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Virion-Associated HIV-1 Vpr: Variable Amount in Virus Particles Derived from Cells upon Virus Infection or Proviral DNA Transfection

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Human immunodeficiency virus type-1 (HIV-1) Vpr is a virion-associated protein implicated to have a role in AIDS pathogenesis. In regard to the amount of Vpr incorporated into virus particles, the published data vary widely. To address this, we quantitated Vpr in virus particles derived from diverse sources that are used to evaluate the biological effect of Vpr. Virus particles from infected cells showed only a small amount of Vpr. Interestingly, virus particles from cells cotransfected with HIV-1 proviral DNA lacking Vpr coding sequences (NLΔVpr) and a Vpr expression plasmid showed a drastic increase (29.4-fold) in the incorporation of Vpr. Furthermore, cotransfection involving NLΔVpr and different concentrations of Vpr expression plasmid resulted in virus particles containing Vpr in proportion to the Vpr expression plasmid used. The differences in virus particles with respect to Vpr as revealed by these studies should be taken into account in assessing the effect of Vpr.

INTRODUCTION

HIV-1 infection in humans is associated with a loss of $CD4^+$ T cells and other target cells (Azad, 2000; Bukrinsky and Adzhubei, 1999; Emerman and Malim, 1998; Levy, 1998). Interestingly, a massive infection of cells in the lymphoid organs has been noted even when there is low viral burden in the peripheral compartments of the body, indicating active viral replication. Hence, the progression of the disease in the context of HIV-1 infection may be linked to the cytopathic effects induced by viral proteins (Azad, 2000; Bukrinsky and Adzhubei, 1999). Recently, it has been suggested that this may be due to a combination of direct killing of the infected cells by virus and indirect effects of viral gene products on bystander cells (Azad, 2000; Bukrinsky and Adzhubei, 1999). The mechanisms that have been proposed in this regard are apoptosis, perturbation of host cell membranes, and a loss of cells through the formation of multinucleated syncytia (Azad, 2000; Bukrinsky and Adzhubei, 1999; Levy, 1998). These effects have been linked to HIV-1-encoded gp120, Tat, Nef, and Vpr (Azad, 2000; Bukrinsky and Adzhubei, 1999). Specifically, Vpr has been shown to result in apoptosis following cell cycle arrest at the G_2 stage (Azad, 2000; Emerman and Malim, 1998). Apoptotic and antiapoptotic effects both have been noted depending on the activation status of the cells and also on the concentration of Vpr (Azad, 2000; Bukrinsky and Adzhubei, 1999). Levy *et al.* (1994 and 1995) reported that Vpr purified from the serum of HIV-1-infected individuals and recombinant Vpr generated through a baculovirus expression system showed an enhancement of HIV-1 production in latently infected and primary cells exposed to HIV-1.

Analysis of HIV-1 particles revealed the presence of several nonstructural proteins of viral origin, in addition to the structural proteins that are responsible for virus morphogenesis (Emerman and Malim, 1998; Levy, 1998). These include Vif, Vpr, and Nef, which are shown to be present in different amounts in the virus particles. It has also been noted that both Vif and Nef are incorporated into heterologous retrovirus particles (Azad, 2000; Emerman and Malim, 1998; Levy, 1998) as well as into particles directed by the HIV-1 structural protein Gag. The incorporation of Vpr, on the other hand, has been shown to be specific, involving the distinct p6 domain of Gag (Emerman and Malim, 1998; Levy, 1998). Besides its essential role in the infection of macrophages by HIV-1, the characteristic features of Vpr include cell cycle arrest at the G2 stage, nuclear localization, nuclear import of the preintegration complex, cation-selective channel activity, and interactions with several candidate cellular proteins (Azad, 2000; Bukrinsky and Adzhubei, 1999; Emerman and Malim, 1998; Levy, 1998). Work carried out

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by our laboratory and others has shown the importance of putative helical domains present in Vpr for its incorporation into virus particles (Mahalingam *et al.,* 1995, 1997; Singh *et al*., 2000a; Subbramanian *et al.,* 1998; Yao *et al.,* 1995). Studies by several investigators indicated that Vpr is a potent activator of transcription from HIV-1 LTR and heterologous promoters including cytokines (Azad, 2000; Bukrinsky and Adzhubei, 1999; Emerman and Malim, 1998).

A central question in understanding the role of Vpr for AIDS pathogenesis through virion-associated, cell-associated, and extracellular Vpr pertains to the amount of Vpr present in each of the compartments. While the level of Vpr expression in virus-infected and HIV-1 proviral DNA transfected cells has not yet been evaluated, there is information available regarding the extracellular Vpr (serum and cerebrospinal fluid) (Levy *et al.,* 1994, 1995). With respect to the amount of Vpr present in the virus particles, it was reported earlier that the number of Vpr molecules may be similar to that of Gag (Cohen *et al.,* 1990). On the other hand, Kobinger *et al.* (1998) reported that the number of molecules of Vpr incorporated into the virus particles is similar in amount to that of reverse transcriptase (RT) molecules. This estimation was based on generating radiolabeled virus particles followed by immunoprecipitation using antibodies specific to Vpr and RT. The underlying assumption with these approaches is that the affinities of the antibodies used for the analysis remain the same for both proteins. However, it is likely that differences in reactivities of the antibodies may lead to conclusions that may not be reflective of the amount of Vpr protein in virus particles. Furthermore, it was also reported that Vpr incorporation occurred at essentially the same level in the virus particles whether Vpr was expressed in *cis* (in context of HIV-1 proviral DNA) or through a heterologous promoter (Hrimech et al., 1999; Poon et al., 1998). Recently, we utilized an epitope tagging approach whereby the flag epitope was added to both Gag and Vpr to monitor the stoichiometry of these proteins in the virus particles. It was determined that the ratio of Gag to Vpr in virus particles, when Vpr-FL is expressed in *cis* (in the context of proviral DNA), is in the range of 150–200:1 (14–18 molecules of Vpr per virion). The expression of Vpr-FL in *trans* through a heterologous promoter showed an efficient incorporation with a Gag to Vpr ratio of 5–7:1 (392–550 molecules) based on 2750 molecules of Gag present in each virion (Lai *et al.,* 2000; Singh *et al.,* 2000). As our results were obtained with Vpr and Gag containing the flag epitope, there is a possibility that the estimated number may be skewed due to the addition of an epitope tag. The goal of the studies presented here was to determine the amount of native Vpr in the virus particles. The results show that there is a drastic difference in the amount of Vpr in the virus particles generated through virus infection or transfection of HIV-1 proviral DNA.

RESULTS AND DISCUSSION

To quantitate the amount of Vpr in the virus particles, we considered virus particles from diverse sources that are routinely used by investigators for functional studies related to Vpr. These include virus particles derived from cells infected with virus, transfected with HIV-1 proviral DNA, or cotransfected with HIV-1 proviral DNA lacking Vpr expression (NLΔVpr) with a Vpr expression plasmid. We used polyclonal Vpr antisera raised against a full-length synthetic Vpr molecule for these studies (Mahalingam *et al.,* 1995). Based on our earlier data (Lai *et al.,* 2000; Singh *et al.,* 2000), we initially subjected the virus particles to immunoblot analysis directly from culture supernatant or purified through a 30% sucrose cushion to determine the sensitivity of the assay and the amount of virus particles to be used. For this purpose, virus particles were generated by cotransfection of HIV-1 proviral DNA lacking Vpr (NLΔVpr) and a Vpr expression plasmid into 293T cells. The virus particles were quantitated by a p24 antigen assay and varying amounts of virus lysate (2–12 ng) were used for immunoblotting. The results shown in Fig. 1A indicate that the intensity of the Vpr band is proportional to the virus concentration tested in the range of 2–12 ng (Fig. 1B). These data support the fact that the assay employed here is sensitive and requires only a minimum amount of virus. We next used 10 ng p24 antigen equivalent of virus to analyze virus particles from diverse sources. The results are shown in Fig. 2A. The virus particles collected from the culture supernatant of the cells infected with virus showed a faint signal with Vpr antibodies (Fig. 2A, lane3) in comparison to virus particles derived from cells transfected with HIV-1 proviral DNA (NL4-3) (lane 2) and also from cells cotransfected with HIV-1 proviral DNA lacking the ability to express Vpr (NLΔVpr) with a Vpr expression plasmid (lane 4). Specifically, an intense signal was observed with the virus particles derived from cells upon cotransfection. The specificity of incorporation of Vpr was evident by the lack of a signal with NLΔVpr-derived virus (lane 1) and also with virus particles derived from cells cotransfected with NLΔVpr and Vpr substitution mutants Vpr F34S, Vpr L39G, and Vpr G43P (data not shown). Densitometry scanning analysis showed that the extent of incorporation of Vpr into the virus particles from cells transfected with proviral DNA was noted to be 2.5 times that of Vpr in the virus particles derived from infected cells (Fig. 2B). Strikingly, a 29.45-fold increase of Vpr incorporation was observed in the virus particles derived from cotransfected cultures. These data are in agreement with the results obtained with an epitope-tagged Vpr and indicate that the extent of incorporation of Vpr can be influenced by the level of Vpr in cells.

Based on this information, we then considered experiments to address whether the amount of Vpr incorporated into the virus particles directly correlates with the

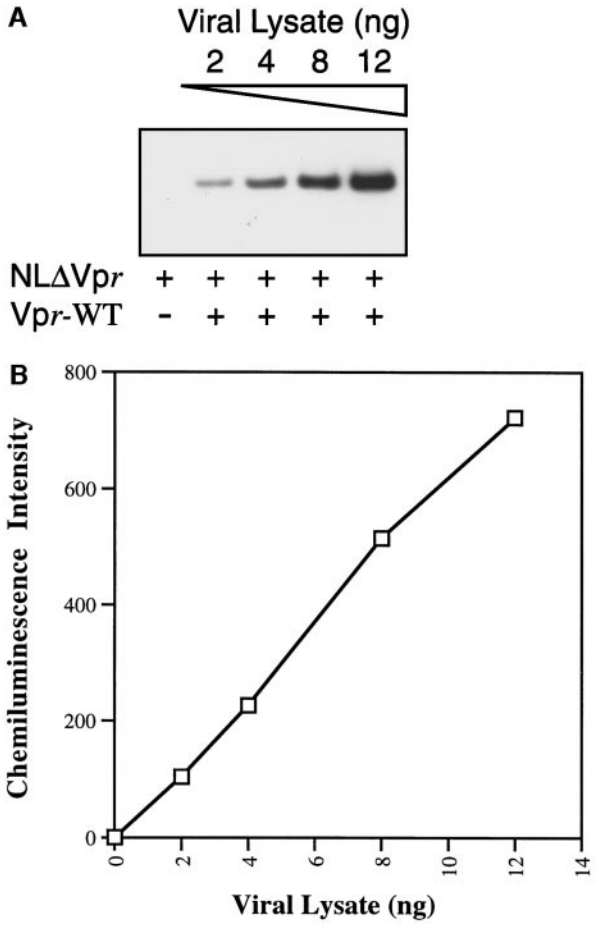

FIG. 1. Immunoblot analysis of virus particles using polyclonal antisera to Vpr. (A) Virus particles, generated by co-transfection of NLΔVpr (5 ug) and Vpr expression plasmid (5 ug) into 293T cells, were collected, centrifuged at 40,000 rpm for 3 h at 4°C through a 30% sucrose cushion, and lysed in lysis buffer. Varying amounts of virus lysate, based on p24 antigen values, were immunoprecipitated with polyclonal antisera to Vpr and immunoreactive proteins were detected using the ECL Plus western blotting detection system. (B) Quantitation of Vpr from (A) was performed by densitometry using Personal Densitometer SI and ImageQuant software. The chemiluminescence intensity of an individual band was plotted against the amount of virus lysate to generate a standard curve.

concentration of Vpr expression plasmid used for analysis. Cotransfection of HIV-1 proviral DNA (NLΔVpr) with varying concentrations of a Vpr expression plasmid was carried out in 293T cells. To verify the expression level of Vpr, cells at 72 h post-transfection were lysed and an aliquot of the lysate was subjected to immunoblot assay. Analysis with 50 ug total cellular protein showed a Vpr band in cells transfected with different amounts of Vpr expression plasmid in a concentration-dependent manner (Fig. 3A). Immunoblot analysis of virus particles revealed that there is a linear increase in the amount of Vpr incorporated into the virus particles when cotransfection with a Vpr expression plasmid was in the range of 1–4 μg (Fig. 3B). Transfection of higher concentrations of the Vpr expression plasmid (6 and 8 μg) showed a reduced level of Vpr incorporation in comparison to virus particles derived from cotransfection involving 4 μg. The densitometric analysis of the band corresponding to Vpr showed a 12-fold increase in Vpr incorporation at 4 μg in comparison to 1 μg Vpr expression plasmid used for cotransfection (Fig. 3C).

Based on the data presented, the conclusions are the following: (i) native Vpr molecules are present in HIV-1 particles in low amounts in relation to the structural protein Gag; (ii) virus particles derived from cells infected with viral isolates showed less Vpr in comparison to virus particles generated through transfection of HIV-1 proviral DNA; and (iii) the extent of incorporation of Vpr into the virus particles, generated through cotransfection of NLΔVpr with a Vpr expression plasmid, is high and linear with respect to the concentration of the Vpr expression plasmid used. The literature published thus far state that HIV-1 Vpr is present in amounts similar to that of Gag protein in the virus particles as noted for Vpx of HIV-2/SIV (Henderson *et al.,* 1988). Recently, it was reported that the amount of Vpr in the virus particles is comparable whether Vpr is expressed in *cis* (through HIV-1 proviral DNA) or in *trans* (through a heterologous promoter) and was the basis to utilize virus particles containing Vpr

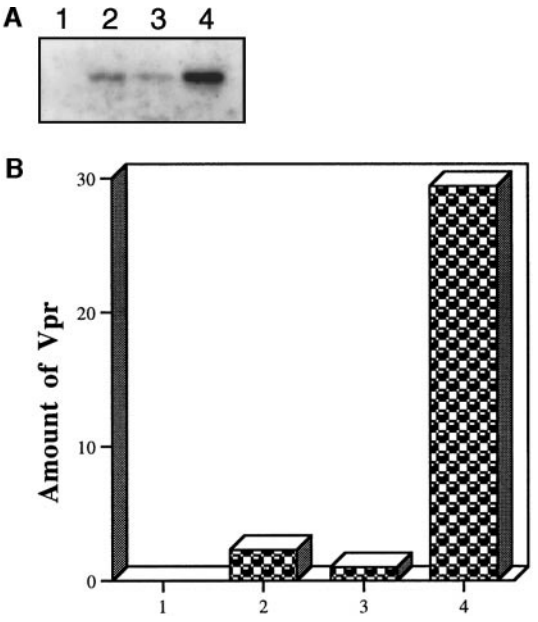

FIG. 2. Comparison of Vpr present in the virus particles. (A) Amount of Vpr in virus particles derived from diverse sources. Transfection of HIV-1 proviral DNA into 293T cells was carried out by the calcium phosphate co-precipitation method. CEMX174 cells were used as target cells for virus infection studies. Virus lysate (10 ng p24 antigen equivalent) was subjected to immunoblot analysis using polyclonal antisera to Vpr and the proteins were detected using the ECL Plus western blotting detection system. (B) Densitometric scanning analysis of Vpr using Personal Densitometer SI and ImageQuant software. Lanes 1 and 2, virus particles derived from cells transfected with NLΔVpr and NL4–3, respectively. Lane 3, virus particles derived from NL4–3 virus infected cells. Lane 4, virus particles from cells cotransfected with NLΔVpr and Vpr expression plasmid.

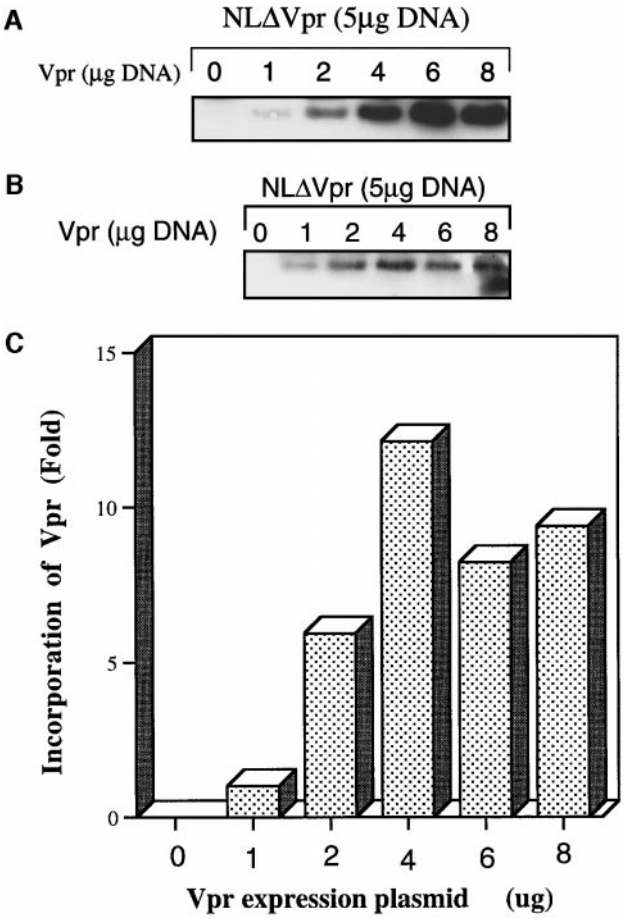

FIG. 3. Effect of varying amounts of Vpr expression plasmid on virion incorporation of Vpr. (A) Expression of Vpr in cells. HIV-1 Vpr was expressed by cotransfection of NLΔVpr (5 ug) with a variable amount of Vpr expression plasmid into 293T cells. Total cellular protein (50 ug) was used for immunoblotting to assess the expression of Vpr. (B) Extent of incorporation of Vpr into the virus particles. Equal amounts of virus lysate, based on p24 antigen values, were immunoprecipitated with polyclonal antisera to Vpr, as described in Materials and Methods. (C) Quantitation of Vpr from (B) using Personal Densitometer SI and ImageQuant software. The fold increase in the incorporation of Vpr was calculated as 1 using data obtained from cotransfection involving 1 ug of Vpr expression plasmid.

(expressed in *trans*) for evaluating the functional role of virion-associated Vpr (Hrimech *et al.,* 1999; Poon *et al.,* 1998). The basis for the discrepancy between the data presented versus other studies may be due to the differences in the techniques used for evaluation, use of antibodies with differing affinities, and the high amount of virus particles used in the analysis. The availability of a large number of HIV-1 proviral DNA molecules in cells due to the transfection process may result in large amounts of Vpr expression, in comparison to the level of Vpr in virally infected cells. This may lead to an increase in the incorporation of Vpr into the virus particles. A 30-fold increase in the virion incorporation of Vpr in the cotransfection setting may be linked to a high level of expression of Vpr by a heterologous promoter. Vpr is considered to be the late gene and its expression is controlled by the regulatory gene product of HIV-1, Rev. However, the placement of Vpr under the control of a heterologous promoter eliminates regulated expression resulting in high levels of Vpr. Considering the cotransfection method involving HIV-1 proviral DNA lacking the ability to synthesize Vpr and a Vpr expression plasmid, it is likely that the resulting virus particles may be a mixture of particles with Vpr and particles without Vpr. It is interesting to note that an immunoblot analysis using equivalent amounts of virus particles from virally infected cells and cotransfected cells showed a high amount of Vpr despite a possible mixture of viruses from cotransfected cells. This indicates that Vpr expression *in trans* through a heterologous promoter leads to an enhanced incorporation of Vpr into the virus particles. NLVpr proviral DNA is ~15 Kb in size and the Vpr expression plasmid is only 5.2 Kb. This would translate into three times more molecules of Vpr expression plasmid as opposed to proviral DNA plasmid. Given this, it is likely that both plasmids may be able to enter the target cells because of the excess of Vpr expression plasmid.

Despite the demonstration that HIV-1 Vpr is a bona fide virion-associated protein, the underlying mechanism for the incorporation of Vpr into the virus particles is not clear. Any model regarding this should take into account the following observations: (a) HIV-1 Gag is a multimeric protein and is present in the range of 2000–2750 copies per virion (Levy, 1998; Turner and Summers, 1999); (b) p6 domain of Gag, which lacks a stable tertiary structure, is essential for virion incorporation (Turner and Summers, 1999); (c) earlier studies indicated that Vpr exhibits oligomerization properties; and (d) the incorporation of Vpr molecules into the virus particles is low when Vpr is expressed in the context of HIV-1 proviral DNA and a high level of incorporation is noted when Vpr is expressed through a heterologous promoter. Based on these observations, it is conceivable that Vpr may interact with multimeric Gag either as a monomer or as an oligomer depending on the expression level of Vpr in cells. In addition, the ability of Vpr to interact with multiple cellular proteins may restrict the availability of Vpr to interact with Gag for its incorporation into the virus particles. The results reported here raise several interesting issues such as (i) how much Vpr is present in producer cells and in virus particles; (ii) what is the ratio of Gag to Vpr in producer cells and in virus particles; and (iii) is there a variation in the amount of Vpr present in virus particles produced by specific target cells such as T-cell lines, PBMCs, and macrophages? The answers to these questions, in our view, are critical to understanding the contribution of Vpr toward AIDS pathogenesis.

MATERIALS AND METHODS

Cell lines

293T, a human transformed primary embryonal kidney cell line, was maintained in Dulbecco's Modified Eagle's Medium (DMEM) and CEMX174 cells were maintained in RPMI 1640 medium (GIBCO BRL Laboratories, Grand Island, NY), supplemented with 1% L-glutamine, penicillin–streptomycin, and 10% fetal bovine serum (FBS) at 37°C in 5% CO_2.

Transfection and generation of virus particles

HIV-1 proviral DNA (pNL4-3) was modified to disrupt the expression of Vpr by an insertion (AATT) between residues 63 and 64 within the Vpr coding region (NLΔVpr). The cloning of wild-type Vpr in pCDNA3 was carried out with primer pairs HKVpr 5′ TCTAGAAGCTTGCCGCCACCATGGAACAAGCCCCAGAAGAC 3′ (forward) and Vpr-XHO 5′ CTAGATGCATGCTCGAGCTAGGATCTACTGGCTCCATT 3′ (reverse) using PCR methodology (Singh *et al.,* 2000). To generate virus particles containing wild-type Vpr, NLΔVpr proviral DNA was cotransfected with Vpr expression plasmid by the calcium phosphate coprecipitation method into RD cells (Singh *et al.,* 2000). Similarly, transfection of NL4-3 containing an intact open reading frame for Vpr was carried out to generate virus particles for assessing the amount of Vpr in transfected cells. The virus particles released into the culture supernatant were collected 120 h after transfection. The culture supernatants were pre-cleared for 10 min at 10,000 rpm and subsequently spun at 40,000 rpm for 3 h using a 30% sucrose cushion. Virus pellets were lysed in lysis buffer (62.5 mM Tris-HCl, pH 6.8, 0.2% SDS, 1% β-mercaptoethanol, 10% glycerol) and p24 antigen assay was used to quantitate the amount of protein present in the virus particles.

Infection of CEMX174 cells

Infection of CEMX174 cells was initiated using an inoculum of 1 ng p24 antigen equivalent of virus generated from proviral DNA NL4-3. After 1 h of infection, the cells were washed twice with PBS to remove the virus and resuspended in RPMI medium. Virus replication was monitored periodically from the medium of the infected cells by a p24 antigen assay. At the end of 10 days, culture supernatant was collected, pre-cleared for 10 min at 10,000 rpm, and subsequently centrifuged at 40,000 rpm for 3 h at 4°C using a 30% sucrose cushion. Virus pellets were lysed in lysis buffer and quantitated using a p24 antigen assay.

Immunoblot analysis

Virus samples, normalized on the basis of p24 antigen values obtained using ELISA (Organon Teknika, Durham, NC), were immunoprecipitated with polyclonal antiserum to Vpr and protein A sepharose CL-4B (Amersham Pharmacia Biotech, Piscataway, NJ) at 4°C overnight. The sepharose beads were then washed and boiled in sample buffer for 5 min and immunoprecipitated proteins were separated on NuPAGE 10% Bis-Tris gel (Invitrogen, Carlsbad, CA) followed by transfer onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% non-fat dry milk and incubated with rabbit polyclonal antiserum to Vpr for 2 h. The membranes were washed three times for 10 min each with TBST (20 mM Tris [pH 7.5], 500 mM NaCl, 0.05% Tween-20) and then probed with secondary antibody (Goat anti-rabbit IgG (H+L) HRP conjugate; Promega, Madison, WI), washed again with TBST, and developed with the ECL Plus western blotting detection system (Amersham Pharmacia Biotech., Piscataway, NJ).

Quantitation of Vpr by densitometry

The intensity of individual bands obtained with immunoblot analysis was used for determination of the amount of Vpr present in the virus particles. Blots were scanned using Personal Densitometer SI and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

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