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Nuclear import and cell cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-2/SIV_{SM}

Thomas M.Fletcher,III, Beda Brichacek[1], Natasha Sharova[1], Margaret A.Newman[2], Gina Stivahtis[3], Paul M.Sharp[4], Michael Emerman[3], Beatrice H.Hahn and Mario Stevenson[1,5]

Department of Medicine, University of Alabama, Birmingham, AL 35294, [1]Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, MA 01605, [3]Division of Molecular Medicine, Fred Hutchinson Cancer Research Center, Seattle, WA 98104, USA and [4]Department of Genetics, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, UK

[2]Present address: Department of Microbiology and Immunology, Baylor College of Medicine, Houston, TX 77030, USA

[5]Corresponding author

The *vpr* genes of human and simian immunodeficiency viruses (HIV/SIV) encode proteins which are packaged in the virus particle. HIV-1 Vpr has been shown to mediate the nuclear import of viral reverse transcription complexes in non-dividing target cells (e.g. terminally differentiated macrophages), and to alter the cell cycle and proliferation status of the infected host cell. Members of the HIV-2/SIV_{SM} group encode, in addition to Vpr, a related protein called Vpx. Because these two proteins share considerable sequence similarity, it has been assumed that they also exhibit similar functions. Here, we report that the functions of Vpr and Vpx are distinct and non-redundant, although both proteins are components of the HIV-2/SIV_{SM} virion and reverse transcription complex. Characterizing SIV_{SM} proviruses defective in one or both genes, we found that Vpx is both necessary and sufficient for the nuclear import of the viral reverse transcription complex. In contrast, Vpr, but not Vpx, inhibited the progression of infected host cells from the G_2 to the M phase of the cell cycle. Thus, two independent functions of the HIV-1 Vpr protein are encoded by separate genes in HIV-2/SIV_{SM}. This segregation is consistent with the conservation of these genes in HIV-2/SIV_{SM} evolution, and underscores the importance of both nuclear transport and cell cycle arrest functions in primate lentivirus biology.

Keywords: accessory genes/primate lentiviruses

Introduction

In addition to the *gag*, *pol* and *env* gene products common to all retroviruses, primate lentiviruses encode 'accessory' proteins which are not required for viral growth in established cell lines, but are likely essential for replication and pathogenesis *in vivo* (Trono, 1995). Among these accessory proteins, Vpr and Vpx, for which no homologs in the non-primate lentiviruses have yet been identified (Myers *et al.*, 1992), are of particular interest because they are packaged into virions in quantities comparable to the viral Gag proteins (Lu *et al.*, 1993; Paxton *et al.*, 1993; Wu *et al.*, 1994; Kewalramani and Emerman, 1996). Their presence and abundance in the virus particle (i.e. at a point preceding *de novo* production of viral proteins) suggests an early role in the virus life cycle.

Human and simian immunodeficiency viruses (HIV/SIV) group into five distinct phylogenetic lineages (Sharp *et al.*, 1994). All of these encode at least one gene in the central viral region (overlapping *vif* and *tat* open reading frames) which is usually termed *vpr*. Interestingly, members of the HIV-2/SIV_{SM}/SIV_{MAC} lineage contain an additional gene in this region which has been termed *vpx*. Since *vpx* and *vpr* share considerable sequence similarity and are juxtaposed in the HIV-2/SIV_{SM}/SIV_{MAC} genome, it has been suggested that they arose from a gene-duplication event (Tristem *et al.*, 1990). This, together with the finding that mutants of SIV_{MAC} which lack functional *vpx* or *vpr* proteins retain their *in vivo* pathogenicity (Gibbs *et al.*, 1995; Hoch *et al.*, 1995), has been taken to suggest that the HIV-2/SIV_{SM}/SIV_{MAC} *vpx* and *vpr* genes have duplicative or at least overlapping functions (Gibbs *et al.*, 1995).

Most information concerning Vpr and Vpx function has been derived from studies of the HIV-1 Vpr protein. Vpr is important for the ability of HIV-1 to infect terminally differentiated macrophages (Bailliet *et al.*, 1994; Heinzinger *et al.*, 1994; Connor *et al.*, 1995) because it facilitates the stability and nuclear localization of the viral reverse transcription complex (Heinzinger *et al.*, 1994). This property overlaps the nuclear transport function of the structural matrix (MA) protein which also mediates infection of non-dividing cells by HIV-1 (Bukrinsky *et al.*, 1993a,b; von Schwedler *et al.*, 1994; Gallay *et al.*, 1995; Bukrinskaya *et al.*, 1996). A second property, unrelated to its role in macrophages, is the induction of cell cycle arrest by HIV-1 Vpr (He *et al.*, 1995; Jowett *et al.*, 1995; Re *et al.*, 1995; Rogel *et al.*, 1995). This effect, which leads to the accumulation of cells in the G_2 stage of the cell cycle, likely explains earlier observations that Vpr influences cell differentiation (Levy *et al.*, 1993).

Redundancy and complementarity in function are not without precedent among the various HIV accessory proteins. For example, Vpu and Nef proteins together with the structural Env protein all modulate the expression of CD4 on the surface of infected cells (Stevenson *et al.*, 1988; Garcia and Miller, 1991; Willey *et al.*, 1992), although their action occurs at different levels. Vpu facilitates the degradation of CD4 in the endoplasmic reticulum (reviewed in Subbramanian and Cohen, 1994), Env prevents the transport of CD4 to the cell surface (Hoxie *et al.*, 1986; Stevenson *et al.*, 1988) and Nef induces lysosomal degradation of CD4 (Aiken *et al.*,

1994). In addition, there is precedent for more than one biological property per viral gene product. For example, Vpu promotes virus particle release (Strebel *et al.*, 1989; Willey *et al.*, 1992) as well as CD4 down-modulation. Thus, HIV/SIV gene products may have multiple as well as partially overlapping functions.

Given the extra *vpx* gene in HIV-2/SIV_{SM}/SIV_{MAC}, we wished to determine whether its protein product plays a redundant role in mediating infection of non-dividing cells and, if so, whether this function is manifest at the same level as Vpr and gag MA proteins. We also investigated whether Vpx interferes with the cell cycle. Our results show that cell arrest and nuclear transport properties, which are encoded by a single gene (i.e. *vpr*) in HIV-1, segregate with two genes (i.e. *vpr* and *vpx*) in HIV-2/SIV_{SM}. Thus, despite their significant homology, HIV-2/SIV_{SM} Vpr and Vpx proteins have little (if any) functional overlap.

Results

Vpx is required for productive SIV_{SM} infection of macaque macrophages

For mutational analyses of *vpx*, *vpr* and the putative nuclear localization signal (NLS) in the gag matrix protein (gag MA), we selected the infectious molecular clone SIV_{SM} PBj1.9 as a representative of the HIV-2/SIV_{SM} group of viruses. This clone is derived from short-term peripheral blood mononuclear cell (PBMC) cultures and thus represents a primary isolate (Dewhurst *et al.*, 1990). It also has a complete set of uninterrupted accessory genes and grows well in macaque macrophages. Unlike most other HIV-2 and SIV_{SM} clones, PBj1.9 thus represents a physiologically relevant virus strain.

Mutations designed to abrogate the translation of *vpx* and *vpr* genes were introduced into the PBj1.9 provirus (Figure 1). In addition, $K^{26, 27}$-T substitutions generated in the MA NLS domain were selected based on effects of similar mutations on the nuclear targeting of HIV-1 gag MA (Bukrinsky *et al.*, 1993a). The ability of these viruses to elicit a spreading infection in dividing (CEMx174; macaque PBMCs) and non-dividing (macaque monocyte-derived macrophages) cell systems was compared.

All viruses, whether containing single or combined mutations in *vpx*, *vpr* or gag MA, grew efficiently and to high titers in CEMx174 cells (Figure 2A). The composition of virions harvested from CEMx174 culture supernatants when reverse transcriptase (RT) counts exceeded 4×10^7 c.p.m./ml did not indicate any mutation reversion. As expected, virions derived from *vpx*- and/or *vpr*-deficient proviruses lacked Vpx and/or Vpr proteins, respectively (Figure 2B). No SIV-reactive proteins were visible by Western blot analysis of uninfected cell extracts using anti-Vpr, Vpx, MA or SIV antibodies (not shown). Interestingly, the packaging of Vpx and Vpr proteins was not interdependent (Figure 2B). For example, the Vpx content of wild-type PBj1.9 was indistinguishable (by Western blot analysis) from that of virions derived from *vpr*-mutant provirus (R2, R2/MA). Similarly, mutations interrupting Vpx expression did not appear to influence the incorporation of Vpr into virus particles (X2, X2/MA). *Vpx*- and/or *vpr*-deficient PBj1.9 viruses also replicated to high titers in primary macaque PBMCs (Figure 3). Thus, as

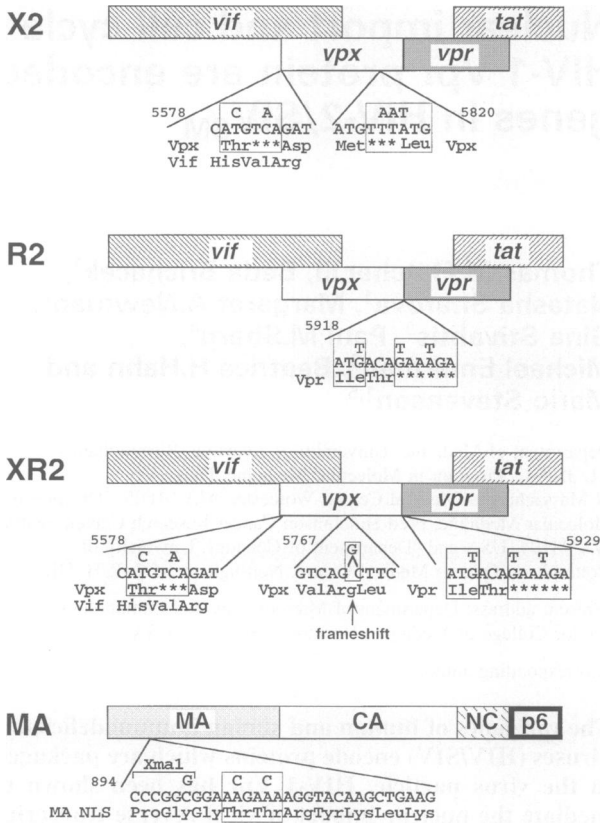


Fig. 1. Vpr and Vpx mutants of SIV_{SM} PBj1.9. X2 and XR2 contain multiple mutations designed to eliminate expression of Vpx from internal methionines. None of the nucleotide substitutions affected the overlapping Vif amino acid sequence. Mutations in PBj1.9 gag MA were selected based on the ability of similar mutations in the context of HIV-1 gag MA to attenuate gag MA-dependent nuclear import of viral DNA in non-dividing cells (Bukrinsky *et al.*, 1993a; von Schwedler *et al.*, 1994).

previously reported for SIV_{MAC} (Gibbs *et al.*, 1995), SIV_{SM} PBj1.9 did not require Vpx and/or Vpr to elicit a spreading infection in dividing host cells.

In contrast to their ability to replicate in dividing target cells (primary PBMCs and CEMx174 cells), PBj1.9 variants with mutations in *vpr*, *vpx* and MA NLS differed considerably in their growth potential in monocyte-derived macaque macrophages (Figure 4A). All PBj1.9 strains lacking a functional Vpx protein (X2, XR2, X2/MA, XR2/MA) were severely impaired in their ability to replicate in these cells. In contrast, the absence of a functional Vpr protein and/or a wild-type matrix NLS domain did not noticeably reduce virus replication. In six independent experiments, mutants lacking Vpx yielded very low RT activities (only 2- or 3-fold higher than mock-infected cultures), while mutants deficient in Vpr and/or the matrix NLS domain grew to titers comparable to wild-type PBj1.9. The residual replication in macrophages by Vpx mutants may have been due to levels of cell division, since human macrophage cultures prepared by adherence from PBMCs contain a small percentage (0.1–0.2%) of dividing cells (M.Stevenson, unpublished data). These data indicate that Vpx, but not Vpr or the putative matrix NLS domain, is required for replication and efficient spread of SIV_{SM} in monocyte-derived macrophages.

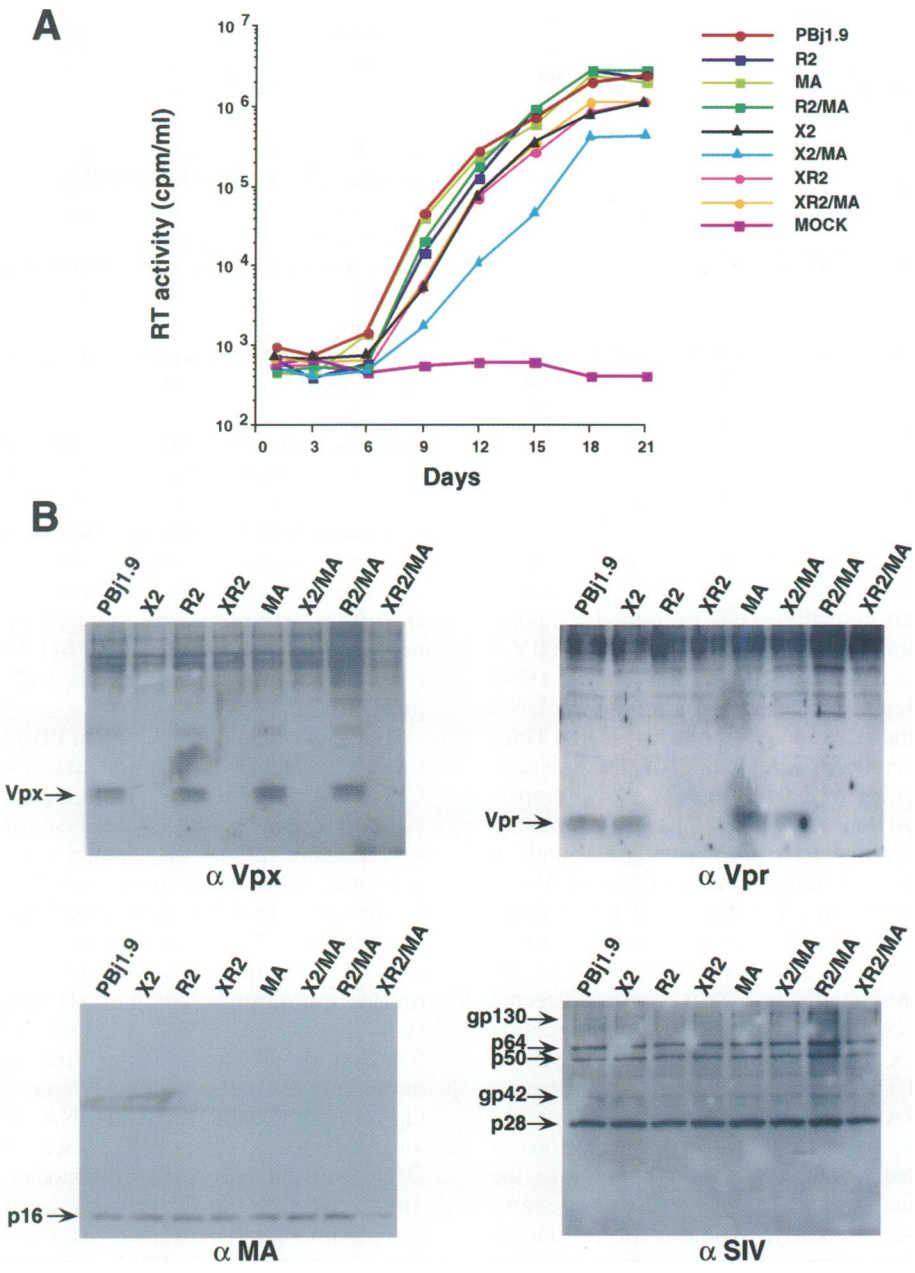


Fig. 2. Vpr and Vpx are dispensable for efficient replication of PBj1.9 in dividing CEMx174 cells. (**A**) Proliferating CEMx174 cells were infected with the indicated PBj1.9 variant (10 ng gag p27/2×10^6 cells). RT activity (c.p.m./ml) at 3 day intervals post-infection is shown on a logarithmic scale. (**B**) Western blot analysis of virion preparations obtained from productively infected human PBMCs reacted with the indicated SIV-specific antiserum. gag MA and pan-reactive SIV$_{MAC}$ antibodies demonstrated equivalent amounts of PBj1.9 protein in each lane.

Despite their markedly reduced growth potential in macaque macrophages, *vpx*-deficient PBj1.9 mutants could be rescued after 21 days by co-cultivation with CEMx174 cells (Figure 4A, shaded area). Thus, in the absence of Vpx, some infectious virus was produced, albeit at very low levels. Indeed, macrophage cultures infected with *vpx*-deficient PBj1.9 produced small (up to 100 pg/ml) but consistently detectable quantities of p27 (Figure 4B). As expected, cultures infected with wild-type PBj1.9, or R2, MA and R2/MA mutants, produced significantly more p27 antigen (up to 10 ng of p27/ml). Western blot (Figure 4C) and sequence analysis (Figure 4D) of CEMx174 rescued viruses (at day 42) confirmed that none had undergone mutation reversion.

To exclude the possibility that the low-level p27 antigen content of *vpx*-mutant cultures represented residual virus from the initial inoculum, we infected identically prepared macaque macrophage cultures with two HIV strains known not to replicate in macrophages. Using the same amount of inoculum (10 ng of p24/27), we did not observe detectable RT activity (Figure 5) or p27 antigen (data not shown) with either HIV-1/SG3 (Ghosh *et al.*, 1993) or HIV-2/7312A (Gao *et al.*, 1994). These results indicated that the p27 antigen content in macrophage cultures infected with *vpx*-deficient PBj1.9 reflected some *de novo* synthesis. However, in the absence of a functional Vpx protein, SIV$_{SM}$ replication in terminally differentiated macaque macrophages was severely impaired.

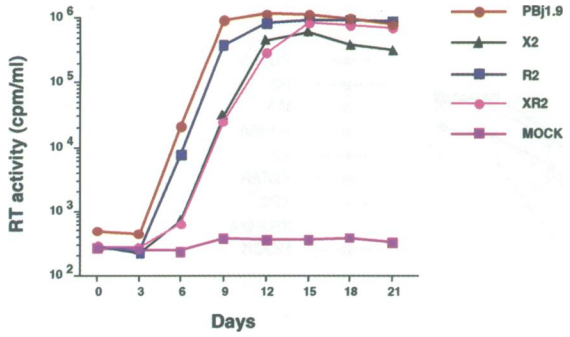


Fig. 3. Vpr and Vpx proteins are not required for an efficient spreading infection of PBj1.9 in macaque PBMCs. The isolation and infection of primary macaque PBMCs are described in Materials and methods. The graph depicts RT activity (logarithmic scale) in culture supernatants at 3 day intervals post-infection.

Vpx facilitates nuclear transport of viral DNA in macrophages

In order to identify the level at which the replication of PBj1.9 *vpx* mutants was restricted in macrophage cultures, early stages in virus infection were examined by polymerase chain reaction (PCR). Previous studies with HIV-1 (Heinzinger *et al.*, 1994; von Schwedler *et al.*, 1994) demonstrated inefficient nuclear localization of viral DNA by gag MA NLS and *vpr* mutants in macrophages. Thus, we examined whether the replication of PBj1.9 *vpx* mutants in macrophages was blocked at the same level by comparing the effect of *vpx* mutations on viral cDNA synthesis and nuclear localization following a single round of replication. Synthesis of minus-strand cDNA (U5′–U3 primers) was detectable in all cultures and found to be roughly equivalent (Figure 6, top of panels). Cultures infected in the presence of 5 μM AZT had no detectable strong-stop DNA products. Thus, cDNA products detected in acutely infected cells were the result of virus infection and *de novo* cDNA synthesis rather than the result of carry-over proviral DNA in the inoculum. The presence of strong-stop DNA products in macrophages infected with the PBj1.9 *vpx* mutants indicated that mutations in *vpx* did not markedly influence virion binding to the cell surface, fusion, uncoating or initiation of reverse transcription *per se*. Similarly, synthesis of full-length cDNA (U5/gag) products was evident by 6 h post-infection in all cultures (Figure 6, second row) which is comparable to the earliest detection of viral cDNA synthesis (5 h) following infection of cells by HIV-1 (Heinzinger *et al.*, 1995). This indicated that *vpx* mutations did not have a major effect on the rate of strong-stop cDNA synthesis in macrophages. Nevertheless, we observed a minor yet reproducible reduction (2- to 3-fold) in the abundance of full-length cDNA products in macrophages infected with the X2 mutant (Figure 6). This reduction was also apparent in macrophages infected with XR2 and X2/MA mutants, but not in macrophages infected with the R2/MA mutant (Figure 6). However, this effect was minor compared to the effect that *vpx* mutations had on 2LTR circle production (U5/U5′ primers). In cultures infected with wild-type PBj1.9 or R2, MA and R2/MA mutants, circle products were detectable as early as 12 h post-infection (Figure 6, bottom row). By contrast, in cultures infected with X2, XR2 and X2/MA mutants, the appearance of 2LTR circle products was markedly delayed and their abundance reduced even at 72 h post-infection (Figure 6). These results thus indicate that the rate and the extent of SIV_{SM} cDNA localization to the nucleus was inefficient in the absence of a functional *vpx* gene.

Vpx and Vpr associate with the reverse transcription complex of SIV_{SM}

We previously demonstrated that the influence of HIV-1 Vpr on nuclear import properties of the viral reverse transcription complex correlated with its presence in that complex (Heinzinger *et al.*, 1994). The effect of *vpx* mutations on nuclear import of PBj1.9 cDNA (Figure 6) as well as the recent findings of Vpx in core structures of HIV-2 (Kewalramani and Emerman, 1996) would predict that Vpx is also associated with the reverse transcription complex. We first compared the density of wild-type virions with that of the *vpx/vpr* double-mutant virions in order to determine whether the absence of these virion-associated proteins altered virion density in such a way so as to affect the resolution of viral nucleoprotein reverse transcription complexes on density gradients. Our results show that the absence of Vpr and Vpx proteins did not markedly alter the density of PBj1.9 virions since wild-type and double-mutant virions had a similar gradient profile (Figure 7A).

To examine the composition of PBj1.9 reverse transcription complexes, cytoplasmic extracts were prepared from CEMx174 cells at 4 h post-infection and resolved on sucrose density gradients. Analysis of gradient fractions by RT assay (Figure 7B) demonstrated two peaks in the gradients. The major peak, which corresponded to a density of 1.16 g/ml, represented residual virions from the inoculum. The second smaller peak with a density of around 1.22 g/ml was consistent with the density of the reverse transcription complex (Bukrinsky *et al.*, 1993b; Heinzinger *et al.*, 1994). Analysis of individual gradient fractions by PCR revealed that viral DNA was distributed throughout the entire gradient (Figure 7B, insert), indicating that pre-existing proviral DNA from supernatants of virus-infected producer cells as well as virion-associated DNA from the high-titer virus stocks (Lori *et al.*, 1992; Trono, 1992) were also present in gradient-fractionated cytoplasmic extracts of acutely infected cells. To remove this 'contaminating' DNA, gradient fractions were dialyzed and examined by an immunoprecipitation/PCR approach (Bukrinsky *et al.*, 1993b; Heinzinger *et al.*, 1994). This immunoprecipitation/PCR approach allows distinction of *de novo* synthesized viral DNA which is associated with viral reverse transcription complexes from the pre-existing viral DNA. Reverse transcription complexes were immunoprecipitated with either gag MA, Vpx or Vpr antibodies, and viral DNA associated with these immune complexes was identified by PCR. In agreement with previous studies on HIV-1 (Bukrinsky *et al.*, 1993b; Heinzinger *et al.*, 1994; Gallay *et al.*, 1995; Bukrinskaya *et al.*, 1996), antibodies to SIV_{SM} gag MA specifically immunoprecipitated viral cDNA from those gradient fractions (fractions 8–12, density 1.18–1.23 g/ml) which corresponded to the density of viral reverse transcription complexes for both wild-type and *vpr/vpx* mutant viruses (Figure 7C and D, upper panels). Importantly, antibodies to Vpx (Figure 7C) and to Vpr (Figure 7D) also immunoprecipitated viral reverse transcription complexes from

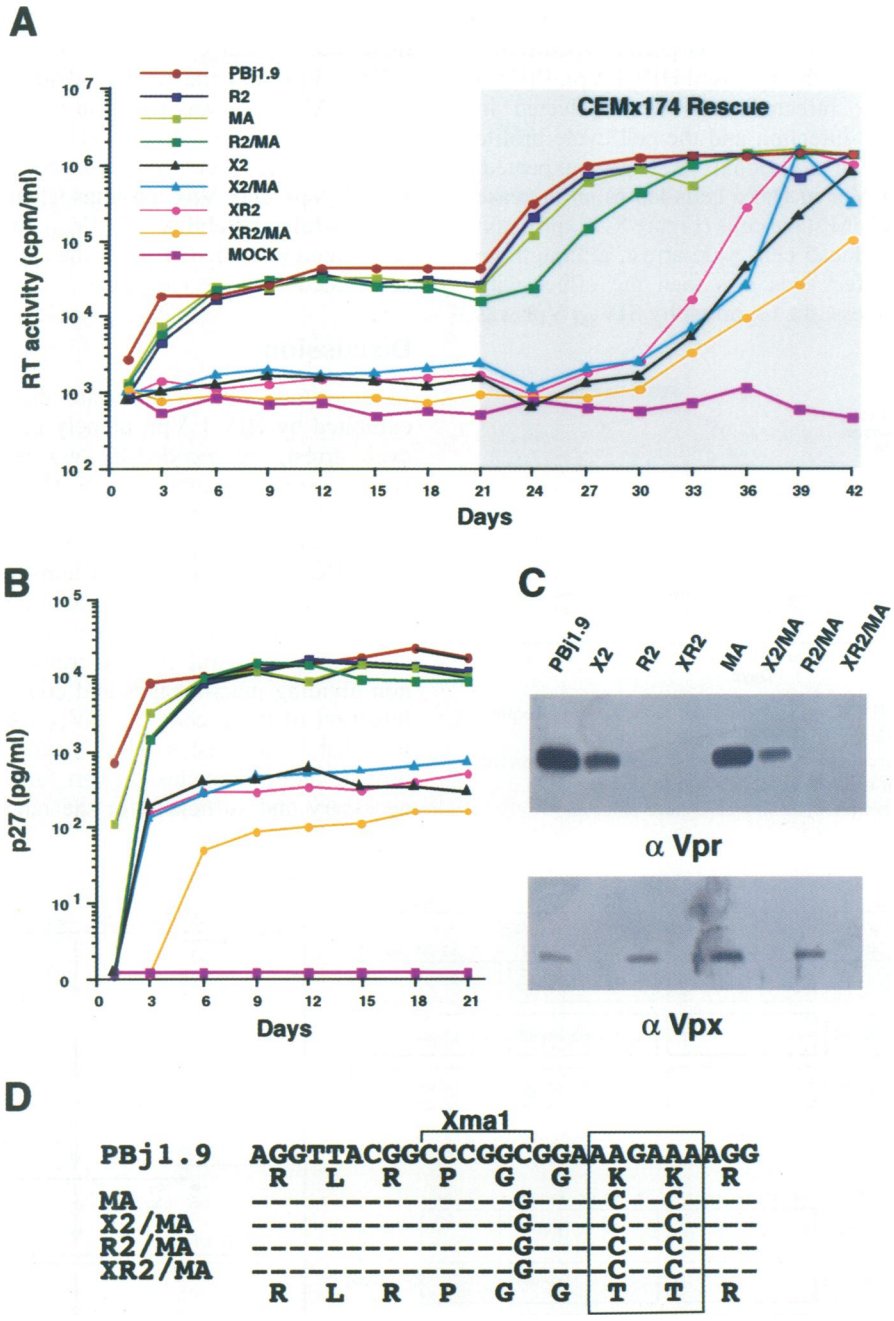


Fig. 4. Vpx is required for PBj1.9 replication in primary non-dividing macaque macrophage cultures. Macaque macrophages were derived from peripheral blood monocytes as detailed in Materials and methods. (**A**) Virus replication was assessed by measuring RT activity and (**B**) gag p27 antigen in culture supernatants at 3 day intervals post-infection. At 21 days post-infection (A), adherent macrophages were co-cultured for 24 h with 1×10^6 CEMx174 cells. Non-adherent cells were then cultured separately and analyzed at 3 day intervals for supernatant RT activity. Western blot analysis (**C**) of viruses rescued by CEMx174 co-culture (day 42) using αVpr and αVpx antibodies shows lack of reversion with respect to Vpx and Vpr proteins, while sequence analysis (**D**) shows lack of reversion in gag MA mutations.

those same gradient fractions of wild-type PBj1.9 (density ~1.20–1.24 g/ml; Figure 7C and D). Neither Vpx nor Vpr antibodies immunoprecipitated viral cDNA from gradient fractionated extracts of cells infected with the *vpx/vpr* double mutant (Figure 7C and D). Use of pre-immune rabbit or mouse antisera led to immunoprecipitation of some small amount of viral cDNA, but this did not correlate with the pattern observed using gag MA antibodies (Figure 7D) and thus verified the specificity of the immunoprecipitation/PCR approach. Taken together, these studies indicate that that SIV$_{SM}$ virion proteins gag MA, Vpr and Vpx associate with *de novo* synthesized viral nucleic acids in the context of a high-molecular-weight nucleoprotein reverse transcription complex.

SIV$_{SM}$ Vpr but not Vpx induces cell cycle arrest

In addition to its role in facilitating infection of non-dividing cells (Heinzinger *et al.*, 1994), HIV-1 Vpr restricts the progression of the host cell from the G_2 to M phase of the cell cycle (He *et al.*, 1995; Jowett *et al.*, 1995; Re *et al.*, 1995; Rogel *et al.*, 1995). We were thus interested in determining whether the cell cycle arrest property of

HIV-1 Vpr was shared by SIV_{SM} PBj1.9 Vpr and Vpx. HeLa cells were infected with VSV-G-pseudotyped retroviral vectors (LXSH) which expressed HIV-1 Vpr, PBJ1.9 Vpr or PBJ1.9 Vpx. Infected cells were selected in hygromycin 24 h post-infection and the cell cycle profile was examined after an additional 72 h. As expected, expression of HIV-1 Vpr in HeLa cells led to an increase in the percentage of G_2/M stage cells (Figure 8). Expression of SIV_{SM} Vpr also induced cell cycle arrest, although the magnitude of this effect was less than the effect with HIV-1 Vpr. Nevertheless, the G_2 arrest by SIV_{SM} Vpr was reproducible in four independent experiments. In contrast, there was no cell cycle arrest in cells expressing SIV_{SM} PBj1.9 Vpx and the relative distribution of cells in G_1 and G_2/M stages was identical to that observed in HeLa cells infected with the LXSH vector only (Figure 8). Similar results were reported recently for SIV_{MAC} and HIV-2 Vpr and Vpx proteins (Planelles *et al.*, 1996). Thus, while the ability of SIV_{SM} to infect macrophages segregated with the *vpx* gene, the cell cycle arrest function segregated with the *vpr* gene.

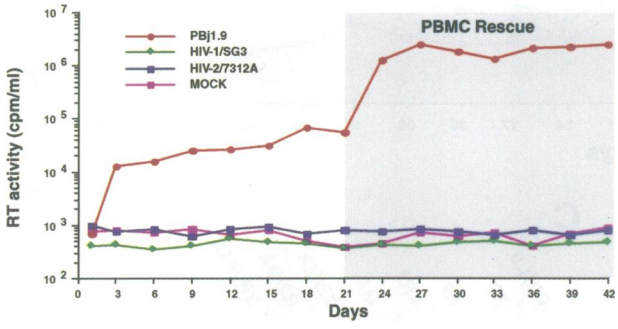


Fig. 5. T-lymphotropic HIV-1 and HIV-2 do not replicate in macaque macrophages. HIV-1/SG3 and HIV-2/7312A were used to infect identically prepared macrophage cultures. No RT activity was detected, indicating the absence of residual virus from the inoculum and of contaminating $CD4^+$ T lymphocytes in macrophage cultures.

Discussion

In this study, we demonstrate that the two properties exhibited by HIV-1 Vpr, namely nuclear import and cell cycle arrest, are encoded by two independent genes, i.e. *vpr* and *vpx* in members of the HIV-2/SIV_{SM} group. Our results show that the PBj1.9 Vpr protein caused cell cycle arrest, yet was dispensable for nuclear localization of viral DNA during infection of non-dividing macrophages. Conversely, PBj1.9 Vpx protein did not influence the host cell cycle, but was necessary for efficient nuclear localization of viral reverse transcription complexes in non-dividing macrophages and consequently for efficient infection of these cells by SIV_{SM}. An additional finding was that in contrast to HIV-1, where gag MA and Vpr provide additive nuclear import functions, Vpx was both necessary and sufficient for the nuclear transport of the

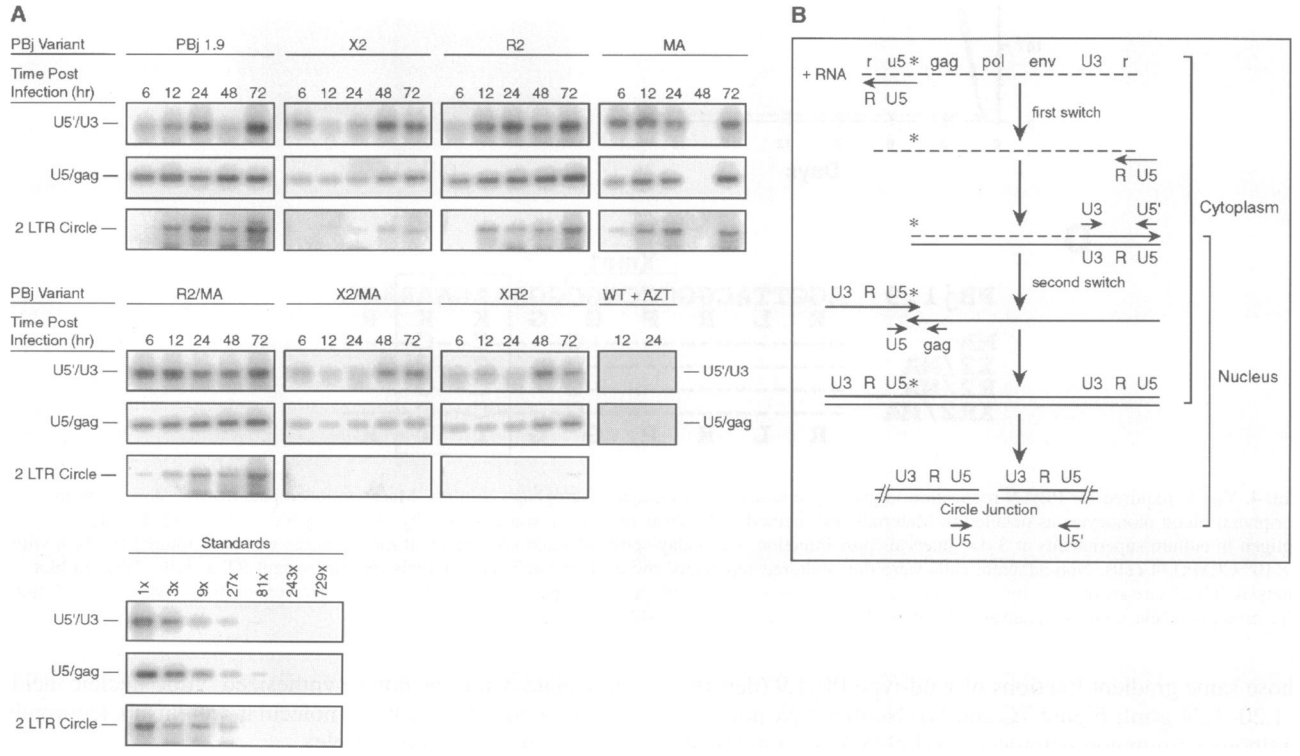
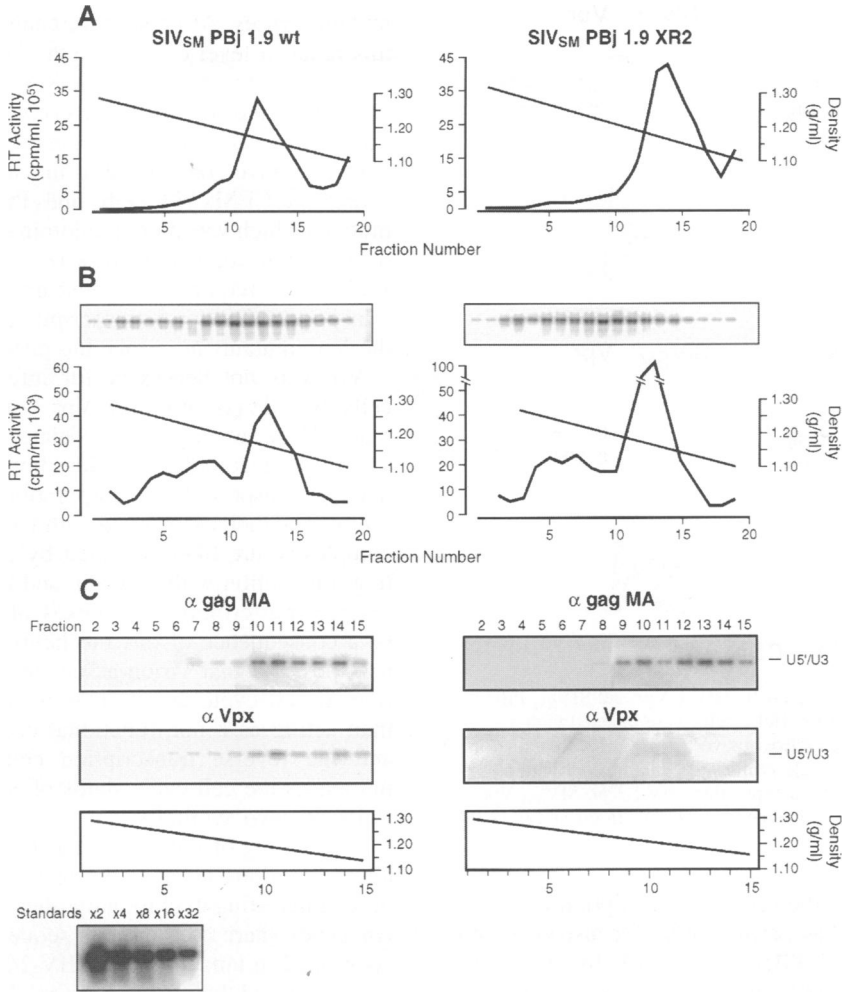


Fig. 6. Vpx is required for efficient nuclear localization of viral reverse transcription complexes. (**A**) Synthesis and nuclear localization of viral DNA by gag MA, *vpr* and *vpx* mutants of PBj1.9 in acutely infected macrophages. At the indicated times post-infection, total cellular DNA was harvested and analyzed by PCR by using SIV_{SM} specific primers. U5/U3 and U5/gag primers amplify predominantly early and late products of reverse transcription, respectively. 2LTR circle forms of viral DNA (amplified using U5′/U5 primers) are formed only after completion of viral DNA synthesis and localization to the nucleus. Prior to infection, pre-existing proviral DNA in the inoculum was removed by treatment of viral stocks with DNase. (**B**) Major products of reverse transcription and location of PCR primers. Hatched and solid lines denote viral RNA and cDNA, respectively, while the primer binding site is indicated by an asterisk. Subcellular location of major steps in reverse transcription denotes that completion of reverse transcription may occur predominantly in the nucleus (Bukrinsky *et al.*, 1993b), although the exact boundaries are not yet defined.



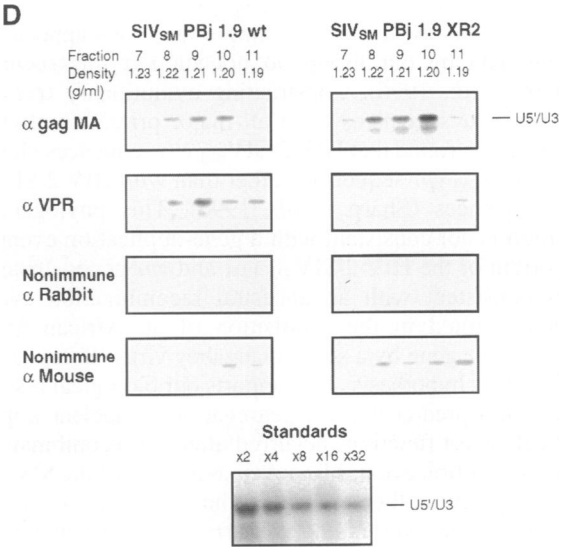


Fig. 7. Association of gag MA, Vpr and Vpx proteins with nucleoprotein reverse transcription complexes of SIV$_{SM}$ PBj1.9. (**A**) Absence of Vpr and Vpx proteins does not influence virion density. Wild-type PBj1.9 virions or XR2 double-mutant virions were pelleted through glycerol (25%) and resolved on a linear 15–60% sucrose density gradient. Both wild-type and the XR2 mutant-derived particles had a density of 1.16 g/ml. (**B–D**) Co-immunoprecipitation of PBj1.9 gag MA, Vpr and Vpx proteins with viral cDNA in high-molecular-weight SIV$_{SM}$ reverse transcription complexes. Hypotonic cell lysates obtained at 4 h post-infection were resolved on a 15–60% sucrose gradient. Aliquots (0.5–1 ml) of each gradient fraction were analyzed for RT activity (B). Gradient fractions were dialyzed and analyzed by PCR (U5′/U3 primers) before (B, inset) and after immunoprecipitation with antibodies to SIV$_{SM}$ gag MA and Vpx (C), to gag MA and Vpr (D) or with non-immune rabbit and mouse antisera (D). (C) and (D) are derived from independent experiments. In these gradients, reverse transcription complexes have a density of ~1.20–1.23 g/ml.

SIV$_{SM}$ reverse transcription complex during infection of non-dividing macrophages. Figure 4 shows that PBj variants lacking functional Vpr protein or containing gag MA mutations shown in the context of HIV-1 gag MA to disrupt NLS function (Bukrinsky *et al.*, 1993a) are still able to infect non-dividing macrophages efficiently. While SIV PBj1.9 gag MA has not been shown biochemically to contain a functional NLS, it nevertheless contains sequences (K^{26}KRYK) which satisfy the minimal requirement for peptide-directed nuclear import (Chelsky *et al.*, 1989). The phosphorylation of HIV-1 gag MA is required for its association with the viral reverse transcription complex (Gallay *et al.*, 1995) and additionally allows its membrane dissociation in the target cell, a requisite for

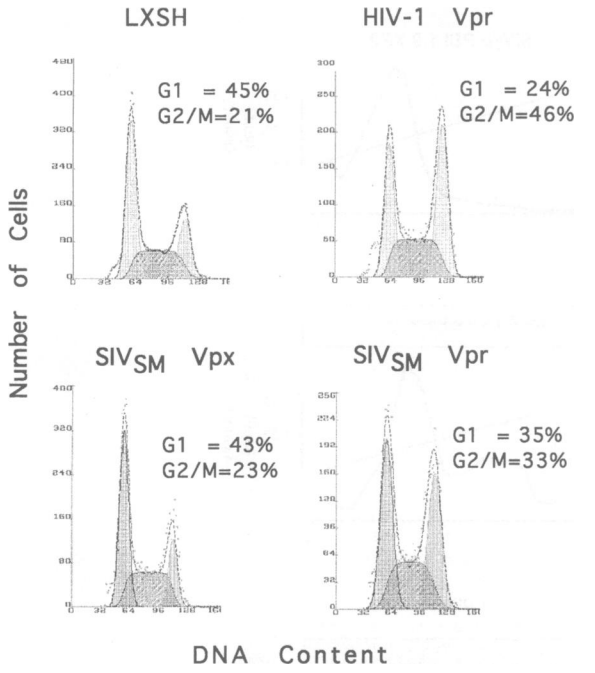


Fig. 8. Induction of cell cycle arrest by HIV-1 Vpr and SIV_{SM} PBj1.9 Vpr, but not SIV_{SM} PBj1.9 Vpx. HeLa cells were infected with a VSV-G envelope pseudotyped retrovirus vector (LXSH) as detailed in Materials and methods. Cell cycle profiles of cells infected with a control vector (LXSH) or vectors expressing HIV-1 Vpr, SIV_{SM} Vpr or SIV_{SM} Vpx together with the percentage of cells in G_1 and in G_2/M are depicted.

subsequent transport of the reverse transcription complex to the nucleus (Bukrinskaya *et al.*, 1996). We also observed phosphorylation of the PBj1.9 gag MA by a virion-associated kinase in highly purified virion preparations (not shown). Although the PBj1.9 MA NLS signal appears not to be involved in SIV_{SM} nuclear transport functions, phosphorylation of PBj1.9 gag MA is not unexpected, since we have demonstrated that phosphorylation of this protein is required for dissociation of the viral reverse transcription complex from the host cell membrane at the point of virus entry (Bukrinskaya *et al.*, 1996) and not for nuclear transport of the complex *per se*. It is possible that $K^{26, 27}$-T mutations were not sufficient in the context of the PBj1.9 gag MA to disrupt the NLS (more extensive mutagenesis of this region will be difficult because of possible effects on virion maturation and release). However, since Vpx was necessary and sufficient for nuclear transport of viral DNA, the issues of gag MA NLS functionality are not important for the outcome of SIV_{SM} infection of macrophages. It is more likely that Vpx exerts a dominant role in nuclear transport of SIV_{SM} reverse transcription complexes and any overlapping effect exerted by gag MA (or Vpr) is minimal in the presence of a functional *vpx* gene. Moreover, since there were no compensatory effects by gag MA or Vpr in the absence of Vpx, any transport properties exhibited by gag MA or Vpr would also be expected to require the presence of Vpx. For example, removal of Vpx could have a global effect on the integrity or stability of the reverse transcription complex, and could thus influence the valency of NLS motifs associated with the complex.

We observed a slight but reproducible reduction in full-length cDNA products in macrophages infected with *vpx* mutant viruses. At present we cannot distinguish whether this reduced level of full-length cDNA products was due to an inherent instability of the SIV_{SM} reverse transcription complex in the absence of Vpx or whether efficient reverse transcription in macrophages requires Vpx. Both effects may also have contributed to the slight reduction in viral spread in CEMx174 cells and PBMCs by SIV_{SM} *vpx* mutants which was more predominant at low multiplicities of infection (data not shown). Additional mutagenesis studies are required to determine whether the nuclear transport and reverse transcription defects exhibited by the Vpx mutants are separable phenotypes.

Vpr was not necessary for infection of non-dividing cells by SIV_{SM}, nor did Vpr compensate for lack of Vpx. Thus, while Vpr associates with SIV_{SM} reverse transcription complexes, it does not directly facilitate their nuclear transport. The incorporation of Vpr and Vpx into virions and their association with viral reverse transcription complexes, are likely dictated by a common mechanism (e.g. interaction with gag p6), and the presence of Vpr in reverse transcription complexes of SIV_{SM} could merely be a consequence of this mechanism. On the other hand, it is possible that virion-associated Vpr has a functional role in cell cycle arrest. This would suggest that rather than acting as a non-functional component of the virion and the reverse transcription complex, incoming Vpr influences the cell cycle status of SIV-infected cells prior to its *de novo* synthesis.

The finding of segregated nuclear import and cell cycle arrest functions in members of the HIV-2/SIV_{SM} lineage poses interesting evolutionary questions. Because *vpx* and *vpr* genes share considerable sequence similarity and are positioned in tandem in the HIV-2/SIV_{SM} provirus, it has been proposed that they are the result of a gene-duplication event subsequent to the divergence of these viruses from the other primate lentiviral lineages (Tristem *et al.*, 1990). Although this explanation is simple (and thus appealing), recent data by our group do not support this scenario (Sharp *et al.*, 1996). Constructing evolutionary trees of *vpr* and *vpx* sequences from all major primate lentivirus lineages, we found that HIV-2/SIV_{SM} *vpx* sequences cluster with SIV_{AGM} *vpr* sequences rather than with HIV-2/SIV_{SM} *vpr* sequences (Sharp *et al.*, 1996). This phylogenetic position is not consistent with a gene-duplication event as the origin of the HIV-2/SIV_{SM} *vpx* and *vpr* genes. Instead, it is consistent with an ancestral recombination event, which resulted in the acquisition of an African green monkey *vpr* gene by a sooty mangabey virus (Sharp *et al.*, 1996). This hypothesis also supports our biological results, and would predict that the segregation of nuclear import and cell arrest functions occurred after the recombination/gene acquisition event. Moreover, as a result of the SIV_{AGM} gene acquisition, there appears to have been no advantage to maintaining overlapping cell arrest and nuclear import functions. Thus, there was a loss of cell arrest function by the acquired *vpx* gene and loss of nuclear transport function by the existing *vpr* gene, and perhaps an additional loss of gag MA function in the ancestral HIV-2/SIV_{SM} virus. We observed far greater defects in nuclear transport by the SIV_{SM} PBj1.9 *vpx* mutant (Figure 6) than by HIV-1 *vpr* mutants (Heinzinger *et al.*, 1994), suggesting that HIV-1 *vpr* is less efficient in facilitating nuclear transport than HIV-2 *vpx*. Thus, in the presence of a strong Vpx

function, the additive gag MA nuclear transport function may not be required.

Finally, it should be noted that the current nomenclature of *vpx* and *vpr* genes is inconsistent. The name *vpr* was originally given to the HIV-1 gene whose product has both nuclear import and cell arrest functions. It has already been suggested that the SIV_{AGM} *vpx* gene should be termed 'vpr' (Tristem *et al.*, 1992), and this can be accepted both on functional grounds (since the gene appears to be similarly bifunctional) and from an evolutionary perspective (since the gene is the ortholog of HIV-1 *vpr*). It is also quite likely that both SIV_{MND} and SIV_{SYK} *vpr* genes are bifunctional and so, until shown otherwise, their naming is appropriate. The HIV2/SIV_{SM} *vpx* gene, having been acquired by recombination and having only one of the two functions, thus merits its distinct name. This leaves only the HIV-2/SIV_{SM} *vpr* gene with an inappropriate nomenclature. Although one could argue to retain its name on evolutionary grounds, it may be better renamed (e.g. 'vpy') to reflect its distinct functional properties. Alternatively, all single genes could be called '*vrx*' to denote the combination of distinct functions, with the *vpr* and *vpx* genes in the HIV-2/SIV_{SM} lineage retaining their current name.

Materials and methods

Construction of PBj1.9 mutant proviruses

All mutational analyses were performed using the infectious molecular clone SIV_{SM} PBj1.9 (Dewhurst *et al.*, 1990). Site-directed mutagenesis (Muta-Gene kit, Bio-Rad) was used to introduce mutations into the PBj1.9 *vpx* and *vpr* genes (subcloned as an internal *Bam*HI–*Sac*I DNA fragment). Mutant X2 was generated using two mutagenic primers (A: 5′-TCCCTGGGATCTTACGTGGCTCACT-3′; B: 5′-CTTGCAATGCAATTACATAGCTTTC-3′), which changed the initiating and one internal methionine codon to threonine and leucine codons, respectively, and introduced translational stop codons at positions 5582 and 5815 (Figure 1). Mutant R2 was made using primer C (5′-TTCTGGAGGTCATTATGTAATTTTATGCTA-3′) which changed the initiating methionine codon to isoleucine and introduced two in-frame stop codons at positions 5924 and 5927, respectively. The double mutant XR2 was constructed by combining these mutations and adding a translational frameshift at position 5772. Mutations in the putative NLS of the PBj1.9 MA protein were performed by PCR mutagenesis. The *gag* region was amplified in two fragments using primers that changed the 5′ two arginine codons of the NLS domain to threonines (positions 903 and 906), and introduced an *Xma*I site at position 898 for subcloning purposes (primer D: 5′-CCCCCCCTCGAGGTCGACGGTATCGCGATA-3′; primer E: 5′-TTTTCTTTCCCCCGGGCCGTAACCTAATTT-3′; primer F: 5′-GGTTACGGCCCGGGGGAACGACAAGGTACAAGC-3′; primer G: 5′-GCCTTCTGATAGCGCTTGAAATCCTG-3′). Mutagenized *vpx/vpr* and MA fragments were reinserted into the PBj1.9 provirus using a series of different subcloning steps (*vpx/vpr* and MA NLS mutations were introduced alone and in combination). None of the introduced nucleotide substitutions resulted in amino acid changes in overlapping reading frames (Figure 1). All PCR amplified fragments, as well as all introduced mutations, were confirmed by DNA sequence analysis.

Cell culture

CEMx174 and Sup T1 T cells were maintained in complete T-cell medium containing RPMI-1640 supplemented with 2 mM L-glutamine, 100 μg/ml gentamicin and 15% fetal bovine serum (FBS). 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FBS. Macaque PBMCs were obtained from heparin-treated blood using lymphocyte separation medium (Organon Teknica), stimulated with phytohemagglutinin (PHA) (4 μg/ml) for 3–4 days and maintained in medium supplemented with interleukin 2 (IL-2) (10 U/ml). Macrophages were purified from unstimulated macaque PBMCs by adherence to plastic as previously described (Banapour *et al.*, 1991). Briefly, 1–3 $\times 10^6$ macaque PBMCs were placed in 12-well tissue culture plates in macrophage medium containing 10% autologous macaque serum and 10% GCT conditioned medium to supply growth factors. Non-adherent cells were removed after a 30 min incubation period at 37°C, followed by extensive washing with phosphate-buffered saline (PBS; six times). Cells were allowed to differentiate in macrophage medium for 10–14 days prior to virus infection.

Western blot analysis

Supernatants from infected PBMC or CEMx174 cultures were clarified of cellular debris by low-speed centrifugation followed by filtration through a 0.45 μm pore filter. Virions were pelleted through a 20% sucrose cushion (125 000 *g*, 2 h, 4°C) and viral pellets were solubilized in sample buffer [62.5 mM Tris–HCl (pH 6.8), 0.2% SDS, 5% 2-mercaptoethanol, 10% glycerol]. Viral samples were denatured by boiling and separated on 12% SDS–polyacrylamide gels. Following electrophoresis, proteins were transferred to nitrocellulose (0.2 μm; Schleicher and Schuell), and incubated with anti-SIV_{SM} Vpr (Newman *et al.*, 1995), anti-HIV-2 Vpx (Kappes *et al.*, 1993), anti SIV_{MAC} MA (Kent *et al.*, 1991) and pan anti-SIV antibodies from experimentally infected macaques. Bound antibodies were visualized with peroxidase anti-Ig followed by ECL.

Transfection and generation of viral stocks

For the generation of viral stocks, 293T cells were transfected with wild-type and mutant SIV PBj1.9 proviral DNAs (25 mg) using a modified calcium phosphate/DNA precipitation method (Stratagene) and incubated at 37°C for 16–18 h. Transfected 293T cells were co-cultured for 24–48 h with 1×10^7 PHA-stimulated human PBMCs in T-cell medium supplemented with IL-2 and PBMCs were then transferred to 75 cm^2 tissue culture flasks. Infected cells were expanded by the addition of 1×10^7 PHA-stimulated human PBMCs and T-cell medium every 5–7 days for 2–3 weeks (human rather than macaque PBMCs were used for expansion because of the large quantities of cells needed for virus amplification). Culture supernatants were analyzed for SIV core antigen (p27) content (Coulter) and by RT activity assay as previously described (Banapour *et al.*, 1991; Li *et al.*, 1992). For acute infection prior to isolation of viral nucleoprotein complexes, viral stocks amplified in Sup T1 or CEMx174 cells were harvested at the peak of virus production (based on RT activity). Culture supernatants were clarified by low-speed centrifugation (2 min, 5000 *g*). Virions were subsequently pelleted (90 min, 35 000 *g*, Beckman Type 19 rotor) and resuspended in 0.01× volumes of culture medium. Concentrated viral stocks were treated with DNase (5 μg/ml) in 10 mM $MgCl_2$ for 30 min (37°C) immediately prior to infection.

Infections

For the analysis of spreading infections, CEMx174 or human PBMCs were infected with supernatants containing 10 ng of gag p27 and incubated for 2–4 h at 37°C in 5% CO_2 and then washed extensively to remove residual virus. Infected cells were resuspended in 10 ml of complete T-cell medium (supplemented with 30 U/ml IL-2 for PBMCs). Culture supernatants were split 1:2 every 3 days with fresh medium and aliquots of culture supernatants were frozen at –70°C for RT determination at the conclusion of the experiment. Terminally differentiated macaque macrophages were infected (10 ng p27; 12-well plate) for 2 h at 37°C in 5% CO_2 and then washed extensively to remove residual virus. Infected cells were adjusted to 2 ml with macrophage medium and incubated at 37°C. The entire 2 ml of medium was collected every 3 days and frozen at –70°C for subsequent RT and p27 determinations. For virus rescue experiments, medium was removed at day 21 post-infection and macrophages were co-cultivated with 1×10^6 CEMx174 (or human PBMC) cells in the appropriate medium for 24 h. Non-adherent cells were transferred to 25 cm^2 tissue culture flasks and incubated for an additional 21 days. Infection of HeLa cells with retrovirus vectors expressing Vpr or Vpx and examination of cell cycle distribution was performed as described (Bartz *et al.*, 1996). Briefly, 293T cells were co-transfected with LXSH-Vpr (a retrovirus vector that expresses Vpr and hygromycin resistance), a plasmid that expresses the MLV gag/pol gene, and a plasmid that expresses the VSV-G protein. Two days later, virus in the cell-free supernatant was concentrated 50× by ultracentrifugation and used to infect HeLa cells. Hygromycin B was added to a concentration of 0.75 mg/ml 1 day after infection to eliminate uninfected cells and, 3 days post-infection, the HeLa cell nuclei were stained with propidium iodide for analysis of DNA content by flow cytometry.

Preparation of SIV reverse transcription complexes

CEMx174 cells (2–5 $\times 10^8$) at a density of 10^8 cells/ml were infected with 2–5 μg (gag p27) of concentrated SIV_{SM} PBj1.9. At 30 min post-infection, cells were adjusted to a density of 2×10^6 cells/ml and incubated at 37°C. At 5–7 h post-infection, SIV reverse transcription complexes were isolated as outlined previously for HIV-1 (Bukrinsky *et al.*, 1992, 1993b). Briefly, cells were subject to hypotonic lysis and cytoplasmic extracts were fractionated on a linear sucrose gradient (15–60% w/w) in a Type SW41 rotor (200 000 *g*, 16 h). Gradient fractions (0.5 ml) were dialyzed against PBS (pH 6.8) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) in a microdialysis chamber (6000 mol. wt cut-off membrane). Dialyzed gradient fractions were incubated with 4 μl non-immune rabbit and mouse serum (Sigma) and 2.5 mg of protein A–Sepharose CL-4B in PBS containing 1% bovine serum albumin (BSA) and 1 mg/ml salmon sperm DNA (Pharmacia), and incubated for 2–4 h at 4° C. Protein A-bound complexes were pelleted (5000 *g*) and clarified supernatants were reacted with either 4 μl polyclonal rabbit anti-Vpr, anti-Vpx, or mouse monoclonal anti-gag MA antibodies. After an overnight incubation at 4°C, an additional 2.5 mg of protein A–Sepharose were added and incubation continued for an additional 2 h. Protein A-bound immune complexes were harvested and washed (three times) with PBS containing 0.1 mM PMSF and 0.1% Triton X-100, and washed once without Triton. Following addition of glycogen (5 μg), DNA was isolated from protein A-bound immunocomplexes using guanidine isothiocyanate (Iso-Quick nucleic acid extraction kit, Bothell, WA) and analyzed by PCR with SIV_{SM}-specific primers.

PCR analysis of SIV_{SM} nucleoprotein immunoprecipitates

SIV_{SM} reverse transcription complexes were isolated from acutely infected cells and resolved on sucrose gradients. These complexes were examined by PCR for the presence of viral nucleic acids after immunoprecipitation with gag MA, Vpr or Vpx antibodies as detailed above. For analysis of viral cDNA in sucrose gradient-fractionated complexes, 100 μl aliquots of each fraction were adjusted to 300 μl with PBS. SDS was added to a final concentration of 0.1% and samples were incubated at 65°C for 1 h. Viral DNA was purified by sequential phenol, phenol/chloroform and chloroform extractions. Early reverse transcripts comprising predominantly minus-strand strong-stop DNA (prior to first template switch) were amplified by PCR using primers to nucleotides 486–503 of U5 (5′-GGCGGCTAGGAGAGATGG) and nucleotides 93–110 of U3 (5′-GGAAGAGGTGCAGAGAAG). Late products of reverse transcription comprising predominantly full-length plus- and minus-strand viral DNA were amplified using primers to nucleotides 535–553 of U5 (5′-GCCCCTGGTCTGTTAGGAC) and nucleotides 967–984 of SIV_{sm} *gag* (5′-CTCCAACAGGCTTTCTGC). Circular products of viral DNA comprising 2 LTRs (Bukrinsky *et al.*, 1993a) were amplified using LTR U5 and LTR U5′ primers. PCR yielded products of 410, 449 and 806 bp for early, late and circle products of reverse transcription, respectively. Early and late reverse transcription products were amplified in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin. Amplification of early/late reverse transcripts proceeded for 30 cycles of PCR (95°C/30 s; 58°C/30 s; 72°C/30 s) followed by a final 5 min extension. 2LTR circle DNA products were amplified in 60 mM Tris–HCl (pH 9.0), 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$ in 35 cycles (94°C/30 s; 62°C/30 s; 72°C/60 s) followed by a final 5 min extension. Cycling conditions were similar to those used for early/late product amplification. PCR standards containing serial dilutions of viral DNA isolated from productively infected CEMx174 cells were used to control for linear amplification conditions. PCR products were Southern blotted onto nylon membranes. PCR products of early reverse transcripts and 2LTR circles were hybridized to an SIV_{SM} PBj1.9 LTR R probe (5′-G^{416} GCTCCACGCTTGCTTG). Late reverse transcription products were hybridized to a 5′ non-translated region probe (5′-G^{725} TGAGGAGCGGGAGTCGG). Hybridized PCR products were visualized on a phosphorimager (Molecular Dynamics, PhosphorImager SF). Residues are numbered according to the sequence of Dewhurst *et al.* (1990) and only 5′ co-ordinates are given for LTR primers.

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