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Analysis of HIV-2 Vpx by Modeling and Insertional Mutagenesis

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Abstract

Vpx facilitates HIV-2 nuclear localization by a poorly understood mechanism. We have compared Vpx to an NMR structure HIV-1 Vpr in a central helical domain and probed regions of Vpx by insertional mutagenesis. A predicted loop between helices two and three appears to be unique, overlapping with a known novel nuclear localization signal. Overall, Vpx was found to be surprisingly flexible, tolerating a series of large insertions. We found that insertion within the polyproline-containing C-terminus destabilizes nuclear localization, whereas mutating a second helix in the central domain disrupts viral packaging. Other insertional mutants in the predicted loop and in a linker region between the central domain and the C-terminus may be useful as sites of intramolecular tags as they could be packaged adequately and retained preintegration complex associated integration activity in a serum starvation assay. An unexpected result was found within a previously defined nuclear localization motif near aa 71. This mutant retained robust nuclear localization in a GFP fusion assay and was competent for preintegration complex associated nuclear import. In summary, we have modeled helical content in Vpx and assessed potential sites of intramolecular tags which may prove useful for protein–protein interactions studies.

Keywords: HIV-2, Vpx, Nuclear localization, Nuclear import

Introduction

Infection of non-dividing cells is a hallmark of the lentiviruses (HIV-1, HIV-2, and the simian immunodeficiency viruses), setting them apart from oncoretroviruses which require mitosis (Lewis and Emerman, 1994). Infection of quiescent cells plays an important role in HIV transmission, dissemination, pathogenesis, and latency. Key to this process is a large nucleoprotein preintegration complex (PIC) which directs the newly reverse transcribed viral cDNA through presumably intact nuclear membranes (reviewed in Greber and Fornerod, 2005, and Fouchier and Malim, 1999). The PIC remains poorly characterized but likely contains viral integrase, matrix, the accessory proteins Vpr or Vpx of HIV-1 or HIV-2/SIV, respectively, and viral cDNA (Bukrinsky et al., 1993, Farnet and Haseltine, 1991, Karageorgos et al., 1993, and Miller et al., 1997). Host barrier-to-auto integration factor, histones, and the DNA-binding protein HMG Y(I) also assist with PIC function (Karageorgos et al., 1993, Chen and Engelman, 1998, Farnet and Bushman, 1997, Harris and Engelman, 2000, Hindmarsh et al., 1999, Li et al., 2000, and Lin and Engelman, 2003).

HIV-2 and other members of the lentivirus family that includes SIVmac and SIVsm encode a small accessory protein called Vpx. This protein is homologous to HIV-1 and SIV Vpr and likely arose by at least one distant gene transfer event (Tristem et al., 1992, Tristem et al., 1998, and Sharp et al., 1996). HIV-2 also encodes a Vpr protein, however, it is Vpx that plays an important role in nuclear targeting similar to that of HIV-1 Vpr (Fletcher et al., 1996, Le Rouzic and Benichou, 2005, Pancia et al., 2000, and Popov et al., 1998a). Thus, the nuclear localization and cell cycle arrest functions of HIV-1 Vpr appear to be split in HIV-2 between Vpx and Vpr, respectively (Fletcher et al., 1996). Furthermore, Vpx is necessary for replication in macrophages (Fletcher et al., 1996 and Pancia et al., 2000). These observations, together with studies linking Vpx (Hansen and Bushman, 1997) or Vpr to PICs, suggest an important role for Vpx in nuclear localization during HIV-2 infection.

The molecular mechanism of HIV PIC nuclear transport remains unclear. Although nuclear localization signals (NLS) have been described in both matrix and integrase, these are neither necessary nor sufficient for nuclear transport (reviewed in Greber and Fornerod, 2005, Stevenson, 1996, and Sherman and Greene, 2002). Both Vpr and Vpx localize to the nucleus or when overexpressed in the absence of Gag (Panico et al., 2000 and Mahalingam et al., 2001), but neither protein contains a classic NLS. Vpr binds to importin-α (Popov et al., 1998a) and can interact with importin-β as well as dock with the nuclear pore (Popov et al., 1998b, Kamata et al., 2005, Gallay et al., 1997, Vodicka et al., 1998, and Fouchier et al., 1998). Despite these findings, there is conflicting evidence implicating the importin-α/β nuclear import complex in Vpr-directed PIC transport (Popov et al., 1998b and Jenkins et al., 1998). HIV PICs have also been observed to use importin 7 (Fassati et al., 2003), although this pathway appears dispensable (Zielske and Stevenson, 2005). Furthermore, Vpr is known to interact with nucleoporins (Le Rouzic et al., 2002 and Popov et al., 1998b). Vpr induces nuclear membrane herniations, which might contribute to the nuclear localization process (de Noronha et al., 2001). Finally, HIV-1 PICs interact with microtubules, which are observed to facilitate their transfer within 75 min after entry to the nuclear membrane region (McDonald et al., 2002).

Various sites in Vpr have been implicated in nuclear localization by mutagenesis (reviewed in Le Rouzic and Benichou, 2005). However, structural studies and the analysis of various mutants suggest that the integrity of a central three helix bundle is important to Vpr function (Morellet et al., 2003). For Vpx, deletions and mutations in the unique C-terminal region, which contains a stretch of polyproline not found in Vpr, are known to destabilize nuclear localization (Panico et al., 2000 and Mahalingam et al., 2001). Interestingly, minimal peptide motifs near aa 65–75 have been shown to be sufficient for nuclear localization when fused to larger proteins (Mahalingam et al., 2001, Rajendra Kumar et al., 2003, and Belshon and Ratner, 2003). Phosphorylation of Vpx has been implicated in nuclear transport of SIV PICs (Rajendra Kumar et al., 2005), as has binding to the host cytoskeleton component actinin (Mueller et al., 2004).

We sought (1) to compare HIV-2 Vpx with HIV-1 Vpr in order to map structural domains and (2) to probe regions by insertional mutagenesis. One goal is to find stable insertion sites that can be used as intramolecular tags. First, we compared a central domain against a three helix structure of HIV-1 Vpr. Next, we created a series of insertions in this region and in the C-terminus and examined Vpx packaging, GFP-tagged nuclear localization, and PIC-associated nuclear import. Vpx was found to be surprisingly flexible, tolerating an array of large insertions. A predicted loop and a linker between the central helical region and the C-terminus appear to be useful sites for engineering intramolecular tags.

Results

Predicting tertiary structure in Vpx

We sought to compare Vpx in a central region to HIV-1 Vpr and noticed that aa 23–87 in Vpx could be simply threaded against a structure of HIV-1 Vpr (Morellet et al., 2003) if five amino acids, Vpx 60–64, were taken into account as a loop (data not shown). This 5-aa sequence is predicted to be coil by various secondary structure prediction algorithms (Figure 1A). Low complexity regions, such as this, can often tolerate variability. We next aligned all of aa 23–87 against Vpr by allowing for a 2-aa adjustment in the low complexity region and submitted the alignment (Figure 1B) for homology modeling by 3D Jigsaw (Bates and Sternberg, 1999 and Bates et al., 2001). The model (schematic shown in Figure 1C) predicts a three helix bundle stabilized by intramolecular hydrophobic contacts and anchored across helices 1 and 3 by the extremely well-conserved histidines, H39 and H82 (Figure 1D) in addition to L40 and V83, which are generally conserved as hydrophobic residues in these positions. However, K68 tends to destabilize the hydrophobic core in this model. For this reason, we sought gap building to better account for the low complexity loop using the Robetta server (Kim et al., 2004, Chivian et al., 2003, and Rohlf et al., 2004), which uses Rosetta (Simons et al., 1997). The process uses energy-minimized constraints by simulated annealing to generate a comparative model and then builds gaps both by ab initio and homology modeling based on peptide fragments. The result (Figs. 1E and F) predicts similar helical content but shifts the loop slightly toward the C-terminus at aa 60–71 compared to the previous models.

Recently, a leucine zipper homodimer has been demonstrated for helix three in HIV-1 Vpr by NMR (Bourbigot et al., 2005). We attempted to model the corresponding region in Vpx (data not shown). While hydrophobic residues L72, I74, and V79 could be modeled, K68 tends to disrupt the di-
mer. Furthermore, although H82 is conserved, Vpx lacks a tryptophan on the opposite helix three with which to make stacking interactions. Thus, a leucine zipper like dimer for Vpx helix three seems unlikely.

The predicted 60–71 loop, which contains five additional amino acids relative to Vpr (Figs. 1E and F), coincides precisely with a recently defined minimal nuclear localization motif, aa 65–72 (Rajendra Kumar et al., 2003 and Belshan and Ratner, 2003). This region contains 68-KYRY-71 which is highly conserved among Vpx sequences from a range of isolates (Figure 1G) but is not found in Vpr. Thus, the Vpx NLS appears to be both structurally and functionally distinct compared to Vpr.

The C-terminal domain can be expected to form a left-handed type II polyproline helix, based on thermodynamic and structural studies of similar peptides (Rath et al., 2005 and Sreerama and Woody, 1994). The linker region between the helical core and the polyproline tail (residues 91–101) can be predicted as a loose coil, given the presence of five glycines and one proline. Overall, such an extended structure is ideal as a protein-binding interface, given potential favorable entropic binding costs.

Figure 1. Vpx/Vpr homology region; Vpx structural predictions. (A) ROD HIV-2 Vpx secondary structure prediction by PsiPred (McGuffin et al., 2000); aa 24–87 are shown; H represents helix, C represents random coil; the remainder of the N- and C-terminal sequences are predicted to be random coil. (B) Alignment of the region in Vpx (top strand) with HIV-1 Vpr (bottom strand), as predicted by Domain Fishing, with manual readjustment of the gap region, as described in Materials and methods. Solid lines indicate identity, dark and light dashes indicate similarity. (C) Vpx aa 1–112 schematic. α-Helical regions predicted by 3DJigsaw, as described in Materials and methods, are indicated as H1–3. Positions H39, P41, and H82 are shown. (D) Remote homology sensing based on multiple Vpx and Vpr sequence alignments; positions H39 and H82 are indicated. (D) Vpx aa 1–112 schematic, as in panel C. α-Helical regions predicted by Robetta, as described in Materials and methods, are depicted. (F) α-Helical region aa 23–87, as in panel E, as modeled by Robetta. The figure was rendered with DeepView (Guex and Peitsch, 1997). (G) Vpx consensus from multiple sequence alignments of HIV-2 and SIV Vpx.
Insertion mutagenesis reveals differing structural requirements for Vpx viral particle recruitment versus nuclear localization

We hypothesized that by creating insertional mutations in Vpx, we could study functional domains and at the same time find favorable sites for intramolecular tagging. A series of 19-aa insertions were generated by transposon-based mutagenesis, and a panel was chosen: Insertion (Ins) 52, at the predicted second helix; Ins 71, within the NLS; Ins 91, at the linker between helix three and the polyproline tail; and Ins 106, within the polyproline motif (Figure 2A). It should be noted that the transposition mechanism leaves small duplications. For example, Ins 52 caused duplication of 51-SW-52, whereas Ins 71 resulted in duplication of 69-YRY-71 (Figure 2A). Ins 106 recreated proline 107, because of the wobble base at codon 106, and had the net effect of duplicating 105-PP-106 (Figure 2A). Next, a His\textsubscript{5} insertion adjacent to H57 was created by site-specific mutagenesis. This site was chosen because early simple threading models predicted flexibility at the end of helix 2.

Figure 2. Insertion mutations. (A) Positions and sequence of transposition-mediated 19-aa insertions and polyhistidine insertion in the Vpx sequence. H1–3 correspond to helices as in Figs. 1F. (B) Western blots of purified viral particles probed for Vpx (upper) or viral capsid (CA) (lower). (C) Cellular lysates used to produce virus as in panel B. Note that a lower resolving SDS-PAGE gel was used, and thus, the migration patterns appear similar for the various mutants; Western blots probed for Vpx or CA. (D) Plot of normalized ratio of Vpx or Vpx mutants:CA localized to viral particles.
The insertion mutants were analyzed for viral particle incorporation by constructing mutant HIV-2 molecular clones and transfecting them into 293T cells. Viral particles were isolated. As shown in Figure 2B, all of the mutant proteins were packaged. After normalizing for total Vpx to HIV-2 capsid (CA) ratios expressed in the cellular lysates (Figure 2C), the efficiency of Vpx recruitment was assessed by taking the ratio of Vpx/CA in the viral particles (Figure 2D). Ins 52 was the most severely compromised, packaged at 6% compared to wild type, whereas Ins 57, 71, 91, and 106 were moderately decreased; 55, 20, 30, and 31%, respectively.

Next, we studied nuclear localization by overexpressing the insertion mutants as GFP fusions. As shown in Figure 3, wild-type Vpx accumulated in the nucleus, as expected. Tiny cytoplasmic inclusion bodies were observed (Figure 3, arrows). Ins 52 localized to the nucleus, also with scattered punctuate cytoplasmic inclusions (Figure 3, arrows). Ins 57 and 106 showed relatively poor nuclear localization, with more diffuse cytoplasmic distribution (Figure 3). Ins 91 consistently localized to the nucleus but caused a toxic appearance to cells (Figure 3). The most striking result was Ins 71, which localized uniformly to the nucleus and did not form inclusion bodies (Figure 3).

Integration activity of the insertion mutants

Next, the insertion mutants were studied in an infection assay in serum-starved cells to test for active nuclear localization in the context of the HIV-2 PIC. In this assay, amphotropic MLV-pseudotyped HIV-2 viruses are used to infect arrested monocytic U937 cells during a short incubation. A nested PCR technique similar to that described by Brussel et al. (2005) counts integration events in the chromosomal DNA, presumably reflecting successful negotiation of the nuclear envelope. The first round of PCR is a linear round using primers against the repetitive and widely distributed Alu chromosomal sequences together with a tagged HIV-2 LTR primer. The second round is a quantitative, real-time, round using primers complementary to the tag and the LTR together with an internal fluorescent probe. As shown in Figure 4, wild-type Vpx virus is much more efficient than ΔVpx for nuclear localization in this assay (lanes 1 and 2), as expected. Ins 106 showed severely compromised function, similar to ΔVpx (Figure 4, lane 7). Ins 52 was strongly reduced (Figure 4, lane 3), whereas Ins 57, 71, and 91 were moderately decreased relative to wild-type virus nuclear localization (Figure 4, lanes 4–6).
Discussion

Structural predictions in Vpx

Because the modeled helical region (Figure 1F) coincides precisely with the most highly conserved sequences between Vpx and Vpr, a three helix bundle structure seems likely. Furthermore, the fact that H39 and H82 are extremely well conserved across all Vpx and Vpr sequences (Figure 1D) points to a structural role for such a configuration. In fact, a recent study showed SIV Vpx H82A to be compromised for nuclear localization and mutant virus that was deficient for replication in macrophages (Mahalingam et al., 2001). Considering diverse Vpx sequences, as shown in Figure 1G, it is apparent that hydrophobic residues predicted to stabilize the central core are well conserved, including residues 25I, 29V, 32I, 37V, 40L, 44L, 45I, 48V, 72L, 74I, and 75M.

Proline at position 41 is predicted to adopt a trans-configuration stabilizing the first turn in the helical bundle, analogous to Vpr P35 (Bruns et al., 2003 and Morellet et al., 2003) (Figure 1F). Proline in this region is highly conserved among Vpx and Vpr sequences, at various positions within the turn. Interestingly, host cyclophilin, a prolyl isomerase, binds to Vpr at proline in the first turn and is important for stable Vpr expression (Zander et al., 2003). Preliminary evidence demonstrates an analogous dependence of HIV-2 Vpx on cyclophilin, substantiating the idea that the conformation of Vpx may be similar to Vpr (data not shown).

At this time, the function of a leucine zipper for Vpr remains unclear. In any event, based on the modeling discussed here, it seems unlikely that Vpx would be able to adopt an analogous dimer involving helix three. This potential structural difference might reflect differing functions. For example, Vpr has been implicated in a wide array of activities not known for Vpx including transcription activation, regulation of apoptosis, and the well-characterized G2 arrest (reviewed in Le Rouzic and Benichou, 2005).

Viral packaging

Surprisingly, the insertion mutations were broadly tolerated for viral packaging in that mutating the helical domain or C-terminus did not completely abrogate this function. However, Vpx Ins 52 was severely compromised, suggesting that either an intact helix two or exposed face of helix two is important for the interaction with Gag. On the other hand, Ins 106 retained viral packaging, suggesting that the polyproline tail is not critical for Gag binding. The helical model (Figs. 1E and F) predicts a loop adjacent to Ins 57 and 71 and a linker region at the site of Ins 91, between the helical domain and the C-terminus. The mutants Ins 57, 71, and 91 retained adequate viral packaging, suggesting that the loop and linker are flexible enough to tolerate variation.

Previous studies have shown that Vpx or Vpr can tolerate a wide range of mutations and still be packaged into virions. For Vpx, the Gag interaction domain would appear to be located between aa 64 and 89, since deletion N- or C-terminal to these positions, respectively, does not abrogate recruitment into particles (Pancio et al., 2000 and Mahalingam et al., 2001). Furthermore, internal deletion of aa 73–89 did not disrupt viral particle incorporation (Jin et al., 2001). This would leave aa 64–73 as a possible minimal Gag interaction domain, which coincides with the loop and the NLS in the model of the protein described here. However, in a previous study, SIV Vpx aa 60–85 fused to GFP was found to be insufficient for viral particle localization (Mahalingam et al., 2001). Ins 71 supports this observation, in that the mutant protein could still be packaged. In terms of the overall three helix bundle, a previous study showed that mutating the extremely conserved histidine at position 82 did not affect viral packaging (Mahalingam et al., 2001), a mutation which is predicted to disrupt the helical bundle structure. Thus, it appears that neither an intact three helix bundle, as modeled here, nor the NLS-loop region is critical for viral packaging. The precise residues involved in viral packaging remain to be determined.

Nuclear localization by GFP fusion assay

The phenotype of Vpx Ins 106 (Figure 3) confirms a previous result in which Vpx was truncated C-terminal to aa 110, resulting in destabilization of nuclear localization (Pancio et al., 2000). Thus, the highly conserved polyproline motif contributes to efficient nuclear localization.

A surprising result was found with Ins 71. This mutation disrupts the NLS motif, 65-SYTKYRYLCIM-75, as defined by Belshan and Ratner (2003). Yet, the mutant localized uniformly to the nucleus in the GFP assay (Figure 3). In the study of Belshan and Ratner, 3 aa at the C-terminal end of the peptide did not seem necessary for the NLS function, given the fact that an 11-aa sequence, aa 61–72, could also mediate nuclear localization. Because the net effect of Ins 71 is to disrupt the motif after position 71, the observations suggest that L72 is not critical for the NLS signal. There may be various reasons contributing to the good performance of Ins 71 in this assay including increased solubility or an extended structure exposing the NLS. In fact, Ins 71 did not form inclusion bodies in the GFP assay (Figure 3). In terms of sequence, the random 19-aa insertion caused duplication of K/R-Y-R-Y (Figure 2A), a motif that is very well conserved among HIV-1/SIV isolates (Figure 1G). Because a recent study of SIV Vpx confirmed aa 61–80 to be sufficient for nuclear entry and that mutation of K68A did not disrupt nuclear localization (Rajendra Kumar et al., 2003), 69-YRY-71 may be the most critical residues. For HIV-2 ROD Vpx, we have recently demonstrated the importance of R70, when mutated together with K68, and Y69 for the nuclear localization signal (Belshan et al., in press).

PIC-associated nuclear localization by Alu integration

In this sensitive, non-dividing cell assay induced by serum starvation, Vpx was observed to enhance PIC nuclear localization (Figure 4, lanes 1 and 2). The relative activities of Ins 52, 57, 71, and 91 in this assay are similar to the relative packaging efficiencies of these mutants as compared
to wild type (compare Figure 4, lanes 3–5 with Figure 2D). Thus, the overall effect of the mutations on PIC activity appears to be minimal. The results confirm those of the GFP nuclear localization assay in the case of Ins 52 and 71. Why Ins 57 behaves poorly in the GFP assay is unclear but might be explained by toxic or artifactual effects of overexpressing the mutant protein. Clearly, overexpression of Ins 91 caused toxicity to the cells in the GFP assay.

Ins 106 appeared to be severely compromised for PIC function in the non-dividing cell assay, suggesting an important role of the polyproline motif in PIC-associated nuclear localization. This result is in agreement with the destabilization of nuclear localization observed in the GFP assay and confirms previous observations mutating this C-terminal region (Pancio et al., 2000).

An alternate test of PIC nuclear localization would be to test the Vpx mutant viruses for infection of macrophages. However, the ROD HIV-2 isolate is defective for efficient macrophage infection for reasons that are unclear at this time.

Summary

One goal of these experiments is to identify regions in Vpx that are suitable for intramolecular tagging. Insertion site 106 appears to be a poor candidate, given the disruption of both PIC associated and GFP fusion nuclear localization. Ins 57 appears to retain PIC activity and incorporates relatively well into viral particles but performed poorly in the GFP assay, which may be subject to artifact given the high levels of overexpressed protein. Indeed, Ins 91 caused toxicity to cells in the GFP assay but appeared to retain PIC integration activity as well as adequate viral packaging. Finally, the phenotype of Ins 71 is unexpected. The mutant sequence suggests that leucine at position 72 is not important to the NLS. The mutant protein showed strong nuclear localization as a free GFP fusion, was non-toxic, did not form inclusion bodies, retained adequate viral packaging, and appeared to be stable for PIC function. In summary, the best candidates for stable insertion sites are clustered around the NLS-loop region, near aa 57–71, or within the helical domain/C-terminus linker, near aa 91.

Viruses have evolved a variety of strategies for harnessing the nuclear pore. For HIV-2, the mechanism of nuclear localization appears unique but remains poorly understood. Characterizing the molecular details of retroviral preintegration complexes and associated host interactions should highlight novel therapeutic targets as well as basic mechanisms of nuclear entry.

Materials and methods

Homology modeling

Amino acids 23–87 in the HIV-2 ROD Vpx primary sequence were aligned with residues 17–76 of HIV-1 Vpr by Domain Fishing (Bates et al., 2001). The alignment resulted in 5 extra aa in Vpx, aa 62–66, coinciding with coil predicted by PsiPred (McCuffin et al., 2000) in the region aa 56–66. Because the region is low complexity, we manually adjusted the alignment by moving the gap to Vpx aa 60–64 and submitted to 3D Jigsaw against PDB 187L (Morellet et al., 2003, Bates and Sternberg, 1999, Bates et al., 2001 and Contreras-Moreira and Bates, 2002). To build the gap, a full-length alignment was submitted to the Rosetta modeling server against Vpr PDB 187L (Kim et al., 2004, Chivian et al., 2003 and Rohl et al., 2004). For the third helix dimerization domain, Vpr coordinates were kindly supplied by Bourbigot et al. (2005). The corresponding region of Vpx was fit manually using Swiss-PdbViewer (Schwede et al., 2003), and the dimer was modeled by Swiss-Model. For Vpx consensus building, the Vpx HIV-2/SIV alignment from the Los Alamos HIV sequence database, [http://www.hiv.lanl.gov/content/hiv-db/mainpage.html](http://www.hiv.lanl.gov/content/hiv-db/mainpage.html), was converted into a sequence logo (Crooks et al., 2004). The height of the letters represents the relative frequency of that residue at the given position (Schneider and Stephens, 1990). For Vpx/Vpr remote homology sensing by hidden Markov models, the SAM-99 server, [http://www.cse.ucsc.edu/research/compbio/sam.html](http://www.cse.ucsc.edu/research/compbio/sam.html) was used (Karplus et al., 1998, Krogh et al., 1994 and Hughey and Krogh, 1996).

Proviral constructs

A subcloning cassette containing a 587 bp fragment of the ROD10 (Bour et al., 1996) proviral clone was used such that a SacI site at bp 5704 has been mutated but does not alter the vpr open reading frame (Pancio et al., 2000). A series of random insertion mutations were made by the method of EZ:Tr (Epicentre, Madison, WI) (Goryshin and Reznikoff, 1998). After sequencing, candidate insertions were chosen, and the bulk of the transposon was removed by restriction digestion with NotI followed by religation. This procedure leaves random 19-aa insertions in every reading frame, with 9 bp of the original DNA sequence duplicated at either end. The modified regions were then digested with SacI and cloned into the ROD10 vector. The polyhistidine mutant was constructed using mutagenic primers coding for five histidines adjacent to the natural histidine at position 57 using the QuikChange method (Stratagene, La Jolla, CA). The ΔVpx construct contains mutations at the initiating and first internal methionines of Vpx such that the reading frame of vpr is intact (Hu et al., 1989).

Transfections, viral particle isolation, and Western blotting

293T cells were transfected with proviral DNA in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum and antibiotics, using TransIT-LT1 (Mirus Bio, Madison, WI) according to the manufacturer’s directions. AT 72 h, the supernatant was cleared by low speed centrifugation, layered on 20% sucrose, and subjected to ultracentrifugation at 26,000×g for 90 min. The pellet was resuspended in SDS-PAGE sample buffer, boiled for 10 min in the presence of DTT, and loaded on Tris–tricine SDS-PAGE. After blotting onto PVDF membranes, the blots were probed with a monoclonal anti-Vpx antibody (NIH AIDS Research and Reference Reagent Program, 6D2.6) or an anti-p24 capsid monoclonal antibody that
cross reacts with HIV-2 p27, both of which had been previously purified on protein A sepharose beads (Sigma, St. Louis, MO) from hybridoma culture. The blots were developed with anti-mouse horseradish peroxidase antibody followed by SuperSignal West Femto substrate (Pierce, Rockford, IL) and imaged and quantitated on chemiluminescent Alpha Imager (Alpha Innotech, San Leandro, CA). For the cellular lysates, the 293T cells were lysed in SDS sample buffer and loaded onto 12% Tris SDS-PAGE, followed by Western blotting.

**GFP fusion nuclear localization**

Insertion mutations were cloned into pEGFP-C1 (Clontech) using BamHI and EcoRI sites, resulting in fusion to the GFP open reading frame at the Vpx N-terminus driven by a constitutive CMV-based promoter. 10^5 HeLa cells grown on coverslips were transfected with 2 μg of DNA in duplicate in 6-well tissue culture plates, DMEM medium supplemented with 10% fetal calf serum and antibiotics, and Transit-LT (Mirus Bio, Madison, WI), according to the manufacturer’s instructions. After 16 h, the cells were fixed with 2% paraformaldehyde, treated with 0.1% Triton X-100 for 5 min, then stained with Hoechst 33258 (Molecular Probes, Eugene, OR). The coverslips were mounted, and the cells were visualized using a fluorescence microscope equipped with an Optronics Magnafire imaging system (Optronics, Goleta, CA). Multiple images were obtained and compared from three independent transfections.

**Alu integration assay**

Amphotropic MLV pseudotyped HIV-2 ROD viruses were prepared by transfection of 293T cells in the presence of 1.5 μg ratio of a CMV driven amphotropic MLV env plasmid DNA to molecular clone ROD DNA using Transit-LT in DMEM medium supplemented with 10% fetal calf serum and antibiotics, according to the manufacturer’s directions. After 72 h, the supernatants were cleared by low-speed centrifugation, and viral particles were quantitated by simian immunodeficiency virus ELISA (Coulter, Miami, FL). U937 monocyte cells (ATCC CRL-1593.2) were plated at 10^5 cells per well in 12-well tissue culture dishes after washing and diluting in 0% fetal calf serum RPMI-1640 media (Gibco, Grand Island, NY) supplemented with antibiotics. After 48 h, 100 ng virus was added, and the reactions were incubated for 4 h at 37 °C in a humidified, CO2 supplemented incubator. The cells were washed in phosphate-buffered saline and genomic DNA was prepared using the Qiagen (Valencia, CA) Mini Blood DNA kit. A linear Probes, Eugene, OR). The coverslips were mounted, and the cells were visualized using a fluorescence microscope equipped with an Optronics Magnafire imaging system (Optronics, Goleta, CA). Multiple images were obtained and compared from three independent transfections.

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