

# HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection

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**HIV-1 Vpr promotes nuclear entry of viral nucleic acids in nondividing macrophages and also causes a G<sub>2</sub> cell-cycle arrest. Consistent with its role in nuclear transport, we show Vpr localizes to the nuclear envelope in both human and yeast cells. Like the importin- $\beta$  subunit of the nuclear import receptor, Vpr also interacts with the yeast importin- $\alpha$  subunit and nucleoporins. Moreover, overexpression of either Vpr or importin- $\beta$  in yeast blocks nuclear transport of mRNAs. A mutant form of Vpr (Vpr F34I) that does not localize at the nuclear envelope, or bind to importin- $\alpha$  and nucleoporins, renders HIV-1 incapable of infecting macrophages efficiently. Vpr F34I, however, still causes a G<sub>2</sub> arrest, demonstrating that the dual functions of Vpr are genetically separable. Our data suggest Vpr functionally resembles importin- $\beta$  in nuclear import of the HIV-1 pre-integration complex and this function is essential for the role of Vpr in macrophage infection, but not G<sub>2</sub> arrest.**

[Key Words: Importin- $\beta$ ; lentivirus; nuclear envelope; HIV; Vpr]

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There is increasing evidence that viruses exploit cellular nuclear import and export pathways to enhance viral replication. Vesicular stomatitis virus inhibits the nuclear export of host cell proteins and RNA (Her et al. 1997); nuclear import is regulated differentially during influenza virus entry and release (Whittaker et al. 1996); and nuclear export of viral mRNA is regulated by retroviruses and pararetroviruses (Goldfarb 1995). The human immunodeficiency virus (HIV) encodes at least three gene products, matrix (MA), integrase (IN), and Vpr, that help target the pre-integration complex to the nucleus, thereby enabling infection of nondividing cells, such as terminally differentiated macrophages (Heinzinger et al. 1994; Gallay et al. 1995, 1997). This characteristic distinguishes lentiviruses from other genera of retroviruses that require mitosis to gain entry to the nucleus and integrate into the host cell genome (Roe et al. 1993; Lewis and Emerman 1994).

The nuclear import of proteins bearing a nuclear localization sequence (NLS) requires two proteins, importin- $\alpha$  and importin- $\beta$ , that comprise a cytosolic NLS receptor (Görlich and Mattaj 1996). The NLS is recognized by importin- $\alpha$  (also called karyopherin- $\alpha$ ), which carries a NLS-binding site. Importin- $\beta$  (also called karyopherin-

$\beta$ ) appears to be responsible for targeting of the complex to the nuclear pore by attachment to nuclear pore proteins called nucleoporins. Transport of the complex through the pore and into the nucleoplasm is not completely understood, but it requires the GTPase Ran and a small protein, p10 (also known as Ntf2) (Melchior et al. 1993; Moore and Blobel 1993, 1994; Paschal and Gerace 1995).

HIV-1 MA and IN carry conventional NLS and probably use the importin- $\alpha/\beta$  pathway for nuclear import (Gallay et al. 1996, 1997). Previous investigators demonstrated the nuclear localization of HIV-1 Vpr (Lu et al. 1993; Di Marzio et al. 1995; Mahalingam et al. 1995), which is a small (14-kD) virion-associated protein common to all primate lentiviruses (Sharp et al. 1994), and it is this karyophilic property that is presumed to be responsible for infection of nondividing cells. In contrast to MA and IN, however, Vpr does not contain a canonical NLS and Vpr-mediated transport is not blocked by NLS peptide (Gallay et al. 1996, 1997), suggesting Vpr karyophilicity is governed by a different determinant of nuclear transport.

In addition to its role in nuclear transport, Vpr also prevents infected cells from entering mitosis by arresting them in G<sub>2</sub> of the cell cycle at a step that is upstream of activation of the mitotic cyclin dependent kinase (He et al. 1995; Jowett et al. 1995; Re et al. 1995; Rogel et al. 1995; Bartz et al. 1996). HIV type 2 (HIV-2) and closely

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related strains of simian immunodeficiency virus (SIV) contain the *vpr* gene and a related gene, *vpx*. In these viruses, the functions of HIV-1 Vpr are split—the G<sub>2</sub>-arrest phenotype segregates with Vpr and the macrophage-infection phenotype segregates with Vpx (Fletcher et al. 1996). This suggests the cell cycle arrest and nuclear targeting functions of HIV-1 Vpr can be separated genetically.

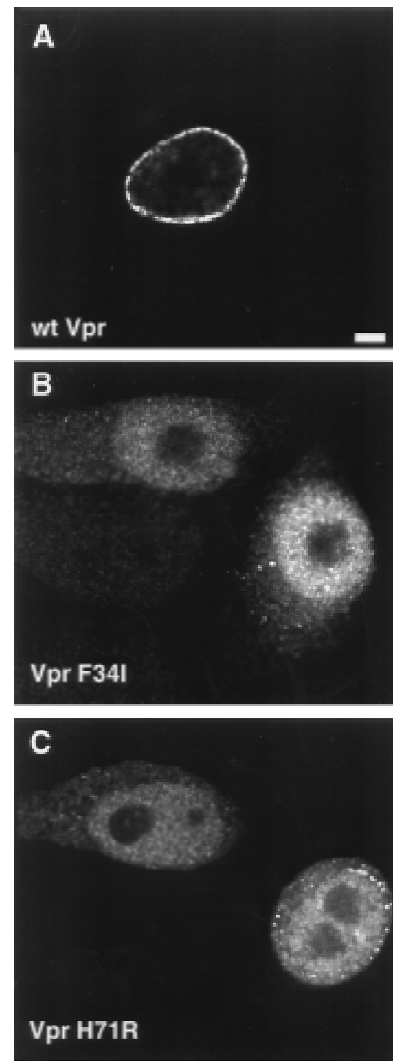
In this report, we show that Vpr present in mammalian cells is associated with the nuclear envelope. Moreover, we describe point mutants of Vpr that segregate the two roles of Vpr and show that nuclear envelope targeting of Vpr is important for the ability of HIV-1 to infect macrophages, but not critical for the ability of Vpr to cause a cell cycle arrest. Expression of Vpr from a galactose-inducible promoter in *Saccharomyces cerevisiae* causes an mRNA export defect indistinguishable from the mRNA export defect resulting from overexpression of yeast importin- $\beta$ . Vpr also binds to transport factors that are known to interact with importin- $\beta$ —importin- $\alpha$  and nucleoporins. These data suggest Vpr promotes nuclear entry of the viral pre-integration complex by tethering it to the nuclear pore in a manner analogous to importin- $\beta$ .

## Results

### *HIV-1 Vpr localizes to the nuclear envelope in human cells*

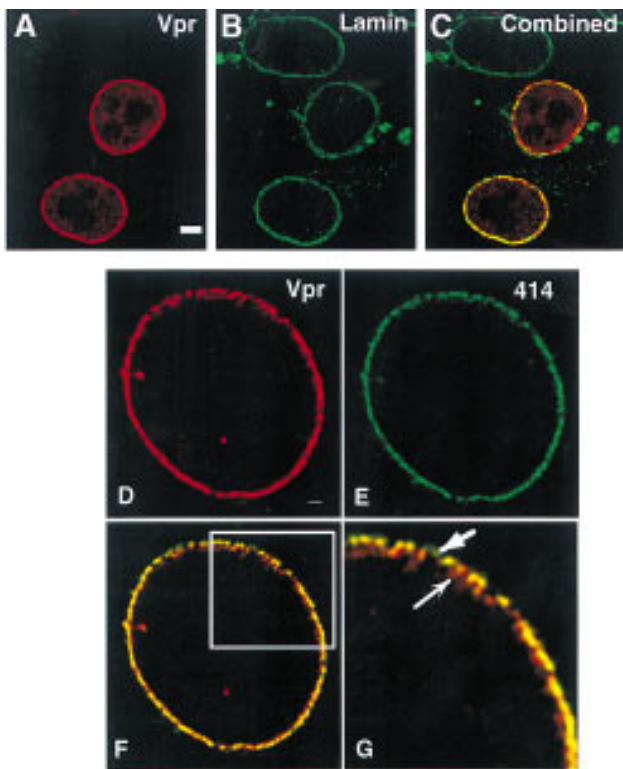
Previous reports using confocal and epifluorescence immunomicroscopy suggested that Vpr localizes to the nucleus (Lu et al. 1993; Di Marzio et al. 1995; Mahalingam et al. 1995). Biochemical fractionation, however, indicated that Vpr associates with membranes (Lu et al. 1993). In view of the somewhat conflicting data, we decided to re-examine Vpr localization in human cells with three-dimensional, deconvoluting microscopy. The resulting images are of higher resolution than is possible with conventional epifluorescence or confocal microscopy (Hiraoka et al. 1987; Agard et al. 1989). Our indirect immunofluorescence staining shows that Vpr forms a ring around the nucleus in greater than 90% of the HeLa cells expressing Vpr after transient transfection with a *vpr* expression vector (Fig. 1A). To examine the specificity of the nuclear rim versus nuclear localization of Vpr, we examined the localization of a subset of Vpr mutants (Selig et al. 1997) and found that two of them, F34I and H71R, have largely lost the perinuclear staining, but are localized more strongly to the nucleus (Fig. 1B,C) than wild-type Vpr (Fig. 1A).

The Vpr staining pattern seen is typical of the localization pattern of proteins found in the nuclear envelope (Gerace et al. 1978; Davis and Blobel 1986). To determine if Vpr colocalizes with nuclear envelope markers, we costained cells with an antibody to detect Vpr and with a polyclonal antibody that reacts with the mammalian nuclear lamins A, B, and C. In optical sections near the middle of the nucleus, antibodies to nuclear lamins form a ring demarcating the perimeter of the nucleus (Fig. 2B),



**Figure 1.** HIV-1 Vpr localizes to the nuclear envelope but point mutants are defective in localization. Indirect immunofluorescence on HeLa cells transfected with HA-Vpr expression constructs using an antibody to the HA-epitope tag on each Vpr expression construct. Staining for wild-type Vpr (A) is brightest in a ring outlining the nucleus; however, the point mutants, F34I (B) and H71R (C), are found in the nucleoplasm but not on the nuclear envelope. Optical sections are near the middle of each nucleus. Scale bar, 4  $\mu$ m.

and Vpr is found in the same ring (Fig. 2A). The colocalization (Fig. 2C) demonstrates that Vpr and the nuclear lamins are in the same plane and localize to the same structure. Note also that some Vpr staining is apparent inside the nucleus (Fig. 2A), but the intensity of this staining is much lower than the nuclear envelope staining. Surface views of these nuclei show mostly nonoverlapping regions of staining between the two antigens (data not shown), suggesting that Vpr does not bind directly to the nuclear lamins. We also performed double staining for Vpr (Fig. 2D) and the monoclonal antibody 414 (mAb 414) (Davis and Blobel 1986), which recognizes a subset of nucleoporins (Fig. 2E). The overlap between



**Figure 2.** HIV-1 Vpr colocalizes with nuclear pores in human cells. (A–C) Optical sections through the nuclei of HeLa cells transfected to express HA-Vpr, stained using indirect immunofluorescence for the HA epitope tag on Vpr (A, red) and antibodies to nuclear lamins (B, green). In the superimposition of staining patterns (C), overlap between the two antigens is visible in yellow. (D–G) Optical section of the nucleus of a HeLa cell expressing Vpr. (E) Staining with mAb 414, which recognizes nuclear pores, outlining the nuclear envelope. (D) Staining for HA epitope tagged Vpr using an antibody to HA, which also outlines the nuclear envelope. (F) The superimposition of nuclear pore staining (green) and Vpr staining (red), visible in yellow, demonstrates the colocalization of the nuclear pore antigens and Vpr. D is a portion of C enlarged; arrows indicate Vpr staining (red) is mostly on the interior, whereas nuclear pore staining (green) is mostly on the exterior faces of the nuclear envelope. Scale bar, 2.5  $\mu\text{m}$  in A–C; 1  $\mu\text{m}$  in D–G.

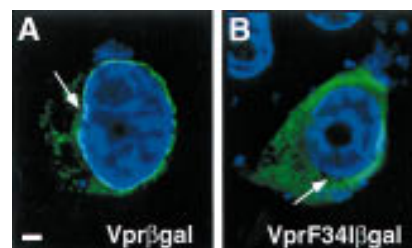
Vpr staining and staining with mAb 414 demonstrates the colocalization of Vpr and the nuclear pore complex (Fig. 2F). A higher magnification view of the overlap (Fig. 2G) shows that Vpr is apparently on the interior of the nuclear envelope compared with the nuclear pore antigen. Higher resolution studies are required, however, to determine precisely Vpr localization vis-a-vis the nuclear pore complex (NPC). In addition, Vpr staining in digitonin-permeabilized cells is indistinguishable from untreated cells (data not shown). Because digitonin treatment of cells liberates cytoplasmic proteins and nuclear envelope proteins not tightly associated with the nuclear envelope (Moroianu et al. 1995b), these results indicate Vpr is integrally associated with the nuclear envelope.

The test of a classical NLS is whether it is sufficient to

confer nuclear localization to a heterologous substrate (Goldfarb et al. 1986; Lanford et al. 1986). The ability of Vpr and Vpr F34I to enter the nucleus on their own (Fig. 1) could be attributable to their small (14 D) size, permitting unrestricted passage through nuclear pores. To test whether Vpr can target a large heterologous protein to the nucleus or whether it targets it to the nuclear envelope, we examined the localization of a Vpr- $\beta$ -galactosidase fusion protein. We found that Vpr- $\beta$ -galactosidase fusion protein did not localize to the nucleus, but rather it was directed to the nuclear envelope (Fig. 3A). In contrast, the F34I mutant fused to  $\beta$ -galactosidase remains cytoplasmic and does not localize to the nuclear envelope, nor does it enter the nucleus (Fig. 3B). As a control for detection of nuclear-targeted proteins, we examined the localization of  $\beta$ -galactosidase with the classical NLS from the T-antigen of SV40 at its amino terminus and found it exclusively in the interior of the nucleus (data not shown). Therefore, the nuclear envelope targeting of wild-type Vpr is specific and yet distinct from classical nuclear localization.

#### *HIV-1 Vpr localizes to the nuclear envelope and affects nuclear transport in yeast*

Because Vpr has been implicated in transport of the pre-integration complex in virally infected cells, and because its nuclear envelope staining is reminiscent of the localization of other nuclear import factors, particularly importin- $\alpha$  and importin- $\beta$  (Chi et al. 1995; Görlich et al. 1995b; Moroianu et al. 1995b), we decided to analyze the nuclear transport properties of Vpr. The function of nuclear transport factors is highly conserved between yeast and vertebrates. In many cases, the yeast homologs to vertebrate nuclear transport factors can substitute for the vertebrate protein both in vitro and in vivo (Aebi et al. 1990; Corbett and Silver 1996). Therefore, we took advantage of established in vivo transport assays in *S.*

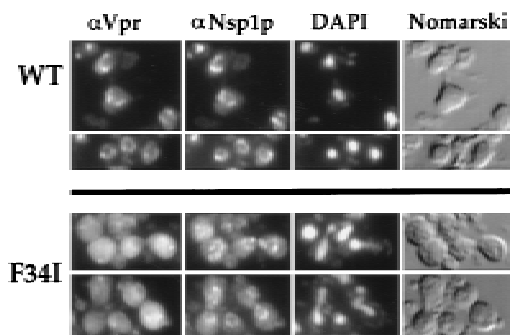


**Figure 3.** Wild-type Vpr but not F34I Vpr targets a fusion protein to the nuclear envelope. HeLa cells transfected to express Vpr- $\beta$ -galactosidase fusion proteins stained with an antibody to  $\beta$ -galactosidase in green, superimposed on fluorescence from DAPI-stained DNA in blue. Arrow in A shows the overlap between  $\beta$ -galactosidase staining in green and DNA staining in blue as aqua, indicating the fusion protein with wild-type Vpr localizes to the nuclear envelope. The lack of aqua overlap in B as indicated by the arrow demonstrates that the F34I mutant fusion protein remains cytoplasmic. Optical sections are near the middle of each nucleus. Scale bar, 2  $\mu\text{m}$ .

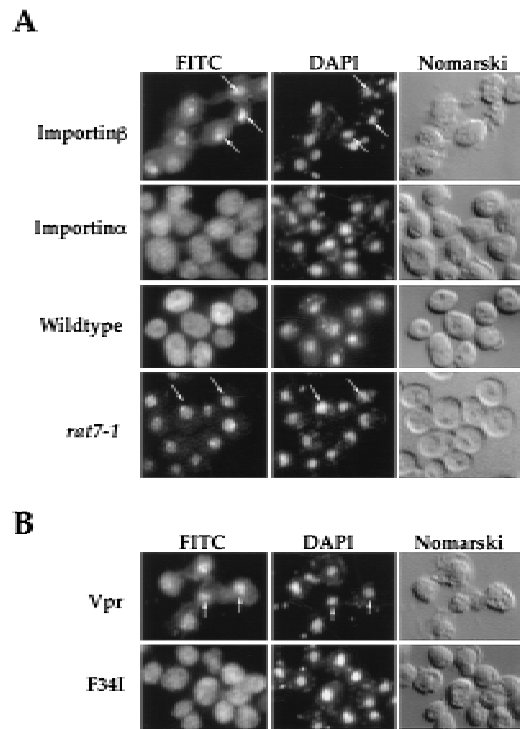
*cerevisiae* and used galactose-inducible constructs to express wild-type Vpr and the F34I mutant (Gu et al. 1997) in *S. cerevisiae*. Yeast cells carrying the Vpr construct were unable to grow on media containing galactose, indicating that expression of Vpr was toxic, as reported previously (Gu et al. 1997). Similar results are obtained if importin- $\beta$  is expressed from a galactose-inducible promoter (data not shown). Expression of the Vpr F34I mutant, however, was not toxic to the yeast cells (Gu et al. 1997).

To determine whether Vpr behaves in yeast as it does in mammalian cells, we examined Vpr localization in yeast by indirect immunofluorescence. Both wild-type Vpr and the F34I mutant were expressed at similar levels after galactose induction (data not shown). As in mammalian cells, the wild-type Vpr localization pattern resembled the localization pattern of nuclear pore proteins and colocalized with the nuclear pore proteins Nsp1p (Fig. 4, top) and Nup159p (data not shown). In contrast, the F34I Vpr mutant protein was observed throughout the cell, with no apparent colocalization with nuclear pore proteins (Fig. 4, bottom). No signal was observed in cells that were not induced to express Vpr (data not shown). Therefore, as in human cells, Vpr localizes to the nuclear envelope in yeast cells.

We have reported previously that members of the importin nuclear import pathway are necessary for mRNA export (Seedorf and Silver 1997). Although loss-of-function mutants in importin- $\beta$  do not exhibit mRNA export defects, suggesting that importin- $\beta$  does not have a direct role in mRNA export (Koepp et al. 1996), we noted that overexpression of importin- $\beta$  from a galactose-driven promoter in wild-type yeast cells causes an accumulation of mRNA in the nucleus (Fig. 5A, top panel) as detected by in situ hybridization with an oligo (dT)-labeled probe (Amberg et al. 1992). Although the importin- $\beta$  export defect is not as complete as that observed in *rat7-1*



**Figure 4.** Vpr is expressed in yeast cells and colocalizes with the nuclear pore protein Nsp1p in yeast. Wild-type and mutant Vpr were expressed in wild-type yeast cells using a galactose-inducible promoter. Wild-type Vpr (top) colocalizes with the Nsp1p staining, whereas the F34I Vpr mutant (bottom) does not. Vpr was visualized using anti-Vpr polyclonal antibodies; Nsp1p was observed using an affinity-purified polyclonal antisera; DNA was visualized by DAPI staining and cells were viewed with Nomarski optics.



**Figure 5.** Expression of Vpr causes an mRNA export defect identical to the defect caused by overexpression of importin- $\beta$ . (A) Importin- $\alpha$  and importin- $\beta$  were expressed in wild-type yeast cells using a galactose-inducible promoter. Cells were then used for the in situ mRNA export assay. Cells expressing importin- $\beta$  (top row) show an accumulation of mRNA in the nucleus, whereas cells expressing importin- $\alpha$  (second row) do not and are indistinguishable from wild-type (third row). *rat7-1* mutant cells at the restrictive temperature (36°C) (bottom row) show the strongest mRNA export defect. (B) Wild-type and mutant Vpr were expressed in wild-type yeast cells and mRNA was visualized using a fluorescence in situ hybridization assay (FISH). Cells expressing wild-type Vpr show a significant nuclear signal, indicating that the mRNA is not being exported properly (top row). Cells expressing the Vpr F34I mutant (bottom row) exhibit staining all over the cell, consistent with the localization of mRNA in wild-type cells. The FITC column shows the mRNA signal; DNA is visualized with DAPI and cells viewed with Nomarski optics. In the FITC column, arrows point to the mRNA signal in the nucleus of cells expressing importin- $\beta$  or wild-type Vpr from the galactose-inducible promoter. In the DAPI column, arrows point to stained nuclei.

mutants (a conditional allele of Nup159 known to block mRNA export; Gorsch et al. 1995; Fig. 5A, bottom panel), there is marked accumulation of mRNA in nuclei compared with wild-type cells (Fig. 5A, third panel). Galactose-driven expression of importin- $\alpha$  in wild-type cells, however, does not cause an accumulation of mRNA in the nucleus (Fig. 5A, second panel), even though its expression is also toxic in yeast cells. This indicates the export defect is specific for the  $\beta$  subunit of the NLS receptor.

If Vpr functions like importin- $\beta$  rather than importin- $\alpha$ , then expression of Vpr from a galactose-inducible pro-

motor should also cause an mRNA export defect. Wild-type yeast cells carrying the Vpr constructs were grown to early log phase under noninducing and nonrepressing conditions and then induced to express the Vpr proteins by the addition of galactose. After a 3-hr induction period, the cells were prepared for the *in situ* mRNA export assay (Amberg et al. 1992). In cells not induced to express Vpr, the mRNA staining was diffuse and found throughout the cell (data not shown), which is identical to the localization of mRNA in wild-type cells. In contrast, cells that expressed wild-type Vpr accumulated signal in the nucleus (Fig. 5B, top panel). In cells that expressed the F34I mutant, however, the mRNA signal was found all over the cell (Fig. 5B, bottom panel), identical to the localization of mRNA in wild-type cells (Fig. 5A, third panel). Nuclear import of an SV40-NLS-invertase protein and the endogenous Npl3 reporter protein were not inhibited by expression of wild-type Vpr in yeast (data not shown), which indicates that not all nuclear transport functions are impaired by Vpr. Therefore, the mRNA export defect of Vpr appears identical to the importin- $\beta$ -induced defect.

#### *Vpr interacts with nuclear transport factors*

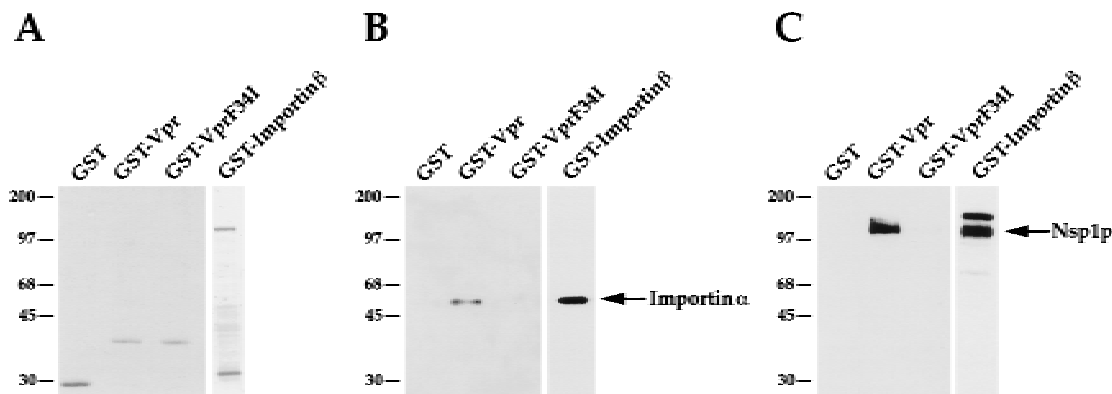
Given the similarity in phenotype between expression of Vpr and importin- $\beta$  in yeast, we next examined the binding of Vpr to nuclear transport factors known to interact with importin- $\beta$ . To purify both wild-type and the F34I mutant Vpr from yeast cells, their respective open reading frames (ORFs) were inserted into a yeast galactose-inducible GST fusion expression vector. The GST fusion proteins were expressed and purified from wild-type yeast cells under nondenaturing conditions (Fig. 6A). Using these samples, Western blots were performed with antibodies to known nuclear transport factors. Interestingly, the wild-type Vpr fusion protein bound both importin- $\alpha$  (Fig. 6B) and the nuclear pore protein Nsp1p (Fig. 6C). On the other hand, the mutant F34I Vpr fusion,

which did not localize to the nuclear envelope (Figs. 1 and 4) bound neither importin- $\alpha$  or Nsp1p (Fig. 6B,C). Using the same approach, we found that importin- $\alpha$  and Nsp1p also copurify with GST-importin- $\beta$  (Fig. 6). We were unable, however, to detect importin- $\beta$  binding to Vpr-GST fusion protein (data not shown). Therefore, the ability of wild-type Vpr to bind nuclear transport factors, whereas the F34I mutant does not, correlates with the induction of an mRNA export defect and proper nuclear envelope localization. Therefore, Vpr mimics both the expression phenotype and binding characteristics of importin- $\beta$ .

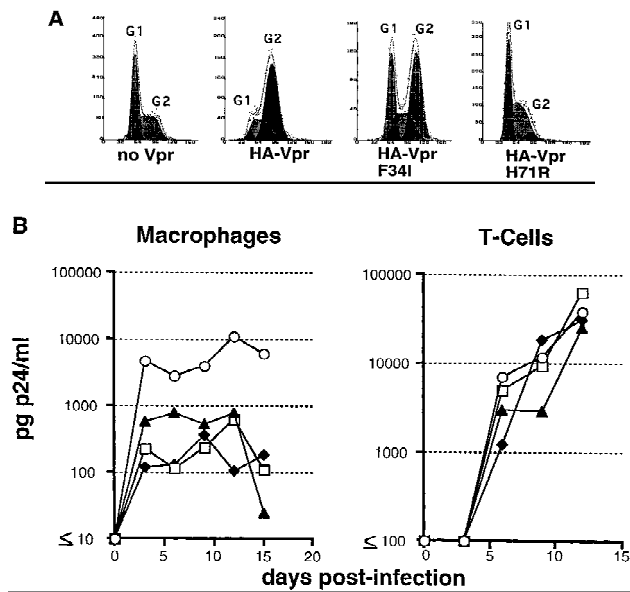
#### *Nuclear envelope localization of Vpr is important for HIV-1 infection of macrophages, but does not correlate with the ability of Vpr to cause G<sub>2</sub> arrest*

We asked whether the involvement of Vpr in the nuclear transport pathway was important for either of the two described functions of Vpr in viral infection—the ability to arrest cells in G<sub>2</sub> of the cell cycle and the ability to infect macrophages efficiently. We assayed Vpr mutants H71R and F34I, which both fail to localize to the nuclear envelope (Fig. 1), for their ability to cause a G<sub>2</sub> arrest when expressed in human cells. Cell cycle profiles of cells that express mutant H71R were not distinguishable from cells without Vpr. The mutant F34I, however, causes cells to accumulate in the G<sub>2</sub> phase of the cell cycle (Fig. 7A), albeit less efficiently than wild-type Vpr. This suggests that nuclear envelope localization is not necessary for Vpr to alter the cell cycle.

To test if nuclear envelope localization of Vpr is important for the function of HIV-1 Vpr in macrophage infection, we cloned the F34I and H71R Vpr mutations into full-length macrophage-tropic HIV-1 proviruses. Because both Vpr and the NLS on MA are implicated independently in targeting the preintegration complex to the nucleus (Heinzinger et al. 1994; Gallay et al. 1995), we constructed Vpr mutant viruses with a defective MA-



**Figure 6.** Vpr interacts with transport factors. Wild-type Vpr and the F34I Vpr mutant were purified from wild-type yeast cells as GST fusions. Samples were resolved by SDS-PAGE and either stained with Coomassie blue dye to demonstrate protein expression (A), and then transferred to nitrocellulose and immunoblotted with anti-importin- $\alpha$  (B) or anti-Nsp1p (C). Importin- $\alpha$  and Nsp1p copurify only with the wild-type Vpr GST fusion and not with the F34I mutant or GST alone. The identical experiment using a GST-importin- $\beta$  fusion is also shown.



**Figure 7.** Effects of wild-type and mutant Vpr on cell cycle progression and macrophage infection. (A) Cell cycle analysis of HeLa cells cotransfected with HA-tagged Vpr constructs and a GFP plasmid. Cells not expressing Vpr  $G_1 = 51\%$ ;  $G_2 = 5\%$ . Cells expressing Vpr,  $G_1 = 5\%$ ;  $G_2 = 69\%$ . Cells expressing Vpr F34I,  $G_1 = 29\%$ ;  $G_2 = 46\%$ . Cells expressing Vpr H71R,  $G_1 = 42\%$ ;  $G_2 = 8\%$ . Analyses are of GFP positive cells. Cell number is on the y-axis and DNA content as measured by fluorescence intensity of propidium iodide staining is on the x-axis. (B) Vpr mutants defective for nuclear envelope localization cannot sustain HIV-1 macrophage infection. (Right) Primary human macrophages were infected with equivalent infectious units of MA-NLS minus HIV-1 containing either wild-type or mutant *vpr*. (Left) Transformed human T cells were infected with the same viruses. Media from each sample were collected every 3 days and replaced completely for macrophage infections, whereas T-cell infections were split 1:2. All samples were assayed for p24<sub>gag</sub> concentration. (x-axis) Days postinfection; (y-axis) p24 pg/ml. (○) HIV-1 MA-NLS<sup>-</sup>, Vpr<sup>+</sup>; (▲) HIV-1 MA-NLS<sup>-</sup>, ΔVpr; (□) HIV-1 MA-NLS<sup>-</sup>, Vpr F34I; (◆) HIV-1 MA-NLS<sup>-</sup>, Vpr H71R.

NLS (Bukrinsky et al. 1993). As a control for viral infectivity, a T-cell line was also infected with the same viral stocks, because unlike in terminally differentiated macrophages, nuclear targeting of the viral preintegration complex should not be a limiting factor in infection of a dividing cell population. As described previously (Bukrinsky et al. 1993), in the absence of MA-NLS, HIV-1 without Vpr infected macrophages less efficiently than virus with Vpr. Compare HIV-1<sub>MA-NLS<sup>-</sup>, ΔVpr<sup>+</sup></sub>, which lacks Vpr, with HIV-1<sub>MA-NLS<sup>-</sup>, Vpr<sup>+</sup></sub>, which contains wild-type Vpr (Fig. 7B). Likewise, virus containing the Vpr F34I and H71R mutants (HIV-1<sub>MA-NLS<sup>-</sup>, VprF34I<sup>+</sup></sub> and HIV-1<sub>MA-NLS<sup>-</sup>, VprH71R<sup>+</sup></sub>, respectively), which are incapable of binding or targeting to the nuclear envelope, displayed markedly decreased infectivity in macrophages compared with the virus that had a wild-type Vpr. The Vpr mutant viruses, however, are capable of promoting a spreading infection in a T-cell line without reduced

infectivity compared with virus with wild-type Vpr (Fig. 7B). Therefore, nuclear envelope localization and targeting of Vpr are important for HIV-1 infection of macrophages but not for Vpr-mediated  $G_2$  arrest.

In SIV from sooty mangabeys (SIV<sub>SM</sub>) and HIV-2, which contain two *vpr*-like genes—*vpr* and *vpx*—the two functions attributed to HIV-1 Vpr are split, with SIV<sub>SM</sub> Vpr retaining the  $G_2$  arrest phenotype and SIV<sub>SM</sub> Vpx facilitating macrophage infection (Fletcher et al. 1996). To see if nuclear targeting was also segregated, we examined the localization of Vpr and Vpx of SIV<sub>SM</sub>. SIV<sub>SM</sub> Vpx was found concentrated on the nuclear envelope and is also apparent in the nucleus in a punctate pattern with discrete spots of staining, whereas SIV<sub>SM</sub> Vpr was found concentrated in the nucleus in a diffuse pattern, and not found on the nuclear envelope (data not shown). Because nuclear envelope localization segregates with SIV<sub>SM</sub> Vpx and infection of macrophages, these data support further the hypothesis that nuclear envelope localization of HIV-1 Vpr is important for the infection of nondividing cells.

## Discussion

We have presented evidence that Vpr interacts with the normal cellular nuclear transport machinery. First, Vpr localizes to the nuclear envelope in both human cells and yeast. Second, Vpr causes an mRNA export defect indistinguishable from the mRNA export defect observed when importin-β is expressed from the same promoter, and third, like importin-β, Vpr interacts with the transport factor importin-α and the nuclear pore protein Nsp1p. Analysis of Vpr mutants shows that interaction with the nuclear transport pathway is important for the role of Vpr in facilitating HIV-1 infection of terminally differentiated macrophages but not for  $G_2$  arrest, indicating that the two roles of Vpr are genetically separable.

A likely explanation for the mRNA export defect observed when Vpr or importin-β is overexpressed in yeast is that both Vpr and importin-β titrate necessary factors required for proper export of mRNA from the nucleus. Because this defect appears to be specific for the β subunit of the NLS receptor, it seems likely that the factor(s) being titrated interacts specifically with importin-β. Obvious candidates for such titrated factors include nuclear pore proteins. Consistent with this hypothesis, the nuclear pore protein Nsp1p copurifies with GST-importin-β fusion protein expressed in yeast cells (Fig. 6). Likewise, Nsp1p and importin-α copurify with GST-Vpr fusion protein from yeast. Because we used cell extracts containing numerous proteins we cannot determine if the binding of Vpr to importin-α and Nsp1p is direct or through unidentified binding partners. Nevertheless, the Vpr mutant F34I, which does not localize to the nuclear envelope or cause an mRNA export defect when expressed in yeast, does not interact with either Nsp1p or importin-α under these conditions. Importin-β has been shown to be required for the import of proteins bearing a canonical NLS in vitro and in vivo (Chi et al. 1995; Enkel et al. 1995; Görlich et al. 1995a; Moroianu et al.

1995a; Koepp et al. 1996). When microinjected into *Xenopus* oocytes, however, dominant-negative mutants of importin- $\beta$  that bind the NPC block the import and export of a number of transport substrates, not simply NLS-bearing proteins (Kutay et al. 1997). These results indicate there are multiple nuclear transport pathways using the same binding sites in the NPC to traverse the pore. One hypothesis to explain our observations is that Vpr binds the docking sites for proteins that carry mRNA/protein complexes out of the nucleus (Seedorf and Silver 1997), therefore preventing the export of mRNA from the nucleus.

The ability of Vpr to cause  $G_2$  arrest in human cells, however, is not attributable to transport or mRNA export defects. Whereas expression of HIV-1 Vpr is toxic in yeast, it does not lead to accumulation in  $G_2$  (Gu et al. 1997), and the mRNA export defect is distinct from cell cycle disruption. Moreover, the mis-localized Vpr F34I mutant produces a significant  $G_2$  arrest in human cells, yet it does not cause an mRNA export defect in yeast (Figs. 6 and 7). This suggests that structures or sequences of HIV-1 Vpr important for its interaction with nuclear transport factors and nuclear pore proteins are not the same as those that lead to the  $G_2$  arrest. On the other hand, nuclear localization (as opposed to nuclear envelope) may be important for the  $G_2$  arrest phenotype because no Vpr mutant has been described that fails to enter the nucleus but retains the  $G_2$  arrest phenotype (Fig. 1; Di Marzio et al. 1995).

Our data suggest that the ability to infect macrophages, which correlates with nuclear envelope localization of Vpr in human cells (Figs. 1 and 7B), is a function of Vpr interactions with transport machinery, specifically importin- $\alpha$  and the nucleoporins. The proteins MA, IN, and Vpr are associated with the HIV-1 preintegration complex and probably work in concert to target the viral complex to the nuclear pore; however, MA is probably less important than IN and Vpr (Fouchier et al. 1997; Gallay et al. 1997). MA and IN bind to importin- $\alpha$  as NLS-bearing substrates (Gallay et al. 1996, 1997). It has been suggested recently that HIV-1 Vpr binds importin- $\alpha$  at a site distinct from the NLS-binding site (Popov et al. 1996), and it was shown that HIV infection of macrophages by Vpr-containing virions is not inhibited by NLS peptide (Gallay et al. 1996, 1997). Therefore, unlike MA and IN, Vpr does not require the importin- $\alpha$  NLS-binding site for its transport function. The nuclear targeting of the pre-integration complex by MA and IN using the classical importin pathway, and by Vpr using a specialized variant of this pathway, act as complementary rather than redundant functions. Notably, a family of importin- $\beta$ -like proteins has been identified recently (Boer et al. 1997; Görlich et al. 1997) that resemble importin- $\beta$  in their ability to bind the NPC but are limited to specific rather than general transport substrates (Aitchison et al. 1996; Pollard et al. 1996; Rout et al. 1997; Seedorf and Silver 1997; Yaseen and Blobel 1997). This suggests that importin- $\beta$ -like proteins function as specific receptors in the nuclear transport of distinct groups of nuclear proteins. We propose that Vpr binds impor-

tin- $\alpha$  as a specialized importin- $\beta$  and docks at specific sites within nuclear pore proteins, tethering its cargo, the HIV-1 preintegration complex, at the nuclear envelope.

## Materials and methods

### *Immunofluorescence and imaging of human cells*

**Single staining** The following plasmids are expression plasmids driven by the HIV-1 LTR. pHAVpr, encodes Vpr from HIV-1<sub>Lai</sub> with the 9-amino-acid epitope tag from influenza hemagglutinin (HA) fused at the amino terminus of Vpr; pHAVprH71R encodes the HA-tagged Vpr H71R mutant; pHAVprF34I encodes the HA-tagged Vpr F34I mutant; pVpr $\beta$ gal encodes the bacterial *lacZ* gene ( $\beta$ -galactosidase) fused to the carboxyl terminus of HIV-1<sub>Lai</sub> Vpr; pF34I $\beta$ gal encodes *lacZ* fused to the Vpr F34I mutant. HeLa cells were plated 18–24 hr before transfection at a density of  $2 \times 10^5$  cells per well in six-well plates. Cells were transfected using LipofectAmine reagent (GIBCO, BRL, Gaithersburg, MD) following the manufacturer's instructions with 0.1  $\mu$ g of pCMV-tat (CMV promoter-driven expression of the HIV-1 *tat* gene) and 0.5–1.0  $\mu$ g of HA-epitope tagged or Vpr- $\beta$ -galactosidase expression plasmids per well.

One day post-transfection HeLa cells were trypsinized and replated onto glass coverslips. The next day the coverslips were washed once in PBS and then fixed in PBS-4% paraformaldehyde for 5 min. Some cells were treated with digitonin (40  $\mu$ g/ml) for 5–15 min before fixation. Fixed cells were permeabilized for 5 min in PBS-0.2% Triton X-100. Blocking was performed in PBS-1% bovine serum albumin (BSA)-20% calf, sheep, or goat serum for 30 min. HA.11 mouse monoclonal antibody (Berkeley Antibody Co., Richmond, CA), which recognizes the HA epitope tag, was diluted 1:100 in PBS and pre-incubated on fixed and permeabilized cells for 30 min to remove any nonspecifically binding antibodies. Preadsorbed HA.11 antibody and anti- $\beta$ -galactosidase (Amersham, Arlington Heights, IL) mouse monoclonal antibody, diluted 1:100 in PBS were incubated on cells for 1 hr at room temperature in a humidified chamber. Coverslips were washed three times, 5–15 min each, in PBS. All secondary antibodies (Jackson ImmunoResearch, Inc., West Grove, PA) were diluted 1:100 in PBS. Fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG was used against the two primary antibodies, HA.11 and anti- $\beta$ -galactosidase, incubated in humidified chambers for 30–60 min. Coverslips were rinsed in PBS twice, stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co., St. Louis, MO) 0.2  $\mu$ g/ml in dH<sub>2</sub>O for 5 min, washed in dH<sub>2</sub>O for 5 min, dehydrated in 100% methanol for 15 min, air-dried, mounted in Vectashield (Vector Laboratories, Burlingame, CA), and sealed with nail polish.

**Double staining** Processing of cells for double staining was similar to that for single staining with the following differences. For Nuclear Lamin staining, the HA.11 was first detected with Texas Red-conjugated goat anti-mouse IgG. A rabbit polyclonal antiserum to nuclear lamins A, B, and C (gift of Larry Gerace, La Jolla, CA) was used at 1:50 dilution and detected with FITC-conjugated goat anti-rabbit IgG. Double staining with HA.11 and mAb 414 (BabCo, Richmond, CA) is problematic because the primary antibodies are from the same host species, mouse, and cross reaction of antibodies may lead to an artifactual apparent overlap of antigens. Several measures were taken to reduce this possibility. HA.11 was first detected with monovalent lissamine rhodamine sulfonyl chloride-conjugated goat anti-mouse IgG Fab. Following the first secondary antibody incuba-



tion, cells were washed three times for 10 min in PBS, refixed, and blocked with unlabeled goat anti-mouse IgG Fab or with serum-containing block to bind to any remaining primary antibody directed toward the first antigen. Then cells were incubated with mAb 414, which was detected with FITC-conjugated sheep anti-mouse IgG. To confirm that this was successful in preventing binding by the second secondary antibody to the first primary antibody, samples were stained for the first antigen and blocked as described, but no second primary antibody was used. Therefore, any fluorescence visible from the second secondary antibody was attributable to binding to the first primary antibody. Control samples processed in this way did not show binding of the second secondary antibody to the first primary (data not shown).

**Imaging** Images of stained cells were observed and collected with the Delta Vision SA3.1 microscope (Applied Precision Inc., Issaquah, WA). DeltaVision uses a mercury lamp and excitation and emissions filters like an ordinary fluorescent microscope, but with computer-controlled filter set changes and stage position (Hiraoka et al. 1991). Using appropriate filters and excitation wavelengths for each fluorophore, a Z-series of 0.2- $\mu$ m sections was collected for each sample. DeltaVision software corrects each image, and then the entire Z-series is deconvoluted using Fourier transforms, removing from any given section, light that is not in focus in that section, so that each section contains the information from all sections (Hiraoka et al. 1990). Volume projections were made for some images, and the reconstructed three-dimensional images used for analysis but attributable to limitations of print media are not reproduced here (data not shown).

#### Cell cycle analysis

HeLa cells were cotransfected with HA-Vpr expression constructs (described above) and pGreenLantern, a modified version of green fluorescent protein (GFP) (Life Technologies, Gaithersburg, MD). Two days post-transfection GFP-positive cells were collected on FACSCAN and analyzed by M-cycle as described previously (Rogel et al. 1995). The  $G_2$  arrest function of the Vpr point mutants F34I and H71R were originally assayed without the HA-epitope tag (Selig et al. 1997).

#### Macrophage-tropic proviruses and HIV-1 infection

All proviruses are composed of the HIV-1<sub>Lai</sub> (Westervelt et al. 1992) backbone with the macrophage-tropic envelope from HIV-1<sub>YU-2</sub> (Li et al. 1991) substituted for the HIV-1<sub>Lai</sub> envelope. pHIV-1<sub>MA-NLS-, Vpr+</sub> contains a wild-type vpr gene with a point mutant in the NLS of MA that abolishes MA nuclear transport ability (Bukrinsky et al. 1993). pHIV-1<sub>MA-NLS-,  $\Delta$ Vpr</sub> contains a frameshift mutation at the vpr Ncol site, creating a null mutant of vpr, and is MA-NLS defective. pHIV-1<sub>MA-NLS-, VprF34I</sub> and pHIV-1<sub>MA-NLS-, VprH71R</sub> are MA-NLS defective and carry the F34I and H71R mutants of Vpr, respectively. Virus was produced by transient transfection of 293T cells with the above plasmids using calcium phosphate precipitation; cell-free media were collected 3 days post-transfection and stored at -80°C as viral stocks.

Monocyte-derived macrophages were obtained by back-washing cells from a leukofilter (obtained from Puget Sound Blood Center, Seattle, WA) with PBS plus 5 mM EDTA. Leukocytes were separated from erythrocytes by ficoll centrifugation. Cells were plated on glass coverslips and nonadherent cells aspirated one day following isolation. Adherent monocytes were cultured in RPMI with 10% human serum for 10–14 days to allow mac-

rophages to differentiate completely before infection. Viral stocks were titered on MAGI cells expressing CCR5 (Vodicka et al. 1997) and equivalent infectious units of each virus were used to infect macrophages for 2 hr, followed by three washes with RPMI. Macrophages were infected at an approximate multiplicity of infection (moi) of 0.1; precise determination of moi is not possible because cell number is estimated as a percentage of cells plated. Media from infected macrophages were collected every 3 days and replaced completely. The data presented (Fig. 7) are typical of results obtained, but variation exists between experiments attributable to donor and cell-plating variation. Although the absolute magnitude of the differences between infectivity varies, the relative infectivities between the viral strains is reproducible.

Jurkat-CCR5 cells, a transformed human T-cell line that was infected with a retroviral vector to express the CCR5 HIV coreceptor (Vodicka et al. 1997), were infected with the same viruses used for macrophage infection at a moi of 0.1, in 300  $\mu$ l of media with a final concentration of 10  $\mu$ g/ml of polybrene. After 2 hr of infection, cells were washed three times. Cells were split 1:2 every 3 days. The amount of gag protein capsid, p24, in each sample was quantified by ELISA (Coulter Immunology, Miami, FL) following the manufacturer's instructions.

#### Indirect immunofluorescence in yeast

Wild-type yeast cells (PSY580) were transformed with plasmids pJ62 and pJ63 (pYES URA3 2 $\mu$ g from Invitrogen Corp, Carlsbad, CA), which contain wild-type Vpr and the F34I Vpr mutant, respectively (Gu et al. 1997). Cells were grown in synthetic media lacking uracil using 2% glucose as a carbon source until saturation and then diluted into synthetic media lacking uracil supplemented with 2% raffinose. When cells reached a density of  $1 \times 10^7$  to  $2 \times 10^7$  cells/ml, galactose was added to 2% to induce the expression of Vpr. After a 3-hr induction at room temperature, cells were fixed with one-tenth volume 37% formaldehyde for 5 min and prepared for immunofluorescence. Briefly, cells were washed once with 1 ml of 0.1 M potassium phosphate at pH 6.5, and once with P solution (1.2 M sorbitol, 0.1 M potassium phosphate at pH 6.5). Yeast cell walls were digested with 15  $\mu$ g/sample of Zymolyase (10  $\mu$ g/ml in P-solution plus 0.5%  $\beta$ -mercaptoethanol (ICN Pharmaceuticals, Costa Mesa, CA) for 30–45 min at 30°C. After digestion, spheroplasts were resuspended in P solution and adhered to a glass slide coated with 0.3% poly-L-lysine. The slide was incubated in ice-cold methanol for 6 min and dried in ice-cold acetone for 30 sec. Cells were washed once in PBS-BSA (PBS, 0.5% BSA) before addition of primary antibody. All primary antibody incubations were carried out overnight. All antibodies were diluted in PBS-BSA. Anti-Vpr was used at 1:250, anti-Nsp1p at 1:250 (gift of M. Stewart, Cambridge, UK), and anti-Nup159p at 1:2000 (Gorsch et al. 1995). After three washes of PBS-BSA, secondary antibody was added for 2 hr. All fluorophore-conjugated secondary antibodies were used at 1:1000 (Jackson ImmunoResearch, Inc., West Grove, PA). DAPI was used at 1  $\mu$ g/ml to visualize DNA. Wells were coated with *p*-phenylenediamine (1 mg/ml in 10% PBS in glycerol, at pH > 8.0) before the addition of a coverslip and sealing with nail polish.

#### Expression of GST-Vpr in yeast

GST-Vpr and GST-VprF34I fusion protein expression constructs were made by inserting the respective vpr sequences into the vector pPS892 (Koepp et al. 1996). The resulting plasmids were transformed into PSY580. Transformants were grown and induced as described for indirect immunofluores-



cence. Induced cells were pelleted and frozen at  $-20^{\circ}\text{C}$  before lysis. Cells were lysed in PBSMT (PBS, 3 mM KCl, 2.5 mM  $\text{MgCl}_2$ , 0.5% Triton X-100) plus protease inhibitors (3  $\mu\text{g}/\text{ml}$  each leupeptin, aprotinin, chymostatin, and pepstatin) by glass bead lysis. Lysates were cleared with a 10-min centrifugation in a microfuge (13,000g) at  $4^{\circ}\text{C}$ . Equal volumes of lysate were mixed with 50  $\mu\text{l}$  (50% slurry) of glutathione-Sepharose (Pharmacia Biotech, Piscataway, NJ) at  $4^{\circ}\text{C}$  for at least 1 hr. Bound samples were washed for two 2-min intervals with 10 volumes of PBSMT at  $4^{\circ}\text{C}$  before the addition of Laemmli buffer.

Immunoblots were probed with anti-importin- $\alpha$  at 1:5000 (gift of D. Görlich, Zentrum für Molekulare Biologie der Universität Heidelberg, Germany), anti-Nsp1p at 1:1000, anti-Rna1p at 1:1000, and anti-Vpr at 1:1000. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Promega, Madison, WI) were used at 1:5000. Bands were visualized using the ECL detection system (Amersham Life Science, Inc., Arlington Heights, IL).

#### mRNA export assay

Wild-type yeast cells (PSY580) were induced to express Vpr as described for indirect immunofluorescence. After induction, cells were fixed in one-tenth volume 37% formaldehyde for 30 min. The assay was performed as described (Amberg et al. 1992). In summary, cells were washed once with 0.1 M potassium phosphate, at pH 6.5, and resuspended in P solution. Cells were spheroplasted with Zymolyase as described for indirect immunofluorescence, adhered to a slide coated with 0.3% poly-L-lysine for 15 min, permeabilized with 0.5% Triton X-100 for 5 min, and then washed in P solution. Standard in situ hybridization procedures were followed with prehybridization for 1 hr at  $37^{\circ}\text{C}$ . Digoxigenin-labeled oligo(dT) (50-mer) was generated using the Genius 6 kit (Boehringer Mannheim, Indianapolis, IN) and used as the probe, added at 1:1000 and incubated on the slide overnight at  $37^{\circ}\text{C}$ . After washes to remove unhybridized probe and blocking for antibody, FITC-conjugated anti-digoxigenin, diluted 1:200, was incubated on the slide for 2 hr, followed by washes and the addition of DAPI (1  $\mu\text{g}/\text{ml}$ ) for 1 min. The slide was washed once after DAPI staining. Wells were coated with *p*-phenylenediamine before the addition of a coverslip and sealing with nail polish.

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