JOURNAL OF VIROLOGY, Nov. 1995, p. 6705–6711 Vol. 69, No. 11
0022-538X/95/$04.00+0

Human Immunodeficiency Virus Type 1 Viral Protein R (Vpr) Arrests Cells in the G_2 Phase of the Cell Cycle by Inhibiting p34^{cdc2} Activity

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Received 25 May 1995/Accepted 31 July 1995

The Vpr accessory gene product of human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus is believed to play a role in permitting entry of the viral core into the nucleus of nondividing cells. A second role for Vpr was recently suggested by Rogel et al. (M. E. Rogel, L. I. Wu, and M. Emerman, J. Virol. 69:882–888, 1995), who showed that Vpr prevents the establishment in vitro of chronically infected HIV producer cell lines, apparently by causing infected cells to arrest in the G_2/M phase of the cell cycle. In cycling cells, progression from G_2 to M phase is driven by activation of the p34^{cdc2}/cyclin B complex, an event caused, in part, by dephosphorylation of two regulatory amino acids of p34^{cdc2} (Thr-14 and Tyr-15). We show here that Vpr arrests the cell cycle in G_2 by preventing the activation of the p34^{cdc2}/cyclin B complex. Vpr expression in cells caused p34^{cdc2} to remain in the phosphorylated, inactive state. p34^{cdc2}/cyclin B complexes immunoprecipitated from cells expressing Vpr were almost completely inactive in a histone H1 kinase assay. Coexpression of a constitutively active mutant p34^{cdc2} molecule with Vpr relieved the G_2 arrest. These findings strongly suggest that Vpr arrests cells in G_2 by preventing the activation of the p34^{cdc2}/cyclin B complex that is required for entry into M phase. In vivo, Vpr might, by preventing p34^{cdc2} activation, delay or prevent apoptosis of infected cells. This would increase the amount of virus each infected cell produced.

The *vpr* accessory gene of human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus encodes a virion-associated, nuclear protein whose role in AIDS pathogenesis is not yet clear. In culture, Vpr does not significantly affect the kinetics of viral replication in transformed cell lines or in primary T lymphocytes (1, 2, 8, 11, 32) but is required for efficient replication in primary monocytes/macrophages (4, 10). In vivo, Vpr appears to increase viral pathogenicity. In one study of macaques infected with wild-type or *vpr*-mutant virus, Vpr appeared to increase both the viral load and the rate at which the animals progressed to AIDS (24). In addition, in some of the *vpr*-mutant virus-infected animals, viral sequences in which *vpr* had reverted to wild type were isolated, suggesting that the presence of an open *vpr* reading frame provided a selective advantage to the virus. In a second study, macaques appeared to progress rapidly to AIDS without reversion of a mutation in *vpr*, although in the presence of an additional mutation in *vpx*, the virus failed to induce disease (13).

At least two, apparently independent roles for Vpr in viral replication have been proposed. In the first, Vpr was proposed to facilitate transport of the virus core into the nucleus of nondividing cells (17). This hypothesis was supported by the finding that shortly after entry, in the absence of a functional Gag MA nuclear localization sequence, *vpr*-mutant virus, but not *vpr*-wild-type virus, failed to enter the nucleus of nondividing cells. The doubly mutated virus maintained its ability to infect dividing cells, further supporting a role for Vpr in nuclear import. This model provides a clear rationale for the inclusion of Vpr in virions, since nuclear import occurs early in infection, prior to the synthesis of new viral proteins.

A second role for Vpr in HIV-1 replication was recently suggested by Rogel et al. (36), who showed that Vpr prevents establishment of chronically infected HIV-1 producer cell lines. This property appeared to be due to the ability of Vpr to block cell division by blocking the mitotic cell cycle in G_2/M. This finding is consistent with an earlier report showing that Vpr expression could prevent cell division and increase the differentiation state of a rhabdomyosarcoma cell line (26). While this model does not explain the role of Vpr in virions, we previously reported results suggesting that Vpr plays a postintegration role in virus replication in monocytes. In that report, we showed that virions containing Vpr molecules but a *vpr*-mutant genome were inefficient in infecting primary monocytes (10).

In eukaryotic cells, the transitions of the cell cycle are driven by cyclin-dependent protein kinases (CDKs) (reviewed in references 28 and 31). The activity of these kinases is regulated both by their phosphorylation state and by their association with cyclins, proteins that bind the CDKs and whose levels fluctuate throughout the cell cycle. CDK activity is activated by association with cyclins; it is inactivated by phosphorylation at specific inhibitory amino acid residues. The transition from G_2 to M phase is regulated by the complex of the CDK p34^{cdc2} with cyclin B. Late in G_2, when cyclin B levels are high, p34^{cdc2} is rapidly dephosphorylated at the inhibitory amino acid residues Thr-14 and Tyr-15. This triggers the initiation of M phase.

Here, we have investigated the biochemical mechanism by which Vpr induces G_2 arrest. We first confirm that cells transfected with Vpr expression vectors arrest in the G_2 phase of the cell cycle. Furthermore, we show that cells infected with HIV-1 are arrested in G_2 as a result of Vpr expression. The arrest was

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accompanied by a nearly complete inhibition of p34^{cdc2}/cyclin B kinase activity. The decrease in p34^{cdc2} kinase activity induced by Vpr was accompanied by an increase in the fraction of the molecules phosphorylated on the two inhibitory phosphorylation sites. The effect was correlated with the ability to induce G_2 arrest, since Vpr molecules containing mutations that prevented G_2 arrest failed to inhibit p34^{cdc2}/cyclin B activity. Finally, we show that the ability of Vpr to inhibit p34^{cdc2}/cyclin B activity accounts for its effect on the cell cycle, since expression of constitutively active p34^{cdc2} mutant molecules overrides Vpr-induced G_2 arrest.

MATERIALS AND METHODS

Plasmids. Vectors for expressing Vpr and amino-terminal influenza hemagglutinin (HA)-tagged Vpr (pc-vpr and pc-5′-tag-vpr) contain the *vpr* open reading frame of HIV-1 NL4-3 inserted into the plasmid pcDNA1/amp (Invitrogen). pcDNA1/amp without an insert is referred to as pcDNA. pc-CD4 consists of pcDNA1/amp into which has been ligated a human CD4 cDNA. Expression of the inserted sequences in these plasmids is driven by the cytomegalovirus early promoter of pcDNA1/amp. Construction and analysis of the protein products expressed by these vectors have been previously described (25, 33). pc-3′-tag-vpr and pc-tag-vprR80A are similar in structure to pc-vpr but encode Vpr molecules with an HA tag at the carboxy terminus and a mutation of Arg-80 to Ala, respectively. Both proteins are stably expressed but are inactive in inducing G_2 arrest (12). pBABE-vpr was constructed by ligating the Vpr coding sequence of pc-vpr to the *Sna*BI and *Xho*I sites of murine leukemia virus retroviral vector pBABE-puro (29).

pHIV-HSA-E$^-$R$^+$ and pHIV-HSA-E$^-$R$^-$ consist of pNL4-3 with the gene for murine heat-stable antigen (HSA), CD24, fused in frame to the *nef* initiator methionine codon. In addition, a frameshift was introduced near the 5′ end of *env* to block production of gp160, limiting the ability of the virus to a single round of replication. These vectors are similar in structure to the HIV-1 luciferase reporter constructs pNL-Luc-E$^-$R$^+$ and pNL-Luc-E$^-$R$^-$, which we previously described (10), except that the luciferase gene in *nef* has been replaced by sequence encoding HSA. To construct pHIV-HSA-E$^-$R$^+$ and pHIV-HSA-E$^-$R$^-$, the murine HSA coding sequence (35) was amplified by PCR using primers containing a 5′ *Not*I and a 3′ *Xho*I site. The PCR product was then cleaved with *Not*I and *Xho*I and ligated to similarly cleaved pNL-Luc-E$^-$R$^+$ and pNL-Luc-E$^-$R$^-$. This resulted in a replacement of the luciferase reporter gene with HSA. pSR-Cdc2, pSR-Cdc2.15F, and pSR-Cdc2.15F14A express enzymatically active HA-tagged wild-type, Tyr-15→Phe, and Tyr-15→Phe/Thr 14→Ala p34^{cdc2}, respectively, and are identical to constructs described previously (16).

Transfections. 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C and 5% CO_2. Cells (2×10^6) seeded the previous day in 10-cm-diameter culture dishes were transfected with 20 μg of plasmid DNA by calcium phosphate precipitation as described previously (6). For cell cycle analyses, 2 μg of pc-CD4 was added to 18 μg of Vpr expression vector or to pcDNA. For preparation of murine leukemia virus stocks, amphotropic packaging cells (34) were transfected with pBABE-puro or pBABE-vpr. Culture medium was harvested and frozen in aliquots 48 h later. To produce amphotropic HIV-1 reporter viruses, 293 cells were transfected with a mixture of pHIV-HSA-E-R$^+$, pHIV-HSA-E$^-$R$^-$, and pSV-A-MLV-env (23). Where indicated, transfected cells were enriched by binding to anti-CD4 magnetic beads (Dynal) according to the manufacturer's recommendation.

Immunoblot analysis. Protein (100 μg) from lysates of the transfected cells was prepared 48 h posttransfection and separated on a sodium dodecyl sulfate. (SDS)–17.5% polyacrylamide gel as described previously (33). The proteins were transferred to an Immobilon filter (Millipore Corp.) and probed with monoclonal antibody 12CA5, anti-p34^{cdc2} (Santa Cruz Biotechnology), anti-cyclin B (Santa Cruz Biotechnology), or rabbit anti-Vpr (10) (Babco) diluted 1:10,000 (0.1 μg/ml) followed by horseradish peroxidase-labeled goat anti-mouse serum diluted 1:7,500. Filters were developed by the chemiluminescence method (Amersham).

p34^{cdc2} kinase assay. p34^{cdc2} kinase activity was measured as previously described (37). Briefly, transfected HeLa cells were lysed in buffer containing 50 mM Tris (pH 8.0), 0.5% Nonidet P-40, 2 mM EDTA, 137 mM NaCl, 10% glycerol, 2 mM sodium vanadate, 100 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates (500 μg of protein) were incubated on ice with 1 μg of anti-cyclin B monoclonal antibody in 1 ml of immunoprecipitation buffer (50 mM Tris [pH 8.0], 0.5% Nonidet P-40, 2 mM EDTA, 137 mM NaCl, 10% glycerol), collected on 20 μl of protein A-agarose beads, and washed three times with immunoprecipitation buffer. The p34^{cdc2}/cyclin B complexes were then incubated for 15 min at 37°C in buffer containing 2.5 μCi of [γ-^{32}P]ATP, 10 μg of histone H1, 100 mM NaCl, 10% Triton X-100, 50 μM ATP, and 10 mM $MgCl_2$. The reaction mixtures were incubated 5 min at room temperature and were analyzed by autoradiography after separation by SDS-polyacrylamide gel electrophoresis (PAGE).

Cell cycle analysis. Cells were harvested 3 days posttransfection and stained for cell surface CD4 by incubation with 1 μg of Leu3a for 30 min on ice in phosphate-buffered saline (PBS) containing 1% fetal calf serum. The cells were washed in PBS and then incubated with 0.5 μg of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin for 30 min on ice. Cells infected with HIV-1 reporter constructs HIV-HSA-E$^-$R$^+$ and pHIV-HSA-E$^-$R$^-$ were stained 3 days postinfection with 0.5 μg of biotinylated anti-HSA (Pharmingen) followed by 0.5 μg of FITC-conjugated streptavidin (Boehringer Mannheim). The cells were then washed in 2 ml of PBS, fixed in 0.25% paraformaldehyde–PBS for 30 min on ice, permeabilized by incubation with PBS containing 0.2% Tween 20 at 37°C for 15 min, and incubated in PBS with 0.03 mg of propidium iodide per ml and 50 μg of RNase A for 60 min at 37°C. The fluorescence of 10,000 cells was analyzed on a FACScan (Becton Dickinson). Data are presented after gating to eliminate CD4$^-$ cells (typically 60% of the cells). G_2/M:G_1 ratios were calculated by CellFit software (Becton Dickinson). Mitotic index was determined as previously described (19). Briefly, transfected cells were harvested, swelled in hypotonic buffer containing 0.075 M KCl for 20 min, fixed in methanol acetic acid, air dried, and stained with Giemsa stain. Mitotic figures were counted in a total of 1,000 cells.

RESULTS

Vpr expressed by transfection arrests cells in the G_2 phase of the cell cycle. Initially, we attempted to establish cell lines that stably expressed Vpr. To do this, we constructed pBABE-vpr, a murine leukemia virus retroviral expression vector based on pBABE-puro (29). We generated amphotropic helper-free pBABE-vpr and pBABE-puro stocks and used these to infect HOS and CEM cells. These cell lines were used because they support high levels of HIV-1 replication and because they are readily selected with puromycin. Following puromycin selection, we found that cells infected with pBABE-puro grew rapidly and were easily visible 3 days after selection. In cultures infected with pBABE-vpr, cells that survived drug selection were easily visible (mock-infected cultures contained no viable cells at this time), but these cells failed to divide and died after 10 to 14 days. During this time, the cells became enlarged and flat (Fig. 1), a finding consistent with that of Levy et al. (26). Infection of the human transformed T-cell line CEM with pBABE-vpr also resulted in cells that failed to divide (not shown). These findings suggested that Vpr might interfere with cell cycle progression; however, because of their failure to divide, we were unable to produce sufficient quantities of cells for further analysis.

To determine whether Vpr prevented cell division by arresting the cells at a defined point in the cell cycle, we established a transient assay in which human cells (293 or HeLa) were cotransfected with a Vpr expression vector and a small amount of a human CD4 expression vector, pc-CD4 (25), that served as a marker plasmid. Three days posttransfection, we stained the cells with anti-CD4, FITC-conjugated secondary antibody, and propidium iodide. We then determined the cell cycle distribution of the transfected cells on a FACScan by measuring the DNA content of the CD4-expressing cells. The results showed that cells transfected with an expression vector encoding HIV-1 Vpr (pc-vpr) or an amino-terminal HA-tagged Vpr molecule (pc-5′-tag-vpr) exhibited an approximate inversion of the G_2/M:G_1 ratio compared with cells transfected with the control vector (Fig. 2A). This finding suggested that the cells had arrested in G_2/M as a result of Vpr expression. In contrast, transfecting with a vector encoding Vpr with a carboxy-terminal HA tag (pc-3′-tag-vpr) had no effect on the G_2/M:G_1 ratio, suggesting that this molecule was inactive in inducing cell cycle arrest (Fig. 2A). This finding was not due to instability of the carboxy-terminal HA-tagged molecule, since it is expressed at a high steady-state level and appears to be correctly folded (12). Apparently, Vpr function requires an unmodified carboxy terminus. Moreover, the ability to arrest cells in G_2 was not restricted to 293 cells. Transfection of HeLa cells with pc-5′-tag-vpr also resulted in G_2 arrest (Fig. 2B).

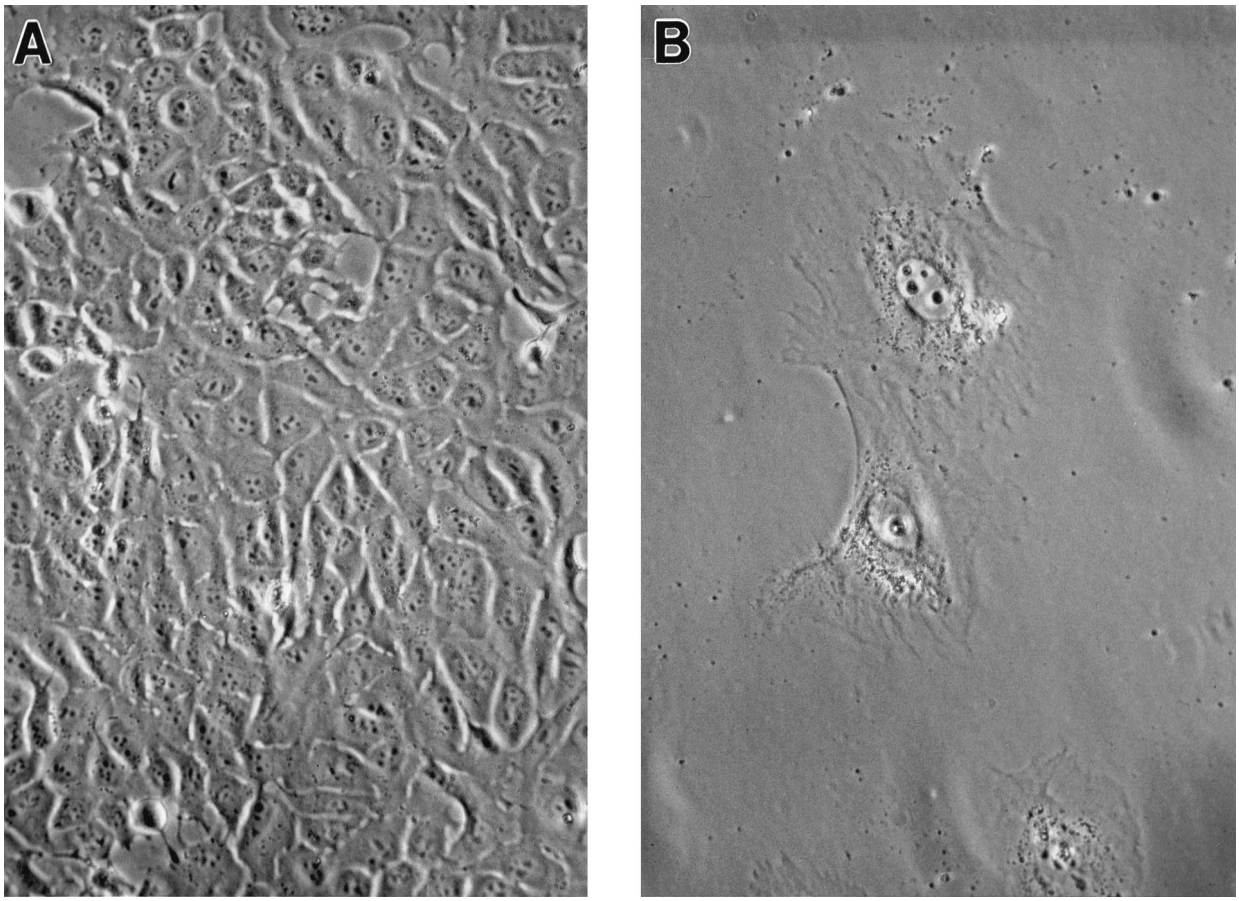

FIG. 1. Morphology of HOS cells stably expressing Vpr. HOS cells were infected with pBABE-puro or pBABE-Vpr helper-free virus stock. The next day, the cells were transferred to selective medium containing 3 mM puromycin. The cells were photographed 5 days later.

Because we were unable to distinguish between G_2 and M-phase arrest by fluorescence-activated cell sorting (FACS) analysis, we tested whether cells expressing Vpr remain in G_2 or proceed to M phase by determining the mitotic index of cells transfected with a Vpr expression vector (19). This analysis showed that Vpr expression did not cause an increase in the number of mitotic cells in the population (14.0 ± 7 for pcDNA-transfected cells and 15.5 ± 0.7 for pc-vpr-transfected cells). Thus, Vpr does not permit cells to proceed to M phase but arrests them in G_2.

HIV-1 infection arrests cells in G_2 as a result of Vpr expression. To test whether cells are arrested in G_2 as a result of Vpr expression in HIV-infected cells, we constructed the single-replication-cycle HIV-1 reporter plasmids pHIV-HSA-E$^-$R$^+$ (*vpr* open) and pHIV-HSA$^-$R$^-$ (*vpr* closed). These proviruses contain, in *nef*, the gene for HSA (CD24), a 30-amino-acid murine cell surface protein (35). Because of a frameshift in *env*, the viruses are capable of only a single round of infection. Following integration and proviral expression, the cells synthesize HSA which can be detected at the cell surface by flow cytometry. We used these constructs to determine whether Vpr arrests the cell cycle in HIV-1-infected cells. To do this, we infected CEM cells with HIV-HSA amphotropic pseudotypes and determined their cell cycle distribution by flow cytometry after staining with an anti-HSA monoclonal antibody and propidium iodide. The results showed that CEM cells infected with pHIV-HSA-E$^-$R$^+$ showed a significantly increased G_2/M:G_1 ratio (Fig. 2C). This was not as pronounced as that found in transfected cells, perhaps because of an overall decrease in the rate at which infected cells progress through the cell cycle. In addition, a small increase in the proportion of cells in G_2/M was observed upon infection with a *vpr*-mutant reporter virus (Fig. 2C), suggesting that viral products other than Vpr may contribute to G_2 arrest. However, Vpr clearly increased the proportion of infected cells in G_2/M.

Vpr expression results in an increase in p34^{cdc2} phosphorylation. In mammalian cells, the transition from G_2 to M phase is driven by the activation of the kinase activity of p34^{cdc2} late in G_2 (31). At this phase in the cell cycle, p34^{cdc2} is activated by two events: increased association with cyclin B and dephosphorylation of two negative regulatory amino acids, Thr-14 and Tyr-15 (20–22). To test whether Vpr interferes with p34^{cdc2}/cyclin B activation, we examined the phosphorylation state and kinase activity of the complex in cells transfected with Vpr expression vectors and a small amount of pc-CD4. The CD4 vector allowed us to enrich the transfected cells on anti-CD4-coated magnetic beads. The effectiveness of this method is demonstrated by the increased amount of Vpr detected in the enriched population (Fig. 3A, bottom panel).

On immunoblots, p34^{cdc2} was visible as two closely spaced bands, the lower of which has been shown to correspond to unphosphorylated p34^{cdc2} and the upper of which has been shown to correspond to Thr-14/Tyr-15-phosphorylated p34^{cdc2} (22) (Fig. 3A, top panel). Expression of Vpr in the transfected cells resulted in a significant increase in the ratio of phosphorylated to unphosphorylated p34^{cdc2} (Fig. 3A, lanes 2 and 3).

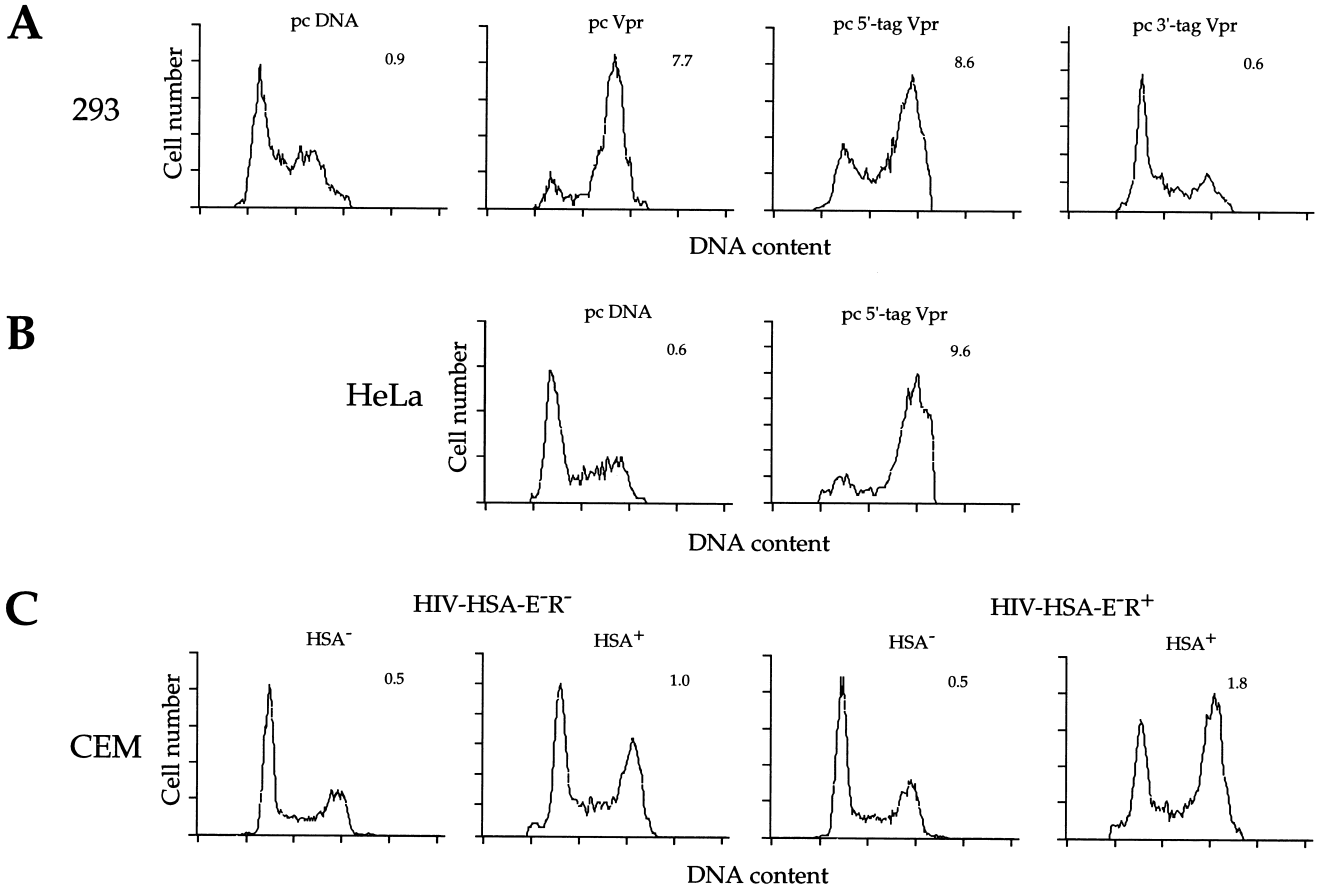

FIG. 2. Transfecting human cells with Vpr expression vector or infecting them with HIV-1 results in G_2/M arrest. (A) Cell cycle analysis of 293 cells expressing Vpr or amino- or carboxy-terminal HA-tagged Vpr. The cells were stained with Leu3a followed by FITC-conjugated goat anti-mouse immunoglobulin and propidium iodide as described previously (38). (B) Cell cycle analysis of HeLa cells expressing amino-terminal HA-tagged Vpr. (C) CEM cells infected with wild-type or mutant Vpr HIV-1 reporter virus (500 ng of $p24^{gag}$) pHIV-HSA-E^-R^+ or pHIV-HSA-E^-R^-. Two days later, the cells were stained with biotinylated anti-HSA and FITC-conjugated streptavidin. The DNA contents of the HSA^+ and HSA^- cells in both cultures are shown. The G_2/M:G_1 ratio, as determined by CellFit (Becton Dickinson) software, is shown at the upper right in each panel. Results shown are representative of at least three independent experiments. Cells not expressing CD4 above the background level (as defined by the FITC fluorescence of parallel mock-transfected cells) were gated out.

This increase was more pronounced in the CD4-enriched cells (Fig. 3A, lanes 4 and 5). Vpr expression also resulted in a small (twofold) increase in the amount of cyclin B in the cells (Fig. 3A, middle panel). This might be expected, given that cyclin B levels increase in G_2. Because $p34^{cdc2}$ is inactive in its phosphorylated state, these findings suggested that Vpr expression might result in inhibition of $p34^{cdc2}$/cyclin B kinase activity.

Vpr expression results in inhibition of $p34^{cdc2}$ kinase activity. To determine whether Vpr expression affects the activation state of $p34^{cdc2}$, we compared the relative kinase activities of $p34^{cdc2}$/cyclin B complexes from cells expressing Vpr with those of control cells. To do this, we transfected HeLa cells with Vpr or a control expression vector. $p34^{cdc2}$/cyclin B complexes were then immunoprecipitated from lysates of the transfected cells and incubated with histone H1 and [γ-^{32}P]ATP. Phosphorylated histone H1 was then detected by autoradiography after separation by SDS-PAGE. The histone H1 phosphorylation detected in this assay was the result of $p34^{cdc2}$ and not other cellular kinases that might have been present in the kinase reaction, since phosphorylated histone H1 was not detected when anti-cyclin B antibody was not added to the immunoprecipitation (not shown). Analysis of lysates from transfected HeLa cells showed that Vpr expression caused a dramatic decrease in $p34^{cdc2}$/cyclin B kinase activity (Fig. 3B,

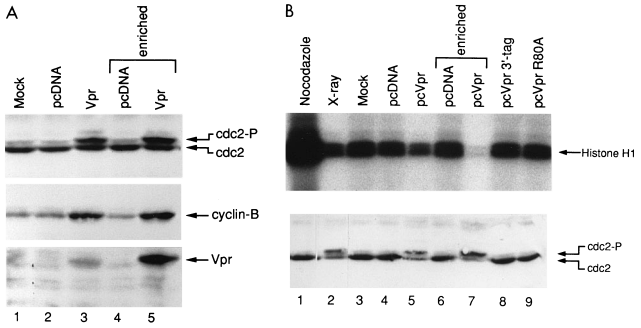

FIG. 3. Vpr increases $p34^{cdc2}$ phosphorylation and inhibits its kinase activity. (A) Immunoblot of HeLa cells transfected with pc-CD4 alone (lane 1), pcDNA (lanes 2 and 4), or pc-vpr (lanes 3 and 5). Filters were probed with anti-$p34^{cdc2}$ antibody (top), anti-cyclin B antibody, (middle) or anti-Vpr serum (bottom). In lanes 4 and 5, the transfected cells were enriched by binding to anti-CD4-coated magnetic beads. $p34^{cdc2}$ appears as a doublet, corresponding to phosphorylated (upper band) and unphosphorylated (lower band) forms (21). (B) Histone H1 kinase activity (top panel) of $p34^{cdc2}$/cyclin B complex immunoprecipitated from HeLa cells transfected with pc-CD4 (25) and the indicated plasmids (lanes 3 to 9). pc-3′-tag-vpr encodes Vpr with a carboxy-terminal HA-tag (lane 8), and pc-tag-vprR80A (lane 9) encodes Vpr containing an Arg-80→Ala mutation. The kinase activities of the $p34^{cdc2}$/cyclin B complex from nocodazole-treated (M-phase) and X-irradiated (G_2-phase) cells are shown for comparison. An immunoblot probed with anti-$p34^{cdc2}$ (bottom panel) shows correlation of the upper $p34^{cdc2}$ band with kinase inhibition. Reactions were analyzed by autoradiography after separation by SDS-PAGE.

top panel). Cells enriched for Vpr expression contained p34^{cdc2}/cyclin complexes which were almost completely inactive in the kinase assay (Fig. 3B, lane 7). These finding are consistent with the increased phosphorylation state of p34^{cdc2} described above (Fig. 3A). Decreased p34^{cdc2} activity could not be attributed to a decrease in p34^{cdc2} or cyclin B levels, since immunoblot analysis of these proteins in lysates of the transfected cells showed that p34^{cdc2} remained constant and cyclin B levels increased slightly (Fig. 3). Instead, decreased kinase activity correlated with increased p34^{cdc2} phosphorylation (Fig. 3B, bottom panel). For comparison, we assayed the p34^{cdc2} kinase activities of cells blocked in M phase with nocodazole and in G_2 with X irradiation. These cells showed the respective increased and decreased p34^{cdc2} kinase activities characteristic of cells in these phases of the cell cycle (Fig. 3B, lanes 1 and 2). Vpr appeared to decrease p34^{cdc2}/cyclin B complex activity more effectively than X irradiation. In cells expressing mutant Vpr molecules that are stably expressed but inactive in cell cycle arrest (12), p34^{cdc2} remained largely dephosphorylated and retained its kinase activity (Fig. 3B, lanes 8 and 9). This finding further strengthened the correlation between Vpr-induced cell cycle arrest and p34^{cdc2} inhibition. Taken together, these findings strongly suggested that Vpr inhibits p34^{cdc2}/cyclin B kinase activity by influencing its phosphorylation at the inhibitory sites (by either increasing the rate of phosphorylation or decreasing the rate of dephosphorylation). Because p34^{cdc2} activation is required for progression from G_2 to M phase, this would lead to the observed G_2 arrest.

G_2 arrest is caused by Vpr-induced inhibition of p34^{cdc2}. To test the hypothesis that p34^{cdc2}/cyclin B complex inactivation accounts for Vpr-induced G_2 arrest, we attempted to override the arrest by coexpressing Vpr and constitutively active mutant p34^{cdc2} molecules. These molecules lack one or both of the inhibitory phosphorylation sites and display increased kinase activity (16, 22). They would not be expected to be subject to Vpr-induced inhibition since they cannot be inactivated by phosphorylation. We therefore cotransfected cells with HA-tagged Vpr expression vector and vectors expressing wild-type p34^{cdc2} and p34^{cdc2} lacking one (Tyr-15→Phe) or both (Tyr-15→Phe/Thr-14→Ala) inhibitory phosphorylation sites. FACS analysis of the transfected cells showed that coexpression of doubly mutated p34^{cdc2} restored the G_2/M:G_1 ratio to close to that of control cells. In contrast, coexpression of wild-type p34^{cdc2} had a small effect, and coexpression of singly mutated p34^{cdc2} (Tyr-15→Phe) partially restored the G_2/M:G_1 ratio (Fig. 4). Immunoblot analysis confirmed that Vpr and p34^{cdc2} proteins had been expressed in the transfected cells (Fig. 4B). Thus, expression of constitutively active p34^{cdc2} overrides Vpr-induced cell cycle arrest. This finding strongly suggested that Vpr induces G_2 arrest by inhibiting p34^{cdc2}/cyclin B kinase activity. Whether this is through a direct interaction of Vpr with p34^{cdc2}/cyclin B or is an indirect effect resulting from an interaction with an upstream regulator of p34^{cdc2}, such as Wee1 kinase or Cdc25 phosphatase (9), is not known.

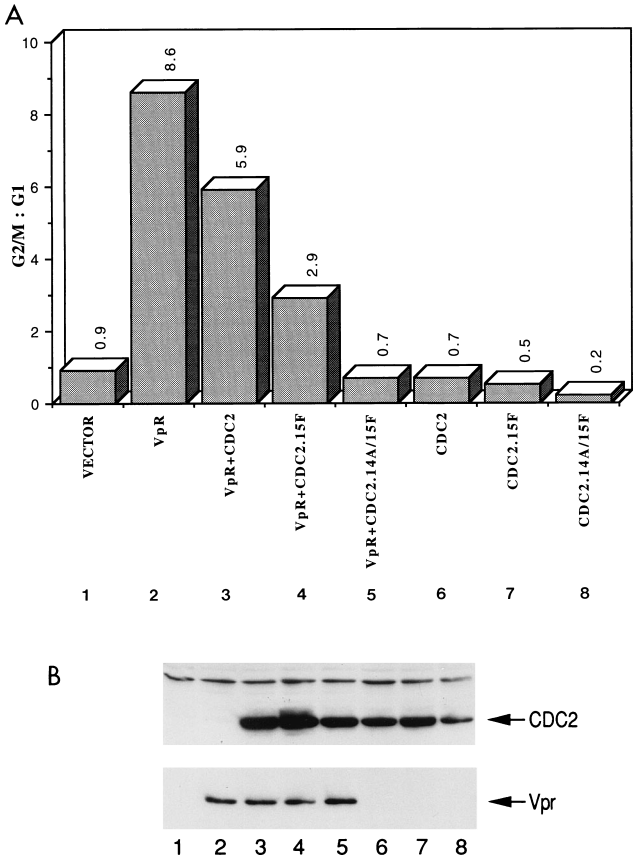

FIG. 4. Expression of constitutively active p34^{cdc2} mutants (Tyr-15→Phe and Tyr-15→Phe/Thr-14→Ala) overrides Vpr-induced cell cycle arrest. (A) Cells were transfected with pcDNA (column 1), pc-5′-tag-vpr (column 2), pc-5′-tag-vpr and pSR-Cdc2 (column 3), pc-5′-tag-vpr and pSR-Cdc2.15F (column 4), pc-5′-tag-vpr and pSR-Cdc2.14A15F (column 5), pSR-Cdc2 (column 6), pSR-Cdc2.15F (column 7), and pSR-Cdc2.14A15F (column 8). pc-CD4 (2 μg) was added to each transfection, and pcDNA was added where necessary to maintain a constant total amount (20 μg) of DNA. HA-tagged Vpr and p34^{cdc2} expression vectors were mixed in equimolar ratios. Cell cycle distribution was determined by FACS 3 days posttransfection. (B) Immunoblots with anti-HA antibody showing expression of HA-tagged p34^{cdc2} and Vpr in the transfected cells described above. Data shown are representative of four independent repetitions of the experiment.

DISCUSSION

We confirm here that human cells transfected with HIV-1 Vpr expression vectors are arrested in G_2 of the cell cycle. In addition, cells infected with HIV-1 are arrested in G_2 as a result of Vpr expression. Mitotic index analysis showed that Vpr expression arrests cells in G_2 and does not permit entry into M phase. Our investigations into the mechanism by which Vpr induces G_2 arrest showed that Vpr expression in transfected cells results in a large increase in the fraction of p34^{cdc2} molecules that are phosphorylated at the inhibitory phosphorylation sites. Vpr expression was also accompanied by a dramatic inhibition of p34^{cdc2}/cyclin B kinase activity. Importantly, we showed that this inhibition appears to account for the cell cycle block, since expression of constitutively active p34^{cdc2} mutants relieved the Vpr-induced G_2 arrest. Taken together, our results suggest that Vpr blocks the dephosphorylation of p34^{cdc2} that normally occurs late in G_2 and in early M phase. Since dephosphorylation is required to activate p34^{cdc2}/cyclin B kinase activity, and its activation is required for progression of the cell cycle from G_2 to M phase, the cells arrest in G_2.

Our experiments do not address the question of whether the effect of Vpr on p34^{cdc2} is direct or indirect. Vpr might bind to p34^{cdc2}, preventing its dephosphorylation. However, our attempts to detect binding of p34^{cdc2} to recombinant Vpr and to show coimmunoprecipitation of Vpr with p34^{cdc2} have been unsuccessful (unpublished observations). Alternatively, Vpr might interact with one of the molecules that regulates p34^{cdc2} phosphorylation. Two of the well-known regulators are Cdc25, a phosphatase that activates p34^{cdc2}, and Wee1, a kinase that inactivates it (9). To prevent p34^{cdc2} activation, Vpr could block Cdc25 or induce Wee1 activity.

The selective advantage provided to the virus by arresting cells in G_2 is not known; however, there are several possibilities. First, blocked cells may produce more virus. This might be the case if transcription factors required for expression of the provirus were more abundant in this phase of the cell cycle. Second, by preventing cell division, Vpr might block the clonal expansion of anti-HIV-specific T cells in an infected individual, dampening the antiviral immune response. However, this is likely to have limited effectiveness since infected cells probably constitute a small part of the antiviral immune response. Furthermore, in vivo, infected cells generally die shortly after infection (18). Third, Vpr expression might be a mechanism for preventing apoptosis or decreasing the rate at which infected cells undergo apoptosis. HIV-1 infection has been shown to induce apoptosis, resulting in a reduction in the total amount of virus that an infected cell produces (3). Delaying the onset of apoptosis would result in an increase in the amount of virus produced by each infected cell. Interestingly, the T cells of HIV-1-infected individuals are more susceptible to apoptosis induction than are those of uninfected individuals (15). It may be that the T cells of an infected individual are primed for rapid apoptosis following infection. Such a virally encoded activity is not unprecedented. Several viruses encode products that modulate apoptosis (reviewed in reference 39). Notably, adenovirus encodes E1B, a protein whose function is to prevent apoptosis that is induced by a second viral protein, E1A (42). In addition, it has recently been shown that HIV-1 gp120 and Tat sensitize T cells to Fas-induced apoptosis (41). It is possible that Vpr evolved to reduce their effects on cell viability.

Induction of apoptosis appears to require progression through the cell cycle (5, 43). For example, arresting a human T-cell line in G_1/S by treatment with aphidicolin prevented the induction of apoptosis following T-cell receptor cross-linking. At least one pathway by which apoptosis is induced involves the premature activation of $p34^{cdc2}$ (27, 40). Expression of Wee1, an enzyme that phosphorylates $p34^{cdc2}$, prevents the induction of apoptosis through this pathway (7). By analogy, Vpr might similarly block apoptosis by preventing $p34^{cdc2}$ activation.

One of the mechanisms by which cytotoxic T lymphocytes (CTL) kill infected target cells is by inducing their apoptosis. CTL-induced apoptosis is, at least in part, mediated by granzymes that activate $p34^{cdc2}$, resulting in a mitotic catastrophe (40; reviewed in reference 14). It has been shown that CTL are less effective in killing noncycling than cycling cells (30, 40). Thus, by blocking the premature activation of $p34^{cdc2}$, Vpr might increase the resistance of infected cells to lysis by CTL.

Protecting infected cells from CTL lysis or from HIV-1-induced apoptosis would not require that the cells be arrested in G_2; it would require only that $p34^{cdc2}$ activation be prevented. Indeed, we found that a large proportion of HIV-1-infected cells were not arrested in G_2. Infected cells not yet arrested in G_2 by Vpr, but in which $p34^{cdc2}$ was held inactive, could still be protected. This hypothesis would provide a rationale for the presence of Vpr in virions. The encapsidated Vpr molecules might, upon introduction into target cells via newly entered virions, provide a block to $p34^{cdc2}$ activation early in infection, prior to the synthesis of new Vpr.

ACKNOWLEDGMENTS

J.H. and S.C. contributed equally to this work.

We thank John Hirst for FACS analysis; Benjamin Chen for constructing pBABE-vpr; Dan Littman and Steven Fabian for the initial HIV-HSA plasmid; Yong Gu for mutant $p34^{cdc2}$ expression vectors; J. Bedford for technical advice; Wendy Chen for graphics; and David Ho, Dan Littman, and Robert Fisher for critical reading of the manuscript.

This work was supported by the Aaron Diamond Foundation and Center for AIDS Research at NYU Medical Center and National Institutes of Health grant R29AI36057 and by a Lotta all' AIDS grant to Paola Di Marzio from the Italian Ministry of Health.

ADDENDUM IN PROOF

Jowett et al. (19a) have shown that Vpr arrests HIV-1-infected cells in G_2/M.

REFERENCES

1. **Adachi, A., N. Ono, H. Sakai, K. Ogawa, R. Shibata, T. Kiyomasu, H. Masuike, and S. Ueda.** 1991. Generation and characterization of the human immunodeficiency virus type 1 mutants. Arch. Virol. **117:**45–58.
2. **Akari, H., J. Sakuragi, Y. Takebe, K. Tomonaga, M. Kawamura, M. Fukasawa, T. Miura, T. Shinjo, and M. Hayami.** 1992. Biological characterization of human immunodeficiency virus type 1 and type 2 mutants in human peripheral blood mononuclear cells. Arch. Virol. **123:**157–167.
3. **Antoni, B. A., P. Sabbatini, A. B. Rabson, and E. White.** 1995. Inhibition of apoptosis in human immunodeficiency virus-infected cells enhances virus production and facilitates persistent infection. J. Virol. **69:**2384–2392.
4. **Balliet, J. W., D. L. Kolson, G. Eiger, F. M. Kim, K. A. McGann, A. Srinivasan, and R. Collman.** 1994. Distinct effects in primary macrophages and lymphocytes of the human immunodeficiency virus type 1 accessory genes vpr, vpu, and nef: mutational analysis of a primary HIV-1 isolate. Virology **200:**623–631.
5. **Boehme, S., and M. J. Lenardo.** 1993. Ligand-induced apoptosis of mature T lymphocytes (propriocidal regulation) occurs at distinct stages of the cell cycle. Leukemia Suppl. **2:**45–49.
6. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
7. **Chen, G., L. Shi, D. W. Litchfield, and A. H. Greenberg.** 1995. Rescue from granzyme B-induced apoptosis by Wee1 kinase. J. Exp. Med. **181:**2295–2300.
8. **Cohen, E. A., E. F. Terwilliger, Y. Jalinoos, J. Proulx, J. G. Sodroski, and W. A. Haseltine.** 1990. Identification of HIV-1 vpr product and function. J. Acquired Immune. Defic. Syndr. **3:**11–18.
9. **Coleman, T. R., and W. G. Dunphy.** 1994. Cdc2 regulatory factors. Curr. Opin. Cell. Biol. **6:**877–882.
10. **Connor, R. I., B. K. Chen, S. Choe, and N. R. Landau.** 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology **206:**936–944.
11. **Dedera, D., W. Hu, H. N. Vander, and L. Ratner.** 1989. Viral protein R of human immunodeficiency virus types 1 and 2 is dispensable for replication and cytopathogenicity in lymphoid cells. J. Virol. **63:**3205–3208.
12. **Di Marzio, P., S. Choe, and N. R. Landau.** Mutagenic analysis of cell cycle arrest, nuclear localization, and virion packaging of human immunodeficiency virus type 1 Vpr. J. Virol., in press.
13. **Gibbs, J. S., A. A. Lackner, S. M. Lang, M. A. Simon, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1995. Progression to AIDS in the absence of a gene for *vpr* or *vpx*. J. Virol. **69:**2378–2383.
14. **Greenberg, A. H., and D. W. Litchfield.** 1995. Granzymes and apoptosis: targeting the cell cycle. Curr. Top. Microbiol. Immunol. **198:**95–119.
15. **Groux, H., G. Torpier, D. Monte, Y. Mouton, A. Capron, and J. C. Ameisen.** 1992. Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals. J. Exp. Med. **175:**331–340.
16. **Gu, Y., J. Rosenblatt, and D. O. Morgan.** 1992. Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. EMBO J. **11:**3995–4005.
17. **Heinzinger, N. K., M. I. Bukrinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman.** 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. USA **91:**7311–7315.
18. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature (London) **373:**123–126.
19. **Jha, M. N., J. R. Bamburg, and J. S. Bedford.** 1994. Cell cycle arrest by colcemid differs in human normal and tumor cells. Cancer Res. **54:**5011–5015.

19a. **Jowett, J. B. M., V. Planelles, B. Poon, N. P. Shah, M.-L. Chen, and I. S. Y. Chen.** 1995. The human immunodeficiency virus type 1 *vpr* gene arrests infected T cells in the G_2 + M phase of the cell cycle. J. Virol. **69:**6304–6313.

20. **Krek, W., J. Marks, N. Schmitz, E. A. Nigg, and V. Simanis.** 1992. Vertebrate p34cdc2 phosphorylation site mutants: effects upon cell cycle progression in the fission yeast Schizosaccharomyces pombe. J. Cell Sci. **102:**43–53.
21. **Krek, W., and E. A. Nigg.** 1991. Differential phosphorylation of vertebrate $p34^{cdc2}$ kinase at the G_1, S and G_2/M transitions of the cell cycle: identification of major phosphorylation sites. EMBO J. **10:**305–316.
22. **Krek, W., and E. A. Nigg.** 1991. Mutations of $p34^{cdc2}$ phosphorylation sites

induce premature mitotic events in HeLa cells: evidence for a double block to p34^{cdc2} kinase activation in vertebrates. EMBO J. **10:**3331–3341.
23. **Landau, N. R., K. A. Page, and D. R. Littman.** 1991. Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. J. Virol. **65:**162–169.
24. **Lang, S. M., M. Weeger, H. C. Stahl, C. Coulibaly, G. Hunsmann, J. Muller, H. H. Muller, D. Fuchs, H. Wachter, M. M. Daniel, R. C. Desrosiers, and B. Fleckenstein.** 1993. Importance of *vpr* for infection of rhesus monkeys with simian immunodeficiency virus. J. Virol. **67:**902–912.
25. **Lenburg, M. E., and N. R. Landau.** 1993. Vpu-induced degradation of CD4: requirement for specific amino acid residues in the cytoplasmic domain of CD4. J. Virol. **67:**7238–7245.
26. **Levy, D. N., L. S. Fernandes, W. V. Williams, and D. B. Weiner.** 1993. Induction of cell differentiation by human immunodeficiency virus 1 vpr. Cell **72:**541–550.
27. **Meikrantz, W., S. Gisselbrecht, S. W. Tam, and R. Schlegel.** 1994. Activation of cyclin A-dependent protein kinase during apoptosis. Proc. Natl. Acad. Sci. USA **91:**3754–3758.
28. **Morgan, D. O.** 1995. Principles of CDK regulation. Nature (London) **374:** 131–134.
29. **Morgenstern, J. P., and H. Land.** 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. **18:**3587–3596.
30. **Nishioka, W. K., and R. M. Welsh.** 1994. Susceptibility to cytotoxic T lymphocyte-induced apoptosis is a function of the proliferative status of the target. J. Exp. Med. **179:**769–774.
31. **Norbury, C. J., and P. Nurse.** 1989. Control of the higher eukaryote cell cycle by p34cdc2 homologues. Biochim. Biophys. Acta **989:**85–89.
32. **Ogawa, K., R. Shibata, T. Kiyomasu, I. Higuchi, Y. Kishida, A. Ishimoto, and A. Adachi.** 1989. Mutational analysis of the human immunodeficiency virus *vpr* open reading frame. J. Virol. **63:**4110–4114.
33. **Paxton, W., R. I. Connor, and N. R. Landau.** 1993. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of *gag* and mutational analysis. J. Virol. **67:**7229–7237.
34. **Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore.** 1994. Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA **90:**8392–8396.
35. **Pirruccello, S. J., and T. W. LeBien.** 1986. The human B cell-associated antigen CD24 is a single chain sialoglycoprotein. J. Immunol. **136:**3779–3784.
36. **Rogel, M. E., L. I. Wu, and M. Emerman.** 1995. The human immunodeficiency virus type 1 *vpr* gene prevents cell proliferation during chronic infection. J. Virol. **69:**882–888.
37. **Rosenblatt, J., Y. Gu, and D. O. Morgan.** 1992. Human cyclin-dependent kinase 2 is activated during the S and G2 phases of the cell cycle and associates with cyclin A. Proc. Natl. Acad. Sci. USA **89:**2824–2828.
38. **Schmid, I., C. H. Uittenbogaart, and J. V. Giorgi.** 1991. A gentle fixation and permeabilization method for combined cell surface and intracellular staining with improved precision in DNA quantification. Cytometry **12:**279–285.
39. **Shen, Y., and T. E. Shenk.** 1995. Viruses and apoptosis. Curr. Opin. Genet. Dev. **5:**105–111.
40. **Shi, L., W. K. Nishioka, J. Th'ng, E. M. Bradbury, D. W. Litchfield, and A. H. Greenberg.** 1994. Premature p34^{cdc2} activation required for apoptosis. Science **263:**1143–1145.
41. **Westendorp, M. O., F. R., C. Ochsenbauer, K. Stricker, J. Dhein, H. Walczak, K. M. Debatin, and P. H. Krammer.** 1995. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. Nature (London) **375:** 497–500.
42. **White, E., P. Sabbatini, M. Debbas, W. S. Wold, D. I. Kusher, and L. R. Gooding.** 1992. The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor alpha. Mol. Cell. Biol. **12:**2570–2580.
43. **Zhu, L., and C. Anasetti.** 1995. Cell cycle control of apoptosis in human leukemic T cells. J. Immunol. **154:**192–200.