

HIV-1 Vpr increases viral expression by manipulation of the cell cycle: A mechanism for selection of Vpr *in vivo*

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The human immunodeficiency virus type 1 (HIV-1) encodes a protein, called Vpr, that prevents proliferation of infected cells by arresting them in G₂ of the cell cycle. This Vpr-mediated cell-cycle arrest is also conserved among highly divergent simian immunodeficiency viruses, suggesting an important role in the virus life cycle. However, it has been unclear how this could be a selective advantage for the virus. Here we provide evidence that expression of the viral genome is optimal in the G₂ phase of the cell cycle, and that Vpr increases virus production by delaying cells at the point of the cell cycle where the long terminal repeat (LTR) is most active. Although Vpr is selected against when virus is adapted to tissue culture, we show that selection for Vpr function *in vivo* occurs in both humans and chimpanzees infected with HIV-1. These results suggest a novel mechanism for maximizing virus production in the face of rapid killing of infected target cells.

Viruses have evolved a number of mechanisms that allow them to interact with the normal host cell cycle. For example, some viruses induce cells to enter the cell cycle from quiescence, while others over-ride checkpoint controls that would otherwise delay or stop cell cycling¹⁻³. Still other viruses prevent cell-cycle progression during the lytic stage of infection^{4,5}. Although the selective advantage for viruses to encourage cell-cycle progression is often linked to their requirement of the host cell's replicative machinery, the selective advantage for viruses to arrest cell-cycle progression is less obvious.

The Vpr protein of HIV-1 is a 14-kDa protein that prevents the proliferation of infected T cells by causing them to accumulate in the G₂ phase of the cell cycle at a step that is upstream of activation of the mitotic cyclin-dependent kinase⁷⁻¹⁰. There is a strong selection against Vpr when HIV is adapted to long-term growth in tissue culture^{11,12}. Nonetheless, conservation of both Vpr sequence¹³ and G₂ arrest function^{14,15} among diverse members of the primate immunodeficiency viruses suggests that it likely confers a selective advantage to this virus family *in vivo* for persistence or transmission.

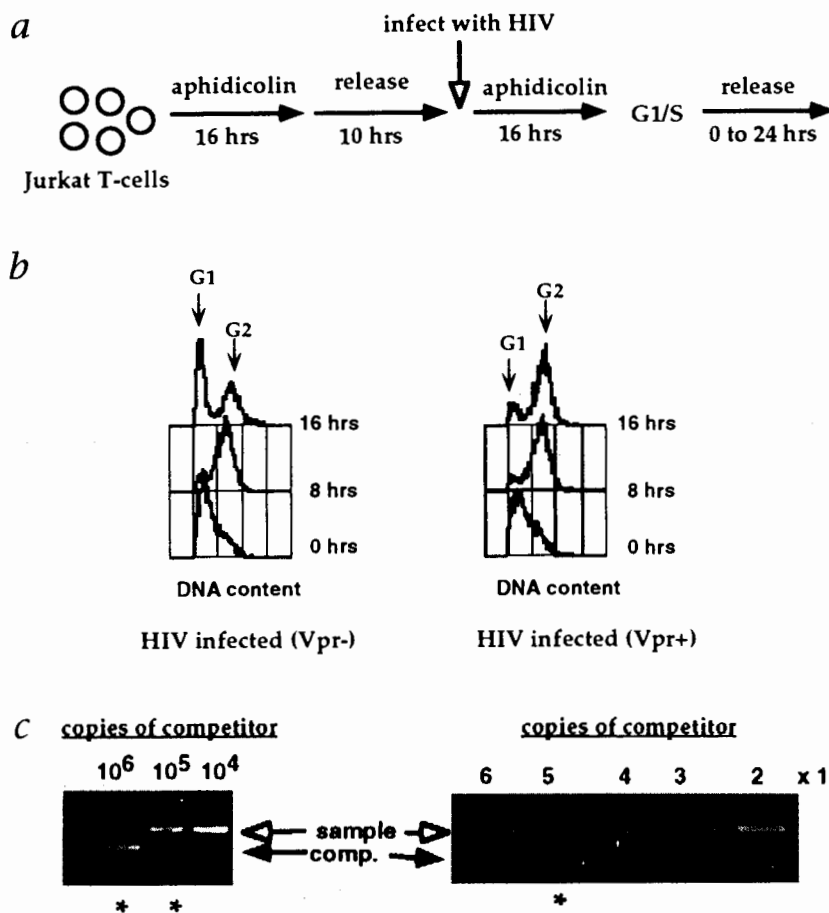
Here, we have attempted to resolve the paradox that Vpr is selected against in tissue culture, yet conserved *in vivo*, by investigating the selective advantage for HIV to encode a protein that causes infected cells to accumulate in the G₂ phase of the cell cycle. We found that expression of HIV RNA is upregulated during G₂ in synchronized populations of infected T cells. Moreover, we show that Vpr is able to increase expression of the HIV-1 long terminal repeat (LTR) indirectly by keeping cells in G₂. Finally, this increased expression due to Vpr results in increased viral production. Because more than 99% of infected cells have a half-life of less than 2 days *in vivo*¹⁶⁻¹⁸, we propose that a function for

Vpr is to maximize viral production *in vivo* by delaying infected cells destined to die in a stage of the cell cycle (G₂) in which transcription of the viral LTR is optimal. Last, we demonstrate that there is selective pressure for Vpr function *in vivo*, because HIV-1 quasispecies that are Vpr-negative revert to wild-type Vpr in both a host in which HIV is pathogenic (humans) and a host where it is relatively nonpathogenic (chimpanzees), and we present a model that can account for the selective pressure for Vpr.

HIV expression in synchronized T cells

We hypothesized that delay or arrest of the cell cycle in G₂ would give a selective advantage to HIV if viral transcription and hence virus production were upregulated in G₂. To examine viral expression in G₁ versus G₂ of the cell cycle, we developed a protocol to infect a synchronized population of Jurkat T cells with HIV-1 that was Vpr-positive or Vpr-negative (Fig. 1a). Infected cells at the G₁/S border were released from an aphidicolin block (Fig. 1a), and allowed to proceed synchronously from G₁/S to G₂ by 8 hours post release (Fig. 1b). By 16 hours after release, most of the cells infected with Vpr⁺ virus remained in G₂, while most of the cells infected with Vpr⁻ virus had passed through mitosis and were in G₁ (Fig. 1b). Later time points showed that the block in G₂ mediated by Vpr was not absolute, but rather Vpr caused cells to delay in G₂ for 12–20 hours (data not shown).

To analyze viral mRNA accumulation in two different phases of the cell cycle, RNA and DNA were then purified from the G₁/S and G₂ time points (Fig. 1b; 0 and 8 hours after release from the second aphidicolin block). The amount of viral RNA and DNA in each population was determined separately by a quantitative-competitive polymerase chain reaction (QC-PCR) protocol, which included an internal standard in each reaction tube (Fig.



1c). Viral expression was then calculated as a ratio of RNA per DNA provirus (Table 1). Although the amount of RNA per DNA provirus varied in each experiment (Table 1), the amount of expression in G₂ was increased relative to the amount of expression in G₁ (the number of copies of RNA/DNA in G₂ divided by the number of copies of RNA/DNA in G₁) in each of four independent experiments. In synchronized cells infected with HIV that was Vpr⁺, there was a 4.0- to 5.0-fold increase (4.6-fold average) in viral RNA per DNA template at the G₂ time point relative to the G₁ time point (Table 1). Moreover, the increased expression in G₂ relative to G₁ was independent of Vpr, because we also observed an increase of expression in G₂ of 2.9- to 4.6-fold (3.5-fold average) in cells infected with virus that was Vpr⁻ (Table 1). We controlled for the fact that the G₂ cells were infected for 8 hours longer than the G₁ cells by measuring viral RNA and DNA in cells that were held at the G₁ block for an additional 8 hours, and although there was some increase, this did not explain the increased expression of RNA from cells in G₂ (data not shown). As an additional control, the expression in G₂ relative to G₁ of a cellular mRNA, the cyclophilin gene, was assessed using a similar QC-PCR strategy on these same extracts. The amount of cyclophilin RNA per viral DNA copy did not change significantly during the cell cycle (Table 1). These results, therefore, are consistent with the hypothesis that HIV expression is increased in the G₂ phase, and that the role of Vpr is to maintain cells in a stage of the viral life-cycle where viral expression is upregulated.

The HIV LTR is upregulated in G₂ of the cell cycle

We then tested whether the increase in LTR activity in the presence of Vpr observed previously¹⁹⁻²¹ was a direct effect or was an

Fig. 1 Analysis of synchronized T cells infected with HIV. **a**, Schematic diagram of protocol for synchronization and infection of Jurkat T cells. T cells were synchronized at the G₁/S border by a double aphidicolin block³⁹, and high-titer VSV-G pseudotype HIV stocks were used to infect 100% of the cells⁷ at the time the second aphidicolin block was applied and were allowed to accumulate at the G₁/S border (time 0). Cells were then released from the second aphidicolin block and allowed to proceed synchronously through the cell cycle. **b**, Cell-cycle profiles of synchronized cells infected with HIV-1 that contained a wild-type Vpr (Vpr⁺; right side) or had a frame-shift mutation that abolished the vpr open reading frame (Vpr⁻; left side). The number of hours after the release from the second G₁/S block (α) is indicated as well as the position of the G₁ and G₂ peaks. **c**, Example of QC-PCR for detection of viral RNA and DNA infected cells. The lower band is the competitor (comp.) plasmid with a small deletion in gag. The upper band is the test sample. The asterisk below the photograph on the left side shows that the sample is between 1 × 10⁶ and 1 × 10⁵ copies. The finer resolution analysis on the right side shows that the point of equivalency between the sample and the competitor is about 5 × 10⁵ copies. This analysis was done to obtain each of the values in Table 1.

indirect effect of the ability of Vpr to delay cells in G₂. Jurkat T cells were cotransfected with an HIV-1 LTR-luciferase plasmid and with a plasmid that expresses Vpr or Vpr mutants. The Vpr mutants had previously been characterized for their ability to cause G₂ accumulation²². Our results show that the ability of Vpr to increase LTR activity correlated with the ability of the Vpr allele to cause a G₂ accumulation; the H33R and H71R mutations, which do not cause G₂ accumulation, also did not increase expression from the HIV-1 LTR; the R90K mutation, which causes only a modest G₂ arrest, also caused only a modest increase in LTR activity, whereas the E24G, F34I, and A89T mutants retained both the ability to cause G₂ accumulation and the ability to transactivate the LTR (Fig. 2a). These results show that the ability of Vpr to increase HIV-1 LTR activity is correlated with its ability to cause G₂ accumulation.

In both the LTRs and in the vpr gene, HIV-1 and HIV-2 share about 50% homology. HIV-2 Vpr is also capable of causing human cells to accumulate in G₂ (ref. 14). Thus, it was of interest to determine whether the ability of Vpr to increase LTR activity was reciprocal between HIV-1 and HIV-2. Indeed, we found that HIV-2 Vpr increased activity of the HIV-1 LTR to the same extent as HIV-1 Vpr (Fig. 2b), and both HIV-1 and HIV-2 Vpr proteins equally increased activity of the HIV-2 LTR (Fig. 2b). These results indicate that there is not a strain-specificity to the transactivation by Vpr, but rather they are consistent with the hypothesis that the ability of Vpr to increase LTR activity is due to its ability to keep cells in G₂.

We next tested whether preventing cells from entering mitosis from G₂ was sufficient for an increase in HIV-1 LTR activity. Transition from G₂ to mitosis in the cell cycle is controlled by the

Table 1 Viral RNA from infected cells increases in G₂

Source of RNA/DNA	Expt. #	Copies of RNA/DNA ^a G ₁ ^b	Copies of RNA/DNA ^a G ₂ ^c	Ratio of expression in G ₂ :G ₁ ^d
<i>vpr</i> ⁺ (<i>gag</i>) ⁺	Expt. 1	67	320	4.8
	Expt. 2	100	500	5.0
	Expt. 3	100	400	4.0
	Expt. 4	33	150	4.5
<i>vpr</i> ⁻ (<i>gag</i>) ⁺	Expt. 1	30	100	3.3
	Expt. 2	27	125	4.6
	Expt. 3	70	200	2.9
	Expt. 4	60	200	3.3
Cyclophilin A ^g	Expt. 3	2330	2600	1.1
	Expt. 3	2666	2571	1.0
	Expt. 4	3333	2000	0.6
	Expt. 4	2000	3000	1.5

^aAbsolute number of copies of RNA and DNA were determined by QC-PCR using internal standards per 2 to 3 × 10⁵ infected cells. The range of RNA copies for HIV *gag* expression ranged from 1 × 10³ to 5 × 10⁶. The range of DNA copies ranged from 1 × 10³ to 1 × 10⁴. The ratios of RNA to DNA copies are presented. The amount of DNA in G₂ was generally twice the amount of DNA in G₁. Therefore, the overall increase in absolute expression in G₂ is twice the amount described by the ratio.

^bTime point 0 h release from the second aphidicolin block (Fig. 1a).

^cTime point 8 h after release from the second aphidicolin block (Fig. 1b).

^dColumn 4 divided by column 3 (G₂ expression normalized per DNA copy divided by G₁ expression normalized per DNA copy).

^eGag RNA and DNA from cells infected with HIV that encoded wild-type Vpr. Results from four independent experiments are shown.

^fGag RNA and DNA from cells infected with HIV that did not encode Vpr. Results from four independent experiments are shown.

^gCyclophilin RNA expression in cells infected with HIV from Expt. 3 and Expt. 4 normalized to the amount of viral DNA. The top line of each experiment is from extracts of cells infected with virus that was Vpr⁺, and the bottom line is from extracts of cells infected with virus that was Vpr⁻.

activity of a cyclin-dependent kinase, p34^{cdc2}. Like Vpr, transfection of a kinase-deficient transdominant mutant of Cdc2 also delays or arrests cells in G₂ (ref. 23). We found that transfection of the transdominant form of Cdc2 increased expression of the HIV-1 LTR to levels similar to those found after transactivation

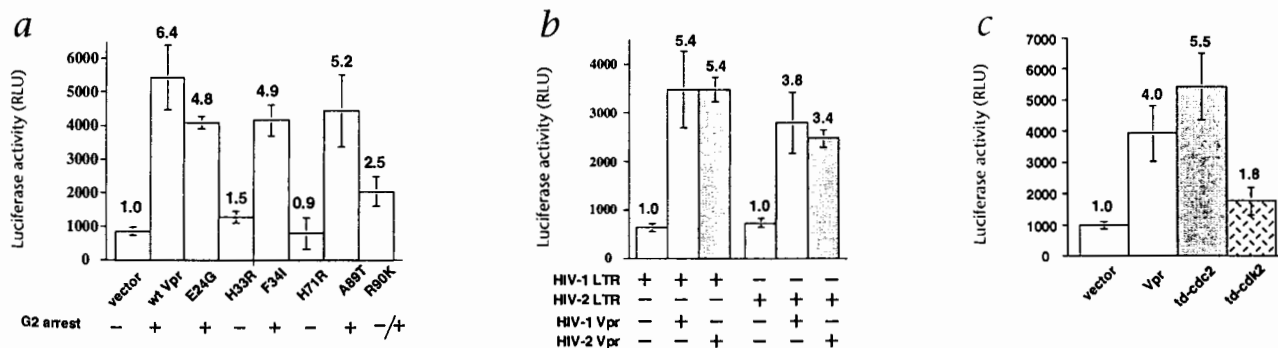


Fig. 2 Transfection of Jurkat T cells with HIV LTR-luciferase plasmids shows upregulation in G₂. The mean value of luciferase activity (expressed as relative light units) for each series of transfection is shown on the y-axis along with the standard deviation of the data. The fold increase in LTR activity relative to HIV-1 LTR luciferase cotransfected with empty vector alone is shown above each bar. All transfections were done in triplicate and normalized either to the amount of β-galactosidase activity from a cotransfected CMV-β-gal plasmid (a and b) or to the amount of total protein (c). **a**, Correlation of G₂ accumulation with Vpr transactivation. Vpr and Vpr mutants were expressed from the murine leukemia virus (MLV) LTR promoter (LXSN-vpr⁺), and were cotransfected into Jurkat T cells with a plasmid that contained the entire HIV-1_{LAI} LTR driving the luciferase gene. The ability of each of these Vpr mutants to cause a G₂ arrest was previously re-

ported²² (except that we now find that the R90K mutation retains some residual G₂ arrest activity) and is shown below the panel. Each transfection contained 0.625 μg of HIV-1 luciferase and 0.625 μg of Vpr expression plasmid (or empty vector). **b**, Cross-transactivation between the HIV-1 and HIV-2 Vpr proteins on the HIV-1 and HIV-2 LTRs. The HIV-2 Vpr is from the HIV-2_{312A} strain, which causes as much G₂ accumulation as HIV-1_{LAI} Vpr (ref. 14). **c**, Transactivation of the HIV-1 LTR by transdominant Cdc2, but not Cdk2. Kinase-deficient transdominant (td) Cdc2 and Cdk2 were expressed from the CMV promoter²³. The amount of transactivation by td-Cdc2 ranged from 3- to 5.5-fold in different experiments, whereas the amount of transactivation by td-Cdk2 was not significantly different from empty vector alone. HIV-1 luciferase plasmid (0.5 μg) was transfected with 1 μg of Vpr, td-Cdc2, td-Cdk2, or empty vector.

Increased virus production in the presence of Vpr

Finally, it was important to determine whether increased LTR expression in G₂ leads to increased virus production. Therefore, we compared the amount of viral antigen secreted into the medium by Jurkat T cells transfected with a replication-defective provirus (to determine single-round production) that expressed *vpr* to one that did not express *vpr*. Virus production from the transfected cells was higher in the presence of Vpr than in the absence of Vpr (Fig. 3a). Moreover, cells infected with a replication-defective virus that was Vpr⁺ secreted more virus than did cells infected with a replication-defective virus that was Vpr⁻ during the first 48 hours after infection (Fig. 3b). Finally, at times shortly after infection, there was greater production of infectious virus from cells infected with replication competent virus that contained Vpr than from cells infected with the same amount of replication competent virus that did not contain Vpr (Fig. 3c). These results indicate that Vpr increases viral production in a single round of viral replication, and that this increase in viral production is correlated with the increased expression in G₂.

Selection for Vpr function *in vivo*

The ability of Vpr to increase viral expression and replication would suggest that there is a selective advantage for Vpr function *in vivo*. However, there is little or no effect of deletions in *vpr* on pathogenesis in either simian immunodeficiency virus (SIV) infection of macaques nor HIV infection of SCID-hu mice²⁴⁻²⁶. To resolve whether Vpr plays a role in the replication of HIV-1 *in vivo*, a retrospective analysis was done to ask whether there is a positive selective pressure for Vpr in both HIV-1-infected chim-

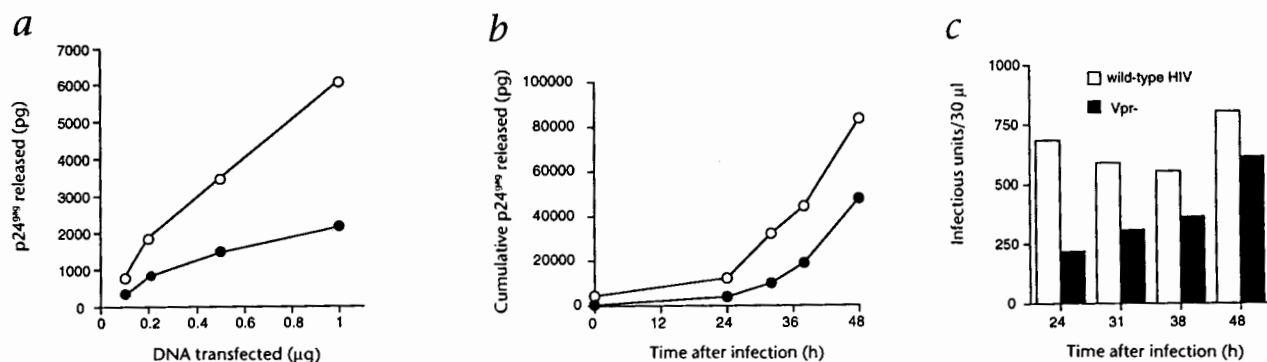


Fig. 3 Increased virus production in a single round in the presence of Vpr. **a**, Proviruses with deletions in *env* (to eliminate second round infections) with and without the endogenous *vpr* gene were transfected into Jurkat T cells. The amount of DNA transfected is indicated on the x-axis and the amount of virus produced in the supernatant of the transfected cells after 48 h is indicated on the y-axis. Virus production was measured 40 h after transfection by an ELISA for p24⁹⁹. A CMV- β -galactosidase plasmid was co-transfected in each case, and the p24⁹⁹ production in the supernatant was normalized for β -galactosidase activity in the cell extracts. Open circles, Vpr⁺ provirus; filled circles, Vpr⁻ provirus. **b**, Jurkat T cells were infected with

HIV (VSV-G) pseudotypes that were either Vpr⁺ (○) or Vpr⁻ (●) at an equal input of virus at a multiplicity of infection of 1.0. The viruses also contained a deletion in *env* to eliminate second round infections. Medium was not changed during the experiment, so that each time point represents the cumulative amount of p24⁹⁹ secreted into the medium. **c**, Jurkat T cells were infected with replication competent HIV that was either wild-type (□) or Vpr⁻ (■) at an equal input of virus. Cells were washed three times after inoculation, and at the times indicated, medium was collected and completely changed so that each time point represents only the accumulation since the previous time point. Infectivity was measured by the MAGI assay³⁷.

panzees, in which the virus is relatively nonpathogenic, and an infected laboratory worker^{27,28}, who received virus containing mutations in *vpr*. Both the chimpanzees²⁹ and the laboratory worker had been infected with virus that originated from the HIV_{11b} stock that encodes an unstable and nonfunctional Vpr because of the insertion of a single thymidine that results in a premature truncation of the protein³⁰.

Sequence analysis of independent clones obtained from PCR products confirmed that the chimpanzee challenge strain of HIV_{11b} was defective in Vpr (Fig. 4a). The single clone from the challenge strain encoding a functional Vpr protein also contained a nonsynonymous mutation at the 5' end of *vpr*, which disrupted the *vif* open reading-frame, and this genotype was not recovered from any subsequent plasma sample. The *vpr* gene was then cloned and sequenced from virion RNA isolated from the plasma of two chimpanzees at two different time points after infection. In both animals, reversion of the *vpr* gene occurred between 6 weeks and 2 years after infection (Fig. 4a).

Because plasma was not available from the infected laboratory worker, we analyzed *vpr* by direct sequencing of the PCR products from DNA of short-term peripheral blood mononuclear cell (PBMC) cultures infected with virus recovered from this individual at three different time points²⁸. Virus isolated from this individual in September 1985 showed that the predominant species of *vpr* gene contained the thymidine (T) insertion that is typical of the frame-shift mutation in the HIV_{11b} strain (Fig. 4b). However, virus isolated 2 years later, in December 1987, and again in May 1988, showed loss of the T insertion and reversion back to an intact *vpr* open reading-frame (Fig. 4b). These data indicate that there is a positive selection for *vpr* function *in vivo*.

Discussion

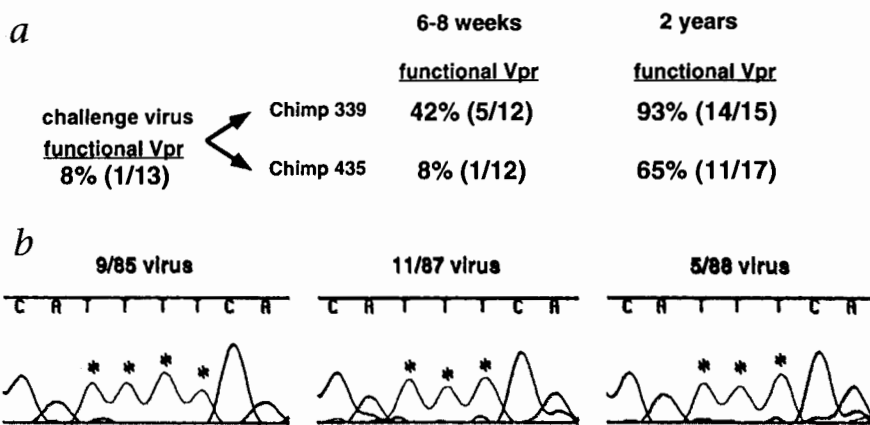
Here we provide evidence that by lengthening the G₂ phase of the cell cycle, HIV has evolved a novel mechanism for indirectly increasing viral production. Specifically, Vpr protein of HIV delays infected cells in the G₂ stage of the cell cycle where the LTR is more active. The increase in expression in G₂ results in higher virus production over a single round of replication and provides

an explanation for the conservation of this function among diverse members of the primate lentivirus family.

We derived a simple model to explain how a virally induced cell-cycle arrest or delay could be selected for *in vivo* on the basis of the increased production of HIV in G₂, the ability of Vpr to extend the G₂ phase of the cell cycle, and the short half-life of infected cells *in vivo* (Fig. 5). In the example shown, the cells infected with virus that is Vpr⁺ spend 18 hours in G₁-S (G₁ and S phase combined) and 12–20 hours in G₂. Thereafter, cells that express Vpr likely undergo cell death by apoptosis³¹. Cells infected with HIV that is Vpr⁻, on the other hand, spend about 18 hours in G₁-S but only 2 hours in G₂ and are capable of undergoing further normal cell divisions. In both cases the rate of virus production in G₂ is at least 4 times the rate of virus production in G₁-S (from Table 1). However, since cells infected with virus that is Vpr⁺ spend more time in G₂, the average rate of virus production for one entire cell cycle from the time that the infected cell begins to produce virus in the presence of Vpr is 2.2–2.6 units (Fig. 5a). The average rate of virus production for one entire cell cycle from the time that the infected cell begins to produce virus in the absence of Vpr, on the other hand, is only 1.3 units (Fig. 5a). Therefore, over a single cell cycle, the average rate of virus production in a population of cells infected with Vpr⁺ virus is lower than the average rate of virus production from a population of Vpr⁻ infected cells. Indeed, the increased rates of virus production in a single round predicted by the model (1.7- to 2.0-fold) are consistent with the experimentally observed values (Fig. 3, a and b).

Iteration of the model over consecutive cell cycles shows that the average rate of virus production from Vpr⁻ infected cells over two cell-cycle times (that is, 40 hours of virus production or one cell division) is 2 units, and the average rate of virus production over three cell-cycle times (that is, 60 hours of virus production or two consecutive cell divisions) is 3 units (Fig. 5b). On the other hand, the average rate of virus production in the presence of Vpr should remain constant (Fig. 5b). However, since the half-life of an infected cell has been estimated to be only 1.6 days¹⁷ (38 hours), an infected T cell could not proceed through more

Fig. 4 Selection for HIV-1 Vpr *in vivo*. **a**, Reversion of *vpr* in infected chimpanzees. The *vpr* gene was amplified by PCR from virion RNA from both the challenge strain and from chimpanzee 339 at 6 weeks post infection and 24 months post infection, and from chimpanzee 435 at 8 weeks post infection and 26 months post infection. The percentage of clones with a functional *vpr* gene (as assayed both by sequence for an open reading frame and by transfection into HeLa cells for the ability to cause G₂ arrest) is shown. In parentheses is the number of functional *vpr* genes obtained out of the number cloned. **b**, Reversion of *vpr* in an HIV-1 infected human. The entire *vpr* gene was sequenced directly from virus isolated at each time point indicated; the portion of the gel from nucleotides 211 to 216 of the *vpr* open reading frame is shown. The four T residues (red) marked with an asterisk for the 9/85 virus demonstrate the frame-shift mutation typical of the HIV_{IIIb} isolate in the *vpr* reading frame³⁰. The *vpr* genes



from both the 11/87 and the 5/88 isolates have only three T residues (marked with an asterisk) at this position.

than two complete cell cycles after virus production has started. Thus, *in vivo*, when the number of cell divisions of infected lymphocytes is limited by their short half-life, we expect selection pressures for higher viral production to maintain Vpr function. The reversion of a Vpr-negative virus to Vpr⁺ takes place over weeks to months in both humans and chimpanzees (Fig. 4). Therefore, the actual net selective pressure on Vpr function in primary cells *in vivo* might be smaller than that calculated here³⁰.

In tissue culture, the selection pressures would be different, because infected cells are able to undergo more cell divisions in the absence of Vpr. On the simplest level, our calculation shows that after two cell divisions a cell infected with a Vpr-negative virus has a greater average rate of virus production than cells infected with virus that is Vpr⁺. In agreement with the model (Fig. 5b), we do find experimentally that although the initial amount of virus produced in the presence of Vpr is more than the amount of virus produced in the absence of Vpr, the rate of production increases over time in the absence of Vpr (Fig. 3c). Therefore, in the absence of continuously added fresh uninfected cells as targets, there would be selection against Vpr, because the infected cells could not divide. Indeed, mutations in *vpr* accumulate rapidly in continuously infected cultures^{12,32}.

Like the HIV LTR, other promoters such as the CDC25C, cyclin B and PLK promoters have increased activity in G₂, and the degree of upregulation in G₂ is about the same as what we have observed for the HIV LTR (refs. 33, 34). The simplest explanation for the increased expression of the HIV LTR in G₂ is that there are transcription factors that are cell-cycle regulated (which may also be T-cell specific) that act on the HIV LTR. Although the overall effect of cell cycle/Vpr on LTR activity is modest, it does result in increased virus production in a single round of replication (Fig. 3), which means that the effect is additive to the viral transactivator, Tat.

In addition to its role in delaying the cell cycle, Vpr is also important for HIV infection of macrophages and other nondividing cells⁶. However, it is likely that these two functions of HIV-1 are not causally linked, because they are phenotypically separated in HIV-2 and SIV_{SM} viruses that encode two related proteins, Vpr and Vpx. In HIV-2/SIV_{SM} the Vpr protein causes a G₂ accumulation, but has no effect on infection of macrophages, and the Vpx protein has no cell-cycle effect, but is important for infection of macrophages³⁵. The experiments described here do

not identify which of these two functions is under more selective pressure *in vivo*. However, even in SIV_{SM}, Vpr provides a selective advantage to the virus³⁶. Finally, our results also demonstrate that the selection for Vpr function *in vivo* is not driven by selection for greater pathogenicity, because Vpr reverted to wild-type in the chimpanzees in the absence of apparent disease. Rather, it seems more likely that the selection is driven by a role for Vpr in maintaining viral persistence.

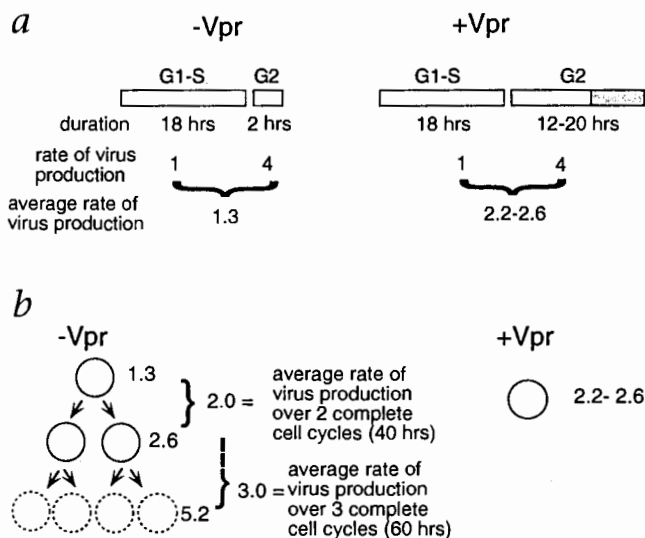
Methods

Infections. High-titer VSV-G pseudotypes of HIVΔenv were made by transient cotransfection of 293T cells with Vpr⁻ and Vpr⁺ HIV proviruses and a VSV-G expression vector as previously described⁷. Titers were determined by the MAGI assay³⁷, and were usually greater than 1 × 10⁹ infectious units per milliliter. p24⁹⁹ production was measured by ELISA (Coulter Immunology, Hialeah, FL) using standards provided by the manufacturer.

Synchronization experiments. Jurkat T cells were grown in RPMI with 10% fetal bovine serum. For synchronization, 2 × 10⁷ cells in 20 ml were incubated for 16 h with 0.75 μM aphidicolin (Sigma), then washed and resuspended in the same amount of medium without drug for 10 h. Cells were resuspended in 300 μl virus stock (at 1.1 × 10⁷ cells/ml) that contained 0.75 μM aphidicolin for a calculated multiplicity of infection of 10 for each pseudotyped virus stock. After 2 h, additional medium with aphidicolin was added to bring the cells to 1 × 10⁶ cells/ml for an additional 16 h. At this point, over 80% of the cells were at the G₁/S border as determined by flow cytometric analysis. The cells were resuspended in medium without drug and harvested at various time points in the cell cycle. For cell-cycle analysis, 5 × 10⁵ cells were fixed in 80% ethanol, stained with propidium iodide, and analyzed as previously described^{7,12}.

Transfection of Jurkat T cells. Vpr expression vectors using plasmids that expressed Vpr from the MLV LTR have previously been described⁷. The entire HIV-1 LTR was cloned into pGL-2 Basic (Promega, Madison, WI) for detection of HIV-1 LTR-directed luciferase activity. For transfections, cells were washed once in PBS, and then 7.5 × 10⁵ cells were resuspended in 1 ml RPMI in a 12-well plate. Transfections were done in triplicate using 1.5 μg of total DNA and 5 μl of Superfect (Qiagen, Chatsworth, CA) reagent. Two days after transfection, cells were washed twice and then resuspended at 1 × 10⁷ cells/ml in luciferase lysis buffer (Promega), and 20 μl of the soluble supernatant was used for luciferase assay with an automated injection apparatus (AutoLumat, EG+G Berthold). Background readings were always less than 5% of the experimental readings. Extract (50 μl) was used for β-galactosidase assay with the fluorescent substrate 4-methylumbelliferyl-β-D-galactosidase, and 5 μl of extract was used for protein determination with a Bradford reagent (Pierce, Rockford, IL).

Fig. 5 Model of how increased viral expression in G₂ can account for a selective advantage for Vpr-mediated cell-cycle arrest. **a**, Average rate of virus production is greater in the presence of Vpr over a single cell cycle. The rate of virus production is given in arbitrary units. The rate of production in G₂ (R_{G2}) is the relative increase in virus production in G₂ over G₁/S. The minimum value for R_{G2} used here is 4, which is the normalized RNA expression in G₂ from Table 1. The time a normal T-cell spends in G₁-S ($T_{G1,S}$) is given as 18 h. The time a normal T-cell spends in G₂ (T_{G2}) is given as 2 h on the basis of our synchronization experiments (not shown). $T_{G1,S}$ is not affected by Vpr, but T_{G2} is extended for 12 to 20 h in the presence of Vpr (denoted by the darkened box). The average rate of virus production (F) throughout the entire cell cycle is $[(T_{G1,S} \times 1) + (T_{G2} \times R_{G2})] / (T_{G1,S} + T_{G2})$. The average rate of virus production in the absence of Vpr for one complete cell cycle is 1.3, and the average rate of virus production in the presence of Vpr for one complete cell cycle is 2.2 to 2.6. **b**, Effect of additional rounds of cell division on the average virus production of a population of infected cells. After one complete cell cycle (20 h), the average rate of virus production in the absence of Vpr is 1.3 from **a**. With two infected cells, the rate is double this amount, or 2.6. However, the average rate over two complete cell cycles (40 h) is 2.0, or $[(1.3 + 2.6)] / 2$. The average rate of the entire population over three complete cell-cycle times is 3.0. The general formula for the average rate of virus production over N cell divisions is $F[2^{(N-1)} - 1] / (N + 1)$. In the presence of Vpr, we assume that the infected cell does not divide. *In vitro*, the supply of uninfected target cells may be limiting; therefore, virus reproduction by cell division will be favored. However, *in vivo*, the average life time of produc-



tively infected cells is short (1.5 to 2 days); therefore, repeated cell cycles are unlikely. Thus, the model explains why *in vivo* conditions select for *vpr*, whereas *in vitro* conditions select against *vpr*.

Quantitative-competitive (QC)-PCR. RNA and DNA were simultaneously isolated from synchronized Jurkat T cells at the G₁/S border or G₂ phase of the cell cycle using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. RNA samples were further treated with RNase-free DNase to ensure that there was no contamination of DNA. RT-PCR without added reverse transcriptase were performed with each run to ensure that DNA carryover had not occurred.

To detect viral RNA or DNA, primers GAG04 and GAG06, corresponding to an internal fragment of the *gag* region, were used³⁸. GAG04: 5'-CATTC-TATTTGTTTCITGAAGGGTACTAG-3', GAG06: 5'-GCITTIAGCCCGAAGTIAT-ACCCATG-3'. In order to quantify viral RNA, QC-RT PCR was performed as described³⁸. A plasmid containing a 70 base-pair deletion in the *gag* region, pGGD70 (kindly provided by Paul Lewis, OHSU) was used to prepare RNA-competing templates. pGGD70 was linearized with *EcoRI* and used as competing templates for DNA QC-PCR. Cyclophilin messages were quantified in a similar manner. Cyclophilin primers and RNA Competicon for QC-RT PCR were purchased from Ambion Inc. (Austin, TX), and PCR reaction conditions were based on the recommendations of the commercial supplier.

Analysis of Vpr from infected chimpanzees. The chimpanzee challenge virus was virus from H9 cells chronically infected with the HIV₁ strain that was subsequently passaged in chimpanzee peripheral blood mononuclear cells (Larry Arthur, NCI). Infected chimpanzees C339 and C435 have been previously described²⁹. Virion RNA was isolated from the initial viral inoculate and from chimpanzee plasma by centrifugation at 100,000g for 2 h at 4 °C followed by lysis of the viral pellet using the Tri-Reagent system (Molecular Research Center, Inc.). After one 75% ethanol wash, cDNA was synthesized by using random primers and amplified by using 30 cycles of PCR for the viral inoculate, and an additional 30 cycle nested PCR for the chimpanzee plasma virus. PCR products were cloned and sequenced. Vpr clones with nonsynonymous mutations were cloned into an expression vector under control of the HIV-1 LTR and assayed for production of functional protein on the basis of their ability to arrest cells in the G₂ phase of the cell cycle, as described previously^{12,22}.

Analysis of Vpr from an infected laboratory worker. Viral isolates from the laboratory worker have been described²⁸. The entire *vpr* region was amplified from infected PBMC genomic DNA by nested PCR using the following primer sets: Round 1: 5'-AAGTACACATCCCACTAGGGGATGC-3' and 5'-AGCATGGTGCCTCCATCTCCACCC-3'. Round 2: 5'-CCTTATTAGGACACATAGTAGCC-3' and 5'-ATGAGCTCTCGTCGCTCTCCG-3'. A touchdown PCR protocol was used which consisted of 40 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min with a decrease in the annealing

temperature of 0.5 °C per cycle. *Taq* Extender (Stratagene, La Jolla, CA) reagents were used in the reactions according to manufacturer's instructions. The amplified products were separated by agarose gel electrophoresis and purified using the Qiaquick gel extraction kit (Qiagen). The purified products were directly sequenced using the inner amplification primers as well as other internal primers on an ABI Prism DNA sequencer (Perkin-Elmer, Foster City, CA).

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