A Conserved LXXLF Sequence Is the Major Determinant in p6^{gag} Required for the Incorporation of Human Immunodeficiency Virus Type 1 Vpr

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The *vpr* gene product of human immunodeficiency virus type 1 (HIV-1) is a virion-associated regulatory protein. A transferable virion association motif for Vpr is located in the p6 domain of the HIV-1 Gag polyprotein. To map the sequences in p6 that are involved in Vpr incorporation, we analyzed the ability of mutant forms of p6 to direct the incorporation of Vpr into chimeric viral particles. Our results show that the determinants which govern Vpr incorporation are largely confined to a C-terminal region of the p6 domain. Within this region, three hydrophobic residues in a highly conserved sequence motif (L-X-S-L-F-G) are absolutely required. Remarkably, the transfer of the conserved motif and of a single flanking residue to a heterologous Gag polyprotein was sufficient to transfer the ability to incorporate Vpr at moderate levels. The transfer of residues 32 to 46 of p6 led to Vpr incorporation levels that were comparable to those obtained with full-length HIV-1 Gag protein, indicating that this region contains essentially all the information required for efficient Vpr incorporation.

In addition to the *gag*, *pol*, and *env* genes found in all retroviruses, the genome of human immunodeficiency virus type 1 (HIV-1) contains at least six open reading frames which code for regulatory proteins, such as Vpr. The presence of at least one homolog of *vpr* in all primate lentiviruses suggests that Vpr contributes to unique features of this subfamily of retroviruses (35). Although Vpr is not required for virus replication in cell culture, viruses that express a functional Vpr replicate more efficiently under some circumstances, particularly in primary monocytes/macrophages (3, 7, 14, 27, 36). It was recently shown that purified Vpr can dramatically increase the permissiveness of cells to HIV-1 replication when added to culture medium, even at very low concentrations (21, 22).

One group of primate lentiviruses, which includes HIV-2 and simian immunodeficiency viruses (SIVs) such as SIV_{mac}239, possess a second *vpr*-like gene, *vpx*, which likely arose by gene duplication (35). It was recently reported that neither Vpr nor Vpx is individually required for progression to AIDS in rhesus monkeys infected with SIV_{mac}239 (11). However, this result may reflect partially redundant roles for Vpr and Vpx in this group of primate lentiviruses, as suggested by the observation that the disruption of both *vpr* and *vpx* prevented disease progression (11). In another study, reversions of point mutations in the *vpr* gene of SIV_{mac}239 were observed in inoculated rhesus monkeys, indicating that Vpr confers some selective advantage in vivo (18).

The *vpr* gene product of HIV-1 is a 96-amino-acid protein whose expression is regulated by Rev (1, 10). Unlike the other regulatory proteins encoded by HIV-1, Vpr is incorporated into virions in substantial amounts (5, 38). Because Vpr has no apparent function in viral morphogenesis, its virion incorporation is suggestive of a role at an early stage of infection, prior

to the de novo synthesis of viral proteins. In the absence of other viral proteins, Vpr is localized primarily to the nucleus, a property which has been attributed to its arginine-rich carboxy-terminal tail (23). It was reported that Vpr is one of the nucleophilic components associated with the HIV-1 preintegration complex, which permit access to the nucleus in the absence of mitosis and enable HIV-1 to replicate in nondividing host cells such as monocyte-derived macrophages (15). An early study revealed a moderate ability of Vpr, consistent with its nucleophilic properties, to transactivate the HIV-1 long terminal repeat as well as several heterologous promoters (6). A role of Vpr in the regulation of gene expression is also suggested by its ability to induce growth arrest and cellular differentiation (20). Vpr alone can affect cell cycle progression and lead to an accumulation in G₂, which may explain why viruses that express a functional Vpr are generally unable to establish a chronic infection of cultured T cells (31). Potential cellular interaction partners for Vpr include a 200-kDa protein (39) and a 41-kDa protein which was found in a complex that also included Vpr and the human glucocorticoid receptor type II (30).

The mechanism by which the nucleophilic Vpr gains access into virions which assemble at the cell surface remains poorly understood. Vpr is not synthesized as a part of a polyprotein and must therefore independently associate with a component of the assembling virion. Several studies have shown that the incorporation of Vpr is independent of *pol* and *env* gene expression and of the encapsidation of viral genomic RNA (19, 23, 28). However, premature termination codons in the *gag* gene which prevented the translation of the C-terminal p6 domain of the Gag polyprotein blocked the incorporation of Vpr (23, 28). We recently reported that the transfer of the HIV-1 p6 domain was sufficient to direct Vpr into heterologous viral particles (16). In the present study, we show that three hydrophobic residues in a highly conserved region near the C terminus of the p6 domain are the major determinants required for Vpr incorporation. We also show that as few as

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seven amino acids from the HIV-1 p6 domain are sufficient to transfer the ability to incorporate Vpr.

MATERIALS AND METHODS

Plasmids. HXBH10-PR⁻ is identical to HXBH10, a *vpu*⁺ variant of the infectious HXB2 proviral clone of HIV-1, except for a point mutation that inactivates the HIV-1 protease (12). HXBH10/NCA_{gag}-PR⁻ (16) contains the murine leukemia virus (MLV) *gag* gene and was used to express the authentic MLV Gag polyprotein Pr65^{gag}. The MLV/HIV-p6 construct (16) expresses the MLV *gag* gene joined precisely at its 3' end to the p6 coding sequence of HIV-1_{HXB2}. Deletions and single-amino-acid substitution mutations in the p6 coding region of MLV/HIV-p6 were introduced by site-directed mutagenesis as described before (17). MLV/HIV-p6(22-46), MLV/HIV-p6(32-46), MLV/HIV-p6(37-46), and MLV/HIV-p6(40-46) are variants of the MLV/HIV-p6(1-46) construct (16) which were generated by deleting p6 codons 1 to 21, 1 to 32, 1 to 36, or 1 to 39, respectively, in MLV/HIV-p6(1-46) by site-directed mutagenesis. In each case, the presence of the expected sequence was confirmed by DNA sequence analysis. The ptrENV construct, which was used for the expression of Vpr *in trans*, is a variant of the HIV-1_{BRU/LAI} molecular clone with large deletions in the *gag*, *pol*, and *env* genes (29).

Cell culture and transfections. COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells (1.4×10^6) were seeded into 80-cm² tissue culture flasks 24 h prior to transfection. The cultures were cotransfected with 25 µg each of Gag and Vpr expression constructs by a calcium phosphate precipitation technique (8).

Viral protein analysis. COS-7 cells were metabolically labeled with [³⁵S]methionine (50 µCi/ml) from 48 to 60 h posttransfection. Viral particles released during the labeling period were pelleted through sucrose cushions (in phosphate-buffered saline [PBS]) for 90 min at 4°C and 26,000 rpm in a Beckman SW28 rotor. Pelleted virions were lysed in RIPA buffer (140 mM NaCl, 8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate [SDS]), and viral proteins were either directly analyzed by electrophoresis through SDS-12.5% polyacrylamide gels or immunoprecipitated prior to electrophoresis with a rabbit anti-Vpr serum (19).

RESULTS

Sequences required for Vpr incorporation are confined to the C terminus of p6. We reported previously that Vpr is efficiently incorporated into viral particles formed by the MLV/HIV-p6 *gag* gene construct, which expresses a Moloney MLV Gag polyprotein that has the 52-amino-acid HIV-1_{HXB2} p6 domain attached to its C terminus (16). Furthermore, residues 1 to 46 of the HIV-1 p6 domain but not residues 1 to 41 were sufficient to transfer the ability to incorporate Vpr (16). These results demonstrated that a highly conserved sequence near the C terminus of p6 (L-X-S-L-F-G) contains essential determinants for Vpr incorporation, while sequences C-terminal to the conserved motif are dispensable (16). To determine whether the N-terminal 41 amino acids of the HIV-1 p6 domain contain additional determinants required for Vpr incorporation, successive internal deletions were introduced into the p6 coding region of MLV/HIV-p6, removing five or six codons at a time (Fig. 1). Each of the chimeric *gag* gene constructs was transfected into COS-7 cells together with ptrENV to provide Vpr *in trans*. The transfected cells were labeled with [³⁵S]methionine from 48 to 60 h posttransfection, and particulate material released during the labeling period was sedimented through sucrose and solubilized in RIPA buffer. Particle-associated viral proteins were analyzed both directly by SDS-polyacrylamide gel electrophoresis (PAGE) and by immunoprecipitation with a rabbit serum that recognizes HIV-1 Vpr prior to electrophoresis.

As shown in Fig. 2A, transfection of each of the mutant MLV/HIV-p6 *gag* gene constructs resulted in the release of chimeric Gag polyproteins in a particulate form. As expected, Vpr was not detectable in particles produced by the authentic MLV Gag polyprotein (Fig. 2, lanes 6 and 12) but was present in MLV/HIV-p6 particle preparations (Fig. 2, lanes 1 and 7). In repeated experiments, deletions of five or six amino acids at a time, which together covered residues 1 to 36 of the p6

FIG. 1. Location of deletions in the HIV-1_{HXB2} p6 domain fused in-frame to the C terminus of the MLV Gag polyprotein. The locations of the highly conserved P-T/S-A-P-P and L-X-R-S-L-F-G motifs within the p6 domain are also indicated. Numbers refer to the positions of residues, counting from the N terminus of the p6 domain. Mutants unable to incorporate Vpr are indicated by shaded boxes. Open boxes indicate that the incorporation of Vpr was not affected. The effects of the two C-terminal truncations illustrated at the bottom on Vpr incorporation have been reported previously (16).

domain, had no reproducible effects on the appearance of Vpr in the particulate fractions (Fig. 2, lanes 2 to 5 and 8 to 10). By contrast, Vpr was not detectable when residues 37 to 41 of the p6 domain were deleted (Fig. 2, lane 11). Together with our previous results (16), these observations show that determinants essential for Vpr incorporation are located exclusively within residues 37 to 46 of the p6 domain (see Fig. 1).

FIG. 2. Effects of deletions in p6 on incorporation of Vpr into chimeric viral particles. COS-7 cells were cotransfected with ptrENV and either with the chimeric Gag polyprotein expression construct MLV/HIV-p6 (lanes 1 and 7), with variants of MLV/HIV-p6 that harbor the indicated deletions in the p6 domain (lanes 2 to 5 and 8 to 11), or with the MLV Gag polyprotein expression construct HXBH10/NCA_{gag}-PR⁻ (lanes 6 and 12). The transfected cells were metabolically labeled with [³⁵S]methionine from 48 to 60 h posttransfection, and material released into the supernatant during the labeling period was pelleted through 20% sucrose and disrupted in RIPA buffer. Aliquots were either analyzed directly by SDS-PAGE to compare the amounts of particle-associated Gag polyprotein (A) or immunoprecipitated with rabbit anti-Vpr serum prior to SDS-PAGE (B).

FIG. 3. Effects of single-amino-acid substitutions in a region critical for Vpr incorporation. COS-7 cells were cotransfected with ptrENV and either with the chimeric Gag polyprotein expression construct MLV/HIV-p6 (lane 1), with variants of MLV/HIV-p6 that harbor the indicated substitutions in the p6 domain (lanes 2 to 11), or with the HIV-1 Gag polyprotein expression construct HXBH10-PR⁻ (lane 12). [³⁵S]methionine-labeled particulate material released into the supernatant was pelleted through 20% sucrose and disrupted in RIPA buffer. Aliquots were either analyzed directly by SDS-PAGE (A) or immunoprecipitated with rabbit anti-Vpr serum prior to SDS-PAGE (B). CyPA, cyclophilin A (34a).

Alanine-scanning analysis of the region required for Vpr incorporation. A sequence comparison shows that the p6 domain is highly variable among different groups of primate lentiviruses and even among different subtypes of HIV-1 (26). However, a short sequence motif near the N terminus of p6 (P-T/S-A-P-P) and a second motif near the C terminus (L-X-S-L-F-G) are remarkably well conserved. While the precise deletion of the conserved P-T/S-A-P-P motif had no effect on the association of Vpr with the particulate fraction, the L-X-S-L-F-G motif falls within the region identified as essential for Vpr incorporation (Fig. 1). To determine the importance of single side chains within this region, p6 domain codons 37 to 46 in the MLV/HIV-p6 construct were individually changed to codons specifying alanine. Alanine was chosen as a replacement because it is an abundant amino acid and is expected not to impose significant steric effects on neighboring side chains. Alanine-scanning mutagenesis has previously been used successfully to identify key residues involved in protein-protein interactions (2, 9).

The mutant *gag* gene constructs were cotransfected into COS-7 cells together with ptrENV, and the protein content of particles released into the supernatant was analyzed directly by SDS-PAGE and by immunoprecipitation with an antiserum directed against Vpr. The alanine scan revealed that only some of the residues that were targeted are critical for Vpr incorporation. Except for the S40→A mutation, alanine substitutions N terminal to the L-X-S-L-F-G motif had no or only moderate

effects on the association of Vpr with viral particles (Fig. 3, lanes 2 to 4). The substitution of Ser-40, which immediately precedes the L-X-S-L-F-G motif, led to a significant reduction in the amount of particle-associated Vpr (Fig. 3, lane 5). Alanine substitutions within the L-X-S-L-F-G motif produced one of two strikingly different phenotypes. The individual substitution of the hydrophobic residues Leu-41, Leu-44, and Phe-45, which occupy positions n , $n + 3$, and $n + 4$ of the L-X-S-L-F-G motif, respectively, totally abolished the ability of the chimeric particles to incorporate Vpr (Fig. 3, lanes 6, 9, and 10). By contrast, alanine substitutions at the nearly equally well conserved positions $n + 2$ and $n + 5$, as well as a substitution at the more variable $n + 1$ position of the motif, had no effect on the incorporation of Vpr (Fig. 3, lanes 7, 8, and 11). The differences in the amounts of particle-associated Vpr were not due to differences in Vpr expression levels, since similar amounts of Vpr could be immunoprecipitated from the lysates of the transfected cells (data not shown). Taken together, our results show that only 3 of the 52 residues of the HIV-1_{HXB2} p6 domain are essential for Vpr incorporation.

Identification of a minimal Vpr virion association domain. The results described above indicated that the determinants needed for Vpr incorporation are located mainly within or just upstream of the conserved L-X-S-L-F-G motif near the C terminus of the p6 domain. To test this idea directly, the C-terminal portions of p6 shown in Fig. 4C were fused in-frame to the MLV Gag polyprotein. Sequences C-terminal to the L-X-S-L-F-G motif were omitted, since we had shown previously that this portion of p6 is not required for Vpr incorporation (16). The MLV/HIV-p6(22–46) and MLV/HIV-p6(32–46) constructs contain about one-half and one-third of the p6 coding sequence, respectively. The MLV/HIV-p6(37–46) construct contains only the region covered by the two deletions which abolished Vpr incorporation (see Fig. 1). Lastly, the MLV/HIV-p6(40–46) construct also lacks the codons for three residues whose individual replacement by alanine did not affect Vpr incorporation.

As shown in Fig. 4A and B, a small amount of Vpr was found associated even with particles produced by the MLV/HIV-p6(40–46) construct (lane 3). The association of Vpr with the chimeric particles was dependent on the presence of the p6 sequences, since particles formed by authentic MLV Pr65^{gag} did not contain Vpr (lane 2). Taking into account the number of methionines in the MLV/HIV-p6(40–46) and HIV-1 Gag polyproteins (3 versus 15), the molar ratio between *vpr* and *gag* gene products in MLV/HIV-p6(40–46) particles, as determined by densitometry, was approximately 10-fold lower than in HIV-1 particles. The inclusion of three additional residues from the p6 domain in the MLV/HIV-p6(37–46) chimera increased the levels of particle-associated Vpr to about 40% of those found in HIV-1 particles (lane 4). The efficiency of Vpr incorporation into MLV particles reached 85% of that obtained with HIV-1 particles when the p6 sequence was extended by another five amino acids, yielding the MLV/HIV-p6(32–46) construct (lane 5). However, beyond that level, only a moderate increase was observed when additional p6 sequences N-terminal to residue 32 were included in the MLV/HIV-p6(22–46) construct (lane 6). Thus, essentially all the determinants required for efficient Vpr incorporation are located within residues 32 to 46 of the p6 domain.

DISCUSSION

Vpr is the only regulatory protein of HIV-1 that is known to be incorporated into virions in significant amounts. We and others reported previously that the virion association of Vpr is

FIG. 4. A C-terminal region of p6 is sufficient for Vpr incorporation. COS-7 cells were cotransfected with ptrENV and with either the HIV-1 Gag polyprotein expression construct HXBH10-PR⁺ (lane 1), the MLV Gag polyprotein expression construct HXBH10/NCA_{gag}-PR⁺ (lane 2), or the indicated chimeric Gag polyprotein expression constructs (lanes 3 to 6). [³⁵S]methionine-labeled particulate material released into the supernatant was pelleted through 20% sucrose and disrupted in RIPA buffer, and aliquots were either analyzed directly by SDS-PAGE (A) or immunoprecipitated with rabbit anti-Vpr serum prior to SDS-PAGE (B). (C) p6 sequences that were attached to the C terminus of the MLV Gag polyprotein. MLV Gag domains in the schematic diagram on top are indicated in white letters against black. The conserved L-X-S-L-F-G motif in p6 is boxed, and residues essential for Vpr incorporation are highlighted in boldface. Numbers refer to the positions of residues in the HIV-1_{HXB2} p6 domain. CyPA, cyclophilin A (34a).

mediated by determinants located in the p6 domain of the HIV-1 Gag polyprotein (16, 23, 28). In the present study, we demonstrate that these determinants are largely confined to a region near the C terminus of the p6 domain. This C-terminal region contains one of two motifs which are invariant in the

otherwise highly variable p6 domains of primate lentiviruses. The more N-terminal of the two conserved motifs (P-T/S-A-P-P) appears to be critical for the role of p6 in the release of budding particles from the cell surface (13). The second conserved motif (L-X-S-L-F-G) is not important for virus release but is essential for the incorporation of Vpr (4, 16). Sequential mutagenesis of the entire p6 region now revealed that sequences outside of the L-X-S-L-F-G motif, with the single exception of Ser-40 immediately N-terminal to the motif, can be altered without significant effects on Vpr incorporation. The sensitivity of Ser-40 to alanine substitution was unexpected, because an alanine is found at this position in some natural isolates of HIV-1 (26). Within the L-X-S-L-F-G motif, each of the three invariant hydrophobic residues (Leu-41, Leu-44, and Phe-45) is essential for Vpr incorporation, but the replacement of the nearly equally well conserved Ser-43 and Gly-46 residues had no effect.

Remarkably, as few as seven amino acids from the HIV-1 p6 domain (Ser-40 plus the conserved L-X-S-L-F-G motif) were sufficient to direct Vpr into heterologous particles. However, efficient incorporation was dependent on the presence of additional p6 sequences upstream of Ser-40. An important role of determinants in less well conserved regions of p6 was previously suggested by the observation that particles formed by the Gag polyprotein of HIV-2_{ROD} incorporate HIV-1 Vpr with very low efficiency despite the presence of the L-X-S-L-F-G motif (16). The present study shows that, at least in the case of HIV-1_{HXB2}, these additional determinants are located primarily between amino acids 32 and 39 of the p6 domain. Each residue in this region could be altered or deleted without appreciable effect on Vpr incorporation; nevertheless, the region as a whole contributed significantly to the efficiency of incorporation when combined with the seven-amino-acid minimal association motif. While the transfer of the latter motif alone (p6 residues 40 to 46) to MLV Gag led to Vpr incorporation levels that were only 10% of those seen in authentic HIV-1 particles, those levels reached 40% upon transfer of p6 residues 37 to 46 and were nearly maximal in the presence of p6 residues 32 to 46.

How can these results be reconciled with our observation that, in the context of the entire p6 domain, the deletion of residues 32 to 36 or the individual substitution of residues 37 to 39 had no effect on Vpr incorporation? One possible interpretation is that in the absence of other p6 sequences, residues 32 to 39 were merely required to separate the Vpr incorporation motif (residues 40 to 46) from the MLV nucleocapsid domain. This interpretation would be compatible with the normal location of the incorporation motif in the HIV-1 Gag polyprotein. However, a model which postulates that the Vpr incorporation motif is formed primarily by highly conserved residues could not easily explain the marked preference for autologous Vpr (16). Therefore, we favor the hypothesis that p6 residues 32 to 39, although not critical at the individual level, nonetheless cooperate to stabilize the interactions that lead to the incorporation of Vpr. Interestingly, a recent study indicates that the incorporation of the HIV-2 Vpr homolog Vpx is mediated by an entirely different region of the p6 domain (37).

What are the structural requirements for Vpr incorporation? In Vpr, an N-terminal region (residues 17 to 34) has been predicted to form an amphipathic α -helix, and mutations expected to disrupt the helix impaired Vpr incorporation (24, 25). Chou-Fasman analysis has also suggested that residues 36 to 86 of Vpr form a β -sheet (25). However, an improved prediction method which combines evolutionary information and neural networks (32-34) strongly suggests a mostly α -he-

- virus type 1 viral protein R localization in infected cells and virions. *J. Virol.* **67**:6542–6550.
24. Mahalingam, S., S. A. Khan, M. A. Jabbar, C. E. Monken, R. G. Collman, and A. Srinivasan. 1995. Identification of residues in the N-terminal acidic domain of HIV-1 Vpr essential for virion incorporation. *Virology* **207**:297–302.
 25. Mahalingam, S., S. A. Khan, R. Murali, M. A. Jabbar, C. E. Monken, R. G. Collman, and A. Srinivasan. 1995. Mutagenesis of the putative α -helical domain of the Vpr protein of human immunodeficiency virus type 1: effect on stability and virion incorporation. *Proc. Natl. Acad. Sci. USA* **92**:3794–3798.
 26. Myers, G., B. Korber, S. Wain-Hobson, R. F. Smith, and G. N. Pavlakis. 1993. Human retroviruses and AIDS 1993: a compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N.Mex.
 27. Ogawa, K., R. Shibata, T. Kiyomasu, I. Higuchi, Y. Kishida, A. Ishimoto, and A. Adachi. 1989. Mutational analysis of the human immunodeficiency virus *vpr* open reading frame. *J. Virol.* **63**:4110–4114.
 28. Paxton, W., R. I. Connor, and N. Landau. 1993. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of *gag* and mutational analysis. *J. Virol.* **67**:7229–7237.
 29. Perez, L. G., M. A. O'Donnell, and E. B. Stephens. 1992. The transmembrane glycoprotein of human immunodeficiency virus type 1 induces syncytium formation in the absence of the receptor-binding glycoprotein. *J. Virol.* **66**:4134–4143.
 30. Refaeli, Y., D. N. Levy, and D. B. Weiner. 1995. The glucocorticoid receptor type II complex is a target of the HIV-1 *vpr* gene product. *Proc. Natl. Acad. Sci. USA* **92**:3621–3625.
 31. Rogel, M. E., L. I. Wu, and M. Emerman. 1995. The human immunodeficiency virus type 1 *vpr* gene prevents cell proliferation during chronic infection. *J. Virol.* **69**:882–888.
 32. Rost, B., and C. Sander. 1993. Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol.* **232**:584–599.
 33. Rost, B., and C. Sander. 1993. Improved prediction of protein secondary structure by use of sequence profiles and neural networks. *Proc. Natl. Acad. Sci. USA* **90**:7558–7562.
 34. Rost, B., and C. Sander. 1994. Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins* **19**:55–72.
 - 34a. Thali, M., A. Bukovsky, E. Kondo, B. Rosenwirth, C. Walsh, J. Sodroski, and H. Göttlinger. 1994. Functional association of cyclophilin A with HIV-1 virions. *Nature (London)* **372**:363–365.
 35. Tristem, M., C. Marshall, A. Karpas, and F. Hill. 1992. Evolution of the primate lentiviruses: evidence from *vpx* and *vpr*. *EMBO J.* **11**:3405–3412.
 36. Westervelt, P., T. Henkel, D. B. Trowbridge, J. Orenstein, J. Heuser, H. E. Gendelman, and L. Ratner. 1992. Dual regulation of silent and productive infection in monocytes by distinct human immunodeficiency virus type 1 determinants. *J. Virol.* **66**:3925–3931.
 37. Wu, X., J. A. Conway, J. Kim, and J. C. Kappes. 1994. Localization of the Vpx packaging signal within the C terminus of the human immunodeficiency virus type 2 Gag precursor protein. *J. Virol.* **68**:6161–6169.
 38. Yuan, X., Z. Matsuda, M. Matsuda, M. Essex, and T.-H. Lee. 1990. Human immunodeficiency virus *vpr* gene encodes a virion-associated protein. *AIDS Res. Hum. Retroviruses* **6**:1265–1271.
 39. Zhao, L. J., S. Mukherjee, and O. Narayan. 1994. Biochemical mechanism of HIV-1 Vpr function: specific interaction with a cellular protein. *J. Biol. Chem.* **269**:15827–15832.