JOURNAL OF VIROLOGY, Apr. 1996, p. 2516–2524 Vol. 70, No. 4
0022-538X/96/$04.00+0

Vpr-Induced Cell Cycle Arrest Is Conserved among Primate Lentiviruses

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Received 25 September 1995/Accepted 20 December 1995

We previously reported that expression of human immunodeficiency virus type 1 strain NL4-3 (HIV-1_{NL4-3}) *vpr* causes cells to arrest in the G_2 phase of the cell cycle. We examined the induction of cell cycle arrest by other HIV-1 isolates and by primate lentiviruses other than HIV-1. We demonstrate that the *vpr* genes from tissue culture-adapted or primary isolates of HIV-1 are capable of inducing G_2 arrest. In addition, we demonstrate that induction of cell cycle arrest is a conserved function of members of two other groups of primate lentiviruses, HIV-2/simian immunodeficiency virus strain sm (SIV_{sm})/SIV_{mac} and SIV_{agm}. *vpr* from HIV-1, HIV-2, and SIV_{mac} induced cell cycle arrest when transfected in human (HeLa) and monkey (CV-1) cells. *vpx* from HIV-2 and SIV_{mac} did not induce detectable cell cycle arrest in either cell type, and SIV_{agm} *vpx* was capable of inducing arrest in CV-1 but not HeLa cells. These results indicate that induction of cell cycle perturbation is a general property of lentiviruses that infect primates. The conservation of this viral function throughout evolution suggests that it plays a key role in virus-host relationships, and elucidation of its mechanism may reveal important clues about pathology induced by primate lentiviruses.

Human immunodeficiency virus type 1 (HIV-1) is a member of a group of lentiviruses that infect humans as well as nonhuman primates (simian immunodeficiency viruses [SIVs]). Five distinct phylogenetic lineages of primate lentiviruses have now been identified: HIV-1/SIV_{cpz}, HIV-2/SIV_{sm}/SIV_{mac}, SIV_{agm}, SIV_{mnd}, and SIV_{syk} (6, 16, 35). HIV-1 and HIV-2 are the etiologic agents for AIDS in humans. SIVs have been isolated from several species of African monkeys in the wild, in which no virus-associated disease has so far been identified, and may cause acquired immunodeficiency syndromes which are similar to AIDS in humans when experimentally or accidentally inoculated into several species of Asian macaques (6, 15, 16). Therefore, primate lentiviruses constitute valuable animal models for the study of virus-induced acquired immunodeficiency syndromes.

HIV-1 is a complex retrovirus which encodes, in addition to the structural genes *gag*, *pol*, and *env*, six accessory genes named *vif*, *vpr*, *tat*, *rev*, *vpu*, and *nef*. The role of the accessory genes in HIV-1-induced pathogenesis is an active area of investigation. Recently, the HIV-1 *vpr* gene has been reported to induce cell growth inhibition (19, 24, 29, 33). Characterization of the growth-inhibitory effect of Vpr demonstrated an increase in the proportion of cells in the gap-2 (G_2) or mitosis (M) phase of the cell cycle in those cells expressing this gene (12, 19, 31, 33). We showed that this perturbation was due to cell cycle arrest at the G_2/M boundary (G_2 arrest) (19). This arrest correlates with inactivation of CDC2 kinase (12, 19, 31), the major regulatory kinase involved in transit from G_2 to M (25). Therefore, it appears that arrest is in G_2 rather than M. Although the functionality and fate of infected cells arrested in G_2 has not yet been determined, it is likely that the effect of *vpr* on the cell cycle perturbs the immune function of infected T cells. Therefore, we have proposed that *vpr* is a key determinant of viral pathogenesis in HIV-1 infection (19). Here we extend our earlier studies to examine cell cycle arrest for other primate lentiviruses, using approaches similar to those described above.

The *vpr* gene is present in HIV-1/SIV_{cpz}, HIV-2/SIV_{sm}/SIV_{mac}, SIV_{mnd}, and SIV_{syk}. Members of the HIV-2/SIV_{sm}/SIV_{mac} group contain an additional gene called *vpx*. *vpx* and *vpr* from HIV-2/SIV_{sm}/SIV_{mac} are thought to have arisen as a duplication of a common ancestor, because they share sequence and functional conservation and are located in tandem in the retroviral genome (37). SIV_{agm} encodes a single gene, named *vpx*, that has sequence homology with both *vpr* and *vpx* (7, 37).

This study compares the *vpr* and/or *vpx* genes from the lentiviruses HIV-1, HIV-2, SIV_{mac}, and SIV_{agm} in the context of their abilities to induce G_2 arrest in primate cells. Our investigations show that expression of *vpr* but not *vpx* from HIV-2 and SIV_{mac} and *vpx* from SIV_{agm} induce G_2 arrest in cells from primates. These results indicate that induction of cell cycle arrest is a conserved function among the three groups of primate lentiviruses studied here. In addition, we demonstrate functional conservation of SIV_{agm} *vpx* with various *vpr* genes, which is consistent with the evolutionary relationships of HIV and SIV.

MATERIALS AND METHODS

Molecular cloning. A linker encoding the influenza virus hemagglutinin (HA) linear epitope consisting of nine amino acids, YPYDVPDYA (38), was constructed by synthesizing and hybridizing two oligonucleotides, 5′-AGCTTCA CCATGTACCCATACGATGTTCCAGATTACGCCAT-3′ and 5′-ATGGCGT AATCTGGAACATCGTATGGGTACATGGTGA-3′. The resulting linker was cloned into the Bluescript 2KS(+) vector (Stratagene, La Jolla, Calif.) previously digested with *Sma*I and *Hin*dIII to generate plasmid Blue-YPYDVPDYA.

The *vpr* and *vpx* genes (except SIV_{mac} *vpr*) from the viral molecular clones indicated below were amplified by PCR (Deep Vent; New England Biolabs, Beverly, Mass.) using the following oligonucleotide primers: HIV-1_{NL4-3} (1), HIV-1_{JR-FL} (21), and HIV-1_{JR-CSF} (21) *vpr*, sense orientation, 5′-CACCATG GAACAAGCCCCAGAAGAC-3′; HIV-1_{NL4-3}, HIV-1_{JR-FL}, and HIV-1_{JR-CSF} *vpr*, antisense, 5′-ATGGATCCAGGGCTCTAGTCTAGGATC-3′; HIV-2_{ROD} (10) *vpr*, sense, 5′-CACCATGGCTGAAGCACCAACAGAGC-3′; HIV-2_{ROD}

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vpr, antisense, 5′-ATGGATCCATGAGTTATTGCATGTTTCTAG-3′; HIV-2_{ROD} *vpx*, sense, 5′-CACCATGGCAGACCCCAGAGAGACAG-3′; HIV-2_{ROD} *vpx*, antisense, 5′-ATGGATCCATTAGACCAGACCTGGAGGG-3′ (this amplification resulted in substitution of threonine at position 2 for alanine); SIV_{mac239} (32) *vpx*, sense, 5′-ACATCATGACAGATCCCAGGGAGAGAATC C-3′; SIV_{mac239} *vpx*, antisense, 5′-ATGGATCCATTTATGCTAGTCCTGG AG-3′ (this amplification resulted in substitution of serine at position 2 for threonine); SIV_{agm}gri677 (18) *vpx*, sense, 5′-ACATCATGACATCAGGAAGA GATCCAAGAGAAC-3′; and SIV_{agm}gri677 *vpx*, antisense, 5′-GATGGATCC TATGCAAGTCCTGGAGGAGGGGG-3′ (this amplification resulted in substitution of alanine at position 2 for threonine).

PCR products generated as described above were digested with either *Bsp*HI or *Nco*I and *Bam*HI and ligated to the Blue-YPYDVPDYA vector previously digested with *Nco*I and *Bam*HI. This resulted in generation of bacterial plasmids carrying *vpr* or *vpx* coding sequences preceded by the HA nonapeptide fused in frame at the amino terminus. These fusion constructs were then digested with *Cla*I and *Not*I, and the resulting fragments were ligated to the vector BSVprThy (19) previously digested with the same enzymes. The mammalian expression vector, BSVprThy, contains two identical transcriptional units consisting of the human cytomegalovirus immediate-early promoter and the siman virus 40 polyadenylation site (19), and these transcriptional units express HIV-1 Vpr and the cell surface marker Thy-1.

An SIV_{mac} *vpr* expression vector was constructed as follows. A linker made of two oligonucleotides, sense (5′-CATGAGCGAAGAAAGACCTCCAGAAAA TGAAGGACCACAAAGGGAACCATG-3′) and antisense (5′-GATCCATGG TTCCCTTTGTGGTCCTTCATTTTCTGGAGGTCTTTCTTCGCT-3′), was ligated to the Blue-YPYDVPDYA vector digested with *Nco*I and *Bam*HI to generate YPYDVPDYA-239Lin. Subsequently, a 350-bp fragment resulting from digestion of the pSS-UP vector with *Nco*I and *Bam*HI was ligated with pYPY-239Lin, which had been digested with the same restriction enzymes. pSS-UP was constructed by cloning an *Sph*I-to-*Sac*I fragment from SIV_{mac239} (32) into pUC18 (New England Biolabs), which was digested with the same restriction enzymes. Cloning of the linker described above resulted in insertion of a serine residue at position 2 of SIV_{mac} *vpr*. A *Cla*I-to-*Not*I 350-bp restriction fragment containing the SIV_{mac} *vpr* open reading frame preceded by the in-frame HA nonapeptide was then subcloned into the BSVprThy vector as described above for other *vpr* and *vpx* constructs.

The resulting vectors direct expression of two genes, *vpr* or *vpx* and murine *thy-1*. For simplicity, these vectors will be referred to with the virus isolate from which they originated, followed by "*vpr*" or "*vpx*."

HIV-1 *vpr*-FS was generated by digestion, end filling, and religation of the *Eco*RI site in the *vpr* coding sequence of the HIV-1 *vpr* vector (see above) and is analogous to the previously described BSVpr-XThy vector (19) except that it encodes the HA nonapeptide. Plasmid pCMV is an expression vector (30) that contains no protein-coding sequences within its mammalian transcriptional unit.

Cells. HeLa cells were cultured in Dulbecco modified Eagle medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% bovine serum (Gibco). CV-1 cells (ATCC catalog no. CCL-70) were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Gibco).

Mammalian cell transfections. Mammalian cell transfections were performed by electroporation as previously described (3). Briefly, 10^7 cells were trypsinized and resuspended in 0.5 ml of ice-cold electroporation buffer (20% fetal calf serum in 1× RPMI [Gibco]) mixed with 10 μg of plasmid DNA, transferred to an electroporation cuvette (Bio-Rad Laboratories [Hercules, Calif.] catalog no. 165-2088), and electroporated in a Gene-Pulser (Bio-Rad) set at 230 V and 960 μF. After electroporation, cells were transferred to a 75-cm^2 tissue culture flask and incubated in culture medium for 72 h.

Cell surface staining, quantification of DNA content, and flow cytometric analysis. A method for cell surface staining simultaneously with quantification of DNA content was adapted from the technique of Schmid et al. (34). Briefly, 10^6 cells were harvested 48 to 72 h posttransfection and resuspended in 100 μl of anti-Thy-1.2 monoclonal antibody conjugated to fluorescein isothiocyanate (Caltag, South San Francisco, Calif.), diluted 1/200 in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline [PBS] with 2% fetal calf serum and 0.01% sodium azide), and incubated for 20 min on ice. Cells were washed with FACS buffer, fixed in PBS with 0.3% paraformaldehyde for 1 h, washed again, and permeabilized in 0.2% Triton X-100 in PBS for 15 min. Cells were washed with PBS, resuspended in FACS buffer containing propidium iodide (10 μg/ml) and DNase-free RNase A (11.25 Kunitz units), and kept on ice until analysis by flow cytometry. At least 2,500 events were collected for flow cytometry. Data acquisition and analysis were performed with Lysis II and Cellfit software (Becton Dickinson, San Jose, Calif.), respectively, with the doublet discrimination module activated. Samples were gated to exclude debris and clumps, and electronic compensation was used to remove residual spectral overlap. Gate settings for distinction between Thy-1-negative and Thy-1-positive cells were based on control stained populations. The mathematical model RFIT was used to calculate the proportions of cells in the G_1, synthesis (S), and G_2+M phases of the cell cycle. For simplicity, G_1/G_2+M ratios have been provided.

Western blotting (immunoblotting). Cell extracts for Western blotting were prepared as follows. Cells (5×10^6) were lysed in Laemmli buffer containing 5% β-mercaptoethanol, kept at 100°C for 5 min, and centrifuged at 12,000 × *g* to remove cell debris. Supernatants were stored at −70°C or subjected to sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then blotted onto a nitrocellulose membrane (Hybond-ECL; Amersham, Arlington Heights, Ill.). The membrane was first incubated with monoclonal antibody 12CA5 (38) and then with goat anti-mouse antibody conjugated to horseradish peroxidase, and proteins were visualized by an enhanced chemiluminescence detection system (Amersham).

RESULTS

Induction of G_2 arrest by *vpr* alleles from diverse HIV-1 isolates. We previously described a transient transfection assay for the study of the influence of *vpr* in the cell cycle (19). This assay is based on the use of an expression vector carrying two independent transcriptional units that direct expression of *vpr* and the reporter gene, *thy-1*, respectively. We previously demonstrated that expression of HIV-1_{NL4-3} *vpr* in human cells (peripheral blood mononuclear, SupT1, and HeLa) as well as cells from African green monkeys (CV-1 and COS) induced cell cycle arrest at the G_2/M boundary (19).

The *vpr* gene was engineered as a fusion protein with a nonapeptide (38) derived from influenza virus HA as a molecular tag to facilitate immunological detection, as previously described by Paxton et al. (28). Amino-terminal addition of the previous epitope tag onto Vpr did not affect virion encapsidation of the fusion protein, protein stability (28), or the ability of Vpr to induce G_2 arrest (reference 19 and data not shown). As a negative control, we engineered a parallel construct encoding a *vpr* mutant (*vpr*-FS) containing a frameshift at codon 64 (19, 29). The mutant construct *vpr*-FS was previously demonstrated not to cause G_2 arrest when introduced into cells either via transfection or via HIV-1 infection (19). HeLa cells transfected with the above-specified constructs were analyzed by direct immunofluorescence for Thy-1 surface expression and with propidium iodide for DNA content (see Materials and Methods). Cells were then analyzed by flow cytometry, and the cell cycle profile of transfected cells (Thy-1 positive) was directly compared with that of untransfected cells (Thy-1 negative) by establishing analysis gates as shown in Fig. 1A. Using this method, we can specifically examine the cell cycle profile of transfected cells expressing *vpr*, even when low efficiencies of transfection (1%), as measured by Thy-1 surface expression, are obtained. Expression of HIV-1_{NL4-3} *vpr* causes an increase in cells in the G_2 or M phase of the cell cycle (Fig. 1A) in Thy-1-positive cells, which is reflected in a decreased G_1/G_2+M ratio (0.2) compared with the ratio for Thy-1-negative cells from the same transfection (2.2), mock-transfected cells (2.8), or cells expressing *vpr*-FS (2.0).

In an effort to determine whether induction of G_2 arrest is a general feature among different HIV-1 isolates, we tested the cell cycle effect of *vpr* alleles from two other clones of HIV-1 (Fig. 1B). Whereas HIV-1_{NL4-3} is a recombinant molecular clone that is considered prototypical among T-cell line-adapted strains, HIV-1_{JR-CSF} and HIV-1_{JR-FL} are primary isolates that replicate efficiently in peripheral blood lymphocytes and monocyte-derived macrophages but not in transformed T-cell lines (21, 26). HIV-1_{JR-CSF} and HIV-1_{JR-FL} Vpr polypeptides differ from HIV-1_{NL4-3} Vpr in four and six amino acid positions, respectively (Fig. 2A). Flow cytometric analysis of HeLa cells transfected with various HIV-1 *vpr* expression constructs demonstrated that *vpr* alleles from HIV-1_{JR-CSF} and HIV-1_{JR-FL} are also capable of inducing G_2 arrest (Fig. 1B). Therefore, the ability of HIV-1 Vpr to induce cell cycle arrest is a general function that is common to a prototypical tissue culture-adapted HIV-1 isolate as well as to two primary isolates.

***vpr*-related genes from various primate lentiviruses induce G_2 arrest.** HIV-1 *vpr* has a significant degree of sequence

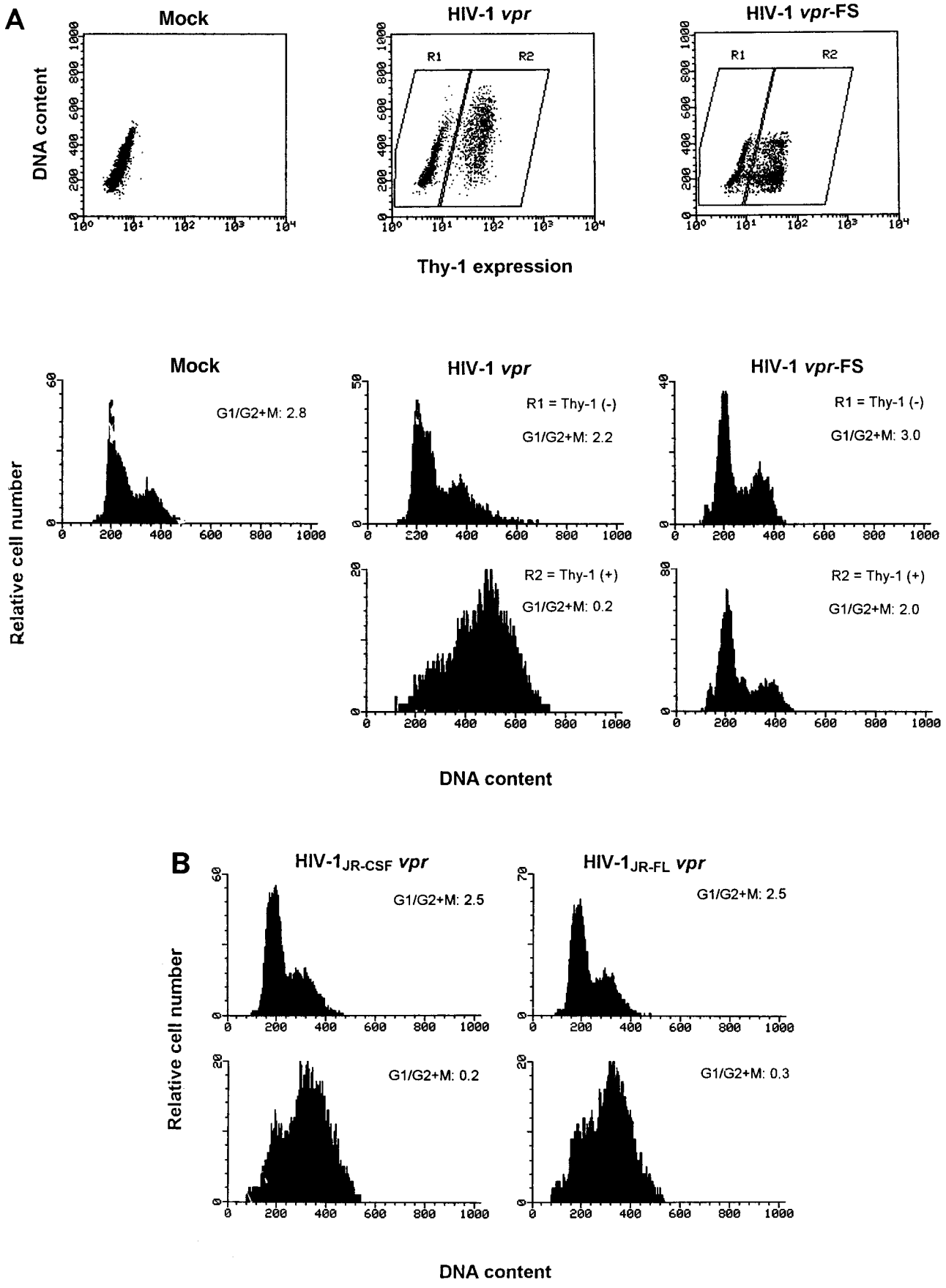
A
Mock
HIV-1 vpr
HIV-1 vpr-FS
DNA content
R1
R2
Thy-1 expression
Mock
HIV-1 vpr
HIV-1 vpr-FS
G1/G2+M: 2.8
R1 = Thy-1 (-)
G1/G2+M: 2.2
R1 = Thy-1 (-)
G1/G2+M: 3.0
R2 = Thy-1 (+)
G1/G2+M: 0.2
R2 = Thy-1 (+)
G1/G2+M: 2.0
Relative cell number
DNA content
B
HIV-1JR-CSF vpr
HIV-1JR-FL vpr
G1/G2+M: 2.5
G1/G2+M: 2.5
G1/G2+M: 0.2
G1/G2+M: 0.3
Relative cell number
DNA content

FIG. 1. Expression of *vpr* from various HIV-1 isolates causes G_2 or M arrest in HeLa cells. (A) HeLa cells (10^7) were transfected by electroporation with 10 μg of control expression vector, pCMV (mock) (30), or an expression vector encoding HIV-1_{NL4-3} *vpr* (HIV-1 *vpr*) or a truncated HIV-1_{NL4-3} *vpr* that contains a frameshift at codon 64 (HIV-1 *vpr*-FS) (19, 29). Expression vectors for *vpr* and *vpr*-FS contained two identical transcriptional units directing expression of *vpr* or *vpr*-FS, respectively, plus the murine surface antigen, Thy-1. Thy-1 is used as a reporter molecule for discrimination of transfected and untransfected cells. *vpr* and *vpr*-FS were expressed as fusion proteins with an amino-terminal influenza virus HA nonapeptide as described previously (28). At 48 h posttransfection, cells were simultaneously stained for surface expression of Thy-1 with fluorescein isothiocyanate-conjugated mouse anti-Thy-1.2-antibody and for DNA content with propidium iodide. Cells were then analyzed by flow cytometry using Lysis II for acquisition and Cellfit (Becton Dickinson) for quantitative analysis of DNA content. Regions for discrimination of Thy-1-negative (R1) and -positive (R2) populations were set as indicated. The G_1/G_2+M ratio is provided for simplicity. The proportion of cells in S phase ranged between 5 and 20%. Transfection efficiencies ranged between 40 and 60%, as measured by Thy-1 surface expression. (B) Expression vectors for *vpr* from HIV-1_{JR-FL} and HIV-1_{JR-CSF} similar to the one described for HIV-1_{NL4-3} were constructed and assayed in HeLa cells as described above.

conservation with *vpr* from other primate lentiviruses (Fig. 2B and C); for example, HIV-1 *vpr* shows 45% amino acid sequence identity with HIV-2 *vpr* and 52% amino acid identity with SIV_{mac} *vpr*. In addition, the Vpr proteins from these viruses can be found in virions interacting with Gag. In view of these structural similarities, we tested whether there was conservation of function among *vpr* genes from HIV-2_{ROD}, SIV_{mac239}, and HIV-1 in terms of cell cycle perturbation. We constructed expression vectors for these genes analogous to that described for HIV-1 *vpr* and studied the effect on the cell cycle of HeLa cells after transient transfection.

Thy-1-positive cells in populations that were transfected with HIV-2 or SIV_{mac} *vpr* showed an increase in cells in the G_2 or M phase of the cell cycle (G_1/G_2+M = 0.7 and 0.9, respectively; Fig. 3A) compared with Thy-1-negative cells from the same transfected population (G_1/G_2+M = 1.7 and 1.7, respectively) or cells transfected with *vpr*-FS (G_1/G_2+M = 2.0; Fig. 3A). A parallel transfection of HIV-1 *vpr* produced G_1/G_2+M ratios of 0.2 and 1.8 in Thy-1-positive and -negative cells, respectively. The effects of HIV-2 and SIV_{mac} *vpr* were not as pronounced as that of HIV-1 *vpr*, although a decrease in the G_1/G_2+M ratio was reproducibly observed (additional data not shown). Therefore, functional conservation was demonstrated among the *vpr* genes of HIV-1, HIV-2, and SIV_{mac}, as shown by their abilities to induce an abnormal increase in cells in G_2 or M.

Influence of *vpx* on the cell cycle of HeLa cells. HIV-1 *vpr* shows sequence conservation with *vpx*, an accessory gene present in HIV-2, SIV_{mac}, and SIV_{agm}. *vpx* from SIV_{agm} shares sequence conservation with *vpr* from HIV-1, HIV-2, and SIV_{mac} and with *vpx* from HIV-2 and SIV_{mac}. We investigated whether *vpx* from HIV-2, SIV_{mac}, and SIV_{agm} have functional homology with *vpr* in the context of induction of cell cycle perturbation (Fig. 3B). Expression of *vpx* from these three viruses did not result in any detectable effects on the cell cycle of HeLa cells when G_1/G_2+M ratios in Thy-1-positive and -negative cells were compared (Fig. 3B).

Influence of *vpr* and *vpx* on the cell cycle of African green monkey cells. The viruses chosen for this study infect various primate hosts. Therefore, it is conceivable that the abilities or inabilities of *vpr* and *vpx* to induce G_2 arrest are regulated by host-specific factors. To examine this, *vpr* and *vpx* constructs described above were tested in CV-1, which is a fibroblast cell line derived from African green monkey kidney. Expression of HIV-1, HIV-2, or SIV_{mac} *vpr* produced cell cycle arrest in CV-1 cells, although the proportion of cells arrested in G_2+M varied with the individual construct (Fig. 4). Expression of HIV-1 *vpr* in CV-1 produced a G_1/G_2+M ratio of 0.9, which is higher than the ratio previously observed in HeLa cells (0.2), indicating that HIV-1 *vpr* is more effective at inducing G_2 arrest in HeLa cells. Expression of HIV-2 and SIV_{mac} *vpr* in CV-1 cells produced G_1/G_2+M ratios of 1.2 and 0.9, respectively. These results support our previous conclusion, from studies using HeLa cells, that there is functional conservation among the *vpr* genes of HIV-1, HIV-2, and SIV_{mac}.

Constructs encoding *vpx* from HIV-2 or SIV_{mac} did not induce detectable cell cycle arrest when transfected into CV-1 cells (Fig. 4), consistent with the lack of effect observed in HeLa cells. Notably, expression of SIV_{agm} *vpx* had no detectable effect on the cell cycle profile of HeLa cells (Fig. 3B) but had a clear effect on CV-1 cells (Fig. 4). Expression of SIV_{agm} *vpx* induced a decrease in the G_1/G_2+M ratio of Thy-1-positive (0.4) compared with Thy-1-negative (1.7) cells. Therefore, there appear to be species-specific factors that mediate the abilities of *vpr* and *vpx* to interfere with the cell cycle. In addition, the finding that SIV_{agm} *vpx* induces G_2 arrest in CV-1 cells suggests that this gene is functionally related to *vpr*.

A

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1 MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETY 50
  ||||||||||||||||||||||||||||||| |||||||||||| ||| |||||
1 MEQAPEDQGPQREPYNEWTLELLEELKNEAVRHFPRIWLHSLGQYIYETY 50
  ||||||||||||||||||||||||||||||| |||||||||||| ||| |||||
1 MEQAPEDQGPQREPYNEWTLELLEELKNEAVRHFPRVWLHSLGQYIYETY 50

51 GDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS 96  HIV-1NL4-3 vpr
   |||||||||||||||||||||||||||||||| ||||||||||||||
51 GDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGITRQRRARNGASRS 96  HIV-1JR-CSF vpr
   |||||||||||||||||||||||||||||| ||||| ||||||||||||
51 GDTWAGVEAIIRILQQLLFIHFRIGCQHSRIGITRQRRARNGASRS 96  HIV-1JR-FL vpr
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B

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.....MEQAP EDQ.....GP QREPYNEWTL ELLEELKSEA VRHFPRIWLH
MAEAPTELPP VDG.....TP LREPGDEWII EILREIKEEA LKHFDPRLLI
....MEERPP ENE.....GP QREPWDEWVV EVLEELKEEA LKHFDPRLLT
MTDPRETVPP GNSGEETIG. ..EAF.AWLN RTVEAINREA VNHLPRELIF
MSDPRERIPP GNSGEETIG. ..EAF.EWLN RTVEEINREA VNHLPRELIF
MASGRDPREP LPGWLEIWDL DREPWDEWLQ DMLRDLNEEA RRHFGMNMLI

NLGQHIYE.. .TYGDTWAG. VE.AIIRILQ QLLFIHFRIG CRH....SRI
ALGKYIYT.. .RHGDTLEG. AR.ELIKVLQ RALFTHFRAG CGH....SRI
ALGNHIYN.. .RHGDTLEG. AG.ELIRILQ RALFMHFRGG CIH....SRI
QVWQRSWRYW HDEQGMSESY TKYRYLCIIQ KAVYMHVRKG CT...CLGRG
QVWQRSWEYW HDEQGMSPSY VKYRYLCLIQ KALFMHCKKG CR...CLGEG
RVWNYCVEEG RRHNTPWNE. IGYKYYRIVQ KSMFVHFRCG CRRRGPFSPY

GVTRQRRARN GASRS....  HIV-1NL4-3 vpr
GQTRGGNPLS AIPTPRNMQ  HIV-2ROD vpr
GQPGGGNPLS AIPPSRSML  SIVmac239 vpr
HGPGGWRPGP PPPPPPGLV  HIV-2ROD vpx
HGAGGWRPGP PPPPPPGLA  SIVmac239 vpx
EERRNGQGGG APPPPPGLA  SIVagm.gri677 vpx
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C

	HIV-1 *vpr*	HIV-2 *vpr*	SIVmac *vpr*	HIV-2 *vpx*	SIVmac *vpx*
HIV-2 *vpr*	45.26				
SIVmac *vpr*	51.58	71.72			
HIV-2 *vpx*	19.79	21.57	29.59		
SIVmac *vpx*	23.26	25.49	29.00	84.82	
SIVagm *vpx*	31.25	33.65	36.36	33.33	32.73

FIG. 2. Comparison of predicted amino acid sequences of *vpr* and *vpx* genes. (A) Alignment of *vpr* alleles from three HIV-1 isolates was performed by using Gap to establish pairwise comparisons (Wisconsin Sequence Analysis Package, version 8; Genetics Computer Group, Inc., Madison, Wis.), and sequences were compiled manually. Vertical lines denote residues that are identical to HIV-1_{NL4-3} *vpr*. (B) Alignment of *vpr* and *vpx* genes was performed by using Pileup (Wisconsin Sequence Analysis Package), using the molecular clones HIV-1_{NL4-3}, HIV-2_{ROD}, SIV_{mac239} and SIV_{agm}gri677. (C) Pairwise comparison in amino acid sequence identity among *vpr* and *vpx* genes was performed by using Bestfit (Wisconsin Sequence Analysis Package).

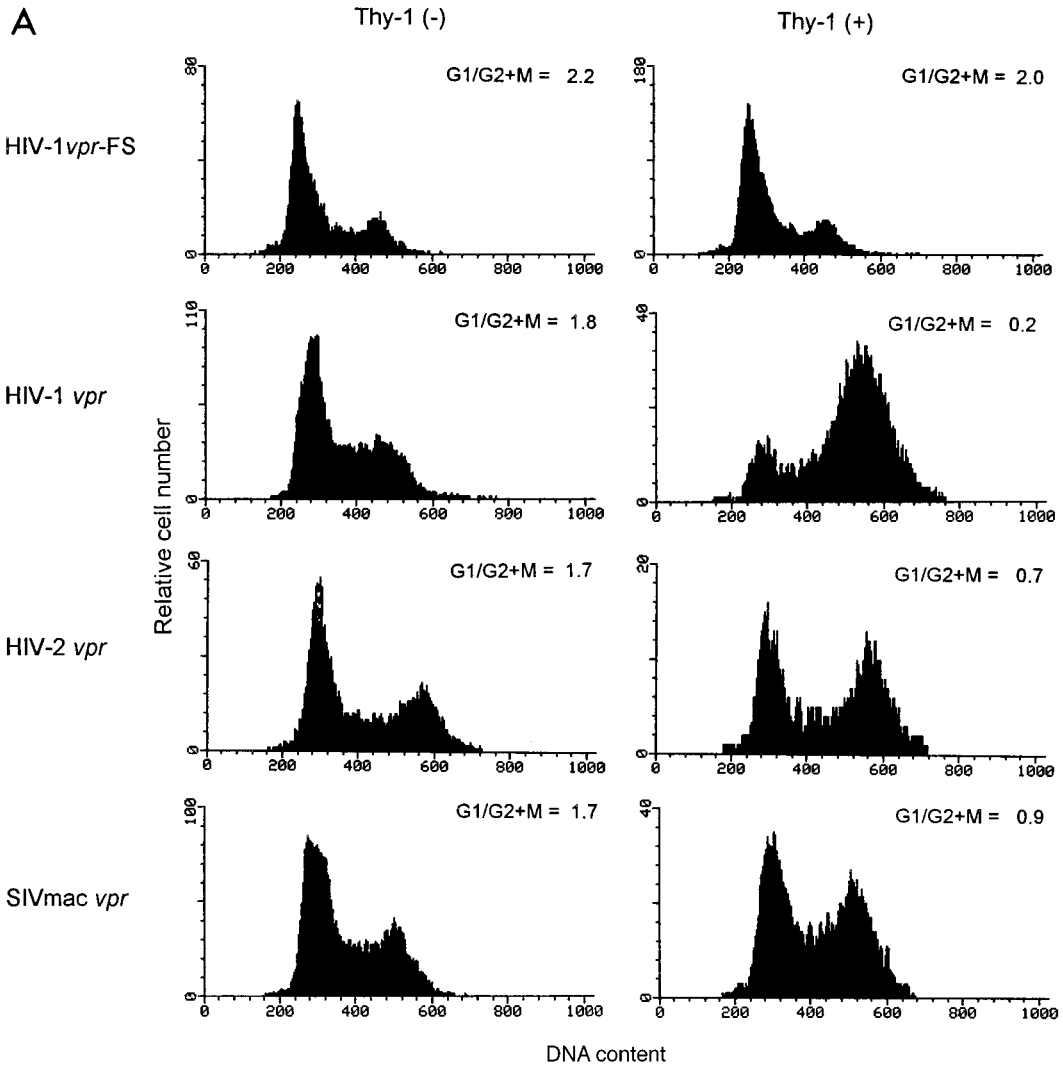

FIG. 3. Effects of *vpr* and *vpx* genes from various primate lentiviruses on the cell cycle of HeLa cells. Expression vectors for various *vpr* and *vpx* genes were constructed as described in Materials and Methods. Plasmid DNA (10 μg) was transfected into 10^7 HeLa cells, and the cell cycle of transfected cells was analyzed 72 h posttransfection as described for Fig. 1. Transfection efficiencies ranged between 40 and 60%, as measured by Thy-1 surface expression. (A) Transfection of various *vpr* genes; (B) transfection of various *vpx* genes. Note that HIV-1 *vpr*-FS and HIV-1 *vpr* are used as controls.

Immunological detection of Vpr and Vpx. We found quantitative differences among *vpr* and *vpx* when measuring effects on the cell cycle. In HeLa cells, HIV-1 *vpr* induced the most marked cell cycle disruption, HIV-2 and SIV_{mac} *vpr* induced an intermediate level of cell cycle disruption, and HIV-2, SIV_{mac}, and SIV_{agm} *vpx* produced no detectable effect. These differences could be due to quantitative differences in the functions of the encoded proteins; alternatively, these differences may be a reflection of levels of expression or biochemical stability of *vpr* and *vpx*. To exclude the latter possibility, extracts from HeLa cells transfected with the above-specified constructs were analyzed by Western blotting (28). All of the above-mentioned constructs except HIV-1 *vpr*-FS yielded proteins of the expected molecular masses (16 to 20 kDa) when analyzed by SDS-PAGE and immunoblotting (Fig. 5). The lack of a detectable protein produced by HIV-1 *vpr*-FS is consistent with previous studies by Paxton et al. (28), in which certain carboxy-terminal truncations of Vpr yielded biochemically unstable proteins. Therefore, we conclude that the failure of HIV-2, SIV_{mac}, and SIV_{agm} *vpx* to induce G_2 arrest in HeLa cells is not a consequence of lack of expression or biochemical stability of their gene products.

DISCUSSION

Induction of G_2 arrest is a general function of *vpr* from diverse HIV-1 isolates. HIV-1_{NL4-3} *vpr* was previously reported to induce cell cycle arrest at the G_2/M boundary of the cell cycle (19). HIV-1_{NL4-3} is a recombinant molecular clone (1) whose biological phenotype is that of a prototypical tissue culture-adapted isolate (26). We established the generality of the induction of G_2 arrest among different HIV-1 strains by showing that HIV-1 clones from two primary isolates, HIV-1_{JR-FL} and HIV-1_{JR-CSF} (21), encode *vpr* alleles that are capable of inducing cell cycle arrest in a manner which is indistinguishable from that of HIV-1_{NL4-3} *vpr*. Therefore, cell cycle arrest induced by *vpr* is a property shared by HIV-1 strains with diverse biological phenotypes.

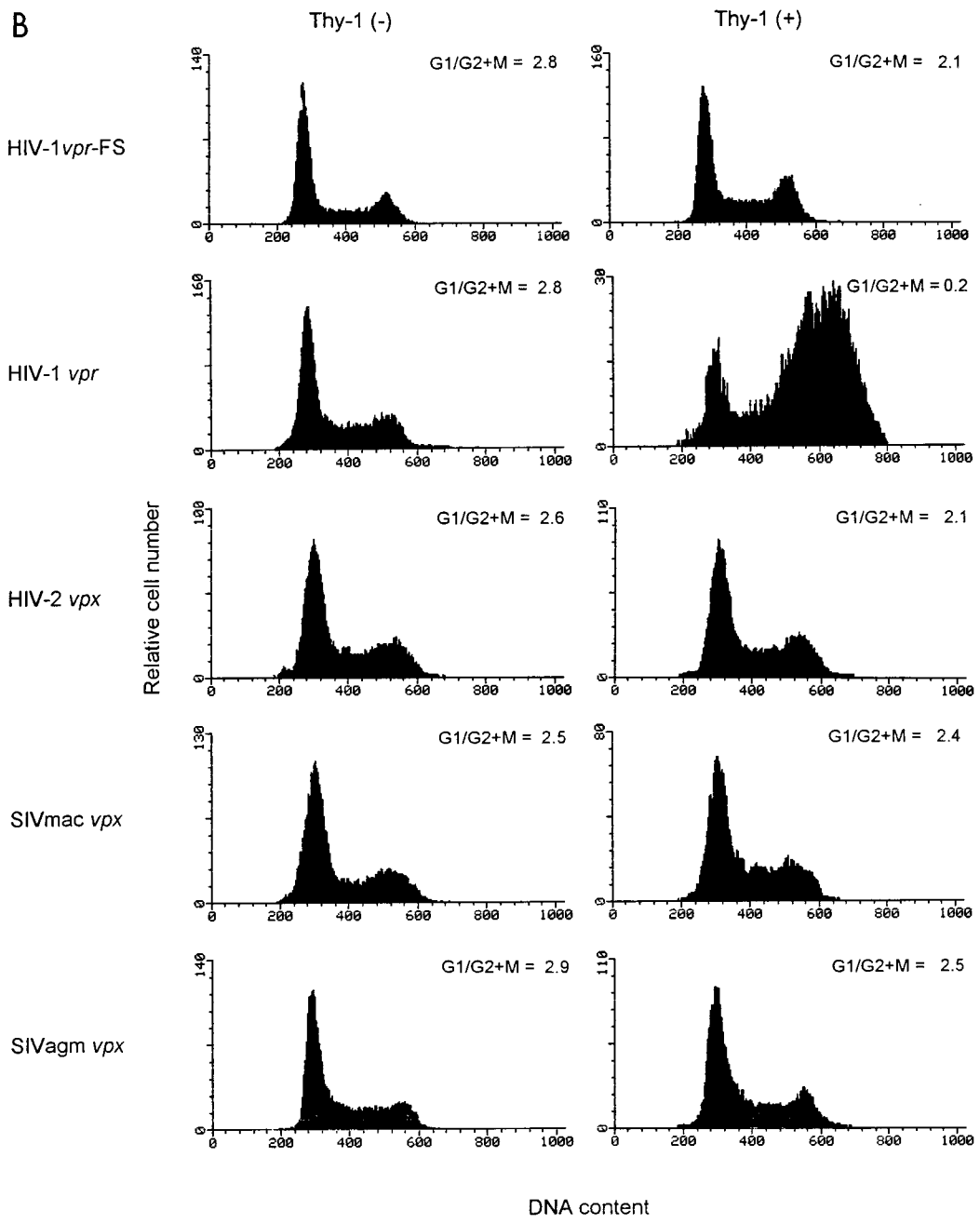

FIG. 3—*Continued*.

Biological functions of *vpr* and *vpx*. All known primate lentiviruses encode *vpr* and/or *vpx*. The *vpr* and *vpx* genes have predicted amino acid sequence homology and encode gene products that are virion encapsidated. In addition, it has been proposed that *vpr* and *vpx* arose as a duplication of an ancestral gene (37). Both of these genes are involved in multiple aspects of the biology of primate lentiviruses, although their precise roles in viral replication are still unclear. Deletion studies in vitro have shown that mutants of *vpr* and/or *vpx* are replication competent and exhibit an array of phenotypes depending on the virus subtype being studied. Deletion of HIV-1 or HIV-2 *vpr* results in viruses with slower replication kinetics in lymphoid cell lines and peripheral blood mononuclear cells but severely attenuated in their abilities to replicate in macrophages (2, 11). Deletion of *vpx* from HIV-2 yielded a virus that is severely attenuated for replication in peripheral blood mononuclear cells (9, 20). Mutation of *vpx* from SIV_{mac} causes significant decreases in virus production in primary cultures of human and rhesus alveolar macrophages (8, 42).

Vpr and Vpx were shown to be present in viral particles as a result of specific interactions with the Gag polypeptides (4, 14, 22, 28, 39, 41, 43). The presence of HIV-1 Vpr and the matrix protein in virions appears to determine nuclear transport of the preintegration complex in nondividing cells (2, 11, 13). This function may play an important role in HIV-1 establishing infection in nondividing cells such as macrophages. In

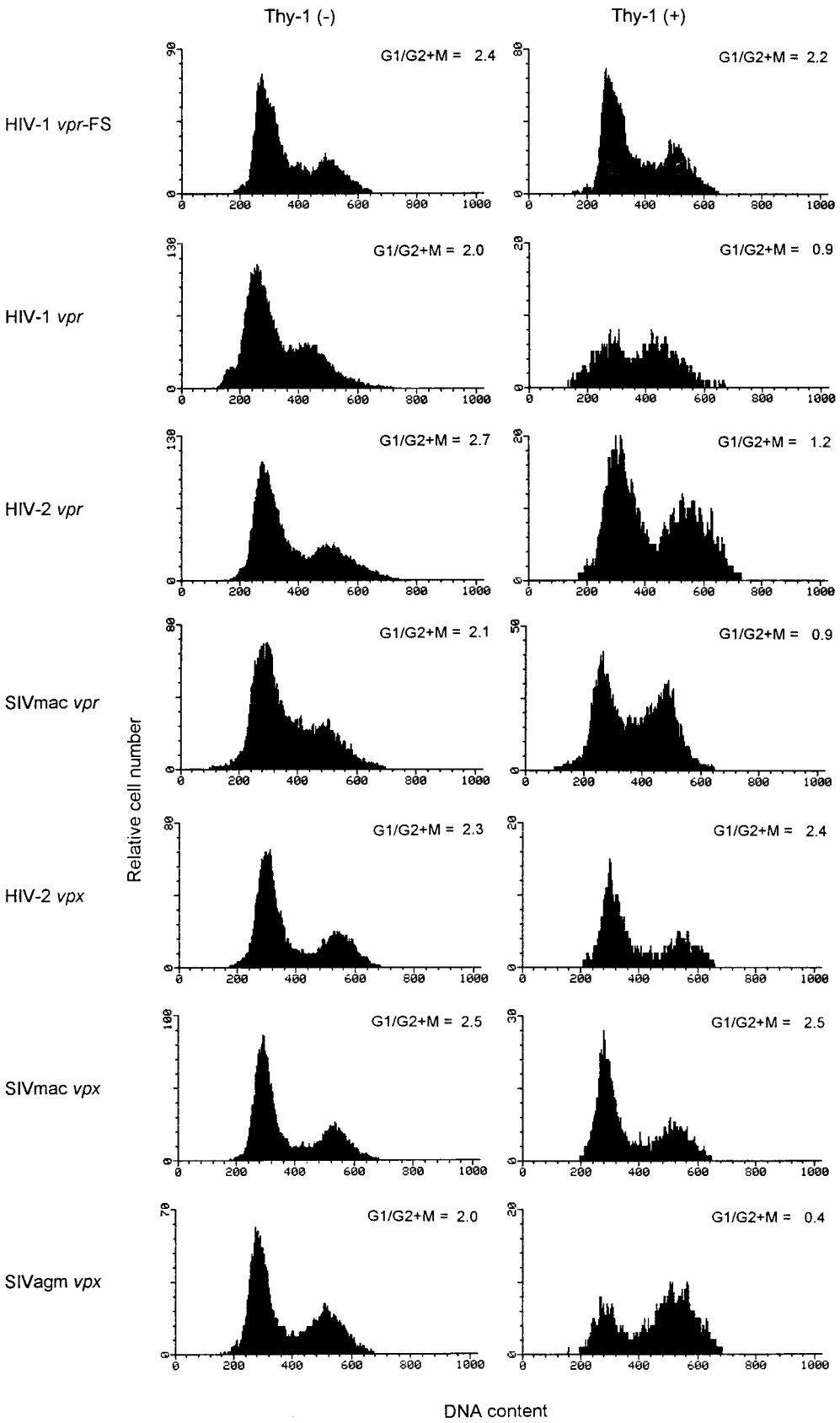

FIG. 4. Effects of *vpr* and *vpx* genes from various primate lentiviruses on the cell cycle of CV-1 cells. Expression vectors for *vpr* and *vpx* (10 μg of plasmid DNA) were transfected into 10^7 CV-1 cells, and analysis was performed 72 h posttransfection as described for Fig. 1. Transfection efficiencies ranged between 5 and 20%, as measured by Thy-1 surface expression.

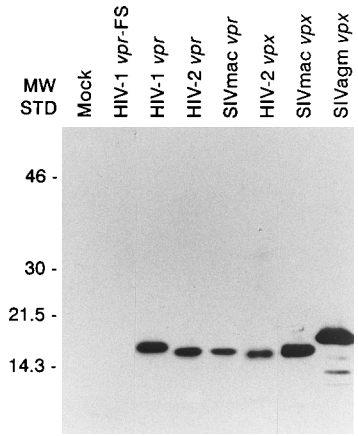

FIG. 5. Detection of *vpr* and *vpx* gene products by immunoblotting. Cells (5×10^6) transfected with various constructs were lysed in 200 μl of Laemmli buffer containing 5% β-mercaptoethanol, incubated at 100°C for 5 min, and centrifuged at 12,000 × *g* to remove cell debris. Supernatants (10 μl; 2.5×10^5 cell equivalents) were subjected to SDS-PAGE. Proteins were blotted onto a nitrocellulose filter and developed with antibody 12CA5 (38) followed by peroxidase-conjugated goat anti-mouse antibody. Bands were visualized by the enhanced chemiluminescence method (Amersham).

HIV-2 and SIV_{mac}, Vpx but not Vpr appears to be involved in the nuclear transport of preintegration complexes (36a). In addition, the *vpr* gene product was shown to act as a weak transcriptional activator of the HIV-1 long terminal repeat (5, 27). Recently, we and others demonstrated that the presence of a full-length *vpr* in HIV-1 precluded establishment of chronic infection in vitro (29, 33) by inducing cell cycle perturbation at the G_2/M boundary (12, 19, 31, 33). We further demonstrated that this cell cycle perturbation was due to a specific arrest of cells at the G_2/M boundary (19).

Here we demonstrate that *vpr* from HIV-1, HIV-2, and SIV_{mac} and *vpx* from SIV_{agm} are functionally related with regard to induction of G_2 arrest, whereas *vpx* from HIV-2 and SIV_{mac} did not have any detectable influence on the cell cycle. Although we have not formally proven here that cell cycle perturbation of *vpr*-related genes is due to cell cycle arrest, it is likely to be the case, given the evolutionary relationship of these genes, and is therefore referred to as such here. The four viruses studied here belong to three of the five phylogenetic groups of primate lentiviruses (6, 16, 35). According to these results, members of these three groups of primate lentiviruses encode a gene (*vpr* from HIV-1 and HIV-2/SIV_{mac} and *vpx* from SIV_{agm}) that is sufficient to produce cell cycle arrest. Thus, ability to induce G_2 arrest appears to be a conserved biological feature among primate lentiviruses and likely represents a key element in the virus-host interaction. The finding that *vpx* from HIV-2 and SIV_{mac} does not induce G_2 arrest suggests that this viral function was retained by only one of the genes (*vpr*) resulting from the proposed duplication (37).

Species-specific factors are involved in induction of G_2 arrest. It is noteworthy that the proportion of cells in G_2 or M induced by HIV-1 *vpr* expression was higher in HeLa cells (G_1/G_2+M = 0.2) than in CV-1 cells (G_1/G_2+M = 0.9). Conversely, SIV_{agm} *vpx* induced marked G_2 arrest in CV-1 cells (G_1/G_2+M = 0.9) but had no detectable effect in HeLa cells (G_1/G_2+M = 2.5). Thus, the magnitude of cell cycle arrest induced by HIV-1 *vpr* and SIV_{agm} *vpx* is higher in cells of the same species from which the corresponding viruses were isolated. These observations indicate that despite sequence and functional conservation, there are quantitative differences in the induction of G_2 arrest that may be associated with cellular factors. Alternatively, these differences may relate to the tissues of origin or the particular cell lines used in this study. Further characterization of potential species-specific differences should involve transfection of lymphocytes from various primates.

We previously demonstrated that the ability of HIV-1 Vpr to induce G_2 arrest correlated with hyperphosphorylation of the CDC2 kinase (19), a key protein controlling the G_2 checkpoint in the cell cycle. The CDC2 kinase is encoded by a highly conserved gene through evolution, as it has 98.7, 96, and 63% amino acid sequence identity to its bovine (40), murine (36), and fission yeast (23) counterparts, respectively. Therefore, given the species-specific cell cycle arrest differences observed in this study, it seems unlikely that Vpr and Vpx affect the cell cycle through direct interaction with the CDC2 kinase. Rather, Vpr and Vpx probably interact with cellular factors other than CDC2 that are divergent among primates.

Importance of *vpr* and *vpx* in in vivo pathogenesis. Experiments to determine the importance of *vpr* and *vpx* in vivo have been performed by inoculating rhesus macaques with mutants of SIV_{mac} (8, 17). These studies demonstrate that mutant viruses with deletion of *vpr* or *vpx* maintain the ability to induce AIDS in macaques. However, a *vpr-vpx* double mutant did not induce disease in rhesus macaques. Therefore, *vpr* and *vpx* constitute viral determinants of pathogenesis in rhesus macaques, although mutation of either *vpr* or *vpx* is not sufficient to render this virus nonpathogenic. The role of SIV_{agm} *vpx* in in vivo pathogenesis is unclear, since the first disease-inducing clone of this virus was isolated only recently (15).

The role that G_2 arrest plays in the pathogenesis of primate lentiviruses is unclear. The fact that SIV_{agm} does not produce disease in its natural host (the African green monkey) suggests that G_2 arrest is not sufficient for pathogenesis. In addition, deletion of *vpr* from SIV_{mac} does not render the mutant virus nonpathogenic, suggesting that G_2 arrest in the SIV_{mac} rhesus macaque system may not contribute to pathogenesis. However, the known sequence conservation of *vpr* and *vpx* throughout evolution, together with the functional conservation we demonstrate here, strongly suggests that G_2 arrest is an important function in the biology of primate lentiviruses. Since primate lentiviruses establish persistence in the absence of disease in their natural hosts, one can hypothesize that *vpr* and/or *vpx* might facilitate persistence by interfering with the cell cycle of $CD4^+$ cells and thus impairing immune surveillance. Elucidation of the primary role of induction of G_2 arrest by primate lentiviruses will require close examination of the biology of these viruses in their natural hosts.

We conclude that induction of G_2 arrest is a conserved function through evolution of primate lentiviruses. Clearly, the inability of infected T cells to proliferate as a result of lentiviral infection has implications for their immune function. We hypothesize that arrest in G_2 leads to functional impairment and possible death of $CD4^+$ lymphocytes. This may constitute a mechanism by which primate lentiviruses escape host immune surveillance and establish persistence. Further comparisons of the *vpr* and *vpx* functions in lentiviral primate systems characterized by immunodeficiency will reveal important clues about progression to AIDS and may ultimately open new avenues for therapy.

ACKNOWLEDGMENTS

We thank L. Antonio and S. Suggs of AMGEN, Inc., and B. Poon and O. Rey for technical assistance, W. Aft for preparation of the manuscript, and G. Feuer for critical reading of the manuscript.

This work was supported by a grant from AMGEN, Inc., grants AI31822 from the National Institutes of Health, and grant AI28697 from the Center for AIDS Research. V.P. was supported by training grant AI07388 from the National Institutes of Health and an AMGEN, Inc., fellowship; I.S.Y.C. was a Scholar of the Leukemia Society of America; and J.B.M.J. was supported by an American Cancer Society fellowship.

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