNA library two-hybrid screening with Vpr as a bait (3, 22).

Five mutants (Vpr*11, -12, -13, -14, and -17) deficient for interaction with UNG that retained binding with clone 22, as well as one mutant (Vpr*215) negative for interaction with clone 22 and positive for UNG binding, were isolated (Fig. 4A). Each of the clones deficient for UNG binding contained multiple mutations (Fig. 4B). However, each contained a common mutation at amino acid 54 in which the tryptophan was replaced by either an arginine or a glycine, while this mutation was not found in the Vpr*215 mutant (Fig. 4B). Since we found two different substitutions, W54R and W54G, these results suggest that W54 is an amino acid residue critical for interaction with UNG.

We then constructed the single mutant W54R, each of the separate mutants found in the first screen, i.e., E17D, E25K, W54R, R88K, A89T, and R90K, and the double mutant N16Y-W18R in both two-hybrid vectors as fusion proteins and in mammalian expression vectors driven by the HIV-1 long terminal repeat (LTR) as native proteins (Fig. 3B and C). The

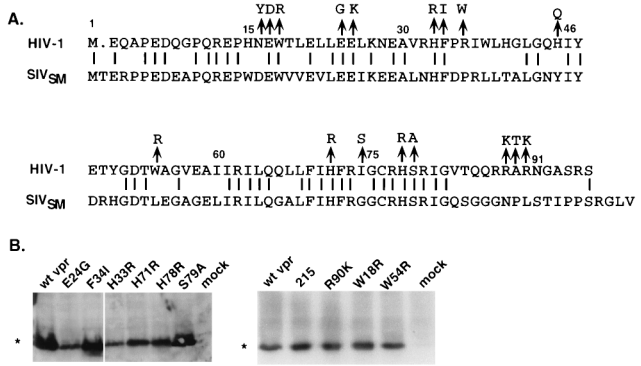


FIG. 2. HIV-1 Vpr mutations. (A) Alignment of HIV-1 Vpr (LAI strain) and $SIV_{SM}$ Vpr (Pbj1.9). Identical amino acids are denoted by lines between the two sequences. Mutations in HIV-1 Vpr used in this study are indicated by arrows. All mutations are single point mutations, with the exceptions of amino acids R36W, H45Q, and I74S, which are combined in one mutant (Vpr215), and amino acids N16W to W18R, which are combined in another (W18R was also made individually). (B) Western blots of representative mutants. All mutants were tested for expression (data not shown). The right and left gels are from separate experiments. 293 cells were transfected in a 35-mm plate with an expression vector that used the HIV-1 LTR as a promoter. Twenty-four hours after transfection, the cells were lysed and 10% of the lysate was run on a 15% polyacrylamide gel, transferred to Immobilin P (Millipore), and probed with a 1:1,000 dilution of a rabbit polyclonal antibody raised to recombinant Vpr (11a). The asterisks indicate the 14-kDa Vpr protein. wt, wild type.

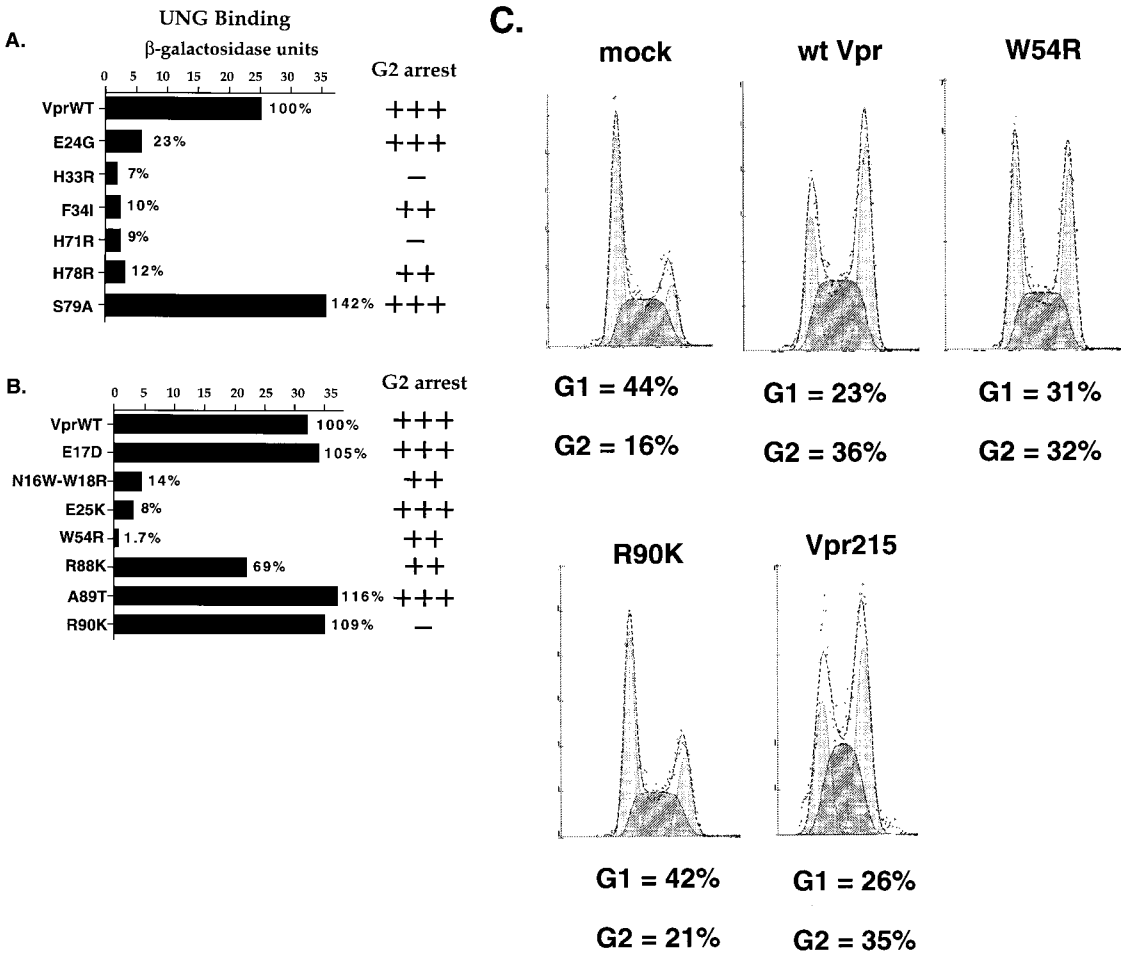


FIG. 3. UNG binding and $G_2$ arrest caused by HIV-1 Vpr mutations. (A) UNG binding and $G_2$ arrest caused by Vpr mutations made in amino acids conserved between HIV-1 Vpr and $SIV_{SM}$ Vpr. UNG binding was quantified as described in the legend to Fig. 1. The numbers to the right of the bars indicate the percentages of β-galactosidase activity (binding) of wild-type Vpr (VprWT) with respect to UNG. Mutations were then subcloned into a mammalian expression vector under the control of the HIV-1 LTR and were used to transfect human HeLa cells. The cell cycle profiles of transfected cells were analyzed as described elsewhere (21), and the percentages of cells in the $G_1$ and $G_2$ parts of the cell cycle were determined. +++, the ratio of $G_2$ to $G_1$ is greater than 1.5; ++, ratio of $G_2$ to $G_1$ of 1.0 to 1.5; +, ratio of $G_2$ to $G_1$ of 1.0 to 0.5; −, ratio of $G_2$ to $G_1$ is less than or equal to 0.5. Each mutant was tested in three separate transfections. (B) UNG binding and $G_2$ arrest of Vpr mutants selected from an HIV-1 Vpr random mutant library. (C) Representative cell cycle profiles for the most critical mutants in this series. The percentages of cells in either $G_1$ or $G_2$ are given beneath the cell cycle profiles. *y* axis, numbers of cells; *x* axis, DNA content. Vpr215 was selected for binding to UNG and lack of binding to clone 22 and contained mutations R36W, H45Q, and I74S (see Fig. 2).

single mutant W54R showed no measurable binding to UNG over background (less than 2% of that with wild type [Fig. 3B]). These results confirm that mutation W54R by itself accounts for the loss of Vpr interaction with UNG. Surprisingly, W54 is not conserved with $SIV_{SM}$ Vpr, although it is located in a cluster of conserved amino acids (Fig. 2A) (see reference 23 for more complete amino acid alignment). Vpr mutants E25K and N16Y-W18R, which have amino acids conserved with $SIV_{SM}$, were also severely reduced in UNG binding efficiency. Previous analyses had shown that the C-terminal end of Vpr is not involved in UNG binding (3). Consistent with this observation, we found that mutations R88K, A89T, and R90K did not affect UNG binding (Fig. 3).

Although mutant W54R had completely lost the ability to bind UNG, this mutant retained the ability to cause $G_2$ arrest, albeit with slightly reduced levels compared to those of wild-type Vpr (Fig. 3B and C). In addition, mutant E25K, which was also reduced in UNG binding to less than 8% of that of wild-type Vpr, caused a $G_2$ arrest that was indistinguishable from that of wild-type Vpr (Fig. 3B). These data indicate that Vpr binding to UNG is not necessary for its ability to cause $G_2$ arrest.

In addition, while the conservative mutant R90K in the C terminus bound UNG as well as wild-type Vpr (Fig. 3B), this mutant failed to cause $G_2$ arrest (Fig. 3C). The steady-state levels of R90K in transfected cells were identical to that of wild-type Vpr (Fig. 2B), indicating that the loss of function is not due to insufficient amounts of protein. These results further indicate that UNG binding is neither necessary nor sufficient for Vpr-induced cell cycle arrest and also confirm another mutational analysis (8) that indicated an important role for the C-terminal domain of Vpr in cell cycle function.

The mutants produced from our mutagenic PCR-generated library were constrained by the requirement that they bind clone 22 but not UNG. Clone 22 encodes an open reading frame of 180 amino acids that shows no significant homology to any cDNA in the database at this time (data not shown). RNA homologous to this gene is found in the thymus and spleen and in peripheral blood mononuclear cells (data not shown). We isolated one mutant, called 215, that failed to bind clone 22

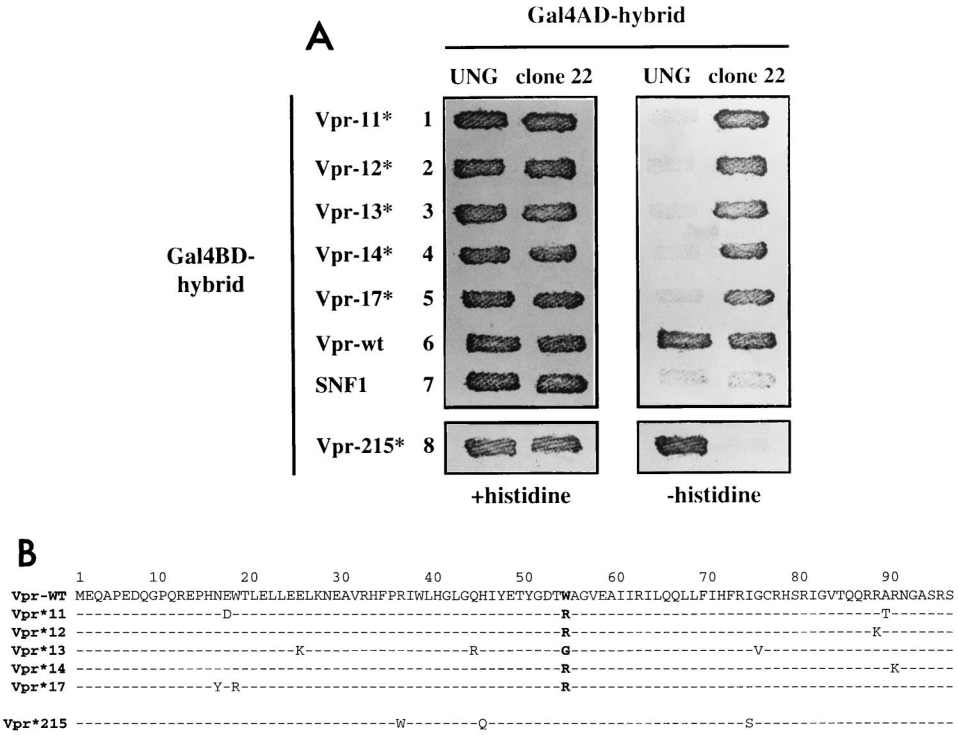


FIG. 4. In vivo selection for mutants that lack binding to UNG. Interaction with UNG and clone 22 of Vpr* mutants (lanes 1 to 5 and 8) selected from an HIV-1 Vpr random mutant library. An HF7c strain expressing the pairs of hybrid proteins fused to the Gal4BD (indicated on the left) and to the Gal4AD (indicated on the top) was analyzed for histidine auxotrophy. Double transformants were patched on selective medium with histidine (left panel) and were then replica plated on medium without histidine (right panel). Interaction of wild-type Vpr (Vpr-wt) with UNG and clone 22 was used as a positive control (lane 6). Lack of interaction of SNF1 with UNG and clone 22 was used as a negative control (lane 7). The *vpr* gene was amplified by error-prone PCR (5) and the fragments were fused to Gal4BD in pGBT10 (4). About $7 \times 10^3$ independent clones were pooled and used to transform the HF7c-MATα yeast strain. The mutant library expressed in HF7c (about 1,500 yeast clones) was then simultaneously screened for selection of mutants both negative with UNG and positive with clone 22, by mating, as previously described (27), with the Y187-MAT**a** yeast strain previously transformed with the Gal4AD-UNG hybrid or Gal4AD-clone 22 hybrid expression plasmids. (B) Amino acid sequences of the Vpr* mutants. Plasmid DNA from each Vpr* mutant, indicated on the left, was recovered, and each insert was completely sequenced. Trp (W) residue in position 54, found in all the Vpr* mutants deficient for UNG binding, is indicated by boldface. WT, wild type.

(0.4% of wild-type Vpr) and yet still bound UNG. Mutant 215 contained mutations R36W, H45Q, and I74S (Fig. 2). Nonetheless, binding of Vpr to clone 22 was also not necessary for $G_2$ arrest, since mutant 215 retained the ability to cause $G_2$ arrest (Fig. 3C).

In conclusion, although two-hybrid studies with HIV-1 Vpr, $SIV_{SM}$ Vpr, and $SIV_{SM}$ Vpx clearly showed that only HIV-1 and SIV Vpr alleles can interact with human UNG while the Vpx protein cannot, a panel of Vpr mutants constructed either by site-directed or by random mutagenesis shows that UNG binding does not segregate with $G_2$ arrest. On the other hand, UNG does not bind to SIV Vpx. Because in SIV the Vpx protein is important for macrophage function while the Vpr protein is involved in $G_2$ arrest (10), this suggests that UNG binding is not necessary for macrophage infection as well.

What, then, is the importance of UNG binding to Vpr? A recent study showed that in the presence of Vpr, the mutation rate of HIV in a single-round assay was reduced by approximately fourfold (17). Recent studies have revealed that incorporation of uracil into viral DNA correlates with reduced replication of equine infectious anemia virus in macrophages (25). Therefore, one possibility is that a third independent function of Vpr (in addition to $G_2$ arrest and transport of the preintegration complex to the nucleus) involves reducing the mutation rate by concentrating UNG in the preintegration complex. The use of the mutants described in this study should allow that hypothesis to be tested.

L. G. and S. B. contributed equally to this work.

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